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Evolutionary Conservation of the *FLOWERING LOCUS C*-Mediated Vernalization Response: Evidence From the Sugar Beet (*Beta vulgaris*)

Patrick A. Reeves,* Yuehui He,[†,1] Robert J. Schmitz,[†] Richard M. Amasino,[†] Lee W. Panella[‡] and Christopher M. Richards*[,2]

**United States Department of Agriculture, Agricultural Research Service, National Center for Genetic Resources Preservation, Fort Collins, Colorado 80521, [†]Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706 and [‡]United States Department of Agriculture, Agricultural Research Service, Northern Plains Area Sugarbeet Research Unit, Fort Collins, Colorado 80521*

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ABSTRACT

In many plant species, exposure to a prolonged period of cold during the winter promotes flowering in the spring, a process termed vernalization. In *Arabidopsis thaliana*, the vernalization requirement of winter-annual ecotypes is caused by the MADS-box gene *FLOWERING LOCUS C* (*FLC*), which is a repressor of flowering. During the vernalization process, *FLC* is downregulated by alteration of its chromatin structure, thereby permitting flowering to occur. In wheat, a vernalization requirement is imposed by a different repressor of flowering, suggesting that some components of the regulatory network controlling the vernalization response differ between monocots and dicots. The extent to which the molecular mechanisms underlying vernalization have been conserved during the diversification of the angiosperms is not well understood. Using phylogenetic analysis, we identified homologs of *FLC* in species representing the three major eudicot lineages. *FLC* homologs have not previously been documented outside the plant family Brassicaceae. We show that the sugar beet *FLC* homolog *BvFL1* functions as a repressor of flowering in transgenic Arabidopsis and is downregulated in response to cold in sugar beet. Cold-induced downregulation of an *FLC*-like floral repressor may be a central feature of the vernalization response in at least half of eudicot species.

In winter-annual ecotypes of *Arabidopsis thaliana*, expression of the MADS-box transcription factor *FLOWERING LOCUS C* (*FLC*) during the first growing season creates a facultative vernalization requirement (Michaels and Amasino 1999; Sheldon *et al.* 1999). During prolonged cold treatments, *FLC* mRNA expression levels decrease, permitting the upregulation of genes that promote flowering (Lee *et al.* 2000; Samach *et al.* 2000). *FLC* is maintained at a repressed level following cold treatment by an epigenetic mechanism that involves histone modifications in *FLC* chromatin (Gendall *et al.* 2001; Bastow *et al.* 2004; Sung and Amasino 2004).

FLC belongs to a clade of six paralogs known from the Arabidopsis genome (Becker and Theissen 2003). All paralogs (*FLC* and *MAF1–MAF5*) function as repressors of flowering. *FLC* and *MAF1–MAF4* are downregulated to varying degrees in response to cold whereas *MAF5* is upregulated (Ratcliffe *et al.* 2001, 2003). Given that *FLC*-like genes share an upstream regulatory complex (He *et al.* 2004; Oh *et al.* 2004; Kim *et al.* 2005) and that at least two genes (*FLC* and *MAF1*) influence the same downstream regulator of flowering, *SOC1* (Hepworth *et al.* 2002; Ratcliffe *et al.* 2003), the control of flowering time via *FLC*-like genes may be administered in a multilocus, dosage-dependent manner.

Summer-annual Arabidopsis ecotypes, which flower rapidly and do not respond to vernalization, have evolved on multiple occasions via disruption of *FLC* regulatory sequences by transposons (Michaels *et al.* 2003) and via deletions and frameshift mutations in *FRIGIDA* (*FRI*) (Johanson *et al.* 2000), a positive regulator of *FLC* (Michaels and Amasino 1999; Sheldon *et al.* 1999). An observed cline in flowering behavior in native European populations of Arabidopsis, characterized by winter annuals in the north and summer annuals in the south, has been attributed to a complex epistatic interaction between functional copies of *FLC* and *FRI* (Caicedo *et al.* 2004). Ironically, the maintenance of this latitudinal cline may be due to natural selection for increased water use efficiency, a pleiotropic consequence of the effect of *FRI* and *FLC* on flowering time (McKay *et al.* 2003), rather than mean winter temperature (Stinchcombe *et al.* 2004).

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. DQ189210–DQ189215 and EF036526.

[1]*Present address:* Department of Biological Sciences, National University of Singapore, Singapore 117543.

[2]*Corresponding author:* USDA-ARS-NCGRP, 1111 South Mason St., Fort Collins, CO 80521. E-mail: crichard@lamar.colostate.edu

FLC-like genes have not been identified in taxa other than Arabidopsis, Brassica, and Raphanus, three genera within the Brassicaceae, in spite of targeted efforts to do so in numerous taxa (Schläppi and Patel 2001; Tadege *et al.* 2003; Hecht *et al.* 2005). Moreover, in wheat, vernalization causes downregulation of a class of flowering repressors distinct from *FLC*, the B-box flowering repressors *VRN2* (*ZCCT1*) and *ZCCT2* (Yan *et al.* 2003, 2004). The phenotypic distinction between "winter" varieties of wheat (which require vernalization to flower) and "spring" varieties (with no intrinsic vernalization requirement) may be a consequence of expression of a nonfunctional form of *VRN2* in "spring" wheat cultivars (Yan *et al.* 2004). Thus the vernalization response in wheat is controlled, at least in part, by a mechanism that is analogous, not homologous, to the mechanism operating in Brassicaceae. While many species outside Brassicaceae or Poaceae (including the focus of this study, the sugar beet, *Beta vulgaris*) exhibit a vernalization response, it has not been established whether the response involves an *FLC*-mediated mechanism (*i.e.*, downregulation of an *FLC*-like repressor of flowering), a *VRN2*-mediated mechanism, or some other, independently evolved mechanism.

The sugar beet and its wild progenitor sea beet (*Beta vulgaris* ssp. *maritima*) are facultative perennials that, under natural growing conditions, exhibit either an annual or a biennial flowering behavior. As in Arabidopsis, a significant association between life form and latitude has been observed among wild populations of *B. vulgaris* ssp. *maritima*. Along the Atlantic and Mediterranean coasts of France, biennial individuals are found with greater frequency in the north, while annuals predominate in the south (Van Dijk *et al.* 1997).

The difference in flowering phenology between annual and biennial sugar beets is determined by the genotype at the *B* locus, known as the "bolting gene" (Munerati 1931; Abegg 1936; Owen 1954; Abe *et al.* 1997). The bolting gene imposes a strict vernalization requirement in homozygous recessive individuals (*bb*) such that, under natural conditions, they perform as biennials. Biennial beets (*bb*) held under artificial long-day conditions will remain vegetative unless exposed to a lengthy period of cold (Stout 1945). A prolonged cold treatment is all that is required to cause biennial beets to flower, although long-day lengths during and after vernalization facilitate the transition (Fife and Price 1953). In annuals (*BB* or *Bb*), long days are necessary and sufficient to cause flowering. Under artificial short-day conditions, annual beets will grow vegetatively for an indefinite period of time without flowering. Thus the pathway to flowering in beet requires specific exogenous cues, whereas Arabidopsis will ultimately flower under most environmental conditions due to promotion by autonomous pathway genes (Koornneef *et al.* 1998).

In this study, we use a phylogenetic approach to identify *FLC* homologs in species outside the Brassicaceae, including the sugar beet. We describe the function and regulation of a sugar beet *FLC* homolog during vernalization and compare the response to *FLC*. Finally, we predict the taxonomic distribution of *FLC*-like genes in the angiosperms and propose that their role in the vernalization response has largely been conserved.

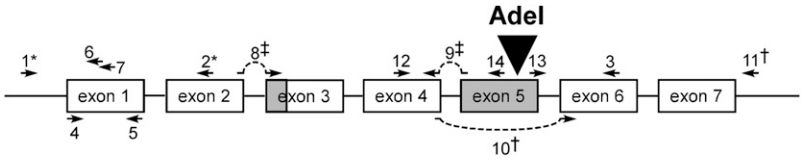
MATERIALS AND METHODS

Identification of FLC homologs by phylogenetic analysis: A consensus MADS-box amino acid sequence was used to query expressed sequence tag (EST) databases from *B. vulgaris*, *Populus* spp., and *Aquilegia formosa* × *pubescens*. In total, five *B. vulgaris* and eight *Populus* spp. ESTs and 19 putative unique gene sequences from *A. formosa* × *pubescens* were analyzed. Twenty-two EST sequences representing *Oryza sativa* MIKC-type MADS-box unigenes were retrieved from the NCBI database. A *Solanum lycopersicum* EST sequence was identified as a possible *FLC*-like gene and was incorporated into the data set. Sixteen additional putative *FLC*-like genes that were distinct from those already integrated into the data set were identified using the Phytome database (Hartmann *et al.* 2006; gene family 51, subfamily 8).

Inferred amino acid sequences of all candidate nucleotide sequences were aligned to the "MIKC^{c}" data set of Becker and Theissen (2003) using DIALIGN-t (Subramanian *et al.* 2005) and ClustalX version 1.83 (Thompson *et al.* 1997). The evolution of lineage-specific motifs within the MADS-box gene family could result in overestimation of branch support when using a global alignment such as that produced by ClustalX. Therefore, the local alignment procedure in DIALIGN-T was used for comparison. Regions of ambiguous alignment were identified by eye and eliminated. As recommended by the findings of Pařenicová *et al.* (2003), two Mδ-type MADS-box sequences (*AGL30* and *AGL94*) were included as outgroups to root trees. The final DIALIGN-T and ClustalX alignments contained 142 and 138 amino acid characters, respectively. Both alignments included the complete MADS-box and the majority of the K-box. A total of 157 sequences were aligned, including a comprehensive sampling of MIKC^{c}-type MADS-box genes from the Arabidopsis genome, as well as numerous characterized genes from other plant taxa, such that all described MADS-box gene lineages were represented. Aligned data sets are available as supplemental material at http://www.genetics.org/supplemental/.

Phylogenetic analysis was used to determine support for relationships between putative *FLC*-like sequences and known MADS-box gene lineages. Bootstrap consensus trees were constructed using neighbor-joining or maximum parsimony. One thousand replicates were used. Maximum parsimony searches used the TBR-M search strategy of DeBry and Olmstead (2000) in PAUP*4.0b10 (Swofford 1999). A mixed amino acid model was used ("aamodelpr=mixed") for Bayesian Markov chain Monte Carlo analysis (Ronquist and Huelsenbeck 2003). Four chains were run for 1.5×10^7 generations. The analysis was repeated to ensure convergence onto a single stationary distribution of trees. A total of 1×10^7 generations were discarded as burn-in, after which 50,000 trees were sampled from each replicate run to determine the optimal consensus tree and posterior probabilities for relevant clades. Complete phylogenetic trees are available as supplemental material at http://www.genetics.org/supplemental/.

Isolation of full-length FLC-like cDNAs from *B.vulgaris*: The inferred amino acid sequences of Arabidopsis *FLC* (AF537203), Arabidopsis *MAF1* (AF342808), and a single



1.	-48F*	GTGAGAGAAATTGCAGCGACGAAG
2.	exon2a*	CCCGTCTGCTGCAGTGGAATCTTGATAG
3.	+19Rex6	CTCATCCTTCAACTGTTCATTCTC
4.	2F	ATGGGAAGAAGAAAAATAGAGATGAARMGNATHGA
5.	10R	TCACAGAGAATAGATAGTTGTCGAGCTTTNTYDAT
6.	1R	GAACGCCGCTTTGAAAATGTAACYTGACGACTAC
7.	2R	GACCAGAACGCCGCTTTGAAAATGTA
8.	A3&A4-5'F‡	CAATAGCTGGTGTTGAAACAGAGAGTTC
9.	A1&A4-3'R‡	TAGATTCTAGCATAAGTTGAGTCTTTCG
10.	A2&A3-5'F†	TACAGACCAGAACCCGAAAGGAGAAG
11.	3'UTR-#1R†	CAAAATGCTGCTCAATGCCGACTCTTAC
12.	+1156Fex2	ATGAGGCTGGAGAGGCAACTAGCTGATG
13.	+34Fex5	GTGAACAGGTATTACTGATTCTCTC
14.	+31Rex5	GTGTTCCGATAGATTCTAGCATAAGT

*,†,‡BvFL1_v1-v4, BvFL1_v2/v3, and BvFL1_v1/v4 qRTPCR primer pairs

FIGURE 1.—Location and sequence of PCR primers targeting the sugar beet *FLC* homolog *BvFL1*. Primers that span intron/exon boundaries are indicated with a dashed line. The unique *Ade*I site in exon 5 is marked with a solid triangle. Putative alternatively spliced regions are shaded.

B. vulgaris EST (BQ595637) identified by phylogenetic analysis to be an *FLC* homolog were used to design consensus-degenerate primers (ROSE *et al.* 1998; HENIKOFF *et al.* 2000) to amplify a short region of *BvFL1* exon 1, from which genomic DNA sequence (GenBank accession nos. DQ189214 and DQ189215) was obtained by genome walking using the Universal GenomeWalker kit (BD Biosciences, San Jose, CA). A complete genomic sequence (EF036526) was subsequently obtained by shotgun sequencing BAC clone SBA02L13 derived from cultivar USH20 (MCGRATH *et al.* 2004). Primer sequences and locations are shown in Figure 1.

Total RNA was isolated from biennial FC606 (PI 590843) (SMITH and RUPPEL 1980) and annual SLC003 (PI 590811) (OWEN 1950) germ plasm. Complete coding sequences of expressed *B. vulgaris FLC*-like genes were amplified using a 3′ RACE procedure (SMART RACE cDNA amplification kit, BD Biosciences) primed with −48F, which targeted the 5′-untranslated region (5′-UTR). PCR products representing full-length cDNAs were cloned using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced. Comparison of genomic and RACE product sequences allowed the prediction of intron/exon boundaries.

Alternative splicing PCR assay: The mRNA variants *BvFL1_v1* and *BvFL1_v4* contained a 42-bp sequence corresponding to exon 5, whereas *BvFL1_v2* and *BvFL1_v3* did not (Figure 2a). To determine whether this distinction was attributable to transcription from multiple distinct genomic loci or alleles, or due to alternative splicing of a single transcribed locus, we performed the following assay: A single *Ade*I restriction site was identified in exon 5 (Figure 1). No other *Ade*I sites were present in the genomic *BvFL1* sequence obtained by genome walking. Total genomic DNA from FC606 and SLC003 was digested with *Ade*I. Primers located in exon 4 (+1156Fex2) and the 3′-UTR (3′UTR-#1R) were used to amplify uncut (Figure 3, lanes 1 and 7) and *Ade*I-digested genomic DNA (lanes 2 and 8). Digestion with *Ade*I prevented amplification of the expected 2.9-kb product. Therefore, there must be an *Ade*I site between +1156Fex2 and 3′UTR-#1R in all amplifiable genomic sequences. To verify that the cut site was in exon 5, PCR was performed with primer pairs +1156Fex2/+31Rex5 and +34Fex5/3′UTR-#1R. +31Rex5 and +34Fex5 were positioned immediately adjacent to the *Ade*I site in exon 5. Appropriately sized products were amplified in both cut and uncut genomic DNA; thus the *Ade*I cut site must be located in exon 5, and not in the intervening regions between exon 5 primers and +1156Fex2 or 3′UTR-#1R, in all amplifiable genomic sequences. We therefore concluded that all amplifiable genomic sequences that contain exon 4 and the 3′-UTR also contain exon 5. This finding supports the hypothesis that variation for the presence of exon 5 seen in *BvFL1* mRNA is attributable to alternative splicing since no genomic sequence that lacked the 42-bp exon 5 region could be identified.

Transformation of Arabidopsis with *BvFL1*: The coding sequences of *BvFL1_v1–v4* were cloned into pMDC32 via Gateway cloning (CURTIS and GROSSNIKLAUS 2003). These constructs were introduced (CLOUGH and BENT 1998) into the Arabidopsis *FLC* null mutant *flc-3* and expressed under the constitutive 35S promoter. Time to flowering was measured in T_1 plants as the total leaf number, including both rosette and cauline leaves, at flowering. Individual T_1 transformants typically represent independent T-DNA insertional events (DESFEUX *et al.* 2000); thus statistical analysis of the effect of *BvFL1* expression on Arabidopsis flowering time was appropriate (*i.e.*, the assumption that repeated measurements were independent was met). The number of T_1 plants measured for each treatment group is shown in Figure 2b. Statistical differences between treatment groups were tested using Welch's *t*-test (UITENBROEK 1997) to correct for unequal variances.

Plant growth conditions and experimental design: Biennial FC606 seeds were planted in growth chambers at 24° with constant illumination by high-pressure sodium and metal halide lamps. Vernalized plants were grown for 30 days in the growth chamber (to the 6- to 10-leaf stage), after which immature leaves were sampled for RNA (RNeasy plant mini kit, QIAGEN, Valencia, CA). Plants were then placed into a photothermal induction chamber and grown at 5° under continuous light for 90 days. Leaves were sampled for RNA after 45 days (at the 15- to 19-leaf stage) and again after 90 days (at the 20- to 24-leaf stage). A 90-day vernalization treatment is sufficient to induce flowering in FC606. Following the vernalization treatment, plants were directly returned to the 24° growth chamber, with no gradual temperature transition. Seventeen days later, a final leaf RNA sample was obtained (at the 27- to 41-leaf stage). Unvernalized plants were grown continuously in the 24° growth chamber and sampled for RNA at comparable developmental stages. Treatment of annual SLC003 plants

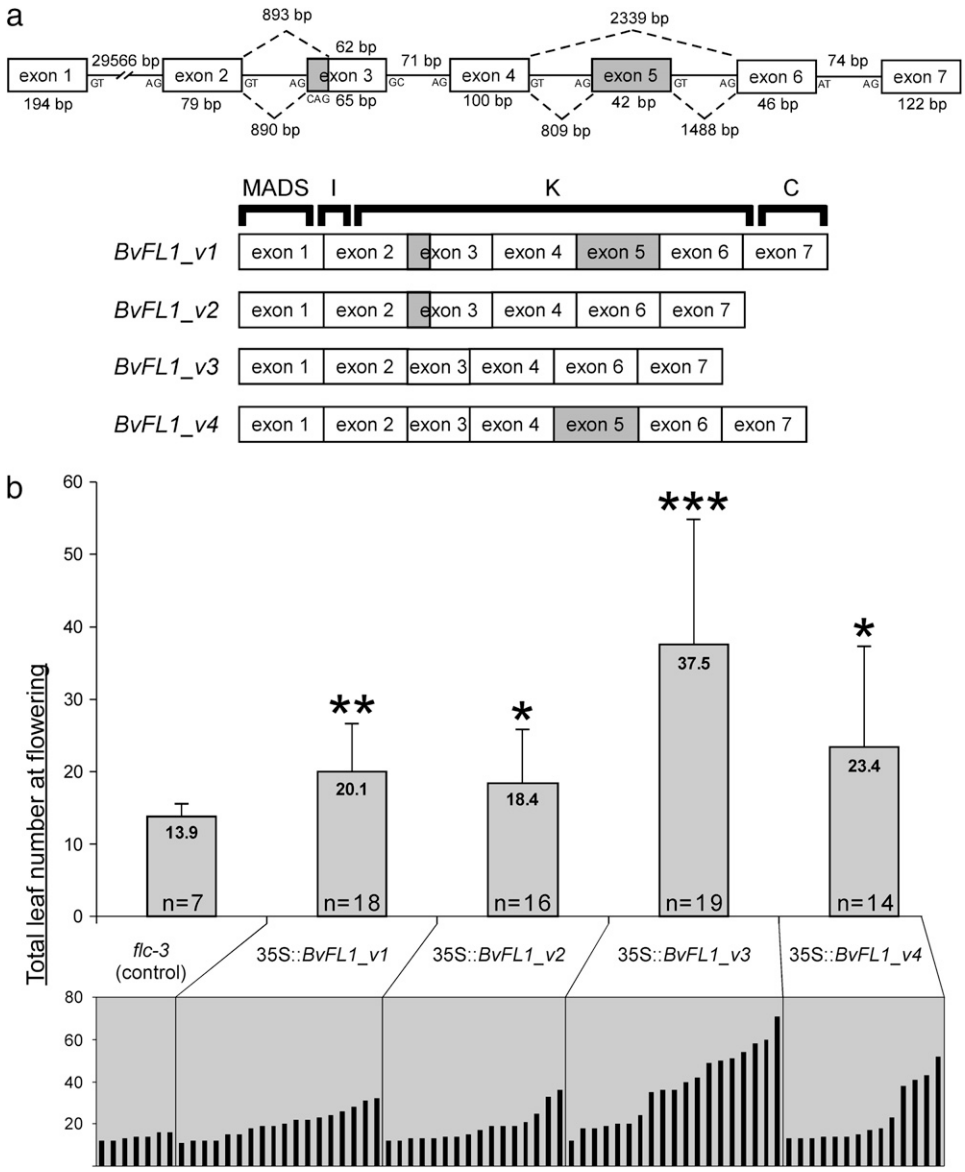


FIGURE 2.—(a) Genomic structure of the sugar beet *FLC* homolog *BvFL1*. Dashed lines indicate the four putative alternative splicing possibilities required to generate mRNA variants *BvFL1_v1–v4*. Coding regions affected by splice site variation are shaded. Approximate boundaries of the MADS, I, K, and C domains are indicated for *BvFL1_v1*. (b) (Top) Alteration of flowering phenology caused by the introduction of *BvFL1* transgenes into Arabidopsis. Expression of *BvFL1_v1–v4* variants caused a statistically significant increase in time to flowering relative to the untransformed control (Welch's *t*-test, *α[0.05], **α[0.01], and ***α[0.001]). Error bars indicate 1 standard deviation from the mean. (Bottom) Distribution of total leaf number at flowering in individual T_1 transformants shows that the only measurable effect of *BvFL1* transgenes was to delay flowering relative to the average baseline value from *flc-3*, an *FLC* null mutant.

was identical except for the timing of RNA sampling, which occurred at earlier leaf stages for the unvernalized treatment group to avoid sampling leaves from bolting individuals.

For shoot apex RNA preparations, unvernalized plants were grown as above, with apical excisions occurring at the two- to four-leaf stage. Vernalized plants were germinated in damp blotters at 24° for 3 days under constant illumination and then transplanted into planting mix when radicles were <2 cm and cotyledons had not yet emerged. Plants were placed into the 5° photothermal induction chamber immediately after transplantation and held for 77 days prior to excision of the shoot apical meristem region, when two to four leaves were present in the basal rosette.

Reverse transcriptase PCR: The Quantitect SYBR Green reverse transcriptase–PCR (RT–PCR) kit and One-Step RT–PCR kit (QIAGEN) were used to perform RT–PCR using 100 ng of total RNA as template in 25-μl reactions. Primers −48F and +19Rex6, which flanked the length variable regions of *BvFL1*, were used. Qualitative assessment of *BvFL1* transcript levels was performed by examining RT–PCR products on native polyacrylamide gels.

Quantitative real-time PCR: Primers used for quantitative real-time PCR are shown in Figure 1. All primer pairs were tested to ensure that they specifically amplified the desired variants using clones containing *BvFL1_v1–v4*. With the exception of the pair targeted to *BvFL1_v1/v4*, all primer pairs were determined not to amplify contaminating genomic DNA using "no reverse transcriptase" controls. For *BvFL1_v1/v4* assays, DNase-treated total RNA was used (Turbo DNA Free, Ambion, Austin, TX). Cytosolic glyceraldehyde-3-phosphate dehydrogenase (*GAPC*) was used as the reference gene (primer gapCex5/6F, GCTGCTGCTCACTTGAAGGGTGG and primer gapCex8R, CTTCCACCTCTCCAGTCCTT). Standard curves were created using three replicates each of five dilutions spanning a three order of magnitude (or greater) dilution series. R^2 values for standard curves were >0.97 for all products. Empirically determined amplification efficiency values were similar among loci (*BvFL1_v1–v4*, $E = 2.01$; *BvFL1_v1/v4*, $E = 2.07$; *BvFL1_v2/v3*, $E = 2.06$; GapC, $E = 2.01$).

Quantitative RT–PCR (qRT–PCR) was performed using the Quantitect SYBR Green RT–PCR kit (QIAGEN) on a Cepheid SmartCycler. A total of 50 ng total RNA was used in each 25-μl qRT–PCR reaction. Cycling profiles for all loci used a 15-sec

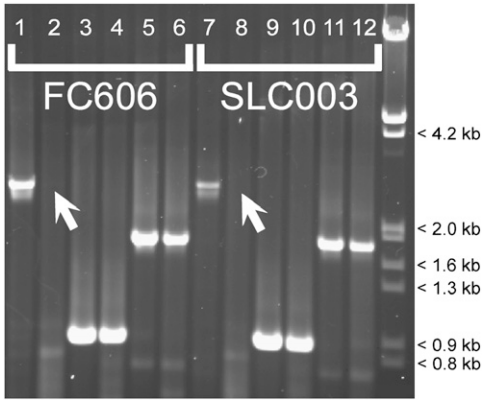


FIGURE 3.—Agarose gel image showing results of alternative splicing PCR assay. Even-numbered lanes are *Ade*I-digested genomic DNA; odd-numbered lanes are uncut. Primers used: +1156Fex2/3′UTR-#1R (lanes 1, 2, 7, 8), +1156Fex2/+31Rex5 (lanes 3, 4, 9, 10), and +31Fex5/3′UTR-#1R (lanes 5, 6, 11, 12). Digestion of genomic DNA with *Ade*I prevented the amplification of a 2.9-kb fragment (open arrows), suggesting that variation for the presence of exon 5 observed in the mature mRNA is not caused by variation for the presence of exon 5 sequence in the encoding genomic DNA. Therefore, the four mature mRNA variants observed (*BvFL1_v1–v4*) are a likely consequence of alternative splicing.

denaturation at 94° and a 30-sec extension at 72°. Annealing times were 30 sec, and temperatures were 60° for *BvFL1_v1–v4* and *BvFL1_v2/v3*, 62° for *BvFL1_v1/v4*, and 55° for *GAPC*. Fluorescence levels were measured for 6 sec at 80° for *BvFL1_v1/v4* and *BvFL1_v2/v3*, at 77° for *BvFL1_v1–v4*, and at 78° for *GAPC*. Two reactions were performed for each primer pair and a mean C_t value was computed (C_t threshold of 5). Reactions were then repeated for a total of four replicates/sample.

The magnitude ("fold change") and statistical significance of differences in expression level between treatment groups were calculated using REST-XL (PFAFFL *et al.* 2002) and are reported in Table 1.

RESULTS AND DISCUSSION

***FLC* homologs are present in the three major eudicot lineages:** To determine whether genes belonging to the *FLC*-like subfamily are present outside Brassicaceae, we identified the MIKC-type MADS-box genes (including putative *FLC*-like sequences) present in EST databases of taxa representing major angiosperm lineages. These included monocots (*O. sativa*, rice; *Triticum aestivum*, wheat), Ranunculales (*A. formosa* × *pubescens*, columbine), caryophyllids (*B. vulgaris* ssp. *vulgaris*, sugar beet; *Mesembryanthemum crystallinum*, iceplant), rosids (*Vitis vinifera*, grape; *Populus tremula* × *tremuloides*, poplar; *Glycine max*, soybean), and asterids (*S. lycopersicum*, tomato; *Solanum tuberosum*, potato; *Lactuca sativa*, lettuce). Phylogenetic analysis of inferred amino acid sequences was used to evaluate relationships between EST sequences/unigenes and all MIKC-type MADS-box genes present in Arabidopsis. We identified ESTs from tomato (AW219962), beet (BQ595637), and poplar (BU887610) that were more closely related to Arabidopsis *FLC*-like genes than to any other MIKC-type MADS-box lineage known from Arabidopsis (Figure 4a and phylogenetic trees supplied as supplemental material at http://www.genetics.org/supplemental/). Likewise, unipeptides from iceplant, grape, soybean, potato, and lettuce, identified in the Phytome database (HARTMANN *et al.* 2006) as putative relatives of *FLC*, were found here to belong to the clade containing Arabidopsis *FLC*-like genes. These relationships were found in the consensus tree regardless of the analytical method or alignment algorithm used. While bootstrap support values for the clade containing *FLC*-like genes were low (44–71%) by some standards, it should be recognized that bootstrap support is a relative measure of character support that is relevant only within a single data set. As shown in Table 2, the support values found here for the *FLC*-like clade were not significantly different from the values that we recovered for the A-, B-, C-, and E-class MADS-box gene lineages, which are generally accepted as monophyletic groups (DOYLE 1994; PURUGGANAN *et al.* 1995; THEISSEN *et al.* 1996; BECKER and THEISSEN 2003). We therefore conclude that a previously undescribed lineage of *FLC* homologs exists. Given the accepted phylogenetic relationships among the taxa included, the lineage of *FLC* homologs must have originated prior to the diversification of the three major core eudicot clades (caryophyllids, rosids, and asterids) (Figure 4b). It should therefore be possible to identify *FLC* homologs in all taxa belonging to the caryophyllids, rosids, and asterids, barring secondary loss of the loci in particular lineages during the course of evolution.

Although the number of sequences present in EST/unigene databases of rice, wheat, and columbine was large (66,238 unipeptides, 88,853 unipeptides, and 84,960 ESTs, respectively), phylogenetic analysis failed to provide any strong indication that *FLC* homologs were present in these species. Weak support for the inclusion of three monocot sequences [rice unigene S16411843 (AK066160), rice FDRMADS5 (AAD38368), and wheat F90-A (CAE53900; CIAFFI *et al.* 2005)] at the base of the clade containing *FLC*-like sequences was found. However, this result was limited to the DIALIGN-T alignments; thus an alignment artifact cannot be ruled out. The two monocot sequences (osat72611 and taes20622) identified by Phytome to be close relatives of *FLC* appeared, in our analyses, to be more closely related to *APETALA 1* and *SQUAMOSA*. While the absence of a sequence from an EST database cannot be held as conclusive evidence that a gene is not present in the genome, the results of our analyses provide an initial suggestion that the *FLC*-like gene lineage may have originated at the base of the core eudicot clade. Accordingly, recognizable *FLC*-like genes may be restricted to core eudicot taxa.

It is premature to describe the newly identified members of the *FLC*-like gene lineage as orthologs of *FLC*

TABLE 1

Statistical analysis of qRT–PCR data

Line[a]	Tissue	*n* (control, sample)[b]	Average fold change[c]	Direction	Significance[d]	Target	Treatment
FC606	Leaf	6, 5	1.113	Down	$P \geq 0.7236$	*BvFL1_v2/v3*	45 days vernalization
FC606	Leaf	6, 6	2.909	Down	$P \leq 0.0377$	*BvFL1_v2/v3*	90 days vernalization
FC606	Leaf	6, 5	2.646	Up	$P \leq 0.0064$	*BvFL1_v2/v3*	17 days postvernalization
FC606	Shoot apex	2, 2	1.222	Down	$P = 0.3323$	*BvFL1_v2/v3*	77 days vernalization
SLC003	Leaf	4, 4	2.948	Down	$P \leq 0.0199$	*BvFL1_v2/v3*	90 days vernalization
SLC003 *vs.* FC606	Shoot apex	2, 2	1.532	SLC003 > FC606	$P = 0.5027$	*BvFL1_v2/v3*	No vernalization
FC606	Leaf	4, 4	1.732	Down	$P \geq 0.1826$	*BvFL1_v1/v4*	90 days vernalization
FC606	Shoot apex	2, 2	1.023	Down	$P = 0.8347$	*BvFL1_v1/v4*	77 days vernalization
SLC003	Leaf	4, 4	1.961	Down	$P \leq 0.0025$	*BvFL1_v1/v4*	90 days vernalization
SLC003 *vs.* FC606	Shoot apex	2, 2	1.089	SLC003 > FC606	$P = 0.8347$	*BvFL1_v1/v4*	No vernalization
FC606	Leaf	4, 4	1.101	Up	$P \geq 0.7356$	*BvFL1_v1–v4*	90 days vernalization
FC606	Leaf	6, 4	1.297	Up	$P \geq 0.4685$	*BvFL1_v1–v4*	No vernalization
SLC003 *vs.* FC606	Leaf	4, 6	1.478	SLC003 > FC606	$P \geq 0.4443$	*BvFL1_v1–v4*	Two- to seven-leaf stage

[a] FC606, biennial; SLC003, annual.
[b] SLC003 used as control for comparisons between lines.
[c] Average magnitude of change, calculated from the two independent qRT–PCR setups.
[d] For statistically significant results ($P < 0.05$), the larger of the *P*-values calculated from replicated qRT–PCR setups is reported. For nonsignificant results, the smallest *P*-value is shown.

because (1) our identification strategy could not result in complete sampling of all MIKC-type MADS-box genes from each taxon and (2) the frequent occurrence of whole-genome duplication events (and subsequent lineage-specific gene extinction events) (Blanc and Wolfe 2004) and the complex birth–death pattern of diversification within the MADS-box gene family (Nam *et al.* 2004) during the evolution of the angiosperms frustrates a strict application of the concept of orthology. Hereafter we refer to the clade containing *FLC* homologs as the *FLC*-like gene subfamily.

High divergence within the *FLC*-like subfamily may explain why previous attempts to identify *FLC* homologs outside Brassicaceae have failed. Methods for homolog identification that rely on overall similarity (*e.g.*, BLAST and Southern hybridization) are most effective within the context of a gene family when sequences diverge in a clock-like manner. Unlike the majority of MADS-box genes, Arabidopsis *FLC*-like genes have evolved under positive Darwinian selection (Martínez-Castilla and Alvarez-Buylla 2003), which can cause rapid divergence among orthologs and substantial deviation from uniform sequence divergence between gene lineages. In such cases, the application of models of sequence divergence that accommodate non-clock-like evolution (*e.g.*, most phylogenetic analysis procedures) will be essential to distinguish putative orthologs from genes belonging to more distantly related paralogous lineages.

Cloning and characterization of the sugar beet *FLC* homolog *BvFL1*: Several examples of gene co-option, or duplication followed by neo- or subfunctionalization, have been documented within the MADS-box gene family (Irish and Litt 2005). Membership of a MADS-box gene within a particular subfamily cannot necessarily be used to predict its function. Because all newly identified members of the *FLC*-like subfamily are uncharacterized [with the exception of the *Brassica napus FLC* ortholog (Tadege *et al.* 2001)], it is not known whether they perform a role similar to Arabidopsis *FLC* during vernalization. We have therefore examined some regulatory and functional attributes of the *FLC* homolog identified in sugar beet for purposes of comparison.

The complete coding sequence for the sugar beet *FLC* homolog corresponding to EST BQ595637 was determined. Four RNA variants were identified in immature leaf and shoot apex cDNA libraries from biennial sugar beet germ plasm FC606. The variants differed by two K-domain indels arranged in all four possible pairwise combinations. The inferred reading frame was identical between variants such that all variants shared the same translation stop codon. There were no nucleotide substitution differences among variants within the region sequenced, which included a portion of the 5′-UTR (24 bp), the complete coding region (603–648 bp), and the 3′-UTR (345 bp).

Comparison of the cDNA sequences with a genomic sequence obtained by genome walking (DQ189215) showed that the 3-bp indel located in exon 3 could be explained by two competing 3′ splice sites in intron 2. The 42-bp indel could be explained by the inclusion or omission of an exon 5 cassette (Figure 2a). A PCR assay that took advantage of a unique *Ade*I restriction site in exon 5 showed that all amplifiable genomic sequences that include exon 4 and the 3′-UTR also include exon 5. Therefore, the absence of exon 5 sequence in two mRNA variants cannot be explained by the absence of

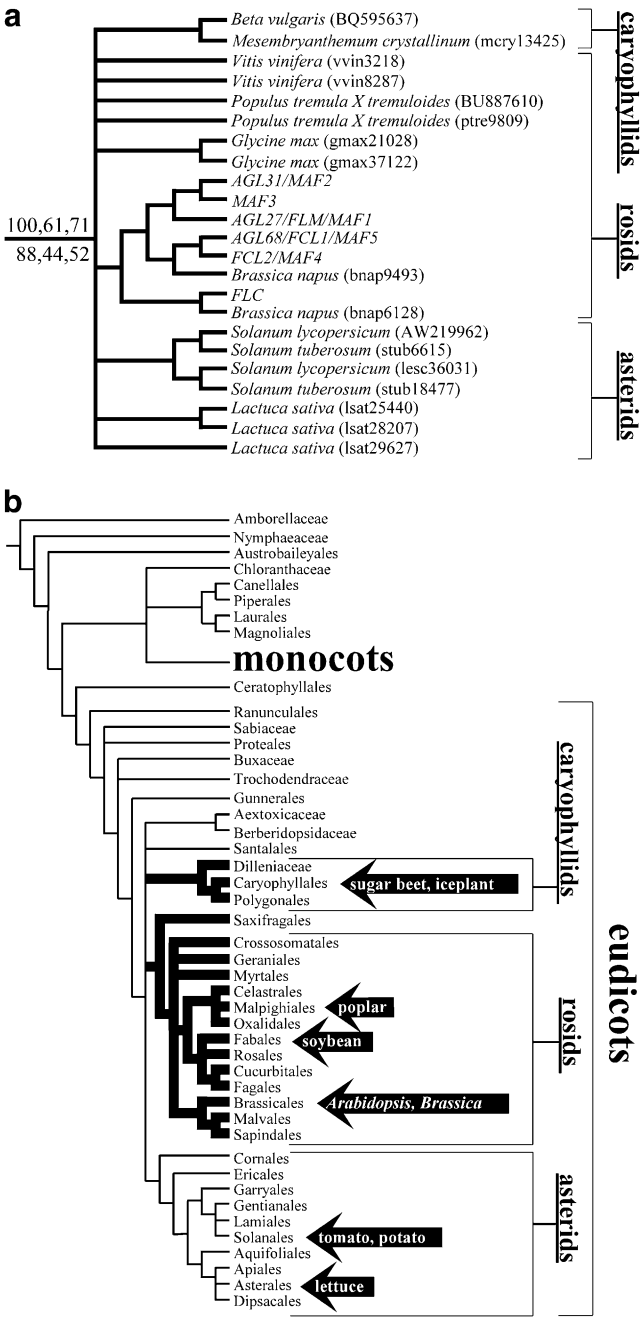


FIGURE 4.—(a) Strict consensus subtree showing the *FLC*-like gene subfamily. Support values (Bayesian posterior probability, maximum-parsimony bootstrap percentage, neighbor-joining bootstrap percentage) are indicated for the node that distinguishes the *FLC*-like subfamily from all other MADS-box gene lineages. Upper values were obtained from a global amino acid sequence alignment (ClustalX); lower values were from a local alignment (DIALIGN-T). GenBank accession numbers or Phytome unipeptide IDs are in parentheses. The presence of sequences from the caryophyllid, rosid, and asterid lineages suggests a core eudicot distribution of *FLC* homologs. (b) Phylogenetic relationships of angiosperms according to the ANGIOSPERM PHYLOGENY GROUP (2003) and JUDD and OLMSTEAD (2004). Based on evidence from sugar beet and Arabidopsis, the predicted taxonomic distribution of vernalization-responsive *FLC*-like repressors of flowering is shown with thick branches. Arrows indicate taxa where *FLC* homologs were found.

exon 5 sequence in the genomic locus that encodes them (Figure 3).

Additionally, we screened 28 sugar beet cultivars and 360 individuals from 30 wild European *B. vulgaris* ssp. *maritima* populations for genotypic variation at two microsatellite loci: one located within intron 1 and a second located ~25 kb upstream of the *BvFL1* start codon (data not shown). We found no evidence that multiple copies of this genomic region are routinely present in wild beet or sugar beet cultivars. Two or fewer alleles amplified in 99.5 and 98.4% of wild individuals for the intron 1 and upstream locus, respectively; two or fewer alleles amplified in all cultivars.

Finally, we screened a BAC library with a total of seven PCR primer pairs distributed across ~60 kb of the sugar beet genome within and around the locus. The library had 6.1× genome coverage, mathematically sufficient to recover any sequence with >99% probability (MCGRATH *et al.* 2004). Three primer pairs targeted transcribed sequence and thus were known to be an exact match for all possible loci. PCR-based screens of the matrix-pooled library identified four BACs, three of which were subsequently determined via DNA sequencing to contain overlapping inserts derived from the same genomic region. The remaining BAC contained a putative pseudogene that lacked sequence-encoding exons 2–7 and exhibited some DNA substitution differences in regions flanking the stretch of sequence homologous to exon 1. Thus we found no evidence for more than one expressed locus in the BAC library.

Taken together, these lines of evidence suggest that the four mature mRNA variants are a result of differential processing of precursor transcripts coded by a single genomic locus. The unlikely alternative hypothesis is that the mRNA variants are encoded by two or more loci that are the result of a duplication event (or events) that occurred so recently that no substitutions have occurred in the transcribed regions and that insufficient change has accumulated at the intron 1 microsatellite locus to permit the identification of multiple loci. Alternative splice variants have been observed for all *FLC*-like genes in Arabidopsis (SCORTECCI *et al.* 2001; RATCLIFFE *et al.* 2003; CAICEDO *et al.* 2004) so the hypothesis is not without precedent. While the possibility that the mRNA variants represent differentially spliced duplicate loci cannot be categorically ruled out, we refer here to the four variants collectively as a single locus, *BvFL1* (*B. vulgaris, FLC-LIKE 1*), and individually as putative splice variants *BvFL1_v1–BvFL1_v4* (DQ189210–DQ189213).

***BvFL1* is a repressor of flowering in transgenic Arabidopsis:** To test the degree of functional conservation between *BvFL1* and *FLC* in a heterologous context, the ability of *BvFL1_v1–v4* to repress flowering in Arabidopsis was evaluated. *BvFL1_v1–v4* constructs driven by the constitutive 35S promoter were inserted into the genome of the Arabidopsis *FLC* null mutant *flc-3* (MICHAELS and AMASINO 1999). All constructs resulted in a statistically

TABLE 2

Comparison of clade support values

Clade/subfamily[a]	Becker and Theissen (2003)	Parsimony, ClustalX	Parsimony, DIALIGN-T	Neighbor-joining, ClustalX	Neighbor-joining, DIALIGN-T	Bayesian, ClustalX[b]	Bayesian, DIALIGN-T
flc	100	61	44	71	52	100	88
squa (A class)	100	32	27	30	38	50	100
def (B class)	99	87	82	82	74	100	98
glo (B class)	54	46	32	61	38	79	36
ag (C class)	100	92	96	99	100	99	100
agl2 (E class)	94	31	74	94	80	73	98
mean (A, B, C, E)	89.4	57.6	62.2	73.2	66.0	80.2	86.3
sd (A, B, C, E)	19.9	29.8	30.9	28.2	27.4	20.7	28.4
p[c]	0.594	0.909	0.556	0.938	0.609	0.339	0.952

[a] Subfamily names are based on Becker and Theissen (2003).
[b] Bayesian posterior probabilities multiplied by 100 for uniformity.
[c] Based on a one-sample *z*-test to determine whether the support value for the *FLC*-like clade was significantly different from the mean of the A-, B-, C-, and E-class clades, which are accepted as monophyletic.

significant delay in the time to flowering (as measured by total leaf number) relative to untransformed *flc-3* (Figure 2b). Therefore, *BvFL1* functions as a repressor of flowering in transgenic Arabidopsis. The strongest repressor was *BvFL1_v3*, which caused a significantly greater ($P < 0.05$) delay in time to flowering than constructs containing the other three putative splice variants. The ability of *BvFL1* to complement *FLC* suggests a likely function as a repressor of flowering in sugar beet. Accordingly, we hypothesize that repression of flowering may be a conserved function of genes belonging to the *FLC*-like gene subfamily. This hypothesis remains to be explicitly tested in sugar beet.

***BvFL1* is downregulated by cold in sugar beet:** We examined whether *BvFL1* was downregulated in response to a cold treatment in beet as most *FLC*-like genes are in Arabidopsis (*i.e.*, *FLC*, *MAF1–MAF4*). Examination of *BvFL1* RT–PCR products in FC606 individuals before and after a 90-day vernalization treatment suggested a substantial decrease in the level of *BvFL1_v2* and *BvFL1_v3* (hereafter *BvFL1_v2/v3*), the two transcripts that did not contain exon 5, relative to *BvFL1_v1/v4* (Figure 5a). RT–PCR products measured at similar developmental stages in individuals that had not been exposed to a vernalization treatment showed no such response (Figure 5b). In contrast to *BvFL1_v2/v3*, qualitative changes in *BvFL1_v1/v4* levels were not marked during cold treatment.

Quantitative RT–PCR confirmed these observations. A statistically significant threefold decrease in *BvFL1_v2/v3* levels (measured simultaneously) relative to the *GAPC* reference gene occurred during the 90-day vernalization treatment, whereas no change in *BvFL1_v1/v4* levels was detected (results of all qRT–PCR analyses are shown in Table 1). However, *BvFL1_v2/v3* expression levels were not significantly different from prevernalization levels after only 45 days of vernalization, in agreement with our observations that a 45-day vernalization treatment is insufficient to induce flowering in most FC606 individuals. Figure 6 shows the relative changes in *BvFL1_v2/v3* expression levels that occurred during the course of the experiment.

These results are consistent with the hypothesis that, in biennial sugar beet, the block on flowering imposed by the strong repressor *BvFL1_v3* is progressively relieved during vernalization by selective downregulation of the *BvFL1_v2/v3* mRNA variants. The vernalization response in biennial beet may therefore involve temperature-sensitive differential production of *BvFL1* variants such that relatively weak repressors containing exon 5 (*BvFL1_v1/v4*) are favored during cold exposure and strong repressors lacking exon 5 (*BvFL1_v3*) predominate during warm growing conditions. This hypothesis requires further examination.

In Arabidopsis, *FLC* expression levels are modulated at the transcriptional level through the interaction of upstream regulators with promoter and intron 1 sequences (Sheldon *et al.* 2002; Bastow *et al.* 2004; Sung and Amasino 2004). While a change in the prevalence of alternative *FLC* transcripts has been observed during vernalization (Caicedo *et al.* 2004), no functional consequence has yet been ascribed to splice variation among Arabidopsis *FLC*-like genes. A possible distinction between beet and Arabidopsis in the regulatory mechanism underlying vernalization-induced promotion of flowering is intriguing because it implies that the same raw materials (*e.g.*, genes belonging to the *FLC*-like subfamily) may come under the control of different regulatory processes during the course of evolution, yet elicit the same general phenotypic response.

The primary response of *BvFL1* to cold occurs in immature leaves, not in the shoot apex: Previous experiments showed that nonvernalized, vegetative shoot apexes of biennial sugar beet could be induced to make the transition to flowering by grafting them onto vernalized biennial stock or annual stock (Stout 1945;

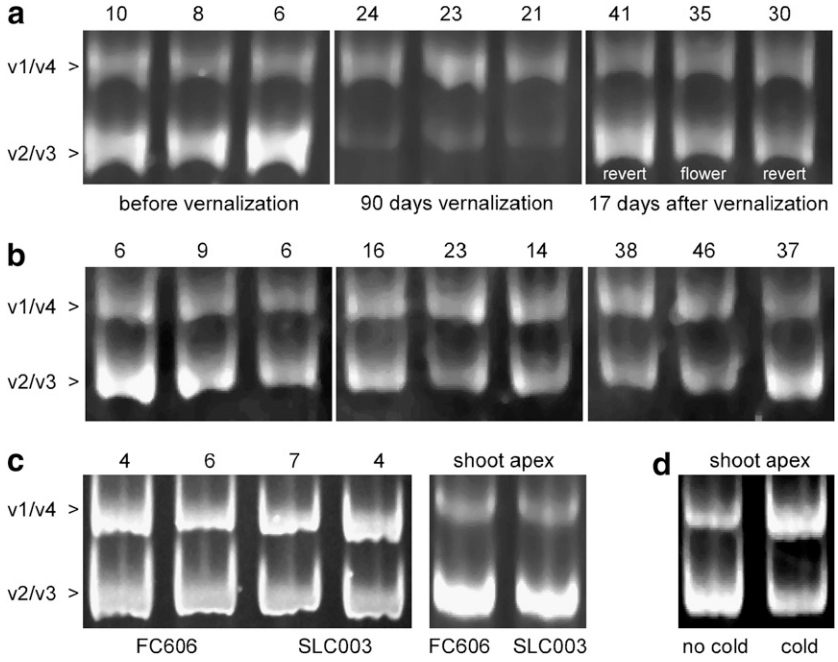


FIGURE 5.—Expression of *BvFL1* in vernalized (a) and unvernalized (b) biennial sugar beet FC606 as measured by RT–PCR. The two resolved bands each contain a pair of comigrating splice variants as labeled on the left. Qualitative expression levels are shown for three individuals, arranged in the same order as in a and b, for both treatments. In a, the center individual flowered following vernalization whereas the others reverted to vegetative growth. Images have been normalized such that the *BvFL1_v1/v4* band is of roughly equal intensity between panels. To compare similar developmental stages between vernalized and unvernalized treatment groups, leaf number, counted upward from the first true leaf, was used. The leaf number from which RNA was taken is shown above each lane. Splice variants *BvFL1_v2/v3* were downregulated relative to *BvFL1_v1/v4* in immature leaves during cold treatment. *BvFL1_v2/v3* was reset to prevernalization levels following cold treatment regardless of whether the plant ultimately flowered or reverted to vegetative growth. (c) Comparison of *BvFL1* expression in immature leaves and shoot apexes of biennial FC606 and annual SLC003. The phenotypic distinction between annual and biennial sugar beets cannot be explained by differences in expression level of *BvFL1* in immature leaves or the shoot apex. (d) The difference in *BvFL1_v1/v4 and BvFL1_v2/v3* expression level between vernalized and unvernalized FC606 shoot apexes was not significant. The response to vernalization of *BvFL1* was primarily confined to immature leaves.

CURTIS *et al.* 1964). These studies demonstrate that the signal to flower is transmissible and that a vegetative shoot apical meristem of biennial sugar beet need not be exposed to cold to be reprogrammed into an inflorescence meristem. The response of *BvFL1* to vernalization at the shoot apical meristem was evaluated using RNA pools derived from vernalized and nonvernalized FC606 individuals. While generally indicative of temperature-sensitive differential RNA processing, the qualitative difference in the *BvFL1* transcript profile between shoot apex treatments was not as marked as it was in leaves (Figure 5d). The difference in expression level, as measured by qRT–PCR, of *BvFL1_v1/v4* and *BvFL1_v2/v3* between meristem RNA pools was not statistically significant ($P = 0.8347$ and $P = 0.3323$, respectively). Thus, in FC606, the response of *BvFL1* to cold was prominent in immature leaves but was not measurable in the shoot apical meristem. This result is consistent with studies showing that immature developing leaves are a site of cold perception in beet (CROSTHWAITE and JENKINS 1993) and with experiments demonstrating that changes in *FLC* expression levels in leaves are an essential component of the Arabidopsis vernalization response (SEARLE *et al.* 2006).

Cold-induced downregulation of *BvFL1* is reversed by warm temperatures: In Arabidopsis the effect of vernalization on *FLC* expression level is largely irreversible because repressed levels are mitotically stabilized by histone methylation (BASTOW *et al.* 2004; SUNG and AMASINO 2004). Maintained repression of *FLC* leads to the so-called "memory of winter" in Arabidopsis wherein previously vernalized plants remain competent to flower long after the initial cold exposure (MICHAELS and AMASINO 2000). A stable vernalized state is common to many species, most notably *Hyoscyamus niger*, where it has been studied extensively (LANG 1965). Vernalized sugar beets, however, are prone to inflorescence reversion—the return of the shoot apical meristem from a state permissive of flowering to a noninduced state of vegetative growth (BATTEY and LYNDON 1990).

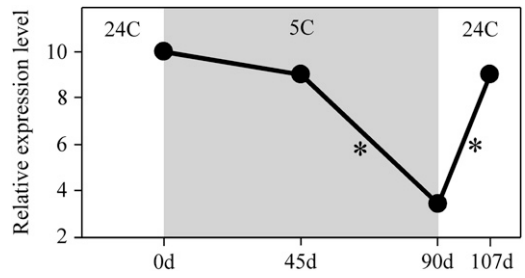


FIGURE 6.—Relative change in *BvFL1_v2/v3* expression levels during and after vernalization treatment of sugar beet germ plasm FC606 as measured by qRT–PCR. The *x*-axis indicates time in days. At 0 days, 30-day-old individuals grown at 24° were sampled for RNA and placed into a 5° photothermal induction chamber. RNA samples were taken after 45 and 90 days at 5°. After 90 days, plants were returned to 24° growing conditions. The *y*-axis indicates mRNA expression level, in relative units, with the starting level arbitrarily set to 10. *BvFL1_v2/v3* levels decreased progressively during cold treatment and then returned to prevernalization levels following vernalization. Asterisks indicate intervals during which statistically significant changes in expression level occurred.

Reversion is dependent on the environmental conditions immediately following vernalization. It is promoted by short days and rapid increase in temperature and suppressed by long days and gradual warming (Chroboczek 1934; Owen 1940). In sugar beet, inflorescence reversion is indicated by the resumption of a rosette pattern of leaf development at the shoot apex following bolting or by the abortion of the elongated bolting stalk prior to the development of flowers and a return to vegetative growth by axillary meristems.

We examined whether repression of *BvFL1_v2/v3* was stably maintained in sugar beet following cold treatment. The *BvFL1* transcript profile examined 17 days after a 90-day vernalization treatment was qualitatively similar to the profile measured prior to the cold treatment (Figure 5a), suggesting that *BvFL1_v2/v3* returns to prevernalization levels after vernalization. Quantitative RT–PCR confirmed a 2.6-fold increase in *BvFL1_v2/v3* expression levels during the 17-day postvernalization period, thereby returning *BvFL1_v2/v3* to a level approximately equal to that observed before vernalization (Figure 6). Moreover, *BvFL1_v2/v3* was upregulated following vernalization regardless of whether the plant ultimately flowered or reverted to vegetative growth (Figure 5a). Thus, in contrast to *FLC* in Arabidopsis, a vernalization treatment does not result in the maintained repression of *BvFL1_v2/v3* in sugar beet: *BvFL1* is quickly reset to the unvernalized state following the return to warm growing conditions. This response is consistent with the propensity for inflorescence reversion observed in sugar beet.

In nature, a resetting *BvFL1* response may prevent biennial *B. vulgaris* individuals from flowering following transient cold periods, akin to the proposed function of Arabidopsis *MAF2* (Ratcliffe *et al.* 2003). Additionally, a vernalization mechanism that resets *BvFL1* expression to a state that represses flowering could promote the resumption of vegetative growth following a single round of flowering in the spring, thereby suggesting one possible mechanism for the facultative perenniality observed in *B. vulgaris*.

***BvFL1* is not the "bolting gene":** Vernalization-responsive winter-annual Arabidopsis ecotypes have higher *FLC* levels than their rapid-flowering, summer annual counterparts (Michaels *et al.* 2003). We examined whether biennial beets exhibit higher *BvFL1* levels than annual beets, which possess no intrinsic vernalization requirement for flowering. The expression level of *BvFL1_v1–v4* (all mRNA variants, measured simultaneously) in immature leaves of biennial FC606 was not significantly different from annual germ plasm SLC003 ($P = 0.444$) when grown under inductive day lengths. Similarly, the relative expression level of *BvFL1_v1/v4* and *BvFL1_v2/v3* in shoot apexes and leaves was indistinguishable between FC606 and SLC003 (Figure 5c). Therefore, the difference in vernalization requirement between biennial FC606 and annual SLC003 cannot be attributed to differences in the abundance of *BvFL1* transcripts in immature leaves or the shoot apex. Furthermore, *BvFL1* was downregulated in response to vernalization in SLC003 in a manner similar to biennial FC606 (Table 1). Thus, at least in terms of *BvFL1* levels, the annual SLC003 retains an intact vernalization response. The lack of clear differences in the quantity or the regulation of *BvFL1* between SLC003 and FC606 suggests that *BvFL1* is not the bolting gene. Moreover, we have mapped *BvFL1* to chromosome 6 of sugar beet. The bolting gene is located on chromosome 2 (Butterfass 1968; Boudry *et al.* 1994); thus *BvFL1* cannot be the bolting gene.

In contrast to Arabidopsis, the presence of a vernalization requirement in sugar beet does not appear to be attributable to a difference in expression level of the *FLC* homolog *BvFL1*. Thus the primary mechanism (upregulation of *FLC* by *FRI*) hypothesized to cause the adaptive delay in flowering in fall-germinating winter-annual Arabidopsis ecotypes (Johanson *et al.* 2000; Michaels and Amasino 2000) may not be responsible for the evolution of the vernalization requirement (which plays a similar ecological role) in wild *B. vulgaris*. This should not be surprising. First, the *FRI/FLC* mechanism is only one component of a complex regulatory network that controls flowering time in Arabidopsis. A number of loci other than *FRI* and *FLC* play independent roles in the Arabidopsis vernalization response (Michaels and Amasino 2001; Ratcliffe *et al.* 2003; Werner *et al.* 2005a,b). During evolution, any number of mechanisms, both within and outside the vernalization pathway, could be recruited to produce a vernalization requirement. Second, by mutagenizing an annual line (*BB*) with ethyl methylsulfonate, Hohmann *et al.* (2005) produced beets that performed as biennials: they had lost the ability to flower under long days alone, but retained an obligate vernalization requirement, flowering only after cold treatment. Hohmann *et al.*'s (2005) results demonstrate that a vernalization requirement may be imposed in beet via loss-of-function mutations with no obvious impact on the vernalization response. Accordingly, we suggest that the bolting gene may lie outside the vernalization pathway. The recessive allele *b* could, for example, represent a loss-of-function allele in the photoperiod pathway. The obligate vernalization requirement of biennial beets could have evolved because of a failure to recognize a long-day flowering cue such that vernalization became the only effective pathway to flowering.

Summary and prospects: Using phylogenetic analysis, we have identified homologs of Arabidopsis *FLC* in the three major eudicot lineages. These data represent the first report of *FLC* homologs outside the Brassicaceae and demonstrate that the *FLC*-like gene subfamily is old, having originated in the common ancestor of the core eudicots or earlier. We have shown that *BvFL1*, an *FLC* homolog in sugar beet, is a repressor of flowering in transgenic Arabidopsis and is downregulated during

vernalization in sugar beet. We therefore propose that a key component of the Arabidopsis vernalization mechanism—downregulation of an *FLC*-like floral repressor—may be conserved in distantly related *B. vulgaris*. This attribute of the vernalization mechanism likewise may be conserved in the broad diversity of taxa descended from the common ancestor of Arabidopsis and *B. vulgaris*.

Higher-level phylogenetic relationships among the taxa shown to contain *FLC*-like genes have been well-studied (Angiosperm Phylogeny Group 2003; Judd and Olmstead 2004; Soltis and Soltis 2004). On the basis of these relationships, it is reasonable to predict that vernalization-responsive, *FLC*-like genes should be found, at a minimum, in the caryophyllid and rosid clades (Figure 4b). Together these two clades comprise approximately half of eudicot species diversity (one-third of angiosperm diversity). Included in the rosids and caryophyllids are numerous fruit, forage, vegetable, and seed crops where, as in sugar beet, flowering time is a trait of critical economic importance. Conservation of the *FLC*-mediated vernalization response creates the opportunity to control flowering time in a broad variety of crop species using molecular approaches or through conventional breeding practices that utilize the natural variation at *FLC*-like loci present in existing genetic resources. Furthermore, flowering time is a trait of clear ecological and evolutionary significance. The evolution of genetically based differences in flowering time is an important mechanism of speciation (Coyne and Orr 2004). Divergence in flowering phenology, for example, has been implicated as the most probable cause of reproductive isolation in the first documented cases of sympatric speciation in plants (Silvertown *et al.* 2005; Savolainen *et al.* 2006). By understanding the molecular mechanisms that determine flowering time in diverse taxa, crucial insight into the genetic basis of plant speciation may also be attained.

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