Parental Age Affects Somatic Mutation Rates in the Progeny of Flowering Plants[1]

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In humans, it is well known that the parental reproductive age has a strong influence on mutations transmitted to their progeny. Meiotic nondisjunction is known to increase in older mothers, and base substitutions tend to go up with paternal reproductive age. Hence, it is clear that the germinal mutation rates are a function of both maternal and paternal ages in humans. In contrast, it is unknown whether the parental reproductive age has an effect on somatic mutation rates in the progeny, because these are rare and difficult to detect. To address this question, we took advantage of the plant model system Arabidopsis (*Arabidopsis thaliana*), where mutation detector lines allow for an easy quantitation of somatic mutations, to test the effect of parental age on somatic mutation rates in the progeny. Although we found no significant effect of parental age on base substitutions, we found that frameshift mutations and transposition events increased in the progeny of older parents, an effect that is stronger through the maternal line. In contrast, intrachromosomal recombination events in the progeny decrease with the age of the parents in a parent-of-origin-dependent manner. Our results clearly show that parental reproductive age affects somatic mutation rates in the progeny and, thus, that some form of age-dependent information, which affects the frequency of double-strand breaks and possibly other processes involved in maintaining genome integrity, is transmitted through the gametes.

In humans, it has long been recognized that the reproductive age of the parents has an influence on the health of their progeny. An older reproductive age of the mother is known to increase the fraction of aneuploid gamete formation (Hurles, 2012). For instance, the risk for a trisomy increases from 2% to 3% for mothers in their 20s to more than 30% for mothers in their 40s (Hassold and Hunt, 2009). The age of the father also has an effect on the frequency of spontaneous congenital disorders and common complex diseases, such as autism and some cancers (Goriely and Wilkie, 2012). Indeed, sperm from 36- to 57-year-old men have more double-strand breaks (DSBs) than those of 20- to 35-year-old individuals (Singh et al., 2003). Similarly, the efficiency of DSB repair was reported to decrease with age in vegetative tissues of the plant model system Arabidopsis (*Arabidopsis thaliana*; Boyko et al., 2006).

Owing to the continuous divisions of spermatogonial stem cells, the male germline of humans is thought to be more mutagenic than the female germline. Indeed, it was shown that the paternal germline is more mutagenic than the maternal one with respect to base substitutions (Kong et al., 2012) and replication slippage errors at microsatellites (Sun et al., 2012). It is also known that carriers of germline mutations in mismatch repair (MMR) genes in humans are prone to get colorectal cancer and that the risk depends on the parent-of-origin of the mutation (van Vliet et al., 2011). The molecular basis of these parental effects is not entirely clear but is likely to involve higher rates of nondisjunction during female meiosis, higher mutation rates during spermatogenesis, and probably additional effects of aging.

In contrast to the effect of parental age on germline mutations, not much is known about potential effects of parental reproductive age on somatic mutation rates in the offspring. However, it has been shown in animal studies that radiation of males can lead to somatic mutations in their progeny—and subsequent generations—that cannot be attributed to mutations in the paternal germline (for review, see Little et al., 2013). Moreover, several recent studies have illustrated the existence of complex parental and transgenerational effects in humans, although their molecular basis is not clear (Grossniklaus et al., 2013). These effects can be of either genetic nature (but the effect is seen even in offspring that did not inherit the genetic variant from their parents; for review, see Nadeau, 2009) or epigenetic nature (where environmental influences can possibly exert effects on subsequent generations; for review, see Pembrey et al., 2006; Pembrey, 2010; Curley et al., 2011). It is currently not

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known whether such parental effects affect the somatic mutation rates in the offspring or whether the effects are modulated by parental age.

Taking advantage of the plant model system Arabidopsis, in which various somatic mutation rates can readily be assessed (Bashir et al., 2014), we investigated the effects of parental reproductive age on somatic mutation rates in the progeny. We report that there is a pronounced effect of parental age on somatic mutation rates in their offspring in a parent-of-origin-dependent fashion. Thus, some form of parental information, which is inherited through the gametes to the next generation, seems to alter the somatic mutation rates in the progeny and changes with parental reproductive age.

RESULTS

To study the effect of parental reproductive age on somatic mutation rates in the progeny, we made use of various Arabidopsis transgenic lines carrying mutated or fragmented versions of the *uidA* reporter gene encoding GUS (Liu and Crawford, 1998; Kovalchuk et al., 2000; Li et al., 2004; Azaiez et al., 2006; Van der Auwera et al., 2008). These mutation reporter lines carrying a nonfunctional *uidA* gene enabled us to score somatic frameshift mutations, base substitutions, intrachromosomal recombination (ICR), and transpositions events, which led to the formation of a functional *uidA* gene. GUS activity, reflecting a mutation event, can easily be monitored in planta using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (Jefferson et al., 1987), the product of which can be detected as blue spots (Supplemental Fig. S1). By scoring the number of these events in large plant populations, we calculated the rates of these various kinds of mutations (blue spots per plant). The age of the plant was counted from the day the seeds were plated on Murashige and Skoog (MS) medium for germination. Independent plants were manually self-pollinated or cross pollinated on four separate occasions representing different age groups.

Mutation Rate Estimation after Normalizing for Variation in Cell Number and Ploidy

Reciprocal C24 $\times$ Columbia-0 (Col-0) Arabidopsis hybrids were reported to have differences in the average cell size and cell number (Fujimoto et al., 2012). Also, hybridization has been shown to alter the cell number and average ploidy per nucleus (Bashir et al., 2014). Therefore, it is important to normalize mutation rates by factoring in cell number, cell size, and average ploidy per nucleus in the progenies derived from parents of different age. For mutation rate estimations, we considered four different parental ages (i.e. 38, 43, 48 and 53 d after sowing [DAS] on MS medium). We used the fourth true leaf (excluding the cotyledons) of Col-0 wild-type plants to measure cell size and cell number. Because leaf size is largely determined by the epidermis (Savaldi-Goldstein et al., 2007; Marcotrigiano, 2010), the number of cells on the adaxial epidermal surface of the leaves was counted in a specified area using scanning electron micrographs. We found no significant variation in cell size among the progenies derived from parents of different age (Fig. 1, A, B, and E). In contrast, the adaxial epidermal cell number decreased with parental age (Fig. 1F). The progeny of 53-DAS parents had a considerably lower cell number compared with those of 38-DAS self-fertilized parents ($P < 0.001$; Fig. 1F). Reciprocal crosses 38 $\times$ 48, 48 $\times$ 38, 43 $\times$ 53, and 53 $\times$ 43 DAS revealed a strong effect on leaf surface area, with progenies from older females having a lower cell number compared with those of younger females ($P < 0.001$; Fig. 1F). With the increase in parental and female reproductive age, we found the average leaf surface area to get smaller (Fig. 1, C, D, and F). The fourth leaves from 48 $\times$ 38 and 53 $\times$ 43 DAS had smaller surface areas compared with the fourth leaves from 38 $\times$ 48 and 43 $\times$ 53 DAS, respectively ($P < 0.001$), indicating that older females have a strong influence on the total leaf surface area of the progeny (Supplemental Fig. S2).

The calculation of mutation rates is based on the reversion of a single *uidA* gene in the genome. Endoreduplication occurs in 90% of angiosperm species (D'Amato, 1984), and it is known that the level of endoreduplication increases with age, such that the ploidy level is highest in the oldest plants (Melaragno et al., 1993). Because this could have an influence on their progeny, it is essential to normalize to the number of genomes per nucleus in the progenies derived from parents of different age. We observed no significant variation in the percentage of nuclei with 2$\times$, 4$\times$, and 8$\times$ ploidy levels among the progenies from crosses of parents at different ages (Fig. 2A), and there was no significant difference in the average ploidy per nucleus in the progenies of parents at different ages (Fig. 2B). Similarly, there was no significant difference in the average ploidy in progenies derived from older mothers (48 $\times$ 38 and 53 $\times$ 43 DAS) compared with that in progenies derived from younger ones (38 $\times$ 48 and 43 $\times$ 53 DAS; Fig. 2B).

Taken together, these data show a significant decrease in cell number in the progenies with both increasing parental age and female reproductive age. Accordingly, mutation rates were corrected for variation in genome number (Table I) by taking into account the average ploidy per nucleus and the cell number in the progenies derived from different parental ages.

Parental Age Increases Frameshift Mutation Rates in the Progeny

To assess the effect of parental reproductive age on frameshift mutation rates in the progeny, Col-0 plants containing an out-of-frame mononucleotide guanine repeat (G10) in the *uidA* reporter gene (Azaiez et al., 2006) were used for crosses between parents of different ages. Frameshift mutations occurring in this repeat can restore the function of the *uidA* gene because of the

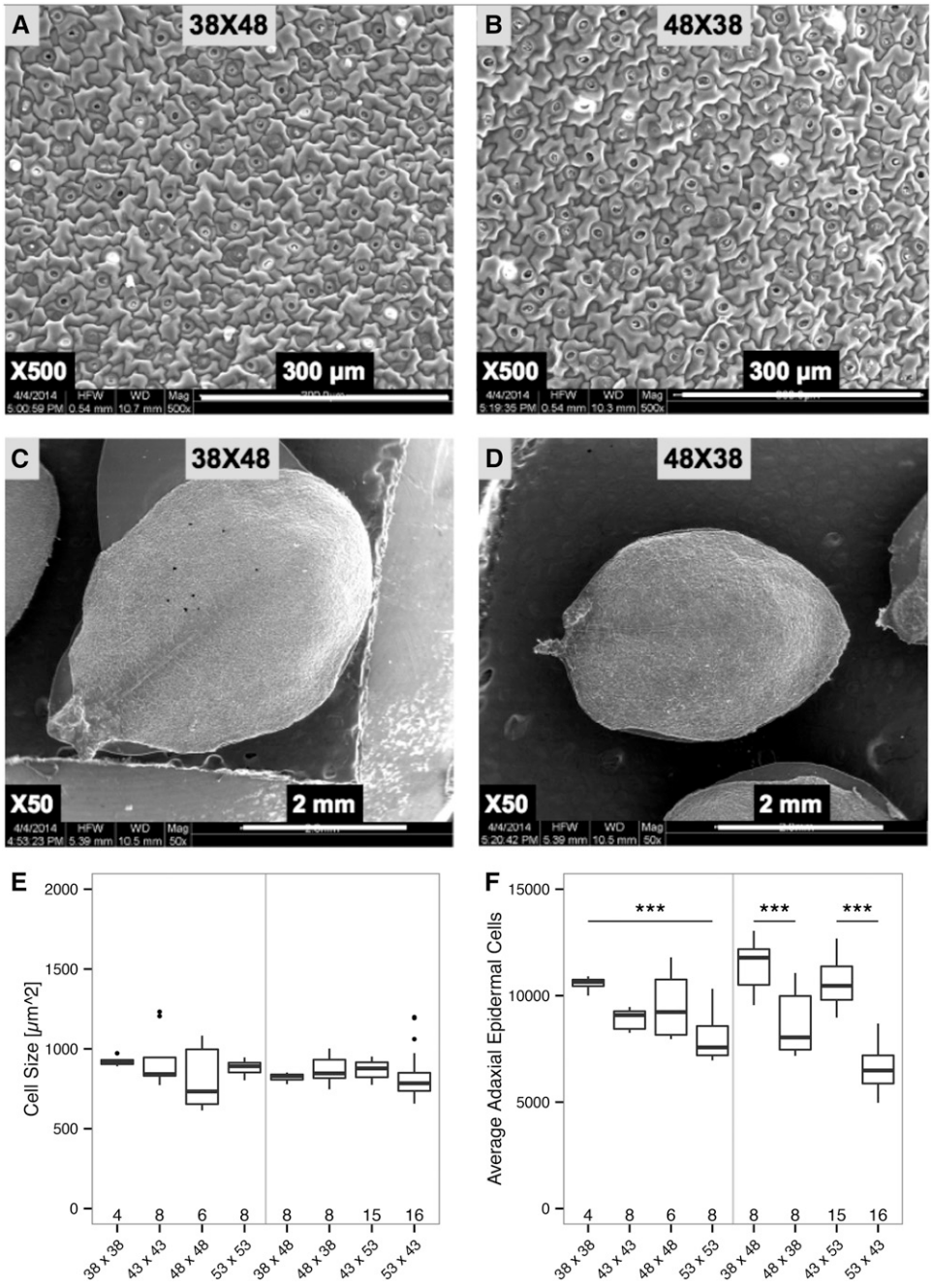

Figure 1. Cell size and cell number. A, B, and E, Cell size does not change with parental age. C, D, and F, Cell number decreases with parental and female reproductive age, resulting in a smaller leaf surface area. A to D, SEM images of the adaxial epidermal cells of the fourth true leaf of a 3-week-old Arabidopsis seedling. E and F, Box plots of cell size and the average number of adaxial epidermal cells of seedlings derived from parents of different ages. The numbers at the bottom of the graph show the biological replicates analyzed. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Materials and Methods" under the heading "Statistical Analysis." No asterisk indicates no significant difference. ***, $P < 0.001$.

addition or deletion of guanine bases. The crosses were carried out soon after the plants started flowering, but the very first set of flowers formed was not used, because they are often (partially) sterile. For the first pollinations, we used flowers from plants 35 to 38 DAS, and crosses were performed over a period of about 2 weeks.

As the parental age increased from 38 to 53 DAS, we observed a rise in the frequency of frameshift mutation events in the progeny ($P < 0.001$; Fig. 3). We would like to point out that the progenies of plants both 49 and 53 DAS displayed a significant increase in frameshift mutation events compared with the progenies of younger parents (38 and 43 DAS). Interestingly, in reciprocal crosses with plants of different ages (38 and 49 DAS), we observed a significant increase ($P < 0.001$) in frameshift mutation events with the age of the mother (Fig. 3). Similar results were obtained when the ages of the parents in reciprocal crosses were 53 and 43 DAS ($P < 0.001$).

These results clearly indicate that the frameshift mutation rate in the progeny goes up with the reproductive age of the parents and that the age of the female parent contributes more towards this increase.

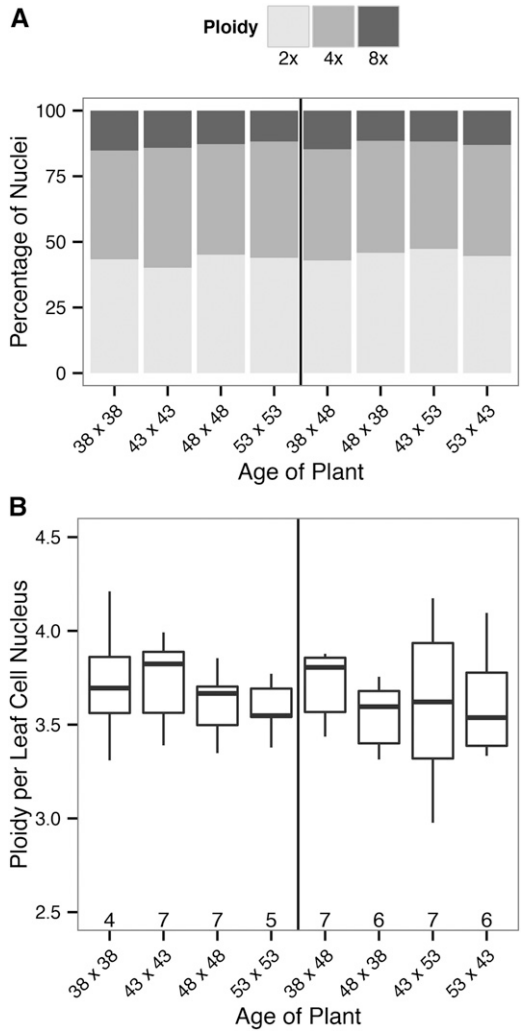

Figure 2. Differences in nuclear ploidy among the progenies from different age groups. A, Percentages of diploid, tetraploid, and octoploid nuclei in leaves; different shades of gray indicate different ploidy. B, Average ploidy per leaf cell nucleus. For each cross, a box plot of the average ploidy per leaf cell nucleus is shown. The numbers at the bottom of the graph show the biological replicates analyzed. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Materials and Methods" under the heading "Statistical Analysis." No asterisk indicates no significant difference.

Parental Reproductive Age Has No Effect on Base Substitution Rates in the Progeny

To study the effect of parental reproductive age on base substitution rates in the progeny, we used two transgenic lines that allowed us to score for T to G transversion and C to T transition mutations (Kovalchuk et al., 2000; Van der Auwera et al., 2008). In line $166_{G\rightarrow T}$, G is mutated to T at the 166th position, and in line $1{,}390_{T\rightarrow C}$, T is mutated to C at the 1,390th position in the open reading frame of the *uidA* gene.

As the age of the parents increased from 38 to 53 DAS, there was no significant difference in T to G transversion (Fig. 4A) or C to T transition rates (Fig. 4B). Also, we did not observe significant changes in base substitution rates in reciprocal crosses with parents aged 38 and 49 and 43 and 53 DAS (Fig. 4). Only a trend of somewhat higher C to T transition rates was observed as parental age increased from 38 to 53 DAS (38 versus 49 DAS, $P = 0.08$; 38 versus 53 DAS, $P = 0.06$; 43 DAS versus 53 DAS, $P = 0.07$; Fig. 4B). A trend for a difference in T to G transversions in reciprocal crosses with slightly higher mutation rates in progenies of older mothers was also observed (43×53 versus 53×43 DAS, $P = 0.06$; Fig. 4A).

In summary, we did not observe any significant changes in filial base substitution rates with increased parental reproductive age, although trends may indicate a slight increase with parental, particularly maternal, age.

Parental Reproductive Age Influences the Rate of ICR Events in the Progeny

To estimate the effect of ageing on somatic ICR frequencies in the progeny, we used transgenic lines R2L1 and R3L30 carrying two inverted catalase introns within the *uidA* gene. A recombination event within the identical sequences of the catalase introns generates a functional *uidA* gene, resulting in GUS activity that can be visualized as blue spots upon histochemical staining (Li et al., 2004).

Using line R2L1, which has a 418-bp inverted intron, ICR rates in the progeny were neither significantly affected by parental reproductive age nor significantly different in reciprocal crosses (Fig. 5A). However, using line R3L30, which has a longer inverted intron of 589 bp, the progeny of 53 DAS plants displayed a significant drop in ICR events compared with that in the progeny of plants 38 ($P < 0.001$), 43 ($P < 0.01$), and 48 DAS ($P < 0.01$; Fig. 5B). Interestingly, reciprocal crosses also showed a difference in ICR rates depending on the parental age. For instance, progeny of 53 and 48 DAS females crossed with 43 and 38 DAS males, respectively, showed a significant increase in ICR rates compared with the reciprocal crosses ($P < 0.001$ and $P < 0.05$, respectively; Fig. 5B). The discrepancy between the results obtained with the two lines may be caused by the different size of the homologous region or, more likely, by the genomic neighborhood of the insertion site, which may reduce the effects of age in line R2L1.

In summary, parental age seems to have a strong effect on ICR rates in the progeny, which may depend on the size of the inverted intron and/or position effects. Similar to what we observed for frameshift mutation rates, the age of the female parent has a stronger influence on the rate of ICR events in the progeny than that of the male parent.

Transposition Rates Increase in Seedlings Derived from Older Parents

To score transposition rates, we used plants carrying the endogenous transposable element *Tag1* inserted between the cauliflower mosaic virus 35S promoter and

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

Progenies of different parents 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 older parental ages (43, 48, and 53 DAS) compared with the youngest parental age (38 DAS), and for reciprocal crosses, the female with higher age (48 × 38 and 53 × 43 DAS) was compared with the female with younger age (38 × 48 and 43 × 53 DAS). 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* is the number of plants analyzed, and median is the median of measurements.

Age	Epidermal Cells				Ploidy per Nucleus				Correction Factor
	n	Median	CIQR	Relative No. of Cells	*n*	Median	CIQR	Relative Ploidy	
DAS									
38 × 38	4	10,645.798	0.028	1	4	3.695	0.081	1	1
43 × 43	8	9,086.439	0.091	0.854	7	3.824	0.085	1.035	0.884
48 × 48	6	9,229.38	0.282	0.867	7	3.666	0.056	0.992	0.86
53 × 53	8	7,568.795	0.182	0.711	5	3.548	0.043	0.96	0.683
38 × 48	8	11,781.776	0.142	1	7	3.805	0.076	1	1
48 × 38	8	8,039.422	0.313	0.682	6	3.596	0.078	0.945	0.644
43 × 53	15	10,463.692	0.15	1	7	3.621	0.17	1	1
53 × 43	16	6,485.826	0.204	0.62	6	3.537	0.11	0.977	0.606

the *uidA* gene, which renders it inactive (Liu and Crawford, 1998). Excision of the *Tag1* element allows expression of the *uidA* gene under the control of the 35*S* promoter, leading to blue spots in histochemical assays.

We observed a gradual increase of transposition rates in the progeny as parental reproductive age increased (e.g. the transposition rates increased in the offspring of 49 DAS [$P < 0.05$] and 54 DAS plants [$P < 0.001$] by 71% and 349%, respectively, compared with the progeny of younger parents [38 DAS]; Fig. 6). Offspring derived from reciprocal crosses involving parents of different age also showed a significant difference in transposition frequencies ($P < 0.001$).

In summary, these results show that the rate of *Tag1* transposition in the progeny increases with parental reproductive age, with a stronger effect through the maternal line.

Seedlings of Older Parents Show a Significant Increase in DSBs

To gain some insights into the molecular basis of altered somatic mutation frequencies in seedlings from parents of different ages, we analyzed whether the frequency of DSBs was affected. To this aim, we performed a neutral comet assay, which is a single-cell gel electrophoresis technique to quantify DNA damage. During electrophoresis, damaged DNA migrates differently from intact DNA and forms the tail of the comet-like structure. The extent of DNA damage can be estimated by the length of the comet tail and its fluorescence intensity relative to that of the comet's head (henceforth referred to as the percentage of tail DNA).

The neutral comet assay allows the detection of DSBs independent of the presence of single-strand breaks (Olive et al., 1991). Intact nuclei of seedlings derived from parents of different ages were isolated, and the comet assay was performed using a commercially available kit. With an increase in parental age, we found a significant increase in the percentage of tail DNA ($P < 0.05$), and this increase depended more on female than male reproductive age based on reciprocal crosses ($P < 0.001$; Fig. 7).

In summary, these results indicate that the frequency of DSBs increases in the progeny of older plants, with a more pronounced effect of the mother's age.

DISCUSSION

Parental age is a major determinant for chromosomal aberrations and other mutations in many organisms. How parental age affects mutation rates in their offspring,

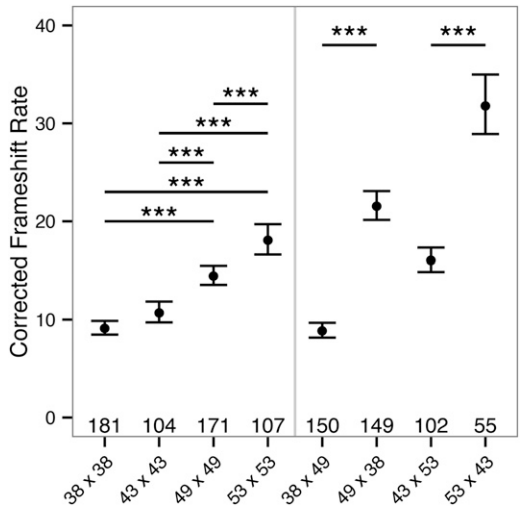

Figure 3. Frameshift mutation rates in Arabidopsis derived from line G10. Frameshift mutation frequency in the F1 progeny of self-fertilized and reciprocally crossed parents of different ages. Point predictions and 95% confidence intervals for frameshift mutation rates are shown. The numbers at the bottom of the graph indicate the numbers of seedlings analyzed. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Materials and Methods" under the heading "Statistical Analysis." No asterisk indicates no significant difference. ***, $P < 0.001$.

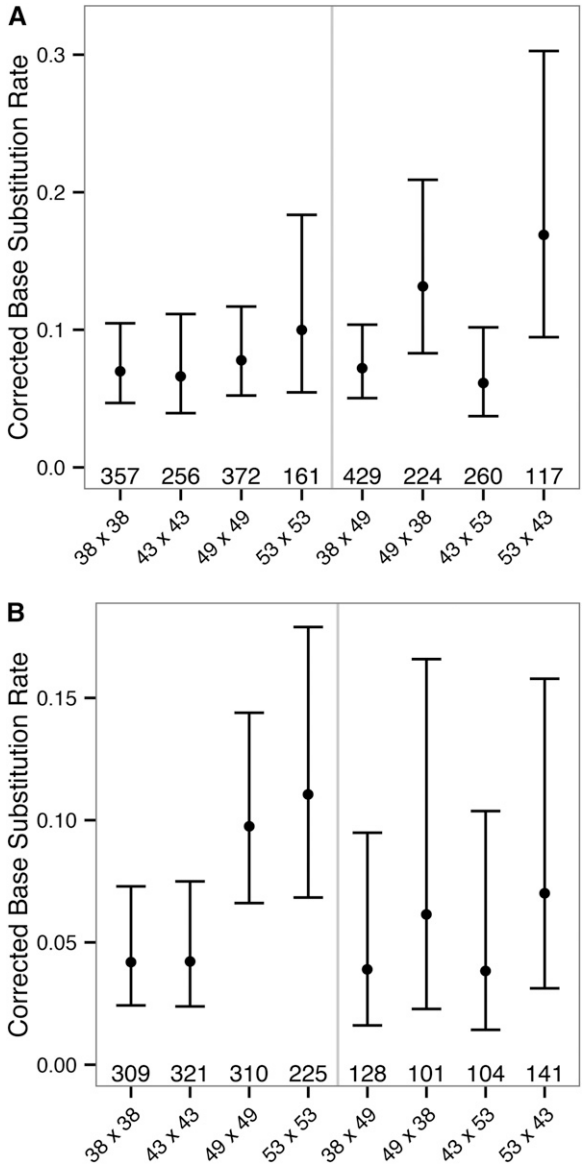

Figure 4. Base substitution rates in F1 progeny of self-fertilized and reciprocally crossed parents of different ages. A, Line $166_{G\rightarrow T}$. B, Line $1{,}390_{T\rightarrow C}$. Point predictions and 95% confidence intervals for base substitution rates are shown. The numbers at the bottom of the graph indicate the numbers of seedlings analyzed. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Materials and Methods" under the heading "Statistical Analysis." No asterisk indicates no significant difference.

however, has rarely been investigated. In plants, there is a chance that somatic mutations are transmitted to the next generation (Walbot and Evans, 2003), and if the frequencies of such spontaneous events are high from plants derived from older parents, this could give rise to increased variation in future generations. Thus, differences in reproductive age between populations could affect their adaptive potential.

Because the level of endoreduplication increases in many organs of both plants and animals with the age of the organism, it is important to take into account the ploidy level, cell size, and cell number (Table I) for a precise estimation of the mutation rates across progenies from parents of different age groups. We found that leaves become significantly smaller with increasing maternal age. Similarly, in mice, 3-month-old male progeny of middle-aged mothers are considerably smaller than those of young mothers (Wang and vom

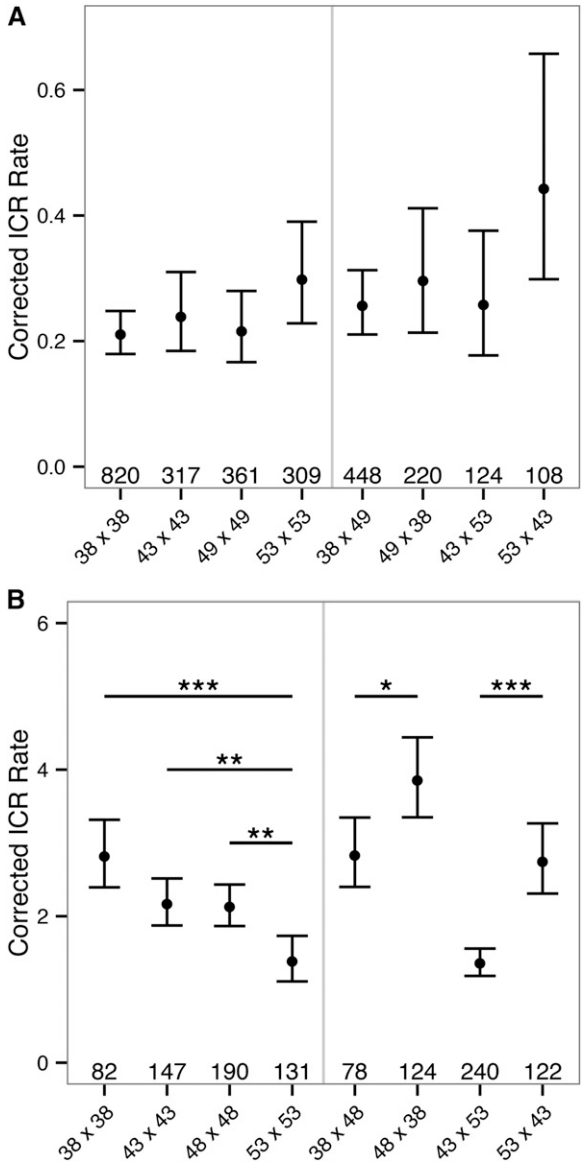

Figure 5. ICR frequencies. A, ICR frequency for line R2L1 in the F1 progeny of self-fertilized and reciprocally crossed parents of different ages. B, ICR frequency for line R3L30 in the F1 progeny of self-fertilized and reciprocally crossed parents of different ages. Point predictions and 95% confidence intervals for ICR rates are shown. The numbers at the bottom of the graph indicate the numbers of seedlings analyzed. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Materials and Methods" under the heading "Statistical Analysis." No asterisk indicates no significant difference. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

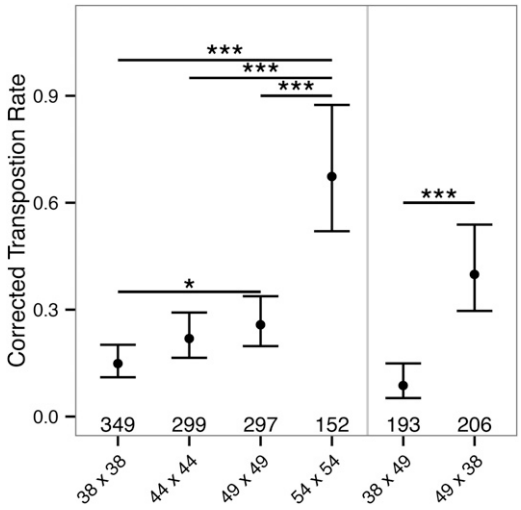

Figure 6. Transposition rates in Arabidopsis plants derived from the *Tag1* line. Transposition frequency in the F1 progeny of self-fertilized and reciprocally crossed parents of different ages. Point predictions and 95% confidence intervals for transposition rates are shown. The numbers at the bottom of the graph indicate the numbers of seedlings analyzed. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Materials and Methods" under the heading "Statistical Analysis." No asterisk indicates no significant difference. *, $P < 0.05$; ***, $P < 0.001$.

Saal, 2000). In fact, effects of maternal age on offspring size are widespread in plants and animals (Kindsvater and Otto, 2014 and refs. therein), but the underlying mechanisms vary and are often not well understood.

Our study shows that parental reproductive age affects the somatic mutation rates in a parent-of-origin-dependent fashion in the progeny. One possible explanation is that certain unknown bioactive compounds or epigenetic changes that accumulate with age are inherited to the progeny and affect somatic mutation rates. Previous work has revealed that DNA polymerase activity decreases in older plants (Bottomley, 1970; Golubov et al., 2010), and if this age-related down-regulation is epigenetically transmitted to the progeny, it may affect somatic mutation rates. Such a down-regulation of gene expression may result from the inheritance of DNA methylation patterns, histone modifications, or small RNAs that mediate gene silencing (Brennecke et al., 2008; Boyko and Kovalchuk, 2010).

Although highly variable and apparently very vulnerable to experimental conditions, effects on somatic ICR rates in the progeny of UV-irradiated or pathogen-infected Arabidopsis parents have been reported, indicating some kind of parental effect that is passed through the gametes to the next generation (Kovalchuk et al., 2003; Molinier et al., 2006; Pecinka et al., 2009). It was observed that this presumably stress-induced response was largely transmitted through the female parent (Boyko and Kovalchuk, 2010). We also saw that somatic mutation rates in the progeny increase with the age of the female parent (Figs. 3 and 5–7), which may indicate the involvement of similar processes. Alternatively, the pronounced maternal effects that we observed may be related to the maternal inheritance of mitochondria or plastids. Indeed, it has been proposed that mitochondrial impairments, which accumulate with age, are an important factor for aging in animals (for review, see Bereiter-Hahn, 2014).

An increase in the microsatellite instability rates is known to go up with increased plant age, and it was found that DNA polymerase activity decreases with increasing plant age (Golubov et al., 2010). Impaired activity of DNA polymerase fidelity, DNA polymerase proofreading, and MMR are potential sources of replication errors. Frameshift mutations can arise as a result of replication errors involving strand slippage (Martina et al., 2012). Our results show an increase of frameshift mutation events in the progeny with increasing parental age, with a stronger effect of female parental age (Fig. 3). These changes might be caused by age-dependent maternal effects on DNA polymerase fidelity, proofreading activity, or MMR efficiency in the progeny.

Although not significant, we observed a trend towards more C→T transitions with increasing parental reproductive age that was not observed for T→G transversions (Fig. 4). A possible increase of C→T base substitution events may be related to cytosine methylation, because methylcytosine is prone to deamination (Mugal and Ellegren, 2011). It is possible that this increase is caused by higher methylcytosine deamination

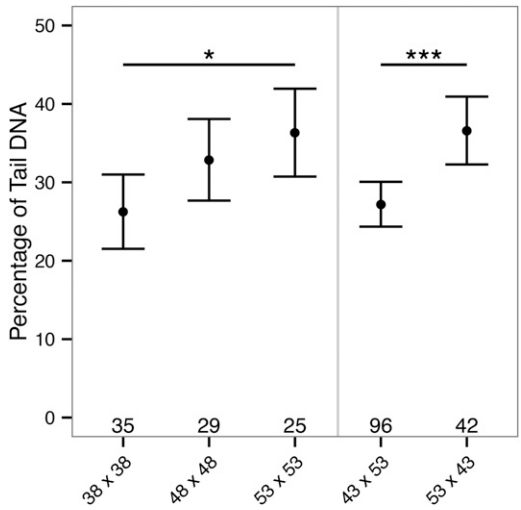

Figure 7. DSBs in seedlings derived from parents of different ages. Percentage of tail DNA was quantified using the neutral comet assay in the F1 progeny of self-fertilized and reciprocally crossed parents of different ages. Point predictions and 95% confidence intervals for the percentage of tail DNA are shown. The numbers at the bottom of the graph indicate the numbers of comets analyzed. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Materials and Methods" under the heading "Statistical Analysis." No asterisk indicates no significant difference. *, $P < 0.05$; ***, $P < 0.001$.

rates in the progeny of old parents or by a more heavily methylated genome in comparison with that of the progeny from young parents.

DSB repair involves homologous recombination and nonhomologous end joining (NHEJ). Homologous recombination repairs DSBs in the G2/M phase of the cell cycle, because it requires a sister chromatid, whereas NHEJ is predominant in the G1/S phase (Mao et al., 2012). Our results show a significant decrease of somatic ICR events in the progeny with increasing parental age (Fig. 5B). Previous studies have shown that somatic ICR decreases with Arabidopsis plant age, whereas NHEJ rates increase (Boyko et al., 2006), concomitant with elevated expression levels of Ku70, a protein involved in NHEJ (Golubov et al., 2010). It is, thus, possible that the state causing the decrease of ICR in old plants is transmitted to the next generation, which could also explain the increase in DSBs in the progeny of older parents (Fig. 7). It was shown that ICR rates increase with the length of the recombination intron substrate present in the *uidA* gene (Li et al., 2004). ICR rates are lower in transgenic line R2L1 carrying a 418-bp intron compared with line R3L30 having a 589-bp intron. This lower ICR rate might explain why we did not observe a significant decrease in R2L1. Alternatively, the ICR reporter construct may be inserted in a region of the genome that is less affected by parental age.

Transpositions are known to increase exponentially with an animal's age (Nikitin and Shmookler Reis, 1997). In *Caenorhabditis elegans*, an age-dependent increase of transposition rates has been observed, and in *Drosophila melanogaster*, an increase of transposition rates is associated with a decrease in lifespan (Egilmez and Shmookler Reis, 1994). Our results show a significant rise of transposition events in the progeny as the parental age increases, and this effect was more prominent with old mothers (Fig. 6). Thus, the effects on transposition are stronger through the female parent. It is possible that parentally inherited small RNAs are involved in controlling transposition events in the progeny (Mosher et al., 2009; Olmedo-Monfil et al., 2010; Autran et al., 2011; Calarco et al., 2012; Ibarra et al., 2012), but such parental effects have not yet been shown at the molecular level.

Although the application of high-throughput whole-genome sequencing technology has allowed the identification of de novo germline mutations in an unbiased manner (Ossowski et al., 2010; Campbell et al., 2012; Lee et al., 2012; Ness et al., 2012), this technology would not allow the detection of the extremely rare somatic mutation events occurring in a population of offspring, which we could analyze using tailor-made mutation detector lines. Whole-exome sequencing has been used to identify rare somatic mutations in endometrial tumors in humans (Le Gallo et al., 2012), which was only possible because they concentrated on limited portions of clonally propagated tumorous cells. Thus, our study presents a unique view on how parental reproductive age affects mutation rates in the progeny, which revealed clear parental effects, often in a parent-of-origin-dependent manner.

Interestingly, the effect of the maternal parent's age is usually more pronounced, which may be related to the manifold ways that the mother can potentially influence the next generation during seed development, for instance through the deposition of bioactive molecules in the gametes, hormonal signals, and nutrient provisioning. With increasing age, small organic molecules, RNAs, and/or proteins may not be deposited in the appropriate amounts in the gametes, having an impact on the somatic mutation rate in the progeny. The unequal deposition of bioactive molecules in sperm and egg cells may account for the parent-of-origin-dependent effects that we observed. Alternatively, the hereditary material itself may be affected by age, and this altered chromatin state could be transmitted to the progeny, thereby altering mutation rates directly and/or affecting the expression of genes involved in creating or repairing mutations. Finally, it is also possible that resource allocation to ovules decreases in older mothers, thus exerting an effect on seed development and, possibly, mutation rates in the progeny after germination. Indeed, leaf age has an impact on the nutrient translocation capacity as the CO_2 fixed per unit of organic matter, as well as the efficiency of transport in older leaves, is considerably lower than in younger ones (Shiroya et al., 1961; Silvius et al., 1978).

CONCLUSION

Little is known about the effect of parental reproductive age on somatic mutation rates in the progeny of any organism. In this study, we had four findings. (1) Frameshift mutations and transposition rates increase with parental age, with a stronger effect through the maternal line. (2) Although base substitution rates are not significantly affected by parental age, in general, C→T transitions are higher than T→G transversions. (3) The rate of ICR events may depend on the size of inverted intron in the transgene. If the intron size is small, the ICR rate does not change, but if the intron is large, the ICR rate decreases with parental age but increases when the female parent is older. The overall decrease of ICR events in the progeny of older parents may result in the higher occurrence of DSBs that we observed. (4) Uniformly, the female's reproductive age has a stronger effect than the male's reproductive age, implying that mutation rates in the progeny depend on a parent-of-origin effect.

MATERIALS AND METHODS

Plant Material

Base substitution detector lines $166_{G\rightarrow T}$ and $1{,}390_{T\rightarrow C}$ were gifts from Igor Kovalchuk and Anna Depicker, respectively (Kovalchuk et al., 2000; Van der Auwera et al., 2008). Transgenic ICR lines (R2L1 and R3L30) carrying inverted catalase introns in the *uidA* gene and the frameshift detector line (G10) were a gift from Francois Belzile (Li et al., 2004; Azaiez et al., 2006). The transposition detection line harboring the transposable *Tag1* element was a gift from Nigel M. Crawford (Liu and Crawford, 1998).

Plant Growth Conditions

Seeds were surface sterilized with 70% (v/v) ethanol followed by 0.5% (v/v) bleach treatment for 3 min. To remove traces of bleach, seeds were washed three times with sterile water and plated on autoclaved MS medium (with 3% [w/v] Suc), pH 5.7, containing 0.05% (v/v) Plant Preservative Mixture (Biogenuix Medsystem Pvt. Ltd.). Seed germination was synchronized by cold treatment of MS plates at 4°C for 48 h in the dark. MS plates were moved to plant growth chambers that had a uniform light intensity of 8,000 lux (under a 16-h-light/8-h-dark cycle). The temperature of the growth chamber (Percival CU-36L6) was maintained at 22°C throughout the course of experimentation, and the humidity inside the plant growth chambers was set to 80%. Two- to 3-week-old seedlings were transferred from MS plates to soil cups inside the growth chamber (Percival AR-36L3) to carry out manual cross pollination and manual self-pollination experiments. The soil mixture consisted of equal proportions of garden soil, peat, perlite, and vermiculite (Keltech Energies Ltd.).

Self-Pollination and Cross Pollination

For cross pollinations, flower buds before pollen maturation were emasculated; 48 h after emasculation, the stigmatic surface of each bud was checked for accidental deposition of pollen grains, and such buds were discarded; only pollen-free stigmas were used for pollinations. Pollen from flowers of the same age was used in self-pollinations, whereas pollen was put on the stigma of plants of a different age in cross pollinations. Different colored threads were used to mark emasculated and crossed buds of different age groups. Between 10 and 25 crosses were performed in three independent replicates. Crossing experiments were performed at 22°C, and seed material was harvested 16 d after pollination.

Histochemical Staining for GUS Activity

Blue spots visualized by an assay for GUS activity (Supplemental Fig. S1), reflecting base substitution reversions, ICR events, frameshift mutations, and transposition events, were counted under a light microscope (Leica KL300). Histochemical staining was performed with 2- to 3-week-old Arabidopsis (*Arabidopsis thaliana*) seedlings as described by Jefferson et al. (1987).

Ploidy Analysis by Flow Cytometry

Ploidy analysis was carried out following the protocol by Dolezel et al. (2007). Four to seven biological replicates were used to determine the percentage of nuclei with 2×, 4×, and 8× ploidy and the average number of genomes per nucleus in the progeny of parents with different parental ages; 60 mg of leaf tissue of a 3-week-old seedling was 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] Tween 20; Sigma-Aldrich) kept at 4°C. Tomato (*Solanum lycopersicum* 'Stupicke') was used as the internal control. 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 mL of Otto I solution. We added 160 mL of Otto II solution (0.4 M $Na_2HPO_4 \cdot 12H_2O$), treated the samples with 50 μg mL^{-1} RNase, and stained the nuclei with 50 μg mL^{-1} propidium iodide. After staining, the samples were analyzed using a BD FACSVerse Flow Cytometer (Becton, Dickinson and Company). Data analysis was carried out by FCS Express 4 Plus De Novo Software and BD FACSuite Software (Becton, Dickinson and Company).

Cell Size and Cell Number Analysis by Scanning Electron Microscopy

For scanning electron microscopy (SEM) of leaves, a wax impression of plant tissue was prepared according to the protocol of Beermann and Hülskamp (2010). We dissected the fourth true leaves (excluding the cotyledons) of 3-week-old Arabidopsis seedlings derived from parents of different ages and deposited onto them a waxy dental material to generate an impression (Coltene PRESIDENT Light Body; Coltene AG). After 5 min, when the wax had hardened, the leaves were gently removed. The negative mold of the leaf was filled with Spurr resin and left overnight for polymerization. The resin containing the leaf impression was removed carefully and coated with gold using a sputter coater (OC Oerlikon). Resins were mounted on SEM stubs with double-sided sticky tape, and the images were taken with FEI Quanta 200 SEM under 20 kV of voltage and 70 Pa of pressure. The total adaxial leaf surface area was analyzed using the SEM images captured at 50× magnification. To estimate cell size and cell number, images were taken at different positions of the leaf at 500× magnification, which corresponds to an area of 258,929 μm^2. The average cell size was estimated by dividing the number of cells observed in a constant area at 500× magnification at different positions of the leaf. To calculate the total number of adaxial epidermal cells, the total area of the leaf was divided by the constant area at 500× magnification and multiplied by the number of cells present in that area at 500× magnification. The number of cells and the total area of the leaf were analyzed by ImageJ; four to 16 biological replicates were taken to determine the average number of adaxial epidermal cells on the fourth true leaf.

Estimating Correction Factors to Calculate Mutation Rates

To calculate mutation rates, the average number of GUS spots per plant was divided by the copy number of the transgene (Kovalchuk et al., 2000). Because the number of cells and average ploidy per nucleus are not identical, the total genome number will not be the same among different progenies. Hence, mutation rates were corrected by considering the change in the number of adaxial epidermal cells of the fourth leaf. The ploidy per nucleus in 3-week-old seedlings (derived from parents 43, 48, and 53 DAS) was compared with that in seedlings derived from self-crossed individuals at 38 DAS. To study maternal age effects, progenies of young mothers (38 × 48 and 43 × 53 DAS) were compared with progenies derived from older mothers (48 × 38 and 53 × 43 DAS).

The correction factor was calculated as

$$\text{Titer} = (P_H \times C_H)/(P_Y \times C_Y)$$

where P_Y is the average ploidy per nucleus. Here, progenies derived from young parents (38 DAS) were used to calculate the differences in parental age. To assess the role of reciprocal crosses, seedlings from 38 × 48 and 43 × 53 DAS parents were used.

P_H is the average ploidy per nucleus in progenies derived from aged parents (43, 48, and 53 DAS for self-fertilization) and older maternal age (48 × 38 and 53 × 43 DAS).

C_Y is the average number of adaxial epidermal cells in the fourth leaf of progenies derived from young parents.

C_H is the average number of adaxial epidermal cells in the fourth leaf of progenies derived from older parents.

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

where GUS is the average number of GUS spots per plant.

In a few instances, parents were 1 d older than controls. For instance, for calculating transposition rates, the parental ages were 44 and 54 DAS; however, the correction factor was based on seedlings derived from parents 43 and 53 DAS, respectively. Similarly, the parental age of frameshift mutation (G10), ICR (R2L1), and base substitution lines was 49 DAS, but the correction factor was based on seedlings derived from parents 48 DAS. A difference of 1 d is much smaller than the age differences we compared, such that we expect only a negligible influence on the estimation of mutation rates.

Comet Assay to Study DSBs at Different Parental Age

With few modifications, a neutral comet assay was performed as per the protocol recommended by the manufacturer (Oxiselect Comet Assay Kit; Cell Biolabs, Inc.); 50 to 100 mg of tissue of 3-week-old Arabidopsis seedlings from different parental ages (38, 48, 53, 43 × 53, and 53 × 43 DAS) was chopped with a razor blade in 1 mL of Otto I solution. Subsequently, the sample was centrifuged at 300 rpm for 5 min, and the pellet was dissolved in 1× phosphate-buffered saline containing 20 mM EDTA. The dissolved sample was mixed with warm low-melting agarose in a ratio of 2:5 and poured onto a slide coated with agarose. A coverslip was placed on top of the sample mix, and the slide was stored at 4°C in the dark for 15 min horizontally. Then, slides were transferred to chilled lysis buffer for 30 to 60 min at 4°C in the dark. The slides were subsequently transferred to cold alkaline solution for 30 min at 4°C in the dark. The samples on the slide were treated with chilled Tris-borate/EDTA buffer for 5 min and transferred to a horizontal electrophoresis chamber containing chilled Tris-borate/EDTA buffer. Electrophoresis was carried out at 1 V cm^{-1} for 15 min. Thereafter, the slides were washed three times with deionized water and treated with 70% (v/v) ethanol for 5 min. After air drying, the sample was stained with vista green, and comets were observed using an upright fluorescent

microscope (Nikon Eclipse 80i) fitted with a fluorescein isothiocyanate filter. Comet analysis was carried using the Comet Assay IV Online Software.

Statistical Analysis

Between 150 and 500, 80 and 800, 60 and 200, and 150 and 400 plants of a population were analyzed to detect base substitutions, ICR events, frameshift mutations, and transpositions, respectively. Staining was performed in at least three replicates. The total number of plants analyzed per population is indicated in the figures.

The numbers of GUS spots are count data, which is why we chose a Quasi-Poisson generalized linear model (GLM) with the log link function (Nelder and Wedderburn, 1972). The Quasi-Poisson GLM was used to account for the overdispersion of the data. The linear predictors were the different age groups and their usage as the female or male parent. The log of the correction factor for cell number and ploidy per leaf cell nucleus was added in the models as a fixed intercept.

In all GLMs, the data from the groups were used for several comparisons. For example, in frameshift mutation line G10, 38 DAS was compared with 43, 48, and 53 DAS; 43 DAS was compared with 48 and 53 DAS; and 48 DAS was compared with 53 DAS. Thus, correction for multiple testing was done to maintain the familywise error rate at 5% (Gabriel, 1969). Therefore, we adjusted P values with a single-step method that considers the joint multivariate t distribution of the individual test statistic (Bretz et al., 2010).

The results are reported with the two-sided P value adjusted for multiple comparisons. All statistical analyses were 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 supplemental materials are available.

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

Supplemental Figure S2. Total leaf surface area in the progenies derived from parents of different ages.

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