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Biological relevance of polyploidy: ecology to genomics
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Flowering time divergence and genomic rearrangements in resynthesized *Brassica* polyploids (Brassicaceae)

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Novel phenotypes often arise in generations immediately following polyploidization. Previous studies have shown that separate lineages derived from a resynthesized *Brassica napus* allopolyploid rapidly evolved heritable differences in flowering time. These early-flowering and late-flowering polyploid lines were expected to be genetically identical because they were derived from a single, chromosome-doubled amphihaploid plant. In this study, we investigated the molecular genetic basis for these flowering time differences. We assessed the diploid *B. rapa* and *B. oleracea* parents and the early- and late-flowering *B. napus* lineages for changes in genome structure, and for changes in transcript levels of four sets of *FLOWERING LOCUS C* (*FLC*) genes. No such changes were observed for *BnFLC1*, but we detected chromosomal rearrangements (e.g. *de novo* non-reciprocal transpositions) and changes in transcript level for *BnFLC2* and *BnFLC3* between the early- and late-flowering *B. napus*. A chromosomal rearrangement of a genomic segment containing *BnFLC3* was responsible for 29% of the phenotypic variation among the *B. napus* lines. Expression of *BnFLC5* was silenced in all polyploids, although no changes in genome structure were detected. An ongoing investigation of 50 identical *B. napus* allopolyploids may further reveal the dynamics of changes in phenotype, genome and transcriptome at the early stages in polyploid evolution.

ADDITIONAL KEYWORDS: *Brassica napus* – chromosomal rearrangements – *de novo* variation – epigenetics – evolution – *FLOWERING LOCUS C* – gene silencing – life history traits – polyploidy – translocations.

INTRODUCTION

Polyploids often possess novel traits that are not present in their diploid progenitors (reviewed in Ehrendorfer, 1980; Levin, 1983; Ramsey & Schemske, 2002). These novel life-history traits, such as drought tolerance, apomixis (asexual seed production), increased organ size and biomass, and changes in pest resistance or flowering time, could allow polyploids to enter new ecological niches (Lumaret, 1988; Thompson & Lumaret, 1992; Segraves & Thompson, 1999; Husband & Schemske, 2000; see also Brochmann *et al*., 2004; Thompson, Nuismer & Berg, 2004 – both this issue). Some polyploids also vary in phenotypic plasticity relative to their diploid progenitors (Emery, Chinnappa & Chmielewski, 1994; Bretagnolle & Lumaret, 1995; Petit, Thompson & Bretagnolle, 1996; Bretagnolle & Thompson, 2001; Schranz & Osborn, 2004).

Despite the observation that newly formed polyploids differ from their diploid parents, the molecular genetic mechanisms by which polyploidization contributes to novel phenotypic variation are not well

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understood. One explanation for the success of polyploids was that the duplicate genes in polyploids may diverge and acquire new functions (neofunctionalization; Force *et al*., 1999; Lynch & Conery, 2000). Although functional divergence of duplicate genes might confer a selective advantage to polyploids over a long evolutionary time period, it is unlikely to provide any immediate advantage to new polyploids. Because immediate phenotypic effects are seen shortly after polyploid formation, other sources of molecular variation must be operating aside from neofunctionalization (reviewed in Soltis & Soltis, 1993; Matzke *et al.*, 1999; Comai, 2000; Wendel, 2000; Liu & Wendel, 2002, 2003; Ramsey & Schemske, 2002; Osborn *et al*., 2003b). Osborn *et al*. (2003b) classified the mechanisms that can lead to novel forms of gene expression in polyploids into three categories: (a) increased variation for dosage-regulated gene expression, (b) altered regulatory interactions and (c) rapid genetic and epigenetic changes.

Investigating the molecular genetic changes that contribute to the difference between polyploids and their diploid progenitors, as well as among polyploids, requires knowledge of both the diploid progenitors and the pedigree (phylogenetic history) of the polyploids. As a result, investigating existing polyploids in their natural environment is problematic because the exact parental genomes are often unknown or have evolved since polyploid formation. In addition to uncertain parentage, untangling the history of natural polyploids can be complicated by multiple-polyploid events and subsequent hybridizations (Soltis & Soltis, 1993; Soltis & Soltis, 1999; see also Abbott & Lowe, 2004; Ainouche, Baumel & Salmon, 2004; Soltis *et al*., 2004 – all this issue). To circumvent these problems, some polyploids can be resynthesized from current forms of diploid progenitors to obtain known pedigrees and genotypes. These synthetic polyploids allow for more precise comparisons with the exact diploid progenitors and a greater range of experimental options. Recent studies have demonstrated rapid genetic changes in resynthesized allopolyploids of *Arabidopsis* (Comai *et al*., 2000; Lee & Chen, 2001; Madlung *et al*., 2002; reviewed by Chen *et al*., 2004 – this issue), *Brassica* (Parkin *et al*., 1995; Song *et al*., 1995; Quijada, 2003; Udall, 2003; Schranz & Osborn, 2004; see also Lukens *et al*., 2004 – this issue) and *Triticum* (Feldman *et al*., 1997; Ozkan, Levy & Feldman, 2001; Shaked *et al*., 2001; Kashkush, Feldman, & Levy, 2002, 2003; reviewed by Levy & Feldman, 2004 – this issue).

The genus *Brassica* is an excellent system for creating and studying resynthesized allopolyploids. In fact, the famous 'Triangle of U' (U, 1935), which illustrates the crossing relationships between three diploid *Brassica* species and their three corresponding allopolyploids, was one of the earliest uses of resynthesized polyploids to infer the evolutionary origins of the naturally occurring polyploids (Karpechenko, 1928; Morinaga, 1931; U, 1935). Since these early biosystematic studies, resynthesized *Brassica* species have been used to illustrate the rapid and extensive evolution of polyploid genomes shortly after polyploid formation (Song *et al*., 1995). Recently, we have used resynthesized *B. napus* lines to investigate the effects of polyploidy on *Brassica* life history traits, with a focus on flowering time (Schranz & Osborn, 2000, 2004).

Although many genes undoubtedly control flowering time in *Brassica* species, *FLOWERING LOCUS C* (*FLC*), a MADS-box gene isolated from *Arabidopsis*, is a key regulator of the autonomous flowering and vernalization pathways in *Brassica* (Kole *et al*., 2001). *Arabidopsis* has a single copy of *FLC* (*AtFLC*) at the distal end of the short arm of chromosome 5 (*At5*) that acts in a dosage-dependent manner to repress flowering (Michaels & Amasino, 1999; Sheldon *et al*., 1999). Diploid *Brassica* species are expected to contain three *FLC* copies, due to a triplication of this *At5* region (Osborn *et al*., 1997; Axelsson, Shavorskaya & Lagercrantz, 2001; Parkin, Lydiate & Trick, 2002; Schranz *et al*., 2002; Parkin, Sharpe & Lydiate, 2003; Lukens *et al*., 2003; see Lukens *et al*., 2004). These three expected homologues of *FLC* were isolated from *B. rapa* (*BrFLC1* on chromosome R10, *BrFLC2* on chromosome R2 and *BrFLC3* on chromosome R3) along with a fourth unexpected homologue, *BrFLC5*, that mapped to a region of R3 without homology to the top of *At5* (Schranz *et al*., 2002). By contrast, only three homologues of *FLC* have been identified in *B. oleracea* (*BoFLC1*, *BoFLC3* and *BoFLC5*), but other genotypes may possess a *BoFLC2* sequence (Schranz *et al*., 2002). Given that *B. napus* is an allopolyploid derived from *B. rapa* and *B. oleracea* (U, 1935), Schranz *et al*. (2002) predicted that *B. napus* should have at least seven *BnFLC* loci, and eight loci were later mapped in *B. napus* with four loci in the *B. rapa* portion of the genome and four loci in the *B. oleracea* portion of the genome (Quijada, 2003; Udall, 2003). Allelic variation for flowering time has been associated with several *BnFLC* loci in crosses of *B. rapa* and *B. napus* (Schranz *et al*., 2002; Quijada, 2003; Udall, 2003; reviewed in Osborn & Lukens, 2003).

In this paper, we report new data on the molecular genetic basis for flowering time variation in early- and late-flowering lineages that were directly derived from a resynthesized *B. napus* allopolyploid (Schranz & Osborn, 2004). Specifically, we measured divergence in flowering time, assayed genotypes to find patterns of rapid genome structural changes (e.g. chromosomal rearrangements) and examined the gene expression of *BnFLC* loci. We also describe ongoing experiments

involving the development and analyses of 50 resynthesized *B. napus* allopolyploids, which may provide further insight on the dynamics of genome changes associated with polyploidization.

MATERIAL AND METHODS

Plant materials and phenotypic analyses

The early-flowering and late-flowering lineages of *Brassica napus* (Schranz & Osborn, 2004) were derived from a single chromosome doubled amphihaploid from a cross between *B. rapa* cv. Reward TO1067 (female parent) and *B. oleracea* TO1434 (male parent, which is a selfed progeny of TO1000). Thus, all polyploid lines within this lineage were expected to be genetically identical because they were derived from a single *B. napus* ancestor (TO1141, see Fig. 1). Reward is an oilseed cultivar and TO1000 is an inbred (S_5) rapid cycling plant derived from CrGC3-3 (Crucifer Genetics Cooperative, Madison, WI, USA). An individual amphihaploid plant (AC) was colchicine treated to generate an S_0 amphidiploid (AACC) by the same process as described by Song, Osborn & Williams (1993). A single S_0 plant (TO1141) was self-pollinated to obtain S_1 seed. A sister lineage to this S_0 resynthesized *B. napus* plant (TO1147) was used as a parent in a genetic mapping study and was found to contain complete genome complements derived from *B. rapa* and *B. oleracea* (Udall, 2003). Two S_1 plants (TO1217 and TO1216) were self-pollinated to generate two pools of S_2 seed. One hundred S_2 plants from each pool were transplanted to a field in Arlington, Wisconsin, in rows spaced 1 m apart and 0.3 m between plants within rows. The hundred S_2 plants were measured for flowering time and selected plants were self-pollinated to generate S_3 seeds. The five earliest and five latest flowering plants for each of the two pools were selected and self-pollinated, giving rise to 20 lines of S_3 seed. Five S_3 seeds from each of the 20 selected lines were individually planted in 10-cm pots and grown in a greenhouse. Two lines of S_3 seeds were utilized in this study: one early-flowering selection (ES98) and one late-flowering line (ES88). These S_3 lines were also used as progenitors for the S_4 generation.

Seeds from selected early- and late-flowering S_4 plants (LL152 and LL149, respectively) were self-pollinated to generate two pools of S_5 seed (Fig. 1). One hundred S_5 plants from each pool were sown in 36-well flats in a growth chamber and grown for 3 weeks. The seedlings were then transplanted to a field in Arlington in double-rows, with 1 m between the double-rows and 0.3 m between plants within rows. All plants were measured for days to flowering when the first flower opened (results shown at bottom of Fig. 1). The five earliest plants from the early-flowering line and the five latest plants from the late-flowering line were individually enclosed within pollination bags.

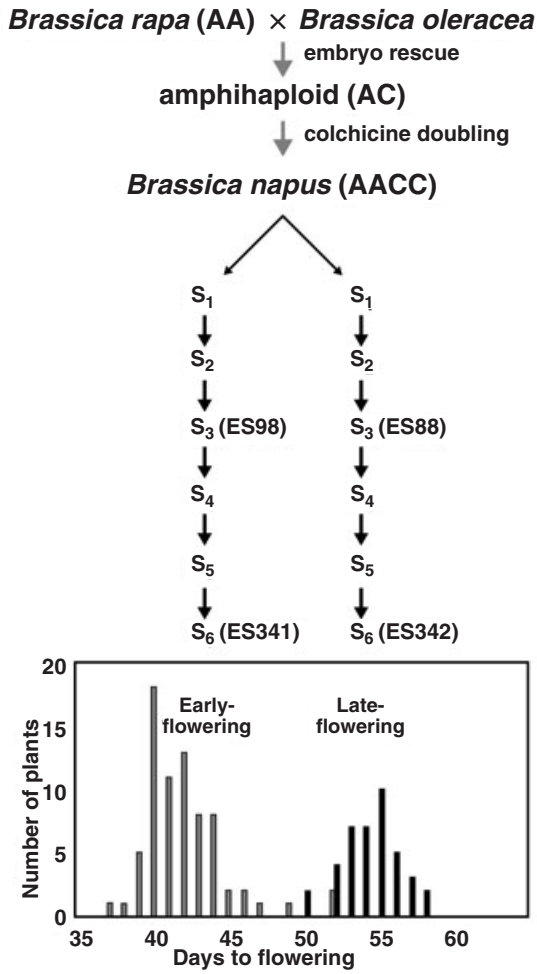

Figure 1. Pedigree of early-flowering and late-flowering allopolyploid *Brassica napus* lines. The parental diploid lines, two early-flowering lines (ES98 at S_3 generation and ES341 at S_6 generation) and two late- flowering lines (ES88 at S_3 generation and ES342 at S_6 generation) were analysed in this study for changes in gene structure and gene expression. The graph at the bottom indicates days to flowering vs. number of plants as analysed at the S_5 generation for early-flowering lines (light histogram bars) and late-flowering lines (dark histogram bars).

Seeds from these selected S_5 plants were grown in a growth chamber, and individual early-flowering (ES341) and late-flowering (ES342) S_6 plants were utilized for genetic crosses. Two reciprocal crosses between these two lines were made (ES342 × ES341 and ES341 × ES342). Individual F_1 seeds derived from both crosses were grown in 15.2-cm pots in a greenhouse. Two F_1 plants, ES349 and ES352 (each from a separate reciprocal cross), were self-pollinated to generate F_2 seeds.

Phenotypic analysis of F_2 generation

For phenotypic evaluation and segregation analysis, 50 F_2 plants from each reciprocal cross, and eight seeds from each F_1 cross, the S_6 polyploid parents, and the original diploid progenitors were grown in PGW-132 growth chambers (Percival Scientific, Boone, IA, USA) under equal light intensity (550 μmol m^{-2} s^{-1}), as measured at mid-canopy height. The reciprocal crosses were grown in flats that were distributed in the growth chambers in a completely randomized design and grown under long day growth conditions of 16 h of light and 8 h of dark. Light measurements were made using an LI-COR spectro-radiometer LI-1800 (LI-COR, Lincoln, NB, USA). The ratio of R:FR was calculated from the intensity of the light from wavelengths of 655–665 nm for red light and 725–735 nm for far-red light. Growth under 28 215-W fluorescent light bulbs and 12 60-W incandescent light bulbs provided the low R:FR ratio of 1.6. Temperature was maintained at a constant 21 °C. Plants were grown in 24-well flats using Jiffy mix⁻ soil (Jiffy Products of America, Batavia, IL, USA) and were watered with 0.5× Hoagland's solution. All plants were measured for days to flowering when the first flower opened.

DNA extraction

Genomic DNA was isolated from lyophilized leaf tissues collected from 12–24 individuals for each genotype using the CTAB procedure described by Kidwell & Osborn (1992) with some modifications (see http://osbornlab.agronomy.wisc.edu/research/protocols/dna.html). DNA was first isolated from six genotypes to be analysed by genetic markers: the two diploid *Brassica* parents, the S_3 and S_6 generations of the early-flowering lines (ES98, ES341), and the S_3 and S_6 generations of the late-flowering lines (ES88, ES342). For these six genotypes, the leaf tissues collected were pools of several individuals. For subsequent DNA isolation from the F_2 population, the leaf tissues were collected from individual plants.

Genetic markers: RFLP analysis

The DNA clones included as probes for analyses by restriction fragment length polymorphisms (RFLP) were the same probes used in previous studies (Ferreira, Williams & Osborn, 1994; Teutonico & Osborn, 1994; Thormann *et al*., 1994; Kole, Vogelzang & Osborn, 1997; Butruille, Guries & Osborn, 1999; Quijada, 2003; Udall, 2003). The procedures used for restriction enzyme digestion, gel electrophoresis, Southern blotting, probe radiolabelling and membrane hybridization are as described by Ferreira *et al*. (1994). DNA clones were from three libraries: a *Pst*I genomic library, a cDNA library and an *Eco*RI library (Thormann *et al*., 1994; Ferreira *et al*., 1994). The probe nomenclature of Parkin *et al*. (1995) and Sharpe *et al*. (1995) was used in which the clones from the *Pst*I and *Eco*RI libraries were renamed 'pW' probes and the clones from the cDNA library were renamed 'pX' probes (see http://osbornlab.agronomy.wisc.edu/research.html for details on these probes). In addition, four homologues of *FLC*, corresponding to *BrFLC1*, *BrFLC2*, *BrFLC3* and *BrFLC5* from *B. rapa* (Schranz *et al*., 2002), were used as probes.

DNA from seven genotypes was analysed by Southern hybridization: the two diploid *Brassica* parents, a DNA mix of the diploid parents, the S_3 and S_6 generations of the early-flowering lines (ES98, ES341), and the S_3 and S_6 generations of the late-flowering lines (ES88, ES342). Blots probed with the four *BrFLC* probes had DNA digested with 12 enzymes (*Eco*RI, *Hin*dIII, *Msp*I, *Hpa*II, *Dra*I, *Xba*I, *Eco*RV, *Pst*I, *Pvu*II, *Hae*III, *Hha*I, *Bam*HI). In addition to the *FLC* probes, 31 other RFLP probes, chosen to cover all the linkage groups of *B. napus*, were hybridized to blots that had DNA digested with four enzymes (*Eco*RI, *Hin*dIII, *Msp*I, *Hpa*II).

Genetic markers: microsatellite analysis

DNA from the same seven genotypes analysed by Southern hybridization was also used in microsatellite analyses. PCR of diluted genomic DNA samples with microsatellite or simple-sequence-repeat (SSR) primers and electrophoresis of amplified DNA was performed according to Senior & Heun (1993) with some slight modifications (de Leon, 2002). A total of 31 PCR primer sequences were chosen to cover all of the linkage groups of *B. napus* except N15 (A. Sharpe & D. Lydiate, pers. comm.).

RNA isolation and cDNA synthesis

RNA was isolated from the same six genotypes analysed by genetic markers: the two diploid *Brassica* parents, the S_3 and S_6 generations of the early-flowering lines (ES98, ES341) and the late-flowering lines (ES88, ES342). These six genotypes were grown in the same growth chambers and environmental conditions as described above for phenotypic analysis. Two RNA samples were extracted per genotype. These two replications were used in all the following experiments in parallel. Each RNA preparation was a pooled sample of 20–24 plants, gathered from six 'four-packs' of plants arranged in a randomized complete block design. Leaf tissue was collected at the four-leaf stage (2- to 3-week old plants) and immediately placed in liquid nitrogen.

Total RNA was extracted from 300 mg of frozen leaf tissue using TRIzol reagent (Sigma, T-9424) according to manufacturer's instructions and treated with RNase-free DNase using the DNA-free kit (Ambion # 1906). Single-strand cDNAs were synthesized using the Superscript First-Strand synthesis system (Invitrogen, #11904–018) and 3.5 μg of total RNA as a template according to the manufacturer's instructions. The cDNA products were diluted 1 : 10 with water, and 5 μL of diluted cDNA was used as template for PCR amplification.

Primer design and gene amplification of *FLC*

Four *FLC* genes were amplified using their corresponding specific primers (Table 1). Primers were designed based on the sequences of *B. rapa* and *B. oleracea* alleles so as to amplify two homoeologous targets that have nucleotide substitutions in the amplified fragment. Forward primers for *BnFLC1* and *BnFLC3* were the same as those used in Schranz *et al*. (2002). The other *FLC* primers were designed specifically for each *FLC* in order to prevent cross-amplification. Initially, *FLC* primer specificity was tested using genomic DNA as a template for PCR to make sure that we were amplifying the correct fragments and to optimize PCR cycling conditions. Subsequently, cDNA of the two diploid parental *Brassica* lines, a mixture consisting of equal amounts of quantified cDNA from the two diploid parents and cDNA from the four allopolyploid *B. napus* lines were analysed by RT-PCR to check the specificity of *FLC* expression levels and to determine the linear stage of amplification.

RT-PCR analysis

For RT-PCR analyses, a constantly expressed ubiquitin gene was used as a control for DNA contamination and PCR cycles. All the reactions were performed in an Eppendorf Mastercycler. PCR reactions were composed of 0.5 μM of each primer, 0.2 μM dNTP, 2 mM $MgCl_2$ and *Taq*DNA polymerase with a total volume of 10 μL. The cycling profile was for 4 min at 96°C, followed by 30 s at 94°C, 45 s at 60°C, 1 min at 72°C and then a final 10 min extension at 72°C. Different PCR cycles were applied to each *FLC*, with 25, 30, 25 and 30 cycles for *FLC1*, *FLC2*, *FLC3* and *FLC5*, respectively. The ubiquitin control had 20 cycles.

Single-strand conformational polymorphism (SSCP) analysis

Qualitative homoeologous gene expression was conducted by cDNA-SSCP analysis (Adams *et al*., 2003; Cronn & Adams, 2003). Briefly, the PCR reaction for SSCP was the same as for RT-PCR except that 0.1 μL ^{32}P dCTP was added to the mixture. Fifty microlitres of loading dye was added to each 10 μL product and 1.7 μL was loaded after denaturing at 95°C for 8 min. SSCP gel was prepared using a 0.5× MDE (Cambrex Bio Science, #50620) gel solution. The gel was run at room temperature at a constant 7 W for 15–19 h to separate each *FLC* homologue. The gel was dried at 70°C for about 1 h and exposed to film for 5–24 h.

Segregation analysis

Segregation analysis was conducted in F_2 populations derived from reciprocal crosses between an early- and a late-flowering line in the S_6 generation. One hundred individuals (50 from each cross) were genotyped by PCR amplification using *FLC2*, *FLC3* and *FLC5* specific primers. PCR profiling for genomic DNA was the same as used for amplifying cDNA, but with 35 cycles. Primers from conserved regions of exon 2 and exon 5, which amplified both *FLC1* and *FLC2*, were used for *FLC2* amplifications to check PCR artefacts. Only *FLC3* fragments, showing homoeologous expression patterns, were further analysed for their association with flowering time by analysis of variance (SAS Institute, 2000).

RESULTS

Phenotypic analyses

The distributions for days to flowering of the early- and late- flowering *B. napus* lineages were distinct and had very little overlap: 37–52 days to flowering, with a mean of 41.9 days for the early-flowering lines;

Table 1. Primer sequences for each *FLC*. *FLC1* F and *FLC3* F are directly from Schranz *et al*. (2002). The remaining primers were designed specifically for this study

	Forward	Reverse
FLC1	5′-CTTGAGGAATCAAATGTCGATAA (in exon 4)	5′-CCATCTGGCTAGCCAAAACAT (in exon 6)
FLC2	5′-AACATGCTGATGATCTTAAGGCTC (in exon 2)	5′-CCCTGGTTCTCTTCTTTCAGCATT (in exon 6)
FLC3	5′-GTGGAATCAAATGTCGGTGG (in exon 4)	5′-AGCCAAAGCCTGATTCTCTTC (in exon 6)
FLC5	5′-CCTCGTTGAGCTAGAAGATCA (in exon 4)	5′-GGAGATTTGTCCAGATGACATCTCT (in exon 7)

50–58 days, with a mean of 54.4 days for the late-flowering lines (Fig. 1). A two-tailed *t*-test indicated that the early- and late-flowering lines were significantly different for days to flowering ($P < 0.01$).

Analyses of *BnFLC*

Each of the four *BnFLC* genes (*BnFLC1*, *BnFLC2*, *BnFLC3*, *BnFLC5*) was used for genome structure and gene expression analyses. In our materials, the four *BnFLC* genes occur at seven loci: four orthologues from *B. rapa* (*BrFLC1*, *BrFLC2*, *BrFLC3*, *BrFLC5*) and three orthologues from *B. oleracea* (*BoFLC1*, *BoFLC3*, *BoFLC5*), because *BoFLC2* was not found in our genotype of *B. oleracea*. The map positions of these loci were determined in previous studies (Schranz *et al*., 2002; Quijada, 2003; Udall, 2003).

Analyses of *BnFLC1*

BnFLC1 in the allopolyploid *B. napus* is derived from homoeologous regions from the diploid parents: *BrFLC1* is on chromosome 10 of *B. rapa* (R10) and *BoFLC1* is on chromosome 9 of *B. oleracea* (O9; Fig. 2A). Southern blots probed with *BrFLC1* revealed that RFLP fragments found in the diploid *Brassica* parents were also found in all of the allopolyploid *B. napus* lines (additive patterns, Fig. 2B). Based on these Southern hybridization results, we constructed ideograms of early-flowering and late-flowering *B. napus* lines for *BnFLC1* on the N10 and N19 chromosome (Fig. 2C). We did not investigate any genotypic markers immediately flanking *BnFLC1*, but other markers on N10 and N19 showed similar additive patterns, including two SSRs on N10, and two SSRs and the RFLP probe pX140 on N19 (data not shown).

Expression analyses of *BnFLC1* showed the same cDNA-SSCP pattern for *B. rapa* and *B. oleracea* (Fig. 2D). All *B. napus* lines also showed this pattern. Owing to the lack of polymorphisms between the diploid parents, we cannot rule out silencing of one of the parental genes in the allopolyploids; however, the expression patterns and the Southern hybridization results provide no evidence that *BnFLC1* contributed to the differences in flowering time between the *B. napus* lines.

Analyses of *BnFLC2*

BnFLC2 in the allopolyploid *B. napus* is expected to be derived from homoeologous regions from the diploid parents: *BrFLC2* is on chromosome 2 of *B. rapa* (R2) and *BoFLC2* is on chromosome 2 of *B. oleracea* (O2;

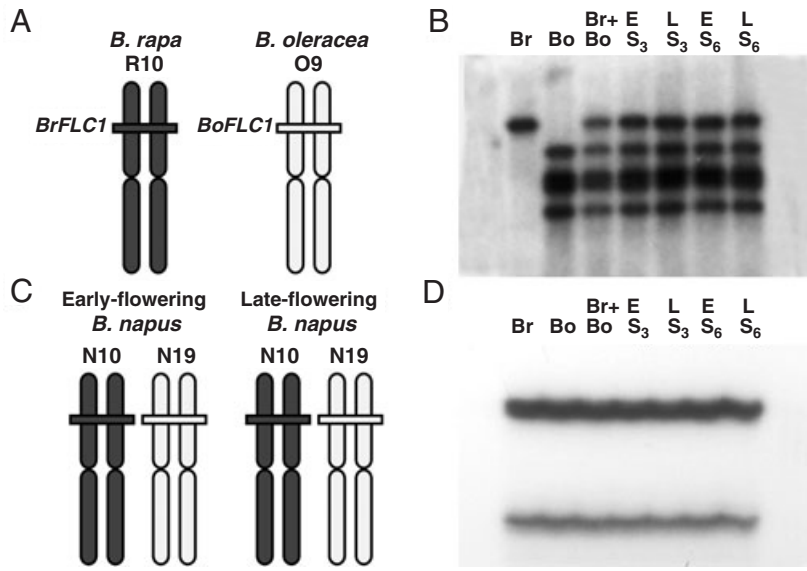

Figure 2. Analyses of *BnFLC1*. A, Ideogram indicating the presence of *BrFLC1* on the R10 chromosome of *B. rapa* (dark shading) and *BoFLC1* on O9 chromosome of *B. oleracea* (light shading). B, Southern blot probed with *BrFLC1* on to *Eco*RI-digested DNA of *B. rapa* (Br), *B. oleracea* (Bo), a pooled mixture of *B. rapa* and *B. oleracea* DNA (Br+Bo), and early-flowering (E) and late-flowering (L) *B. napus* lines from the S_3 and S_6 generations (ES_3 = ES98, ES_6 = ES341, LS_3 = ES88, LS_6 = ES342). C, Ideogram indicating the putative location of *BnFLC1* on the N10 and N19 chromosome of early-flowering and late-flowering *B. napus* lines based on RFLP analysis. Both lineages have a *BrFLC1* locus from *B. rapa* (dark shading) and a *BoFLC1* locus from *B. oleracea* (light shading). D, Expression analysis of *BnFLC1* using cDNA-SSCP technique indicating no expression differences between the early-flowering and late-flowering *B. napus* lineages.

Schranz *et al*., 2002; Quijada, 2003; Udall, 2003). However, Southern blots probed with *BrFLC2* revealed RFLP fragments only in *B. rapa*, the pooled diploid mixture and the early-flowering *B. napus* lines (Fig. 3A, B). Based on these Southern hybridization results, we constructed ideograms of early-flowering and late-flowering *B. napus* lines for the putative location of *BnFLC2* on the N2 or N2 and N12 chromosomes of the early-flowering lines but the absence of *BnFLC2* in late-flowering *B. napus* lines (Fig. 3C). The absence of *FLC2* on N2 in the late-flowering line can be explained by the occurrence of a non-reciprocal transposition involving a segment of N12 to the homoeologous position N2. This explanation is supported by Southern blot results using the probe pW241 that detected a marker locus flanking *FLC2* on R2, and in the homoeologous position on O2 (Fig. 3A). The late-flowering lines are missing the N2 locus and appear to have two copies of the N12 locus based on a visual inspection of fragment intensity (Fig. 3B). Results from pW241 also suggest that a non-reciprocal transposition occurred between the S_3 and S_6 generations of the early-flowering lines. The S_3 early-flowering line contains both the *B. rapa* and the *B. oleracea* pW241 loci, whereas the S_6 early-flowering line is missing the *B. oleracea* locus and appears to have two copies of the *B. rapa* locus (Fig. 3B). The S_6 early-flowering line also appears to have two copies of *BrFLC2*, based on fragment dosage (Fig. 3B). Two microsatellites (SSRs) on N2 and one SSR on N12 that are well below the mapped locations for *BnFLC2* showed an identical additive pattern for all polyploids (data not shown), indicating that the hypothesized rearrangements involved only an upper portion of the N2 and N12 chromosomes.

The results from expression analyses of *BnFLC2* using RT-PCR were consistent with the Southern blot results. Gene expression was observed in *B. rapa*, the pooled mixture and the early-flowering *B. napus* lines, but not in *B. oleracea* or the late-flowering lines, which were missing copies of *FLC2* (Fig. 3D). The S_6 early-flowering line appeared to have more *BnFLC2* transcripts, consistent with a double-dose of the *BrFLC2* gene

If the early-flowering parent had two copies of *BrFLC2* and the late-flowering parent had zero, we

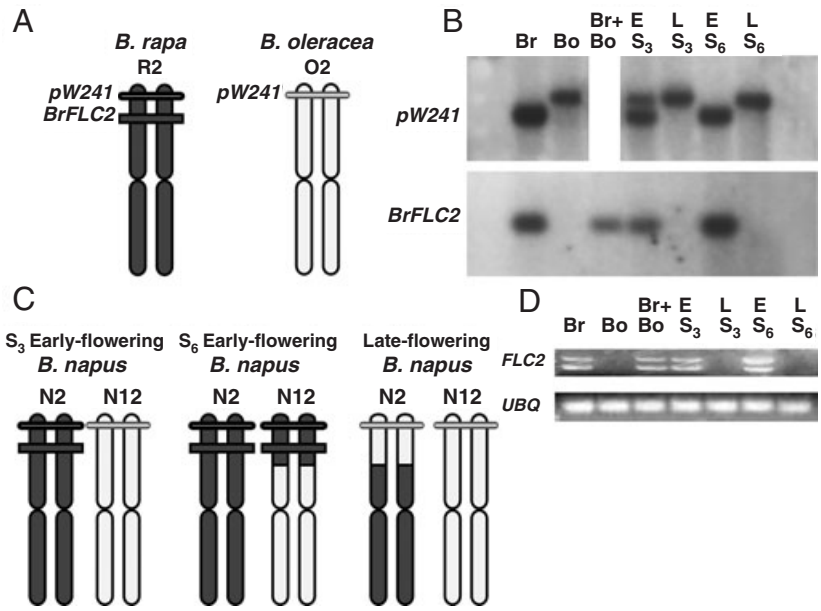

Figure 3. Analyses of *BnFLC2*. A, Ideogram indicating the presence of *BrFLC2* on the R2 chromosome of *B. rapa* (dark shading) and the absence of *BoFLC2* on the O2 chromosome of *B. oleracea* (light shading). B, Southern blot probed with *BrFLC2* on to *Bam*HI-digested DNA of *B. rapa*, *B. oleracea*, a pooled mixture of *B. rapa* and *B. oleracea* DNA, and early-flowering and late-flowering *B. napus* lines (same lines as in Fig. 2). RFLP fragments are found only in *B. rapa*, the pooled mixture and the early-flowering *B. napus* lines. C, Ideogram indicating the putative location of *BnFLC2* on the N2 and N12 chromosome of early-flowering and late-flowering *B. napus* lines based on RFLP analysis. The early-flowering line has a *BrFLC2* locus from *B. rapa* (dark shading) and none from *B. oleracea* (light shading). The late-flowering line has no copies of *FLC2* because a putative non-reciprocal transposition gives only *B. oleracea* (light shading). A neighbouring RFLP marker (pW241) supports a non-reciprocal transposition hypothesis. D, Expression analysis of *FLC2* using RT-PCR indicates expression differences among the diploid *Brassica* species and the early-flowering and late-flowering *B. napus* lineages (ubiquitin expression also shown as a control).

would expect a digenic segregation ratio with 1 : 16 having no *FLC2*. We analysed the F_2 population for segregation of *BrFLC2* and found zero F_2 individuals with no copies of the gene. Thus, we could not test for an association of the *BnFLC2* changes with flowering time. The lack of fit between the observed and expected segregation ratios may be due to unusual segregation associated with the chromosomal rearrangement.

Analyses of *BnFLC3*

BnFLC3 in the allopolyploid *B. napus* is derived from homoeologous regions from the diploid parents: *BrFLC3* is on chromosome 3 of *B. rapa* (R3) and *BoFLC3* is on chromosome 3 of *B. oleracea* (O3; Fig. 4A). Southern blots probed with *BrFLC3* revealed an additive pattern for the late-flowering lines (both *BrFLC3* and *BoFLC3* present); however, the early-flowering *B. napus* lines have lost *BoFLC3* and appear to have a double-dose of *BrFLC3* (Fig. 4B). These results can be explained by a non-reciprocal transposition of an N3 segment to the homoeologous position on N13 in the early-flowering lineage (Fig. 4C). Further evidence for the rearrangement was provided by probe pX133, which identified loci adjacent to *BnFLC3* on N3 and N13 (Fig. 4A, C) having similar patterns to *BnFLC3* in the early- and late-flowering lines. Other markers on N3 or N13, including one SSR on the bottom of N3, two SSRs on the middle and bottom of N13, and several RFLP probes that hybridized to loci on N3 and N13 (including *BnFLC5*), detected additive patterns in all polyploids, indicating that the rearrangements involved only a distal portion of N13 (data not shown).

The results from expression analyses of *BnFLC3* using cDNA-SSCP were consistent with the Southern hybridization results. The early-flowering lines did not express *BoFLC3* and appeared to have higher levels of *BrFLC3* expression (Fig. 4D). We also observed a decrease in expression of *BrFLC3* between the S_3 and S_6 late-flowering lines. This may be explained by a change in gene dosage (from 2 *BoFLC3* : 2 *BrFLC3* to 3 *BoFLC3* : 1 *BrFLC3*) owing to a non-reciprocal transposition event in the S_5 generation that had not yet become homozygous. This explanation is supported by results from the Southern blot showing a dosage shift between the S_3 and S_6 generations (Fig. 4B).

We performed a segregation analysis of *BnFLC3* on the F_2 population. Figure 5A shows a model of inher-

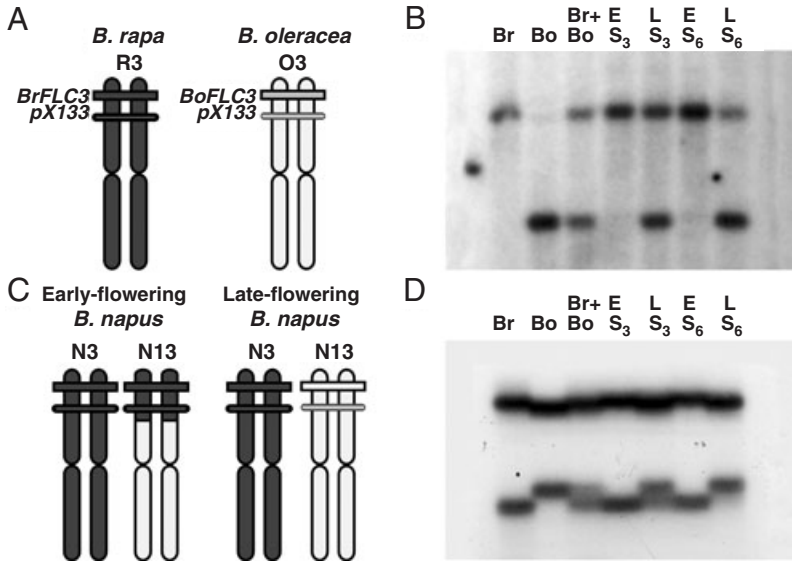

Figure 4. Analyses of *BnFLC3*. A, Ideogram indicating the presence of *BrFLC3* on the R3 chromosome of *B. rapa* (dark shading) and *BoFLC3* on the O3 chromosome of *B. oleracea* (light shading). B, Southern blot probed with *BrFLC3* on to *Bam*HI-digested DNA of *B. rapa*, *B. oleracea*, a pooled mixture of *B. rapa* and *B. oleracea*, and early-flowering and late-flowering *B. napus* lines (same lines as in Fig. 2). RFLP analysis reveals the loss of *B. oleracea* fragments in the early-flowering *B. napus* lines. C, Ideogram indicating the putative location of *BnFLC3* on the N3 and N13 chromosome of early-flowering and late-flowering *B. napus* lines based on RFLP analysis. The early-flowering line has two *BrFLC3* loci from *B. rapa* (dark shading) and none from *B. oleracea* (light shading) due to a non-reciprocal transposition. The late-flowering line has one *BrFLC3* locus from *B. rapa* (dark shading) and one *BoFLC3* locus from *B. oleracea* (light shading). The non-reciprocal transposition was verified by flanking RFLP markers (pX133). D, Expression analysis of *FLC3* using cDNA-SSCP indicated expression differences between the early-flowering and late-flowering *B. napus* lines, as well as between generations of the late-flowering *B. napus* lines.

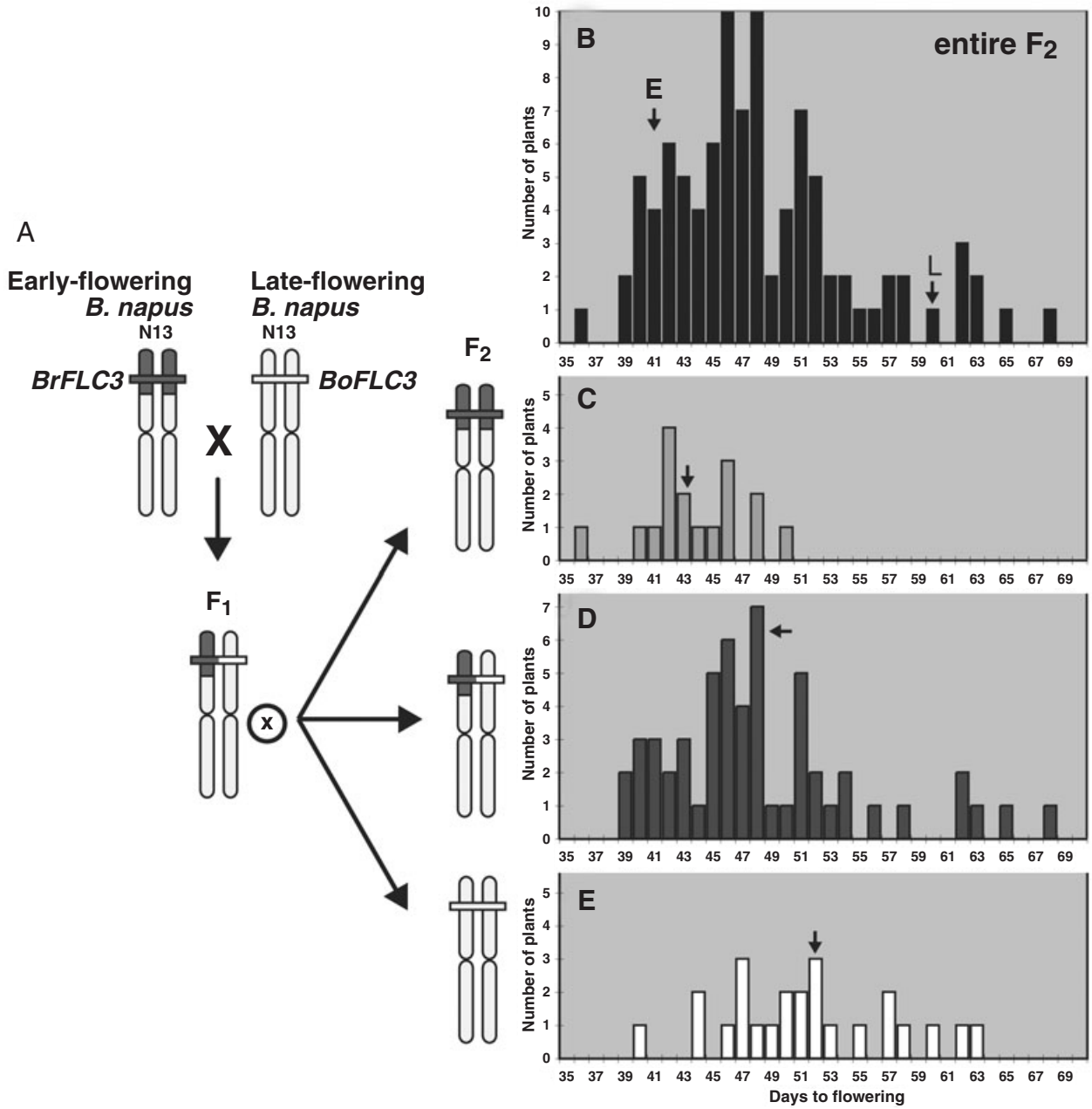

Figure 5. Segregation analysis of *FLC3*. A, Development of segregating F_2 population from early-flowering (ES341) and late-flowering (ES342) *B. napus* lines. Ideograms show presence of *FLC3* on N13 chromosomes from *B. rapa* (dark shading) and *B. oleracea* (light shading). B, Frequency distribution of days to flowering vs. number of plants for the entire F_2 population. The range in flowering time of the F_2 populations was 36–68 days, with a mean of 47.9 days, and was approximately normally distributed ($\chi^2 = 2.6$). The mean of the early-flowering parent (ES341) was 41.0 days (E) and for the late-flowering parent (ES342) the mean was 60.3 days (L). Hence, the distribution for days to flowering of the F_2 plants was mostly between the allopolyploid parents. The F_1 plants took an average of 44.8 days to flower, similar to the mean of the resulting F_2 populations. Days to flowering of the progenitor diploid lines were 35.0 for the *B. rapa* parent and 38.1 for the *B. oleracea* parent. C, Frequency distribution of days to flowering vs. number of plants for genotypes homozygous for *B. rapa* alleles (dark shading). Arrow indicates mean of Br/Br: 43.88 days to flowering. D, Frequency distribution of days to flowering vs. number of plants for genotypes heterozygous for *B. rapa* alleles (dark shading) and *B. oleracea* alleles (light shading). Arrow indicates mean of Br/Bo: 48.43 days. E, Frequency distribution of days to flowering vs. number of plants for genotypes homozygous for *B. oleracea* alleles (light shading). Arrow indicates mean of Bo/Bo: 52.12 days. Note that these three genotypes had significant differences in flowering time.

itance for *BnFLC3* based on the F_2 from the cross between the S_6 early- and late-flowering lines. The distributions of the two reciprocal F_2 populations for flowering time were almost identical (means of 46.2 and 49.5 days). Thus, there does not appear to be a maternally inherited effect for the control of flowering time. Given this, and the fact that the flowering time–population interactions were not significant ($P = 0.93$), we combined the individuals from the two populations for all subsequent analyses (Fig. 5B–E). The distribution of days to flowering for the *c*. 100 individuals in the two F_2 populations was mostly between the flowering times of the allopolyploid parents (Fig. 5B).

The segregation ratio for *BnFLC3* on N13 was not statistically different from the expected 1 : 2 : 1 ratio for allelic segregation of *BrFLC3* and *BoFLC3* (17 *Br*/*Br* : 53 *Br*/*Bo* : 24 *Bo*/*Bo*; $\chi^2 = 2.6$, $P = 0.27$). The segregation of *BnFLC3* was associated with flowering time. Plants with two *B. rapa* alleles flowered significantly earlier (mean of 43.9 days to flowering, Fig. 5C) than those with two *B. oleracea* alleles (mean of 52.1 days to flowering, Fig. 5E) ($P < 0.0001$). Thus, the expected effect of substituting one *B. rapa* allele for one *B. oleracea* allele was a delay in flowering of 4 days. Segregation of *BnFLC3* explained 29% of the phenotypic variation for days to flowering in the ANOVA of the F_2 population.

Analyses of *BnFLC5*

BnFLC5 in the allopolyploid *B. napus* is derived from homoeologous regions from the diploid parents: *BrFLC5* is on chromosome 3 of *B. rapa* (R3) and *BoFLC5* is on chromosome 3 of *B. oleracea* (O3; Fig. 6A). Southern blots probed with *BrFLC5* revealed an additive pattern for the early- and late-flowering lines (both *BrFLC5* and *BoFLC5* present, Fig. 6B). Based on this result and the map positions of *FLC* loci from other studies (Quijada, 2003; Udall, 2003), the break point of the transposition on N13 in the early-flowering line appears to be between *BnFLC3* and *BnFLC5* (Fig. 6C).

RT-PCR analysis of *BrFLC5* indicated expression only in the *B. rapa* parent. We did not detect *BoFLC5* expression in *B. oleracea* or in any of the allopolyploid *B. napus* lines (Fig. 6D). Unlike other *BnFLC* loci, the expression patterns differed from the RFLP patterns seen on Southern blots (Fig. 6B). We might expect silencing of *BoFLC5* in the allopolyploid lines, because this was not expressed in the diploid parent, but silencing of *BrFLC5* in the allopolyploid line was

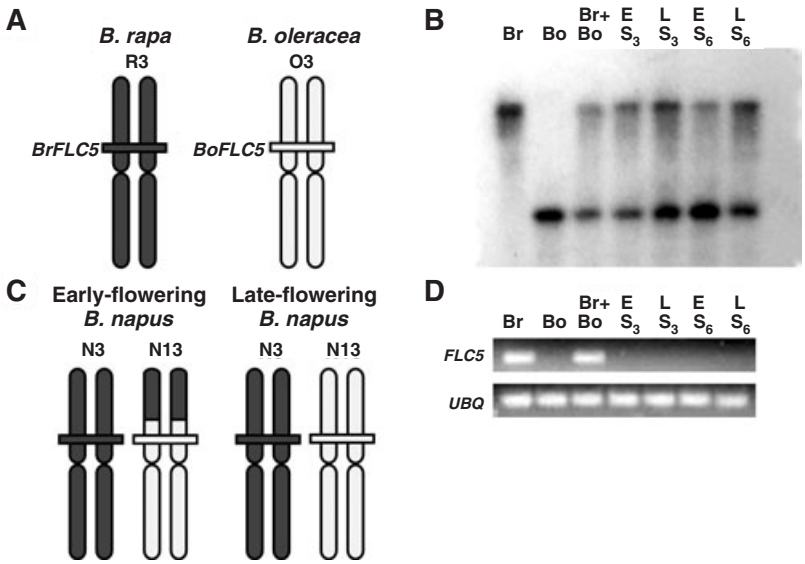

Figure 6. Analyses of *BnFLC5.* A, Ideogram indicating the presence of *BrFLC5* on the R3 chromosome of *B. rapa* (dark shading) and *BoFLC5* on the O3 chromosome of *B. oleracea* (light shading). B, Southern blot probed with *BrFLC5* on to *Eco*RI-digested DNA of *B. rapa*, *B. oleracea*, a pooled mixture of *B. rapa* and *B. oleracea*, and early-flowering and late-flowering *B. napus* lines (same lines as in Fig. 2). RFLP fragments are found in all lanes. C, Ideogram indicating the putative location of *BnFLC5* on the N3 and N13 chromosome of early-flowering and late-flowering *B. napus* lines based on the Southern hybridization results. The early-flowering line and late-flowering line each had one *BrFLC5* locus from *B. rapa* (dark shading) and one *BoFLC5* locus from *B. oleracea* (light shading). D, Expression analysis of *BnFLC5* using RT-PCR indicated expression differences among the diploid *Brassica* species and the early-flowering and late-flowering *B. napus* lineages (ubiquitin expression also shown as a control).

unexpected. However, given additivity of the gene and absence of expression in both early- and late-flowering lines, we have no evidence that *BnFLC5* expression differed among the polyploids or had an affect on the divergence in flowering time.

DISCUSSION

The results from our study show that selection among the progeny of a resynthesized allopolyploid *B. napus* was effective in creating lineages with divergence in flowering time. Within six generations, there was almost no overlap of these lineages in the time to initiation of flowering, and in nature this rapid evolution may be sufficient for ecological separation and sympatric speciation. The phenotypic differentiation between these lines was more fully assessed in another study (Schranz & Osborn, 2004), in which significant differences were found for eight life-history traits. What mechanisms could cause the phenotypic differences between these early- and late-flowering lineages in *B. napus*? Based on previous studies (Michaels & Amasino, 2000; Schranz *et al*., 2002; Schranz & Osborn, 2004), we chose four pairs of *BnFLC* genes as candidates to begin our investigation into the molecular mechanisms that could cause these rapid phenotypic changes. We found that three of the four loci (*BnFLC2*, *BnFLC3*, *BnFLC5*) had undergone structural and/or expression changes in the allopolyploids, and have strong evidence that genomic rearrangements at one locus (*BnFLC3*) were associated with the divergence of flowering time.

BnFLC1 has been shown to have a large impact on flowering time in *B. napus* (Tadege *et al*., 2001) as well as *B. rapa* (Kole *et al*., 2001). However, we found no qualitative differences for *BnFLC1* between the early- and late-flowering lines for either gene structure or gene expression, and thus no evidence that *BnFLC1* is a cause of differences in flowering time between the lines. However, we cannot rule out quantitative differences in expression levels of *BnFLC1* or differences in post-transcriptional modifications.

For *BnFLC2*, a structural change occurred between the early- and late-flowering lineages that was associated with expression differences among the lineages. The early-flowering lines had *BrFLC2* from N2 duplicated on N12 whereas the late-flowering lines had the chromosomal fragment from N12 (which lacks a corresponding *BoFLC2*) duplicated on to N2. Thus, *BrFLC2* was deleted from the late-flowering lines, resulting in no detectable expression for this gene. This finding is surprising because the early-flowering lines show expression of *FLC2*, a repressor of flowering, and the late-flowering lines did not. One might expect the opposite pattern, in which the expression of *FLC2* would be seen in the late-flowering lines and not the early-flowering lines. It is possible that this rearrangement does not affect the flowering time differences in these lines because their *FLC2* genes are non-functional or have diverged to another function. It is also possible that *BrFLC2* is non-functional but it interferes with the function of other *FLC* genes. In this case, *BrFLC2* might act as a negative repressor of flowering and its presence would be associated with early-flowering as was observed in our study. However, we did not obtain data that would allow us to test for a genetic association between the changes involving flowering time.

For *BnFLC3*, gene structure and expression changes were discovered and these changes were related to differences in flowering time based on a genetic segregation analysis. In the early-flowering lines, *BoFLC3* was lost and *BrFLC3* was duplicated from N3 to N13. The late-flowering lines had both *BrFLC3* and *BoFLC3*, although the S_6 generation may have been heterozygous for a rearrangement on N3 (Fig. 4D). The segregation analysis confirmed that replacement of the *BoFLC3* allele on N13 with *BrFLC3* was associated with earlier flowering. Although the *BoFLC3* gene came from a rapid-cycling line, the diploid *B. rapa* parent flowered earlier than the *B. oleracea* parent and it is possible that *BoFLC3* expresses at higher levels or is a more effective inhibitor of flowering than *BrFLC3*.

The results for *BnFLC5* are interesting because a parental gene is silenced within the allopolyploids. The patterns of gene expression clearly do not match the gene structure data. The RFLP results indicate that each of the diploid parents has *FLC5* and that all the polyploid lines have both *BrFLC5* and *BoFLC5*. The lack of *BoFLC5* expression in the parent and polyploids may be due to a pre-existing mutation, but the silencing of *BrFLC5* in the polyploids would have to be due to genetic or epigenetic changes that arose with or after polyploid formation. We did not observe any changes in DNA methylation of *BrFLC5* in the polyploids compared with the *B. rapa* parent based on comparing restriction fragment patterns after digesting with methylation-sensitive and -insensitive enzymes (*Msp*I and *Hpa*II). It is possible that further analyses will reveal methylation changes or some other chromatin modification that silenced the gene. Alternatively, the process of polyploidization may have altered the regulatory network that allows expression of *BrFLC5* (Osborn *et al*., 2003b).

Our results indicate that extensive genomic rearrangements (e.g. *de novo* non-reciprocal transpositions) can be an important source of novel phenotypic variation in resynthesized *B. napus*. *De novo* chromosomal rearrangements have been observed in previous studies of segregating populations derived from natural and/or resynthesized *B. napus* that involve the

transposition of segments between homoeologous chromosomes (Parkin *et al*., 1995; Sharpe *et al*., 1995; Osborn *et al*., 2003a; Quijada, 2003; Udall, 2003). These studies suggest that there are 'hot spots' for homoeologous recombination in the *B. napus* genome resulting in three classes of transposition products found in mapping studies: segregating reciprocal transpositions (RTs), segregating non-reciprocal transpositions (NRTs) and *de novo* NRTs. Quijada (2003) and Udall (2003) found 50 *de novo* NRTs and several segregating NRTs in four mapping populations of *B. napus* DH lines, and some segregating NRTs were associated with variation in flowering time (e.g. *FLC2* associated with an N2/N12 transposition). Our study found that a chromosomal rearrangement was associated with novel variation in flowering time. Specifically, we found that a *de novo* N3/N13 transposition that contained *BnFLC3* accounted for a significant portion of the phenotypic variation between the *B. napus* lines. Although *FLC* is a likely candidate for the observed flowering time affects associated with this rearrangement, other flowering time genes that are linked to *FLC3* on N3/N13 (e.g. *CONSTANS*, Axelsson *et al*., 2001) also could contribute to this effect. Changes in other flowering time genes (Levy & Dean, 1998; Mouradov, Cremer & Coupland, 2002; Simpson & Dean, 2002) in other genomic regions also may have contributed to the divergence between the *B. napus* lines.

The study of synthetic polyploids allows us to recognize the phenotypic changes and the molecular genetic components that may influence them following polyploidization. Despite the fact that we only examined a single trait, a single gene and two lineages derived from a single resynthesized polyploid, after a few generations we observed very large phenotypic changes, gene loss and possible epigenetic regulatory changes. Important questions remain: how broad are these phenomena? Are the same genes, genic regions and traits sensitive to polyploid-induced changes to the same degree? To address these questions, we are currently performing a survey of phenotypes, genomes and transcriptomes (see Chen *et al*., 2004, for detecting genome-wide expression changes in *Arabidopsis* and *Brassica* polyploids) for a set of 50 replicates of *B. napus* synthetic polyploids created *de novo* from better-known diploid parents. The progeny of the 50 independently derived S_0 *B. napus* should be genetically identical, and this gives a strong null hypothesis for observations and statistical tests. In theory, any changes observed should be due to the processes of hybridization or polyploidization.

Our study of early- and late-flowering lines has shown that genetic changes associated with polyploid formation can lead to divergence in a phenotype with important agricultural and ecological implications. Far from being a 'dead end' (Wagner, 1970), polyploidy is associated with remarkable rapid evolution and variation. Using a replicated set of 50 polyploid lines, we will broaden this view by examining the frequency of dynamic changes. Ultimately, our goal is to relate studies of resynthesized polyploids back to the mechanisms that occur in natural polyploids.

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