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Evidence for homology of flowering-time genes *VFR2* from *Brassica rapa* and *FLC* from *Arabidopsis thaliana*

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Abstract Variation in flowering time is important for the adaptation of plant species to different natural and agricultural environments. We previously identified *VFR2* as one of two major quantitative trait loci (QTLs) controlling *v*ernalization-responsive *f*lowering time in a segregating population derived from a cross of annual and biennial *Brassica rapa*. The region containing *VFR2* is homologous to a region in *Brassica napus* that controls the same trait, and also to a region on chromosome 5 of *Arabidopsis thaliana* that contains several flowering-time loci. In order to determine precisely the allelic effects and map position of *VFR2*, we backcrossed the late allele into an early flowering line and obtained monogenic segregation for flowering time in a BC_3S_1 population. The two homozygous genotypic classes differed by 43 and 95 days to flowering in the field and growth chamber, respectively; and the effect of the late allele was almost completely additive. DNA probes that were previously shown to detect RFLP loci in the *VFR2* region, or in the homologous regions of *B. napus* or *A. thaliana* (including two DNA clones of flowering-time genes) were used to construct a high-resolution map around *VFR2*. An RFLP detected by an *A. thaliana* cDNA clone of *flowering locus C* (*FLC*) co-segregated exactly with *VFR2* in 414 gametes analyzed (<0.24 cM). *FLC* is a repressor of flowering and is required for the winter-annual habit of late-flowering ecotypes of *A. thaliana*. The regulation of *FLC* RNA in *B. rapa* was consistent with that seen in *A. thaliana*; RNA levels were up-regulated in the biennial parent and down-regulated by cold treatment. Thus, *VFR2* appears to be homologous to *FLC* and may control flowering time though a similar mechanism as in *A. thaliana***.**

Keywords *Brassica rapa* · *Arabidopsis thaliana* · Comparative mapping · Flowering-time genes · Vernalization

Introduction

Genetic variation for flowering time is important for the adaptation of plant species to different environments, and for the selection of crop plants that meet specific cultivation and consumer needs. Within the cultivated *Brassica* species, annuals and biennials have been selected as two extreme forms of flowering habit. Biennials have an absolute requirement for cold treatment before flowering can occur (vernalization). This flowering behavior is critical for producing some vegetable forms, such as cabbages of *Brassica oleracea*, and for adaptation to certain agricultural practices, such as the fall planting of oilseed *Brassica napus* and *Brassica rapa* (syn. *campestris*).

We previously studied the genetic differences in flowering time between an annual (‘R500’) and biennial (‘Per’) cultivar of oilseed *B. rapa* by analyzing quantitative trait loci (QTLs) in an F_2 population (Teutonico and Osborn 1995) and a recombinant inbred population (Osborn et al. 1997). In both studies, most of the variation in flowering time was attributed to two QTLs, named *VFR1* and *VFR2* for *v*ernalization-responsive *f*lowering time in *B. rapa*. The two genomic regions containing these QTLs were shown to be homologous to two regions in *B. napus* which contain QTLs (*VFN1* and *VFN2*) controlling vernalization-responsive flowering time in segregating populations derived from annual and biennial oilseed cultivars (Osborn et al. 1997; Butruille et al. 1999). The

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Brassica regions containing *VFR2* and *VFN2* were also shown to be homologous to a region at the top of chromosome 5 in the related crucifer *Arabidopsis thaliana*, where the flowering-time genes *CONSTANS* (*CO*), *EMBRYONIC FLOWER 1* (*EMF1*), *FY* and *FLOWERING LOCUS C* (*FLC*) are located (Osborn et al. 1997).

VFR2 was estimated to have a large effect on flowering time, and this effect could be due to a gene homologous to any one or a combination of the four genes *CO*, *EMF1*, *FY*, and *FLC* in *A. thaliana*. *FLC* is a good candidate because: (1) by interacting with *FRI* on chromosome 4 it causes the latest flowering phenotypes observed in *A. thaliana*, (2) plants with late-flowering alleles respond to vernalization, and (3) natural allelic variation for this locus exists among ecotypes of *A. thaliana* (Koornneef et al. 1994; Lee et al. 1994). *FLC* was isolated recently by positional cloning and found to encode a MADS-domain protein (Michaels and Amasino 1999; Sheldon et al. 1999). The other three flowering-time genes were identified by mutational analyses. *CO* and *FY* mutant alleles delay flowering and *FY* mutants respond to vernalization (Koornneef et al. 1991). *CO* has been cloned and it encodes a protein with similarities to zinc-finger transcription factors (Putterill et al. 1995). Mutant alleles of *EMF1* cause precocious flowering (Yang et al. 1995) and wild-type alleles are believed to encode a floral inhibitor (Sung et al. 1992). Natural allelic variation has not been observed for *CO*, *FY* and *EMF*.

Because *VFR2* was analyzed as a QTL, we can not make precise linkage map comparisons to *A. thaliana* genes. Therefore, we backcrossed the late-flowering allele of *VFR2* into an early flowering genotype in order to achieve monogenic segregation for flowering time. In this article, we report on a high-resolution map of *VFR2* that allows more precise comparison to *A. thaliana* flowering-time genes. We present evidence from mapping data and from RNA-blot analyses that *VFR2* is homologous to *FLC* from *A. thaliana*.

Materials and methods

Backcrossing and phenotypic analysis of *VFR2*

A recombinant inbred line from a previously described population (Kole et al. 1997) was selected that had alleles from the biennial parent Per for RFLP marker loci flanking the *VFR2* region and alleles from the annual parent R500 for RFLP marker loci flanking the *VFR1* region. This line was backcrossed to R500 followed by two successive backcrosses with selection for late-flowering plants having Per alleles at marker loci flanking *VFR2*. One BC_3 plant heterozygous at *VFR2* was self-pollinated and BC_3S_1 plants were grown to evaluate the phenotypic effects of *VFR2* and to develop a high-resolution map.

A set of 426 BC_3S_1 plants was grown in a chamber at 21°C under a 16-h photoperiod and a light intensity of 1200 $\mu E/m^2$ per s from fluorescent and incandescent bulbs. A second set of 66 plants was grown under the same conditions for 3 weeks and then vernalized for 3 weeks in a cold room (4–6°C) under a constant cool-white fluorescent irradiance of 30–35 $\mu E/m^2$ per s before returning to the growth chamber. A third set of 92 BC_3S_1 plants was grown in a field in Madison, Wis. Seed was sown in flats on April 23, 1998, and germinated under fluorescent lights (21°C, 16-h photoperiod). After 22 days, plantlets were transplanted to the field as spaced plants and grown to maturity. Days to flowering (DTF), measured from seed sowing to the first open flower, and leaf number (LN), measured as the number of nodes on the main stem to the first flowering node, were recorded for up to 144 days after sowing in the non-vernalized population grown in the chamber; however, some late plants selected for marker genotyping were observed for up to 160 days until flowering. These traits were recorded for all of the vernalized plants and the field-grown plants.

RFLP analysis and linkage mapping

Samples of total genomic DNA from the 115 earliest flowering and 21 randomly selected late-flowering BC_3S_1 plants from the non-vernalized growth chamber experiment, and of R500, Per and the BC_3 parents, were digested with *Eco*RI, *Eco*RV or *Hin*dIII and used to prepare Southern blots. Blots were probed with eight DNA clones from *Brassica* species used previously for developing RFLP maps of the *VFR2* region (Osborn et al. 1997) and four DNA clones from *A. thaliana*, including a cDNA clone of the flowering time gene *CO* (Putterill et al. 1995) and a portion of a cDNA clone of FLC containing exons 2–6 (Michaels and Amasino 1999). DNA isolation, preparation of blots and hybridizations were carried out essentially as described by Teutonico and Osborn (1994), except that the final washing for probes from *A. thaliana* was done in 0.5× SSC for 10–20 min and for probes from *B. rapa* or *B. napus* in 0.2–0.5× SSC for 20–30 min, using higher concentrations with more re-uses of the blots.

Genotypes of the 115 early flowering plants, or of a subset having recombinant chromosomes in the *VFR2* region, were scored for the 12 marker loci. A high-resolution map around *VFR2* was constructed using the MAPMAKER v 2.0 program with the Haldane mapping function (Lander et al. 1987; Lincoln et al. 1992). Southern blots containing *Eco*RI-digested DNAs from all of the plants grown in the field were probed only with the *FLC* cDNA clone.

RNA gel-blot analysis

Plants were grown on agar-solidified medium containing 0.65 g/l of Peters Excel 15–5-15 fertilizer (Grace Sierra, Milpitas, Calif.) for the duration of the experiment. Plants which were not cold-treated were grown for 10 days in warm conditions (22°C under continuous light provided by cool-white fluorescent bulbs). Cold-treated plants were grown for 5 days under warm conditions before being transferred to cold conditions (2°C under 8-h photoperiods) for 3 or 6 weeks. Following cold treatment, plants were grown in warm conditions for 5 additional days. Whole plants were then harvested for RNA isolation.

Total RNA was isolated using RNA Isolator (Genosys Biotechnologies, The Woodlands, Tex.) according to the manufacturers instructions. For RNA blots, 15–20 µg of RNA was separated by denaturing-formaldehyde-agarose gel electrophoresis as described elsewhere (Sambrook et al. 1989). RNA blots were probed with a ^{32}P-ATP-labeled cDNA fragment of *FLC* that did not contain the conserved MADS-box domain. Blots were also probed with an 18S rRNA probe as a control for the quantity of RNA loaded.

Results

Phenotypic effects of *VFR2*

The 426 non-vernalized BC_3S_1 plants grown in a chamber segregated distinctly into 115 early flowering plants, and 311 late- or non-flowering plants. The early group had a range of 37 to 50 days to flowering (DTF) and a mean of 41.7 DTF, which was similar to the mean of the

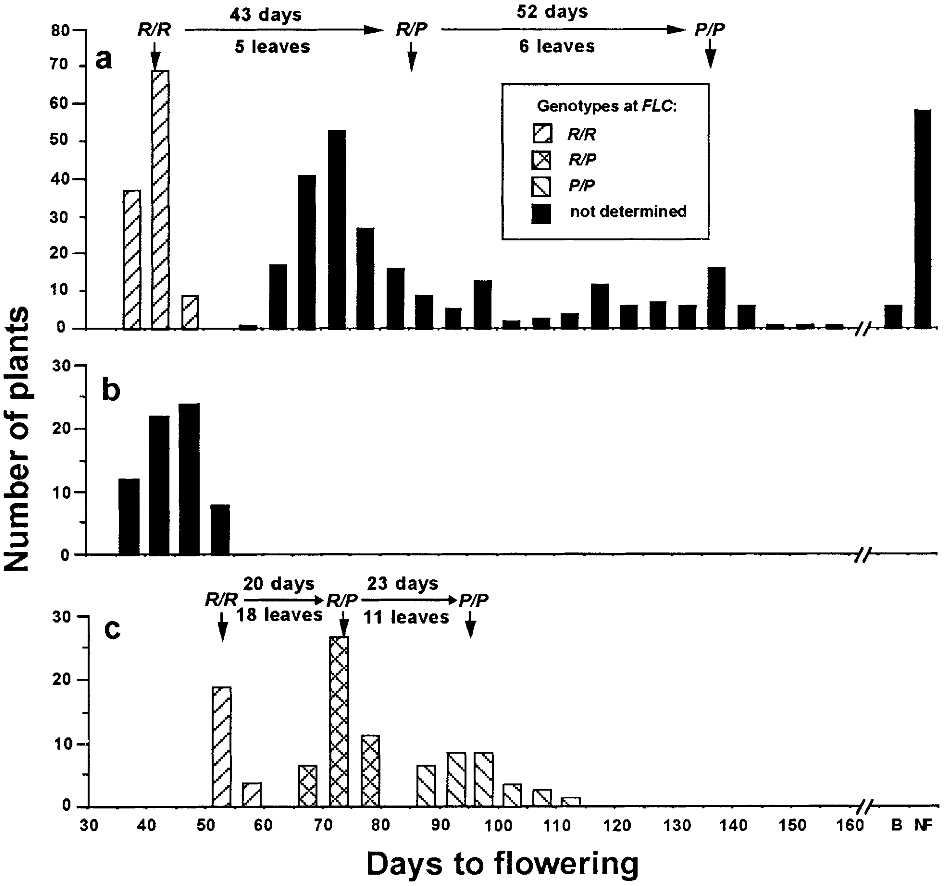

Fig. 1a–c Distribution of BC$_3$S$_1$ plants segregating at *VFR2* for days to flowering. **a** 426 non-vernalized plants grown in a chamber [some plants were still in bud (B) or had no sign of flowering (NF) at the end of the experiment]; **b** 66 plants vernalized for 3 weeks and grown in a chamber (the time in vernalization was subtracted); and **c** 92 plants grown in the field. The effects of allele substitution on days to flowering and number of leaves to first flower are indicated for non-vernalized plants (**a** and **c**) having homozygous R500 (*R*/*R*), homozygous Per (*P*/*P*) or heterozygous (*P*/*R*) genotypes at *VFR2*. The RFLP genotypes detected by *FLC* for distinct phenotypic classes in **a** and **c** are shown by different shadings of the bars

annual parent, R500 (39 DTF) (Fig. 1a). There was a gap of 10 days in the distribution and then a group of 244 plants exhibited 60 to 144 DTF. On the 144th day, 67 plants had not flowered, including six that had buds. The late parent Per showed no sign of flowering on the 144th day after sowing. The number of plants observed in the two classes, early and late- or non-flowering, fit the 1:3 ratio expected for the segregation of two alleles at a single locus, for which only one of the two homozygous classes can be distinguished ($\chi^2=0.9$, $P>0.3$).

Days to flower was significantly correlated (r=0.75; $P<0.01$) with leaf number (LN) at flowering. The early group had a low LN (18.2) similar to R500 (16.5). The late group had a mean LN of 24.0 that equalled the biennial parent Per on the 144th day after sowing.

All plants that were vernalized for 3 weeks and then grown in the chamber flowered in 42 to 58 days (Fig. 1b). The mean DTF (50.3) was similar to vernalized R500 (44.0 DTF) and also close to the early plants in the non-vernalized set (41.6 DTF). These plants had a mean LN of 22.8, similar to vernalized R500 (21.0 LN) and to the early group in the non-vernalized population (18.2 NL).

The 92 BC$_3$S$_1$ plants grown in the field segregated into three distinct groups for flowering time. The early group flowered in 50 to 56 days (mean of 52.7 DTF), the middle group flowered in 67 to 78 days (mean of 72.5 DTF), and the late group flowered in 87 to 97 days (mean of 95.8 DTF) (Fig. 1c). The observed number of plants in each category (21 early, 43 mid, and 28 late) fit the 1:2:1 segregation ratio expected for two alleles at a single locus, where all three genotypic classes can be phenotypically distinguished ($\chi^2=1.46$, $P>0.25$).

Genetic linkage of *VFR2*

The 12 marker loci that were analyzed for the 115 early flowering plants spanned 20.7 cM in the *VFR2* region of linkage group 8 (Fig. 2). Eight of these probes came from *Brassica* species and had been used previously to map RFLP loci in *B. rapa* or the homologous region in *B. napus* (Osborn et al. 1997). For one of these probes (TG1G9), all of the 115 early plants had the R500 genotype. Based on the number of plants analyzed, this marker locus is very tightly linked to *VFR2* (<0.44 cM).

We selected 21 plants at random from the late- and non-flowering group and analyzed these with the TG1G9 probe to estimate the genotypic effects of the late allele from Per on leaf number and flowering time. We used the genotype at marker locus *tg1g9* that co-segregated with *VFR2* as an indication of the *VFR2* genotype. Seven plants were homozygous for the allele from Per, and these had a mean DTF of 136.7 and a mean LN of 28.7. Fourteen plants were heterozygous, and had a mean of 84.9 DTF and a mean NL of 22.6 (Fig. 1).

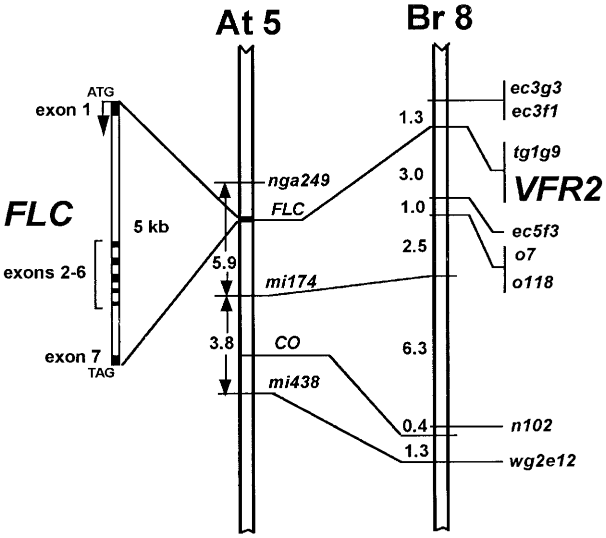

Fig. 2 A portion of *B. rapa* linkage group 8 (Br 8) around a vernalization-responsive flowering time gene (*VFR2*) and the corresponding region in *A. thaliana*. The names of RFLP loci are on the right and linkage distances in cM are on the left. The positions of RFLPs detected by four *A. thaliana* DNA clones (FLC, mi174, CO and mi438) in *B. rapa* are indicated by lines connecting their positions on chromosome 5 of *A. thaliana* (At 5). The map positions of loci on At 5 are from linkage analyses in a recombinant inbred population (*nga249*, *mi174* and *mi438*) or from physical map data (Lister and Dean 1993; http://genome.www3.stanford.edu/cgi-bin/AtDB/). *CO* and *FLC* are cloned flowering-time genes. An RFLP locus detected in *B.rapa* by exons 2–6 of a *FLC* cDNA co-segregated exactly with the *VFR2* phenotype in 414 gametes

Four of the 12 probes came from *A. thaliana*, and their map positions are known based on genetic mapping in a population of RI lines (*mi174* and *mi438*), or physical mapping (*CO* and *FLC*) (Lister and Dean 1993; Putterill et al. 1995; Michaels and Amasino 1999; http://genome.www3.stanford.edu/cgi-bin/AtDB/). The four loci span a genetic distance of approximately 5–10 cM and a physical distance of 3.3 Mb at the top of *A. thaliana* chromosome 5. These probes detected four colinear loci in the high-resolution map of *VFR2* that spanned a distance of 14.5 cM (Fig. 2). The cDNA clone of *CO* detected an RFLP locus 13.2 cM from *VFR2*, indicating that *VFR2* does not correspond to *CO*. The cDNA clone of *FLC* detected only three restriction fragments on Southern blots: a monomorphic 17-kb fragment present in all BC_3S_1 genotypes (and in Per and R500) and two allelic fragments of 6 kb (from Per) and 9 kb (from R500) that segregated in the BC_3S_1 population (data not shown). All plants in the early flowering group were homozygous for the R500 allele detected by *FLC* (Fig. 1).

Southern blots of the 92 BC_3S_1 plants that were evaluated for flowering time in the field were also probed with the *FLC* cDNA. The three genotypic classes detected by this probe corresponded exactly with the three phenotypic classes observed in the field. All plants in the earliest-flowering group were homozygous for the R500 allele detected by *FLC*, all plants in the latest-flowering group were homozygous for the Per allele, and all plants in the intermediate-flowering group were heterozygous (Fig. 1). Thus, we observed exact co-segregation of the flowering time phenotype and the RFLP locus detected by *FLC* for 414 gametes (2×115 gametes in the chamber experiment plus 2×92 gametes in the field experiment).

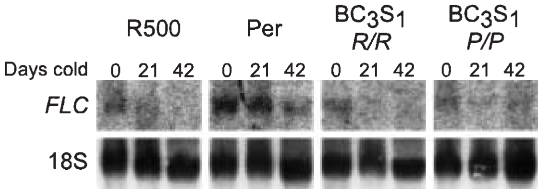

Fig. 3 Expression of RNA homologous to *A. thaliana FLC* in *B. rapa* genotypes. RNAs were isolated from the annual R500, the biennial Per, and BC_3S_1 plants homozygous for the R500 (*R*/*R*) or Per (*P*/*P*) alleles of *VFR2* following 0, 21, or 42 days of cold treatment and RNA gel blots were probed with an *FLC* cDNA

Expression of *FLC* homologs in *VRF2* genotypes

The levels of RNA homologous to *FLC* were determined in the biennial parent Per, the annual parent R500, and two BC_3S_1 lines homozygous for either the Per or R500 alleles of *VFR2* (Fig. 3). RNA was isolated from whole plants, which were vernalized for 0, 21, or 42 days. In the absence of vernalization the *FLC* transcript was detected in all genotypes. The biennial parent Per, however, showed higher levels of expression than R500 and both BC_3S_1 lines. *FLC* transcript levels in all lines were reduced following vernalization. In R500 and both BC_3S_1 lines, transcript levels were nearly undetectable after 21 days of vernalization, while in Per the *FLC* transcript level was reduced, but still detectable, after 42 days of vernalization.

Discussion

The flowering locus we analyzed in this study, *VFR2*, was originally identified by QTL mapping in a population in which the late allele from Per delayed flowering by a mean of 33.4 days (mean difference between two homozygous classes) in a field experiment (Osborn et al. 1997). After three generations of backcrossing to the annual parent, R500, we observed Mendelian segregation of the BC_3S_1 population for flowering time, in which the two homozygous genotypic classes differed by 43 days in the field and by 95 days in the growth chamber. Homozygous early genotypes could be distinguished phenotypically in both environments. Heterozygous genotypes could be phenotypically distinguished from the two homozygous genotypes only in the field, perhaps because spaced-planting allowed for less competition among late-flowering plants. Based on the phenotypes of these heterozygotes and the estimated mean phenotype of heterozygotes from the chamber experiment, we conclude that the effect of the late allele was almost completely additive. The delay in flowering was significantly correlated with an increase in leaf number at the time of flowering, indicating that the late allele delays reproductive maturity rather than simply slowing plant growth rate.

BC_3S_1 plants that were homozygous for the early allele took longer to flower in the field (52.7 DTF) than the same genotypes in the growth chamber (41.7 DTF). This difference could be due to differences in light quality during early growth in the two experiments. The chamber-grown plants were continually exposed to a mixture of incandescent and fluorescent lights, whereas the field-grown plants were germinated and grown under only fluorescent light for 3 weeks before transplanting to the field. Fluorescent lights contain less far-red light than do incandescent lights, and reduced far-red conditions can delay flowering in *A. thaliana* (Martinez-Zapater and Somerville 1990; Lee and Amasino 1995). However, plants that were homozygous for the late allele flowered more quickly in the field (96 DTF) than in the growth chamber (137 DTF). This could be due to a slight vernalizing effect of cool springtime temperatures in the field, or to some other environmental difference, such as light intensity, light quality, and/or day length.

The genetic characteristics and phenotypic effects of *VFR2* in *B. rapa* and *FLC* in the related crucifer *A. thaliana* suggest that these loci may be homologous. The loci are each involved in the late-flowering, vernalization-responsive growth habits of biennial *B. rapa* or winter-annual *A. thaliana*, and naturally occurring allelic variation exists in both species. The results of genetic and transgenic studies in *A. thaliana* show that *FLC* regulates flowering by a rheostat-like mechanism: increasing dosage and expression of *FLC* leads to correspondingly later flowering (Lee et al. 1994; Michaels and Amasino 1999). Similarly, *VFR2* in *B. rapa* also delays flowering in a dosage-dependent manner.

Our results from comparative linkage mapping further suggest that *VFR2* is homologous to *FLC*. The *VFR2* phenotype co-segregated exactly with an RFLP locus detected by a cDNA clone of *FLC* (no recombinants in 414 gametes, corresponding to a genetic distance of <0.24 cM). *B. rapa* has a nuclear DNA content of approximately 500 Mb/1 C (Arumuganathan and Earle 1991), and total genetic map distances ranging from 890 to 1876 cM have been reported (Song et al. 1991; Chyi et al. 1992; Teutonico and Osborn 1994; Kole et al. 1997). Thus, if the *B. rapa* genome has an average of 270–560 kb/cM, 0.24 cM would correspond to physical distance of 65–134 kb; however, the exact physical distance could vary greatly depending on the level of recombination in this specific genome region.

The portion of the cDNA used for this analysis (exons 2–6; Fig. 2) hybridized to the *VFR2* locus and at least one other locus. This is not surprising, because the *B. rapa* genome is replicated (Song et al. 1991; Teutonico and Osborn 1994), perhaps due to polyploidy in its ancestry (Lagercrantz and Lydiate 1996). One of these *FLC* homologs maps to linkage group 2 (our unpublished data), and we are investigating it's possible correspondence with the flowering-time QTL detected on this linkage group (Osborn et al. 1997).

Lagercrantz et al. (1996) observed a region of the *B. nigra* genome that was co-linear with the top of *A. thaliana* chromosome 5 and was associated with variation in flowering time in a segregating population. Based on results from mapping an RFLP locus detected by a cDNA clone of *CO* from *A. thaliana* and the QTL for flowering time in *B. nigra*, they concluded that *CO* was a likely candidate gene for the *B. nigra* QTL. However, they did not consider other *A. thaliana* flowering-time genes at the top of chromosome 5, and they did not have a precise map position for the *B. nigra* flowering-time locus. Our results clearly indicate that *VFR2* from *B. rapa* does not correspond to *CO*.

In *A. thaliana*, *FLC* acts to inhibit flowering and is up-regulated in winter-annual strains. Vernalization suppresses the late-flowering phenotype of these strains and causes a mitotically stable repression of *FLC* expression. This up-regulation and flowering-time effect of *FLC* in winter-annual strains is due to the presence of a second gene, *FRI*. Strains that contain a naturally occurring recessive *flc* allele in the presence of *FRI* flower early; however, expression of the recessive allele is still up-regulated in these strains (Michaels and Amasino, unpublished data). In the presence of weak or loss-of-function *fri* alleles, strains containing either the *FLC* or a naturally occurring *flc* allele have low and indistinguishable levels of *FLC* transcript and very little difference in flowering time (Michaels and Amasino 1999). Thus, the expression of *FLC* and its effect on flowering in winter-annual strains of *A. thaliana* requires the presence of *FRI*, and the differential effect of the naturally occurring early and late-flowering *FLC* alleles is not due to differences in transcript levels.

In *B. rapa*, *FLC* regulation is similar to that seen in *A. thaliana*. *FLC* transcript is detected at higher levels in the biennial Per than in the annual R500 and is down-regulated by vernalization. After backcrossing into R500, BC_3S_1 lines with the early (R/R) and late- (P/P) flowering alleles of *VFR2* both had low and indistinguishable levels of *FLC* expression, similar to that of the R500 parent. Thus, as is the case in *A. thaliana*, the higher levels of expression seen in Per are most-likely dependent upon other modifier loci in the biennial background. However, unlike *A. thaliana*, the late-flowering allele of *VFR2* had a large effect on flowering time in the background of an early flowering, annual genotype. This effect does not appear to be due to a higher level of *FLC* transcript, but could be due to a higher level or different function of the protein encoded by this allele. Although *FRI* is responsible for the up-regulation of *FLC* in naturally occurring winter-annual strains of *A. thaliana*, loss-of-function mutations in several other flowering genes in the autonomous pathway cause a vernalization-reversible up-regulation of *FLC* (Michaels and Amasino 1999; Sheldon et al. 1999). Thus, candidates for loci that up-regulate *FLC* in biennial *B. rapa* could be homologs of *FRI* or of other genes in the autonomous flowering pathway.

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