Hybridization Alters Spontaneous Mutation Rates in a Parent-of-Origin-Dependent Fashion in Arabidopsis[1][W]

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Over 70 years ago, increased spontaneous mutation rates were observed in *Drosophila* spp. hybrids, but the genetic basis of this phenomenon is not well understood. The model plant Arabidopsis (*Arabidopsis thaliana*) offers unique opportunities to study the types of mutations induced upon hybridization and the frequency of their occurrence. Understanding the mutational effects of hybridization is important, as many crop plants are grown as hybrids. Besides, hybridization is important for speciation and its effects on genome integrity could be critical, as chromosomal rearrangements can lead to reproductive isolation. We examined the rates of hybridization-induced point and frameshift mutations as well as homologous recombination events in intraspecific Arabidopsis hybrids using a set of transgenic mutation detector lines that carry mutated or truncated versions of a reporter gene. We found that hybridization alters the frequency of different kinds of mutations. In general, Columbia (Col) × Cape Verde Islands and Col × C24 hybrid progeny had decreased T→G and T→A transversion rates but an increased C→T transition rate. Significant changes in frameshift mutation rates were also observed in some hybrids. In Col × C24 hybrids, there is a trend for increased homologous recombination rates, except for the hybrids from one line, while in Col × Cape Verde Islands hybrids, this rate is decreased. The overall genetic distance of the parents had no influence on mutation rates in the progeny, as closely related accessions on occasion displayed higher mutation rates than accessions that are separated farther apart. However, reciprocal hybrids had significantly different mutation rates, suggesting parent-of-origin-dependent effects on the mutation frequency.

In plants, somatic mutations are an important source of genetic variability, which can, in principle, be passed on as heritable changes to the progeny of an individual (Baake and Gabriel, 1999). Plants have higher point mutation rates than animals (Kovalchuk et al., 2000) and do not have a fixed germline, such that there is a chance for somatic mutations to be transmitted to the next generation (Walbot and Evans, 2003). In contrast, animals cannot transmit somatic mutations to their progeny, as their germline cells are set aside early during development.

Mutation rates have been estimated in several organisms, including bacteria and mammals (Kovalchuk et al., 2000). The easiest phenotype for estimating mutation rates in plants is chlorophyll deficiency or albinism. In barley (*Hordeum vulgare*), it was estimated that albino phenotypes can result from mutations in about 300 different nuclear genes (Klekowski, 1992). Mutations leading to chlorophyll deficiency in barley and buckwheat (*Fagopyrum esculentum*) occur at rates of 3.2×10^{-4} and 3.1×10^{-4} events per nuclear genome per generation, respectively. In long-lived red mangrove (*Rhizophora mangle*) plants, this rate was found to be 25 times higher, which led to the prediction that long-lived plants have higher mutation rates per generation than short-lived ones, possibly resulting from more mutations in the lineage giving rise to the germline (i.e. the meiocytes; Klekowski and Godfrey, 1989).

Spontaneous point mutation rates in Arabidopsis (*Arabidopsis thaliana*) are in the range of 10^{-7} to 10^{-8} events per base pair and generation. These estimates are based on functional reversions of a mutated *uidA* gene encoding GUS (Swoboda et al., 1994; Kovalchuk et al., 2000). Sequencing the genome of five Arabidopsis mutation accumulation lines revealed a spontaneous point mutation rate of 7×10^{-9} base substitutions per site per generation (Ossowski et al., 2010). This study is in agreement with the whole-genome sequencing results from mammalian cancer cells, where C→T transitions were more frequent than any other type of point mutation (Ossowski et al., 2010; Pleasance et al., 2010). This is likely related to the fact that C in mammals and plants can be methylated and that methyl-C is prone to C→T transitions.

Frameshift (FS) mutations result from the addition or deletion of bases, altering the frame of triplet codons. The DNA replication and repair machinery makes use

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of a mismatch repair mechanism to correct mispaired bases after polymerase slippage during replication. Impaired mismatch repair can result in FS mutations via the insertion or deletion of nucleotides (Leonard et al., 2003). FS mutation rates in microsatellites depend on the length of the repetitive tract and the base composition. Tracts with G and C runs are more mutable than those with A and T runs, and longer microsatellite tracts make polymerase slippage events more frequent (Azaiez et al., 2006). FS mutations within a G7 tract, carrying seven G nucleotides in the *uidA* gene in the Arabidopsis genome, are in the range of 1.45×10^{-8} events per genome per generation, which is 5-fold higher than in tomato (*Solanum lycopersicum*), suggesting that mutation rates differ between plant species (Azaiez et al., 2006). In Arabidopsis, FS mutation rates of G13 and G7 repeats in the *uidA* gene are found to be 2×10^{-6} and 2×10^{-7} events per cell division, respectively.

Homologous recombination (HR) is involved in the reactivation of stalled DNA replication forks, is important for DNA repair, helps in the segregation of homologous chromosomes in meiosis I, and is essential for telomere maintenance (Sung and Klein, 2006). Double-stranded DNA breaks are also repaired by HR, where information at the site of the lesion is copied from the sister chromatid (Ilnytskyy et al., 2004). In Arabidopsis, HR frequencies are on the order of 10^{-6} to 10^{-7} events per genome per generation, estimated by functional recombinants of the *GUS* reporter gene in transgenic lines carrying two overlapping, truncated *GUS* fragments (Puchta et al., 1995). Sequence divergence decreases recombination frequency (e.g. 0.16% sequence divergence led to a 3-fold decrease of the HR rate; Opperman et al., 2004).

Close to 10% of animals and approximately 25% of plants hybridize with at least one other species (Mallet, 2007). It is known that hybridization triggers rapid genomic rearrangements in plants (e.g. intraspecific sunflower [*Helianthus* spp.] hybrids have 50% more nuclear DNA than their parents; Baack et al., 2005). Hybrid plants also exhibit differential gene expression patterns (Meyer et al., 2007), and transposon-mediated gene silencing effects are pronounced in hybrids compared with inbred parental lines (Baack and Rieseberg, 2007). Over 70 years ago, it was reported that hybrids derived from crosses between *Drosophila melanogaster* and *Drosophila simulans* display an increased somatic mutation frequency (Belgovsky, 1937). Similarly, offspring of the backcrosses from hybrids between two races of *Drosophila pseudoobscura* exhibit increased mutation rates (Sturtevant, 1939). Interestingly, crosses between geographically separated strains of *D. melanogaster* also show an enhanced mutation frequency in the hybrids (Thompson and Woodruff, 1980). Furthermore, mutation rates in hybrid *D. melanogaster* males are 15 times higher than in nonhybrid males (Simmons et al., 1980). However, all this early work on flies was based on eye color, body color, and bristle shape defects, and the precise molecular nature of the mutations is unknown.

Despite hybridization occurring frequently in nature and being important for the origin of new plant species, little is known about spontaneous mutation rates in plant hybrids. Kostoff (1935) reported increased somatic mutation rates in interspecific tobacco (*Nicotiana* spp.) hybrids. When red-flowering *Nicotiana tabacum* plants were crossed with the white-flowering species *Nicotiana alata*, a few hybrid flowers had white stripes on a red background. It was proposed that the dominant red color gene changed to its recessive allelic form, giving rise to striped flowers. Interestingly, reversions from recessive to dominant alleles were reported to occur at higher frequencies, and striped flowers were only seen in crosses between distantly related *Nicotiana* spp. Thus, hybridization may produce conditions where an otherwise stable allele behaves like a mutable one (Kostoff, 1935). The molecular basis of these observations is not known, but it may be due to the mobilization of a transposon in the neighborhood of a gene responsible for floral pigmentation in the hybrids or the involvement of posttranscriptional gene-silencing events (Gerats et al., 1990; Napoli et al., 1990).

Here, we describe the effects of intraspecific hybridization on mutation rates in the model plant Arabidopsis. We examined (1) the frequency of different classes of spontaneous mutations in intraspecific hybrids, (2) whether mutation rates in hybrids are correlated with the overall genetic distance between the parents, and (3) whether the direction of the cross influences the mutation rates (maternal and paternal effects). We found that hybridization alters somatic mutation rates in the F1 progeny, but it does not seem to be related to the overall genetic distance between the parents. Surprisingly, reciprocal hybrids had significantly different mutation rates, indicating a parent-of-origin-dependent effect on the generation or repair of mutations in the hybrids.

RESULTS

To determine the frequencies of mutations in hybrids, we made use of various transgenic lines carrying mutated or fragmented versions of the *GUS* reporter gene (Kovalchuk et al., 2000; Li et al., 2004; Azaiez et al., 2006; Van der Auwera et al., 2008) and looked for reversion, FS, or HR events leading to the production of functional GUS enzyme. GUS activity can be monitored in planta using a chromogenic substrate, and each individual blue spot reflects a mutation event (Supplemental Fig. S1). By scoring such events among a large number of plants, we arrived at the mutation frequency (blue spots per plant) in the hybrid progeny after applying appropriate correction factors (see "Materials and Methods"). Mutation rates in hybrids obtained from transgenic lines in the Columbia (Col) background were compared with Col × Col F1 plants (control) derived from hand pollination between a wild-type Col parent and transgenic detector lines that carried GUS constructs, allowing us to score point mutations, FS mutations, and HR events.

Table I. *Normalization of mutation rates by cell number and ploidy per nucleus*

Different Arabidopsis hybrids and the Col × Col control were analyzed for their adaxial epidermal cell count and their ploidy per leaf cell nucleus to obtain the correction factor. The coefficient of interquartile range (CIQR) was calculated as a nonparametric measurement of variance in the style of the coefficient of variance. The relative number of cells and the relative ploidy are the normalization values for the hybrid, normalized to the Col × Col control. The correction factor was calculated by multiplying the two normalization values. This correction factor was used to correct the number of GUS spots before analysis. *n*, Number of plants analyzed; Median, median of measurements; CIQR, interquartile range/median.

Cross	Epidermal Cells				Ploidy Per Nucleus				
	n	Median	CIQR	Relative No. of Cells	*n*	Median	CIQR	Relative Ploidy	Correction Factor
Col × Col	14	36,616	0.465	1.000	4	4.12	0.064	1.000	1.000
Col × C24	14	41,047	0.234	1.121	13	4.03	0.081	0.977	1.096
C24 × Col	14	45,671	0.234	1.247	14	4.15	0.273	1.007	1.256
Col × Cvi	12	38,081	0.063	1.040	20	3.94	0.104	0.957	0.996
Cvi × Col	27	63,175	0.317	1.725	21	4.32	0.028	1.048	1.808
Col × Ws	10	45,933	0.219	1.254	10	3.79	0.036	0.919	1.153
Ws × Col	11	55,158	0.527	1.506	10	3.31	0.047	0.804	1.211
Col × L*er*	9	59,745	0.198	1.632	5	4.02	0.074	0.976	1.593
L*er* × Col	9	69,330	0.346	1.893	12	4.17	0.048	1.013	1.919

Cell Number and Average Ploidy per Nucleus Differ between Parents and Hybrids

The mutation rates we determined were based on reversion events from a single *GUS* gene in both the intraaccession and interaccession hybrid progeny. All the transgenic lines used in this study carried a single *GUS* reporter gene per genome. For comparison of the mutation rates between parents and various hybrids, we had to correct the observed reversion events by putting them in relation to the total copy number of the *GUS* reporter gene (i.e. the number of genomes). Because hybridization may affect cell number and cell size, as well as the degree of endopolyploidization, these factors have to be taken into account to calculate the number of genomes. It has long been known that the large size of hybrid maize (*Zea mays*) and hybrid tomato seeds is primarily due to an increase in cell number (Kiesselbach, 1922; East, 1936; Ashby, 1937). An effect on cell size and cell number was also reported for Arabidopsis C24 × Col hybrids (Fujimoto et al., 2012). Therefore, we determined cell number, cell size, and ploidy in hybrid plants in order to normalize the number of events we observed per plant by the number of cells and the average ploidy level per nucleus.

As the size of a leaf is primarily determined by its epidermis (Savaldi-Goldstein et al., 2007; Marcotrigiano, 2010), we examined the epidermis of the sixth true leaf to estimate differences in cell size and cell number. We made scanning electron micrographs of adaxial leaf epidermal surfaces and counted the number of cells in a specified area (Table I; for details, see "Materials and Methods"). In general, intraaccession Arabidopsis hybrids resulted in leaves with more cells that were larger than those of the Col × Col control (Fig. 1; Supplemental Fig. S2). We found that the crosses Cape Verde Islands (Cvi) × Col ($t = 4.43$, $P < 0.001$), Col × Landsberg *erecta* (L*er*; $t = 3.16$, $P = 0.011$), and L*er* × Col ($t = 5.00$, $P < 0.001$) had more epidermal cells than the Col × Col control leaves (Fig. 1C). Hybrids derived from the cross Wassilewskija (Ws) × Col had larger epidermal cells ($t = 3.68$, $P = 0.002$) than the Col × Col control plants (Supplemental Fig. S2A). All hybrids except the ones derived from crosses Col × C24 and Col × Cvi had a larger leaf surface area than the control Col × Col (Supplemental Fig. S2B). We attribute the larger leaf surface area to the increased cell number and, in the case of the Ws × Col hybrid, to larger epidermal cells.

To correct for the number of genomes present in hybrids versus their respective parents, we also had to consider the level of endopolyploidization, which may differ between parents and their hybrid offspring. Although a previous study had not found significant ploidy differences between reciprocal Col × C24 hybrids and their parents (Banaei Moghaddam et al., 2010), it was important to check this for all hybrids used in this study. We estimated the average genome number (ploidy) per nucleus by flow cytometry (Supplemental Fig. S3). The percentage of nuclei with ploidy levels of 2×, 4×, and 8× varied between hybrids and the Col × Col control (Fig. 2A). Plants derived from the cross Ws × Col ($t = 3.44$, $P = 0.003$) had reduced average ploidy per nucleus compared with Col × Col (Fig. 2B).

Taken together, these data indicate that, in general, hybridization leads to an increase in cell number. An increase in cell number in conjunction with a slight decrease in the average ploidy per nucleus as seen in some hybrids is consistent with higher cell division rates, as suggested (Miller et al., 2012), but its extent is accession dependent.

Intraspecific Hybridization Affects Spontaneous Point Mutation Rates

To determine if point mutation rates in hybrids are significantly different from Col × Col controls and to examine whether hybridization favors one class of point mutations over others, we examined several lines carrying a point-mutated *GUS* reporter gene, allowing

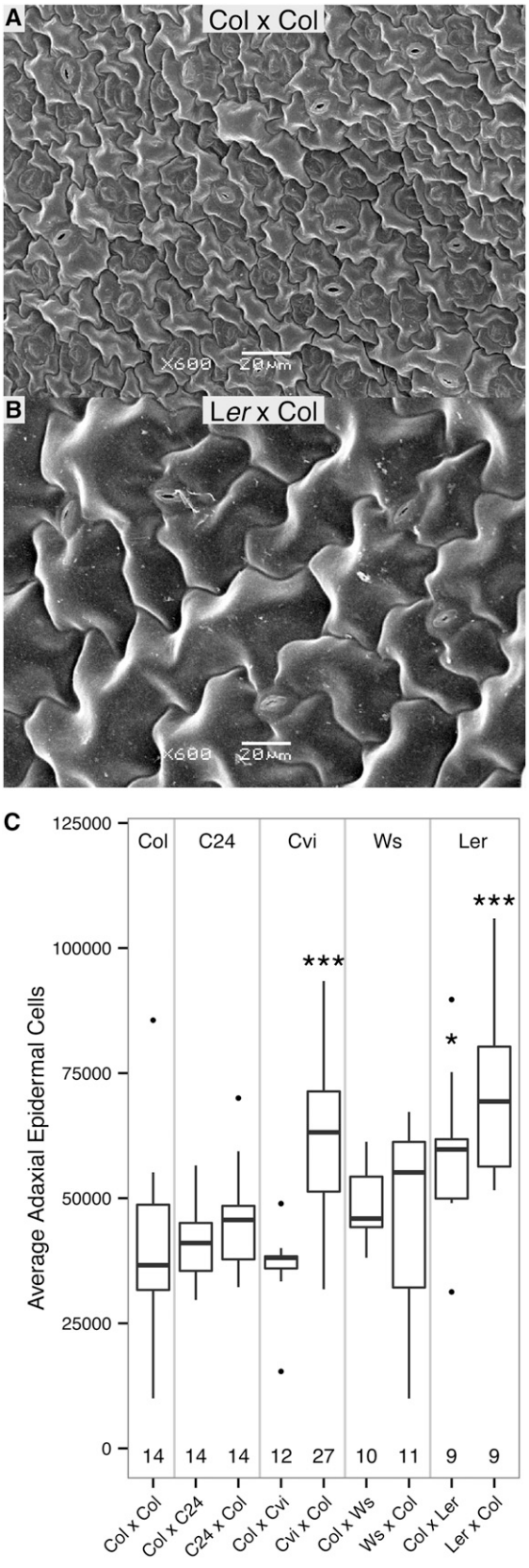

Figure 1. Cell number in Arabidopsis hybrids and parents. A and B, Scanning electron microscopic images of the adaxial epidermal cells of the sixth true leaf of a 3-week-old Arabidopsis plant. In general, hybrids (B) have larger cells than the wild-type parents (A). C, For each cross, a box plot of the number of adaxial epidermal cell number in the sixth leaf of 3-week-old Arabidopsis plants is drawn. The numbers at the bottom of the graph show the number of leaves analyzed. The whiskers represent 1.5 × interquartile range and the dots are outliers. The asterisks above the box plots indicate whether the specific cross is significantly different from the control Col × Col (*$P < 0.05$, ***$P < 0.001$). *P* values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis."

us to score reversion events. Plants homozygous for nonsense mutations within the *uidA* reporter gene were used to generate hybrids (Kovalchuk et al., 2000). In lines used for scoring T-to-G reversions in Arabidopsis hybrids ($112_{G\rightarrow T}$ and $166_{G\rightarrow T}$), the base G is mutated to base T at the 112th and 166th positions in the open reading frame of *uidA* gene, respectively. In addition, we also used missense mutants designed to find C:G-to-T:A transitions, where the base T is mutated to base C, at the 1,390th position in the open reading frame of the *uidA* gene (Van der Auwera et al., 2008).

A point mutation reversion event restores the function of the *uidA* gene, as scored by the presence of blue spots in seedlings after histochemical staining for GUS activity (Supplemental Fig. S1; Jefferson et al., 1987). The mutation rates we determined were corrected for differences in the total number of genomes, based on the data obtained from average ploidy per nucleus and differences in cell number (Figs. 1 and 2; Table I). All mutation rates presented below have been adjusted accordingly.

In hybrids derived from line $1390_{T\rightarrow C}$, C→T point mutation events were either unaffected or increased depending on the hybrid combinations (Fig. 3A; Supplemental Table S1). If the transgenic line was used as a pollen donor, the mutation rate increased by 98% in the cross C24 × Col ($t = 3.33$, $P = 0.007$; Fig. 3A). In contrast, a decrease of T→G mutations in line $166_{G\rightarrow T}$ of 40% was observed in C24 × Col ($t = -2.79$, $P = 0.033$; Fig. 3B; Supplemental Table S2). In hybrids derived from line $112_{G\rightarrow T}$, a decrease of T→G mutations by 85% for C24 × Col ($t = -7.64$, $P < 0.001$) and by 87% for Cvi × Col hybrids ($t = -9.81$, $P < 0.001$) was observed (Fig. 3C; Supplemental Table S3). A 74% decrease in T→A point mutations ($t = -5.93$, $P < 0.001$) was observed for Cvi × Col using line $118_{T\rightarrow A}$ (Fig. 3D; Supplemental Table S4).

In conclusion, our data indicate a general decrease of transversion events (T→G and T→A mutations) and an increase of transition rates (C→T). This increase of C→T point mutations may be due to the increased methylation of C residues as a result of hybridization (Groszmann et al., 2011; Shen et al., 2012). In both plants and animals, methylated C nucleotides are prone to deamination in vivo, which can lead to C→T transitions (Ossowski et al., 2010; Mugal and Ellegren, 2011). In general, hybridization seems to affect different classes of mutations in opposite ways: it promotes C→T transitions but suppresses T→G transversions.

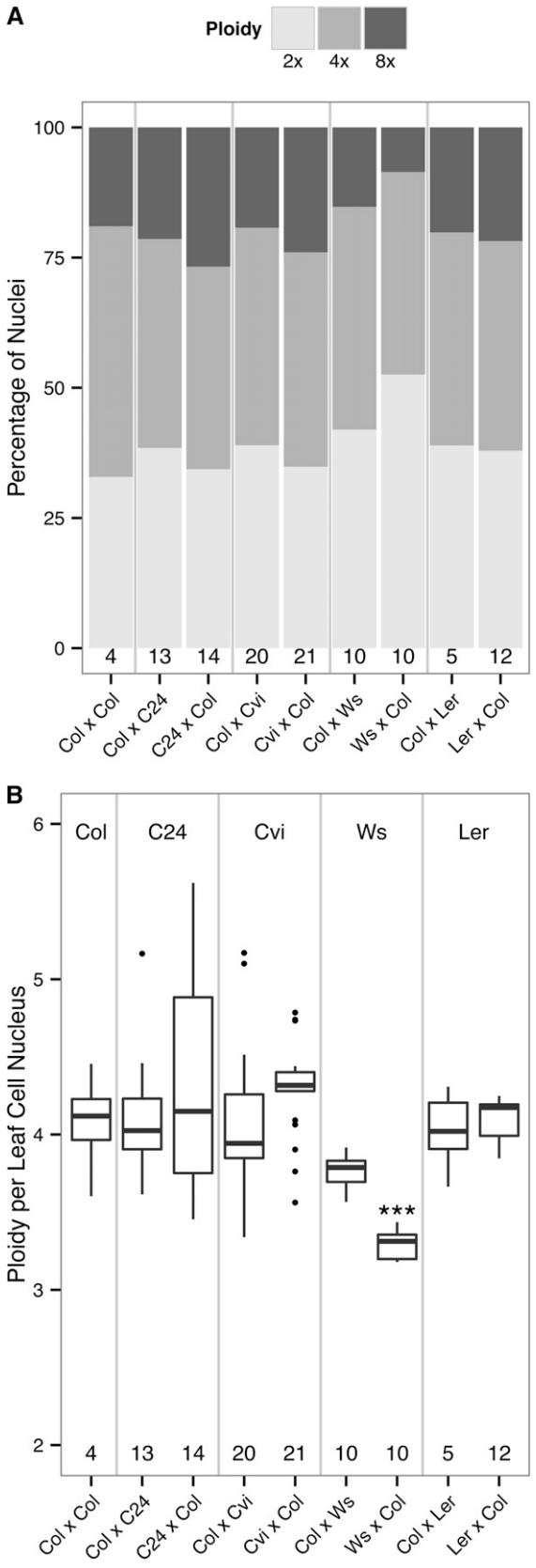

Figure 2. Ploidy distribution of nuclei in Arabidopsis hybrids and parents. A, Percentage of diploid, tetraploid, and octoploid nuclei in leaf cells. The different shades indicate diploid, tetraploid, and octoploid nuclei. B, Average ploidy per leaf cell nucleus. Accessions are indicated at the top of the graph. For each cross, a box plot of the average ploidy per leaf cell nucleus is drawn. The numbers at the bottom of the graph show the number of leaves analyzed. The whiskers represent 1.5 × interquartile range and the dots are outliers. The asterisks above the box plots indicate whether the specific cross is significantly different from the control Col × Col (***$P < 0.001$). P values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis."

Altered Rates of Insertion/Deletion of Nucleotides Change FS Mutation Events in Hybrids

In order to assess the effect of hybridization on FS mutation rates, Col plants containing out-of-frame mononucleotide G repeats (G7 and G10) in the *uidA* reporter gene were crossed to the C24, Cvi, and Ws accessions. FS mutations arise as a result of insertion or deletion of nucleotides within these DNA repeats, which restore the function of the *uidA* gene (Azaiez et al., 2006). Compared with Col × Col reference plants, no significant change in FS mutation rates was observed in hybrids of line G7 (Fig. 4A; Supplemental Table S5). However, FS mutation rates were higher in line G10 compared with line G7 (Fig. 4B; Supplemental Table S6). Using the transgenic line G10 as a pollen donor, we found that FS rates increased by 94% ($t = 7.26$, $P < 0.001$) for the C24 × Col hybrid.

FS mutations occur largely due to DNA replication slippage events (Viguera et al., 2001). In hybrids, FS rates may vary depending on the number of repeats in the insert and also the parental genotype.

The Frequency of HR Events in Hybrids Depends on the Specific Parental Combination

To estimate the frequencies of HR events in hybrids, we used transgenic lines R2L1, R3L30, and R3L66 (in the Col background), which carry two inverted catalase introns within the *uidA* gene. A recombination event within the identical sequences of the catalase introns makes the gene functional, resulting in blue spots upon histochemical staining (Li et al., 2004). In lines carrying the R2 and R3 constructs, the DNA sequences common to both the inverted introns within the *uidA* gene were 418 and 589 bp, respectively. To study the impact of sequence divergence on the recombination rates in hybrids, a homeologous reporter line was used (Li et al., 2004). The transgenic line R3m11L85 carries 11 sequence polymorphisms (1.9% sequence divergence) within the 589-bp recombination substrate of the two inverted catalase introns.

Using the reporter line R3m11L85 as mother, the HR rates were different for all the crosses analyzed. The HR rates increased by 360% ($t = 12.37$, $P < 0.001$) and 57% ($t = 2.83$, $P = 0.032$) for Col × C24 and Col × Ws, respectively (Fig. 5A; Supplemental Table S7). A decrease of the HR rate by 49% ($t = -3.14$, $P = 0.012$) was found for Col × Cvi. Using R3m11L85 as a pollen donor, we found the HR rate to increase by 259% ($t = 6.34$, $P < 0.001$) for C24 × Col (Fig. 5A; Supplemental Table S7).

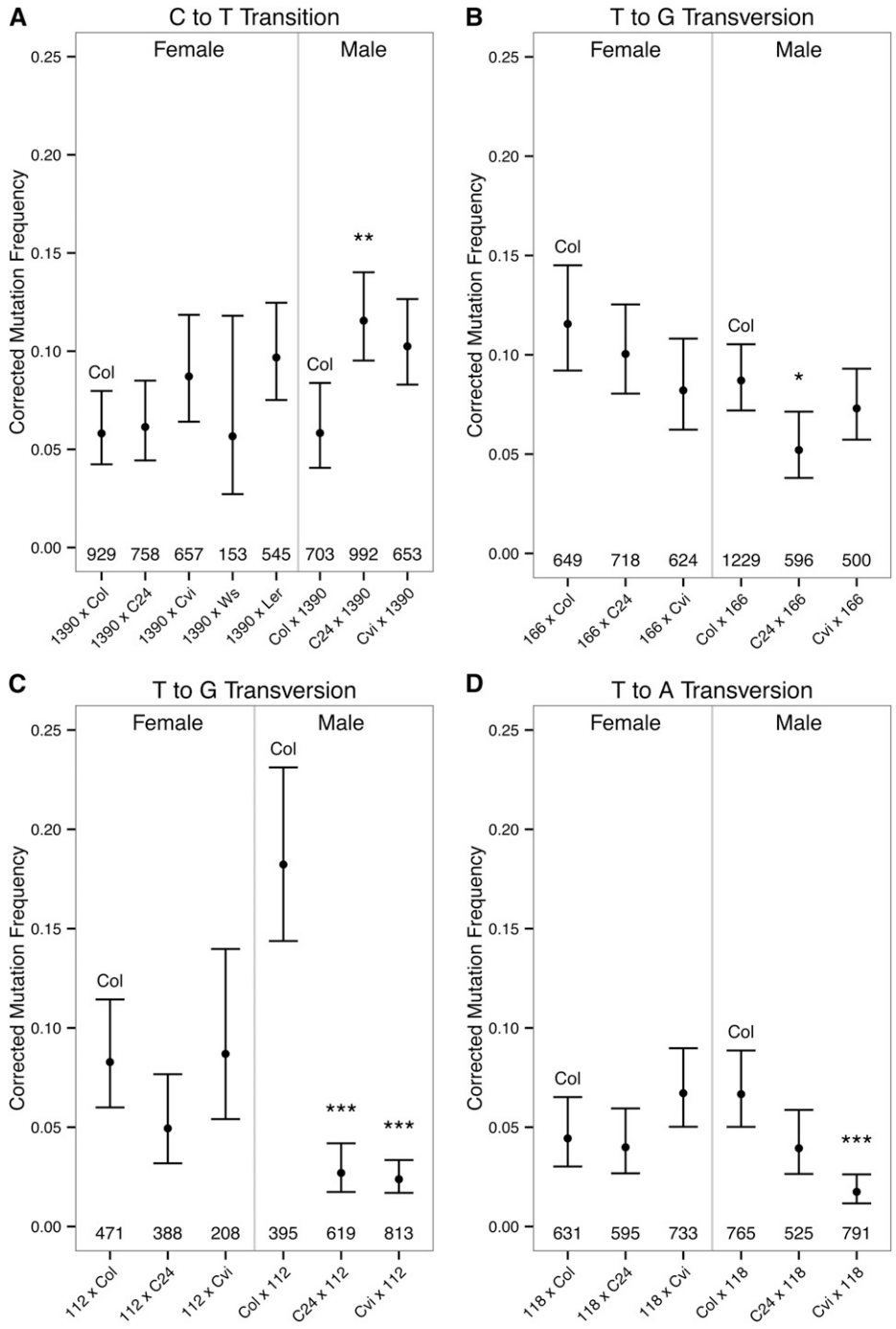

Figure 3. Point mutation rates in Arabidopsis hybrids. Point predictions and 95% prediction intervals for mutation rates in Arabidopsis hybrids derived from $1390_{T\rightarrow C}$ (A), $166_{G\rightarrow T}$ (B), $118_{T\rightarrow A}$ (C), and $112_{G\rightarrow T}$ (D) are drawn. Numbers at the bottom of the graph show the number of seedlings analyzed. The asterisks above the interval indicate whether the specific cross is significantly different from the corresponding control indicated with "Col" above the interval (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). P values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis."

A 92% increase in the HR rate was observed when R3L66 was used as the mother ($t = 8.91$, $P < 0.001$) for Col × C24 (Fig. 5B; Supplemental Table S8). However, using it as a pollen donor, the HR rates dropped in both hybrids by 31% ($t = -4.39$, $P < 0.001$) and 69% ($t = -10.78$, $P < 0.001$) for C24 × Col and Cvi × Col, respectively (Fig. 5B; Supplemental Table S8).

Using R3L30 as a mother, the HR rates increased by 44% ($t = 4.51$, $P < 0.001$) for Col × C24 but decreased by 36% ($t = -3.83$, $P = 0.001$) for Col × Cvi (Fig. 5C; Supplemental Table S9). When R3L30 was used as a pollen donor, a decrease of the HR rate by 70% ($t = -12.45$, $P < 0.001$) was observed for Cvi × Col (Fig. 5C; Supplemental Table S9).

Crosses with all accessions tested showed a decrease in HR rates if crossed with R2L1 (Fig. 5D; Supplemental Table S10). Using R2L1 as a mother, the HR rates in Col × C24, Col × Cvi, and Col × Ws hybrids decreased by 49% ($t = -6.77$, $P < 0.001$), 61% ($t = -8.58$, $P < 0.001$), and 41% ($t = -4.05$, $P < 0.001$), respectively. When used as a pollen donor, HR rates decreased in Cvi × Col by 83% ($t = -11.66$, $P < 0.001$).

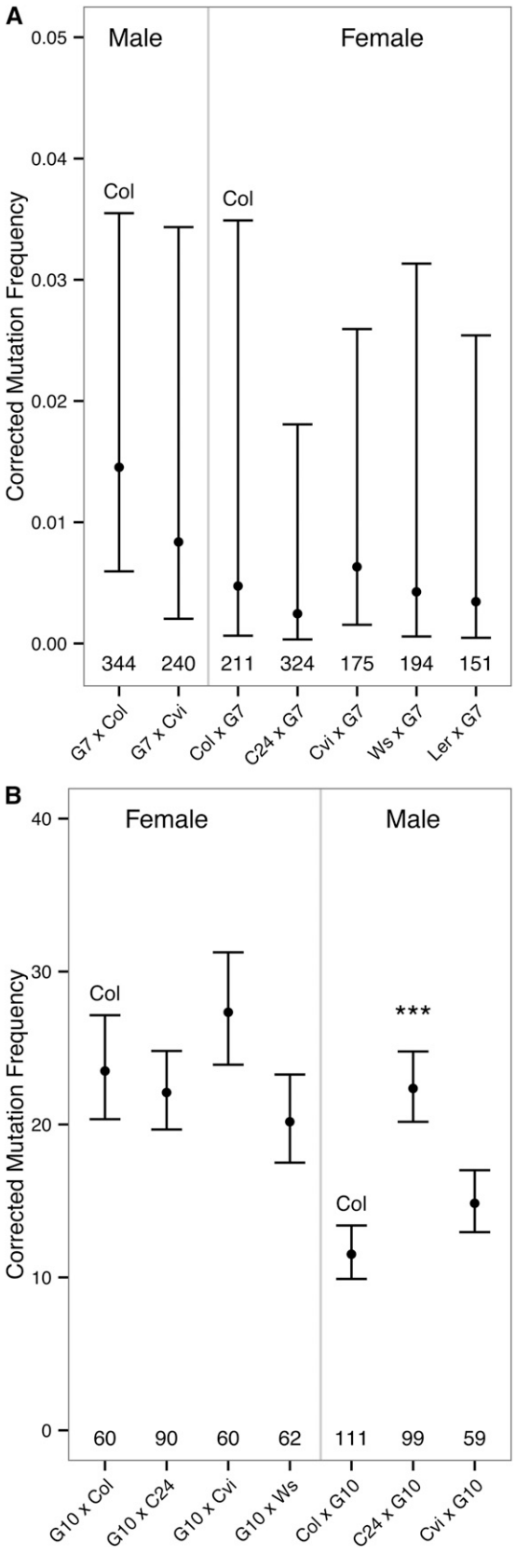

Figure 4. FS mutation rates in Arabidopsis hybrids. FS mutations were analyzed in hybrids derived from G7 (A) and G10 (B) lines in the Col background. Point predictions and 95% confidence intervals for FS mutation rates are drawn. Numbers at the bottom of the graph show the number of seedlings analyzed. The asterisks above the interval indicate whether the specific cross is significantly different from the corresponding control indicated with "Col" above the interval (***$P < 0.001$). *P* values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis."

Our data show that HR rates are strongly affected by hybridization, but whether they increase or decrease appears to depend on the particular combination of parents and reporter construct used. For instance, line R2L1 behaves quite differently from all other lines tested, since it showed decreased HR rates in all crosses analyzed, independent of the direction of the cross. This may be because R2L1 had the shortest homology track of the constructs analyzed or may indicate an effect of the local genomic environment of the insertion site on the HR rate in this line. Line R3m11L85 carries the same HR substrate as R3L66 or R3L30, except for 1.9% sequence divergence. In crosses with C24, for which the highest number of combinations was analyzed, R3m11L85 behaves differently from the other two lines. Either the effect on HR rates is much stronger or it goes in the opposite direction (Fig. 5, A–C), indicating an effect of sequence divergence on HR rates in these hybrids.

Mutation Rates in Hybrids Do Not Correlate with the Overall Genetic Distance of the Parents

We studied the role of genetic distances between the parents on mutation rates in the hybrid progeny. Mutation detector lines in the Col background were crossed with C24, Cvi, Ws, and L*er*, which differ based on their patterns of polymorphisms. L*er* and Ws are very similar to each other, being assigned to the same subpopulation based on an analysis of population structure, and are quite closely related to Col (Schmid et al., 2003; Nordborg et al., 2005). In contrast, C24 and Cvi are both rather distinct from Col, with Cvi being very different from all other accessions analyzed (Nordborg et al., 2005).

We found no obvious effect of the overall genetic distance between the parents on the frequency of mutation events in the F1 hybrids (Fig. 3). However, Cvi had effects on the frequency of HR events that were strikingly different from those of other accessions (e.g. a strong reduction in HR events), whereas crosses with C24 and Ws had either no or opposite effects (Fig. 5, A and C).

In conclusion, it is evident that the particular accession used to generate the hybrids has a strong effect on the mutation rates. However, these effects can go in opposite directions even for closely related accessions and do not appear to be correlated with the overall genetic distance of the parents.

Maternal and Paternal Effects on the Mutation Frequency in Hybrids

To investigate whether there are parental effects on somatic mutation rates in hybrids, we generated reciprocal

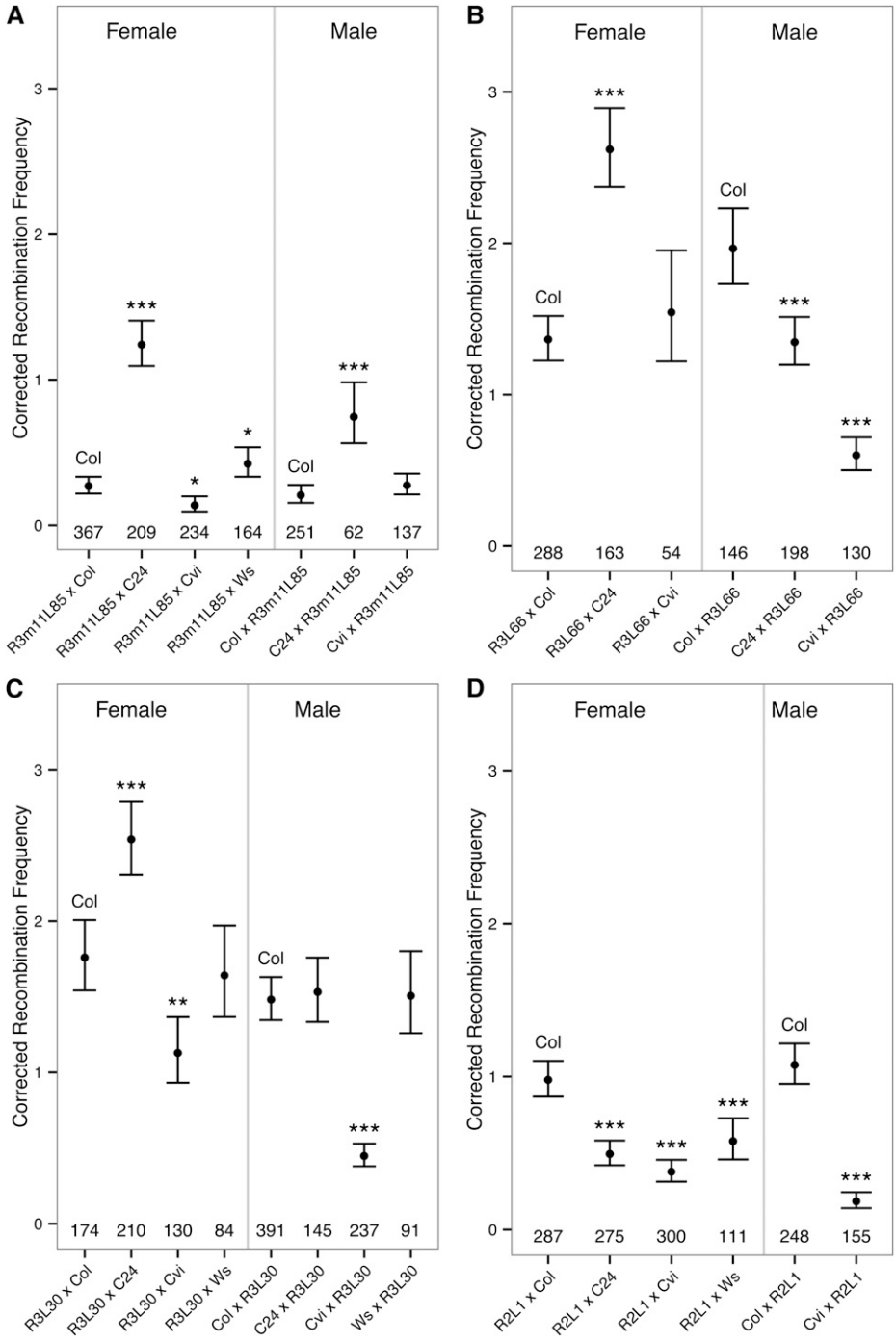

Figure 5. HR rates in Arabidopsis hybrids. HR rates in Arabidopsis hybrids derived from the transgenic lines R3m11L85 (A), R3L66 (B), R3L30 (C), and R2L1 (D) in the Col background are shown. Point estimates and 95% confidence intervals for HR frequencies are drawn. Numbers at the bottom of the graph show the number of seedlings analyzed. The asterisks above the interval indicate whether the specific cross is significantly different from the corresponding control indicated with "Col" above the interval (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). P values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis."

hybrids from the transgenic lines used for scoring point mutations, FS mutations, and HR events. We observed strong parental effects on point mutation frequencies in Col × C24 and Col × Cvi hybrids, as reciprocal hybrids often had significant differences in their mutation frequencies (Fig. 6, A–D). Using the C24 accession, we found parental effects for crosses with line $1390_{T\rightarrow C}$ ($t = 3.41$, $P = 0.007$) and $166_{G\rightarrow T}$ ($t = 3.41$, $P = 0.004$). For the Cvi accession, we found parental effects for crosses with lines $118_{T\rightarrow A}$ ($t = 5.39$, $P < 0.001$) and $112_{G\rightarrow T}$ ($t = 4.43$, $P < 0.001$). For line $112_{G\rightarrow T}$, we found parental effects of the transgene in the intraaccession control cross Col × Col ($t = 3.94$, $P < 0.001$).

Reciprocal hybridization had no effect on FS mutation rates but HR rates differed, suggesting parental effects on somatic HR. Parental effects were found in all interaccession crosses (Fig. 6, E–H). Using the C24 accession, we found a decrease in HR frequencies when the transgenic line was used as a pollen donor ($t = 3.35$, $P = 0.006$; $t = 8.69$, $P < 0.001$; and $t = 6.02$,

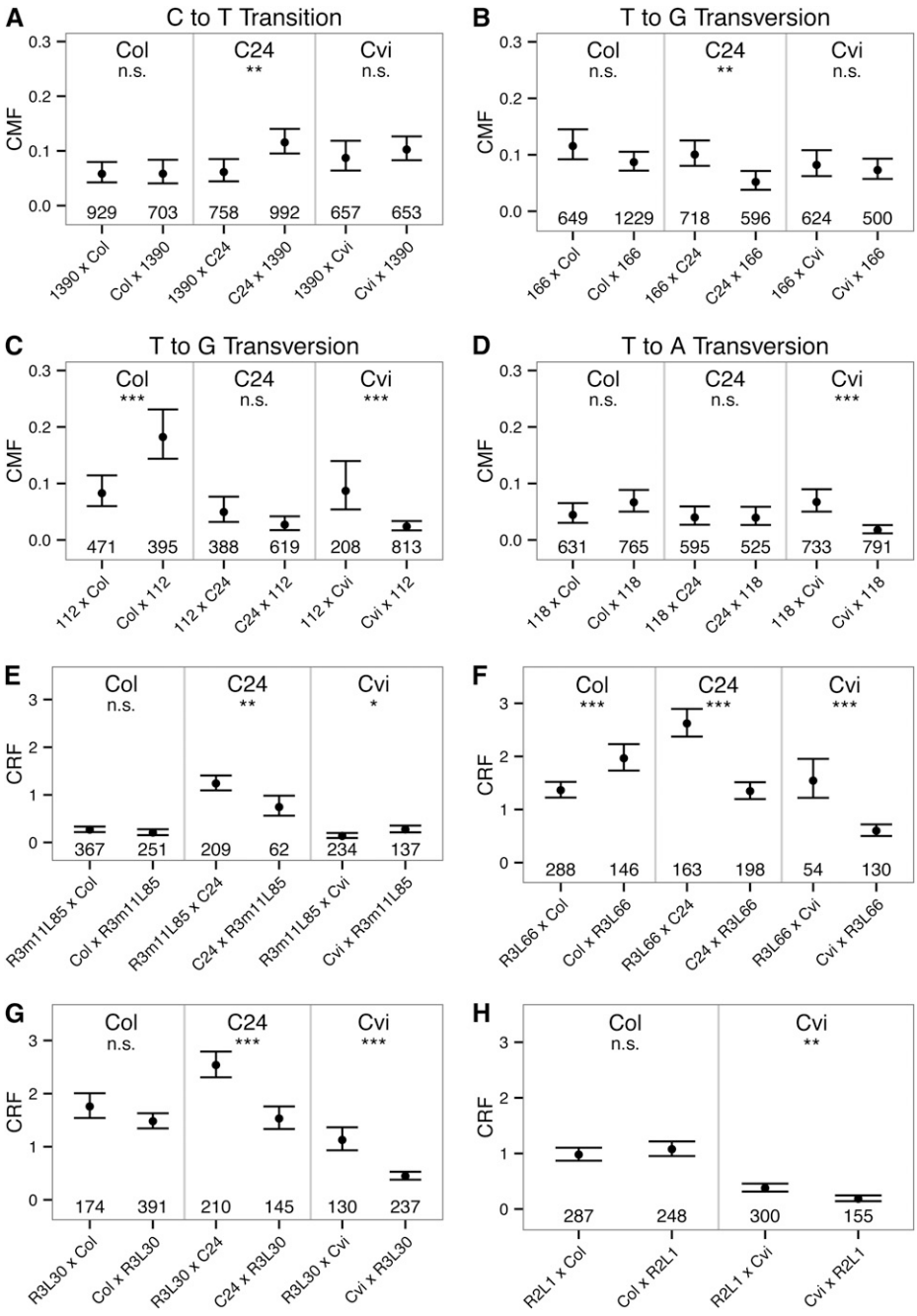

Figure 6. Parental effects on point mutation and HR frequencies in reciprocal hybrids. Point mutation (A–D) and HR (E–H) rates differ depending on which parent contributed the reporter construct. Point estimates and 95% confidence intervals for the mutation and HR rates are drawn. Numbers at the bottom of the graph show the number of seedlings analyzed. The asterisks indicate whether the specific comparison in significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). P values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis." n.s., Not significant; CMF, corrected mutation frequency; CRF, corrected recombination frequency.

$P < 0.001$ for R3m11L85, R3L66, and R3L30, respectively). However, using the Cvi accession, we found an increase in HR rates for the transgenic line R3m11L85 ($t = 3.06$, $P < 0.016$), while a decrease was observed for the other transgenic lines when they were used as pollen donors ($t = 6.38$, $P < 0.001$; $t = 7.29$, $P < 0.001$; and $t = 4.27$, $P < 0.001$ for lines R3L66, R3L30, and R2L1, respectively).

We also observed parental effects in the intraaccession control cross (Col × Col) for line R3L66 ($t = 4.39$, $P < 0.001$; Fig. 6, F and G). For the same line, interaccession crosses with C24 showed opposite parental effects, compared with the intraaccession cross, while Cvi showed the same effects.

In summary, these results show that there are strong parental effects on the frequency of point mutation and HR events in both interaccession and intraaccession crosses.

DISCUSSION

Interspecific hybridization is known to result in extensive chromosomal rearrangements, which can lead

to drastic changes of genome size and potentially in reproductive isolation (Fontdevila, 2005; Lai et al., 2005). In contrast, little is known about the effect of intraspecific hybridization on genome integrity in plants. By making use of a set of transgenic detector lines, we determined point mutation, FS mutation, and HR rates in reciprocal hybrids. As the offspring of intraspecific crosses show hybrid vigor; cell size, cell number, and the level of endopolyploidization may differ between parents and hybrids. In order to compare mutation rates between them, we corrected the number of observed events per plant taking these parameters into account (Table I). This detailed analysis showed that hybridization has an effect on somatic mutation rates, and the use of specific reporter lines allowed us to identify distinct effects of hybridization on point mutations, FS mutations, and somatic HR rates.

Application of high-throughput whole-genome sequencing allows the identification of de novo mutations in an unbiased manner. This was done after allowing the accumulation of mutations for multiple generations or by the sequencing of parents and their offspring in different organisms, such as *Escherichia coli*, *Chlamydomonas reinhardtii*, Arabidopsis, and humans (Ossowski et al., 2010; Campbell et al., 2012; Lee et al., 2012; Ness et al., 2012). Whole-exome sequencing has been successfully used to identify somatic mutations in endometrial tumors in humans (Le Gallo et al., 2012). Despite these advances, it is still difficult to measure somatic mutation events like HR by whole-genome sequencing approaches, and the use of various reporter constructs (Puchta et al., 1995) becomes important to study such events. Since there is always a chance of the transmission of a small fraction of somatic mutations to the next generation in plants, it is important to investigate such effects arising as a result of hybridization, which may contribute to genetic diversity in subsequent generations.

Our results show that, in general, transversion (T→G and T→A) rates decrease significantly in hybrids while transition (C→T) rates tend to increase (Fig. 3). Spontaneous transversions often occur due to oxidative stress, via the interaction of DNA with reactive oxygen species or the products they generate (De Bont and van Larebeke, 2004). The reduced frequency of transversions in hybrids may be connected to a change in the redox state in comparison with the parents. Although not yet investigated in detail, there are reports indicating differences in redox state in hybrids of wheat (*Triticum aestivum*; Bykova et al., 2011; Liu et al., 2012). It is interesting that T→G transversion reversion rates were different for the $112_{G\rightarrow T}$ and $166_{G\rightarrow T}$ lines, which illustrates a position effect on the transgene, possibly due to different insertion sites within chromosomes. In contrast to the general decrease in transversions, C→T transitions showed an increase, the largest being 2-fold in C24 × Col hybrids with the $1390_{T\rightarrow C}$ line (Fig. 3A). This could be related to the increase in genome-wide C methylation in hybrids (Groszmann et al., 2011; Shen et al., 2012), which can lead to C→T transitions due to the higher frequency of deamination of methyl-C (Ossowski et al., 2010; Mugal and Ellegren, 2011).

DNA polymerases are important for new strand synthesis, and any change in their sequence could have an impact on mutagenesis rates. For instance, mutations in DNA polymerase δ are responsible for a significant proportion of spontaneous mutations in selected cancer cell lines (Daee et al., 2010). The maintenance of genetic information, structural integrity, and stability of genomes requires error-free replication of DNA to prevent mutations. Replication of DNA is a complex high-fidelity reaction involving many enzymes, and it is reported that on average one wrong base gets incorporated per 10^{10} nucleotides synthesized (Minnick and Kunkel, 1996). The selectivity of DNA polymerases and their proofreading activity, along with the mismatch repair mechanism, ensures that the newly synthesized DNA strand is error free (Kunkel, 1992; Schaaper, 1993; Kroutil et al., 1996; Azaiez et al., 2006). The altered mutation rates in hybrids could possibly arise due to the heterozygous nature of the hybrid genome, resulting in a hybrid composition of the DNA polymerases, their interacting proteins, and the DNA repair complexes. The hybrid composition of subunits with slightly different amino acid sequences from the two parents might alter the fidelity of replication and/or repair, thus generating new genetic variation. The fact that in some hybrids we also found an effect on the frequency of FS mutations (Fig. 4B), for which DNA polymerase fidelity and DNA repair enzymes play a prime role, further supports the above hypothesis.

HR rates were strongly affected in the hybrids. However, while up to a 3.6-fold increase was observed in Col × C24 hybrids, the HR rate decreased up to 3.3-fold in Col × Cvi hybrids (Fig. 5). The reason for this difference is not clear, since Cvi and C24 accessions are rather distant from Col (Zhao et al., 2007). These effects on HR rates could also be caused by specific interactions of different isoforms, encoded by the two divergent genomes in the hybrids, which participate in the various multimeric complexes involved in HR. Alternatively, hybridization may lead to specific changes in gene expression. For instance, it was shown that reduced expression of the catalytic DNA polymerase δ led to an increase in HR rates (Schuermann et al., 2009). Such effects on gene expression may differ in the two hybrids that show opposite effects.

Interestingly, we found strong parent-of-origin-dependent effects for both point mutations and HR rates. This was unexpected, because the reciprocal hybrids have an exactly identical genetic constitution, with the exception of the parental origin of the reporter construct, but a similar effect had also been reported in *Drosophila* spp. (Simmons et al., 1980). Thus, parental effects have to be postulated. These could be of epigenetic nature; for instance, the changes in DNA methylation upon hybridization may not affect maternal and paternal genomes equally (Shen et al., 2012). Furthermore, the epigenetic constitution, including DNA methylation states, is known to differ between maternal and paternal genomes (Calarco et al., 2012; Ibarra et al., 2012). This can result in expression differences

(Autran et al., 2011), which may influence the isoform composition of multimeric protein complexes important for DNA replication and repair as outlined above. Alternatively, these parental effects may be related to nuclear-cytoplasmic interactions, as the reciprocal hybrids also differ with respect to the cytoplasm, and thus mitochondria and plastids, that contains the hybrid genome. Given that mitochondria play a central role in the redox state of the cell, it is possible that the cytoplasm of an inbred is optimized for its respective genome with respect to oxidative stress and mutation frequency, and that this balance is altered when a genome of another accession is introduced. Clearly, further studies at the molecular and biochemical levels will be required to get a better understanding of these intriguing parental effects on mutation rates.

CONCLUSION

Hybridization leads to highly heterozygous genomes compared with those of the inbred parents they are derived from. Although it was discovered over 70 years ago that hybridization has an effect on mutation rates in the offspring, little was known about which types of mutations hybridization induces and how the parental genotypes affect mutation rates. Using a set of mutation detector lines in the model plant Arabidopsis, we analyzed various kinds of mutations in different reciprocal hybrids and estimated their frequencies. We studied whether (1) hybridization promotes one particular class of mutations (point mutations, FS mutations, and somatic HR), (2) there is an effect of the genetic distance of the parents on the mutation frequencies, and (3) there are parental effects on mutation rates. We observed that hybridization affects point mutation and HR rates in a parent-of-origin-dependent manner.

MATERIALS AND METHODS

Plant Material

Point mutation reporter lines ($118_{A\rightarrow T}$, $112_{G\rightarrow T}$, and $166_{G\rightarrow T}$) were a gift from Igor Kovalchuk (Kovalchuk et al., 2000). The subline 699-2 represents the reporter line $118_{A\rightarrow T}$. Line 693 represents $112_{G\rightarrow T}$ detector, and the subline 747-3 represent the $166_{G\rightarrow T}$ reporter line. Line $1390_{T\rightarrow C}$ was provided by Anna Depicker (Van der Auwera et al., 2008). HR reporter lines (R2L1, R3L30, R3L66, and R3m11L85), harboring inverted catalase introns in the *uidA* gene, and FS reporter lines (G7 and G10) were a gift from Francois Belzile (Li et al., 2004; Azaiez et al., 2006). Based on previous analyses, all transgenic lines used appear to have a single insertion of the transgene. Arabidopsis (*Arabidopsis thaliana*) accessions Col, C24, L*er*, Ws, and Cvi were procured from the Arabidopsis Biological Resource Center (Ohio State University).

Plant Growth Conditions

Seeds were surface sterilized with 70% (v/v) ethanol, followed by 0.5% (v/v) bleach, for 2 min each, by vortexing in 1.5-mL Eppendorf tubes. Subsequently, the seeds were washed twice with autoclaved water and plated on sterile Murashige and Skoog (MS) medium (with 3% [w/v] Suc), pH 5.7, containing 0.05% (v/v) Plant Preservative Mixture (Biogenuix Medsystem). To synchronize seed germination, MS plates with seeds were kept at 4°C for 48 h in the dark and then moved to a plant growth chamber, having a uniform light intensity of 8,000 lx under a 16-h-light/8-h-dark cycle. The temperature was maintained at 22°C throughout the experiments, and the humidity was set at 80%. Three-week-old seedlings were transplanted from MS plates to soil and transferred back to the growth chamber. We used a 1:1 proportion of garden soil and peat, perlite, and vermiculite (Keltech Energies).

Plant Hybridization

For generating hybrids, flower buds prior to pollen maturation were emasculated in all the transgenic lines. After 48 h, the stigmatic surface of the buds was checked for pollen grains due to accidental cross-pollination, and such buds were discarded. Only pollen-free stigmas were used for pollination experiments with the Col, C24, Cvi, Ws, and L*er* accessions as pollen donors. Similarly, reciprocal crosses were performed by emasculating wild-type plants of these accessions, and the transgenic lines served as pollen donors. Colored thread labels were used to identify emasculated and crossed buds. Between 10 and 30 emasculations were performed in three to five replicates, independently. All crossing experiments were done at 22°C, and care was taken to ensure uniform plant age at the time of crossing to minimize possible variations of mutation frequencies in the progeny. Seed material was harvested 14 to 16 d after cross-pollination.

Histochemical Staining for GUS Activity

GUS staining was performed with 2- to 3-week-old plants as described (Jefferson et al., 1987). Staining buffer contained 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (Biosynth), 100 mM sodium phosphate buffer (pH 7.0), 0.1% Triton X-100, and 50 μg mL^{-1} kanamycin. The GUS staining solution (10 mL) was added to six-well plates (50 plants well^{-1}) and later vacuum infiltrated twice for 10 min each. After 48 h of incubation at 37°C, stained plants were cleared with 70% (v/v) ethanol. Blue spots reflecting point mutation reversion, recombination, and FS mutation events were counted using a light microscope (Supplemental Fig. S1).

Cell Number and Cell Size Analysis by Scanning Electron Microscopy

The sixth leaf of a 3-week-old Arabidopsis plant was dissected using the MZ8 stereo zoom microscope (Leica Microsystems), vacuum infiltrated for 5 min, and fixed overnight at 4°C in a solution of 0.1 M cacodylic acid and 2% (v/v) glutaraldehyde (pH 7). After fixation, the samples were rinsed with 0.1 M cacodylic acid and later dehydrated through a graded acetone series (30%, 50%, 70%, 90%, and 100% [all v/v]; 15 min for each step). The last step of dehydration in 100% acetone was repeated twice, and samples were left in it before critical point drying. The leaves were critical point dried with CO_2 using the CPD Polaron E 3000 (Quorum Technologies), later the samples were gold coated with a Balzers Union sputter coater (OC Oerlikon Balzers), and finally, leaves were mounted on the scanning electron microscope stubs aided by double-sided sticky tape and examined with a JSM-6360 LV scanning electron microscope (JEOL) under an accelerating voltage of 20 kV. The total adaxial leaf area was determined from the scanning electron microscopy leaf images captured at 6× magnification by using Adobe InDesign CS5 software (Supplemental Fig. S4). For cell size measurement, the total area (155 × 125 μm^2; under which the cells were counted for each leaf sample) was divided by the cell number obtained in that region. To count cells, images were captured at 600× magnification from four different locations of the same leaf, and the cells were manually counted by identifying the cell boundaries with the help of a marker on the printed images. Cells were counted from each leaf under a fixed area of 155 × 125 μm^2 (Supplemental Fig. S4). To obtain the total adaxial epidermal cell count, the entire adaxial leaf area was divided by the fixed area of 155 × 125 μm^2, and this number was multiplied with the cell count of the area of 155 × 125 μm^2. Ten to 15 biological replicates were used for the determination of the average number of adaxial epidermal cells on leaf 6.

Flow Cytometry to Determine Ploidy Distribution

The percentages of nuclei having 2×, 4×, and 8× ploidy were analyzed by flow cytometry with up to 20 biological replicates per hybrid or accession. The number of nuclei with various ploidy levels is represented by the area under their respective 2×, 4×, and 8× peaks (Supplemental Fig. S3). The average genome number per nucleus in hybrids and parents was analyzed using a slightly modified protocol of Dolezel et al. (2007). Leaf tissue (approximately

50 mg) and internal standard (tomato [*Solanum lycopersicum*]) were chopped with a razor blade in a petri dish containing 1 mL of ice-cold Otto solution I (0.1 M citric acid and 0.5% [v/v] Triton X-100; Sigma-Aldrich) kept at 4°C. The homogenate was mixed by pipetting and filtered through a 42-μm nylon mesh. The filtrate was centrifuged at 200*g* for 5 min to sediment the nuclei, and the pellet was resuspended in 40 μL of Otto I solution. The nuclei were stable in it for 2 d at 4°C, which enabled us to sample and analyze them later. For flow cytometry, we used the Cell Lab Quanta SC flow cytometer (Beckman Coulter International). We added 160 μL of Otto II solution (0.4 M $Na_2HPO_4 \cdot 12H_2O$, 0.02 μL mL^{-1} mercaptoethanol, and 5.48 μg mL^{-1} 4′,6-diamidino-2-phenylindole) to the nuclei using the flow cytometer robotics, and the flow cytometric measurements were recorded upon initialization.

Estimating Correction Factors for Calculating the Mutation Rates

Mutation rates were estimated by dividing the average number of GUS spots per plant by the copy number of the transgene (Kovalchuk et al., 2000). Because the number of cells and the average ploidy level per nucleus differ between hybrids and controls, the total genome number per plant will not be identical. Thus, the mutation rates were corrected based on the relative changes in the number of adaxial epidermal cells in the sixth leaf and leaf ploidy measurements, compared with a Col × Col control cross. The correction factor (titer) was derived as follows:

$$\text{titer} = (G_H \times C_H)/(G_C \times C_C)$$

where G_C = average ploidy per nucleus in Arabidopsis Col × Col F1 plants (control), G_H = average ploidy per nucleus in the hybrid, C_C = average number of adaxial epidermal cells in the sixth leaf of Col × Col Arabidopsis F1 plants (control), and C_H = average number of adaxial epidermal cells in the sixth leaf of the Arabidopsis hybrid.

$$\text{mutation rate} = \text{GUS}/\text{titer}$$

where GUS = average number of GUS spots per plant.

Statistical Analysis

In our experimental design, the measured phenotypes were compared between different hybrids (crosses). First, the number of adaxial epidermal cells per leaf was compared between crosses. Because these numbers of cells per leaf are count data, we chose a Poisson generalized linear model (GLM) with log link function (Nelder and Wedderburn, 1972). The linear predictors were the different hybrids. Furthermore, the cell sizes and the average number of ploidy per nucleus were compared using Gaussian GLMs with the log link and the same linear predictors. The Gaussian family with log link (and not a linear model with log-transformed response variable) was chosen, since the residuals showed an increased variability for larger fitted values.

In a second step, the previously described mutation rates between the crosses were compared using a Quasi-Poisson GLM (log link). The Quasi-Poisson GLM was chosen to account for overdispersion in the data. In contrast to the previous models, the linear predictors group the observations based on the usage of the transgenic line as female or male parent and the different hybrids. Since it is more likely to detect mutations in crosses with a high number of cells and a high average ploidy per nucleus, a correction factor was added in the models as a fixed intercept. The correction factor was calculated as log of the cell number times the average ploidy per leaf cell nucleus.

In all GLMs, the data from the reference group was used in several comparisons; for example, for the transgenic line 166, Col × Col (with line 166 as female parent) was compared against Col × C24 (with line 166 as female parent), Col × Cvi (with line 166 as female parent), and Col × Col (with line 166 as male parent). Thus, the same reference, namely Col × Col (with line 166 as female parent), was used three times, which requests a correction for multiple testing in order to maintain the family-wise error rate at 5% (Gabriel, 1969). Therefore, we adjusted the *P* values with a single-step method that considers the joint multivariate *t* distribution of the individual test statistics (Bretz et al., 2010).

The results are reported with the value of the *t* statistic and the corresponding (two-sided) *P* value adjusted for multiple comparisons. All statistical analysis was carried out in R (R Developmental Core Team, 2010). To adjust the *P* values for multiple testing, the R package multcomp was used with the test specification "single-step" (Bretz et al., 2010). Graphs were produced using the ggplot2 package (Wickham, 2009).

Supplemental Data

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

Supplemental Figure S1. Functional GUS reversion of a point mutation event resulting in a blue spot on the leaf of a 3-week-old Arabidopsis seedling.

Supplemental Figure S2. Average cell size in Arabidopsis hybrids and Col × Col control and total leaf surface area of the sixth true leaf of 3-week-old Arabidopsis parental and hybrid plants.

Supplemental Figure S3. Distribution of nuclear ploidy as analyzed by flow cytometry.

Supplemental Figure S4. Scanning electron microscopy images of the adaxial surface of the sixth true leaf in Arabidopsis hybrids and parents.

Supplemental Table S1. C→T transition events scored in Arabidopsis hybrids using the transgenic line $1390_{T\rightarrow C}$.

Supplemental Table S2. T→G transversion events scored in Arabidopsis hybrids using the transgenic line $166_{G\rightarrow T.}$

Supplemental Table S3. T→G transversion events scored in Arabidopsis hybrids using the transgenic line $112_{G\rightarrow T.}$

Supplemental Table S4. T→A transversion events scored in Arabidopsis hybrids using the transgenic line $118_{A\rightarrow T.}$

Supplemental Table S5. FS mutation events scored in Arabidopsis hybrids using the transgenic line G7.

Supplemental Table S6. FS mutation events scored in Arabidopsis hybrids using the transgenic line G10.

Supplemental Table S7. Recombination rates measured in Arabidopsis hybrids using the transgenic line R3m11L85.

Supplemental Table S8. Recombination rates measured in Arabidopsis hybrids using the transgenic line R3L66.

Supplemental Table S9. Recombination rates measured in Arabidopsis hybrids using the transgenic line R3L30.

Supplemental Table S10. Recombination rates measured in Arabidopsis hybrids using the transgenic line R2L1.

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