Characterization and Effects of the Replicated Flowering Time Gene *FLC* in *Brassica rapa*

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ABSTRACT

Functional genetic redundancy is widespread in plants and could have an important impact on phenotypic diversity if the multiple gene copies act in an additive or dosage-dependent manner. We have cloned four *Brassica rapa* homologs (*BrFLC*) of the MADS-box flowering-time regulator *FLC*, located at the top of chromosome 5 of *Arabidopsis thaliana*. Relative rate tests revealed no evidence for differential rates of evolution and the ratios of nonsynonymous-to-synonymous substitutions suggest *BrFLC* loci are not under strong purifying selection. *BrFLC1*, *BrFLC2*, and *BrFLC3* map to genomic regions that are collinear with the top of At5, consistent with a polyploid origin. *BrFLC5* maps near a junction of two collinear regions to Arabidopsis, one of which includes an *FLC*-like gene (*AGL31*). However, all *BrFLC* sequences are more closely related to *FLC* than to *AGL31*. *BrFLC1*, *BrFLC2*, and *BrFLC5* cosegregate with flowering-time loci evaluated in populations derived by backcrossing late-flowering alleles from a biennial parent into an annual parent. Two loci segregating in a single backcross population affected flowering in a completely additive manner. Thus, replicated *BrFLC* genes appear to have a similar function and interact in an additive manner to modulate flowering time.

DUPLICATION of genes, as chromosomal blocks, individually, or by whole genome polyploidization, is thought to be a major mechanism for creating new genetic and phenotypic diversity. The impact of paralogous genes on diversification is particularly striking in flowering plants where as many as 70% of species, including many of our most important crop plants, show evidence for polyploidy (Masterson 1994). The selective advantage of genetic redundancy is not well understood, but having multiple copies of genes could contribute to phenotypic diversity through the functional divergence of redundant genes. Although examples of this have been discovered, many duplicated genes appear to retain their original function (see Wendel 2000 for review). The lower than expected frequency of duplicate gene silencing (*e.g.*, Nadeau and Sankoff 1997) also suggests that maintenance of duplicated gene function is an important feature in evolution. Force *et al.* (1999) have hypothesized subfunctionalization as one explanation for retention of duplicated gene function—that is, the accumulation of different mutations in duplicated genes that cause each locus to control only a subset of the functions of the single ancestral gene. However, by this mechanism, the retention of function would contribute little to phenotypic diversity.

A mechanism by which retention of duplicated gene function could impact phenotypic diversity is if each gene copy contributed to the control of the phenotype in a dosage-dependent manner. Increases in enzymatic activity and gene expression are associated with increasing ploidy (*e.g.*, Roose and Gottlieb 1980; Guo *et al.* 1996), and a study of Hox group 3 genes in mice found that paralogous loci can act in a dosage-dependent manner to affect phenotype (Manley and Capecchi 1997). In plants, changes of regulatory genes are believed to be particularly important for the diversification of plant phenotypes (Doebley and Lukens 1998; Shepard and Purugganan 2002), and alleles at several key regulatory genes controlling developmental processes are known to interact in an additive manner (*e.g.*, *Tb1*, Lukens and Doebley 1999; fw2.2, Frary *et al.* 2000; *CO*, Koornneef *et al.* 1991; and *FLC*, Michaels and Amasino 2000). These additive or dosage-dependent effects at a single regulatory locus could be expanded through gene replication if multiple copies of the genes also interacted in an additive or dosage-dependent manner.

Brassica species, which include several important crops with a wide range of morphologies, are hypothesized to be ancient polyploid relatives of *Arabidopsis thaliana* (Lagercrantz 1998). A major component of the morphological diversity in Brassica species is variation in flowering time. In *A. thaliana*, many genes have been

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identified that control flowering time, but much of the natural variation involves allelic variation at *FLOWERING LOCUS C* (*FLC*) or at *FRIGIDA* (*FRI*), a regulator of *FLC* expression (Michaels and Amasino 1999; Sheldon *et al.* 1999; Johanson *et al.* 2000). *FLC* acts in a dosage-dependent manner to delay flowering (Michaels and Amasino 1999; Sheldon *et al.* 1999). Alleles that cause late flowering produce intermediate phenotypes when heterozygous with early flowering alleles, and transgenic expression of additional *FLC* genes leads to even later flowering phenotypes (Michaels and Amasino 2000). Diploid Brassica species contain three copies of the genomic region that corresponds to the top of chromosome 5 in *A. thaliana* (At5) where *FLC* is located (Osborn *et al.* 1997; Lagercrantz 1998; Parkin *et al.* 2002). QTL having large effects on flowering time have been mapped to these genome regions in *Brassica rapa* (Teutonico and Osborn 1994; Osborn *et al.* 1997), *B. napus* (Ferreira *et al.* 1995; Osborn *et al.* 1997; Butruille *et al.* 1999), *B. oleracea* (Bohuon *et al.* 1998; Lan and Paterson 2000), *B. nigra* (Lagercrantz *et al.* 1996), and *B. juncea* (Axelsson *et al.* 2001). Thus, multiple copies of a gene homologous to a flowering-time gene on At5, such as *FLC*, could contribute to the wide range of variation in flowering time observed in Brassica species.

Axelsson *et al.* (2001) hypothesized that the QTL that they identified in several Brassica species correspond to homologs of *CO*, another flowering-time gene at the top of At5 that is involved in photoperiod regulation of flowering (Putterill *et al.* 1995). Their evidence was based on confidence intervals for QTL and map positions or hypothesized map positions for *CO* and *FLC* homologs. Kole *et al.* (2001) provided strong evidence that *VFR2*, a flowering-time locus in *B. rapa*, is allelic to a *B. rapa FLC* homolog. *VFR2* was originally identified as a QTL in a segregating population derived from annual and biennial oilseed *B. rapa* parents (Osborn *et al.* 1997). However, after backcrossing the late-flowering allele into the annual parent, the flowering-time effects conferred by *VFR2* segregated as a single Mendelian locus that mapped 13 cM from a *CO* homolog but cosegregated exactly with a *FLC* homolog (Kole *et al.* 2001). The effect of the late allele was almost completely additive and was nearly eliminated by 3 weeks of vernalization. These data strongly suggest that *VFR2* is a *B. rapa* homolog of *FLC*. Tadege *et al.* (2001) subsequently reported the cloning of five *FLC* homologs (*BnFLC1*–*BnFLC5*) from the allopolyploid *B. napus* ($n = 19$) by screening a cDNA library. Expression of these cDNA with a 35S promoter in *A. thaliana* delayed flowering, and the expression of the five *BnFLC*s in *B. napus* was reduced by vernalization. Their results are consistent with *B. napus* having multiple functional homologs of *FLC*; however, the total number and origins of *FLC* homologs and their effects through allelic variation in Brassica species are not known. This information could provide important new insight into the evolution of replicated genes.

In this study we report on the cloning of four genomic *FLC* genes from the diploid *B. rapa* ($n = 10$) and three genes from *B. oleracea* ($n = 9$). These genes were compared to each other and to *A. thaliana* genes by sequence analysis and comparative mapping. Phenotypic effects associated with the four *BrFLC* sequences were determined by evaluating flowering-time variation in backcross populations segregating for *FLC* loci individually or in combinations. Our results provide evidence that polyploidy has contributed to phenotypic variation for flowering time in *B. rapa* through replication of *FLC*, an important regulatory gene that acts in a dosage-dependent manner.

MATERIALS AND METHODS

Cloning and sequence analysis of Brassica *FLC* genes: Plants of the biennial *B. rapa* oilseed cultivar, Per, were grown in a growth chamber for 2 weeks under long-day (LD) conditions (16 hr light:8 hr dark) at 21°. Total RNA was extracted from leaves using the TRI reagent (Sigma, St. Louis) as directed by the manufacturer. First strand of cDNA was synthesized with the SuperScript II reverse transcriptase (Life Science Technology, Gaithersburg, MD) using the poly(dT)-M13 primer (5′-GTA AAA CGA CGG CCA GTC CCT TTT TTT TTT TTT T-3′). Synthesized first strands of cDNA were used as templates to amplify *BrFLC* cDNA by using the FLC44 primer (5′-CGG CTT AGA TCT CCG GCG ACT-3′) and the poly(dT)-M13 primer. The PCR products were cloned into pGEM-Teasy vectors (Promega, Madison, WI) and sequences were analyzed. All cDNA corresponded to a single *BrFLC* gene.

To isolate additional genomic Brassica *FLC* genes, conserved primers were designed by aligning the *BrFLC* cDNA with *A. thaliana FLC* cDNA (AF116527; Figure 1a, exon 2 and exon 7 primers) and used for 35 cycles of PCR with genomic DNA from doubled haploid lines of *B. rapa* (IMB218) and *B. oleracea* (TO1000). PCR products were excised from the gel, purified using the GFX PCR DNA and gel band isolation kit (Amersham Biosciences, Piscataway, NJ), and cloned into pGem T-Vectors (Promega).

Plasmid inserts were sequenced by ABI PRISM dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA). At least two independent clones from separate PCR reactions were sequenced for each locus. Sequencing contigs were assembled using the Sequencher software package (GeneCodes, Ann Arbor, MI). After sequence analysis (see below) locus-specific primers were designed from a variable region of exon 4 of the *B. rapa* sequences (Figure 1a).

FLC sequences from *B. rapa* (AY115675–AY115678), *B. oleracea* (AY115672–AY115674), and *A. thaliana* (AF116528) were aligned using the Multiple Alignment Program (Huang 1996) and by eye. Exon and intron boundaries were identified by comparison to *A. thaliana* mRNA sequence (AF116527) and by checking boundary consensus sequences (Brown *et al.* 1996). The coding sequences for *FLC* from *A. thaliana*, *B. rapa*, *B. oleracea*, and *B. napus* (AY036888–AY036892) and for two *FLC*-like genes from *A. thaliana*, *AGL27* (AF312665) and *AGL31* (AY052229), were aligned using CLUSTALW (Thompson *et al.* 1994) with manual adjustments.

Phylogenetic analyses were done using PAUP*, version 4.0 (Swofford 2000) with both maximum-parsimony and maximum-likelihood methods. For maximum-likelihood analyses,

the transition:transversion ratio (ts/tv) was set at the default value of 2.0. The base frequencies and the gamma shape parameter α were both determined empirically from the data. Heuristic searches were done with tree-reconnection branch swapping. Bootstrap support values (BS) were estimated by doing 10,000 "fast" replicates using the parsimony criterion.

The rate of molecular evolution of the *B. rapa FLC* genes was tested by a relative rate test (Tajima 1993) with *A. thaliana FLC* sequence as the out-group, using the MEGA2 software package (Kumar *et al.* 2001). Similarly, *AGL27* and *AGL31* were compared using *FLC* as an out-group. The method of Nei and Gojobori (1986) was used to calculate the number of synonymous substitutions per synonymous site (*d*S) and the number of nonsynonymous substitutions per nonsynonymous site (*d*N) and their ratio (*d*N/*d*S) using the codeml program in the PAML software package (Yang 2000).

Genetic mapping and map comparisons: Two regions containing flowering-time QTL, *FR1* and *FR2*, were mapped using two backcross populations. The populations were derived from two recombinant inbred (RI) lines from a previously described *B. rapa* population (Kole *et al.* 1997) created from a cross between Per and R500, an annual sarson. For *FR1*, an RI line (PQ3) had Per alleles at restriction fragment length polymorphism (RFLP) loci flanking the *FR1* region on linkage group R2 (Osborn *et al.* 1997) and R500 for RFLP loci flanking other flowering-time QTL. This line was backcrossed to R500 for three generations with selection at each generation for plants having Per alleles at marker loci flanking *FR1*. One BC_3 plant heterozygous at *FR1* was self-pollinated and 78 BC_3S_1 plants were grown in the field, along with 20 replicates of both early (R500) and late (PQ3) parents, under the same conditions as described by Kole *et al.* (2001). For *FR2*, an RI line (IMB1061) was selected that had Per alleles at RFLP markers on the linkage group R3 flanking *FR2* and that had R500 alleles at RFLP markers flanking other known flowering-time QTL (Osborn *et al.* 1997). The RI line was crossed to R500, and a hybrid plant (genetically equivalent to a BC_1) was selfed. One hundred resulting BC_1S_1 plants and five replicates of the early parent (R500) and the late parent (PQ1050) were grown in a growth chamber under LD conditions at 21°.

Linkage maps for the *FR1* and *FR2* regions were generated using RFLP and simple sequence repeat (SSR) marker loci. DNA was extracted as described in Kidwell and Osborn (1992) and were analyzed for RFLP by Southern blot hybridizations as described by Teutonico and Osborn (1994). Probes hybridized to blots included DNA clones found on linkage groups containing flowering-time QTL (Osborn *et al.* 1997), the four isolated *B. rapa FLC* genomic clones, and the *A. thaliana FLC* and *CO* clones used by Kole *et al.* (2001). In addition, several PCR markers were used. The exon 7 primer with the exon 4 locus-specific primers for *BrFLC2* and *BrFLC3* (Figure 1a) gave polymorphic PCR products (Figure 1b). Also, several SSR markers provided by D. Lydiate (personal communication) were used. In total, 14 marker loci per population were utilized. Linkage maps for the *FR1* (R2) and the *FR2* (R3) regions were constructed by analyzing segregation data from the two backcross populations using JoinMap 3.0 (CPRO-DRO, Wageningen).

Brassica probes used for RFLP analyses in this study (R2 and R3) and in previous studies (R10 = LG8, Kole *et al.* 2001; N2, N3, and N10, Osborn *et al.* 1997 and Butruille *et al.* 1999) were sequenced to infer the position of putative homologs within the *A. thaliana* genome. The sequences were compared to the *A. thaliana* genomic sequence as provided on February 15, 2001, on the TAIR database (http://www.arabidopsis.org). Sequences were compared by BLASTn analysis (Altschul *et al.* 1997), and putative homology relationships were established if pairwise comparison BLAST scores were ≥82 (L. Lukens, F. Zou, D. Lydiate, I. Parkin and T. Osborn, unpublished results).

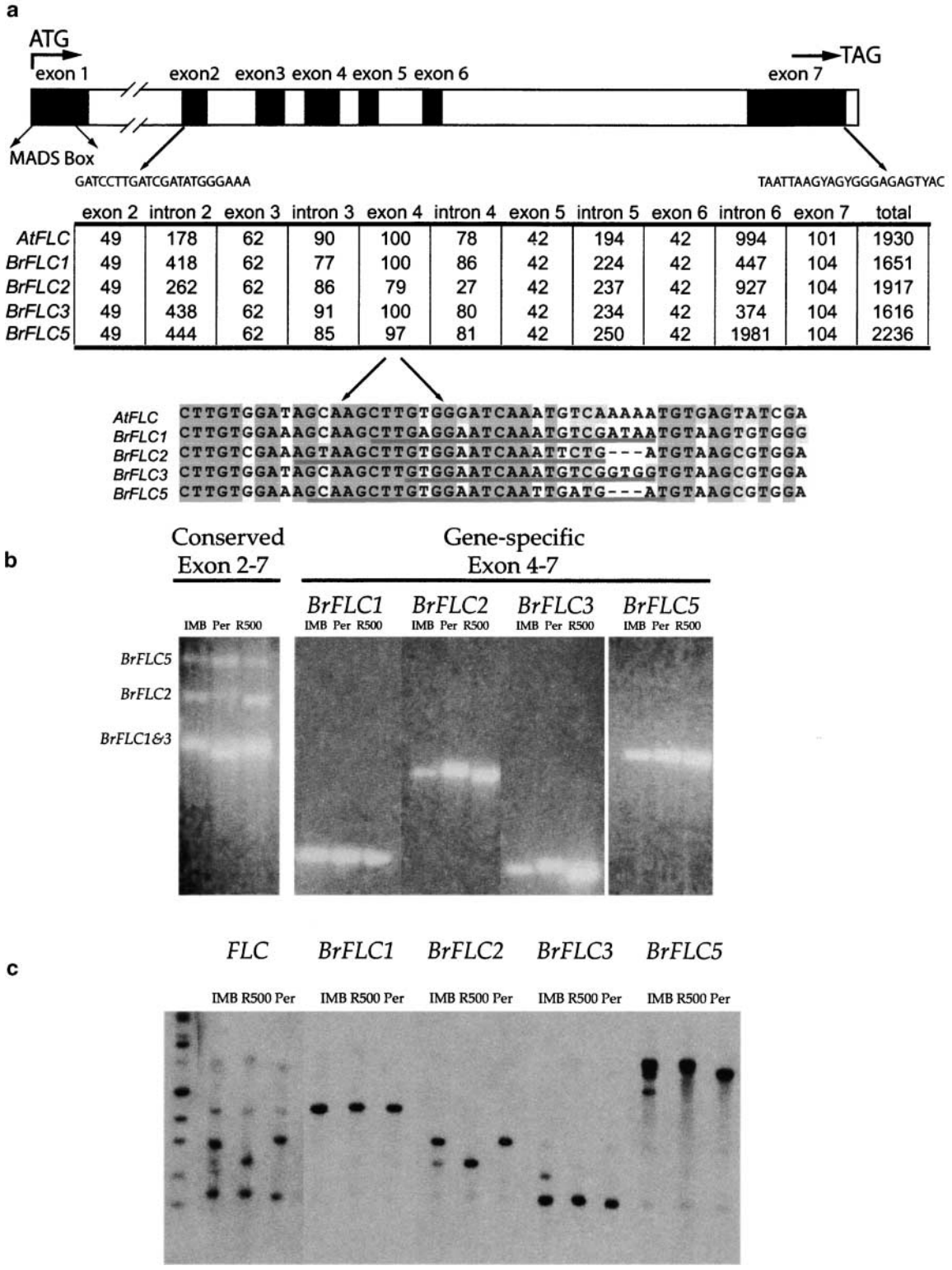
Flowering-time evaluation, QTL mapping, and gene interaction analysis: The 78 BC_3S_1 plants segregating for *FR1*, the 100 BC_1S_1 plants segregating for *FR2*, and the 326 F_2 plants segregating for both *FR1* and *VFR2* (described below) were evaluated for flowering time by counting the number of days after sowing to the first open flower (DTF) and the number of leaves on the main axis at flowering (LN). Using the linkage maps constructed for the *FR1* and *FR2* populations, QTL for flowering time were analyzed using QTL Cartographer (Basten *et al.* 2001) with a 10-cM covariate window for composite interval mapping (CIM). For both *FR1* and *FR2* populations, the broad-sense heritability for flowering time was estimated from variance components using the average variance of the parents and the hybrid as an estimate of the environmental variance and the variance of the segregating populations as an estimate of the phenotypic variance.

To study the interactions of two putative *FLC* loci, an F_2 population (326 plants) that included both *VFR2* (described in Kole *et al.* 2001) and *FR1* in an R500 background was created by crossing two BC_3S_1 homozygous plants (*fr1/fr1, VFR2/VFR2* × *FR1/FR1,vfr2/vfr2*). The F_2 population was grown in the field under conditions as reported in Kole *et al.* (2001). The 326 F_2 plants were screened by RFLP for *VFR2* with the *BrFLC1* clone and for *FR1* with the *BrFLC2* PCR polymorphism. To test the effects of the two putative *FLC* loci and their interactions, a two-factor analysis of variance was done using Proc MIXED of SAS (Littell *et al.* 1996) with means weighted according to the frequency of individuals in each two-locus class.

RESULTS

Cloning and analysis of Brassica *FLC* sequences: *Cloning and sequence characterization:* Five cDNA clones were analyzed by sequencing. Four of these clones were identical (*BrFLC* cDNA1) and contained 896 bp (coding 196 amino acids) corresponding to the seven exons of *A. thaliana FLC* with 75% identity (85% for the coding region). The fifth clone (*BrFLC* cDNA2) was 100% identical to *BrFLC* cDNA1 for exons 1–6; the final exon and the 3′-untranslated region (3′-UTR) were highly divergent and had no significant homology to any other sequence in GenBank. This was apparently a splicing variant of the same gene as *BrFLC* cDNA1, as explained below.

Alignment of *BrFLC* cDNA1 and *FLC* allowed us to design highly conserved primers in exons 2 and 7 (Figure 1a), and amplification with these primers yielded three distinct fragments after gel separation (Figure 1b). Cloning and sequencing of these PCR products resulted in the identification of four *BrFLC* genes (*BrFLC1*, *BrFLC2*, *BrFLC3*, and *BrFLC5*) and three *B. oleracea* genes (*BoFLC1*, *BoFLC3*, and *BoFLC5*). Locus names are based on their similarity to *BnFLC* cDNA sequences reported by Tadege *et al.* (2001; see below). Southern blot analysis using *FLC* as a probe identified four prominent restriction fragments in *B. rapa* (Figure 1c). The correspondence between the four *FLC* restriction fragments and our four cloned *BrFLC* genes was confirmed by locus-specific Southern hybridization analyses (Figure 1c).



	exon 2	intron 2	exon 3	intron 3	exon 4	intron 4	exon 5	intron 5	exon 6	intron 6	exon 7	total
AtFLC	49	178	62	90	100	78	42	194	42	994	101	1930
BrFLC1	49	418	62	77	100	86	42	224	42	447	104	1651
BrFLC2	49	262	62	86	79	27	42	237	42	927	104	1917
BrFLC3	49	438	62	91	100	80	42	234	42	374	104	1616
BrFLC5	49	444	62	85	97	81	42	250	42	1981	104	2236

FIGURE 1.—Cloning and analysis of *B. rapa FLC* homologs. (a) Summary of genomic structure of the *A. thaliana FLC* gene showing sequences of conserved primers from exons 2 and 7 used to clone *B. rapa* homologs. The chart lists intron and exon sizes (in base pairs) for *FLC* and the four *B. rapa FLC* homologs (*BrFLC1–BrFLC5*) cloned from a rapid-cycling doubled-haploid line of *B. rapa* (IMB). The nucleic acid alignment revealed a highly variable region of exon 4 used to design gene-specific forward primers (sequences underlined). (b) PCR amplification of genomic *FLC* sequences in IMB and the *B. rapa* cultivars Per and R500. Conserved primers (exon 2 and exon 7) amplify all four paralogs. Separation of *FLC1* and *-3* is not seen because of similar lengths (1616 and 1651 bp, respectively). Amplification specificity is shown by using gene-specific forward primers with a conserved exon 7 reverse primer. (c) Southern blot hybridizations of *Msp*I-digested DNA of IMB, R500, and Per hybridized with exon 2 through exon 7 genomic probes of *FLC* and of *BrFLC1–BrFLC5*. The *A. thaliana* probe hybridizes to all four Brassica genes, whereas the gene-specific probes show very little cross-hybridization.

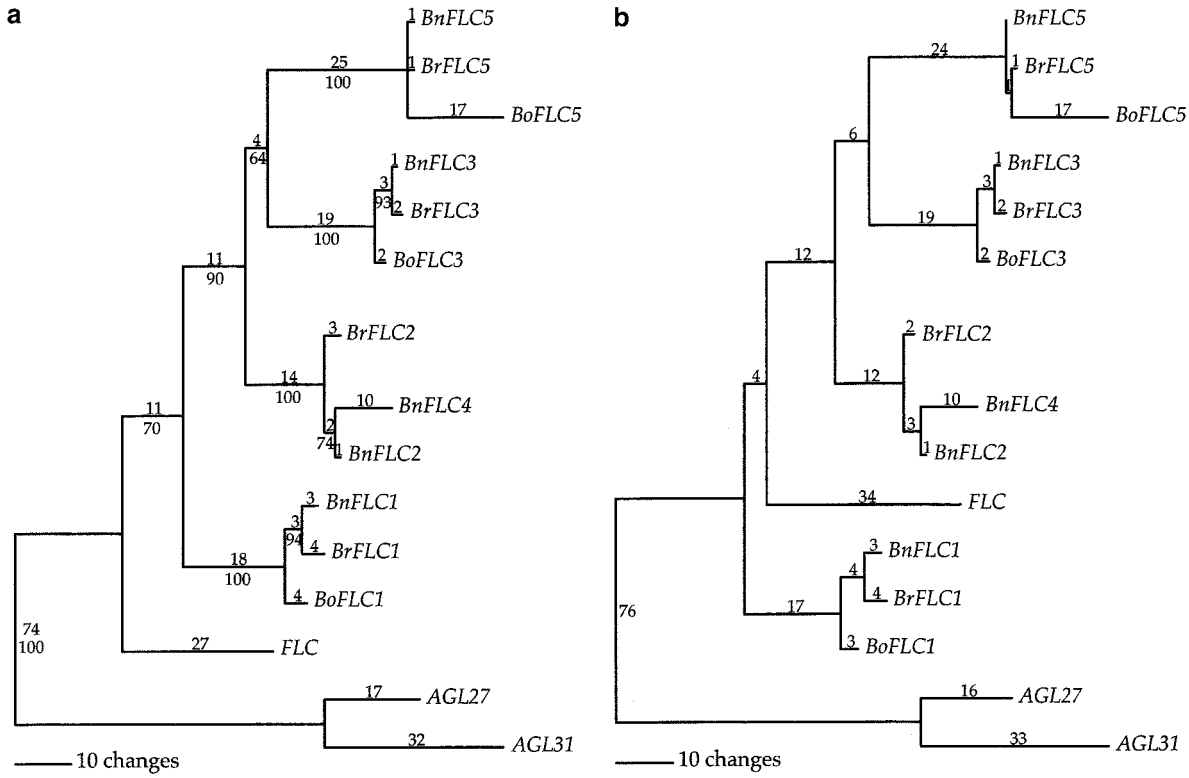


FIGURE 2.—Phylogenetic analyses of Brassica *FLC* homologs. Aligned coding sequences of *FLC* homologs from *B. napus* (*Bn*; TADEGE *et al.* 2001), *B. rapa* (*Br*), *B. oleracea* (*Bo*), and the *FLC*-like genes *AGL27* and *AGL31* were used for phylogenetic analyses. Phylogenetic analyses were done using both maximum-parsimony (a) and maximum-likelihood (b) methods. The number of nucleotide changes is shown on the top of each branch and bootstrap support values are shown below the branch of the consensus tree from the two most parsimonious trees (a). Analyses show four well-supported Brassica *FLC* clades, represented by each of the four *BrFLC* sequences. The *BnFLC* sequences are sisters to the *BrFLC* sequences. Three clades form a monophyletic Brassica group (*BrFLC2*, *BrFLC3*, and *BrFLC5* clades). The analyses show all *FLC* sequences are monophyletic with respect to the *FLC*-like sequences *AGL27* and *AGL31*. However, the two analyses differ in the placement of *BrFLC1*; parsimony gives a monophyletic Brassica clade (a) and maximum likelihood shows a paraphyletic relationship (b).

Exon and intron boundaries were identified by comparison to the *A. thaliana* cDNA sequence and by checking boundary consensus sequences (data summarized in Figure 1a). The *BrFLC* coding regions were 81.8–84.6% identical to *FLC*. Exon size was highly conserved among the Brassica and *A. thaliana FLC* sequences. The one exception was exon 4 of *BrFLC2* for which both the IMB218 and the R500 alleles had a 56-bp deletion (established by partial sequencing of the Per and R500 *BrFLC2* alleles) that eliminated part of exon 4 (18 bp) and intron 4 (38 bp).

Several introns were conserved in length and sequence. In particular, intron 3 was highly conserved and was the only intron whose sequences could be confidently aligned with 74.4–81.3% sequence similarity to *FLC*. Other introns were more polymorphic. Intron 2 varied 1.7-fold in length. Per and R500 alleles of *BrFLC3* had two indels of 17 and 21 bp relative to one another in intron 2 (established by partial sequencing of these alleles). Intron 1 was not cloned because of its large size in *A. thaliana* (3.5 kb). However, PCR analyses of *B. rapa* genomic DNA revealed that *BrFLC3* has a relatively small intron 1 (~1140 bp), while the other loci also have large (>3 kb) intron 1 sequences (data not shown). Intron 6 was highly variable in length (5.3-fold). Sequence comparisons with the 3′ sequence of *BrFLC* cDNA2 revealed that it contained a portion of intron 6 of *BrFLC5* that was in frame with the exon 6 sequence, but excluded exon 7. Hence, *BrFLC* cDNA1 and *BrFLC* cDNA2 are alternate splice variants of the same locus, *BrFLC5*. A putative 51-bp insertion of noncoding mitochondrial DNA (92% similarity) was also identified in intron 6 of *BrFLC1*. The sequencing of the *A. thaliana* genome revealed 14 such insertions, ranging in size from 94 to 3500 bp (ARABIDOPSIS GENOME INITIATIVE 2000). The mitochondrial insertion was not present in *BoFLC1*.

FLC phylogeny: Phylogenetic analyses were conducted using a total of 451 bp of aligned coding sequence from exons 2–7 (Figure 1b), excluding indels. A total of 206 sites were polymorphic, and 145 were phylogenetically informative. Maximum-parsimony analysis resulted in two most parsimonious trees with a length of 308 (consistency index of 0.85; retention index of 0.87; consensus

TABLE 1

d_N and d_S substitutions among Brassica *FLC* sequences compared to *A. thaliana FLC*

Gene	d_N	d_S	d_N/d_S
BrFLC1	0.098	0.340	0.289
BnFLC1	0.094	0.341	0.277
BrFLC2	0.079	0.368	0.215
BnFLC4	0.114	0.368	0.311
BnFLC2	0.090	0.352	0.257
BrFLC3	0.109	0.303	0.362
BnFLC3	0.110	0.320	0.343
BrFLC5	0.108	0.359	0.302
BnFLC5	0.104	0.379	0.275

TABLE 2

Ratio (d_N/d_S) of d_N and d_S substitutions among *BrFLC* sequences

	BrFLC1	*BrFLC2*	*BrFLC3*	*BrFLC5*
BrFLC1				
BrFLC2	0.313			
BrFLC3	0.535	0.341		
BrFLC5	0.332	0.328	0.460	

tree shown in Figure 2a). Maximum-likelihood analysis yielded a tree with ln $L = -1905$, an estimated ts/tv ratio of 1.382 and with rate variation estimated among nucleotide sites as gamma shape parameter $\alpha = 1.53$ (Figure 2b).

The phylogenetic analysis of *FLC* and *FLC*-like sequences showed several interesting relationships (Figure 2). First, all *FLC* sequences from Brassica species fall into four well-supported clades, each of which we refer to by the *BrFLC* sequence included in the clade. Sequences from each of the three species are present in each clade with the exception of a *B. oleracea FLC* in the *BrFLC2* clade. Second, one sequence, at most, from a base Brassica diploid is found in any one group, suggesting that there has not been recent gene duplication. Third, both analyses (Figure 2) give a monophyletic group including the *BrFLC2*, *BrFLC3*, and *BrFLC5* clades with high-parsimony bootstrap support (90% BS), but with poor resolution within the group [only 64% BS for a *BrFLC3/BrFLC5* clade with parsimony (Figure 2a) and weak support for a *BrFLC2/BrFLC3* clade with maximum-likelihood analysis (Figure 2b)]. Fourth, parsimony and likelihood analyses differ with respect to the placement of the *BrFLC1* clade—being monophyletic with the other Brassica *FLC* sequences with parsimony, but paraphyletic with likelihood. Fifth, three of the five *BnFLC* sequences (*BnFLC1*, *BnFLC3*, and *BnFLC5*) cloned by Tadege *et al.* (2000) are sisters to the *BrFLC* sequences. Finally, both parsimony and likelihood analyses suggest that the Brassica sequences are more closely related to *FLC* than to the paralagous *AGL27* and *AGL31*.

FLC sequence analyses: Duplicate loci that have diverged in function can show differential rates of evolution. Tajima's relative rate tests comparing the *BrFLC* sequences to one another and using *A. thaliana FLC* as an outgroup gave chi-square values between 0 and 1 with all values being nonsignificant. Hence, we find no evidence that one locus is evolving more rapidly or more slowly than the others. Comparative mapping studies raised questions about the relationship of *BrFLC5* to *AGL31* (see below). Hence, we wanted to resolve several issues regarding the *AGL31* cluster of genes, designated *FLC*-like sequences 2, 3, and 4 (*FLCL2-4* by Tadege *et al.* 2001). *FLCL2–4* genes are 62.2–74.6% identical to *FLC* for predicted coding regions. Although *FLC* and the *AGL31* cluster are paralogous due to a larger chromosomal duplication event (Arabidopsis Genome Initiative 2000), *FLCL2–4* are more similar (67.7–82.7%) to *AGL27* than to *FLC*. *AGL27* is located in a nonduplicated region of chromosome 1. Relative rate tests were used to test for functional divergence of *AGL31* by comparison to *AGL27* using *FLC* as an outgroup. The test showed that *AGL31* is not significantly different from *AGL27*.

The number of d_S and the number of d_N and their ratio (d_N/d_S) were calculated for *BrFLC* and *BnFLC* sequences as compared to *FLC* (Table 1). A ratio of 0 is evidence for strong amino acid conservation and purifying selection and a ratio of ≥ 1.0 suggests neutral or positive selection. The d_N/d_S ratios for the Brassica *FLC* sequences compared to *FLC* ranged from 0.26 to 0.36 (Table 1) and from 0.31 to 0.53 when *BrFLC* sequences were compared to one another (Table 2). These values are similar to the ratios found between Brassica and *A. thaliana CO* genes (0.39–0.44), but much higher than the average of 0.10 (Lagercrantz and Axelsson 2000) and 0.14 (Tiffin and Hahn 2002) for other Brassica genes compared to their *A. thaliana* homologs. The ratio for Brassica *FLC* sequences is also higher than the average for the K-box and C regions of several MADS-box genes (Purugganan *et al.* 1995).

Genetic mapping and map comparisons: *BrFLC1* was determined to be the *FLC* locus mapped onto linkage group R10 on the basis of comparisons to results reported by Kole *et al.* (2001). The position of *BrFLC2* was mapped onto R2 using the 78 BC_3S_1 plants. The R2 map included a total of 14 genetic marker loci spanning 58.9 cM. Both *BrFLC3* and *BrFLC5* were mapped onto R3 using the 100 BC_1S_1 plants. The R3 map contained 14 marker loci covering 54.9 cM.

Comparative mapping between *A. thaliana* and linkage groups from *B. rapa* (R2, R3, and R10) and their homologs in *B. napus* (N2, N3, and N10) confirmed extensive synteny and collinearity among these groups and with chromosome 5 of *A. thaliana* (At5; Figure 3). The collinearity consisted of two blocks, one having homology to the top of At5 and the second with inverted

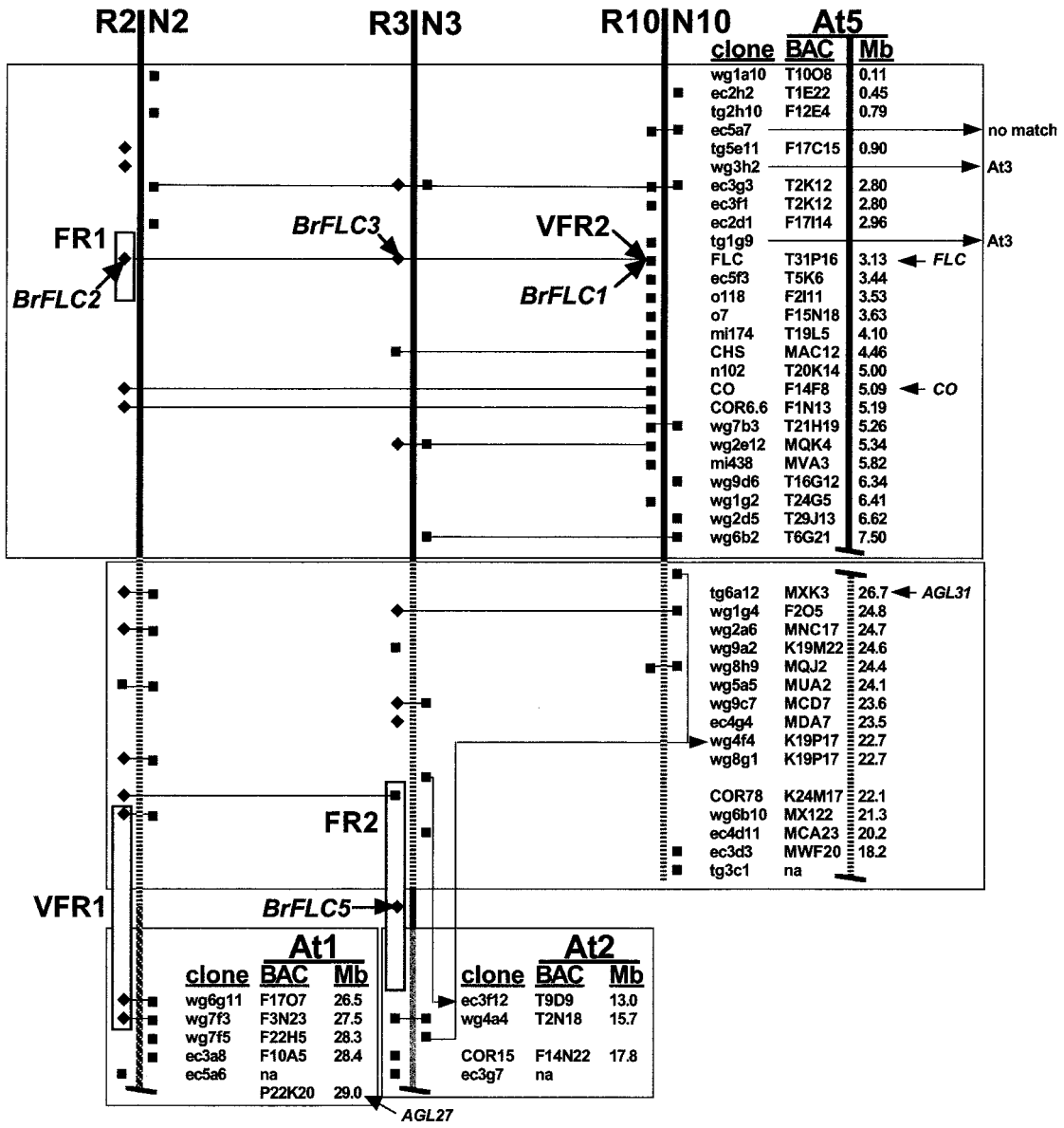


FIGURE 3.—Comparative map of Brassica linkage groups containing *FLC* homologs. Vertical lines represent homologous linkage groups in *B. rapa* (R) and *B. napus* (N). ■, relative positions (but not relative distances) of RFLP loci mapped in previous studies on R2, R3, and R10 (*B. rapa* linkage groups equivalent to LG2, LG3, and LG8 of KOLE *et al.* 1997; OSBORN *et al.* 1997; KOLE *et al.* 2001) and on N2, N3, and N10 (*B. napus* linkage groups from OSBORN *et al.* 1997; BUTRUILLE *et al.* 1999). ◆, relative positions of markers used in this study. Markers connected with horizontal lines are RFLP loci detected with the same probe. Homology of RFLP markers to *A. thaliana*, based on comparative mapping (OSBORN *et al.* 1997; KOLE *et al.* 2001) or on BLAST searches using DNA sequences of RFLP probes (L. LUKENS, F. ZOU, D. LYDIATE, I. PARKIN and T. OSBORN, unpublished results), is shown by the name of the *A. thaliana* genomic bacterial artificial chromosomes and genomic positions in megabases of DNA. Brassica genome regions that are collinear with four *A. thaliana* genome segments are enclosed in boxes. Loci *wg4f4* on N3 and N10 and *ec3f12* on N3 are not in collinear positions, but their map positions are based on a consensus map from five populations (BUTRUILLE *et al.* 1999) and may be incorrect. Open bars indicate the positions of QTL for flowering time based on backcross populations in this study (*FR1*, *VFR1*, and *FR2*) and one qualitative trait locus (*VFR2*; KOLE *et al.* 2001). Map positions of *B. rapa* homologs of the *A. thaliana* flowering-time gene *FLC* are shown, three of which correspond to the positions of flowering-time genes. Three of the *BrFLC* genes (*BrFLC2*, *BrFLC3*, and *BrFLC1*) map to positions on R2, R3, and R10 that show collinearity to the top of At5 where *FLC* is located. However, *BrFLC5* maps to an interval between regions of collinearity to the bottom of At5 and to At2.

orientation to a region on the bottom of At5. The first region from marker *wg1a10* to *wg6b2* corresponded to 0.11–7.50 Mb of At5. The second region from marker *tg6a12* to *ec3d3* corresponded to 26.7–18.2 Mb of At5. After the second shared region of collinearity to the bottom of At5, R2 then shared homology to At1 (26.6–29.0 Mb), R3 shared homology to At2 (13.0–17.8 Mb), and R10 terminated. Hence, all three linkage groups

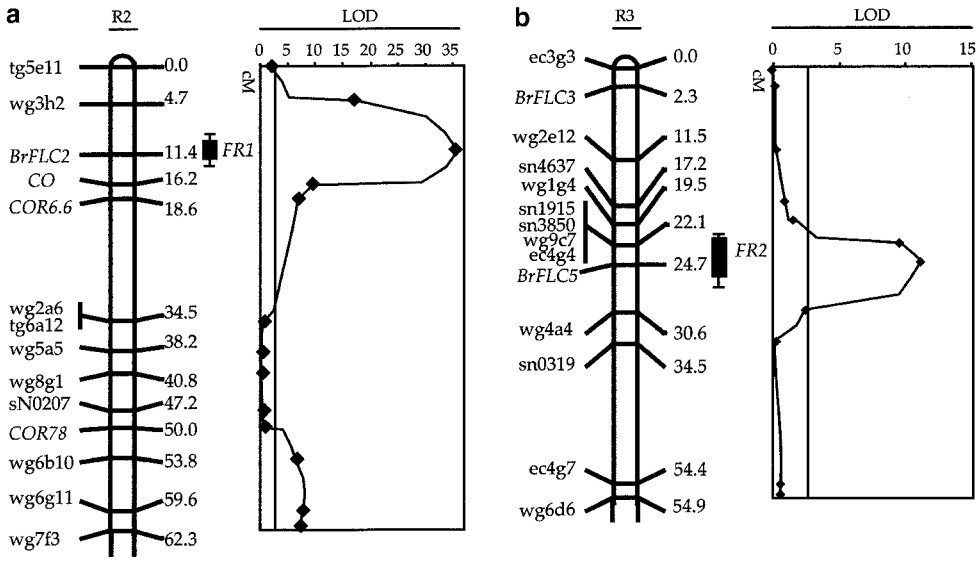


FIGURE 4.—Genetic mapping and QTL analyses of *B. rapa* chromosomes R2 and R3. The effects on flowering time associated with *BrFLC2*, *BrFLC3*, and *BrFLC5* were analyzed in two populations derived by backcrossing alleles from a biennial (Per) into an annual (R500) *B. rapa* cultivar. Marker names, distances in centimorgans, LOD plots, and QTL positions with 1 and 2 LOD confidence intervals are shown for R2 (a) and R3 (b). (a) Seventy-eight BC_3S_1 plants were screened with 14 RFLP markers on R2, including *BrFLC2* and *CO*. The largest QTL (R^2 = 80%; LOD score = 35) was centered on *BrFLC2*. *CO* was outside the confidence interval. (b) One-hundred BC_1S_1 plants were screened with 10 RFLP markers on R3, including *BrFLC3* and *BrFLC5*, and with 4 SSR markers. The largest QTL (R^2 = 39.0%; LOD score = 10.6) was centered on *BrFLC5*. No QTL effect was identified with *BrFLC3*. A *CO* locus on R3 was not identified in this population.

(R2, R3, and R10) appear to share common chromosomal breakpoints compared to *A. thaliana*.

The *BrFLC2*, *BrFLC3*, and *BrFLC1* all mapped to the expected collinear region (3.13 Mb on At5 where *FLC* is located) of R2, R3, and R10, respectively. However, *BrFLC5* did not map to a region having collinearity to the top of At5. It mapped to the interval between the runs of collinearity with the bottom of At5 and At2 (Figure 3).

Flowering time, QTL, and gene interaction analyses: *R2 (FR1) population:* The 78 BC_3S_1 plants grown in a field required 51–83 DTF and formed 14–35 LN, with means of 63.3 DTF and 25.7 LN. The flowering-time variation was greatly reduced in another set of BC_3S_1 plants after 3 weeks of vernalization (data not shown). DTF was significantly correlated with LN (r = 0.78; $P < 0.01$). The average DTF and LN for 20 plants of the early flowering parent (R500) were 51.5 and 17.9, respectively. For the late flowering parent (PQ3) the means were 75.8 DTF and 29.4 LN. Fourteen genetic marker loci, including *BrFLC2* and spanning 58.9 cM of R2, were used for QTL analysis (Figure 4a). CIM revealed two QTL. The major QTL (*FR1*, LOD = 34.7) centered on the *BrFLC2* locus, explained 80.6% of the variation, and had an additive effect of 9.4 DTF. The correlation of *BrFLC2* genotypes with flowering time is summarized in Figure 5b. A second, smaller QTL (*VFR1*, LOD = 8.1) centered at 53.9 cM and explained 14.0% of the variation in the population with an additive effect of 4.1 DTF. The broad-sense heritability for flowering time of this population was estimated to be 0.96.

R3 (FR2) population: The 100 unvernalized BC_1S_1 plants grown in a growth chamber had a range of flowering times from 46 to 92 DTF and from 22 to 44 LN, with averages of 66.4 DTF and 28.9 LN. The variation was greatly reduced in another set of BC_1S_1 plants after three weeks of vernalization (data not shown). DTF was significantly correlated with LN (r = 0.74; $P < 0.01$). Based on averages of 5 plants the early flowering parent (R500) had values of 43.8 DTF and 20.8 LN, the hybrid had values of 69.4 DTF and 27.8 LN, and the late flowering parent (PQ1050) had values of 92.5 DTF and 37.3 LN. Fourteen genetic marker loci spanning 54.9 cM of R3 including *BrFLC3* and *BrFLC5* were used for QTL analysis (Figure 4b). There was segregation distortion for *wg4a4* ($P < 0.01$) with fewer plants having the homozygous Per genotypes. The *BrFLC1* and *sn0319* loci were similarly distorted ($P < 0.05$). Composite interval mapping (CIM) gave a single QTL (LOD = 10.64) centered on the *BrFLC1* locus. This QTL explained 39.0% of the variation in flowering time with an additive effect of 8.0 DTF. The broad-sense heritability for flowering time of this population was estimated to be 0.95.

R2 (FR1) and R10 (VFR2): We analyzed interactions between two putative *FLC* genes by comparing two BC_3S_1 populations segregating for *FR1* (Figure 5a) and *VFR2* (Figure 5b) alone, with an F_2 population segregating for both *FR1* and *VFR2* that was derived by crossing two BC_3S_1 homozygous plants (*fr1/fr1,VFR2/VFR2* × *FR1/FR1,vfr2/vfr2*; Figure 5c). The days to flower for the 78 BC_3S_1 plants segregating for *FR1* discussed above were plotted by genotype at the *BrFLC2* locus (Figure 5a). Similarly, the days to flower for the BC_3S_1 plants segregating for *VFR2* reported in KOLE *et al.* (2001) were plotted by genotype at the *BrFLC1* locus (Figure 5b). The 326 F_2 plants had a mean DTF of 92.8 with a range of 50–150 DTF, and the mean LN was 36.5 with a range of 14–53 LN. At 150 days after planting, the experiment

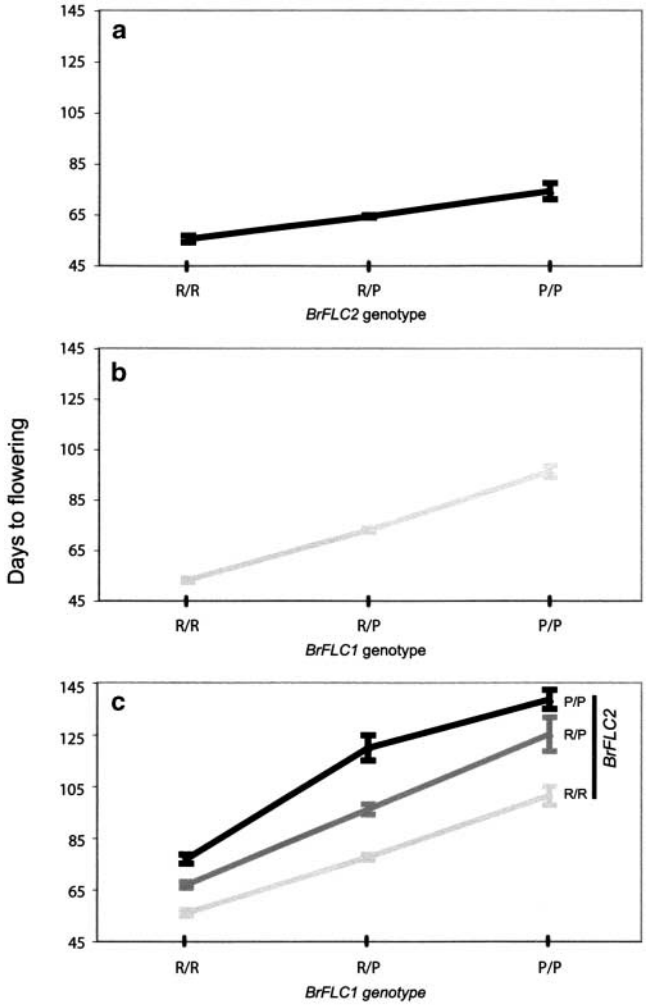


FIGURE 5.—Genetic effects for late-flowering alleles at *FR1* (*BrFLC2*) and *VFR2* (*BrFLC1*). (a) Days to flowering for *FR1* genotypes segregating in a BC_3S_1 population and scored as *BrFLC2* marker classes. R/R, R/P, and P/P are homozygous R500, heterozygous, and homozygous Per genotypes, respectively. Error bars represent the 95% confidence interval for the mean flowering time associated with each genotypic class. (b) Days to flowering for *VFR2* genotypes segregating in a BC_3S_1 population and scored for *BrFLC1* marker classes, as reported by KOLE *et al.* (2001). (c) Days to flowering for the nine genotypes of *FR1* and *VFR2* loci segregating in a single population and scored as *BFLC1* and *BFLC2* marker classes. Each line represents a *FR1* genotypic class at the three genotypes of *VFR2*. Data are from an F_2 population (326 plants) that included both QTL segregating in an R500 background, derived from a cross of two BC_3S_1 homozygous plants (*fr1fr1VFR2VFR2* × *FR1FR1vfr2vfr2*). Additive effects at the two QTL explain 98% of the genetic variation for flowering time, suggesting that these QTL are duplicate copies of the same gene that have retained similar function.

was terminated with 18 plants having never flowered. DNA from all plants was used to genotype at *BrFLC1* and *BrFLC2*, giving nine genotypic classes. The days to flower of the 326 F_2 plants segregating for both *FR1* and *VFR2* were plotted on the basis of genotype at both *BrFLC1* and *BrFLC2* (Figure 5c).

To test the main and interaction effects of two *BrFLC* loci on flowering time, the F_2 data were subjected to a two-factor analysis of variance. The full genetic model explained 87% of the flowering-time variation. Ninety-eight percent of this genetic variation was due to the individual additive effects of *BrFLC1* (72.2%) and *BrFLC2* (25.4%), similar to the results for the populations with each gene segregating alone (Figure 5). Dominance at *BrFLC1* was significant in the F_2 population, as were some of the epistatic interactions, but in total these nonadditive effects explained only 2.4% of the genetic variation.

DISCUSSION

Brassica species contain a wide range of morphological variations that have been selected for use as vegetables, oilseeds, and condiments. The expression of these variations may be due, in part, to allelic variation at redundant copies of key regulatory genes controlling developmental processes. Genes that affect phenotypes in a dosage-dependent manner would be particularly effective at expanding phenotypic diversity if they contained allelic variation at multiple functional copies. Our findings suggest that *FLC* is such a gene in *B. rapa*.

Cloning and analysis of Brassica *FLC* sequences: Using a PCR-based cloning approach, we identified four *FLC* homologs from *B. rapa* (named *BrFLC1*, *BrFLC2*, *BrFLC3*, and *BrFLC5*; Figure 1) and three *B. oleracea* homologs (*BoFLC1*, *BoFLC3*, and *BoFLC5*). Our ability to accurately identify and distinguish the different homologs was established by locus-specific PCR (Figure 1b) and by Southern blot analysis (Figure 1c). Southern blot hybridization with the four individual *BrFLC* clones accounted for all the restriction fragments detected by hybridization with an *A. thaliana FLC* probe (Figure 1c). We were not able to clone a *BoFLC2* sequence, and Southern blot analysis suggested that this locus does not exist or is highly diverged in the rapid cycling *B. oleracea* TO1000 (data not shown). However, additional loci are likely in *B. oleracea*, including a tandem duplication of *BoFLC1* (A. MILLAR, G. KING and N. SALATHIA, personal communication).

Our results using Tajima's relative rate test do not support the hypothesis of differential rates of evolution of the different Brassica *FLC* loci. Thus, we assumed that differential rates of evolution would not complicate our phylogeny reconstructions. Our phylogenetic analyses provide several interesting hypotheses for the origins of the duplication events giving rise to multiple *FLC* loci in Brassica. If the duplication events in the Brassica lineage all took place following the divergence from the Arabidopsis lineage, then the Brassica clade would be monophyletic. Both analyses (Figure 2) give a monophyletic clade of *BrFLC2*, *FLC3*, and *FLC5* (but with poor internal resolution). However, parsimony analysis (Figure 2a) and the maximum-likelihood analysis (Figure 2b)

differ in their placement of the *BrFLC1* clade. Parsimony analysis has the *BrFLC1* clade as being monophyletic with the *BrFLC2*, *FLC3*, and *FLC5* clades (but with only 68% BS support) and maximum likelihood has the *BrFLC1* clade as sister to *A. thaliana FLC* and to the remaining Brassica *FLC* sequences. Hence, the phylogeny does not resolve whether the duplication event leading to the *BrFLC1* clade and the ancestor of the *BrFLC2*, *-3*, and *-5* clades occurred before or after the divergence of the Brassica and Arabidopsis lineages.

Our phylogeny reconstruction also shows that three of the five *BnFLC* sequences (*BnFLC1*, *BnFLC3*, and *BnFLC5*) cloned by Tadege *et al.* (2001) are sisters to the *BrFLC* sequences, suggesting that these and probably *BnFLC2* (Figure 2) are *B. rapa FLC* homologs from *B. napus*. The homology of *BnFLC4* is uncertain since we did not detect a *BoFLC2*. *B. napus* ($n = 19$) is an interspecific allopolyploid between *B. rapa* ($n = 10$) and *B. oleracea* ($n = 9$). We obtained partial sequence of four *B. rapa* and three *B. oleracea FLC* sequences, suggesting that at least seven *FLC* loci should be in *B. napus*. Tadege *et al.* (2001) obtained evidence for only five *FLC* loci in *B. napus* on the basis of their cDNA library screening. Our unpublished mapping data with natural *B. napus* show at least seven *FLC* loci; thus the likely *B. olereacea FLC* homolog sequences in *B. napus* have yet to be identified and merit additional study.

Previous studies have established homology between the top of chromosome 5 of *A. thaliana* and three Brassica linkage groups (Lagercrantz *et al.* 1996; Osborn *et al.* 1997; Bohuon *et al.* 1998; Parkin *et al.* 2002). Our analysis confirms these previous results, showing strong collinearity between regions of three *B. rapa* linkage groups (R2, R3, and R10) and the top of At5. We also mapped *FLC* loci (*BrFLC2*, *BrFLC3*, and *BrFLC1*) at the predicted collinear regions of R2, R3, and R10, respectively (Figure 3). We attempted to map *CO* loci in the At5 homologous regions of *B. rapa* because others have argued that QTL mapping to these regions is due to alleles of this gene (Bohuon *et al.* 1998; Axelsson *et al.* 2001). We identified polymorphisms that mapped to R2 and to R10, but none that mapped to R3. This could simply be due to a lack of allelic variation at or near *CO* in our cross; however, efforts to clone *CO* homologs resulted in only two cloned *CO* orthologs from *B. nigra* (*Bni COa* and *BniCOb*; Lagercrantz and Axelsson 2000) and only a single pair of homeologous *CO* sequences from N2 and N12 of polyploid *B. napus* (Robert *et al.* 1998). Differences in the copy number and organization of Brassica *CO* and *FLC* loci, including the presence of a fourth Brassica *FLC* locus (*BrFLC5*), suggest that the two genes located only 2 Mb apart in the *A. thaliana* genome may have had different evolutionary pathways in Brassica species.

Whereas *BrFLC1*, *BrFLC2*, and *BrFLC3* map within collinear regions, *BrFLC5* maps on R3 to the interval between two stretches of collinearity with At2 and with the bottom of At5 (Figure 3). Analyses of the *A. thaliana* genome sequence found that the region around and including *FLC* (2.9–3.3 Mb) on the top of At5 was duplicated to the region from 26.4 to 27.1 Mb on the bottom of At5 (Arabidopsis Genome Initiative 2000). This duplicated region contains four similar tandem copies of a MADS-box gene (*FLCL2–4* of Tadege *et al.* 2001), including *AGL31*, that are all presumably paralogs of *FLC*. Although the proximity of *BrFLC5* to the collinear region containing the *AGL31* cluster suggests that *BrFLC5* could be orthologous to one of these loci, several observations suggest that *BrFLC5* is an ortholog of *FLC* and not of *AGL31*. First, we tested if *BrFLC5* might have several tandem copies of the gene, as the *AGL31* region does. We observed no evidence for this on the basis of Southern blot analysis of Per and R500 DNA using six restriction enzymes (*Dra*I, *Bam*HI, *Msp*I, *Cfo*I, *Eco*RV, and *Xba*I) and the *BrFLC5* sequence as a probe (data not shown). Second, the comparative mapping data showed that the orientations of the collinear regions between R3 and the bottom of At5 are inverted relative to one another (Figure 3). To explain the orthology of *BrFLC5* and one of the *AGL31* genes, one would have to hypothesize that the inversion event occurred after the fusion of the At2 and At5 regions, leaving the *AGL31* ortholog at the breakpoint or some other complex chromosomal rearrangement. Third, phylogenetic analyses clearly show that *BrFLC5* is more closely related to *FLC* than to *AGL31* (Figure 2). Finally, *BrFLC5* does not appear to have changed more rapidly than any of the *BrFLC* sequences, and relative rate tests of *AGL31* with *AGL27*, using *FLC* as an out-group, did not give evidence of differential rates of evolution. Hence, all lines of evidence suggest that *BrFLC5* is an ortholog of *FLC* and not of *AGL31*. Determination of how an *FLC* ortholog was duplicated and inserted in this location on R3 will require additional experimental work.

Functional constraints may be reduced for duplicate genes, and we found mixed evidence for this for *BrFLC* genes. The higher dN/dS ratios for *BrFLC* sequences compared to the average of other MADS-box genes and the large variation in intron length suggests that they are not under strong purifying selection. Hence, the proteins have some flexibility to allow new amino acid sequences. However, except for the deletion in *BrFLC2*, there is strong conservation for exon size, with only a few changes in amino acid chain length (Figure 1a). The flexibility for allowing amino acid substitutions, reflected in the high d_N/d_S ratios, could indicate that the *BrFLC* sequences are undergoing rapid evolution, as Lagercrantz and Axelsson (2000) argue for Brassica *CO* sequences. The higher ratio can be interpreted to mean either relaxed sequence constraint while maintaining function or selection for diverse sequences and function. To test for conservation in function of the different *BrFLC* loci, we determined the phenotypic ef-

fects associated with alleles at *BrFLC* loci by using the sequences as candidate genes in segregation analyses.

Effects of *FLC* regions on flowering time: We found that three of our four cloned *B. rapa FLC* homologs, *BrFLC1*, *BrFLC2*, and *BrFLC5*, cosegregate with loci controlling flowering time in populations derived by backcrossing alleles from a biennial *B. rapa* into an annual *B. rapa*. *BrFLC1* cosegregates exactly with the *VFR2* locus on R10 reported by Kole *et al.* (2001). *BrFLC2* and *BrFLC5* map within confidence intervals of flowering-time QTL *FR1* on R2 and *FR2* on R3, respectively (Figure 4). These results generally agree with two previous QTL studies using F_2 (Teutonico and Osborn 1995) and RI (Osborn *et al.* 1997) populations derived from the same parental lines, although there were differences in the magnitude and positions of effects. Populations used in the previous studies segregated for many loci affecting flowering time, and the QTL estimations may have been biased by chance associations of unlinked genomic regions. We used backcrossing in the current study to eliminate allelic variation at nontarget QTL, minimizing the bias that could be created by other segregating regions. This appeared to be very effective for the two QTL on R2, which were estimated in a population derived after three generations of backcrossing and whose combined effects closely matched the heritability estimate of the population. It was less effective for the QTL on R3, which were estimated in a BC_1S_1 population and accounted for only about one-half of the heritable variation of this population. A QTL effect on R3 near *BrFLC3* was not detected in this or previous studies; however, the parents may have *BrFLC3* alleles with small differential effects on flowering time that could be detectable after additional backcrossing.

Other researchers have found flowering-time variation associated with these same genomic regions in *B. rapa* and other Brassica species (Bohuon *et al.* 1998; Axelsson *et al.* 2001; Österberg *et al.* 2002), and this variation was attributed to replicated *CO* loci (Axelsson *et al.* 2001). Our results indicate that most of the difference in flowering time between the annual and biennial *B. rapa* that we analyzed is controlled by replicated *FLC* loci. One of these flowering loci (*VFR2*) mapped as a single Mendelian locus precisely with *BrFLC1* (Kole *et al.* 2001), and the other two were mapped as QTL in defined backcross populations to regions containing *FLC* homologs within the QTL confidence intervals. Further evidence that these loci correspond to *FLC* came from the reduction in flowering-time effects after vernalization. Finally, we tested the combined effects of alleles segregating at two loci, *FR1* and *VFR2* (*BrFLC1*), and found little evidence for epistasis; additive effects of the two loci accounted for 98% of the genetic variation for flowering time. This result supports our hypothesis that *FR1* and *VFR2* are duplicate copies of the same gene that have maintained a similar function. The overexpression of *BnFLC* in *A. thaliana* by Tadege *et al.* (2001) also provides evidence that multiple *FLC* loci encode functional gene products, but it does not demonstrate the allelic effects of these loci in Brassica. This is important for determining the role of replicated genes in phenotypic diversity and could be further demonstrated by analyzing the phenotypic effects of additional alleles derived from diverse genotypes, by studying their gene expression pattern, and by transformation experiments using Brassica *FLC* alleles expressed from native promoters.

Michaels and Amasino (2000) presented the "flowering rheostat" model to explain the additive effects of *FLC* alleles at endogenous and transgenic *FLC* loci in *A. thaliana*. In their model, additional copies of *FLC* act in an additive manner to increase the time to flowering, like settings on a rheostat, until biennialism is obtained. Our results from analyzing the interaction effects of two *FLC* loci fit this model and illustrate how replicated copies of *FLC* could expand the rheostat-like effect of the gene. The effects of gene dosage on an important trait like flowering time explains why replicated *FLC* genes have been retained and have apparently maintained ancestral function. Retention of replicate gene function has been observed at higher than expected frequencies (Lynch and Conery 2000). For genes that act in a dosage-dependent manner, the expansion of phenotypic variation through gene replication may be one reason for widespread success of polyploids and for the retention of duplicate gene function.

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