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Evolution

Rapid genome change in synthetic polyploids of *Brassica* and its implications for polyploid evolution

(hybridization/restriction fragment length polymorphism/molecular evolution/cytoplasmic–nuclear interaction)

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ABSTRACT **Although the evolutionary success of polyploidy in higher plants has been widely recognized, there is virtually no information on how polyploid genomes have evolved after their formation. In this report, we used synthetic polyploids of *Brassica* as a model system to study genome evolution in the early generations after polyploidization. The initial polyploids we developed were completely homozygous, and thus, no nuclear genome changes were expected in self-fertilized progenies. However, extensive genome change was detected by 89 nuclear DNA clones used as probes. Most genome changes involved loss and/or gain of parental restriction fragments and appearance of novel fragments. Genome changes occurred in each generation from F_2 to F_5, and the frequency of change was associated with divergence of the diploid parental genomes. Genetic divergence among the derivatives of synthetic polyploids was evident from variation in genome composition and phenotypes. Directional genome changes, possibly influenced by cytoplasmic–nuclear interactions, were observed in one pair of reciprocal synthetics. Our results demonstrate that polyploid species can generate extensive genetic diversity in a short period of time. The occurrence and impact of this process in the evolution of natural polyploids is unknown, but it may have contributed to the success and diversification of many polyploid lineages in both plants and animals.**

Polyploidy is one of the most distinctive and widespread modes of speciation in higher plants. Thirty to 70% of angiosperms, including many important crop plants, are estimated to have polyploidy in their lineages (1–6). The success of polyploid species has been attributed to their ability to colonize a wider range of habitats and to survive better in unstable climates compared with their diploid progenitors (7–10), presumably due to increased heterozygosity and flexibility provided by the presence of additional alleles (11–13). Genome multiplicity also provides a genetic buffer against the effects of individual alleles; and thus, new mutations are expected to contribute less to the evolution of polyploids compared to diploids (6). However, this hypothesis assumes that diploids and polyploids have equal mutation rates. It is possible that genome change is greatly accelerated in new polyploids derived from interspecies hybrids, due to greater instabilities created by the interaction of diverse genomes. Such changes could result in rapid genetic divergence of newly formed polyploids and might have contributed to the evolutionary success of many polyploid lineages.

The potential contribution of genome change to the evolution of polyploids has been overlooked, mainly due to lack of information on how polyploid genomes have evolved after their formation. There is some indirect evidence for genetic changes in polyploids after their formation. For example, molecular data on the cultivated *Brassica* species indicate that the polyploid genomes of *Brassica juncea*, *Brassica napus*, and *Brassica carinata* are different from the genomes of their diploid progenitors *Brassica rapa*, *Brassica nigra*, and *Brassica oleracea*, as reflected by alterations in restriction fragment length polymorphism (RFLP) patterns (14) and the linkage orders of RFLP loci (15). However, these and other studies on polyploid evolution (5, 16) have compared natural polyploids, which are usually hundreds or thousands of years old, to present forms of hypothesized progenitors. Thus, it was not possible to distinguish between genome change after formation of the polyploid and genome divergence within the diploid progenitor species or to determine how quickly newly formed polyploid genomes evolve.

Synthetic polyploids provide a model system to study early events in the evolution of polyploid genomes. Because the exact progenitors for a synthetic polyploid are known, we can determine precisely whether extensive genome changes occur after synthesis of polyploids and if so, the timing and processes of genome changes. We recently developed a series of synthetic *Brassica* polyploids by reciprocal interspecific hybridizations between the diploid species, followed by chromosome doubling of the F_1 hybrids (17). We now report direct evidence for nuclear genome changes in these synthetic polyploids on the basis of comparing RFLP patterns of synthetic polyploids and their self-pollinated progenies by using a large number of cloned DNA probes.

MATERIALS AND METHODS

Plant Materials. The synthetic polyploids used in this study were derived from reciprocal interspecific hybridizations between single plants of the diploid species *B. rapa* (A genome), *B. nigra* (B genome), and *B. oleracea* (C genome) (17) and are designated as AB (A × B), BA (B × A), AC (A × C), and CA (C × A). AB and BA are reciprocal hybrids analogous to the natural polyploid *B. juncea*, and AC and CA are reciprocal hybrids analogous to the natural polyploid *B. napus*. The colchicine doubled hybrids were bud self-pollinated to form F_2 progenies (17). For each synthetic polyploid, nine F_5 plants were derived from a single F_2 plant by controlled bud self-pollination in isolation.

Fertility, Cytology, and Molecular Characterization. The fertilities of F_5 plants were determined by number of seeds per self-pollination based on at least 100 pollinations and expressed as percentages of seed set per self-pollination of cultivars (*B. napus* cv. Westar for AC/CA and *B. juncea* cv. Domo for AB/BA). Chromosome counting and meiotic analyses of F_5 plants were conducted by using described methods (17). RFLP analyses were conducted as described (17) by using DNAs of the parental lines A, B, and C (isolated from 15 to 20 plants that were self-pollinated progenies of the original parental plants) and DNAs of synthetic polyploids (isolated

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Abbreviation: RFLP, restriction fragment length polymorphism.

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from single plants). DNA samples were digested with restriction endonucleases *Eco*RI or *Hin*dIII unless indicated otherwise, and Southern blots were probed with 19 anonymous nuclear DNA clones, 63 cDNA clones, 7 cloned nuclear genes of known function, 6 chloroplast DNA clones, and 5 mitochondrial DNA clones. Some of the nuclear DNA clones were used previously for RFLP mapping (15), and a list of the names and sources of clones are available upon request.

Data Analysis. Each restriction fragment detected by the 89 nuclear DNA probes using a single restriction enzyme was scored as present or absent for single plants of each synthetic polyploid and the diploid parents. The percentages of dissimilar restriction fragments were calculated for pairs of individuals using binary data sets of ones and zeros for the presence and absence of restriction fragments. Average genetic distances were calculated as the mean percentage dissimilarity for all pairs of plants.

RESULTS AND DISCUSSION

We analyzed four synthetic polyploids, AB, BA, AC, and CA, for rapid genome changes by comparing RFLP patterns between single F_2 plants and their self-pollinated F_5 progenies. Parental diploid species also were included in the RFLP analysis to verify the donor genome of individual fragments. Some fragments present in parental diploid lines were not present in the corresponding F_2 polyploid plants, probably because the original single parental plants were heterozygous at some loci and they transmitted only one of the alleles at those loci. However, since each synthetic polyploid was completely homozygous after chromosome doubling, differences in RFLP patterns between the F_2 and F_5 plants should be indications of genome changes instead of resulting only from segregation.

Patterns, Timing, and Frequency of Genome Change. RFLP analyses using six chloroplast and five mitochondrial DNA probes indicated that all F_5 plants examined had the same patterns as those of their F_2 parents, and these patterns matched those of the female diploid parents. In contrast, a wide range of changes was observed in the nuclear genomes of F_5 plants. Most nuclear genome changes involved loss or gain of parental restriction fragments, as well as appearance of novel fragments in F_5 plants (Table 1). In some cases, restriction fragments appeared in F_5 plants that were the same (based on molecular weight) as fragments present in diploid parents, but these fragments were not observed in the F_2 plants (Fig. 1*A*; designated as gains of parental types in Table 1). In one case, we observed a fragment in five BA F_5 plants that was not found in either the A or B genome parents, but an identical size fragment was present in the C genome parent and all AC and CA F_5 individuals (Fig. 1*B*).

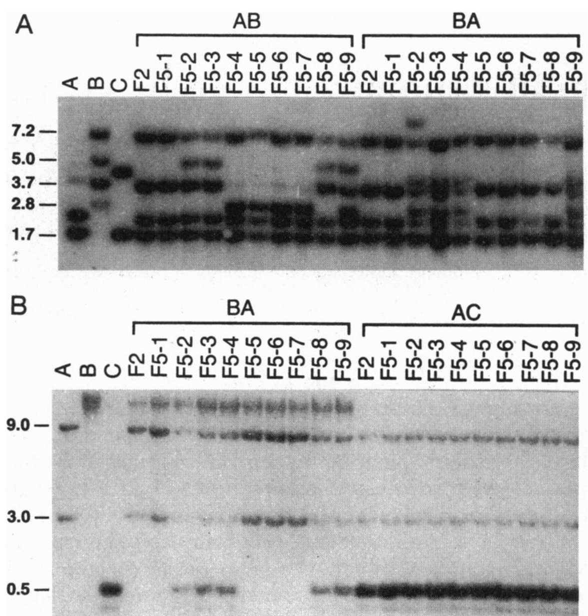

FIG. 1. Examples of genome change in nine F_5 plants derived from single F_2 plants of synthetic polyploids AB, BA, and AC. Diploid parent genotypes are labeled A, B, and C. (*A*) *Hin*dIII-digested DNAs probed with EZ3 (a genomic DNA clone of an anonymous open reading frame from *Arabidopsis thaliana* provided by Howard Goodman, Massachusetts General Hospital, Boston), showing loss of fragments and gain of diploid parental type fragments (5.0 kb and 2.8 kb) in some F_5 plants. (*B*) *Hpa* II-digested DNAs probed with EC3C8 (an anonymous cDNA clone from *B. napus*) showing gain of a 0.5-kb fragment in five BA F_5 plants that does not exist in either the A or B parental genome, but the same size fragment is present in the C genome parent and all AC F_5 plants. These same RFLP patterns also were detected with *Msp* I, suggesting that changes in methylation did not cause these differences. Both *A* and *B* have been repeated three times using the same DNAs and enzymes but different blots.

These results suggest that some genome changes resulted in restriction fragments that were pre-existing in a parent or in a related genome.

The timing of genome change was examined by testing individuals from all generations with several probes that detected genome changes. Changes in RFLP patterns were detected in each generation from F_2 to F_5 (data not shown). Therefore, the numbers listed in Table 1 represent the total changes accumulated from the F_2 to the F_5 generations.

The frequencies of genome change were different between the two polyploid species. Twice as many genome changes were detected in the AB and BA polyploids as in the AC and CA

Table 1. Frequencies and types of genome changes in F_5 progenies of synthetic polyploids compared to F_2 parents

					Types of fragment changes†					
					Loss/gain				Loss	Gain
Polyploid line*	Plants, no.	Probes, no.	Probes detecting changes, no.	Fragments changed, no.	A	B	C	Shared fragments	F_2 fragments	Novel fragments
AB F_5	9	82	43	96	9/13	25/12		9/1	9	19
BA F_5	9	82	59	95	8/12	14/0		4/1	5	51
AC F_5	9	89	23	38	7/1		19/4	3/0	4	1
CA F_5	9	89	31	51	15/1		16/5	7/0	3	4

Genome changes were assessed for each polyploid line by direct comparisons between nine F_5 plants and a single F_2 parental plant for nuclear RFLP patterns from *Eco*RI or *Hin*dIII digests, and data from the nine F_5 plants were pooled.

*AB, BA, AC, and CA are synthetic polyploids derived from hybridizations of diploid parents A × B, B × A, A × C, and C × A, respectively.

†Loss = fragments present in diploid parent and in the F_2 but not present in F_5 plants; gain = diploid parental fragments absent in F_2 plants but present in F_5 plants. A, B, and C = fragments specific to A, B, or C parental genomes; shared fragments = fragments shared by both diploid parents; F_2 fragments = fragments found in the F_2 but not found in either diploid parent; novel fragments = fragments found only in F_5 plants.

polyploids (Table 1). Previous studies have shown that *B. rapa* (A genome) is substantially more closely related to *B. oleracea* (C genome) than to *B. nigra* (B genome) (14, 18–20). Therefore, higher frequencies of genome change were associated with higher degrees of divergence between the parental diploid genomes.

Potential Causes of Genome Changes. The high frequency of genome change we observed was presumably due to genetic instabilities in the new polyploids that were manifested after several generations of self-pollination. Results from analyzing inbred genotypes of the diploid *B. rapa* suggest that inbreeding alone is unlikely to have caused this high level of genome change. Five F_5 plants derived by self-pollination from an F_1 hybrid of the cultivars Per and R500 were compared to their F_2 parent plants for RFLP patterns detected by 35 nuclear DNA clones, and no changes were detected other than loss of restriction fragments at heterozygous loci.

The changes we observed could have resulted from several different processes, such as chromosome rearrangement, point mutation, gene conversion, DNA methylation, and others yet to be described. Loss of chromosomes was not an important factor because all F_5 plants had the expected chromosome numbers, except for one plant (CAF5-9), which appeared to be missing one chromosome (data not shown). Our preliminary data suggest that chromosome rearrangements involving intergenomic (nonhomologous) recombination could be a major factor contributing to genome change. In the F_2 and F_3 generations (17) and in the F_5 generation of the synthetic polyploids, we observed a high frequency of aberrant meioses with chromosome bridges, chromosomes lagging, and multivalents. These aberrant meioses probably indicate intergenomic chromosome associations and could have resulted in loss of RFLP fragments through subsequent segregation of recombined or broken chromosomes. A small frequency of these events could result in gain of novel fragments due to recombination events within the probed regions.

Intergenomic associations also could provide the opportunity for gene-conversion-like events, and as evidence for this, we observed simultaneous loss/gain of parental restriction fragments for some of the probes. For example, using the probe EZ3, we found that the gain of a 2.8-kb *Hin*dIII fragment in plants ABF5-4, -5, -6, and -7 was accompanied by the loss of a 3.7-kb fragment (Fig. 1*A*). Similarly, plants ABF5-2, -3, -8, and -9 gained one copy of a 5.0-kb fragment and appeared to lose one copy of a 7.2-kb fragment based on reduced signal intensity (Fig. 1*A*). Further analysis of F_6 progenies from ABF5-2 and -8 using EZ3 indicated that the 7.2-kb and 5.0-kb fragments segregated as alleles, and concerted gain/loss of the 2.8-kb/3.7-kb fragments occurred in some of these F_6 plants. These types of changes can be explained by gene-conversion-like events, which have been shown to occur in yeast through a mechanism involving nonhomologous recombination (21, 22). However, further characterization is needed to determine the mechanism leading to these changes.

Changes in DNA methylation also could account for changes in restriction fragments. The restriction enzymes we used (*Eco*RI and *Hin*dIII) do not recognize the symmetric CpG or CpNpG sites that are predominant DNA methylation sites in both animals and plants (23, 24), and although *Eco*RI and *Hin*dIII can be inhibited by DNA methylation (25), there is no direct evidence showing methylation at these restriction sites in nuclear genomes of higher plants. Therefore, most genome changes that we observed were probably not due to changes in DNA methylation. To test the potential contribution of DNA methylation to genome changes, we used 26 of the DNA clones that detected genome changes in *Eco*RI and/or *Hin*dIII digestions as probes on blots containing DNA restricted with *Hpa* II and *Msp* I. Because *Hpa* II and *Msp* I recognize the same restriction site, CCGG (one of the predominant DNA methylation sites), but have different sensitivities to methylation positions (25), differences in RFLP patterns revealed by the two enzymes should indicate changes in DNA methylation at specific positions. Seven probes detected changes in RFLP patterns in the F_5 plants that were identical or very similar for the two restriction enzymes. Only two probes detected changes that appeared to be due to changes in methylation (Fig. 2). These results indicated that changes in DNA methylation could contribute to genome changes, but this was not a major factor.

Genetic Consequences of Genome Change. For each of the synthetic polyploids, genome changes resulted in rapid genetic divergence of its derivatives from each other and from the original F_2 plant. The relative magnitudes of divergence were calculated by using pairwise genetic distance estimates based on the percentage of dissimilar restriction fragments. The average genetic distances between F_5 plants and their F_2 parents were 9.6% for AB, 8.2% for BA, 4.1% for AC, and 3.7% for CA, and the average distances among F_5 plants were 7.7% for AB, 9.4% for BA, 2.1% for AC, and 2.5% for CA. Phenotypic variation among F_5 plants within each polyploid provided additional evidence for genetic divergence. Fertilities among F_5 plants ranged from 0% to 24.9% for the AB/BA synthetics and from 0% to 100% for the AB/BA synthetics. F_5 plants also varied for morphological traits, such as leaf shape and color, branching patterns, and number of side shoots.

Directional Genome Change and Cytoplasmic Effect. The synthetic polyploids were analyzed for directional changes in their component genomes by comparing genetic distances of F_2 and F_5 individuals to each of their diploid parents (Fig. 3). For the AB polyploid, the nine F_5 plants showed significant directional change away from the B genome parent but not from the A genome parent (Fig. 3, AB). Most of the directional change was probably due to greater loss of B genome fragments than A genome fragments (25 vs. 9, respectively, Table 1). For the BA polyploid, there was no significant directional change when all nine F_5 plants were included in the analysis (Fig. 3, BA). However, five of the plants (BAF5-2, -3, -4, -8, and -9) derived from two F_4 plants showed much higher levels of change, and these five plants deviated significantly from both the A and B diploid parents. In four of the five plants, the A genome changed more than the B genome, an opposite situation to that observed in the AB polyploid.

Because the synthetic polyploids we developed contained maternally donated cytoplasm (17), differences in cytoplasmic–

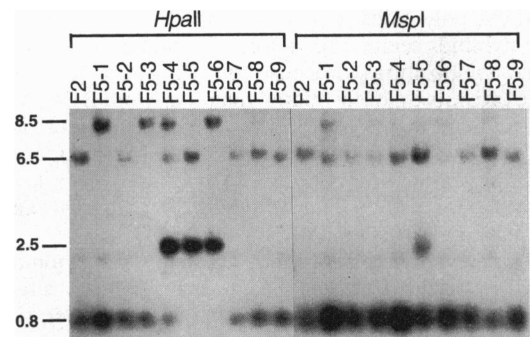

FIG. 2. Evidence for DNA methylation changes in AC synthetic polyploids. DNAs from nine F_5 plants of the AC polyploid and their parental F_2 plant were digested with *Hpa* II and *Msp* I restriction enzymes and probed with EC2F2 (an anonymous cDNA clone from *B. napus*). A 0.8-kb fragment is present in DNAs from all plants digested with *Msp* I but is absent or faint in the *Hpa* II digestion of F5-4, -5, and -6 DNAs, suggesting methylation at the C5mCGG position in these plants. Appearance of a 2.5-kb fragment in the *Msp* I-digested DNA of F5-5 suggests a partial DNA methylation at the 5mCCGG position. Appearance of an 8.5-kb fragment in F5-1, -3, -4, and -6 digested with *Hpa* II might represent another change in DNA methylation, but this fragment is faintly present in the same DNAs digested with *Msp* I. This result was obtained twice with different blots.

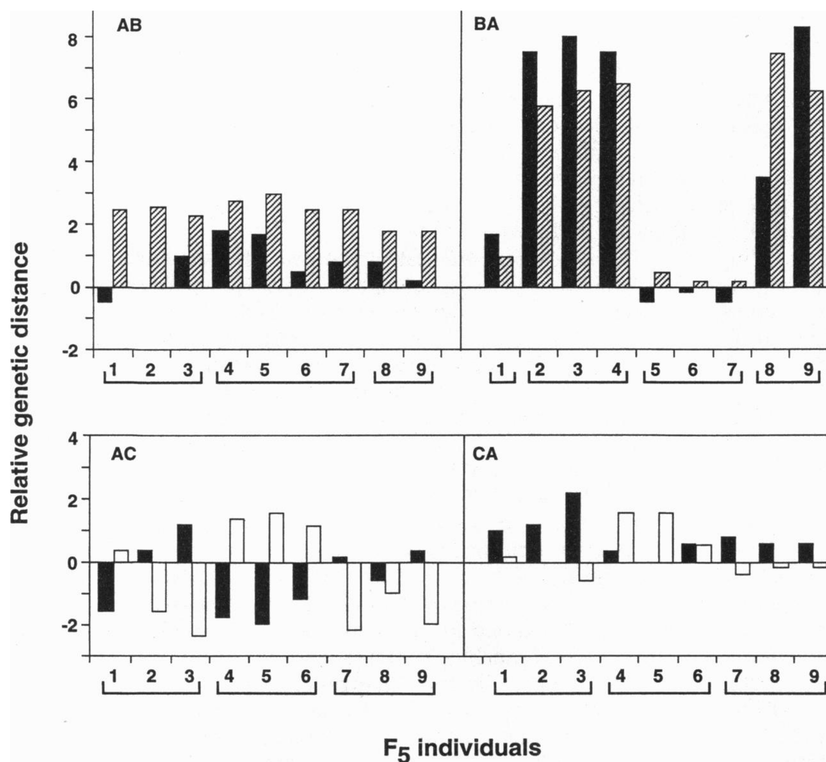

FIG. 3. Relative genetic distances between F_5 individuals of the four synthetic polyploids (AC, CA, AB, and BA) and the diploid parental genomes A (black bars), B (hatched bars), and C (open bars). The F_5 individuals within a bracket are derived from the same F_4 plant, whereas plants in different brackets are from different F_4 plants. For each polyploid, relative genetic distances were calculated as (% dissimilarity between an F_5 plant and its diploid parent) − (% dissimilarity between the F_2 plant and its diploid parent). A positive value means that the overall genome composition of the F_5 plant is more distant from the diploid parent than is the F_2 plant, and a negative value means that the F_5 is more similar to the diploid than is the F_2. Statistical tests were performed to determine whether the mean relative genetic distance of a polyploid deviated significantly from zero (z = mean distance/SD). The mean distance (SD) of AC to A = −0.55 (1.1), $P > 0.31$; AC to C = −0.51 (1.6), $P > 0.38$; CA to A = 0.82 (0.6), $P > 0.10$; CA to C = 0.29 (0.8), $P > 0.35$; AB to A = 0.7 (0.7), $P > 0.18$; AB to B = 2.4 (0.4), $P < 0.0001$; BA to A = 3.9 (3.9), $P > 0.15$; and BA to B = 3.8 (3.2), $P > 0.12$. For BAF5-2, -3, -4, -8, and -9 the mean relative distance of BA to A = 7.1 (2.0), $P < 0.001$ and BA to B = 6.0 (1.1), $P < 0.0001$.

nuclear interactions of specific genomes could have contributed to differences in the extent and direction of genome changes. The AB polyploid contained the A genome cytoplasm, and in these plants the paternally donated nuclear genome (B genome) showed significant directional change, whereas the maternally donated nuclear genome (A genome) did not. These results are consistent with our previous study showing that the AB nuclear genome of the natural *B. juncea* polyploid, which has the A cytoplasm, is more similar to *B. rapa* (A genome) than to *B. nigra* (B genome) (14). Interpretation of results from the BA polyploid was hindered by the highly variable rates of genome changes among F_5 progenies; however, the paternally donated nuclear genome (A genome) also showed significant directional change in a subset of rapidly changing F_5 plants.

Significant directional changes were not observed for the AC and CA polyploids (Fig. 3, AC and CA). Thus, changes in these polyploids seemed to cause only random fluctuation of genome compositions. Because the A and C cytoplasmic genomes are more closely related than the A and B cytoplasmic genomes (14), the absence of directional changes and overall lower frequencies of genome change in the AC and CA vs. AB and BA polyploids may be due, in part, to higher levels of cytoplasmic–nuclear genome compatibility in the AC and CA polyploids.

Implications of Rapid Genome Change for Polyploid Evolution. Using synthetic polyploids, we have demonstrated that extensive genome change can occur in the early generations of *Brassica* polyploids. Genetic diversity accumulated among self-fertilized progenies, even when the starting materials were completely homozygous. We do not know whether these types of changes or this extent of change has occurred in the early generations of natural *Brassica* or other polyploid species. However, our molecular results, when combined with variation in fertility and other morphological traits observed in our synthetic polyploids and in previous studies (26, 27), suggest that rapid genome change in newly formed polyploids can produce many novel genotypes that would provide new genetic variation for selection. Thus, rapid genome change could accelerate evolutionary processes among progenies of newly formed polyploids, and this may, in part, account for the success and diversification of many ancient polyploid lineages in both plants and animals.

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