RESEARCH ARTICLE

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How diploidization turned a tetraploid into a pseudotriploid[1]

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PREMISE OF THE STUDY: Despite being highly fertile and occupying a large geographic region, the North American heartleaf bittercress (*Cardamine cordifolia*; Brassicaceae) has a puzzling triploid-like chromosome number ($2n = 3x = 24$). As most triploids are sterile, we embarked on a detailed analysis of the *C. cordifolia* genome to elucidate its origin and structure.

METHODS: Mitotic and meiotic chromosome complement of *C. cordifolia* was analyzed by comparative chromosome painting using chromosome-specific BAC contigs of *Arabidopsis thaliana*. Resulting chromosome patterns were documented by multicolor fluorescence microscopy and compared with known ancestral and extant Brassicaceae genomes.

KEY RESULTS: We discovered that *C. cordifolia* is not a triploid hybrid but a diploidized tetraploid with the prevalence of regular, diploid-like meiotic pairing. The ancestral tetraploid chromosome number ($2n = 32$) was reduced to a triploid-like number ($2n = 24$) through four terminal chromosome translocations.

CONCLUSIONS: The structure of the pseudotriploid *C. cordifolia* genome results from a stepwise diploidization process after whole-genome duplication. We showed that translocation-based descending dysploidy (from $n = 16$ to $n = 12$) was mediated by the formation of five new chromosomes. The genome of *C. cordifolia* represents the diploidization process *in statu nascendi* and provides valuable insights into mechanisms of postpolyploidy rediploidization in land plants. Our data further suggest that chromosome number alone does not need to be a reliable proxy of species' evolutionary past and that the same chromosome number may originate either by polyploidization (hybridization) or due to descending dysploidy.

KEY WORDS Brassicaceae; centromere loss; chromosome fusion; chromosome translocation; diploidization; dysploidy; karyotype evolution; polyploidy; whole-genome duplication

Polyploidy (whole-genome duplication, WGD) is frequently and almost always followed by diploidization. Diploidization is an overarching term encompassing a variety of processes transforming a polyploid genome into a (pseudo)diploid one. Cycles of polyploidization followed by diploidization across land plants have been reviewed recently (e.g., Cui et al., 2006; Soltis et al., 2009; Jiao et al., 2011, 2012; Vanneste et al., 2014). Diploidization is self-evident as a drop in ploidy level and chromosome number to a diploid-like state (i.e., typically the lowest ploidy level and chromosome number known for a given taxonomic unit and/or related taxa). Rediploidization processes conceal past WGDs, and their identification requires an integrative approach.

Genome diploidization simultaneously operates on multiple hierarchical levels. Probably most information on diploidization mechanisms was gained through whole-genome sequencing of plant genomes. Each round of polyploidization leads to genomic redundancy, which can be both advantageous and unfavorable. Duplicated genes can be retained and subjected to subfunctionalization or neofunctionalization, whereas most paralogs are lost. The dosage balance theory suggests that retention and removal of duplicated genes is nonrandom (Freeling, 2009; De Smet et al., 2013; Conant et al., 2014). Fractionation, removing most of the duplicated genes and reverting the duplicated genome into a diploid-like genome, seems to be biased toward one of the duplicated (sub)genomes in many ancient polyploids (e.g., Schnable et al., 2011; Wang et al., 2011; Tang et al., 2012). Garsmeur et al. (2014) suggested that biased fractionation and genome dominance (i.e., the least fractionated subgenome is more expressed) are

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characteristic for ancient allopolyploids, whereas unbiased fractionation and subgenome equivalence in gene expression point to ancient autopolyploidy.

Postpolyploidy genome fractionation is associated with genome downsizing, particularly through loss of low-copy sequences and repeats (Ma et al., 2004; Freeling et al., 2012; Renny-Byfield et al., 2013), and structural chromosomal changes leading to the reduction of chromosome number, i.e., descending dysploidy (Mandáková et al., 2010a, b; Cheng et al., 2013; Hohmann et al., 2015; Murat et al., 2015). Karyotypic variation in several natural and synthetic allopolyploids likely reflects the "adjustment" of two or more parental genomes within a polyploid nucleus and may initiate rediploidization of the polyploid genome. Natural populations of the neoallopolyploid *Tragopogon miscellus* Ownbey show frequent aneuploidy, intergenomic translocations, and compensation of homeologous chromosomes (Chester et al., 2012), and similar patterns were revealed in resynthesized *Brassica napus* L. allopolyploids (Xiong et al., 2011). In newly synthesized tetraploid wheats, Zhang et al. (2013) showed that some genome combinations exhibit remarkable karyotype stability, whereas other combinations showed extensive intergenomic rearrangements and aneuploidy. Here, the stability of new allopolyploid genomes is given by intrinsic compatibility of the hybridizing parental genomes, whereby only genome combinations analogous to natural tetraploid wheats manifest karyotype stability. In the allotetraploid genome of *Cardamine flexuosa* With. (Brassicaceae), although both parental subgenomes remained stable overall, a reciprocal translocation between two homeologous chromosomes may indicate the start of diploidization of the allotetraploid genome (Mandáková et al., 2014).

The hallmark of diploidization is a drop in the number of chromosomes (i.e., linkage groups), and consequently in the number of centromeres and telomeres. That reciprocal chromosome translocations among homologous or homeologous chromosomes is the mechanistic basis of descending dysploidy in both diploids and polyploids has been known for a long time (Darlington, 1937). However, only recently have tools and resources become available to reconstruct events of chromosome number reduction in different groups of plants. Whole-genome assemblies, bioinformatic tools, and comparative cytogenetic maps allow for reconstructions of ancestral polyploid genomes and their subsequent evolution. This multidisciplinary approach gained particularly interesting data for the mustard family (Brassicaceae). Several recent studies suggest that the Brassicaceae-specific paleopolyploidization (At-α) was followed by either diploidization and remarkable karyotype stasis (Hu et al., 2011; Slotte et al., 2013), diploidization associated with chromosome number reduction leading in its extreme form to the reduced *Arabidopsis thaliana* (L.) Heynh. genome (AGI, 2000), or by a number of younger and clade-specific mesopolyploid WGDs. Depending on the degree of polyploidization (tetraploidy vs. hexaploidy), time of origin, and tempo of diploidization, mesopolyploid genomes have been rediploidized to a different extent. Whereas in the mesohexaploid genome ($n = 20$) of *Camelina sativa* (L.) Crantz the three subgenomes are still retained (Kagale et al., 2014), the number of ancestral chromosomes has been reduced by 1.06-fold in *Cardamine pratensis* L. (from $n = 32$ to $n = 30$; Mandáková et al., 2013), 1.6-fold in *Pachycladon* Hook. f. (from $n = 16$ to $n = 10$; Mandáková et al., 2010a), 1.7-fold in *Biscutella laevigata* L. (from $n = 16$ to $n = 9$; Geiser et al., 2016), 2.1-fold in *Brassica rapa* L. (from $n = 21$ to $n = 10$; Wang et al., 2011; Cheng et al., 2013), and up to 4-fold in *Stenopetalum nutans* F. Mull. (from $n = 16$ to $n = 4$; Mandáková et al., 2010b).

Here we focus on reconstructing patterns of genome evolution through postpolyploidy diploidization in *Cardamine cordifolia* A. Gray (heartleaf bittercress, Brassicaceae). This rhizomatous, perennial, herbaceous plant is self-compatible (N. K. Whiteman, personal observation) but primarily outcrossing (Louda, 1984; Louda and Rodman, 1996), and highly fertile (N. K. Whiteman, personal observation). Butterflies appear to be the primary pollinators (Louda and Rodman, 1996; Fig. 1A). The species is a dominant riparian forb preferring moist, shaded microhabitats associated with snowmelt streams and bogs (Louda, 1984). It is found growing at >600 m in 10 states of the United States and in British Columbia, Canada (Fig. 1B). *Cardamine cordifolia* occupies two primary distribution areas: one in the northern Rocky Mountains, Cascade Mountains, and northern Sierra Nevada mountains and one in the southern Rocky Mountains. The Snake River provides a major biogeographic barrier between two varieties, *Cardamine cordifolia* var. *lyallii* (S.Watson) A.Nelson & J.F.Macbride in the west and *Cardamine cordifolia* var. *cordifolia* O.E.Schulz in the east, though these varieties are not currently recognized due to a lack of defining morphological characters (eFloras, 2015; I. A. Al-Shehbaz, Missouri Botanical Garden, personal communication). *Cardamine cordifolia* is an ecological model plant species to study the effect of herbivory on plant growth and habitat distribution in natural settings and the ecological consequences of interactions between plant defenses, herbivores, and bacteria (Louda, 1984; Louda and Rodman, 1996; Humphrey et al., 2014).

Since the chromosome count of $2n = 24$ was established in *C. cordifolia* (Bell, 1965; Mulligan, 1965), an apparent paradox arose because the triploid chromosome number (based on $x = 8$ typical for *Cardamine* L.) was not associated with the expected (semi)sterility of these allegedly triploid plants. Mulligan (1965, p. 666) proposed, "It seems more likely that *C. cordifolia* is tetraploid with the base number $x = 6$ rather than triploid with the base number $x = 8$." But the origin of this polyploid genome has not been satisfactorily explained to this date. Therefore, we embarked on a detailed analysis of the origin and genome evolution of *C. cordifolia* by means of cross-species chromosome painting using *A. thaliana* BAC contigs. We show here that the enigmatic chromosome number of *C. cordifolia* is not the result of a hybridization event, but rather that the *C. cordifolia* genome has originated by diploidization of an ancestral tetraploid genome.

MATERIALS AND METHODS

Plant material—Material from two populations of *C. cordifolia* were used in the current study. Collection sites were chosen near the center of the distribution of the formerly recognized varieties *C. cordifolia* var. *cordifolia* (Copper Lake, Colorado [CO], USA; GPS coordinates: 39°00′17.8″N, 106°56′34.5″W) and *C. cordifolia* var. *lyallii* (Umatilla County, Oregon [OR], USA; GPS coordinates: 45°42′41.8″N, 117°59′29.6″W). Rhizomes were collected from both sites in July 2011 and regrown in a glasshouse at the University of Arizona, Tucson, Arizona, USA. Inflorescences formed in the spring under natural lighting.

Chromosome preparation—Young inflorescences were harvested in the field and greenhouse, fixed in freshly prepared ethanol–acetic

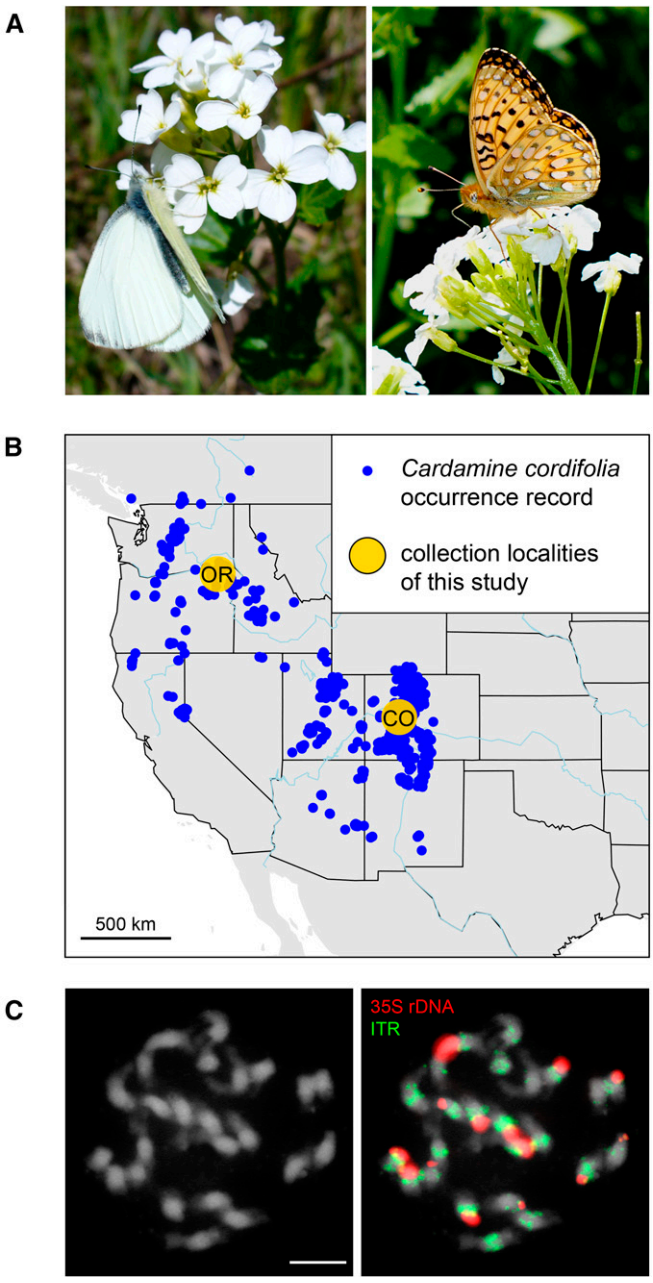


FIGURE 1 Pollination biology, distribution, and chromosome number of *Cardamine cordifolia*. (A) Butterflies appear to be primary pollinators of *C. cordifolia* (Louda and Rodman, 1996). Photographs were taken near the Rocky Mountain Biological Laboratory, Colorado, USA. (B) *C. cordifolia* is widely distributed across high elevation sites in western North America. The map shows locations of all occurrence records documented in the Intermountain Regional Herbarium Network, Consortium of Pacific Northwest Herbaria, and Global Biodiversity Information Facility online databases (accessed September 2015). Symbols CO and OR refer to the analyzed populations from Colorado and Oregon, respectively. (C) Twenty-four DAPI-stained mitotic metaphase chromosomes of *C. cordifolia* and localization of 16 loci of 35S rDNA (red fluorescent signals) together with interstitial telomeric repeats (ITRs; green fluorescent signals). Scale bar: 5 μm.

acid fixative (3:1, v/v) overnight, and subsequently stored in 70% ethanol at −20°C until use. Selected flower buds were rinsed in distilled water and citrate buffer (10 mM sodium citrate, pH 4.8) and incubated in an enzyme mix (0.3% cellulase, cytohelicase, and pectolyase; all Sigma, St. Louis, Missouri, USA) in citrate buffer at 37°C for 5 h. After digestion, individual flower buds were placed on a microscopic slide, disintegrated by a needle in a small drop of citrate buffer, and the material spread in 20 μL of 60% acetic acid on a hot plate (50°C). After the material was fixed on the slide using 100 μL of the ethanol–acetic acid fixative, the slide was tilted and dried using a hair dryer. The preparations were staged using a phase contrast microscope, and suitable slides containing tapetal mitoses and/or meiosis I chromosomes were postfixed in 4% v/v formaldehyde in distilled water for 10 min and air-dried.

DNA probes—For comparative chromosome painting (CCP) in *C. cordifolia* (CO), chromosome-specific BAC clones of *A. thaliana* grouped into contigs according to 22 genomic blocks (Lysak et al., 2016) were used (Fig. 2A). The combinations of *A. thaliana* BAC contigs localized on chromosomes of plants from the CO population were used as painting probes in plants from the OR population. The *A. thaliana* BAC clone T15P10 (AF167571) containing 35S rRNA genes was used for in situ localization of nucleolar organizer regions (NORs), and *A. thaliana* clone pCT4.2 (M65137), corresponding to a 500-bp 5S rRNA repeat, was used for localization of 5S rDNA loci. The *Arabidopsis*-type telomere repeat (TTTAGGG)n was prepared according to Ijdo et al. (1991). All DNA probes were labeled with biotin-dUTP, digoxigenin-dUTP, or Cy3-dUTP by nick translation as described by Lysak and Mandáková (2013).

Comparative chromosome painting—Chromosome preparations were treated with 100 μg/mL RNase (AppliChem, Darmstadt, Germany) in 2× sodium saline citrate (SSC; 20× SSC: 3 M sodium chloride, 300 mM trisodium citrate, pH 7.0) for 60 min and with 0.1 mg/mL pepsin (Sigma) in 0.01 M HCl at 37°C for 5 min; then postfixed in 4% formaldehyde in 2× SSC for 10 min, washed in 2× SSC twice for 5 min, and dehydrated in an ethanol series (70%, 90%, and 100%, 2 min each). Selected labeled BAC clones were pooled and ethanol-precipitated. The pellet was resuspended in 20 μL of hybridization mix (50% v/v formamide and 10% w/v dextran sulfate in 2× SSC) per slide. The probe and chromosomes were denatured together on a hot plate at 80°C for 2 min and incubated in a moist chamber at 37°C overnight. Posthybridization washing was performed in 20% formamide in 2× SSC at 42°C. Amplification and detection of fluorescent signals followed the protocol of Lysak and Mandáková (2013). Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 2 μg/mL) in Vectashield antifade (Vector Laboratories, Burlingame, CA, USA) and photographed using Olympus BX-61 epifluorescence microscope equipped with a Zeiss CoolCube camera. Images were acquired separately for all four fluorochromes using appropriate excitation and emission filters (AHF Analysentechnik, Tübingen, Germany). The four monochromatic images were pseudocolored and merged using the Adobe Photoshop CS2 software (Adobe Systems, San Jose, CA, USA).

RESULTS

Comparative karyotype—All analyzed plants of *C. cordifolia* had 24 chromosomes ($2n = 24$, Fig. 1C). Based on our previous results

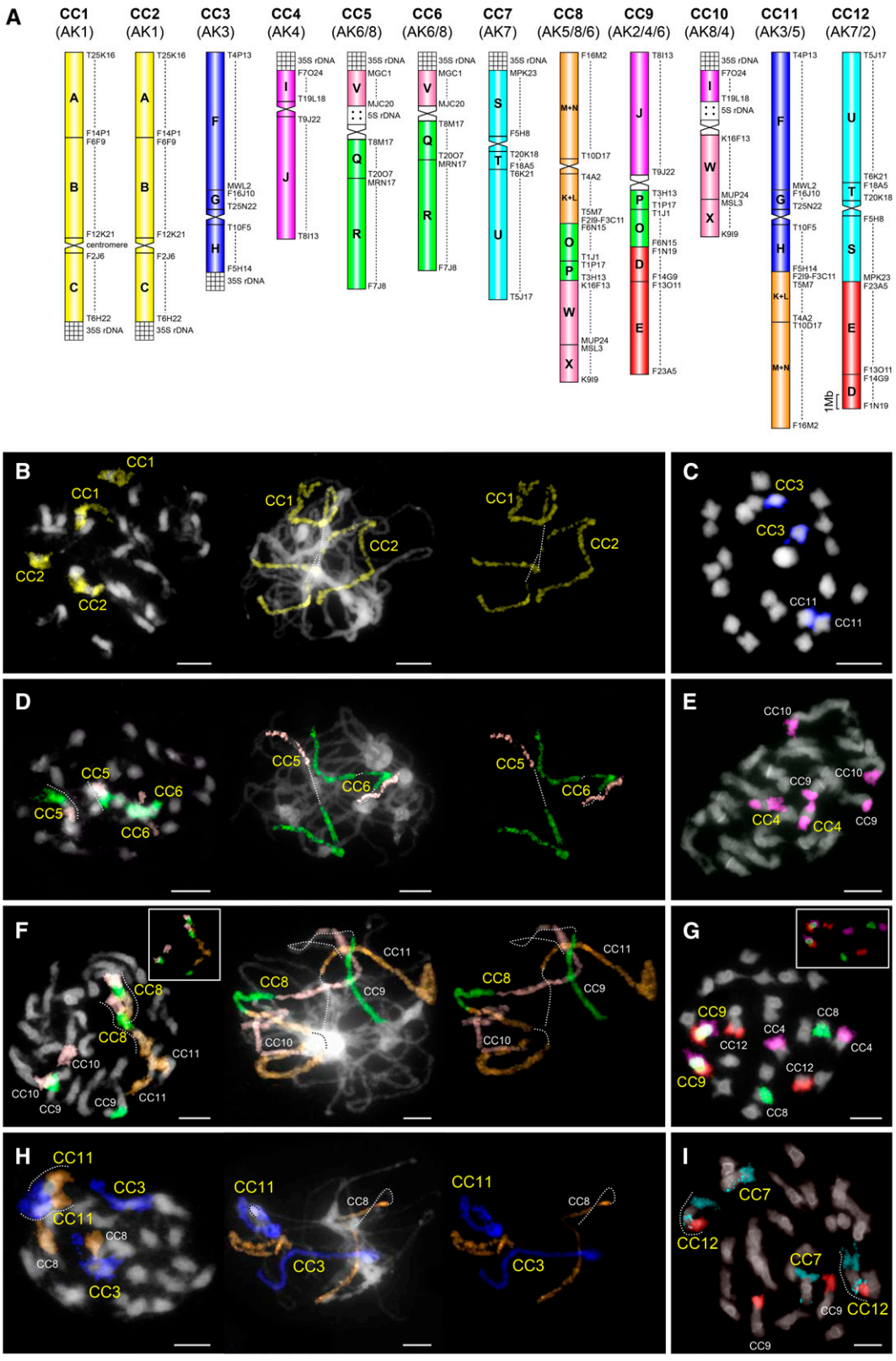


FIGURE 2 Genome structure of *Cardamine cordifolia* based on comparative chromosome painting (CCP) analysis. (A) Comparative karyotype based on CCP data showing the composition of 12 *C. cordifolia* chromosomes (CC1–CC12), position of 44 genomic blocks (A–X) and localization of rDNA loci. Chromosome IDs in parentheses and eight different colors correspond to homeologous chromosomes in the ancestral crucifer karyotype (ACK). Centromeres with an uncertain origin as compared with chromosomes of the tetraploid *Cardamine* ancestor and ACK are white. BAC clones of *A. thaliana*

in other *Cardamine* species (e.g., Hay et al., 2014; Mandáková et al., 2013, 2014) painting probes were designed following the structure of surmised eight ancestral *Cardamine* chromosomes comprising 22 genomic blocks (Appendix S1, see Supplemental Data with the online version of this article; Lysak et al., 2016): the structure of chromosomes 1 to 5 and chromosome 7, were considered as identical to that of chromosomes AK1 to AK5, and AK7 in the ancestral crucifer karyotype; two other *Cardamine* chromosomes were thought to result from a whole-arm reciprocal translocation between chromosomes AK6 and AK8 (chromosomes AK6/8 and AK8/6). Following this premise, chromosome-specific *A. thaliana* BAC contigs (see Fig. 2A) corresponding to the 22 genomic blocks and eight ancestral *Cardamine* chromosomes (Appendix S1) were applied as painting probes to pachytene bivalents and tapetal mitotic chromosome spreads (Fig. 2B–I). For verifying the completeness and centromere-to-telomere orientation of the hybridized BAC contigs, each contig was polarized by BAC subcontigs and/or individual BACs labeled by three different fluorochromes (see Materials and Methods). The design of painting probes has been subsequently modified to elucidate the actual genome structure of *C. cordifolia*. On the basis of initial experiments, we hypothesized that despite the triploid-like chromosome number, the genome of *C. cordifolia* is derived from a tetraploid ancestor. Indeed, cytogenetic analyses allowed us to unambiguously identify all the 22 genomic blocks to be duplicated to build the 12 *C. cordifolia* chromosomes (Fig. 2A). Seven *C. cordifolia* chromosomes (CC1–CC7) shared the same structure as the ancestral *Cardamine* chromosomes including two copies (CC5 and CC6) of the *Cardamine*-specific translocation chromosome AK6/8. The remaining five chromosomes (CC8–CC12) combine genomic blocks of two or three ancestral chromosomes and are further referred to as translocation chromosomes (Fig. 3).

Fluorescent in situ hybridization (FISH) of ribosomal RNA genes (rDNA) revealed nucleolar organizer regions (NORs) harboring 35S rDNA in the terminal position on short arms of seven nontranslocation chromosomes and one translocation chromosome (Figs. 1C and 2A), and 5S rDNA at pericentromeres of two nontranslocation chromosomes (Fig. 2A). Localization of *Arabidopsis*-type telomere repeats revealed low to high copy numbers of interstitial telomeric repeats (ITRs) at all 12 (peri)centromeres (Fig. 1C). Size variation of ITR arrays was not correlated with the chromosome structure (e.g., translocation vs. ancestral chromosomes).

Origin of translocation chromosomes and karyotype symmetry—Relationships between the 12 chromosomes of *C. cordifolia* and the 16 chromosomes of the inferred tetraploid *Cardamine* ancestor are displayed in Fig. 3A. On the basis of these comparisons, we reconstructed the origin of the five translocation chromosomes in *C. cordifolia* (Fig. 3B). Chromosome CC8 originated by a terminal chromosome translocation (TCT) between chromosomes AK5 and AK8/6 followed by a loss of the AK8/6 centromere. Chromosome CC10 was formed as a result of a whole-arm reciprocal translocation between short arms of AK4 and AK8/6; the second translocation product (AK4/6) experienced another TCT with chromosome AK2 to form chromosome CC9 (the AK2 centromere was eliminated). A TCT event between short arms of AK3 and AK5 has formed chromosome CC11, whereby the AK5 centromere was eliminated. And finally, TCT involving chromosomes AK2 and AK7 resulted in the origin of CC12 and loss of the AK2 centromere. As by CCP, we were unable to discriminate between two parental subgenomes forming the tetraploid *C. cordifolia* genome (see comparable fluorescence intensities in Fig. 2B–I), it cannot be determined whether the inferred chromosomal rearrangements involved nonhomologous chromosomes from one or two subgenomes. However, comparable fluorescence intensities of all homeologous genomic blocks suggest an autopolyploid origin of the tetraploid *C. cordifolia* ancestor followed by rediploidization.

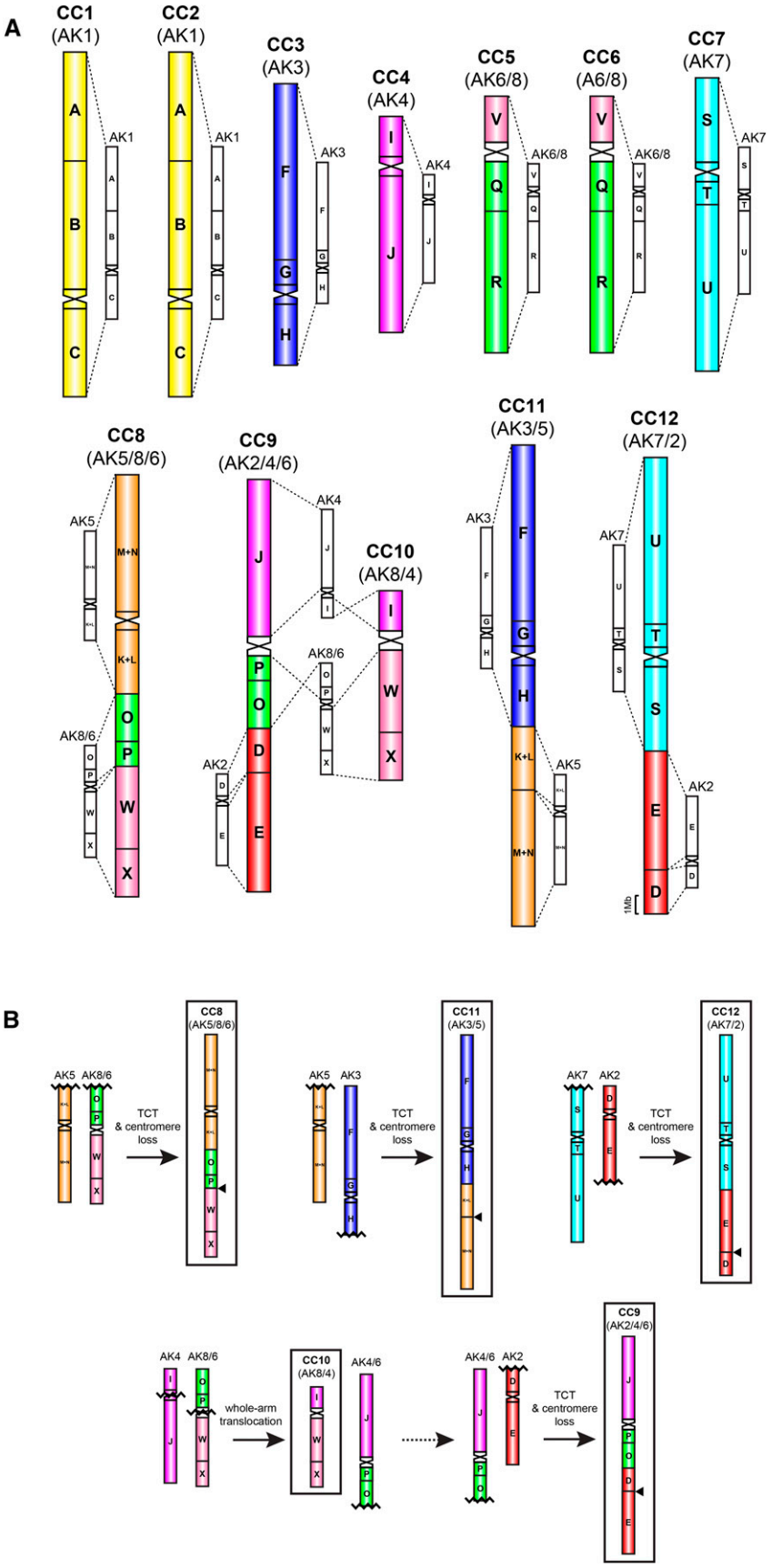
The inferred TCT events resulted in inactivation and/or loss of four ancestral centromeres (Fig. 3; Appendix S2, see online Supplemental Data) and the origin of four longer and more metacentric translocation chromosomes (CC8, CC9, CC11, and CC12). Whereas the average chromosome size (expressed as length of *A. thaliana* BAC contigs in Mb) was 11.8 Mb in the ancestral tetraploid genome, the average chromosome size in *C. cordifolia* equals 15.7 Mb. These events also made the karyotype of *C. cordifolia* more symmetric as shown by the increased mean centromeric index (CI; length of the short arm to the total chromosome length × 100): 27.2% in the tetraploid ancestor vs. 30.1% in *C. cordifolia*. In *C. cordifolia*, translocation chromosomes have a mean CI of 39.6% and average chromosome size of 21.1 Mb, whereas the remaining eight chromosomes have a mean CI of 25.4% and average chromosome size of 13 Mb.

Intraspecific karyotype stasis—To understand whether the genome structure and particularly the five translocation chromosomes are stable throughout the distribution area of *C. cordifolia*, we analyzed two localities more than 1000 km apart (Fig. 1B). Painting probes designed according to the karyotype of the Colorado (CO) population were used in the Oregon population (OR). These analyses proved that genomes of both populations are structurally identical (online Appendix S3).

Chromosome pairing in pollen meiosis I—Despite the likely autopolyploid nature of the *C. cordifolia* genome, homologous chromosomes usually pair regularly as bivalents (65.2%; $n = 181$). Because translocation chromosomes share homeology with one or two other chromosomes (see Fig. 2A), we analyzed meiotic pairing of translocation chromosomes CC8, CC9, CC11, and CC12. Chromosomes CC8, CC11, and CC12 form predominately bivalents (71–80%, Fig. 4A, F, and H) and less frequently they pair as quadri- and multivalents with chromosomes without shared ancestral homeology—CC8: 25% (Fig. 4B), CC11: 27% (Fig. 4G), CC12: 16% (Fig. 4I). Chromosome CC9 forms bivalents only in 36% of meiotic configurations (Fig. 4C) but quadrivalents with a chromosome without shared homeology in 47% (Fig. 4D) and with chromosome CC4 (sharing block J with CC9) in 11% (Fig. 4E). Apart from the

←

defining each genomic block as well as painting probes used are listed along the chromosomes. (B–I) The structure of the 12 *C. cordifolia* chromosomes reconstructed by CCP on mitotic prometaphase/metaphase and pachytene chromosomes. (B) Chromosomes CC1 and CC2, (C) CC3, (D) CC5 and CC6, (E) CC4 and the upper arm of CC10, (F) CC8 and the bottom arm of CC10, (G) CC9, (H) CC11, and (I) CC7 and CC12. Fluorescent signals of Alexa 488, Cy3, and Texas Red were pseudocolored to match the eight colors in (A). Note that due to the whole-genome duplication the used BAC contigs also identified homeologous chromosomes. Chromosomes were counterstained with DAPI. All scale bars: 5 μm.


A
CC1
(AK1)
CC2
(AK1)
CC3
(AK3)
CC4
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CC5
(AK6/8)
CC6
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CC8
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CC9
(AK2/4/6)
CC10
(AK8/4)
CC11
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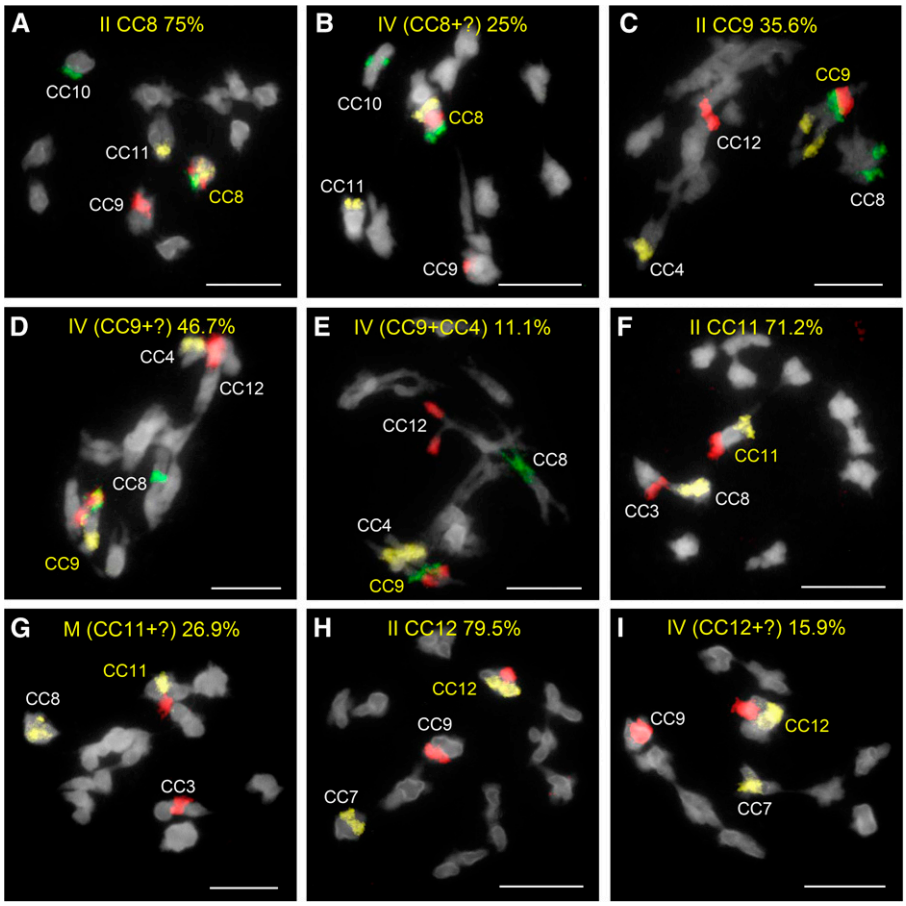

FIGURE 4 Analysis of meiotic pairing configurations of translocation chromosomes in *Cardamine cordifolia* (CO). Chromosomes in diakinesis were hybridized with marker *A. thaliana* BAC clones identifying chromosomes CC8 (A, B), CC9 (C, E), CC11 (F, G), and CC12 (H, I). Percentage of translocation chromosomes forming bivalents (II), tetravalents (IV) and multivalent (M) is given. Note that the used BAC clones hybridized also to homeologous regions of other chromosomes. All scale bars: 10 µm.

large-scale CC9–CC4 homeology (see Figs. 2A and 3A), the reasons for these pairing configurations of CC9 remain inconclusive and can be attributed to incomplete diploidization of this translocation chromosome.

DISCUSSION

Heartleaf bittercress (*C. cordifolia*) is a sexually reproducing species distributed in a large part of the North American Intermountain West and Northwest, yet its chromosome number is puzzling. Given the ancestral (base) chromosome number for the genus *Cardamine*, the chromosome complement of 12 chromosomes ($2n = 24$) can be interpreted as triploid arising through hybridization between two diploid ($2n = 2x = 16$) or between diploid and tetraploid ($2n = 4x = 32$) species, or as tetraploid based on $x = 6$ (Mulligan, 1965). Here we showed that neither hypothesis is correct and that the pseudotriploid genome of *C. cordifolia* originated through diploidization of a primary tetraploid ancestral genome. Hence, *C. cordifolia*, while being a functionally diploid species, arose from a tetraploid genome. The extant genome of *C. cordifolia* originated from its tetraploid progenitor through descending dysploidy, whereby the origin of four translocation ("fusion") chromosomes reduced the original number of linkage groups from 16 to 12. These events eliminated an equivalent of one genome ($x = 8$), i.e., one quarter of the tetraploid genome (Fig. 5). Although we do not know whether the present-day genome of *C. cordifolia* was formed once or polytopically, the identical genome structure of the two analyzed populations points to a monotopic origin of the genome. However, analysis of a representative number of populations is required to corroborate the karyotype stasis and monotopic origin of the diploidizing descending dysploidy. The case of *C. cordifolia* further illustrates how misleading chromosome number can be as the sole criterion for interpreting the origin and evolutionary past of a species or clade (see also Lysak et al., 2007; Considine et al., 2012; Cusimano et al., 2012; Mandáková et al., 2013).

Mechanism of descending dysploidy—Two essential processes were identified as a universal mechanism reducing chromosome number in *C. cordifolia*: terminal chromosome translocation (TCT, Lysak, 2014) and centromere elimination (Fig. 3B). A TCT resulted from two breaks at terminal regions of two different chromosomes followed by recombination combining the two chromosomes. Because the breaks usually occurred on short chromosome arms (but see the origin of CC12) and the NORs (35S rDNA loci) are absent on the translocation chromosomes (Fig. 2A), we hypothesize that the sequence homology of rDNA repeats might have mediated some of the TCT events. Due to the relatively large distance between the two centromeres on the resulting translocation chromosomes (Fig. 3B), one of the centromeres must become inactive or eliminated to avoid segregation problems of dicentric chromosomes (Schubert and Lysak, 2011; Lysak, 2014). Because both genomic copies of ancestral chromosomes AK2 and AK5 were involved in TCT events, we conclude that these translocations were based on illegitimate recombination

FIGURE 3 Origin of the *Cardamine cordifolia* genome from a tentative ancestral *Cardamine* karyotype. (A) Relationships between the 16 chromosomes of the ancestral tetraploid *Cardamine* genome and the 12 chromosomes of *C. cordifolia*. (B) Proposed most parsimonious origins of five *C. cordifolia* translocation chromosomes from nine chromosomes of the tetraploid *Cardamine* ancestor. Centromeres with an uncertain origin as compared with chromosomes of the tetraploid *Cardamine* ancestor and ACK are white. Arrowheads indicate positions of inactive/lost centromeres. TCT: terminal chromosome translocation. In both (A) and (B), chromosome IDs in parenthesis correspond to homeologous chromosomes of the *Cardamine* ancestor, the eight colors correspond to chromosomes and genomic blocks of ACK (online Appendix S1).

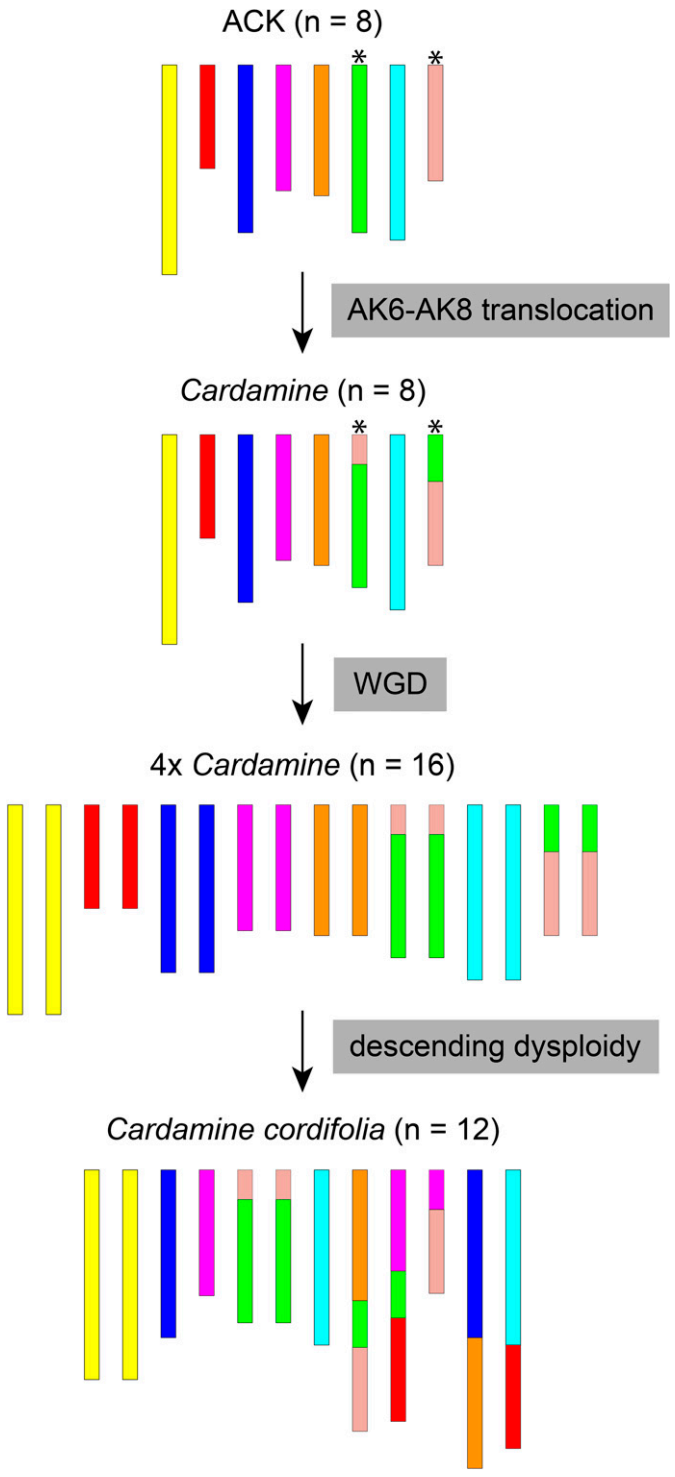

FIGURE 5 The inferred origin and evolution of the *Cardamine cordifolia* genome. The ancestral *Cardamine* genome (n = 8) originated from the ACK genome (n = 8) through a reciprocal translocation between chromosomes AK6 and AK8 (asterisks). The ancestral diploid genome has undergone a whole-genome duplication (WGD) resulting in a (meso) tetraploid *Cardamine* genome (n = 16). This genome was then diploidized through descending dysploidy mediated by interchromosomal translocations toward the extant 12 chromosomes of *C. cordifolia*.

between nonhomeologous chromosomes of the two parental (sub) genomes. The four TCT events mediated a stepwise reduction of chromosome number from 32 to 24 (Fig. 3B). However, at each step, plants with reduced chromosome numbers should become homozygous and fertile, and ultimately, plants with 24 chromosomes had to gain a selective advantage and become dominant. Alternatively, population bottlenecks could facilitate the changes in genome structure through genetic drift. Assuming that TCT is a predominant mechanism of karyotype evolution in *C. cordifolia*, we might hypothesize that the number of chromosomes will further decrease by TCT between the remaining eight chromosomes (CC1–CC7 and CC10) to a pseudodiploid ploidy level ($2n = 2x = 16$).

Descending dysploidy in Cardamine—Multiple and independent auto- and allopolyploidization events are not rare in the genus *Cardamine* (Carlsen et al., 2009), with 68% taxa being entirely polyploid or having both diploid and polyploid cytotypes (Kučera et al., 2005). Further, a growing body of data suggests that descending dysploidy is also not rare in *Cardamine*. Lawrence (1931) was the first to suspect that the hypotetraploid chromosome number ($2n$ = 30) in *C. pratensis* might have resulted from "chromosome fusion" in otherwise tetraploid species ($2n = 4x = 32$). The origin of the "fusion chromosome" was recently confirmed by Mandáková et al. (2013) as a translocation between two nonhomeologous chromosomes accompanied by centromere elimination. It is equally conceivable that the 28 chromosomes documented for some populations of *C. pratensis* (e.g., Lippert and Heubl, 1988) also result from descending dysploidy in the primary tetraploid plants.

Diploidization of an autotetraploid or allotetraploid?—The *C. cordifolia* genome originated through diploidization of an ancestral tetraploid *Cardamine* genome. Was the tetraploid genome autopolyploid or allopolyploid in origin? Comparable fluorescence intensities observed for all painting probes (Fig. 2B–I) can be interpreted as evidence for autopolyploidy or hybridization between two species of a similar age and genomic make-up. The identical structure of homeologous chromosomes and chromosomal regions (Fig. 2A) could again point to an autotetraploid origin. However, *Cardamine* species do not seem to be differentiated by many large-scale chromosomal rearrangements (Hay et al., 2014; Mandáková et al., 2013, 2014). Because the genus *Cardamine* is dominated by the base number x = 8 (Kiefer et al., 2014) and aneuploid chromosome numbers in *Cardamine* species are explainable by descending dysploidies (Lawrence, 1931; Mandáková et al., 2013 and the current study), an allopolyploid origin of *C. cordifolia* through hybridization between two taxa with $2n$ = 12 is unlikely.

CONCLUSION

The extant structure of the pseudotriploid genome of *C. cordifolia* is reflecting ongoing rediploidization turning a polyploid genome into a diploid one. This example illustrates how the diploidization process based on descending dysploidy might have reshuffled paleopolyploid genomes inferred for several lineages of land plants.

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LITERATURE CITED

AGI [Arabidopsis Genome Initiative]. 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* 408: 796.

Bell, C. R. 1965. Documented plant chromosome numbers 65: 3. *Sida* 2: 168–170.

Carlsen, T., W. Bleeker, H. Hurka, R. Elven, and C. Brochmann. 2009. Biogeography and phylogeny of *Cardamine* (Brassicaceae). *Annals of the Missouri Botanical Garden* 96: 215–236.

Chester, M., J. P. Gallagher, V. V. Symonds, A. V. Cruz da Silva, E. V. Mavrodiev, A. R. Leitch, P. S. Soltis, and D. E. Soltis. 2012. Extensive chromosomal variation in a recently formed natural allopolyploid species, *Tragopogon miscellus* (Asteraceae). *Proceedings of the National Academy of Sciences, USA* 109: 1176–1181.

Conant, G. C., J. A. Birchler, and J. C. Pires. 2014. Dosage, duplication, and diploidization: Clarifying the interplay of multiple models for duplicate gene evolution over time. *Current Opinion in Plant Biology* 19: 91–98.

Considine, M. J., Y. Wan, M. F. D'Antuono, Q. Zhou, M. Han, H. Gao, and M. Wang. 2012. Molecular genetic features of polyploidization and aneuploidization reveal unique patterns for genome duplication in diploid *Malus*. *PLoS One* 7: e29449.

Cui, L., P. K. Wall, J. H. Leebens-Mack, B. G. Lindsay, D. E. Soltis, J. J. Doyle, P. S. Soltis, et al. 2006. Widespread genome duplications throughout the history of flowering plants. *Genome Research* 16: 738–749.

Cusimano, N., A. Sousa, and S. S. Renner. 2012. Maximum likelihood inference implies a high, not a low, ancestral haploid chromosome number in Araceae, with a critique of the bias introduced by 'x'. *Annals of Botany* 109: 681–692.

Darlington, C. D. 1937. Recent advances in cytology. J. & A. Churchill, London, UK.

De Smet, R., K. L. Adams, K. Vandepoele, M. C. Van Montagu, and S. Maere., and Y. Van de Peer. 2013. Convergent gene loss following gene and genome duplications creates single-copy families in flowering plants. *Proceedings of the National Academy of Sciences, USA* 110: 2898-2903.

eFloras. 2015. Primary floras. eFloras.org, website http://www.efloras.org [accessed 16 October 2015], Missouri Botanical Garden, St. Louis, Missouri and Harvard University Herbaria, Cambridge, Massachusetts, USA.

Freeling, M. 2009. Bias in plant gene content following different sorts of duplication: Tandem, whole-genome, segmental, or by transposition. *Annual Review of Plant Biology* 60: 433–453.

Freeling, M., M. R. Woodhouse, S. Subramaniam, G. Turco, D. Lisch, and J. C. Schnable. 2012. Fractionation mutagenesis and similar consequences of mechanisms removing dispensable or less-expressed DNA in plants. *Current Opinion in Plant Biology* 15: 131–139.

Garsmeur, O., J. C. Schnable, A. Almeida, C. Jourda, A. D'Hont, and M. Freeling. 2014. Two evolutionarily distinct classes of paleopolyploidy. *Molecular Biology and Evolution* 31: 448–454.

Geiser, C., T. Mandáková, N. Arrigo, M. A. Lysak, and C. Parisod. 2016. Repeated whole-genome duplication, karyotype reshuffling and biased retention of stress-responding genes in Buckler mustards. *Plant Cell* 78: 1–15.

Hay, A. S., B. Pieper, E. Cooke, T. Mandáková, M. Cartolano, A. D. Tattersall, R. D. Ioio, et al. 2014. *Cardamine hirsuta*: A versatile genetic system for comparative studies. *Plant Journal* 78: 1–15.

Hohmann, N., E. M. Wolf, M. A. Lysak, and M. A. Koch. 2015. A time-calibrated road map of Brassicaceae species radiation and evolutionary history. *Plant Cell* 27: 2770–2784.

Hu, T. T., P. Pattyn, E. G. Bakker, J. Cao, J. F. Cheng, R. M. Clark, N. Fahlgren, et al. 2011. The *Arabidopsis lyrata* genome sequence and the basis of rapid genome size change. *Nature Genetics* 43: 476–481.

Humphrey, P. T., T. T. Nguyen, M. M. Villalobos, and N. K. Whiteman. 2014. Diversity and abundance of phyllosphere bacteria are linked to insect herbivory. *Molecular Ecology* 23: 1497–1515.

Ijdo, J. W., R. A. Wells, A. Baldini, and S. T. Reeders. 1991. Improved telomere detection using a telomere repeat probe (TTAGGG)n generated by PCR. *Nucleic Acids Research* 19: 4780.

Jiao, Y., J. Leebens-Mack, S. Ayyampalayam, J. E. Bowers, M. R. McKain, J. McNeal, M. Rolf, et al. 2012. A genome triplication associated with early diversification of the core eudicots. *Genome Biology* 13: R3.

Jiao, Y., N. J. Wickett, S. Ayyampalayam, A. S. Chanderbali, L. Landherr, P. E. Ralph, L. P. Tomsho, et al. 2011. Ancestral polyploidy in seed plants and angiosperms. *Nature* 473: 97–100.

Kagale, S., C. Koh, J. Nixon, V. Bollina, W. E. Clarke, R. Tuteja, C. Spillane, et al. 2014. The emerging biofuel crop *Camelina sativa* retains a highly undifferentiated hexaploid genome structure. *Nature Communications* 5: 3706.

Kiefer, M., R. Schmickl, D. A. German, T. Mandáková, M. A. Lysak, I. A. Al-Shehbaz, A. Franzke, et al. 2014. BrassiBase: Introduction to a novel knowledge database on Brassicaceae evolution. *Plant & Cell Physiology* 55: e3.

Kučera, J., I. Valko, and K. Marhold. 2005. On-line database of the chromosome numbers of the genus *Cardamine* (Brassicaceae). *Biologia* (Bratislava) 60: 473–476; database available online at http://147.213.100.117/cardamine/.

Lawrence, W. J. C. 1931. The chromosome constitution of *Cardamine pratensis* and *Verbascum phoeniceum*. *Genetica* 13: 183–208.

Lippert, W., and G. R. Heubl. 1988. Chromosomenzahlen von Pflanzen aus Bayern und angrenzenden Gebieten. *Berichte der Bayerischen Botanischen Gesellschaft* 59: 13–22.

Louda, S. M. 1984. Herbivore effect on stature, fruiting, and leaf dynamics of a native crucifer. *Ecology* 65: 1379–1386.

Louda, S. M., and J. E. Rodman. 1996. Insect herbivory as a major factor in the shade distribution of a native crucifer (*Cardamine cordifolia* A. Gray, bittercress). *Journal of Ecology* 84: 229–237.

Lysak, M. A. 2014. Live and let die: Centromere loss during evolution of plant chromosomes. *New Phytologist* 203: 1082–1089.

Lysak, M. A., K. Cheung, M. Kitschke, and P. Bureš. 2007. Ancestral chromosomal blocks are triplicated in Brassiceae species with varying chromosome number and genome size. *Plant Physiology* 145: 402–410.

Lysak, M. A., and T. Mandáková. 2013. Analysis of plant meiotic chromosomes by chromosome painting. *Methods in Molecular Biology* 990: 13–24.

Lysak, M.A., T. Mandáková, and M.E. Schranz. 2016. Comparative paleogenomics of crucifers: ancestral genomic blocks revisited. *Current Opinion in Plant Biology* 30: 108–115.

Ma, J. X., K. M. Devos, and J. L. Bennetzen. 2004. Analyses of LTR retrotransposon structures reveal recent and rapid genomic DNA loss in rice. *Genome Research* 14: 860–869.

Mandáková, T., P. B. Heenan, and M. A. Lysak. 2010a. Island species radiation and karyotypic stasis in *Pachycladon* allopolyploids. *BMC Evolutionary Biology* 10: 367.

Mandáková, T., S. Joly, M. Krzywinski, K. Mummenhoff, and M. A. Lysak. 2010b. Fast diploidization in close mesopolyploid relatives of *Arabidopsis*. *Plant Cell* 22: 2277–2290.

Mandáková, T., K. Marhold, and M. A. Lysak. 2014. The widespread crucifer species *Cardamine flexuosa* is an allotetraploid with a conserved subgenomic structure. *New Phytologist* 201: 982–992.

Mandáková, T., R. Shimizu Inatsugi, J. Zozomová-Lihová, K. Shimizu, A. Kovařík, K. Marhold, and M. A. Lysak. 2013. The more the merrier: Recent hybridization and polyploidy in *Cardamine*. *Plant Cell* 25: 3280–3295.

Mulligan, G. A. 1965. Chromosome numbers of the family Cruciferae. II. *Canadian Journal of Botany* 43: 657–668.

Murat, F., R. Zhang, S. Guizard, H. Gavranović, R. Flores, D. Steinbach, H. Quesneville, E. Tannier, and J. Salse. 2015. Karyotype and gene order evolution from reconstructed extinct ancestors highlight contrasts in genome plasticity of modern rosid crops. *Genome Biology and Evolution* 7: 735–749.

Renny-Byfield, S., A. Kovarik, L. J. Kelly, J. Macas, P. Novak, M. W. Chase, R. A. Nichols, et al. 2013. Diploidization and genome size change in allopolyploids is associated with differential dynamics of low- and high-copy sequences. *Plant Journal* 74: 829–839.

Schnable, J. C., N. M. Springer, and M. Freeling. 2011. Differentiation of the maize subgenomes by genome dominance and both ancient and ongoing gene loss. *Proceedings of the National Academy of Sciences, USA* 108: 4069–4074.

Schubert, I., and M. A. Lysak. 2011. Interpretation of karyotype evolution should consider chromosome structural constraints. *Trends in Genetics* 27: 207–216.

Slotte, T., K. M. Hazzouri, J. A. Ågren, D. Koenig, F. Maumus, Y. L. Guo, K. Steige, et al. 2013. The *Capsella rubella* genome and the genomic consequences of rapid mating system evolution. *Nature Genetics* 45: 831–835.

Soltis, D. E., V. A. Albert, J. Leebens-Mack, C. D. Bell, A. H. Paterson, C. Zheng, D. Sankoff, et al. 2009. Polyploidy and angiosperm diversification. *American Journal of Botany* 96: 336–348.

Tang, H., M. R. Woodhouse, F. Cheng, J. C. Schnable, B. S. Pedersen, G. Conant, X. Wang, et al. 2012. Altered patterns of fractionation and exon deletions in *Brassica rapa* support a two-step model of paleohexaploidy. *Genetics* 190: 1563–1574.

Vanneste, K., G. Baele, S. Maere, and Y. Van de Peer. 2014. Analysis of 41 plant genomes supports a wave of successful genome duplications in association with the Cretaceous–Paleogene boundary. *Genome Research* 24: 1334–1347.

Wang, X., H. Wang, J. Wang, R. Sun, J. Wu, S. Liu, Y. Bai, et al. and Brassica rapa Genome Sequencing Project Consortium. 2011. The genome of the mesopolyploid crop species *Brassica rapa*. *Nature Genetics* 43: 1035–1039.

Xiong, Z., R. T. Gaeta, and J. C. Pires. 2011. Homoeologous shuffling and chromosome compensation maintain genome balance in resynthesized allopolyploid *Brassica napus*. *Proceedings of the National Academy of Sciences, USA* 108: 7908–7913.

Zhang, H., Y. Bian, X. Gou, Y. Dong, S. Rustgi, B. Zhang, C. Xu, et al. 2013. Intrinsic karyotype stability and gene copy number variations may have laid the foundation for tetraploid wheat formation. *Proceedings of the National Academy of Sciences, USA* 110: 19466–19471.