Genomic Changes in Resynthesized *Brassica napus* and Their Effect on Gene Expression and Phenotype

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Many previous studies have provided evidence for genome changes in polyploids, but there are little data on the overall population dynamics of genome change and whether it causes phenotypic variability. We analyzed genetic, epigenetic, gene expression, and phenotypic changes in ~50 resynthesized *Brassica napus* lines independently derived by hybridizing double haploids of *Brassica oleracea* and *Brassica rapa*. A previous analysis of the first generation (S0) found that genetic changes were rare, and cytosine methylation changes were frequent. Our analysis of a later generation found that most S0 methylation changes remained fixed in their S5 progeny, although there were some reversions and new methylation changes. Genetic changes were more frequent in the S5 generation, occurring in every line with lines normally distributed for number of changes. Genetic changes were detected on 36 of the 38 chromosomes of the S5 allopolyploids and were not random across the genome. DNA fragment losses within lines often occurred at linked marker loci, and most fragment losses co-occurred with intensification of signal from homoeologous markers, indicating that the changes were due to homoeologous nonreciprocal transpositions (HNRTs). HNRTs between chromosomes A1 and C1 initiated in early generations, occurred in successive generations, and segregated, consistent with a recombination mechanism. HNRTs and deletions were correlated with qualitative changes in the expression of specific homoeologous genes and anonymous cDNA amplified fragment length polymorphisms and with phenotypic variation among S5 polyploids. Our data indicate that exchanges among homoeologous chromosomes are a major mechanism creating novel allele combinations and phenotypic variation in newly formed *B. napus* polyploids.

INTRODUCTION

Polyploidy is thought to play a significant role in flowering plant evolution, and 30 to 80% of extant species are considered polyploid (Masterson, 1994; Ramsey and Schemske, 1998; Otto and Whitton, 2000; Meyers and Levin, 2006; Rieseberg and Willis 2007). Recent evidence suggests that most major lineages of angiosperms have undergone genome doubling over evolutionary time, suggesting that most or all diploids may be ancient polyploids (Cui et al., 2006). Polyploids can often be distinguished from their presumed progenitors with respect to morphology, ecology, cytology, and physiology (Levin, 1983; Ramsey and Schemske, 2002). Variation in these and other phenotypic traits may contribute to niche exploitation and speciation (Rieseberg and Willis, 2007) and might also enhance the utility of polyploids for agriculture. This variation is difficult to study in natural polyploids that often have extinct or unknown parents, while newly resynthesized allopolyploids with known parents permit more critical comparisons of polyploids and progenitors. There is growing evidence from such comparisons that polyploids have novel phenotypic variation and that polyploidy can lead to changes in gene expression through gene dosage effects, altered regulatory interactions, and genetic and epigenetic changes (reviewed in Soltis and Soltis, 1995; Guo et al., 1996; Ramsey and Schemske, 2002; Osborn et al., 2003b; Soltis et al., 2004; Adams and Wendel, 2005; Chen and Ni, 2006).

Evidence for genomic changes in nascent polyploid taxa comes from observations of resynthesized polyploids (e.g., *Arabidopsis suecica, Brassica napus*, wheat [*Triticum aestivum*], cotton [*Gossypium hirsutum*], *Nicotiana tabacum*, and *Triticale*) and natural polyploid species that have well-documented parentage (e.g., *Tragopogon, Senecio, Sparta*, and *Glycine*). Among these examples, there have been reports of deletion events (Ozkan et al., 2001; Shaked et al., 2001; Ma and Gustafson, 2006; Tate et al., 2006), gene conversion events (Wendel et al., 1995; Kovarik et al., 2004, 2005), rDNA loci changes (Joly et al., 2004; Pontes et al., 2004), transposon activation (Kashkush et al., 2002, 2003; Madlung et al., 2005), chromosomal rearrangements (Kenton et al., 1993; Parkin et al., 1995; Lim et al., 2004, 2006; Pires et al., 2004; Udall et al., 2005), and epigenetic phenomena (Chen and Pikaard, 1997a, 1997b; Adams et al., 2003; Hegarty et al., 2005; Madlung et al., 2005; Salmon et al., 2005; Lukens et al., 2006; Wang et al., 2004). However, little is known regarding the mechanisms leading to these changes, their population dynamics, and their impact on gene expression and phenotype in new polyploids.

Genetic changes, including chromosomal rearrangements, transpositions, and deletions, can lead to altered gene expression in allopolyploids (Osborn et al., 2003b; Chen and Ni, 2006).
Pires et al. (2004) detected homoeologous rearrangements in resynthesized *B. napus* that altered the expression of parental FLC genes. In *Tragopogon*, the loss of DNA fragments was shown to be the major cause of cDNA amplified fragment length polymorphisms (AFLPs) (Tate et al., 2006). Epigenetic changes, including DNA methylation, histone modifications, and RNA interference, also may lead to changes in gene expression in newly formed polyploids (Chen and Pikaard, 1997a; Comai, 2000; Liu and Wendel, 2003; Osborn et al., 2003b; Madlung and Comai, 2004; Adams and Wendel, 2005). Studies in wheat and *Arabidopsis* allopolyploids have found that changes in cytosine methylation correlate with altered gene expression (Lee and Chen, 2001; Kashkush et al., 2002) and reactivation of silent transposons (Madlung et al., 2005). Genetic and epigenetic changes are predicted to affect phenotypic variation, and phenotypic studies in newly formed *B. napus* allopolyploids previously demonstrated the emergence of heritable de novo variation in flowering time and other life history traits (Schranz and Osborn, 2000; Pires et al., 2004; Schranz and Osborn, 2004). Studies have suggested that homoeologous rearrangements might be responsible for altering variation in seed yield (Osborn et al., 2003a), flowering time (Pires et al., 2004), and disease resistance (Zhao et al., 2006). However, most previous studies on polyploidy have lacked enough independent polyploid events to understand the population dynamics of such changes and have been unable to decipher mechanisms of genetic change due to a lack of mapped, genome-wide, dosage-sensitive molecular markers. There has also been a paucity of integrated genotypic, gene expression, and phenotypic data.

In this study, we investigated genetic, epigenetic, transcriptional, and phenotypic changes among ~50 independently resynthesized *B. napus* lines derived by hybridizing double haploid lines of *Brassica oleracea* and *Brassica rapa*. This relatively large population allowed for a comprehensive analysis of population-wide and genome-wide changes following polyploidization. Previously, we analyzed this population at the S₀ generation and found that genetic changes were rare and cytosine methylation changes were frequent (Lukens et al., 2006). Here, we analyzed the same population of resynthesized polyploids at the S₀ and S₅ generations using simple sequence repeat (SSR), restriction fragment length polymorphism (RFLP), cDNA single-strand conformation polymorphism (SSCP), and cDNA-AFLP markers, and we measured 13 phenotypic traits in a replicated experiment. We present evidence for a mechanistic link between genetic changes in newly resynthesized *B. napus* and changes in gene expression and phenotypic variation.

**RESULTS**

**Genetic Changes in S₅ Resynthesized *B. napus* Allopolyploids**

Analysis of 368 HindIII, EcoRI, or Dral RFLP markers and 65 SSR markers in 47 S₅ lines of resynthesized *B. napus* revealed fragment losses in every line, with 33% of the RFLP markers and 71% of the SSR markers showing changes among the S₅ lines. Approximately 48% of A-genome–specific (*B. rapa*) and 34% of C-genome–specific (*B. oleracea*) RFLP and SSRs showed changes. The S₅ lines were highly variable (Figure 1) and normally distributed (Shapiro-Wilk test; \( P = 0.3824 \)) for total number of fragment changes. All but two lines lost both A- and C-genome markers. There was a marginal significant difference between the average number of A-genome losses per S₅ line (mean 6.6, SD = 4.3) and the average number of C-genome losses per S₅ line (mean 8.6, SD = 5.6; \( t \) test, \( P = 0.048 \)), and the proportion of A- and C-genome fragment losses among S₅ lines was unequal (4.1 and 4.7%, respectively; \( \chi^2 = 3.92, P = 0.047; \) Table 1). There were no differences between the mean number of changes per colchicine-doubled S₅ line (mean 15.6, SD = 7.1) and the mean number of changes per spontaneously doubled S₅ line.

![Figure 1](image-url)  
*Figure 1.* Number of DNA Fragment Losses in Each S₅ Line of Resynthesized *B. napus*. The sum of all fragment losses (RFLP and SSR) is shown for each of 42 colchicine-doubled and 10 spontaneously doubled lineages (asterisks). Lineages connected by arrows were derived from the same S₀ plants, one by colchicine doubling (lines 17 and 19) and one by spontaneous doubling (lines 28 and 39). Line numbers 28 and 39 were considered nonindependent measures and were not included in the statistical analyses. Three lines died out at the S₁, S₃, and S₅ generations and are labeled accordingly. These lines were not included in statistical analyses of the S₅ generation. The portion of markers lost from the A (*B. rapa*) and C (*B. oleracea*) genomes of *B. napus* are shown with gray and black bars, respectively.
Table 1. Summary of Genetic Changes among 47 Resynthesized S5 B. napus Allopolyploids

<table>
<thead>
<tr>
<th>Marker Type</th>
<th>Genome Doubling</th>
</tr>
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<tbody>
<tr>
<td>Total</td>
<td>SSR</td>
</tr>
<tr>
<td>Total no. DNA fragments expected&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20,167</td>
</tr>
<tr>
<td>Total no. DNA fragments lost (%)</td>
<td>713 (3.5)</td>
</tr>
<tr>
<td>No. A fragments expected&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>7,589</td>
</tr>
<tr>
<td>No. A fragments lost (%)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>308 (4.1)</td>
</tr>
<tr>
<td>No. C fragments expected&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>8,597</td>
</tr>
<tr>
<td>No. C fragments lost (%)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>405 (4.7)</td>
</tr>
<tr>
<td>No. AC fragments expected&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>3,981</td>
</tr>
<tr>
<td>No. AC fragments lost&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>No. AC fragments lost&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Method of allohaploid genome doubling. Colch., colchicine; Spont., spontaneously.

<sup>b</sup>Expected number of fragments is equal to the product of total markers scored and the number of lines, minus missing data, and is the number expected under parental additivity.

<sup>c</sup>In 71% of the 509 cases in which an RFLP fragment was lost, it was associated with intensification of a fragment from the other parental subgenome, suggesting that a loss/duplication had occurred. These data were not collected for SSR markers because PCR products are not easily quantifiable.

<sup>d</sup>Designations A (B. rapa), C (B. oleracea), and AC (common to both) indicate the genome of origin of the markers scored.

Approximately 71% of RFLP marker fragment losses (Table 1) occurred concomitantly with an intensification of a homoeologous RFLP, consistent with loss of one locus and duplication of the other (Figure 2). SSR markers detected the loss of parental markers, but they could not be used to detect the duplications. Patterns of loss and intensification often occurred at linked markers on homoeologous linkage groups (Figure 2), suggesting that the changes resulted from exchanges between homoeologous chromosomes leading to homoeologous nonreciprocal transpositions (HNRTs) (Pires et al., 2004; Udall et al., 2005). The large number of markers available for A1 and C1 allowed an in-depth analysis of the HNRTs present in the S5 generation (A1 is B. rapa linkage group 1 and is equivalent B. napus linkage group N1; C1 is B. oleracea linkage group 1 and is equivalent B. napus linkage group N11; see Methods for explanation of Brassica linkage group nomenclature). Twenty-nine of the 47 S5 lines showed genetic changes on these chromosomes (Figure 3). Changes in marker hybridization patterns often affected blocks of multiple linked markers. Loss of markers in a block was typically associated with intensification of markers on the homoeologous block (e.g., bottom of A1/C1). Exceptions include SSR markers (explained above) and some RFLP markers, for which the homoeologs may not have been detectable due to gel migration pattern or divergence or absence of the homoeologous sequence in one of the parents. Most of the altered segments included markers at the ends of chromosomes (e.g., bottom of C1 in first three lines of Figure 3), although some interstitial changes were observed (e.g., top of C1 in line 2 and bottom of C1 in line 9 of Figure 3).

Dynamics of Genetic Changes across the B. napus Chromosomes

Genetic changes (fragment losses and loss duplications) were detected on 36 of 38 chromosomes of B. napus despite the limitation that only 133 RFLP and SSRs (31% of all markers) could be assigned a map location (see Methods). The frequency of genetic changes across linkage groups was not equal (Wald $\chi^2 = 172.7$, $P < 0.0001$). The linkage group with the highest frequency of changes was C1 (20.8%), one linkage group could not be assigned any markers (A4), and one (A8) showed no changes (see Supplemental Table 1 online). Homoeologous linkage groups A1/C1, A2/C2, and A3/C3 are each syntenic along their entire length (Parkin et al., 1995, 2005; Udall et al., 2005). Neither the A2/C2 nor A3/C3 homoeologous pairs showed significant biases in the frequency of B. rapa or B. oleracea–derived fragment losses. However, at A1/C1, there was a significant bias toward C1 losses (20.8%) over A1 losses (7.9%) ($\chi^2 = 17.52$, $P < 0.0001$). No evidence for nullisomic-tetrasomic aneuploids was found among any of the 47 independent lines. However, we cannot rule out the loss of a chromosome after an exchange occurred.

Transgeneration and S1 Segregant Analyses Support a Recombination-Based Mechanism

To determine when rearrangements occurred between the S0 and S5 generations, we conducted a transgenerational analysis of six allopolyploid lineages for markers on A1/C1. We detected genetically fixed HNRTs in bulked DNA samples as early as the S2 generation and as late as the S5 generation (see Supplemental Figures 1 and 2 online). We then genotyped 14 to 16 individuals in the generation prior to our detection of a fixed HNRT and detected an average of 3.6 (range 1 to 6) individuals carrying a homozygous rearrangement. Regardless of whether an HNRT in a line appeared interstitial or terminalized in the S5 analysis, we frequently observed variable lengths or forms of the expected rearrangement segregating in the prior generation (see
Supplemental Figure 3 online). We did not detect HNRTs fixed for the other homoeolog segregating in the generation prior to a fixed rearrangement that would have derived from an intact reciprocal exchange; however, we did in some cases detect partial reciprocal rearrangements segregating that could have derived from a reciprocal exchange with additional recombinations (see Supplemental Figure 4 online).

In a previous study, evidence for chromosomal rearrangements (HNRTs) was not observed in the S0 generation (Lukens et al., 2006). To see if rearrangements arose after the first meiosis, we analyzed four markers that spanned three pairs of homoeologous chromosomes (A1/C1, A2/C2, and A3/C3) in 10 to 16 S1 progeny from each of eight S0 allopolyploids. Of the 118 segregants analyzed among the eight lines, three had evidence of a rearrangement (see Supplemental Figure 5 online).

DNA Methylation Changes in S5 Resynthesized B. napus Allopolyploids

Analysis of 337 HpaII and 320 MspI RFLP markers revealed methylation changes in every S5 line, with 60% of the HpaII and 54% of the MspI markers showing changes. The same markers were analyzed in the S0 generation (Lukens et al., 2006), and the results were compared (see Supplemental Figure 5 online). The 47 independent S5 lines were normally distributed for number of methylation changes (Shapiro–Wilk test; P = 0.1054). The proportion of total HpaII fragment changes among S5 lines (loss and reversions; 2.1%) was greater than the proportion of MspI fragment changes (0.1%) (χ² = 249.8, P < 0.0001; data not shown). Thus, CpG methylation changes were observed at nearly a 20-fold greater rate than CpNpG changes in the S5 generation. The proportion of total A-genome fragment changes (1.8%) among S5 lines was significantly greater than the proportion of total C-genome fragment changes (1.3%) (χ² = 9.003, P = 0.003; data not shown). The proportion of fragment changes in colchicine-doubled lines (1.0%) was not different from the proportion of changes among spontaneously doubled lines (1.4%) (χ² = 2.765, P = 0.096; data not shown).

Loss of Specific DNA Markers and Transcripts among S0 and S5 Polyploid Lines

We assayed for transcriptional changes in three gene targets for which we detected HNRTs among S5 polyploid lines. The RFLP probe pW225 detected HNRTs between linkage groups A1 and C1 in many polyploids (Figures 2 and 3). Rearrangement at this locus was perfectly correlated with the loss and intensification of homoeologous pW225 RNA transcripts among S5 polyploids.
Evidence for changes in CpG methylation occurred in two lines probed with pW225, but there was no evidence for changes in pW225 transcripts in these lines (data not shown). RFLP and PCR analyses of the Brassica FLC-3 and FLC-5 genomic loci also identified HNRTs among S5 polyploids. FLC-3 DNA marker losses were correlated with the loss of parental transcripts (all SSRs and some RFLPs), and black bars indicate missing data. Markers are listed to the left and were assigned to linkage groups A1 and C1 based on mapping data: *, markers with identical fragments mapped in B. oleracea, B. rapa, and B. napus populations (our unpublished data; Udall et al., 2005); **, marker assigned to linkage group based on mapping studies with the same probe for which a single locus was predicted (our unpublished data; Udall et al., 2005; D. Lydiate and A. Sharpe, personal communication); *** marker assigned to linkage group based on mapping data with the same probe for which two or more loci were predicted; these markers were assigned to this linkage group based on coincident losses in other markers. Some differences exist between mapping data and these marker orders (e.g., pW145Ef and pW220 on C1 are in the opposite order of that suggested by our unpublished data). These differences may be explained by the relatively short centimorgan distance between these markers and because we had so few lines and markers for analysis.

DNA Marker Losses Correlate with the Loss of cDNA-AFLP Fragments among S5 Polyploid Lines

Analysis of 360 cDNA-AFLP markers in resynthesized B. napus revealed that 4% of all S0 markers showed changes among the lines. Approximately 5% of the A-genome cDNA-AFLP markers and 6% of C-genome cDNA-AFLP markers showed changes in the S0 generation. All changes were fragment losses confirmed in two biological replicates (see Supplemental Figure 8 online). We did not observe reproducible nonparental (novel) cDNA-AFLP marker fragments. In the S0 generation, the proportion of A-genome fragments that were lost (0.2%) was not different from the proportion of C-genome losses (0.3%) ($\chi^2 = 0.31, P = 0.576$;
Table 2). The average number of cDNA-AFLP fragment losses in the S0 was 0.63 per line. Twenty-nine of 31 cDNA-AFLP fragments that had disappeared in the S0 generation appeared in the S5. There was no difference between the proportion of fragments lost in colchicine (0.2%) and spontaneously (0.1%) doubled lines ($\chi^2 = 0.10$, $P = 0.747$; Table 2).

Analysis of 360 cDNA-AFLP markers in the S5 generation revealed that 42% showed changes among the lines. Approximately 60% of the A-genome markers and 54% of the C-genome markers showed changes in the S5 generation. Only two markers common to both parental transcriptomes (2.1% of total) showed changes in the S5 generation. In the S5 generation, the proportion of A-genome fragment losses (4.0%) was not significantly different from the proportion of C-genome fragment losses (4.3%) ($\chi^2 = 0.72$, $P = 0.395$; Table 2). The total number of cDNA-AFLP fragment losses was normally distributed among S5 lines (Shapiro-Wilk test; $P = 0.0889$). The average number of cDNA-AFLP fragment losses in the S5 generation was 10.6 per line. There was no difference in the frequency of fragment losses between colchicine (3.0%) and spontaneously doubled lines (3.0%) ($\chi^2 = 0.002$, $P = 0.964$). The proportion of total fragments lost in the S0 generation (0.2%) was significantly different from the proportion of losses in the S5 generation (3.0%) ($\chi^2 = 432.91$, $P < 0.0001$; Table 2).

No relationship between genetic or epigenetic changes and cDNA-AFLP fragments losses was detected in the S0 generation; however, in the S5 generation, DNA fragment losses were significantly correlated with cDNA-AFLP fragment losses ($r^2 = 0.55$, $P < 0.0001$; Table 3; see Supplemental Figure 9 online). Methylation changes were not correlated, suggesting that genomic rearrangements in S5 lines were largely responsible for loss of cDNA-AFLP markers.

Figure 4. DNA and RT-PCR SSCP Analysis of Resynthesized B. napus Polyploids. Comparison of pW225 DNA and transcripts from parental lines of B. rapa (IMB218; A-genome) and B. oleracea (TO1000; C-genome), a 1:1 mixture of parental cDNA or DNA, and samples from both S0 and S5 generations of six synthetic B. napus polyploid lines (9, 21, 31, 17, 35, and 49). Two biological replicates of RNA for each RT-PCR sample and two technical replicates of each DNA PCR sample were resolved in adjacent lanes (1 and 2). To control for genomic DNA contamination in RT-PCR reactions, adjacent reactions were conducted on genomic DNA, which amplified larger genomic fragments not detected in RT reactions (not visible in figure). pW225 DNA and gene expression was additive for all lines in the S0 generation. The loss of homoeologous DNA and transcripts was observed in many of lines at the S5 generation; two lines shown demonstrate evidence for the loss of B. rapa DNA and transcripts (17 and 35) and two demonstrate the loss of B. oleracea DNA and transcripts (9 and 21). Six total bands were detected by DNA and cDNA SSCP from each parent, indicating that three highly similar targets were amplified (fragments connected by arrows represent complementary strands of the same DNA sequence). For each line that had a rearrangement at this locus, the loss of a parental cDNA was associated with an intensification of the homoeologous cDNA fragment, matching the HNRT patterns observed in both RFLP and DNA SSCP markers (see Figures 2 and 3).
Phenotypic Variation among Selfed Polyploid Lineages

Overall phenotypic variability (measured as the sum of SD across all traits) increased between the S0 and S5 generations (Figure 5A), and most individual traits exhibited a significant increase in phenotypic variation (examples of a few traits shown in Figures 5B and 5C; see Supplemental Table 2 online). One S5 allopolyploid (line 9) displayed a dwarf-like phenotype (Figure 5D). There was also variation in leaf morphology and cuticular waxiness among S5 polyploid lines (Figure 5E).

The numbers of DNA and cDNA fragment losses per line in the S5 generation were both significantly correlated with our metric for overall phenotypic variability in the S5 (Table 3). Total methylation changes were not significantly correlated with phenotypic variability. A multivariate analysis of variance (MANOVA) joint test confirmed that the number of DNA fragment losses had an overall effect on the means of all phenotypes in the S5 generation (Wilks' Lambda; \( F = 2.3; P = 0.0317 \)). No significant overall effects on phenotypes were detected for cDNA fragment losses or total methylation changes in either generation. Univariate tests detected a significant effect of DNA fragment losses on the number of open flowers at first flower in the S0 generation (\( P = 0.0249 \)) and effects of cDNA fragment losses on the number of open flowers at first flower and raceme height in the S5 generation (\( P = 0.032 \) and 0.017, respectively).

Using RFLP marker data from FLC loci (FLC-1, FLC-2, FLC-3, and FLC-5), a multiple linear regression (MLR) was performed to measure whether or not rearrangements involving any of these flowering time loci were associated with flowering time variation among the polyploid lines (Figure 5B). The analysis indicated that the presence or absence of B. oleracea homoeoalleles of FLC-1 and FLC-3 together explained 28% of the variation in flowering time among advanced polyploids (\( P < 0.01 \)).

DISCUSSION

Genetic and Epigenetic Changes in B. napus

Since these allopolyploid lines were developed from doubled haploid genotypes of B. oleracea and B. rapa, our null hypothesis was that all lines would have identical genomes. This hypothesis was rejected in the S0 generation due to DNA methylation changes (Lukens et al., 2006). In the S5 generation, most of the earlier methylation changes remained fixed (see Supplemental Figure 6 online), but a small proportion reverted and some new changes were observed. In the S5 generation, the null hypothesis was rejected primarily due to genetic changes. Every line lost RFLP or SSR fragments by the S5 generation (Figure 1). Although some losses may represent deletions, many of the RFLP

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**Table 2. Summary of cDNA-AFLPs between 48 S0 and 47 S5 Resynthesized B. napus Polyploid Lines**

<table>
<thead>
<tr>
<th></th>
<th>S0 Generation</th>
<th>S5 Generation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Colch. Spont.</td>
<td>Total Colch. Spont.</td>
</tr>
<tr>
<td>Total no. fragments expected(b)</td>
<td>17,020</td>
<td>16,680</td>
</tr>
<tr>
<td>Total no. fragments lost (%)</td>
<td>31 (0.2)</td>
<td>504 (3.0)</td>
</tr>
<tr>
<td>No. A fragments expected(b,c)</td>
<td>6,428</td>
<td>6,298</td>
</tr>
<tr>
<td>No. A fragments lost (%)(b,c)</td>
<td>14 (0.22)</td>
<td>250 (4.0)</td>
</tr>
<tr>
<td>No. C fragments expected(b,c)</td>
<td>5,984</td>
<td>5,870</td>
</tr>
<tr>
<td>No. C fragments lost (%)(b,c)</td>
<td>17 (0.3)</td>
<td>252 (4.3)</td>
</tr>
<tr>
<td>No. AC fragments expected(b,c)</td>
<td>4,608</td>
<td>4,512</td>
</tr>
<tr>
<td>No. AC fragments lost (%)(b,c)</td>
<td>0</td>
<td>2 (0.04)</td>
</tr>
</tbody>
</table>

\(a\) The table is split by fragments scored in the S0 or S5 generation and is further divided into total marker data and data collected from colchicine (Colch.) or spontaneously (Spont.) doubled lines.

\(b\) Expected number of fragments is equal to the product of total markers scored and the number of lines, minus missing data, and is the number expected under parental additivity.

\(c\) Designations A (B. rapa), C (B. oleracea), and AC (common to both) indicate the transcriptome of origin of the cDNA-AFLP markers scored.

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**Table 3. Pearson Correlations and P Values for Pairwise Comparisons of Marker and Phenotypic Data Sets among the S5 Generation Resynthesized B. napus Polyploids**

<table>
<thead>
<tr>
<th></th>
<th>No. DNA Losses</th>
<th>No. cDNA Losses</th>
<th>No. Methylation Changes</th>
<th>Phenotypic Variability</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. DNA losses(a)</td>
<td>(r = 0.742)</td>
<td>(P &lt; 0.0001)</td>
<td>(P = 0.7544)</td>
<td>(P = 0.0041)</td>
</tr>
<tr>
<td>No. cDNA losses(a)</td>
<td>(r = 0.048)</td>
<td>(r = 0.065)</td>
<td>(P = 0.6716)</td>
<td>(P = 0.0134)</td>
</tr>
<tr>
<td>No. methylation changes(a)</td>
<td>(r = 0.424)</td>
<td>(r = 0.370)</td>
<td>(P = 0.2127)</td>
<td>(r = 0.192)</td>
</tr>
</tbody>
</table>

\(a\) DNA = total DNA fragment loss/line; cDNA = total cDNA fragment loss/line; methylation = total methylation changes (sum of fragment loss, fragment reversions, novel fragments across HpaII and MspI blots)/line; phenotypic variability = sum of SD from the mean of all traits/line.
fragment losses were associated with an intensification of a homoeologous fragment, suggesting HNRTs. The large size of our resynthesized allopolyploid population allowed us to address the dynamics of genome-wide changes. The 47 lines were normally distributed for genetic, epigenetic, and expression changes, as would be expected if the changes occurred independently among lines. Although genetic changes occurred on nearly every chromosome, they were not equally distributed among chromosomes, occurring at higher frequencies on chromosomes with large regions of homoeology between the A- and C-genomes (e.g., A1/C1, A2/C2, and A3/C3). A detailed analysis of HNRT between A1 and C1 demonstrated various combinations of nonreciprocal events on the ends of chromosomes and in interstitial regions (Figure 3).

Figure 5. Examples of Phenotypic Variation among Resynthesized B. napus Polyploids.

(A) to (C) Frequency distributions of S0 (gray bars) and S5 (black bars) generations for total phenotypic variability represented as summation of all trait values in SD units (A), flowering time (B), and the number of fertile siliques (C). The values for the diploid parents B. rapa (line IMB218; A-genome) and B. oleracea (line TO1000; C-genome) are indicated with arrows in (B) and (C). Note that this measure of fertile siliques was conducted in the absence of pollinators and that while the TO1000 is self-compatible it does not readily self-pollinate.

(D) Photographs of one S5 line (no. 9) displaying a dwarfed growth habit that was pronounced by the S4 generation.

(E) Variation in shape of the fourth leaf from diploid parents (IMB218 far left, and TO1000 at far right) and some S5 polyploid lines (shown between parental samples). Bar = 20 cm.
Genetic changes in the S5 occurred more frequently in the C-subgenome (although the difference was marginally significant), while cytosine methylation changes occurred more frequently in the A-subgenome. In a previous study with resynthesized B. napus, Song et al. (1995) analyzed reciprocal allopolyploids (AACC and CCAA) and did not detect significant directional changes among the A- and C-subgenomes; however, they included only a few lineages derived from a single hybrid of each reciprocal cross. We analyzed only CCAA polyploids, but we included many independently derived polyploid events. Importantly, we observed large variation among independent lines for both the extent and direction of genomic change (Figure 1), highlighting the importance of analyzing many polyploid events to accurately assess the dynamics of genome change. Genomic changes directed toward one parental genome have been observed in Brassica juncea, in which the direction was dependent on the lineage analyzed (Song et al., 1995), and in Triticale polyploids, in which the rye (Secale cereale) subgenome tended to be preferentially modified (Ma et al., 2004; Ma and Gustafson, 2006). The cause of variation in number of rearrangements observed among our S5 lines is not known; however, it appears to be unrelated to variation in methylation changes in the S0 or S5 observed among our S5 lines. The cause of variation in number of rearrangements to be preferentially modified (Ma et al., 2004; Ma and Gustafson, 2006). The cause of variation in number of rearrangements observed in some segregating individuals and most likely resulted from recombination among homoeologs following successive meioses (see Supplemental Figure 3 online). This is supported by our detection of partial reciprocal rearrangements among some segregants (see Supplemental Figure 4 online) and interstitial rearrangements (Figure 3). Therefore, the fixation of HNRTs observed in the later generations suggests that stochastic forces and segregation during selfing can fix both forms of a given rearrangement. This is illustrated in Figure 3, which shows that both forms of HNRTs have segregated from reciprocal exchanges and become fixed among 52 lineages. In sum, our data support the role of a recombination-based mechanism for chromosomal rearrangements (HNRTs).

Genetic evidence suggests that chromosomal rearrangements (HNRTs) occur in both natural and resynthesized B. napus (Sharpe et al., 1995; Song et al., 1995; Pires et al., 2004; Udall et al., 2005; Leflon et al., 2006; Liu et al., 2006; Nicolas et al., 2007). Homoeologous exchanges were observed cytologically for A7/C6 reciprocal transpositions in previous studies with B. napus allopolyploids (Osborn et al., 2003a). B. oleracea and B. rapa share extensive similarity among homoeologous chromosomes (Parkin et al., 2005), which may promote recombination. This is supported by studies in B. napus haploids (AC) and triploids (AAC), which have demonstrated that pairing and exchange among homoeologs occurred at a significant rate and was under the control of genetic loci (Jenczewski et al., 2003; Leflon et al., 2006; Liu et al., 2006; Nicolas et al., 2007).

Although deletions have been reported in allopolyploid wheat, Triticale, and Tragopogon (Ozkan et al., 2001; Shaked et al., 2001; Han et al., 2005; Ma and Gustafson, 2006; Tate et al., 2006), none of these studies report duplication of homoeologous regions associated with deletion events. Some of these studies have relied on PCR-based marker systems that are not dosage sensitive; thus, loss-duplication events may occur in other polyploid species but have not been detected. Dosage-sensitive RFLP markers were used for two studies on resynthesized B. juncea polyploids (Song et al., 1995; Axelsson et al., 2000), and neither one provided direct evidence for exchanges between the A- and B-subgenomes, which are more divergent than the A- and C-subgenomes. Axelsson et al. (2000) found no evidence for any genomic changes in a mapping study. Song et al. (1995) observed high frequencies of DNA fragment losses and gains in self-pollinated lineages, but marker data consistent with HNRTs (loss intensification of homoeologous fragments) were not presented. Thus, other mechanisms of genomic change may predominate in B. juncea.

**Potential Mechanisms for Chromosomal Rearrangement by HNRT**

RFLP markers allow for the detection of both the loss and the duplication of homoeologous markers (Parkin et al., 1995; Sharpe et al., 1995; Pires et al., 2004; Udall et al., 2005). When loss-intensification patterns are observed among linked homoeologous markers, chromosomal rearrangements (HNRTs) can be discriminated from deletions. The mechanism leading to HNRTs most likely involves recombination. In our previous study, we found that genomic rearrangements were rare in the S0 generation of these resynthesized allopolyploids when bulked DNA samples of 8 to 12 individuals were analyzed (Lukens et al., 2006). In this study, we analyzed individual S1 plants and detected rearrangements segregating in the S1 progeny at a low frequency (3/118 segregants were observed in three of eight lines analyzed), indicating that meioses were required for their frequent occurrence by the S5. If a reciprocal exchange occurred among a pair of homoeologs during meiosis, then opposite forms (fixed for one homoeolog or the other) might each be expected at a frequency of 1/16 segregants. This estimate is consistent with the observation that 1/14 of the segregants derived from line 17 (S0) carried the opposite form of a HNRT fixed in later generations for that line (data not shown) and that 1/16 of the segregants derived from line 48 (S0) carried an HNRT (see Supplemental Figure 5 online).

Further evidence for a recombination mechanism for chromosome rearrangements was detected in our transgenerational analysis of ancestors of lines genetically fixed for A1/C1 HNRTs (see Methods). The results suggested that an individual picked for selfing two generations prior to fixation was heterozygous for the nonreciprocal transposition. Rearrangements of variable length were observed in some segregating individuals and most likely resulted from recombination among homoeologs following successive meioses (see Supplemental Figure 3 online). This is supported by our detection of partial reciprocal rearrangements among some segregants (see Supplemental Figure 4 online) and interstitial rearrangements (Figure 3). Therefore, the fixation of HNRTs observed in the later generations suggests that stochastic forces and segregation during selfing can fix both forms of a given rearrangement. This is illustrated in Figure 3, which shows that both forms of HNRTs have segregated from reciprocal exchanges and become fixed among 52 lineages. In sum, our data support the role of a recombination-based mechanism for chromosomal rearrangements (HNRTs).

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**Genome Rearrangements Alter Gene Expression in B. napus Polyploids**

We detected a correlation between genome rearrangements and changes in the expression of targeted genes and anonymous transcript markers. For pW225 transcripts, multiple SSCP markers were detected that maintained consistent signal intensity while a single pair of homoeologous products revealed loss and intensification patterns that mirrored RFLP patterns (Figures 2 and 4). These patterns might have been predicted if the level of transcript derived from each homoeologous sequence was proportional to its copy number. Although FLC-3 and FLC-5
had HNRTs, we could only infer the loss of homoeologous FLC-3 and *B. rapa* FLC-5 transcripts using RT-PCR (see Supplemental Figure 7 online). Nevertheless, genome rearrangements altered the contribution of parental alleles to the polyploid transcriptomes and were an important mechanism generating allelic variation.

Our genome-wide expression survey of the S0 generation revealed a similar frequency of cDNA-AFLP markers displaying changes as was detected in the early generations of resynthesized wheat and *Arabidopsis* (Comai et al., 2000; Lee and Chen, 2001; Kashkush et al., 2002). Neither genetic changes nor cytosine methylation changes (detectable by HpaII and MspI) were correlated with cDNA-AFLP changes in the S0 generation; however, it is possible that some of these lost transcripts resulted from other epigenetic modifications. This is supported by our observation that some missing cDNA-AFLP marker fragments among S0 polyploids appeared in the S5 generation, which would presumably occur only if the silencing mechanism was reversible.

The cDNA-AFLP marker losses in the S5 generation were highly correlated with DNA fragment losses, suggesting that genome rearrangements were the major cause of gene expression changes (Table 3). Studies in wheat have also detected the loss of cDNA-AFLP fragments following intergeneric hybridization and confirmed that sequence polymorphisms were responsible for some expression changes (Kashkush et al., 2002). In *Tragopogon* allopolyploids, the majority of cDNA-AFLP marker losses resulted from genetic changes (Tate et al., 2006). As in the S0 generation, we did not detect a relationship between cytosine methylation changes and cDNA-AFLP fragment losses in the S5 generation. This result suggests that cytosine methylation change was not an important mechanism on a genome-wide scale causing qualitative changes in gene expression in resynthesized *B. napus*, as has been observed for specific loci in studies with *Arabidopsis* and wheat (Chen and Pikaard, 1997a; Kashkush et al., 2002). However, it is possible that cytosine methylation changes caused quantitative transcriptional changes or led to qualitative silencing of only a few loci. It is also possible that other DNA and chromatin modifications might explain additional variation in the loss of cDNA-AFLP fragments (Osborn et al., 2003b; Chen and Ni, 2006). We detected no overall bias in the frequency of A- or C-genome-specific cDNA-AFLP changes among S5 polyploids. In cotton, organ-specific biases in the silencing of homoeologs has been observed; however, there is no overall bias in the expression of transcripts from one parental cotton genome over the other (Adams et al., 2003, 2004; Adams and Wendel, 2004). Conversely, in *Arabidopsis suecica* allopolyploids, silencing of *A. thaliana* homoeologs occurred more frequently than those derived from *Arabidopsis arenosa* (Wang et al., 2006). As with genomic changes, we observed large variation among our lines for the extent and direction of gene loss or silencing, again highlighting the importance of analyzing a large number of lineages.

**Genome Rearrangements and de Novo Phenotypic Variation**

Phenotypic variation increased with generation advancement and was significantly correlated with DNA fragment losses (Table 3), suggesting that chromosomal rearrangements may broadly impact phenotypes in resynthesized *B. napus* polyploids. Phenotypic variation was also significantly correlated with cDNA fragment losses, although it was not as high as the correlation with DNA loss, and there was no significant overall effect of cDNA-AFLP losses on phenotypes (MANOVA results). The weaker association of phenotypic variation with transcript loss compared with DNA loss may be due to sampling variation for DNA and cDNA losses. It is also possible that since the DNA losses may reflect rearrangements that include large numbers of genetically linked transcript changes, they more accurately reflect effects on phenotype than do cDNA losses. In addition to cis effects, homoeologous chromosomal rearrangements may have trans effects that cause changes in the expression of genes located outside of rearranged chromosomal segments. This would be particularly true of genes lying within rearrangements that are components of regulatory complexes and that have diverged significantly in the progenitor species, such that a genetic loss or loss duplication leads to altered dosage and regulatory interactions (Osborn et al., 2003b; Riddle and Birchler, 2003; Birchler et al., 2005). Thus, the inclusion of genome-wide estimates of quantitative variation in gene expression, in addition to estimates of qualitative transcript loss, may better reflect the overall effects of expression changes on phenotypic variation.

Schranz and Osborn (2000, 2004) previously described de novo phenotypic variation in resynthesized *B. napus* polyploids and concluded that only a few genetic changes might be necessary for its induction, particularly if they involved important genes. Genetic variation generated by the replacement of one homoeoallele with another might result in novel phenotypes when the two homoeoalleles have different magnitudes of effects on a trait and act in an additive manner or result in the unmasking of recessive alleles. Studies in *B. rapa* characterized FLC alleles with additive effects, suggesting that allelic variation among replicated FLC loci may contribute to variation in flowering time in *Brassica* (Schranz et al., 2002). Pires et al. (2004) detected rearrangements, including FLC, that affected flowering time in a cosegregation analysis with resynthesized *B. napus* polyploids, a result supported by our findings that HNRTs at FLC-3 and FLC-1 were associated with flowering-time variation among S5 polyploids. Both reciprocal exchanges and nonreciprocal transpositions have been observed in natural populations of *B. napus*, suggesting that intergenic rearrangement may be an important mechanism generating phenotypic variation in this species (Parkin et al., 1995; Sharpe et al., 1995; Osborn et al., 2003a; Osborn, 2004; Pires et al., 2004; Udall et al., 2005). In fact, studies in natural *B. napus* have reported HNRTs with phenotypic effects on seed yield (Osborn et al., 2003a) and *Sclerotinia* resistance (Zhao et al., 2006). Importantly, some of the chromosomes altered by HNRTs in these studies with natural *B. napus* also incurred rearrangements in our study of resynthesized *B. napus* allopolyploids (e.g., A1/C1, A2/C2, and A3/C3), and some RFLP markers that have detected HNRTs in previous studies detected them in our population of polyploids (i.e., pW239; see Udall et al., 2005). Therefore, it is likely that genomic hot spots for chromosome rearrangement exist in *B. napus*, some of which have specific phenotypic effects. Variation might also be created if genes are deleted or duplicated in the regions of an exchange due to misalignment during recombination.
Although natural *B. napus* has lower frequencies of de novo rearrangements than resynthesized lines (Parkin et al., 1995; Sharpe et al., 1995; Song et al., 1995; Pires et al., 2004; Udall et al., 2005), it is possible that chromosome pairing in natural *B. napus* was initially more unstable and that stronger disomic pairing evolved with selection for improved seed set. In our study, we intentionally maintained all lines during the advancement of generations, including lines that had decreased fertility or pollen viability (Figure 5C), and these lines would have been quickly selected against in a natural environment. We cannot rule out the possibility that natural *B. napus* initially acquired stronger genetic control over chromosome pairing from one of its diploid progenitors. It is also possible that parents of natural *B. napus* differed for homoealleles at loci controlling pairing and that homoeologous exchanges contributed to variation for pairing control and the evolution of more stabilized pairing. Regardless, our results show that this mechanism can be particularly prevalent in the early generations after polyploid formation, when novel phenotypic variation may be critical for the survival and propagation of a new species. Further research will be necessary to determine to what extent homoeologous chromosome exchanges contribute to novel variation in other polyploid species.

**METHODS**

**Plant Material**

More than 50 resynthesized *Brassica napus* allopolyploid plants (CCAA) were developed by hybridizing doubled haploid *Brassica oleracea* line TO1000 (egg donor; C-genome) with doubled haploid *Brassica rapa* line IMB218 (pollen donor; A-genome) as described previously (Lukens et al., 2006). Forty-two amphidiploid lines were generated by colchicine treatment, and the remaining 10 lines underwent spontaneous chromosome doubling. Lineages were advanced by single plant descent. At each generation following self-fertilization, six seeds were planted for each genotype to ensure germination. Two of the six plants were chosen at random and self-fertilized to ensure adequate seed set for each genotype. If both plants produced >50 seeds, one was chosen at random for advancement to the next generation; otherwise, the plant with the higher seed set was selected. Line number designations used in all figures are based on those shown in Figure 1 (see Supplemental Data Set 1 online for lines, primers, probes, and genotype numbers). Two lines were mosaics of spontaneous allopolyploid (CCAA) and hybrid allopolyploid tissue (CA).

**DNA Gel Blotting and Microsatellite Analysis**

Genomic DNA was extracted from leaf tissue from young plants using the cetyl-trimethyl-ammonium bromide method (Kidwell and Osborn, 1992). Each sample was comprised of a bulk of eight to 12 S6 plants. DNA from each sample was digested with the following restriction endonucleases: EcoRI, HindIII, DraI, MspI, and HpaII. DNA gel blotting, probe labeling, and hybridizations were performed using 76 *Brassica* cDNA and genomic DNA probes as described in previous studies (Ferreira et al., 1994; Butruille et al., 1999; Udall et al., 2005; Lukens et al., 2006). Some probes were not used in either the S0 or S5 analyses (see Supplemental Data Set 1 online for lines, primers, and probes). Probe nomenclature of previous studies was used (Parkin et al., 1995; Sharpe et al., 1995; Udall et al., 2005). Thirty-seven SSR primer pairs were used to assay changes in microsatellites. Twenty-eight of these primer pairs were developed by D. Lydiate and A. Sharpe and were amplified by PCR and resolved by polyacrylamide electrophoresis as previously described (Lukens et al., 2006). Nine SSRs used in a recent mapping study were PCR amplified and resolved by electrophoresis in 3% agarose gels (our unpublished data; Uzunova and Ecke, 1999; Suwabe et al., 2002). For SSRs, all polyploid samples were run adjacent to reactions containing parental DNAs, a mix of parental DNAs, and a reaction with no DNA.

**Genome Fragment Analysis**

To assay for genetic changes, fragment losses were scored if they were confirmed in at least two of the following methylation-insensitive enzymes: EcoRI, HindIII, and DraI. In some cases, changes were observed in only one of these enzymes but were also present in both MspI and HpaII digests with no evidence for a DNA methylation change and were scored as genetic changes. An estimate of the total number of markers for each probe was determined by the restriction enzyme (EcoRI, HindIII, or DraI) that generated the largest number of marker fragments. Markers that generated identical patterns for a given probe were scored as a single marker fragment. Fragment losses (SSR or RFLP) were recorded, and HNRT was inferred if the loss of one RFLP fragment occurred with an intensification (as estimated by eye) of signal at a homoeologous RFLP fragment. This type of loss/intensification dosage pattern has previously been inferred to result from HNRTs (Udall et al., 2005). Losses that were not confirmed in multiple enzymes (as stated above) and unreadable fragments were coded as missing data.

**Mapping Genome Rearrangements**

Map positions of 133 markers were assigned using data from other *B. rapa*, *B. oleracea*, and *B. napus* mapping populations (our unpublished data; Udall et al., 2005; D. Lydiate and A. Sharpe, personal communication). For most of these markers (94), the fragment from our resynthesized *B. napus* was confirmed to be equivalent to a fragment mapped in populations using the same parental lines and probes (TO1000 or IMB218). For 27 of the markers, only one locus or one pair of loci (A- and C-genomes) had been mapped with the same probe, and these assignments were used. For 12 marker loci, mapping data predicted two or more potential loci within a diploid genome, and we assigned these marker loci to the linkage group containing linked markers that showed coincident losses among our polyploid lines. For homoeologous linkage groups A1 and C1, JoinMap was used to approximate the order of assigned markers using marker polymorphism data derived from the polyploidy lines. The *Brassica* linkage group nomenclature is undergoing a change: *B. rapa*, *B. oleracea*, and *B. napus* linkage groups were previously designated R1-R10, O1-O9, and N1-N19, respectively. This nomenclature is being replaced by linkage groups that refer to the A (*B. rapa*) and C (*B. oleracea*) genomes. Thus, the new nomenclature is A1-A10 (≈ R1-R10), C1-C9 (≈ O1-O9) for diploids, and in allopolyploid, *B. napus* N1-N19 is replaced by A1-A10 and C1-C9 designations (see http://www.brassica.info/information/lg_assignments.htm).
Methylation Analysis

DNA methylation changes were scored by comparing HpaII and MspI blots (for 67 probes) as previously described (Lukens et al., 2006). The absence of a parental MspI or HpaII fragment in an allotriploid was interpreted as a change in CpgCpG or CpG methylation, respectively. Changes observed in MspI or HpaII blots that were observed in any methylation-insensitive restriction digests and changes observed in both MspI and HpaII blots not observed in the other restriction digests were scored as missing data. DNA methylation changes in the S5 generation were categorized as fixed (having occurred in the S0 generation and remained unchanged), reversions of previous methylation changes, or were categorized as novel fragment changes (fragment losses + novel fragments). The total number of S5 methylation changes was taken as the sum of reversions and new fragment changes in the S5. Only lines and probes in common between the S0 and S5 generations were analyzed for methylation data in this article.

Transgeneration and S1 Segregant Analysis

Six lines (2, 26, 8, 22, 17, and 48) that revealed interstitial or terminalized rearrangements on homoelogous linkage groups A1 and C1 (see Figure 3) were genotyped with linked SSCP and SSR markers (pW225, pX135, SORC20, and SORD38) across all generations. SSCP markers pW241, pW225, and pX135 were designed from RFLP probe sequences available online at GenBank (see Supplemental Data Set 1 online for SSCP primer sequences and RFLP probes). Bulked DNA samples (of 16 individual plants per line) were genotyped to identify in which generation rearrangements were fixed in these lines, and the number of S5 generation DNA samples was analyzed in the prior generation.

To determine if rearrangements had occurred following meiosis of the S0 generation, 10 to 16 individual S1 plants derived from each of eight S0 plants (26, 17, 22, 18, 2, 8, and 27) were genotyped using a combination of SSRs (SORC20) and SSCP markers designed from RFLP probes (FLC3, pW241, and pW225) that spanned homoelogous linkage groups A1/C1, A2/C2, and A3/C3 (see Supplemental Data Set 1 online for lines, primers, and probes).

cDNA-AFLP Analysis

Seed was sown in Metro Mix Soil in flats containing 14 rows of seven cells each (cells were ~3.81 cm in diameter and ~20 cm deep). Flats were organized into two replicate blocks in a 100× foot indoor grow room at the University of Wisconsin Biotron Facility. Outer rows of each flat were seeded with border plants, and each of the remaining inner 12 rows were seeded in random order with an individual S0, S5, or parental line of B. rapa or B. oleracea (each cell was double seeded and thinned to one plant). Each block contained 10 flats, and each line was represented once per block. Within each block, the positions of flats were randomized daily.

Plants were watered daily and fertilized every other day with dilute (1 tablespoon/20 liters) Peters Professional Peat Lite Special 20-10-20. Temperature was maintained at 21°C, humidity at 60%, and light maintained at ~450 μmol m⁻² s⁻¹ for 16 h each day.

All plants were harvested at the same developmental stage, when the second and third true leaves were outstretched from the meristem. Plants were harvested at the same time of day (11:00 AM to 12:00 PM CST). Leaves two and three were bulked from the seven plants comprising a given line replicate and were flash-frozen in liquid N₂ and stored at ~80°C.

Total RNA was extracted using Tri-Reagent (Molecular Research Center) according to the manufacturer’s protocol. RNA samples were DNase treated with Ambion Turbo DNA-free DNase and quantified using a Bio-Rad Smart Spec 3000. mRNA was purified from ~50 μg of total RNA sample using the Qiagen Oligoex mRNA purification kit according to the manufacturer’s protocol. Each mRNA sample was eluted with 40 μL of elution buffer. First-strand cDNA synthesis was conducted as follows: 20 μL of eluted mRNA was combined with 1 μL of oligo(dT) primer (20 μM) and heated to 70°C for 5 min; samples were snap-cooled on ice and added to 18 μL of distilled deionized water, 5 μL of 10× RT buffer, 5 μL of 10 mM deoxynucleotide triphosphate (dNTP), and 1 μL of M-MLV reverse transcriptase (New England Biolabs); and reactions were incubated at 37°C for 45 min. Second-strand cDNA synthesis was performed as follows: 50 μL of first-strand cDNA was combined with 38.5 μL of distilled deionized water, 0.5 μL of RNase H, 1 μL of Escherichia coli DNA Polymerase I, and 10 μL of New England Biolabs buffer 2; and reactions were incubated at 16°C for 2 h. Thirty-four microliters of distilled deionized water, 1 μL of E. coli DNA ligase, and 15 μL of DNA ligase buffer (New England Biolabs) were added, and reactions were incubated at room temperature for 15 min. Double-stranded cDNAs were purified using phenol/chloroform extraction and resuspended in 20 μL of distilled deionized water. Ten microliters of double-stranded cDNA was digested with TaqI and Asel restriction enzymes according to the manufacturer’s protocol (New England Biolabs). Primers and adapters were designed using published protocols (Bachem et al., 1996, 1998) and contained either a TaqI or Asel restriction site. All adapter, preamplification, and selective primer sequences can be found in Supplemental Data Set 1 online. Adapters were ligated to restriction-digested double-stranded cDNA samples using T4 DNA Ligase (New England Biolabs), samples were diluted 1:20, and 1 μL was used as templates in preamplification reactions. PCR reaction conditions were as follows: 1 μL of template was combined with 35 μL of distilled deionized water, 5 μL of 10× PCR Buffer, 4 μL of 25 mM MgCl₂, 2.5 μL of 2 mM dNTP, 1 μL of each primer (5 μM), and 0.5 μL of Taq polymerase. Reactions were placed in a thermocycler under the following program: (1) 72°C for 30 s, (2) 94°C for 3 min, (3) 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and (4) 72°C for 5 min. For selective amplifications, TaqI-selective primers were labeled with F35 using T4 polynucleotide Kinase (New England Biolabs) according to the following procedure: 10.8 μL of TaqI primer (5 μM) was combined with 7.8 μL of distilled deionized water, 3 μL of 10× polynucleotide kinase buffer, 6 μL of γ-3P-dCTP, and 2.4 μL of polynucleotide kinase and was incubated at 37°C for 30 min (see Supplemental Data Set 1 online for lines, primers, and probes). Selective PCR amplifications were set up by combining 2 μL of 1:20 diluted preamplification, 1 μL of 10× PCR buffer, 0.8 μL of 25 mM MgCl₂, 1 μL of dNTP (2 mM), 1 μL of Asel primer (5 μM), 0.25 μL of labeled TaqI primer, 0.5 μL of Taq polymerase, and 3.5 μL of distilled deionized water. Reactions were thermocycled according to the following PCR program: (1) 94°C for 3 min, (2) 11 cycles of 94°C for 1 min, 65°C for 1 min (~0.7°C/cycle), and 72°C for 2 min, (3) 22 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and (4) 72°C for 5 min. PAGE was conducted at 65 W of constant power according to standard protocols. All cDNA-AFLP reactions were separated with samples of parental and parental mix cDNA and a water control. To control for DNA contamination, DNA-AFLPs were conducted in parallel on parental samples and two random polyploid lines and were used as controls beside cDNA-AFLPs for the five primer sets analyzed. DNA-AFLP controls generated many more bands than cDNA-AFLPs and had unique profiles. Additionally, (RT-) controls were performed by screening samples of mRNA by PCR using two gene-specific primer sets (FLC-3 and FLC-5; see below). cDNA-AFLP results that were not repeatable in both biological replicates were scored as missing data. Sixteen primer combinations generated a total of 360 transcript loci, of which 137 were of B. rapa origin, 127 were of B. oleracea origin, and 96 were common to both parental transcriptomes (see Supplemental Data Set 1 online for lines, primers, and probes).

RT-PCR and DNA SSCP Analysis

The same mRNA preparations used for cDNA-AFLP analysis were used for cDNA synthesis in RT-PCRs. First-strand synthesis was conducted using 250 ng of mRNA, M-MLV reverse transcriptase (New England
MgCl₂, 1 

Brassica

the AT4G32551 annotation (CDS sequence 1941 to 2136 bp) and Resource WU-Blast. Primers were designed in exons 13 and 14 based on gene AT4G32551 [p(N) 
dopsis (accession number CZ906459), which was highly similar to the 

Three specific primer sets (FLC3, FLC5, and pW225) were used in RT-PCR SSCP analyses (see Supplemental Data Set 1 online for primer sequences and Pires et al., 2004 for a study that previously used our FLC primers). pW225 primers were designed from the RFLP probe pW225 (accession number CZ906459), which was highly similar to the Arabidopsis gene AT4G32551 [p(N) = 4.3e-66. The Arabidopsis Information Resource WU-Blast]. Primers were designed in exons 13 and 14 based on the AT4G32551 annotation (CDS sequence 1941 to 2136 bp) and Brassica nucleotide sequence. pW225 PCR products were sequenced to verify their identity. PCR amplifications were set up by combining 1.5 μL of 1:10 diluted cDNA, 1 μL of 10× PCR Buffer, 0.8 μL of 25 mM MgCl₂, 1 μL of dNTP (2 mM), 1 μL of F primer (5 μM), 1 μL of R primer (5 μM), 0.5 μL of Taq polymerase, 0.1 μL of α-dCTP32, and 3.1 μL of H₂O. Reactions were thermocycled according to the following PCR program: (1) 96°C for 4 min, (2) 30 cycles of 94°C for 30 s, 58°C for 45 s, and 72°C for 1 min, (3) 72°C for 10 min, and (4) 4°C hold. α-dCTP32 was incorporated during PCR reactions using Taq polymerase, and the samples were resolved by MDE electrophoresis (Cambrex Bio Science) using Bio-Rad sequencing gels according to the manufacturer’s protocol. Gels were run for 17 to 25 h at 7 W constant power. All RT-PCR SSCP reactions were run beside samples of parental and parental mix cDNA and DNA, a water control.

Phenotypic Analysis

Seed was sown in 6-inch pots in Metro Mix soil. Pots were placed on four 14.5 × 5-ft ebb and flow tables in an indoor greenhouse room at the University of Wisconsin Biotron Facility. A randomized complete block design was used, and each of four ebb and flow tables was considered a block. Within each block, each of the S0 and S5 polyploid lines, parental genotypes, and four natural B. napus genotypes were represented once, and every 2 weeks plant positions within blocks were randomized. Temperature was maintained at 21°C and a combination of natural light and supplemental lighting provided an average intensity of 678 μmole/m²/s across the greenhouse. Each table had two 600-W HPS lights centered over it, which operated 16 h a day. Ebb and flow tables were flooded twice per day for 10 min with half-strength Hoagland fertilizer.

Thirteen phenotypic characters were measured in an analysis of variation among the polyploid lines. Flowering time was measured at the time the first flower bud opened. At this time, a number of other traits was measured, including leaf number, the total number of open flowers at first flower, plant height, raceme height, number of sertations on the margin of the fourth true leaf, width of the fourth true leaf at its widest point, length of the petiole of the fourth true leaf, length of the leaf blade of the fourth true leaf, and the length of petiole displaying leaf wings when present. Two weeks after flowering initiated, we estimated variation in flower size by measuring the length and width of the open face of the seventh flower from the top of each raceme (flower petals were spread open and measured from end to end). At harvest, the number of fertilized siliques (having at least one developing seed) was counted on each plant, and the number of secondary branches was counted as the number that broke bud.

Statistical Analyses of Genetic and Transcriptional Markers and Phenotypic Data

For RFLP, SSR, and cDNA-AFLP fragment data analyses, the prop.test function of R statistical software (R Development Team, 2006) was used for two sample tests of equal proportions where applicable. Microsoft Excel was used for simple t tests and histograms. SAS Version 9.1 for Windows (SAS Institute, 2006) was used for all tests of normality (PROC UNIVARIATE NORMAL). The PROC LOGISTIC and PROC GENMOD functions were used to test the null hypotheses of equal proportion of genetic changes across all linkage groups. SAS Analyst was used for F tests of equal variance, correlation analyses, and MLRs. MLRs were conducted using total transcript markers lost/line as the dependent variable and the number of total DNA fragment losses/line and total number of DNA methylation changes/line as independent variables. An MLR was also performed using FLC markers (FLC1-oleracea, FLC3-oleracea, FLC5-oleracea, FLC2-rapa, FLC3-rapa, and FLC5-rapa) as independent variables and days until flowering as a dependent variable. For all MLRs, models were built using stepwise selection, and the criteria for entering and staying in a model were both set at P < 0.01. To estimate overall phenotypic variability among synthetic polyploids across both the S0 and S5 generations, the following metric was calculated: for each phenotype, least-squares means for all polyploid lines (S0 and S5) were used to calculate a grand mean and S0 for that trait. Each line’s deviation from the grand mean was calculated in SD units. Standard deviations were summed across every phenotypic character analyzed, giving rise to a measure of how phenotypically variable each line was relative to the entire population of polyploids across both generations. MANOVA was conducted using SAS Version 9.1 and was used to test whether or not total DNA fragment losses, cDNA fragment losses, and methylation changes had effects on the means of all phenotypic traits jointly. Our model treated the 13 phenotypic traits as dependent variables and the three marker data sets as independent variables. Univariate tests for each trait were performed and then the manova h = all function was used to test the overall effect of the independent variables on all traits simultaneously.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers CZ906459, CZ906477, CZ906395, DT469125, DT469153, CZ906400, CZ906385, DT469132, CZ906392, CZ906429, DT469129, CZ906379, CZ906384, AY115673, AY115675, AY115678, and AY115676. A complete list of accession numbers for all RFLP probes can be found in Supplemental Data Set 1 online.

Supplemental Data

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

Supplemental Figure 1. Analysis of DNA Samples across Generations.

Supplemental Figure 2. DNA SSCP and SSR Gel Analyses of DNA Samples across Generations for Two Lineages.

Supplemental Figure 3. DNA SSCP and SSR Analyses of Line 2 (S2) Segregants.

Supplemental Figure 4. DNA SSCP and SSR Analyses of Line 26 (S1) Segregants.

Supplemental Figure 5. DNA SSCP Analysis of FLC-3 in Line 48 (S0) Segregants.

Supplemental Figure 6. Summary of Methylation-Sensitive RFLP Changes Detected among S0 and S5 Polyploids Relative to the Parents.

Supplemental Figure 7. RT-PCR Analysis of FLC-3 and FLC-5.

Supplemental Figure 8. cDNA-AFLP Display of Resynthesized Brassica napus Polyploids.

Supplemental Figure 9. Relationship between Total Number of Genome Fragment Losses and Total Number of cDNA Fragment Losses in Resynthesized Brassica napus Polyploids.

Supplemental Table 1. Summary of the Distribution of Marker Changes among Brassica Linkage Groups.

Supplemental Table 2. F Tests for Equal Phenotypic Variance among Lines of Resynthesized Brassica napus Polyploids in the S0 and S5 Generations.

Supplemental Data Set 1. Lines, Primers, and Probes.
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