Allopolyploidization Lays the Foundation for Evolution of Distinct Populations: Evidence From Analysis of Synthetic *Arabidopsis* Allohexaploids

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ABSTRACT Polyploidization is an important mechanism for introducing diversity into a population and promoting evolutionary change. It is believed that most, if not all, angiosperms have undergone whole genome duplication events in their evolutionary history, which has led to changes in genome structure, gene regulation, and chromosome maintenance. Previous studies have shown that polyploidy can coincide with meiotic abnormalities and somatic cytogenetic mosaics in *Arabidopsis* allotetraploids, but it is unclear whether this phenomenon can contribute to novel diversity or act as a mechanism for speciation. In this study we tested the hypothesis that mosaic aneuploidy contributes to the formation of incipient diversity in neoallopolyploids. We generated a population of synthesized *Arabidopsis* allohexaploids and monitored karyotypic and phenotypic variation in this population over the first seven generations. We found evidence of sibling line-specific chromosome number variations and rapidly diverging phenotypes between lines, including flowering time, leaf shape, and pollen viability. Karyotypes varied between sibling lines and between cells within the same tissues. Cytotypic variation correlates with phenotypic novelty, and, unlike in allotetraploids, remains a major genomic destabilizing factor for at least the first seven generations. While it is still unclear whether new stable aneuploid lines will arise from these populations, our data are consistent with the notion that somatic aneuploidy, especially in higher level allopolyploids, can act as an evolutionary relevant mechanism to induce rapid variation not only during the initial allopolyploidization process but also for several subsequent generations. This process may lay the genetic foundation for multiple, rather than just a single, new species.

POLYPLOIDY is a major force in shaping angiosperm evolution and plant biodiversity (Ohno 1970; Stebbins 1971; Hegarty and Hiscock 2008; Leitch and Leitch 2008; Buggs *et al.* 2011). Polyploids are organisms with two or more complete sets of chromosomes. Polyploidy is frequent in nature and evidence shows that most, if not all, angiosperms have undergone at least one ancient genome doubling event in their evolutionary history (Bowers *et al.* 2003; Blanc and Wolfe 2004; Cui *et al.* 2006; Soltis and Soltis 2009; Jiao *et al.* 2011). Two major forms of polyploidy exist: autopolyploidy, which describes multiple genomes derived from a single species, and allopolyploidy, which refers to species in which genome doubling occurred concomitantly with the hybridization of two or more species.

Newly formed allopolyploids (neoallopolyploids) are subject to multiple changes from their progenitors in response to genome duplication, including structural chromosomal change, aneuploidy, genome rearrangement, epigenetic remodeling, and transcriptional change (Madlung *et al.* 2002; Henry *et al.* 2005; Huettel *et al.* 2008; Lim *et al.* 2008; Wright *et al.* 2009; Salmon *et al.* 2010; Chen 2010). Plants are generally quite plastic and can tolerate variation in their cytological composition (Leitch and Leitch 2008), including stable aneuploidies, supernumerary- and B chromosomes (Grant 1971). For example, in the eudicot *Claytonia virginica*, aneuploid cytotypes have been described with chromosome number variations between 12 and 191, depending on the geographic location of the population (Lewis *et al.* 1967). One hypothesis for why aneuploidy is tolerated in plants with higher ploidy levels is that the high degree of chromosomal duplication acts as a buffer to effects that would be more deleterious in diploids (Stebbins 1971).

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The allopolyploid *Arabidopsis suecica*, which is derived from *A. thaliana* and *A. arenosa* (O'Kane *et al.* 1996), is an ideal model for studying polyploidy. In resynthesized *A. suecica* genetic and genomic changes are frequently deleterious to the neoallopolyploids in their early generations. Allopolyploidization in these plants frequently results in moderate meiotic instabilities and in reduced fertility and fecundity (Comai *et al.* 2000; Madlung *et al.* 2005). In resynthesized *A. suecica*, allopolyploidization is also associated with stochastic changes in gene transcription that correlate, at least in part, with epigenetic modifications (Wang *et al.* 2004, 2006b; Madlung *et al.* 2005). These responses can result in reduced embryo viability and create an evolutionary bottleneck effect (Comai *et al.* 2000). In *Tragopogon*, resynthesized allopolyploid siblings vary in morphology, fertility (Tate *et al.* 2009), and tissue-specific transcriptional patterning (Buggs *et al.* 2010), possibly providing novel material upon which natural selection can act. Allopolyploidization can also lead to rapid advantages to the new species. Work by Ni *et al.* 2009 demonstrated that epigenetic changes in circadian-mediated pathway genes led to increases in photosynthetic output and overall hybrid vigor in the F_7/F_8 generation of a resynthesized *A. suecica* line as compared to the progenitors.

Mitotic chromosomal abnormalities, including aneuploidies, have been reported in both resynthesized and natural *A. suecica*, suggesting that these instabilities can arise during the early stages of allopolyploid formation and persist during the establishment of the species (Wright *et al.* 2009). Natural accessions of *A. suecica* display stable phenotypic variability despite low genetic diversity among accessions (Madlung *et al.* 2012). Work in polyploids of *Tragopogon* showed that chromosomal aberrations, such as intergenomic translocations, and mono- or trisomies, were variable between populations (Lim *et al.* 2008; Chester *et al.* 2012). Given the mostly stochastic nature of genomic changes during allopolyploidization that lead to genomic variability in allopolyploid offspring, we hypothesized that allopolyploidy not only leads to the formation of a single new species but to many potentially different variants, effectively promoting instant radiation. To test this hypothesis in a higher level allopolyploid, we produced populations of an *Arabidopsis* allohexaploid and analyzed cytogenetic stability in several distinct lines over eight generations. Here we report much greater cytological instability in early generation allohexaploids than previously reported in allotetraploids, somatic karyotypic variability within individuals, incipient establishment of various cytological groups among the different sibling lines tested, and correlative changes in phenotypes. Together, our data support the notion that higher allopolyploidization can result in cytologically variable sister lines of the new allopolyploid species, provide the raw material on which natural selection can act, and possibly lay the foundation for the subsequent evolution of distinct populations.

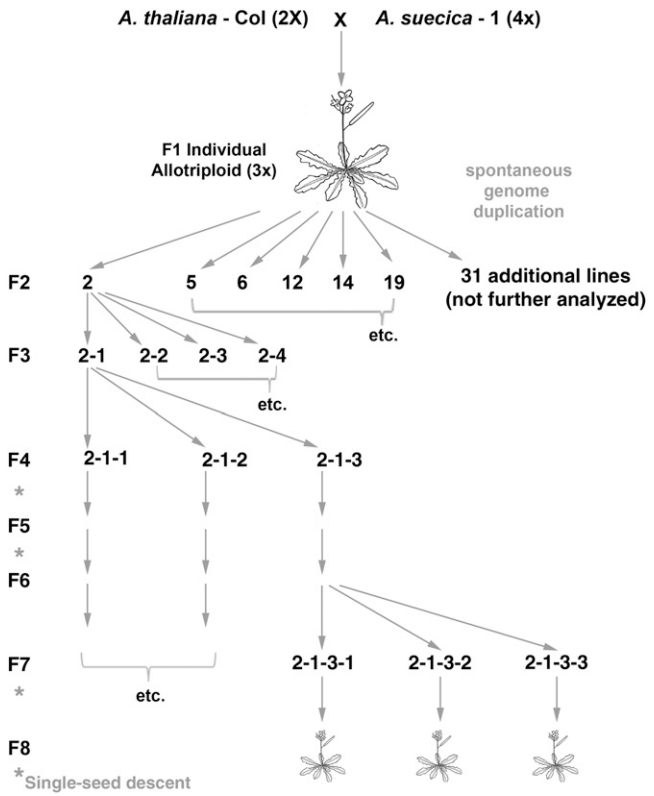

Figure 1 Pedigree and crossing scheme. Diploid Col *A. thaliana* was crossed with a tetraploid *A. suecica* to produce a triploid F_1 generation individual, which spontaneously duplicated its genome, generating a set of allohexaploid siblings. Siblings were selfed to yield six distinct lines (2, 5, 6, 12, 14, and 19).

Materials and Methods

Plant material

Arabidopsis allohexaploids were synthesized by crossing diploid *A. thaliana* ($2n = 2x = 10$), accession Columbia (*At*) as the maternal parent, with allotetraploid *A. suecica* (*As*, Sue-1, TAIR accession CS22505, $2n = 4x = 26$), as the paternal parent (Figure 1). One branch of the sterile triploid F_1 hybrid spontaneously duplicated its genome and produced 37 viable hexaploid seeds, which were used as single-seed founders of individual, separate lines numbered 1–37. Lines were propagated, seeds harvested, and per F_2 line one to several offspring were planted to produce the subsequent generation. Numbering followed the general scheme whereby the first number represents the original line. Additional numbers were used as needed to designate specific sibling lines. All offspring was self-fertile. All plants were grown in soilless peat mix. F_1 and F_2 plants were grown in a growth chamber at the University of Washington at 22° ± 3° under 16 h of light (TL80 fluorescent bulbs, Philips, Eindhoven, The Netherlands) and 8 h of darkness. F_3–F_6 and F_8 plants were grown in a greenhouse at the University of Puget Sound under supplemental metal halide lamps (PL Light Systems, Beamsville, Ontario, 140–180 μmol m^{-2} s^{-1}). The F_7 generation was grown at the University of Missouri

(Columbia, MO) first in a growth chamber (16/8 h light/dark cycle, 20°), and then in a greenhouse under natural daylight conditions (~16/8 light/dark) and temperatures ranging between ~20° and 30°. Seeds were not cold treated (vernalized) before germination. For FISH analysis both flower buds and root cells were collected. For the mitotic analysis, buds were harvested premeiotically. Later-stage flower buds were used for meiotic analysis. Root cell analysis was performed on root tips of 1-week-old seedlings grown on wet filter paper.

Flow cytometry

Nuclei were isolated from 4-week-old plants and stained with propidium iodide as described (Henry *et al.* 2005). The resuspended nuclei were spiked with 2.5 μl chicken erythrocyte nuclei (BioSure, Grass Valley, CA), which have a genome size of 1.05 billion bp (International Chicken Genome Sequencing Consortium 2004) per haploid genome. The nuclei were kept on ice in the dark for 2–4 hr and analyzed using a Becton-Dickinson FAC Scan (San Jose, CA). We collected 50,000 events per extract and the median fluorescence of each peak including the internal control was calculated using CellQuest software (Becton-Dickinson). Genome sizes were calculated and averaged for those preparations that were run in duplicate (N = 1–2).

Fluorescence in situ hybridization with CEN probes

Fluorescence in situ hybridization (FISH) was performed as described (Kato *et al.* 2004; Wright *et al.* 2009) with the following modifications: After fixing the tissue and before digestion with pectolyase and cellulase, premeiotic or meiotic floral bud tissue was washed twice with 100 μl of 1× citrate buffer (10 mM sodium citrate, 10 mM EDTA, pH 5.5). Root cells were digested using the same protocol, except that following enzymatic digestion, individual root tips were placed and directly squashed on the glass slide.

Probes for species-specific centromeric repeats were amplified from genomic DNA and labeled as described (Comai *et al.* 2003; Wright *et al.* 2009). Cells were randomly selected from FISH results that were interpretable with high confidence. In most cases, digestion had not fully removed the cytoplasm outlines of the cell, allowing us to exclude the possibility that chromosomes had been lost or gained from a cell spread during the slide preparation process producing artifacts. Cells were counted conservatively and cells with overlapping or uninterpretable signals were not counted.

From the original 37 F_2 plants six sibling lines were randomly selected for analysis. FISH was used to determine the number of chromosomes from the two parental species in all analyzed offspring plants. Centromeric repeats from *At* were painted with fluorescein and from *Aa* with Texas Red. Thus, the diploid *A. thaliana* parent had 10 green chromosomes, and the tetraploid *A. suecica* parent had 10 green chromosomes from its *A. thaliana* and 16 red chromosomes from its *A. arenosa* progenitor. In an allohexaploid cell with fully additive genomes there should thus be 20 green *At* and 16 red *Aa* chromosomes (hereafter referred to as 20/16).

Generations F_3–F_5 were analyzed with a Zeiss fluorescence microscope. Digital images were acquired with a MicroPublisher 3.3 digital camera and QCapure software 2.71 (both from Quantitative Imaging, Surrey, British Columbia, Canada). Whole images were cropped and linearly adjusted for contrast, brightness, and color using Adobe Photoshop 7.0.1 (Adobe Systems, San Jose, CA). The F_6 and F_7 generations were analyzed using an Olympus BX41 and an Olympus BX61 equipped with F-View Soft Imaging system and Microsuite Basic Edition (Olympus) software, which was used to adjust whole images for contrast and brightness.

Chromosomes were counted on the basis of the number of centromeric signals. Eighteen plants were analyzed in the F_3 and ~10–21 cells were analyzed for each plant, (n = 204 cells, average = 11 cells/plant). Of the F_6 generation, 18 plants were analyzed and 4–10 cells were counted in each plant (n = 171 cells, average = 5 cells/plant). Thirty-four plants were analyzed in the F_7 generation and 4–40 cells were counted from each plant (n = 650 cells, average = 19 cells/plant). For root analysis a Nikon Eclipse Ti confocal microscope was used fitted with EZ-C1 3.9 acquisition software and NIS Elements BR 3.1 imaging software for analysis.

Determining and characterizing aneuploidy

Two measurements were collected to assess the degree of aneuploidy and genome stability. The first measurement was the number of cells that did not contain the expected additive number of chromosomes contributed from the two parents. Because a large proportion of cells displayed aneuploidy, a second method of analysis was developed on the basis of the assumption that some lines or individuals had established new adjusted chromosome numbers that were different from the additive value of the parents (the "adjusted euploid" *vs.* the "original euploid" value). These numbers, thus, reflect the fact that in some lines of later generations, euploidy, as defined by the expected additive genomes of the parent species, was no longer the predominant state. Means of chromosome counts for individuals or for sister plants from a common line were compared either within or between generations. Statistical significance of difference in chromosome numbers was assigned using either ANOVA or a two-tailed Student *t*-test.

Phenotypic analysis

Rosette leaf formation, fecundity, flower formation, and plant height were recorded for allohexaploids of the F_7 and F_8 generations. Pollen viability was estimated by live/dead staining of pollen from individual flowers with acetocarmine (45% acetic acid, 0.5% carmine w/v). The six anthers were extracted from each flower, placed in 20 μl acetocarmine, and squashed to release the pollen. Pollen was stained for 5 min and then viewed under a dissecting microscope. Ten separate fields of view of each slide were analyzed for live (stained red) or dead (not stained) pollen. Pollen stainability was statistically compared between the hybrid plants on

the one hand and the combined parent species on the other hand, using an independent-samples Student *t*-test.

Transcriptional analysis

Reverse transcription PCR was performed as described previously (McCullough *et al.* 2010) with the exception that leaf RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA). Absence of genomic DNA in the c-DNA preparation was verified by using intron-spanning β-tubulin (Tub_P1: ATCCGT GAAGAGTACCCAGAT, Tub_P2: TCACCTTCTTCATCCGCA GTT) (Yu *et al.* 2002), and *FLOWERING LOCUS C* (*FLC*) primers (AtFLCF: GCTGATGATCTTAAAGCCTTGG, AtFLCR: ATCTGGCTAGCCAAAACCTGG). *FLC* primers amplified both copies of *FLC* from *A. arenosa* and the single copy from *A. thaliana* (Wang *et al.* 2006a). The amplicon also contained a *Cla*I restriction site for genotyping the parental origin of each transcript using CAPS analysis. This allowed the distinction between the single *A. thaliana*-, and the two *A. arenosa*-derived alleles. Treatment with *Cla*I resulted in two fragments of the *A. thaliana* allele, while the *A. arenosa*-derived *A. suecica* allele was not digested (Wang *et al.* 2006a). A total of eight F_8 or F_9 plants of equal age were sampled.

Statistical analysis

All statistical analyses were performed using SPSS versions 14 and 19, (SPSS, Chicago, IL), Microsoft Excel (Redmond, WA), and Graphpad (La Jolla, CA). Statistical significance was accepted at the $P < 0.05$ level.

Results

To verify ploidy levels in our experimental population, we measured the genome size of the triploid F_1 and hexaploid F_2 generations. Our genome size measurements of diploid *A. thaliana* (143 Mb) and 4*x A. suecica* (344 Mb) confirmed earlier measurements for *A. thaliana* (Davison *et al.* 2007) and *A. suecica* (Madlung *et al.* 2012). The F_1 individual's genome size (249 Mb) was as expected very close in size to the average of the two parental genomes (244 Mb), suggesting an additive triploid genome. Measurements for F_2 plants (Figure 2A) showed slight variations between sibling lines with an average of 547 Mb (± SEM 4 Mb), which was ~10% higher than would have been expected from a duplication of the F_1 genome. Microsatellite analysis with species-specific markers showed additive signals of both parents in the hybrid (data not shown). F_1 pollen FISH analysis of tetrads from the infertile branches of the F_1 plant showed irregular numbers of chromosomes and chromosome laggards between developing pollen cells (Figure 2, B and C).

Allohexaploids display frequent and sustained aneuploidy

To determine whether the genome of the neoallohexaploid was karyotypically uniform and stable, we analyzed cell spreads from the population for the occurrence of aneuploidy. Compared to the additive chromosome number (20/16), plants from the three generations analyzed displayed between 78 and 96% aneuploid cells per generation (Figures 3 and 4). Analysis of variance (ANOVA) suggested that the overall degree of aneuploidy between the F_3, F_6, and F_7 generations was not significantly different ($F = 2.722$, $P = 0.073$, d.f. = 2). Each plant exhibited different levels of aneuploidy that ranged from 18 to 100% although the majority of the plants (57 of 68 plants) exhibited >80% aneuploid cells in their premeiotic tissues (Supporting Information, Table S1). Importantly, somatic cell chromosome numbers were generally not constant within a plant but instead formed mosaics within the premeiotic tissue. We observed abnormally dividing mitotic, and in older buds rare meiotic cells (Figure S1). In these cells bridges and laggards can be seen between daughter cells, suggesting a mechanism for the observed occurrence of aneuploidy.

On the basis of the great amount of variation in chromosome numbers from cell to cell within individuals, one question was how the distribution of *At* and *Aa* chromosomes varied from plant to plant within sister lines, across lines, and across generations. To determine whether some lines lost chromosomes more frequently than others from generation to generation, and whether the chaotic chromosome constitution would stabilize over generations, we displayed our data as chromosome frequency charts for each individual analyzed (Figure S2), and determined from them the modal number of *At* and *Aa* chromosomes separately per cell per individual. This method of analysis assumed that each plant established a new adjusted chromosome number possibly distinct from the expected additive number (20/16), and we considered a cell as euploid (with respect to its new adjusted chromosome numbers) if it contained the modal numbers of both *At* and *Aa* chromosomes (Table S3; Figure S2). The modal chromosome numbers varied substantially between siblings of the same generation as well as between generations of the same line. For instance, some plants, especially in the F_7 generation, had cells in which the number of *At* and *Aa* chromosomes were reduced (hypoploid) to 16 and 15 chromosomes, respectively (Table S3). Conversely, several plants had chromosome numbers above (hyperploid) the original euploid number (Figure 3, B, C, and F; Table S3). Other variants of the same generation exhibited chromosome numbers that ranged from 12 to 17 *Aa* chromosomes and from 13 to 23 *At* chromosomes in each cell (Figure 3 and data not shown). After determining modal chromosome numbers for each plant, there were 11 different cytotypes in the F_3 and F_6 generations, and 18 different cytotypes in the F_7 generation (Table S3). Analysis of variance (ANOVA) based on the adjusted euploid values, suggested that the overall degree of aneuploidy was statistically significantly lower in the F_6 generation compared to both the F_3 and F_7 generations, but the F_3 and F_7 did not show a significant difference between each other ($F = 14.735$, $P < 0.001$, d.f. = 2) (Figure 4).

To determine whether somatic mosaic aneuploidy is unique to immature floral tissue or is pervasive throughout

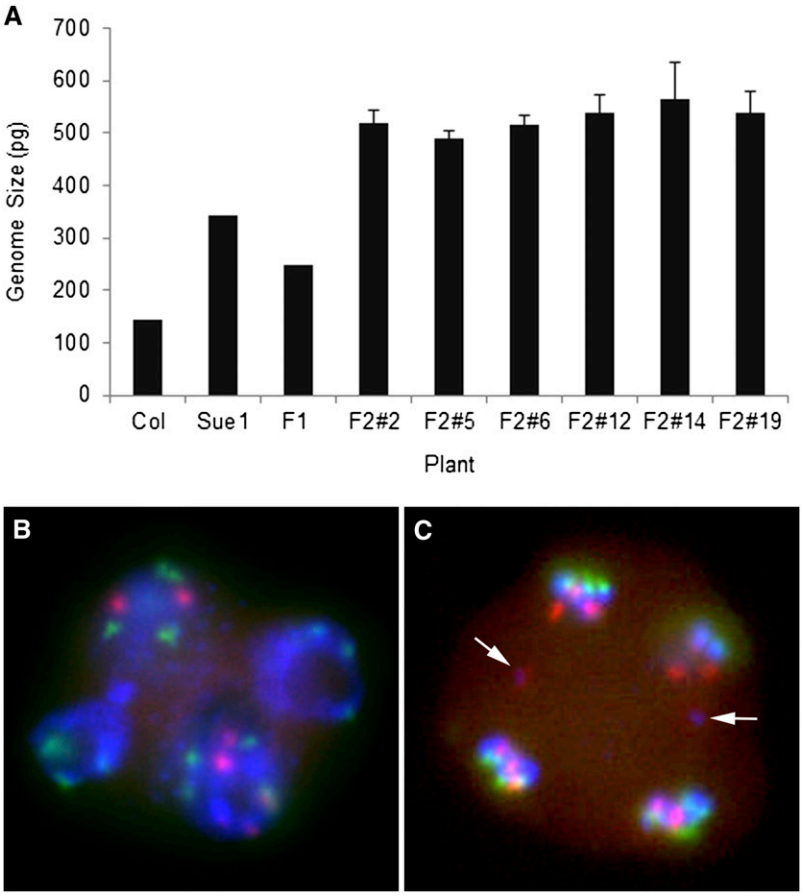

Figure 2 Genome size of parental species, triploid F_1, and allohexaploid F_2 individuals. (A) Genome sizes of 37 F_2 plants (6 of them are shown) were measured using flow cytometry. Error bars represent SE, $N = 1–2$. (B) FISH of F_1 pollen tetrad with irregular chromosome numbers. (C) Chromosome laggards (arrows) between developing tetrads of F_1 triploid. Chromosomes were labeled with *At*- (green) or *Aa* (red)-specific centromeric probes and counterstained with DAPI.

the plant, we performed root squashes in four sibling lines of the F_7 generation. All individuals tested showed mosaic aneuploidy, with chromosome numbers varying to a similar degree as seen in the premeiotic floral tissue (Figure S3).

To test whether chromosomes from one progenitor were more or less likely to contribute to aneuploidy, we compared chromosome loss or gain between chromosomes inherited from *At* or *Aa* origin. A majority of cells exhibited the original euploid number of 16 *Aa* chromosomes; however, proper inheritance of the 20 *At* chromosomes was much less common (Figure 5; Table S1; Table S2). Of the 1035 cells assayed, an average of 2.62 ± 0.07 (SE) *At* chromosomes were lost or gained while *Aa* loss/gain on average occurred at a rate of only 1.34 ± 0.05 (SE) chromosomes per cell. Using an unpaired *t*-test, we tested whether aneuploidy was preferentially caused by loss/gain of *At* chromosomes on the one hand, or loss/gain of *Aa* chromosomes on the other hand (Figure 5). We observed a statistically significant difference indicating that *At* chromosomes were more frequently the cause of aneuploidy than *Aa* chromosomes ($P < 0.001$; $t = 14.85$; d.f. = 2068).

To test whether cells were more likely to lose or gain additional chromosomes, we classified all analyzed cells as either hyperploid (containing >36 chromosomes) or hypoploid (containing <36 chromosomes) and compared the frequency of the two states. Cases of hyperploidy (regardless of the parental origin of the extra or missing chromosomes) were statistically significantly less frequent than cases of hypoploidy, as compared by an unpaired *t*-test (Figure 6, for raw data see Table S1; $P < 0.001$, $t = 14.8$, d.f. = 270). Of the 136 plants surveyed, 8% of cells showed simultaneous hyperploidy of both parental types of chromosomes, and 59% of cells showed simultaneous hypoploidy of both *At* and *Aa* chromosomes (Table S1). Some sister lines were more prone to hyperploidy. For example, line 6 frequently displayed *At* chromosome numbers up to 23 (Table S1). The complete FISH data set can be found in Table S4.

Cytogenetic stability of allohexaploids increases as generations progress

To assess the overall genetic stability of the allohexaploids in relation to their parent species, we compared the level of aneuploidy in each generation of the allohexaploids with that of its progenitors, *A. thaliana* and *A. suecica*. While diploid *A. thaliana* is very stable and does not display somatic aneuploidy, mosaic aneuploidy has been reported in premeiotic floral tissues for *A. suecica* at levels of 0–20%, depending on the assayed population (Wright *et al.* 2009). We compared the degree of aneuploidy (on the basis of

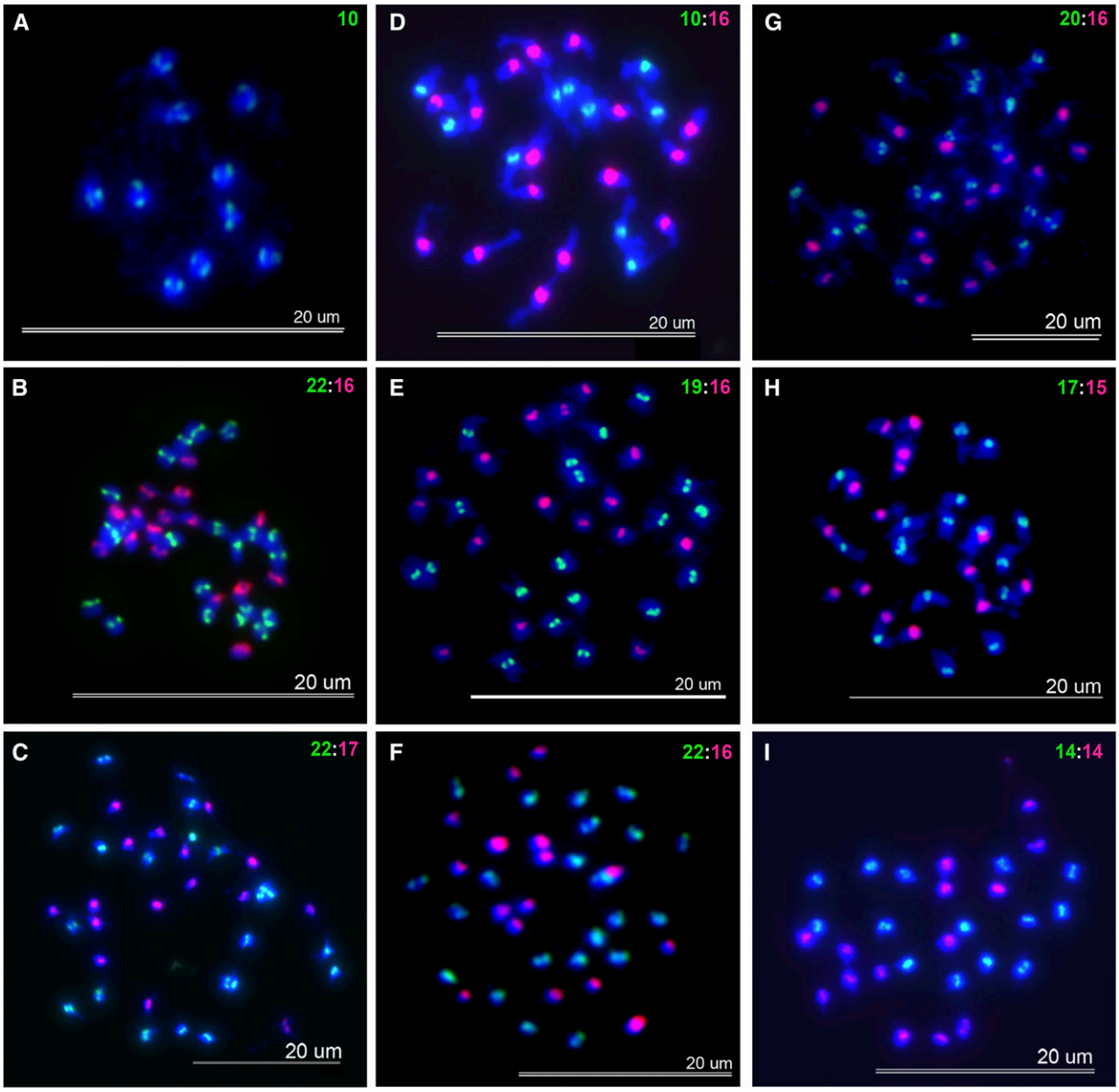

Figure 3 Mitotic chromosomes of somatic cells from parents and allohexaploids. Chromosomes were labeled with *At*- (green) or *Aa* (red)-specific centromeric probes and counterstained with DAPI (blue). (A) Maternal parent *A. thaliana*. (B) F_7 6-1-2-3. (C) F_7 6-1-2-3. (D) Paternal parent *A. suecica*. (E) F_6 19-1-9. (F) F_7 19-1-10-1. (G) F_3 14-8 (example of euploid cell). (H) F_6 14-4-4. (I) F_7 14-4-4-1. Green and red numbers represent the chromosome count of that particular cell.

adjusted euploid value) of the allohexaploids to the basal level of aneuploidy in these populations of *A. suecica*. As a conservative estimate, we assumed that any offspring of a cross between *A. suecica* and *At* could thus have an innate base level of aneuploidy of up to 20%. Any plant with >20% aneuploidy was considered to be more unstable than the progenitor species with respect to chromosome composition. While all of the F_3 plants ranged in their level of aneuploidy from 60 to 100%, the F_6 and F_7 generations included many plants with levels of aneuploidy between 0 and 60% (Figure 7). The F_6 generation exhibited the least amount of aneuploidy, making it the most genetically stable of the three generations.

Pollen stainability in allohexaploids is reduced

The fertility of 15 randomly selected F_7 allohexaploid plants was assessed by pollen staining with acetocarmine and compared to that of the parent species (*At* and *A. suecica*) (Table 1; Figure S4). In general, pollen stainability of the allohexaploids was lower than in the parent species. There was no obvious correlation between the degree of aneuploidy and pollen stainability, suggesting that chromosomal aberrations

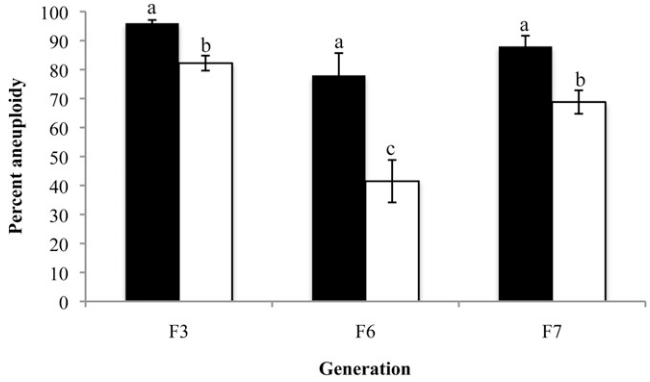

Figure 4 Somatic aneuploidy in individual cells of allohexaploids. The degree of aneuploidy was determined using FISH analysis (as in Figure 3) of plants from six sister lines over three generations. Aneuploidy was calculated on the basis of either divergence from the additive chromosome number (original euploid) of the two progenitors (solid bars) or adjusted modal (adjusted euploid) chromosome numbers (open bars) of each allohexaploid (see text for details). Error bars reflect the SEM. $N = 204$ cells (F_3), $N = 171$ cells (F_6), $N = 626$ cells (F_7). Means between generations were compared within each type of analysis (solid or open bars) using ANOVA tests followed by Tukey post hoc analysis. Bars not connected by the same letter are statistically significantly different at $P < 0.001$ (adjusted euploid). (Original euploid comparisons (solid bars) $P = 0.073$, $F = 2.72$, d.f. $= 2$; adjusted euploid comparisons (open bars): $P < 0.001$, $F = 14.74$, d.f. $= 2$.

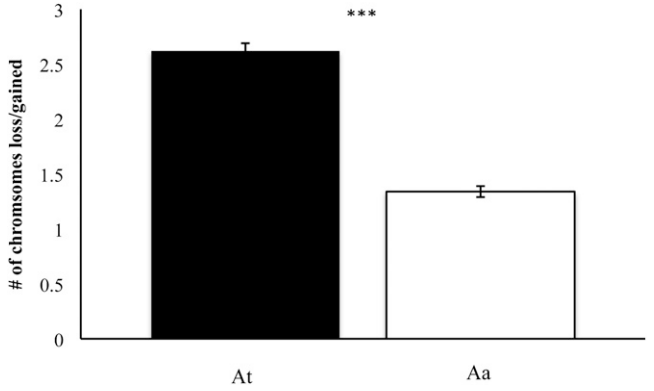

Figure 5 Most frequent origin of chromosomes lost or gained in aneuploid allohexaploids. The contribution to aneuploidy by *At* or *Aa* chromosomes was compared in the three investigated generations. Chromosomes from the *At* genome were more likely to be lost/gained than those from the *Aa* genome (error bars: SE; $N = 1035$ cells; $P < 0.001$, $t = 14.85$, d.f. $= 2068$). The difference was significant at $P < 0.05$.

were not directly linked to gamete viability. Interestingly, the allohexaploid with the greatest pollen stainability (F_6 5-1-6-2) also had sustained the greatest overall loss of chromosomes from the original euploid number. The other hexaploids displayed lower pollen stainability than did the parents. The combined averages of the pollen stainability of the hexaploid plants (25 ± 5.6%, SE; $N = 16$) on the one hand, and the combined parent species (71 ± 2.5%, SE, $N = 2$) on the other hand, were statistically significantly different using an independent-samples Student *t*-test ($P = 0.012$, $t = 2.84$, d.f. $= 16$). Individual values for stainability are reported in Table 1.

Although we did not quantify fecundity of the allohexaploids, visual observation of the amount of collected seeds showed a steady decline in some but not all sibling lines over the seven generations. All six original lines used in this study were still represented by germinating seeds in the F_8. However, when considering all 21 F_7 sibling sublines within these main six original lines (see pedigree in Figure 1), only eight produced germinating seed in the F_8 generation (data not shown).

Phenotypes of different allohexaploid lines vary substantially between sister lines

To assess whether there was any correlation between variation in the adjusted modal chromosome numbers and variation in novel phenotypes of the plants, we made visual inspections for plant height, flower size, leaf morphology, leaf size, leaf number, flowering time, and phenotype of the inflorescence. Flowering time varied widely between sister lines with some lines (lines 2 and 5) lagging behind a full generation. To determine whether the observed differences in flowering time between sibling allohexaploid lines were due to steady state levels of the floral inhibitor *FLC*, we performed RT–PCR analysis in eight individuals, of which six were flowering and two were not flowering at the time of analysis. *FLC* was expressed approximately equally in all tested plants, indicating that *FLC* expression was not likely to be the primary driving force behind the differences in flowering time (Figure S6). Digestion of the RT–PCR product with *Cla*I indicated that in all eight individuals, at least one *thaliana*- and one *arenosa/suecica*-derived allele was transcribed (data not shown).

Variation in leaf morphology was particularly pronounced. Some lines displayed smaller, more rounded leaves that resembled those of *A. thaliana*, others showed long, slender, and highly serrated leaves, more similar to *A. arenosa*, while others had intermediate serration and a slightly wider leaf body, typical for *A. suecica*. To assess whether variation increased within sister lines derived from a single original 6*x* seed, we compared leaf shape and growth habit in four F_7 sister lines of the original line 5 and one from line 14 (Figure 8). Flowering time varied between these lines by ~4 weeks between the earliest and the latest flowering line (Figure 8A). Leaf coloration varied from light green (5-1-1-5) to dark green (5-1-1-4). Phenotypes varied from slightly spinose, not quite entire leaf margins (lines 5-1-6-1 and 5-1-1-5) to pinnatifid (pinnately lobed) leaves in line 5-1-6-4, to leaves that were lobed on the proximal half and serrated on the distal half of the leaf (5-1-1-4) (Figure 8B). Leaf length among the more serrated leaves varied from short (14-3-3-1) to long (5-1-6-4). Cauline leaves were serrated in 5-1-6-4 but smooth in 5-1-6-1 (Figure 8A). Several individuals from separate sister lines displayed very long, spindly leaves early on in development (Figure 8, D and E) compared to the usually rounded and only slightly serrated leaves commonly found in the paternal

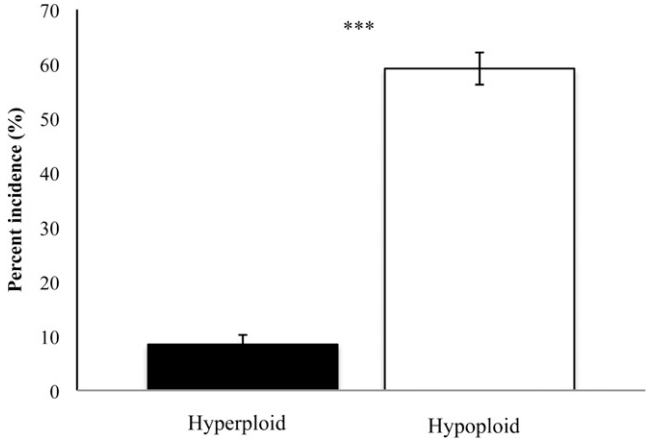

Figure 6 Incidence of hyperploidy and hypoploidy among all allohexaploid cells analyzed. The frequency of cells displaying chromosome numbers above the original additive chromosome number of 20/16 was significantly lower than for cells with fewer than 20/16 chromosomes in cells of allohexaploid plants ($P < 0.001$, $t = 14.8$, d.f. $= 270$).

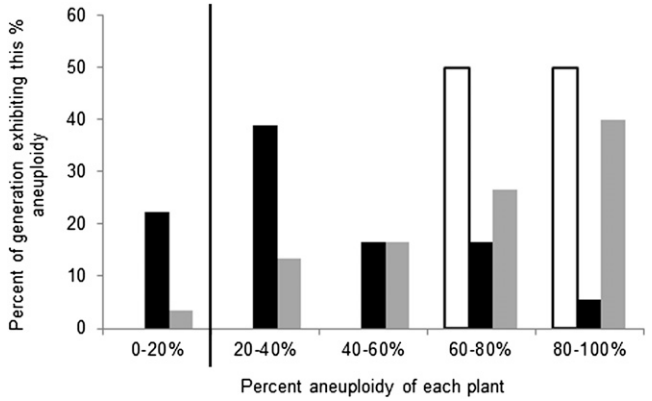

Figure 7 Frequency of plants with high levels of aneuploidy compared by generation. Multiple cells from each individual plant were assessed for their level of aneuploidy and grouped into five bins on the basis of the frequency of aneuploid cells in their tissue. Frequencies of plants belonging to each bin are graphed separately by generation. Open bars, F_3; solid bars, F_6; shaded bars, F_7. Previous results in natural *A. suecica* populations (Wright *et al.* 2009) display aneuploidy in 0–20% of cells. Therefore, as a conservative estimate of aneuploidy contributed by the paternal parent, bins with >20% of aneuploid cells are separated from the background noise aneuploidy by the vertical line.

parent of the allohexaploids (Figure 8C). Some of the plants with spindly leaves died at the early rosette stage (data not shown), while others developed into fertile adult plants (Figure 8, G and H), albeit with a later flowering time than most of their same-age cohort siblings (Figure 8F).

We correlated chromosome loss or gain with changes in leaf morphology. Table 2 shows that plants with a 20/16 cytotype have the most *A. suecica*-like phenotype with medium sized, oval, and slightly serrated leaves. An increase in *At* chromosomes seems to coincide with smaller, more rounded *A. thaliana*-like leaves, while a loss of chromosomes had several effects, including a variety of leaf shapes and sizes but no clear trend toward one specific phenotype. It must be noted that our analysis was not able to distinguish exactly which chromosomes were lost or gained and whether or not different lines with the same overall chromosome number had lost (or gained) the same or different chromosomes.

Aside from leaf phenotypic variation, overall growth habit and development varied widely (Figure S5). One plant (F_6 5-1-1-1) produced several flowers with five, rather than the normal four petals (Figure S5). We compared cells harvested from buds adjoining the flowers with the abnormal petal number to cells from a bud cluster on a separate side shoot of the same plant with normal petals. We found that buds neighboring the five-petaled flowers had an overall higher degree of aneuploidy (36%) than buds grown in proximity to the normal flowers (16%), but found no difference in their modal chromosome numbers (17 *At*/16 *Aa*; Table S2).

Discussion

Genetic diversity within a population allows natural selection, which promotes adaptation and evolution of a species (Seehausen 2004; Mallet 2007). Allopolyploidization leads to massive stochastic changes in gene activity and the genome landscape of the new allopolyploid species compared to its progenitors (Otto and Whitton 2000; Charlesworth and Wright 2001; Osborn *et al.* 2003; Adams and Wendel 2005; Comai 2005; Chen 2007, 2010; Soltis and Soltis 2009; Buggs *et al.* 2010, 2011). This sudden increase in novel variation lays the foundation for subsequent selection and evolutionary change. While molecular events shaping genomic change during and immediately after allopolyploidization have been studied intensely (Chen 2010), less

Table 1 Pollen viability of F_6 and F_7 allohexaploids and their parent species

Plant number	Viability (%)	No. of chromosomes		Total no. of chromosomes
		At	*Aa*	
Col 2*x*	68	10	—	10
Sue 1	75	10	16	26
F_6 5-1-1-1 5p[a]	34	17	16	33
F_6 5-1-1-1 4p[a]	34	17	16	33
F_6 5-1-6-1	44	16	16	32
F_6 5-1-6-2	85	13	14	27
F_7 6-1-2-2	15	21	15	36
F_7 6-1-2-3	5	21	16	37
F_7 6-1-2-4	62	20	16	36
F_7 6-1-8-1	7	16	16	32
F_7 6-1-8-3	13	21	16	37
F_7 6-1-8-4	20	23	16	39
F_7 6-1-9-3	20	20	16	36
F_7 12-19-1-1	12	18	16	34
F_7 12-19-1-2	12	17	16	33
F_7 12-19-1-4	25	19	16	35
F_7 12-19-1-5	15	19	15	34
F_7 14-4-4-1	2	16	15	31

[a] 5p, five-petaled inflorescence; 4p, four-petaled inflorescence on the same plant.

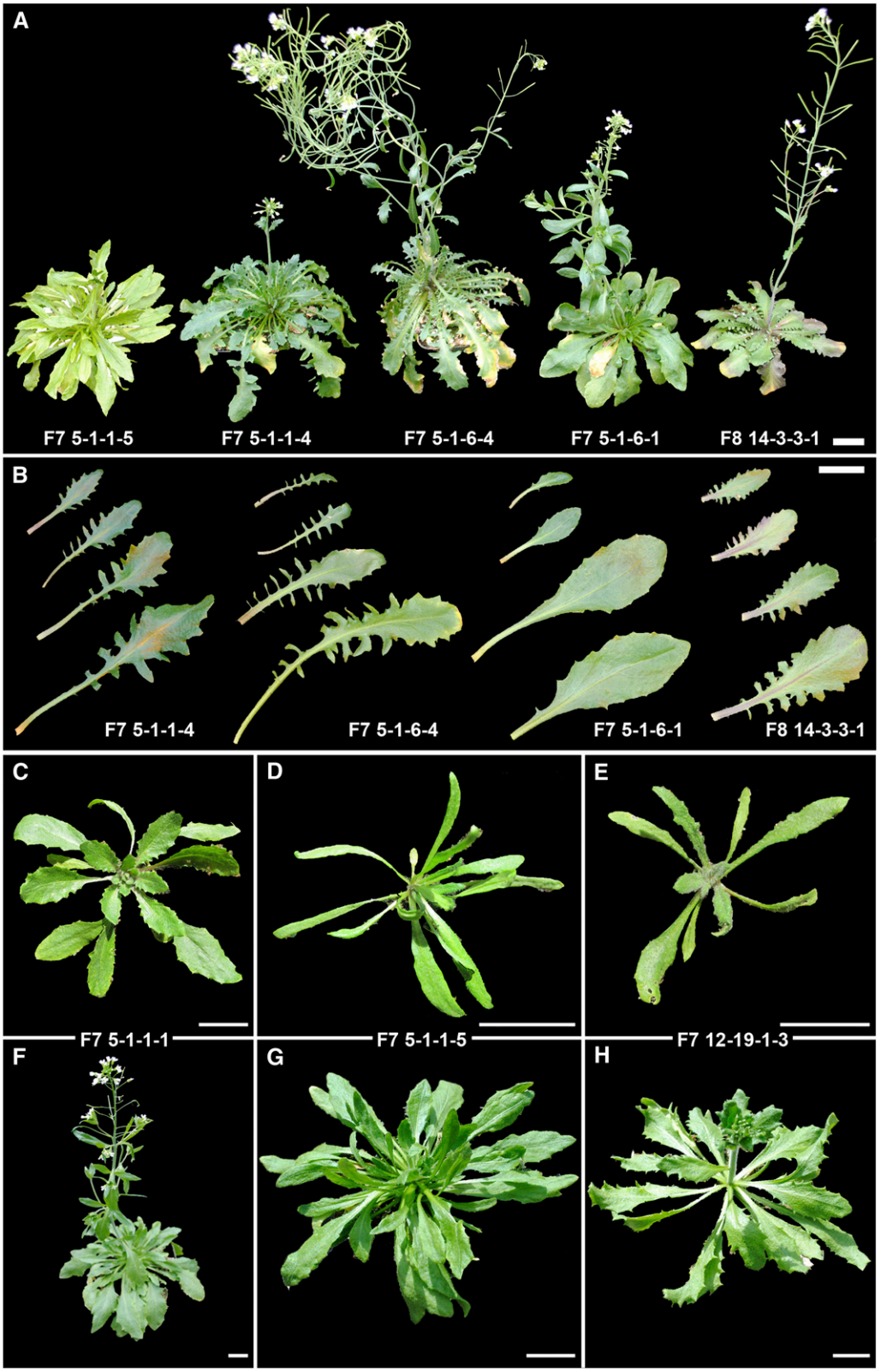

Figure 8 Phenotypes of select F_7 and F_8 allohexaploids. (A) Flowering times varied between F_7 siblings of the same line by up to 4 weeks. Note overall growth habit, light green foliage in individual 5-1-1-5, and variation in leaf morphology. For comparison with the most common leaf phenotype, an individual from line 14 is shown. (B) Variation in leaf serration between siblings of line 5 and an individual of line 14. (C–H) Developmental changes in three individuals between early rosette stage (~3 weeks old) and onset of flowering (C/F; D/G; E/H). Note the slender leaves in D and E compared to the more common leaf shape in C. All plants were grown in the same conditions, were of the same age, and were photographed at the same times. Bars, 2 cm.

experimental attention has been given to the question of whether allopolyploidization leads to the formation of a single new species or whether it can result in evolutionary radiation. In the example of the natural allotetraploid *A. suecica* genetic variation among populations is low (Lind-Hallden *et al.* 2002) and phenotypic differences between accessions are subtle (Madlung *et al.* 2012). By contrast, in resynthesized *A. suecica* genomic variation is frequent but newly

Table 2 Rosette leaf morphology of select F_6 and F_7 plants and their modal chromosome numbers

	Current modal chromosome nos.		Rosette leaf phenotype			
Plant no.[a]	*At*	*Aa*	Leaf size	Leaf edge	Additional	
F_7 6-1-8-4	23	16	Small, circular	Smooth	—	Smaller
F_7 6-1-8-3	21	16	Small, circular	Smooth	—	Circular
F_7 6-1-2-3	21	16	Medium, oval	Serrated	Round tips	Smooth
F_7 6-1-6-4	21	16	Medium, oval	Serrated	Curly stem	
F_7 6-1-2-2	21	15	Medium, oval	Slightly serrated	—	
F_7 19-1-10-1	20	16	Small, oval	Serrated	—	
F_7 6-1-6-2	20	16	Medium, oval	Slightly serrated	—	
F_7 6-1-2-4	20	16	Large, oval	Serrated	—	
F_7 6-1-2-1	18	17	Long, oval	Slightly serrated	—	
F_7 6-1-8-2	18	11	Small, oval	Serrated	Pointy tips	
F_7 12-19-1-1	18	16	Medium, thin	Serrated	Pointy tips	
F_6 5-1-1-1	17	16	Long, thin	Slightly serrated	Pointy tips	
F_6 5-1-6-1	16	16	Very small	—	—	
F_7 14-4-4-1	16	15	Long, very thin	Smooth	—	Longer
F_7 19-1-10-2	14	12	Short, oval	Serrated	—	Thinner
F_6 5-1-6-2	13	14	Very small	—	—	Serrated

[a] Plants are ordered from highest to lowest chromosome number.

formed allopolyploids also display poor fertility, fecundity, and viability (Comai *et al.* 2000), indicating that allopolyploidization presents a severe bottleneck. To our knowledge this is the first report using FISH in a controlled pedigree of resynthesized allopolyploids to address the question to what degree changes in karyotype concurrent with allopolyploidization provide the raw material for natural selection on other traits in a tractable, well-studied genetic system like *Arabidopsis*.

Somatic mosaicism and cytotypic variation is prevalent in early-generation allohexaploids

Synthetic allohexaploids of *A. thaliana* and *A. suecica* display extensive and lasting mosaic aneuploidy in somatic floral and root tissue. We observed frequent cytotypic variation between different lines, different siblings of the same line, and different cells of the same plant (Figure 3; Table S1). Variable numbers of chromosomes had previously been reported for several Finnish *A. suecica* accessions (Harmaja and Pellinen 1990) and somatic mosaics had been found in most natural and in one resynthesized *A. suecica* accessions (Wright *et al.* 2009). Chromosomal mosaics have been described in a number of polyploid plants such as synthetic hexaploid potatoes (Venkateswarlu and Krishna Rao 1969; Ramanna and Hermsen 1971), polyploids of various *Triticinae* (Sachs 1952; Pohlendt 1958), tobacco (Nuti Ronchi *et al.* 1981), and *Rubus* (Thompson 1962). The fact that somatic aneuploid mosaics have predominantly been reported in polyploids suggests that the additional homeologous chromosomes in polyploids provide the plant with added genomic flexibility. This increased genetic variation in the organism has been suggested as a mechanism for evolutionary change (Gill *et al.* 1995) but direct evidence for this hypothesis has been sparse. In human embryos, mosaic aneuploidy arising via nondisjunction of chromosomes during mitosis (Daphnis *et al.* 2005) or via premature centromere division (Furukawa *et al.* 2003) has mostly been associated with abnormal development.

When based on adjusted euploid chromosome numbers, the level of aneuploidy in our study fell statistically significantly from the F_3 to the F_6 generation, but that trend toward genomic stability was not continued in the F_7 generation, and levels of aneuploidy remained overall relatively high (Figure 4). On the basis of original chromosome numbers, all three generations showed continually high levels of aneuploidy (Figure 4). To assess changes of karyotype stability throughout the generations more closely, we compared each plant's adjusted aneuploidy level with the previously reported level of aneuploidy in the parent species *A. suecica*-1 (Wright *et al.* 2009). Any plant that exhibited aneuploidy levels higher than the highest observed levels in *A. suecica* accessions (20%) was considered genetically unstable. Under these analytical conditions, all plants of the F_3 generation were unstable, whereas the F_6 and F_7 generations contained plants that showed both higher and lower levels of aneuploidy (Figure 7). Judging from this analysis we conclude that there is an increase in relative karyotype stability in the later generations compared to F_3. However, we also note that stability was highest under this criterion in F_6, and somewhat lower in the F_7 generation. Our data are consistent with the notion that somatic aneuploidy and genome instability are greatest upon allopolyploidization, and this instability becomes less pronounced in subsequent generations. We note that the F_7 generation was grown in a different location from where the other generations were grown and where slightly different greenhouse conditions might have had an influence on plant development. Should growth conditions play a role in genomic stability, the observations for the F_7 generation might be viewed in this light. In any case, our experiments show that by the time these plants had reached the F_7 generation, there was still a considerable amount of cytogenetic instability present.

Stable (nonmosaic) aneuploidy can lead to a variety of consequences. Blakeslee *et al.* (1920) described phenotypic variances in all possible trisomics of *Datura* noting that these aneuploids were less vigorous than diploids but viable. The loss or gain of a single gene on a mono- or trisomic chromosome can either lead to a gene dosage effect where affected gene products show changes in expression levels, or gene expression levels can be unaffected as a consequence of dosage compensation (Birchler 2010). Extrapolating from these findings, it appears possible that in somatic mosaics like in the population investigated here, each cell has somewhat different gene expression levels, either showing gene dosage effects or dosage compensation.

It is interesting to note that *At* chromosomes were more frequently lost than *Aa* chromosomes in the allohexaploids (Figure 5; Table S1; Table S2). In tetraploid *A. suecica* we had previously found *Aa* chromosomes to be less stably transmitted than *At* chromosomes (Wright *et al.* 2009). The karyotypic constitution of the allohexaploids used in this study contains the equivalent chromosome complement of autotetraploid *A. thaliana* and diploid *A. arenosa*. It is possible that the greater instability of autotetraploid meiosis compared to that of diploid meiosis in *A. thaliana* (Wright *et al.* 2009) is the reason for the higher transmission instability of *At* chromosomes.

Cases of chromosome loss were more prevalent than cases of chromosome gain (Figure 6). A possible mechanism for aneuploidy in mitotic cells is nondisjunction during mitosis (Daphnis *et al.* 2005), which would explain both chromosome losses and gains. Additionally, the formation of laggards, as observed in this material (Figure S1), can account for chromosome loss during cell division (Thompson 1962; Nuti Ronchi *et al.* 1981; Ford *et al.* 1988).

Phenotypic variation

The various allohexaploid lines showed widely varying phenotypes (Figure 8). Some of these phenotypes were loosely correlated with the degree of aneuploidy observed (Table 2). Sublines (Figure 1) that died out during the first seven generations sometimes displayed obvious developmental abnormalities, such as dwarfism or the inability to flower (data not shown). Other unsuccessful lines produced less or mostly inviable (nonstainable) pollen (Table 1; Figure S4) or did not set seed (data not shown). It was surprisingly difficult to obtain high-quality meiotic chromosome spreads from this material, which is consistent with the notion that pollen formation was reduced in all except one line. In the absence of sufficient meiotic data, it is difficult to speculate on chromosome pairing behavior in these allohexaploids.

The most conspicuous difference in phenotype between sister lines was the time needed until flowering. Previous microarray analysis work with resynthesized allotetraploid *A. suecica* had shown increased levels of the floral repressor *FLC* in late-flowering allopolyploid material (Wang *et al.* 2006a,b). *FLC*-mediated flower repression is alleviated by cold treatment (vernalization) (Sheldon *et al.* 2008; Amasino and Michaels 2010). Some *A. thaliana* ecotypes have low levels of *FLC* even without vernalization (Sheldon *et al.* 2000), and in nonvernalized *A. thaliana FLC* expression can be found in floral structures (Sheldon *et al.* 2008). The ability of a plant to flower is not solely controlled by *FLC*, as several other pathways are also involved in the control of flowering (Amasino and Michaels 2010). In allopolyploid *Brassica* species, flowering time variation between populations is correlated with chromosomal rearrangements involving *FLC*-carrying chromosome segments and changes in *FLC* transcription (Pires *et al.* 2004). To begin to address the molecular basis of the observed variability in flowering time between early and late flowering lines in this hexaploid population, we tested transcriptional activity of *FLC* but found no significant expression differences (Figure S6). CAPS analysis showed that alleles from both original parents were transcriptionally active in all tested lines (data not shown). Since the plants in our experiments were not vernalized, *FLC* expression differences may not have played a decisive role in flowering time (Sheldon *et al.* 2008). Given the observed flowering time differences and the lack of evidence that *FLC* is directly responsible for the phenotypes, it appears that flowering time variation is regulated in a more complex manner in these allohexaploids than solely via *FLC* gene dosage differences. In allopolyploids of *Arabidopsis* genomic rearrangements have been described at only a few loci (Madlung *et al.* 2005) and no evidence has been reported for *bona fide* homeologous exchanges.

Despite the current lack of data explaining the underlying molecular reasons for phenotypic variation in the allohexaploid sibling lines, our analysis brings up several important points. First, we have shown that allopolyploidization in a cross between *A. thaliana* and *A. suecica* does not produce a single homogeneous population but leads to an aneuploid swarm that displays cytogenetic heterogeneity, phenotypic variation, and variability in individuals' fertility. In a relatively short period of time, lines have begun to separate from each other, displaying typical new chromosome numbers (Figures 4 and 7) and phenotypic characteristics (Figure 8; Figure S4; Figure S5). This novel variation incurred via allopolyploidy could thus represent the foundation for evolutionary radiation that may propel the new populations to produce many, rather than just a single new allopolyploid species. Second, our data show that neoallohexaploids, when compared to neoallotetraploids (Comai *et al.* 2000; Wright *et al.* 2009) produced from the same progenitor genomes, display a much greater degree of somatic aneuploid mosaicism and cytogenetic variability. This mosaicism is systemic and found both in root and shoot tissues (Figure 3; Figure S1; Figure S3). While phenotypic diversity is subtle in allotetraploid *A. suecica* (Comai *et al.* 2000; Madlung *et al.* 2012), it is more pronounced in allohexaploids, possibly due to the fact that the greater number of homeologs in the genome allows a greater degree of flexibility in genome reshuffling. Third, our study suggests that allopolyploidization may not be a singular bottleneck event incurred only during the

first meiosis, in which the genome is rearranged, and which is followed by slow genomic recovery (Cifuentes *et al.* 2010). Instead, at least in allohexaploids of *Arabidopsis*, somatic aneuploidy appears to promote the reorganization of the genome in somatic cells for at least seven generations.

Despite their differences in cytotypic make up and physical appearance, most of these lines appear to be genomically and phenotypically still unstable. We cannot predict from the material at its current state if all or any of these lines will be able to survive and stabilize. Phenotypic variation, observed in tetraploid resynthesized *A. suecica* allopolyploids (Comai *et al.* 2000), was able to give rise to several stable, vigorous lines (Ni *et al.* 2009). On the other hand, previous work with 50 resynthesized allotetraploid *Brassica napus* lines showed over a period of 10 generations that these plants became less, rather than more stable. However, in this case the instability was likely due to homeologous transpositions (Gaeta *et al.* 2007; Gaeta and Pires 2010), and homeologous chromosome replacement (Xiong *et al.* 2011), but not to aneuploidy.

While we did not attempt in this study to assign specific chromosome losses or gains to corresponding phenotypes, it is intriguing to speculate on such relationships. We are currently testing BAC FISH markers that would allow us in the future not only to distinguish aneuploid cells, but to assign complete karyotypes to each cell. This might then allow the correlation of dosage effects of specific chromosomes with observed phenotypes.

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Allopolyploidization Lays the Foundation for Evolution of Distinct Populations: Evidence From Analysis of Synthetic *Arabidopsis* Allohexaploids

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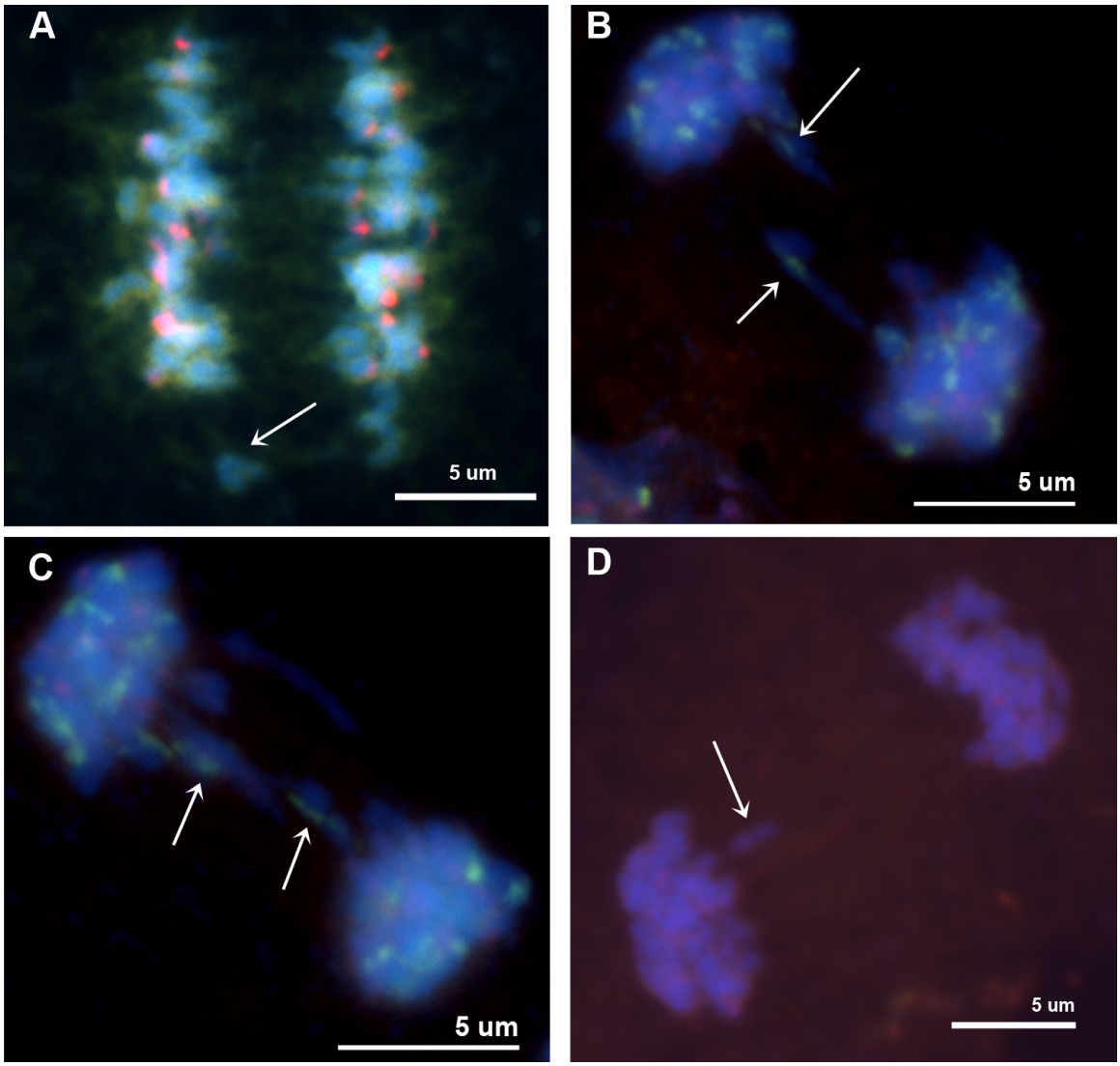

Figure S1 Laggards and bridges in meiotic (A) and mitotic (B-D) cells of allohexaploids. Cells were prepared for FISH as described in the methods and centromeres labeled with fluorescein (green, *At*) or Texas Red (red, *Aa*). Arrows point to bridges or laggards between dividing cells. A: F9 12-3-5-2, B: F8 2-8-5-3, C: F8 2-8-5-3, D: F8 2-8-5-3.

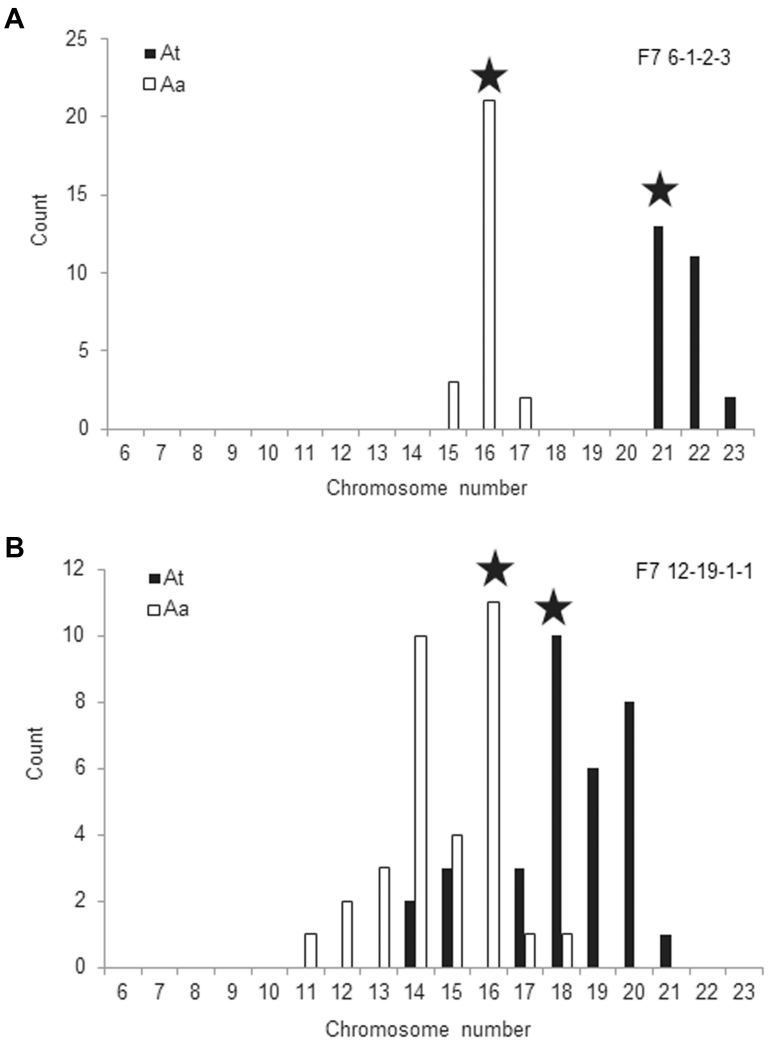

Figure S2 Somatic mosaicism in F7 *Arabidopsis* allohexaploids. Two examples illustrating the adjusted euploid number in two individuals (12-19-1-1 and 6-1-2-3) from separate sibling lines. N= 33 analyzed cells (A), N= 26 analyzed cells (B). The modal chromosome numbers ("adjusted euploid" values) are denoted by stars for each parental genome. Original euploid chromosome numbers (additive from the progenitors) were 20/16.

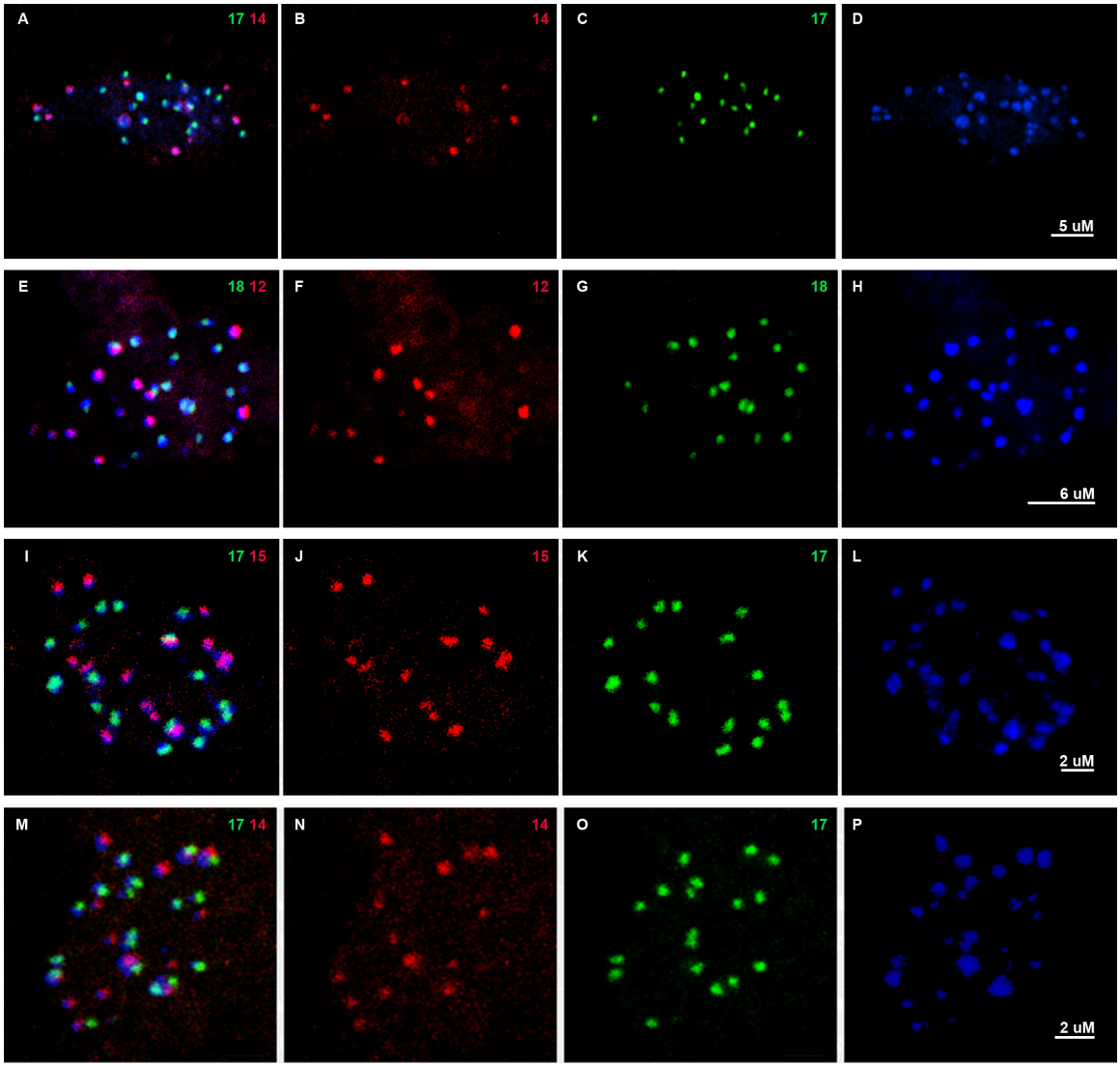

Figure S3 Mitotic chromosomes in root tip cells of allohexaploids. FISH was performed as described in Fig. 3. Composites as well as separate Texas Red, Fluorescein, and DAPI channels are shown for 4 examples A-D: F6 6-1-8, E-H: F7 6-1-9. I-L: F7 19-1-9-1. M-P F7 19-1-9-1.. Numbers in top right corner of each panel indicate the chromosome number of that particular cell (green: *At* chromosomes; red: *Aa* chromosomes). In panel O the *At* chromosome number maybe 17 or 18, but either case indicates hyperploidy.

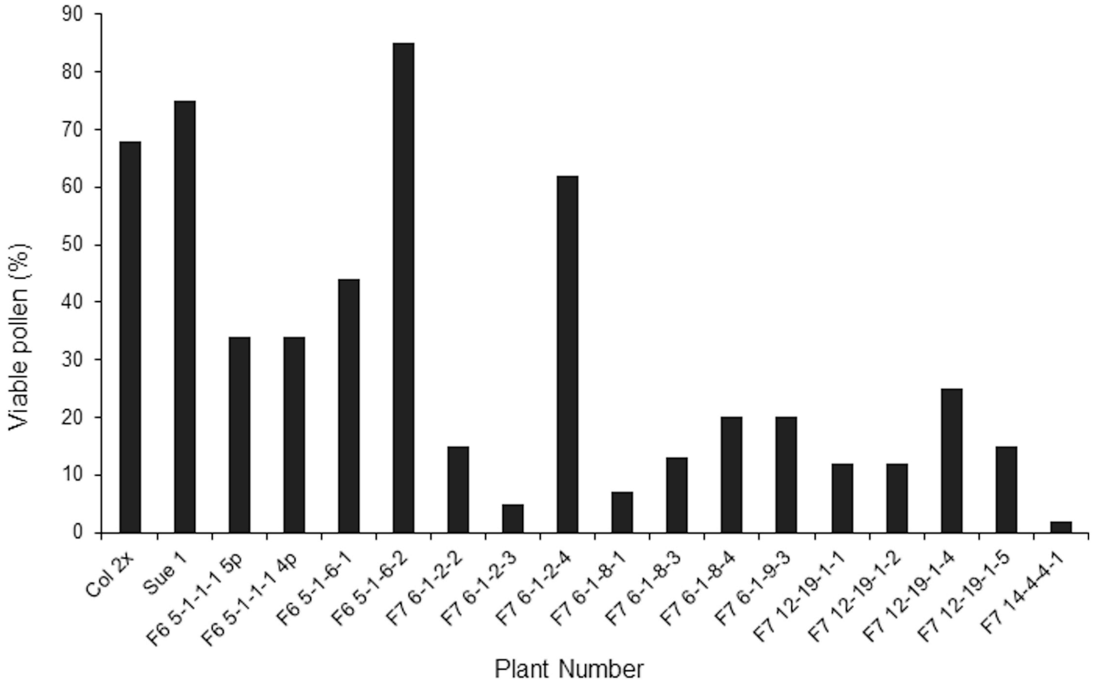

Figure S4 Pollen viability in allohexaploids and progenitors. Live/dead staining of pollen was performed using acetocarmine. The average percent viability for the parent species was 71±2.5%, (SE, N=2) while the overall average for allohexaploids was 25±5.6% (SE, N=16), which was statistically significantly different (p=0.012, t=2.84, df=16).

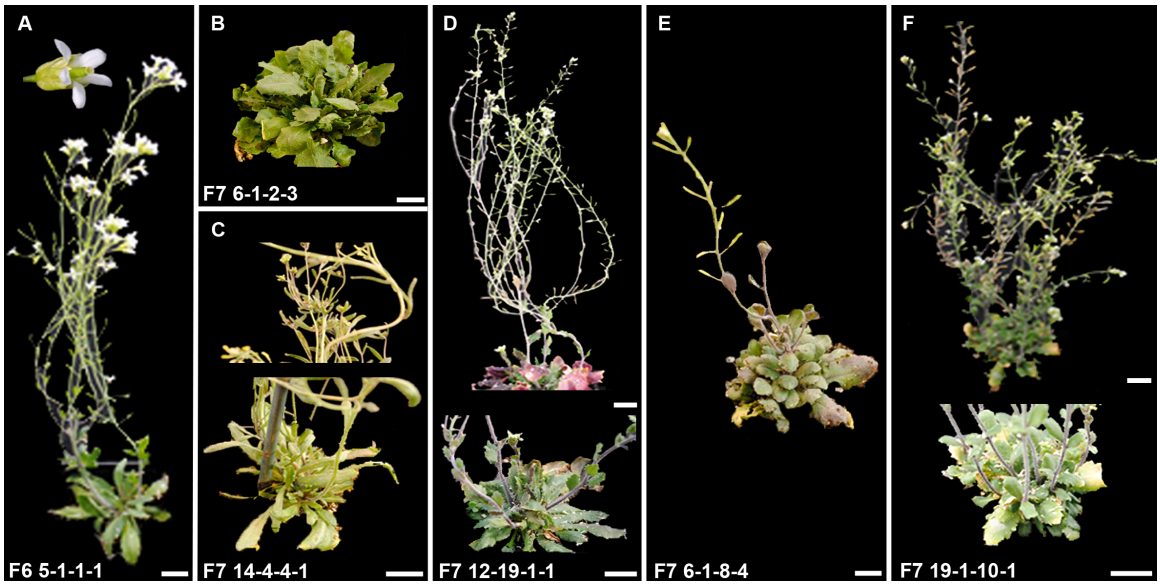

Figure S5 Phenotypic variation in F6 and F7 allohexaploids. A) F6 5-1-1-1 individual with 5 petals. B) Rosette of F7 6-1-2-3. C) F7 14-4-4-1. D) F7 12-19-1-1 with close-up of rosette. E) F7 6-1-8-4. F) 19-1-10-1 with close-up of rosette. Size bar = 2cm.

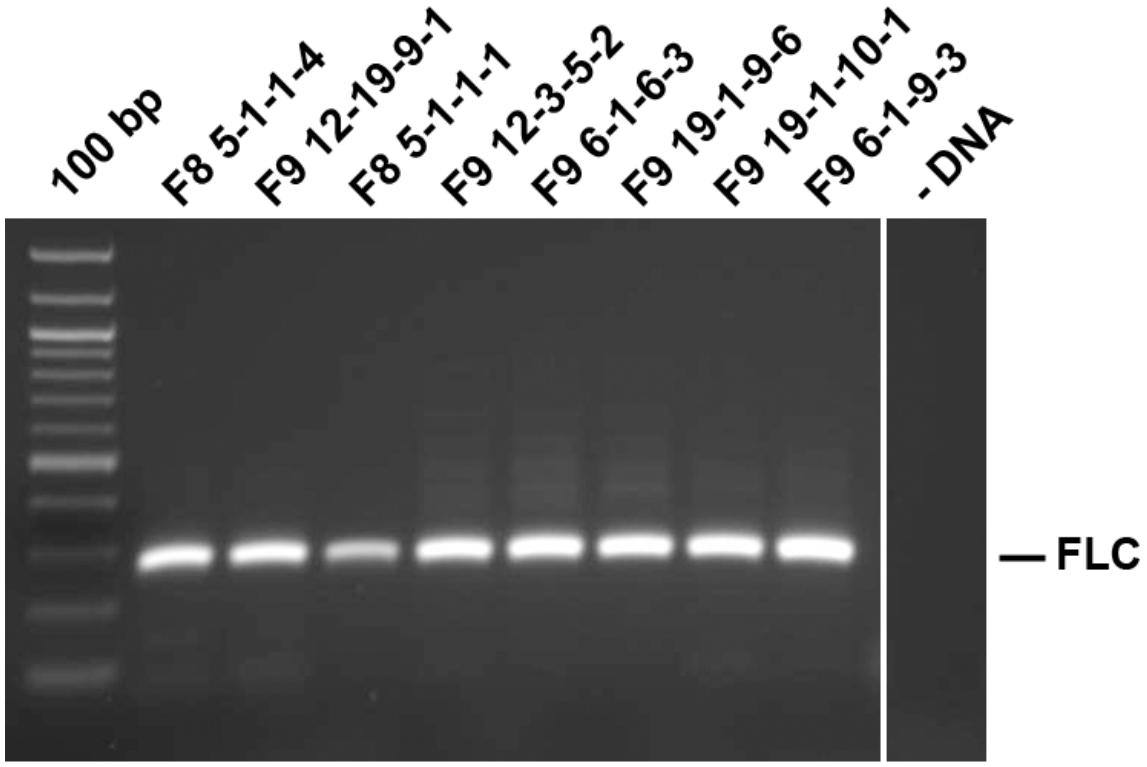

Figure S6 Steady state expression of *FLC* in early and late flowering allohexaploids. RNA was isolated from two late flowering (5-1-1-4; 12-19-9-1) and six early flowering allohexaploid individuals, DNAse-treated, and reverse transcribed. Intron-spanning primers for *FLC* were used in RT-PCR (809 bp for genomic DNA, 266 bp for cDNA). Loading control experiments to ascertain that similar amounts of cDNA were loaded were performed with actin and tubulin (data not shown). PCR products were digested with *ClaI* which uses a polymorphic site that distinguishes between *FLC* alleles derived either from *A. thaliana* or from *A.arenosa/suecica*. *ClaI* digest showed that both types of alleles were expressed (data not shown). The data suggest that *FLC* expression in these non-vernalized plants is not the primary cause for the difference in flowering time.

Table S1 Frequency of Aneuploidy in Allohexaploids over 3 Generations Based on Expected Chromosome Numbers*.

Generation	Plant Number	Cells analyzed	Aneuploid cells (%)	Cells with expected At chromosome number (%)	Cells with expected Aa chromosome number (%)	At hyperploid** chromosomes (%)	Aa hyperploid** chromosomes (%)	At hypoploid chromosomes (%)	Aa hypoploid chromosomes (%)
F3	**2-1**	10	100	0	30	20	20	80	50
	2-5	10	90	20	40	0	10	80	50
	2-8	11	100	0	27	0	27	100	45
	5-1	10	90	10	30	0	0	90	70
	5-2	10	90	10	40	10	0	80	60
	5-3	14	100	0	93	0	0	100	7
	6-1	11	91	18	45	9	18	73	36
	6-5	11	100	0	64	0	9	100	27
	6-10	11	91	27	9	0	27	73	64
	12-3	12	92	25	30	0	17	75	50
	12-11	10	90	20	50	0	10	80	40
	12-19	11	91	9	55	0	0	91	45
	14-2	10	100	0	10	0	10	100	80
	14-4	10	100	30	40	10	30	60	30
	14-9	21	95	10	24	5	0	86	76
	19-1	10	100	0	30	0	0	100	70
	19-3	11	100	0	18	0	0	100	82
	19-5	11	100	18	18	18	9	64	73
	Average		**96**	**11**	**36**	**4**	**10**	**85**	**53**

Generation	Plant Number	Cells analyzed	Aneuploid cells (%)	Cells with expected At chromosome number (%)	Cells with expected Aa chromosome number (%)	At hyperploid** chromosomes (%)	Aa hyperploid** chromosomes (%)	At hypoploid chromosomes (%)	Aa hypoploid chromosomes (%)
F6	**2-8-3**	1	100	0	100	0	0	100	100
	2-8-5	9	44	78	78	0	0	33	22
	5-1-1-15	25	96	25	72	0	0	96	68
	5-1-1-14	25	100	25	88	0	0	96	12
	5-1-6-1	26	100	26	35	0	0	65	58
	5-1-6-2	6	100	6	33	0	0	100	50
	5-1-6-4	13	100	13	77	0	8	100	15
	6-1-2	8	50	63	75	13	13	25	13
	6-1-6	7	71	29	71	0	0	71	43
	6-1-8	7	100	0	86	100	0	0	14
	6-1-9	4	50	50	100	50	0	0	0
	12-19-1	10	100	0	100	0	0	20	0
	12-19-9	1	0	100	100	0	0	0	0
	14-4-3	2	100	0	100	0	0	100	0
	14-4-4	7	100	0	0	0	0	100	100
	19-1-4	7	14	86	100	0	0	14	0
	19-1-9	6	100	0	100	0	0	100	0
	19-1-10	7	86	14	100	71	0	14	0
	Average		**78**	**29**	**79**	**13**	**1**	**58**	**27**

Generation	Plant Number	Cells analyzed	Aneuploid cells (%)	Cells with expected At chromosome number (%)	Cells with expected Aa chromosome number (%)	At hyperploid** chromosomes (%)	Aa hyperploid** chromosomes (%)	At hypoploid chromosomes (%)	Aa hypoploid chromosomes (%)
F7	**6-1-2-1**	7	100	7	14	0	86	100	0
	6-1-2-2	24	100	13	4	88	0	0	96
	6-1-2-3	26	100	0	81	100	8	0	12
	6-1-2-4	6	50	50	83	0	0	50	17
	6-1-6-2	11	18	82	100	0	0	18	0
	6-1-6-4	4	100	0	75	75	0	25	25
	6-1-8-1	38	89	38	50	3	0	74	50
	6-1-8-2	9	100	11	11	11	11	78	78
	6-1-8-3	13	92	15	46	62	0	23	54
	6-1-8-4	28	93	7	68	71	0	18	32
	6-1-9-1	28	96	7	18	0	0	93	89
	6-1-9-3	13	92	31	38	15	8	54	54
	6-1-9-4	11	100	0	55	0	0	100	73
	12-1-9-4	38	95	8	16	0	0	92	84
	12-3-5-1	9	89	9	45	0	0	89	44
	12-19-1-1	33	82	24	33	3	6	73	61

Generation	Plant Number	Cells analyzed	Aneuploid cells (%)	Cells with expected At chromosome number (%)	Cells with expected Aa chromosome number (%)	Cells with At hyperploid** chromosomes (%)	Cells with Aa hyperploid** chromosomes (%)	Cells with At hypoploid chromosomes (%)	Cells with Aa hypoploid chromosomes (%)
F7	**12-19-1-2**	27	89	26	63	0	4	74	33
	12-19-1-4	15	93	7	87	0	0	93	87
	12-19-1-5	11	100	0	9	0	0	100	91
	12-19-9-1	7	29	86	86	14	0	0	14
	14-3-3-1	17	100	17	12	0	6	94	82
	14-4-4-1	25	100	25	4	0	0	100	96
	19-1-9-1	35	100	35	11	0	6	100	83
	19-1-9-2	9	100	9	22	0	0	100	78
	19-1-9-3	26	100	27	22	0	4	96	70
	19-1-9-4	28	82	18	43	0	0	79	57
	19-1-9-5	20	100	20	30	0	0	100	70
	19-1-9-6	20	100	20	10	0	5	100	85
	19-1-9-7	17	100	17	6	0	6	76	88
	19-1-9-9	15	100	15	33	0	0	93	67
	19-1-10-1	40	70	40	60	40	5	15	35
	19-1-10-2	16	62	16	50	0	0	63	50
	Average		**89**	**24**	**34**	**3**	**2**	**80**	**68**

* Expected chromsome number is: *At* - 20*; Aa* - 16

** Hyperploid chromosomes are those that exceed the expected number and hypoploid chromosomes are those that are fewer than the expected number

Table S2 Frequency of Aneuploidy in Allohexaploids over 3 Generations Based on Modal Chromosome Numbers.

Generation	Plant number	Cells analyzed	Modal chromosome numbers*		Aneuploid (%)
			At	Aa	
F3	**2-1**	10	18	16	90
	2-5	10	18	16	90
	2-8	11	19	16	91
	5-1	10	17	16	100
	5-2	10	17	14	80
	5-3	14	19	16	64
	6-1	11	19	16	73
	6-5	11	16	16	73
	6-10	11	16	13	91
	12-3	12	17	16	75
	12-11	10	19	16	70
	12-19	11	19	16	64
	14-2	10	16	15	80
	14-4	10	20	16	100
	14-9	21	17	15	86
	19-1	10	19	15	80
	19-3	11	19	14	91
	19-5	11	19	16	82
	Average				**82**
F6	**2-8-3**	1	19	16	0
	2-8-5	9	20	16	56
	5-1-1-1 5p **	26	17	16	36
	5-1-1-1 4p**	25	17	16	16
	5-1-6-1	26	16	16	77
	5-1-6-2	6	13	14	100
	5-1-6-4	13	15	16	31
	6-1-2	8	20	16	50
	6-1-6	7	20	16	100
	6-1-8	8	23	16	38
	6-1-9	4	20	16	50
	12-19-1	10	19	16	20
	12-19-9	1	20	16	0

	14-4-3	2	18	16	0
	14-4-4	7	17	15	71
	19-1-4	7	20	16	43
	19-1-9	6	19	16	17
	19-1-10	7	21	16	43
	Average				**41**

Generation	**Plant number**	**Cells analyzed**	**Modal chromosome numbers**		**Aneuploid (%)**
			At	**Aa**	
F7	**6-1-2-1**	7	18	17	57
	6-1-2-2	24	21	15	29
	6-1-2-3	26	21	16	58
	6-1-2-4	6	20	16	50
	6-1-6-2	11	20	16	18
	6-1-6-4	4	21	16	50
	6-1-8-1	38	16	16	84
	6-1-8-2	9	18	11	89
	6-1-8-3	13	21	16	85
	6-1-8-4	28	23	16	46
	6-1-9-1	28	18	14	93
	6-1-9-3	13	20	16	92
	6-1-9-4	11	19	15	82
	12-1-9-4	38	17	14	95
	12-3-5-1	9	19	16	78
	12-19-1-1	33	18	16	91
	12-19-1-2	27	17	16	81
	12-19-1-4	15	19	16	27
	12-19-1-5	11	19	15	27
	12-19-9-1	7	20	16	29
	14-3-3-1	17	16	14	88
	14-4-4-1	25	16	15	76
	19-1-9-1	35	16	14	92
	19-1-9-2	9	14	12	67
	19-1-9-3	26	16	15	77
	19-1-9-4	28	16	16	82
	19-1-9-5	20	16	16	80

19-1-9-6	20	16	12	90
19-1-9-7	17	18	15	76
19-1-9-9	15	17	16	73
19-1-10-1	40	20	16	70
19-1-10-2	16	20	16	69
Average				**69**

* Modal chromosome numbers were determined as the most common chromosome number for *At* and *Aa* individually for each plant.

** 4p and 5p denotes cells from different floral clusters of the same plant that either produced 5-petaled or 4-petaled flowers.

Table S3 Complete FISH Data Set

Generation	Plant Number	Cell Number	At	Aa	Total Number
F3	2-1	1	18	15	33
		2	22	21	43
		3	17	14	31
		4	14	13	27
		5	22	20	42
		6	19	12	31
		7	16	16	32
		8	18	15	33
		9	18	16	34
		10	17	16	33
	2-5	1	18	18	36
		2	19	14	33
		3	18	15	33
		4	18	15	33
		5	17	13	30
		6	20	16	36
		7	18	16	34
		8	19	16	35
		9	20	15	35
		10	16	16	32
	2-8	1	19	17	36
		2	18	13	31
		3	19	16	35
		4	15	15	30
		5	19	15	34
		6	17	16	33
		7	16	13	29
		8	17	18	35
		9	16	18	34
		10	11	13	24
		11	18	16	34
	5-1	1	17	15	32
		2	17	12	29
		3	18	16	34
		4	16	14	30
		5	17	12	29

	6	20	16	36
	7	19	14	33
	8	16	11	27
	9	18	15	33
	10	19	16	35
5-2	1	18	16	34
	2	17	12	29
	3	17	14	31
	4	23	16	39
	5	20	16	36
	6	13	14	27
	7	18	14	32
	8	17	16	33
	9	16	14	30
	10	17	14	31
5-3	1	18	16	34
	2	19	16	35
	3	19	16	35
	4	18	16	34
	5	17	16	33
	6	17	16	33
	7	19	16	35
	8	19	15	34
	9	18	16	34
	10	17	16	33
	11	19	16	35
	12	17	16	33
	13	17	16	33
	14	19	16	35
6-1	1	21	13	34
	2	19	16	35
	3	19	16	35
	4	20	16	36
	5	19	16	35
	6	19	11	30
	7	18	12	30
	8	19	15	34
	9	19	17	36
	10	18	16	34

	11	20	17	37
6-5	1	14	14	28
	2	18	16	34
	3	15	17	32
	4	15	16	31
	5	13	16	29
	6	17	15	32
	7	16	16	32
	8	17	16	33
	9	15	15	30
	10	16	16	32
	11	16	16	32
6-10	1	20	13	33
	2	19	14	33
	3	19	17	36
	4	20	16	36
	5	16	12	28
	6	18	15	33
	7	15	17	32
	8	20	17	37
	9	16	15	31
	10	16	13	29
	11	19	13	32
12-3	1	18	15	33
	2	20	16	36
	3	17	16	33
	4	19	15	34
	5	20	14	34
	6	15	17	32
	7	18	13	31
	8	19	15	34
	9	17	16	33
	10	17	17	34
	11	17	16	33
	12	20	15	35
12-11	1	18	18	36
	2	19	13	32
	3	20	15	35
	4	20	16	36

	5	19	16	35
	6	19	16	35
	7	19	16	35
	8	17	15	32
	9	18	14	32
	10	17	16	33
12-19	1	19	15	34
	2	17	15	32
	3	19	16	35
	4	19	16	35
	5	19	16	35
	6	19	16	35
	7	18	15	33
	8	17	15	32
	9	20	16	36
	10	18	16	34
	11	17	15	32
14-2	1	13	12	25
	2	17	13	30
	3	16	14	30
	4	13	18	31
	5	18	15	33
	6	18	15	33
	7	18	15	33
	8	16	15	31
	9	16	15	31
	10	16	16	32
14-4	1	20	14	34
	2	20	17	37
	3	21	16	37
	4	20	18	38
	5	18	16	34
	6	17	17	34
	7	19	15	34
	8	19	16	35
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