Differential Regulation of FLOWERING LOCUS C Expression by Vernalization in Cabbage and Arabidopsis¹

Shu-I Lin, Jhy-Gong Wang, Suk-Yean Poon, Chun-lin Su², Shyh-Shyan Wang, and Tzyy-Jen Chiou*


Vernalization is required to induce flowering in cabbage (Brassica oleracea var Capitata L.). Since FLOWERING LOCUS C (FLC) was identified as a major repressor of flowering in the vernalization pathway in Arabidopsis (Arabidopsis thaliana), two homologs of AtFLC, BoFLC3-2 and BoFLC4-1, were isolated from cabbage to investigate the molecular mechanism of vernalization in cabbage flowering. In addition to the sequence homology, the genomic organization of cabbage FLC is similar to that of AtFLC, except that BoFLC has a relatively smaller intron 1 compared to that of AtFLC. A vernalization-mediated decrease in FLC transcript level was correlated with an increase in FT transcript level in the apex of cabbage. This observation is in agreement with the down-regulation of FT by FLC in Arabidopsis. Yet, unlike that in Arabidopsis, the accumulation of cabbage FLC transcript decreased after cold treatment of leafy plants but not imbibed seeds, which is consistent with the promotion of cabbage flowering by vernalizing adult plants rather than seeds. To further dissect the different regulation of FLC expression between seed-vernalization-responsive species (e.g. Arabidopsis) and plant-vernalization-responsive species (e.g. cabbage), the pBoFLC4-1::BoFLC4-1::GUS construct was introduced into Arabidopsis to examine its vernalization response. Down-regulation of the BoFLC4-1::GUS construct by seed vernalization was unstable and incomplete; in addition, the expression of BoFLC4-1::GUS was not suppressed by vernalization of transgenic rosette-stage Arabidopsis plants. We propose a hypothesis to illustrate the distinct mechanism by which vernalization regulates the expression of FLC in cabbage and Arabidopsis.

The transition from the vegetative to reproductive phase is essential for completion of the life cycle of flowering plants. This transition is particularly important in agriculture in terms of productivity. The timing of the reproductive transition is determined by developmental status and environmental conditions. A combination of these two factors ensures that flowering occurs at appropriate times, with an accumulation of sufficient nutrients and favorable environmental conditions (Levy and Dean, 1998; Reeves and Coupland, 2000; Mouradov et al., 2002; Simpson and Dean, 2002). Genetic analysis has revealed that multiple pathways, such as the photoperiod and autonomous pathways, are involved in regulating this transition (Koornneef et al., 1998; Levy and Dean, 1998; Simpson et al., 1999). In addition, exposure to an extended period of low temperature is needed to promote flowering in many species (Chourad, 1960). This process is known as vernalization (Napp-Zinn, 1987). Vernalization is a natural adaptation ensuring that flowering occurs only after winter, in order for flowers and seeds to develop under favorable conditions.

During the past few years, two major loci, FRIGIDA (FRI) and FLOWERING LOCUS C (FLC), have established a vernalization requirement in Arabidopsis (Arabidopsis thaliana; Burn et al., 1993; Lee et al., 1993; Clarke and Dean, 1994; Koornneef et al., 1994; Johanson et al., 2000). FRI acts upstream of FLC to positively regulate FLC expression (Michaels and Amasino, 2000). In addition to FRI, other floral repressors, FRL1 and FRL2 (Michaels et al., 2004), VIP3 and VIP4 (Zhang and van Nocker, 2002; Zhang et al., 2003), ESD4 (Reeves et al., 2002), HOS1 (Lee et al., 2001), ART1 (Poduska et al., 2003), and PIE1 (Noh and Amasino, 2003), were subsequently identified as promoting the expression of FLC. By contrast, several genes in the autonomous pathway, such as LD, FCA, FY, FLK, FPA, FLD, and FVE, have been shown to repress FLC expression (Michaels and Amasino, 1999; Sheldon et al., 1999, 2000; Simpson, 2004).

FLC encodes a MADS-box transcription factor that functions as a repressor of the floral transition (Michaels and Amasino, 1999; Sheldon et al., 1999). In different vernalization-responsive ecotypes and flowering-time mutants of Arabidopsis, the levels of FLC mRNA and protein correlate well with flowering time in response to cold treatment (Michaels and Amasino, 2000; Sheldon et al., 2000). Also, the duration of vernalization has been shown to be proportional to the degree of down-regulation of FLC (Sheldon et al., 1999, 2000).
These results suggest that FLC is a major determinant of the vernalization response. Two genes, FT and SUPPRESSOR OF CO 1 (SOC1, or AGAMOUS-LIKE 20), functioning downstream of CONSTANS (CO), a flowering activator in the photoperiod pathway, were identified as being negatively regulated by FLC (Lee et al., 2000; Onouchi et al., 2000; Samach et al., 2000). Evidence supports the direct binding of the FLC protein to the CAReG box of the SOC1 promoter, which results in the suppression of SOC1 expression (Hepworth et al., 2002). This regulation by FLC is antagonistic to the positive regulation of CO in the expression of SOC1.

The repression of AtFLC by vernalization is mitotically stable, which means that the effect caused by cold temperature can be maintained even if treated plants are returned to a warm temperature, which suggests an epigenetic repression (Wellensiek, 1964; Michaels and Amasino, 2000; Sheldon et al., 2000; Henderson et al., 2003; Henderson and Dean, 2004). However, this effect is reset in the next generation after meiosis (Michaels and Amasino, 2000; Sheldon et al., 2000). Modification of chromatin structure, such as deacetylation and methylation of histone 3, recently involved in the initiation of histone deacetylation in FLC during vernalization (Henderson et al., 2003; Henderson and Dean, 2004). Interestingly, the DNA regions associated with these changes in histone modification are located within the first intron of AtFLC and its promoter in Arabidopsis (He et al., 2003; Bastow et al., 2004; Sung and Amasino, 2004). VIN3 is involved in the initiation of histone deacetylation in AtFLC chromatin during prolonged cold treatment, and VIN3, VRN1, and VRN2 are required for histone methylation and the creation of a stable inactive state (Henderson and Dean, 2004; Sung and Amasino, 2004). Interestingly, the DNA regions associated with these changes in histone modification are located within the first intron of AtFLC and its promoter in Arabidopsis (He et al., 2003; Bastow et al., 2004; Sung and Amasino, 2004), in agreement with the previous observation that intron 1 is required for the maintenance of FLC repression (Sheldon et al., 2002).

In addition to Arabidopsis, other species in the family of Brassicaceae rely on vernalization to promote flowering (Friend, 1985). Vernalization-responsive flowering-time loci of Brassica species segregate as two major quantitative trait loci that are colinear with the regions of FRI and FLC in the Arabidopsis genome (Osborn et al., 1997). This observation indicates that homologs of FRI and FLC genes are likely to be important in the control of flowering time through vernalization in other Brassica species. In fact, several FLC homologs have been isolated from Brassica species, such as Brassica napus (Tadege et al., 2001) and doubled haploid lines of Brassica rapa and Brassica oleracea (Schranz et al., 2002). Moreover, genetic manipulation of FLC expression has been proven to modify flowering time in both Arabidopsis (Michaels and Amasino, 1999; Sheldon et al., 1999) and B. napus (Tadege et al., 2001).

Vernalization can be classified into two types according to the age of the plant that senses low temperature (Friend, 1985). One is the seed-vernalization-responsive type, in which plants can sense low temperatures during seed germination; the other is the plant-vernalization-responsive type, in which plants need to reach a certain developmental stage before they become sensitive to low temperatures. Biennial plants that grow vegetatively in the first year and flower in the following year after winter usually belong to the plant-vernalization-responsive type. Some species in the Brassicaceae family, such as Arabidopsis and B. rapa, are the seed-responsive type, but several varieties in B. oleracea are plant responsive (Friend, 1985).

Cabbage (B. oleracea var Capitata L.) is one of the most important and popular vegetable crops in the Brassicaceae family and is a plant-vernalization-responsive type (Ito et al., 1966; Friend, 1985). Cabbage normally requires 6 to 8 weeks of low temperature (5°C) to induce flower initiation at the stage of 7 to 9 leaves or when the stem diameter reaches 6 mm (Ito et al., 1966; Friend, 1985). It is of interest to understand the mechanisms involved in flowering in seed- versus plant-vernalization-responsive types. Hossain et al. tried to transfer the seed-vernalization character from Chinese cabbage (B. rapa) into cabbage via ovule culture (Hossain et al., 1990). The F2 progeny of this interspecies hybrid showed seed-vernalized characteristics. In our study, we cloned the FLC homologs from cabbage and characterized their expression in response to vernalization. Moreover, the cabbage FLC gene was introduced into Arabidopsis to investigate the different regulatory mechanisms involved in the seed- and plant-vernalization responses. Our results support a distinct machinery in regulating FLC expression being responsible for the different vernalization responses between seed- and plant-responsive types.

**RESULTS**

**Molecular Cloning and Characterization of Cabbage FLC Genes**

Cabbage, like Arabidopsis, is a vernalization-dependent plant in the family Brassicaceae. Since AtFLC was identified as a major flowering repressor in the vernalization pathway in Arabidopsis (Michaels and Amasino, 1999; Sheldon et al., 1999), an FLC homolog was hypothesized to exist in cabbage and function similarly. Primers were designed according to the C-terminal coding sequence of AtFLC without the MADS-box domain to amplify the cabbage FLC cDNA. An inbred cabbage line, TNSS42-12, was chosen for RNA isolation because of its high degree of vernalization requirement (S.-S. Wang, unpublished data). A partial cDNA of one FLC homolog was cloned from cabbage via reverse transcription-PCR. A full-length cDNA clone was subsequently isolated after screening a cabbage cDNA library constructed from young leaves with a probe generated from the partial cDNA sequence. This clone was designated as BoFLC4-1 (GenBank accession no. AY306122). With use of
genomic walking to isolate the BoFLC4-1 promoter, another FLC homolog was found. This second FLC gene was named BoFLC3-2 (GenBank accession no. AY306123). The nomenclatures of BoFLC4-1 and BoFLC3-2 were based on their deduced amino acid sequence similarity to BnFLCs from B. napus (Tadete et al., 2001). BoFLC4-1 and BoFLC3-2 share 88% and 83% identity in nucleotide and deduced amino acid sequences, respectively. The deduced amino acid sequences of BoFLC4-1 and BoFLC3-2 show 79% to 81% identity with AtFLC and up to 98% identity with the BnFLCs from B. napus. In addition, sequence comparison with the partial sequences of BrFLCs and BoFLCs from B. rapa and B. oleracea, respectively (Schranz et al., 2002), showed high similarity at the amino acid level. The phylogenetic relationship among them is illustrated in Figure 1. BoFLC4-1 is most similar to BrFLC4, whereas BoFLC3-2 has a closer relationship to BoFLC3, BrFLC3, and BnFLC3. The genomic sequences of both genes were subsequently obtained by PCR and genomic walking (GenBank accession nos. AY306124 for BoFLC4-1 and AY306125 for BoFLC3-2). The genomic organization of BoFLC4-1 and BoFLC3-2 is similar to that of AtFLC and the partial genomic sequences of BrFLCs and BoFLCs (Schranz et al., 2002). They all consist of seven exons in similar sizes. However, BoFLC4-1 and BoFLC3-2 have a relatively small intron 1 (approximately 1.1 kb) compared to AtFLC (approximately 3.5 kb; see Fig. 5 for details).

Repression of BoFLC Expression by Vernalization

To determine whether cabbage FLCs are involved in controlling flowering via the vernalization process, RNA gel-blot analysis was carried out to investigate the FLC expression pattern in response to vernalization. Eight-week-old cabbage plants from 3 different varieties, TNSS42-12, Yehsen, and YSL-0, were vernalized at 4°C for 2 to 6 weeks. Vernalization requirements for these three varieties are different. TNSS42-12 requires at least 45 d at 5°C and Yehsen needs about 60 d at 5°C, whereas YSL-0 can flower after the local winter season in the lowlands of Taiwan, where the average winter temperature is 17°C to 20°C from December to February (http://www.cwb.gov.tw/V4; S.-S. Wang, unpublished data). BoFLC transcripts were analyzed in different tissues (apex, young leaf, mature leaf, stem, and root) before and after cold treatment with use of a BoFLC4-1 cDNA probe lacking the MADS box (Fig. 2). In all three varieties, FLC transcripts were most abundant in the apex before cold treatment. The FLC transcript level was decreased mainly in the apex, young and mature leaves, and to a lesser extent in the stem and root during cold treatment. This observation is consistent with the decrease in Aflc expression in all tissues after vernalization in Arabidopsis (Sheldon et al., 2000). Nevertheless, the expression of FLC in the root and stem was not as strong as that in the apex and leaf. Low expression levels of BoFLC transcripts in the roots of Yehsen and YSL-0 and a lesser response in the roots of TNSS42-12 indicated that roots may not be involved in the vernalization process. This is in agreement with the previous observation in pie1 mutants, in which root expression of FLC did not affect flowering (Noh and Amasino, 2003).

The initial transcript levels of FLC in the apex, young leaf, and mature leaf before cold treatment are similar in these three varieties (Fig. 2D); however, the down-regulation of FLC in YSL-0 was more rapid than in TNSS42-12 and Yehsen after cold treatment. The FLC transcript level was significantly reduced in YSL-0 after 2-week treatment and barely detectable after 4-week treatment but remained substantial in the other 2 varieties during the same period of cold treatment (Fig. 2, A–C), which suggests that the repression of FLC in YSL-0 is more sensitive to cold temperature compared to the other 2 varieties. This observation may explain the shorter vernalization requirement for YSL-0 to flower.

Because in Arabidopsis FT is down-regulated by FLC (Samach et al., 2000), we examined the expression of the FT homolog in parallel with FLC expression in cabbage. A distinct band that cross-hybridized with the Arabidopsis FT probe was detected in cabbage tissues, and we refer to this putative FT homolog as...
The expression of BoFT showed an inverse expression pattern compared with that of FLC (Fig. 2A). This observation is in agreement with the down-regulation of FT by FLC in Arabidopsis (Samach et al., 2000). In contrast with the apex, expression of BoFT in young and mature leaves, stems, and roots did not increase during vernalization, even though the expression of BoFLC decreased, which suggests that other regulators participate in regulating the expression of BoFT in these tissues.

The expression of BoFLC4-1 in TNSS42-12 was monitored in different developmental stages by RNA gel-blot analysis (Fig. 3A). Since the apex was shown previously to have the highest expression of BoFLC4-1, apex tissue was harvested from 1- to 8-week-old cabbage plants for further examination. The entire aboveground tissue, including apex and cotyledon, was collected and indicated as "shoot" for the first week of sampling. Cotyledons were collected separately from the apex of 2-week-old seedlings but not at the 4- and 8-week-old stages since they were senescent. The expression of BoFLC4-1 was very weak in the shoot at the 1-week-old stage but significantly increased at the 2-week-old stage and had a dramatic boost at 4 to 8 weeks (Fig. 3A). Thus, FLC expression strongly increases with age. The FT homolog was highly expressed in the shoot of 1-week-old seedlings, whereas FLC expression was barely detectable at the same stage. However, in older plants, the expression of FT in the apex gradually decreased to an almost undetectable level at the 8-week-old stage, whereas the expression of FLC increased. This result again supports the down-regulation of FT by FLC (Samach et al., 2000).

Previous studies had shown that FLC expression was down-regulated and flowering promoted after vernalizing Arabidopsis seeds (Michaels and Amasino, 1999; Sheldon et al., 2000). Unlike Arabidopsis, in cabbage seed vernalization is not able to induce flowering. Vernalization is effective only when cold treatment is applied to mature cabbage plants (Ito et al., 1966; Friend, 1985). As shown in Figure 2, a decrease in expression of BoFLC was observed in vernalized 8-week-old cabbage plants. However, BoFLC does not respond to seed vernalization: cold treatment of cabbage seeds (4°C for 4 weeks) did not alter the expression pattern of FLC or FT (compare Fig. 3, A and B). This observation is in agreement with seed vernalization not being effective in promoting flowering in cabbage, which is in contrast with the down-regulation of AtFLC in Arabidopsis when seeds are vernalized.

To determine whether the change of signal intensity shown in these RNA gel blots was attributable to
BoFLC4-1 or BoFLC3-2, a 3′-untranslated region (UTR) specific sequence of BoFLC3-2 was used as a probe for hybridization. No BoFLC3-2 specific signal was detected in all tissues examined (data not shown). This result indicated that the expression level of BoFLC3-2 was very low, and signals observed in the RNA gel-blot analysis were mainly derived from BoFLC4-1, although we cannot exclude cross-hybridization to other unidentified BoFLCs. DNA gel-blot analysis revealed that both BoFLC4-1 and BoFLC3-2 exist as single-copy genes in cabbage (data not shown). Nevertheless, more cabbage FLC genes are likely to exist based on the pattern of multiple bands from low stringency hybridization. Previously, Schranz et al. cloned three FLC genes (BoFLC1, BoFLC3, and BoFLC5) from a doubled haploid line of B. oleracea (Schranz et al., 2002; Fig. 1), which supports the existence of at least one additional member of the FLC gene family in cabbage.

**Figure 3.** RNA gel-blot analysis of BoFLC4-1 and BoFT in response to different developmental stages and seed vernalization. Cabbage seeds were subjected without (A) or with (B) seed vernalization treatment. Seed vernalization was carried out at 4°C for 4 weeks. Various tissues were harvested at different stages from 1- to 8-week-old cabbage plants. The methylene blue staining of 25S rRNA in each blot is shown as a loading control.

**Comparison of Promoter and Intron 1 Sequences of BoFLCs and AtFLC**

To further study the regulation of BoFLC gene expression in response to vernalization, the promoters of both BoFLC4-1 and BoFLC3-2 were isolated by genome walking. Genomic fragments 2.0 kb and 1.5 kb upstream from the translational start sites of BoFLC4-1 and BoFLC3-2, respectively, were cloned. Comparing promoter sequences to that of AtFLC identified three conserved regions (Fig. 5A). The first conserved sequence (P1) includes the 5′ UTR and a sequence up to −200 bp upstream from the translational start site. The second conserved sequence (P2), consisting of 51 bp, was found at −219 to −269 bp of AtFLC. The third conserved sequence (P3) was located at about −2.4 kb and −2.0 kb of AtFLC and BoFLC4-1.
respectively, but at about -0.4 kb upstream of BoFLC3-2 from the translational start site. A partial open reading frame encoding a 3-keto-acyl-ACP dehydratase was identified in this 1.5-kb upstream sequence of the BoFLC3-2 gene (Fig. 5A), which indicates that the promoter of BoFLC3-2 could be truncated and this may account for the lack of detectable BoFLC3-2 mRNA.

As mentioned earlier, the introns 1 of BoFLC4-1 and BoFLC3-2 (approximately 1.1 kb) are relatively smaller than that of AtFLC (approximately 3.5 kb), and the intron 1 sequence of AtFLC was identified to be important in the epigenetic repression of AtFLC through chromatin modification (Sheldon et al., 2002; He et al., 2003; Bastow et al., 2004; Sung and Amasino, 2004). Thus, the intron 1 sequences of cabbage and Arabidopsis were compared. The sequence comparison revealed that several segments in the AtFLC intron 1 could be aligned with the sequences in the BoFLC4-1 intron 1 (I1–I6 in Fig. 5B). An approximately 50-bp sequence, indicated by an arrow in the conserved segment of I3 (Fig. 5B), was found to be organized as an imperfect direct repeat in the intron 1 of BoFLC4-1. Intriguingly, a continuous 2.6-kb segment of the AtFLC intron 1 could not identify any homologous sequence in the BoFLC4-1 intron 1 (Fig. 5B). A similar result was obtained after comparing the intron 1 sequences of BoFLC3-2 and AtFLC, except there was no 50-bp repeat in the BoFLC3-2 intron 1. Thus, size differences between introns 1 of AtFLC and BoFLC is mainly due to an additional 2.6-kb segment close to the 3' end of the AtFLC intron 1.

Vernalization Responses of BoFLC4-1 in Transgenic Arabidopsis

Since the promoter and intragenic sequences were reported to be crucial for the regulation of AtFLC in response to vernalization (Sheldon et al., 2002), the complete genomic sequence of BoFLC4-1, including the 2-kb upstream promoter and complete sequences of 7 exons and 6 introns, was fused to the β-glucuronidase (GUS) reporter gene (pBoFLC4-1::BoFLC4-1::GUS) and transformed into Arabidopsis to examine its expression pattern and vernalization responses in Arabidopsis. GUS staining was observed in the apex, young leaves, and roots of transgenic Arabidopsis seedlings (Fig. 6A). Interestingly, pollen grains and germinating pollen showed strong GUS staining (Fig. 6, B–D).

The transgenic T2 lines were examined for the response to seed or plant vernalization. Seeds or 10-d-old rosette plants were treated at 4°C for 2, 4, 6, and 8 weeks. Vernalized seedlings were returned to the normal growth temperature for another 10 d before harvest. Vernalized rosette plants were harvested immediately after treatment. Total RNAs from seedlings without vernalization or with seed-vernalization (Fig. 7A) or plant-vernalization treatment (Fig. 7B) were isolated, and the transcript levels of the endogenous AtFLC and BoFLC4-1::GUS transgenes were inspected.

Upon seed vernalization, the expression of endogenous AtFLC deceased significantly and was not detectable after a 4-week cold treatment (Fig. 7A). Endogenous AtFLC in plant-vernalized seedlings were

![Figure 5. Sequence comparison of AtFLC, BoFLC4-1, and BoFLC3-2 sequences. A, Three conserved segments (P1, P2, and P3) were identified among the 5' upstream sequences of three FLC genes. The organization and size (bp) of exon and intron (shown as a solid triangle) are shown. The arrow indicates the translational start site. The arrow bar shown in the upstream sequence of BoFLC3-2 indicates an additional open reading frame encoding a 3-keto-acyl-ACP dehydratase. B, Comparison of intron 1 sequence between BoFLC4-1 and AtFLC revealed several conserved regions: I1, I2, I3, I4, I5, and I6. The arrow bar in the I3 region represents a direct repeat (see text for detail). Lines a and b in A and lines c and d in B indicate the regions previously identified to be associated with chromatin modification in the epigenetic repression of FLC by vernalization in Arabidopsis.](image-url)
also down-regulated during cold treatment. Thus, both seed- and plant-vernalization treatments were effective, although the repression during plant vernalization was not as complete as that during seed vernalization (Fig. 7B). However, the down-regulation of the BoFLC4-1::GUS transcript by seed vernalization showed heterogeneity among the 11 transgenic T2 lines examined. Three groups of T2 lines were classified, and one independent line from each group was selected for representation in Figure 7. In the 4 independent lines in group 1, the transcript levels of BoFLC4-1::GUS did not change significantly during seed vernalization (line 1 in Fig. 7A). In the 3 independent lines of group 2, transcripts of BoFLC4-1::GUS were undetectable after 4- and 6-week treatments but reappeared after 8-week treatment of seed vernalization (line 2 in Fig. 7A). In the remaining 4 independent lines in group 3, transcripts of BoFLC4-1::GUS were undetectable at the 4- or 6-week time points, but the expression of BoFLC4-1::GUS was restored after that (line 3 in Fig. 7A). The heterogeneity among several transgenic lines and fluctuation of the BoFLC4-1::GUS transcript during vernalization indicate that the suppression of BoFLC4-1::GUS was incomplete and unstable when transgenic Arabidopsis seeds were vernalized. By contrast, none of transgenic lines exhibited reduced BoFLC::GUS expression after plant-vernalization treatment, even though the level of endogenous AflC transcript was gradually reduced during the treatment (Fig. 7B). The instability of repression by seed vernalization and lack of repression of BoFLC::GUS after plant vernalization in transgenic Arabidopsis demonstrate that the repression of BoFLC4-1 by vernalization cannot be properly regulated in Arabidopsis. This result suggests that the regulatory machinery controlling FLC expression by vernalization in cabbage is different from that in Arabidopsis.

DISCUSSION

In this study, two FLC homologs, BoFLC4-1 and BoFLC3-2, were isolated from cabbage (Fig. 1). The expression of BoFLC4-1 and its regulation by vernalization were analyzed. The FLC transcripts were highest in the apex, as was observed in Arabidopsis (Michaels and Amasino, 1999). Steady-state levels of the BoFLC transcript decreased during vernalization of cabbage plants, mainly in the apex and leaf (Fig. 2, A–C). It is of interest to note that the three cabbage varieties (TNSS42-12, Yehsen, and YSL-0) with different vernalization requirements showed similar transcript levels of FLC before vernalization (Fig. 2D) but a different degree of reduction of FLC expression after vernalization (Fig. 2, A–C). Suppression of FLC in YSL-0, a short-vernalsation requirement variety, occurred much more rapidly than that of the other two varieties, which require longer exposure to cold.

FT and SOC1 are recognized as key integrators of multiple floral pathways in Arabidopsis (Simpson and Dean, 2002). FT and SOC1 expression is repressed by FLC (Lee et al., 2000; Onouchi et al., 2000; Samach et al., 2000). When Arabidopsis FT and SOC1 cDNAs were used as probes to search for homologous genes in cabbage, the SOC1 homolog was not detected in cabbage tissues tested, but an FT homolog was detected. The reduction of BoFLC expression by vernalization corresponded to the increased level of BoFT in the apex (Fig. 2A). Moreover, BoFT also showed an expression pattern opposite to that of FLC in the apex from different developmental stages of nonvernalized cabbage (Fig. 3). This observation implies that Arabidopsis and cabbage may regulate flowering similarly in terms of the correlation between FT and FLC. Nevertheless, BoFT was strongly expressed in both nonvernalized young and mature leaves, and the expression of BoFT was not up-regulated in leaves after vernalization, even though the expression of FLC was already down-regulated (Fig. 2A). This observation suggests that the expression of BoFT in the leaf is not sufficient to promote flowering, and the increase in BoFT expression in the apex during vernalization is a prerequisite for the induction of flowering in cabbage. This observation is in conflict with the recent study that misexpression of FT in a wide range of
tissues activates flowering in Arabidopsis (An et al., 2004). Decreased FLC expression accompanies increased expression of FT in the apex, which suggests that the apex is the main location mediating flowering by vernalization in cabbage. This suggestion is in agreement with early observations that the apex is sensitive to low temperatures and must be exposed to cold for vernalization to occur (Chourad, 1960; Ito et al., 1966).

MADS-box proteins are thought to form dimers through the interaction of the K domain at the center and bind to DNA through the MADS-box domain at the N terminus (Schwarz-Sommer et al., 1992; Mizukami et al., 1996; Riechmann et al., 1996). Recent evidence indicates that certain MADS-box proteins may associate as tetramers (Favaro et al., 2003). ag and ap3 mutant phenotypes were generated by transforming the wild-type Arabidopsis with mutant forms of AG or AP3 lacking the C- and N-terminal regions, respectively (Mizukami et al., 1996; Krizek et al., 1999). Moreover, a dominant-negative mutation was successfully created using heterologous AP3 genes from two plant species (Tzeng and Yang, 2001). Here, we demonstrate another example of generating a dominant-negative mutation by use of genes from different plant species. Both DN1 (lack of MADS box) and DN2 (lack of C domain) constructs (Fig. 4) retain the K domain presumably involved in protein-protein interaction. Transgenic Arabidopsis overexpressing either N-terminal truncated (DN1) or C-terminal truncated (DN2) BoFLC4-1 flowered earlier than wild-type plants (Fig. 4). The early flowering phenotype could result from the formation of a nonfunctional multimer between the truncated BoFLC4-1 and endogenous AtFLC proteins. Overexpression of the truncated FLC protein through a dominant-negative effect may provide an alternative approach to regulating flowering time in vernalization-dependent plant species.

Comparison of promoter sequences upstream of BoFLC4-1, BoFLC3-2, and AtFLC revealed three conserved regions (P1, P2, and P3; Fig. 5A). The alignment of these conserved regions suggests that a large sequence deletion between the P2 and P3 regions occurred in the promoter of BoFLC3-2. The truncated promoter of BoFLC3-2 may explain its low level of transcripts in cabbage. Positive and negative regulatory elements between the P2 and P3 regions were identified in the promoter of AtFLC (Sheldon et al., 2002). Whether there are any cis-acting elements located between the P2 and P3 regions that are responsible for the expression of BoFLC3-2 remains to be studied. The P2 conserved region (51 bp) is located within the 75-bp promoter sequence of AtFLC that was reported to be essential for AtFLC expression in nonvernalized plants (Sheldon et al., 2002). About 500 bp upstream to the P3 region of BoFLC3-2, a gene encoding 3-keto-acyl-ACP dehydratase was identified (Fig. 5A). Coincidently, this gene can be aligned with a similar gene (At5g10160.1) that is one locus away from the AtFLC (At5g10140.1) in the Arabidopsis genome. This suggests that this genomic organization had been preserved between cabbage and Arabidopsis during evolution.

Cabbage flowering can be promoted by vernalizing adult plants but not seeds (Friend, 1985). Cabbage must pass through the juvenile phase to be able to sense cold and induce flowering. We showed that vernalization of 8-week-old cabbage plants was effective in repressing the expression of BoFLC4-1 (Fig. 2); however, cold treatment of cabbage seeds did not alter the expression level of BoFLC4-1 (Fig. 3). The regulation of BoFLC4-1 expression by vernalization of adult cabbage plants but not seeds is correlated with the
effectiveness of vernalization. This situation is different from that in Arabidopsis in which FLC is downregulated and flowering is promoted by seed vernalization, which suggests that the regulatory mechanism of FLC expression in plant-vernialization-responsive species is different from that in seed-vernialization-responsive species. To further characterize the differences in vernalization responses between the 2 species types, the whole genomic sequence of BoFLC4-1 containing the 2-kb promoter and 7 exons was fused to the GUS reporter gene (pBoFLC4-1::BoFLC4-1::GUS) to examine its localization and vernalization response in Arabidopsis. It is of interest to note that GUS staining was strongly detected in pollen grains and germinating pollen of the transgenic plants (Fig. 6, C and D). Indeed, the BoFLC4-1 transcript also was detected in cabbage flowers, and transgenic cabbage overexpressing a double-stranded RNAi construct of BoFLC4-1 produced very few seeds (J.-G. Wang, S.-Y. Poon, S.-S. Wang, and T.-J. Chiou, unpublished data). Moreover, transgenic Arabidopsis expressing an AtFLC antisense construct showed reduced fertility (Sheldon et al., 1999), and overexpressed AtFLC was defective in floral morphology and produced less than normal quantities of pollen or no pollen (Hepworth et al., 2002). These results indicate that FLC may participate in pollen development or pollination in addition to controlling flowering time. However, the observation of normal seed setting in flc null mutants (Michaels and Amasino, 2001) disagrees this hypothesis. Whether the difference between transgenic manipulation and mutation in endogenous gene gives rise to this contradiction remains to be resolved.

When transgenic Arabidopsis seeds were subjected to vernalization, the BoFLC4-1::GUS transcript did not show significant changes in some of the transgenic lines (e.g. line 1 in Fig. 7A), but its transcript was undetectable at one or two time points of the treatment in other lines (e.g. lines 2 and 3 in Fig. 7A). However, the down-regulation of the BoFLC4-1::GUS transcript was not sustained after prolonged cold treatment, which indicated that down-regulation was not stable. This observation is inconsistent with the quantitative relationship between the duration of cold treatment and the extent of down-regulation of AtFLC, as observed in Figure 7 and in a previous report (Sheldon et al., 2000). Because a 2-kb promoter region and complete exon and intron sequences were included in the transgene construct, it is not likely that critical sequences for vernalization responses were left out of this construct. Nevertheless, we cannot rule out that the BoFLC4-1::GUS construct would not be properly regulated in cabbage.

The mechanism involved in inactivation of AtFLC during vernalization recently was demonstrated to be associated with chromatin modification, such as histone deacetylation and methylation (Bastow et al., 2004; Sung and Amasino, 2004). Because chromatin immunoprecipitation with a specific antibody recognized the acetylated or methylated histone H3, several regions in the AtFLC gene associated with the modified histone H3 were identified. Those sequences were highlighted and designated as the four regions, a, b, c, and d, in Figure 5, A and B. Regions a and b are located in the promoter, while regions c and d are located in intron 1. The sequence of region a was previously identified to

Figure 8. A working hypothesis of the vernalization responses in cabbage (A) and Arabidopsis (B). The solid-bold and dashed lines indicate vernalization of plants and seeds, respectively. The triangle indicates the increase or decrease of FLC or FT expression after vernalization. See text for description.
consist of a negative regulatory element of AtFLC (Sheldon et al., 2002). Region b is across the conserved segments of P1 and P2. Region c overlaps with the conserved segments I1 and I2 in intron 1, and region d is located within the 2.6-kb region that is missing in the BoFLC intron 1. In BoFLC4-1, similar b and c sequences were identified, but no homologous sequences of a and d were found (Fig. 5). We suspect that lack of counterpart sequences to these regions a and d in BoFLC4-1 may cause the unstable repression of BoFLC4-1::GUS after seed vernalization in transgenic Arabidopsis; however, we do not exclude the possibility that other unidentified cis-regulatory elements in AtFLC, which may not be present in BoFLC4-1, are also important for responding to seed vernalization in Arabidopsis.

The expression of BoFLC4-1::GUS was not inactivated when transgenic rosette plants were subjected to vernalization treatment. We postulate that certain cabbage-specific trans-regulatory factors required for the suppression of BoFLC4-1 during cabbage vernalization do not exist in Arabidopsis. The different responses in terms of seed or plant vernalization are likely due to the distinct interactions between specific cis- and trans-regulatory elements in these two different vernalization-responsive types. Nevertheless, it is also possible that the homologs of upstream regulators of AtFLC such as VIN3 (Sung and Amasino, 2004) may be involved in the differential responses of seed versus plant vernalization in cabbage. For example, a cabbage VIN3 homolog may only be induced during vernalization of mature cabbage plants but not in vernalized seedlings to down-regulate the expression of FLC. However, the interaction between FLC and its upstream regulators in Arabidopsis and cabbage may not be identical since the expression of BoFLC4-1 in transgenic Arabidopsis was not suppressed when endogenous AtFLC was completely inactivated (Fig. 7A). It was surprising to find that the BoFLC4-1::GUS transcript was increased after plant vernalization treatment in most of the transgenic lines (Fig. 7B), but we have no explanation for this observation.

In Figure 8, a working hypothesis is proposed for the different mechanisms in controlling cabbage and Arabidopsis flowering by vernalization. The BoFLC transcript gradually accumulates along with a decreased level of BoFT in the apex during cabbage growth. Vernalization of cabbage seeds does not alter the expression of FLC and FT. The presence of vernalization repressors or the absence of vernalization activators could be responsible for the ineffectiveness of seed vernalization in cabbage. The expression of repressors or activators may depend on age and be regulated coordinately in an opposite manner. During development, the loss of repressors or accumulation of activators may be associated with increased competence to sense cold temperature. The accumulated vernalization activators, together with cold temperature, in mature cabbage plants can efficiently down-regulate the expression of FLC (Fig. 8A). Without vernalization, cabbage remains in vegetative growth. The expression of FT shows an opposite pattern to that of FLC, and increased FT expression in the apex after plant vernalization may be critical to induce cabbage flowering (Fig. 2A). However, seed vernalization is more effective than plant vernalization in down-regulating FLC expression in Arabidopsis (Fig. 7). This observation is consistent with a previous report that cold treatment at the rosette stage is less effective in promoting flowering than seed cold treatment in Arabidopsis (Nordborg and Bergelson, 1999). We hypothesized that fewer vernalization activators or the presence of vernalization repressors in the rosette stage may reduce the competence of the vernalization response and result in later flowering (Fig. 8B). Nevertheless, vernalized Arabidopsis rosette plants show accelerated flowering compared to non-vernalized plants.

In summary, we show that cabbage BoFLC4-1 plays a similar role to Arabidopsis FLC in controlling flowering. However, AtFLC responds to seed vernalization but BoFLC4-1 does not. Comparison of promoter and intragenic sequences between BoFLC4-1 and AtFLC revealed differences that may be specifically involved in both the seed or plant vernalization response. Further characterization of these sequences may provide an opportunity to dissect different regulatory mechanisms of the vernalization response in seed versus plant-responsive species.

**MATERIALS AND METHODS**

**Plant Material and Treatment**

Cabbage (Brassica oleracea var. Capitata L.) was grown in growth chambers with a 16-h photoperiod (with cool fluorescence light at 150-200 μE m⁻² s⁻¹) and a 24/20°C light/dark temperature cycle. Three cabbage varieties, TNSS42-1 and YSL-0, two inbred lines, and Yehsen, an open-pollinated local variety, provided by the Tainan District Agricultural Research and Extension Station, were used for vernalization treatment (Wang et al., 2000). Arabidopsis (Arabidopsis thaliana) ecotype C24, purchased from Lehle Seeds (Round Rock, TX), was used for all experiments. Seed vernalization of Arabidopsis was carried out by transferring 8-week-old plants to 4°C for 2 to 8 weeks, as described by the research group in the Tainan District Agricultural Research and Extension Station (Wang et al., 2000). Arabidopsis (Arabidopsis thaliana) ecotype C24, purchased from Lehle Seeds (Round Rock, TX), was used for all experiments. Seed vernalization of Arabidopsis was carried out by transferring 8-week-old plants to 4°C for 2 to 8 weeks, as described by the research group in the Tainan District Agricultural Research and Extension Station (Wang et al., 2000).

**Cloning of BoFLC4-1 Genes and Sequence Analysis**

The cDNA of BoFLC4-1, lacking the MADS-box domain, was cloned by reverse transcription-PCR using primers designed from the AtFLC sequence (forward primer 5'-gggataacagctgcattc-3' and reverse primer 5'-taaacaagtctgctcattc-3'). The full-length BoFLC4-1 gene was subsequently cloned after screening a cabbage cDNA library constructed by the SMART cDNA library construction kit (CLONTECH, Palo Alto, CA). PCR and genomic walking (GenomeWalker kit; CLONTECH) were carried out to obtain the genomic and cDNA sequences of BoFLC4-1 and BoFLC3-2 genes. The promoters of both BoFLC4-1 and BoFLC3-2 also were cloned with use of the same genomic walking approach. The phylogenetic relationship of the partial amino acid sequences of BoFLC3-2 and BoFLC4-1 and other FLC proteins from
RNA Extraction and RNA Gel-Blot Analysis

Total RNA was isolated from various tissues using TRIzol reagent (Invitrogen, Carlsbad, CA). An amount of 15 μg of total RNA was run on a 2.0-kb formaldehyde-agarose gel and blotted onto a nylon membrane (Roche, Mannheim, Germany). The membrane was stained with reversible methylene blue (Sigma B-1177; St. Louis) to check the loading and transfer of RNA in each sample. BoFLC and BoFLC-1 cDNA, lacking the MADS-box, and AtFT cDNA (AV563203, a EST clone from Kazusa DNA Research Institute) were used to probe the samples. The signal was analyzed 5 min at room temperature, and in 0.1× SSC, 0.1% [w/v] sodium lauroyl sarcosine, 0.02% [w/v] SDS, 2% [w/v] blocking reagent overnight, and then washed in 2× SSC/0.1% (w/v) SDS twice for 15 min at room temperature, and in 0.1× SSC/0.1% (w/v) SDS twice for 15 min at 68°C. Detection was performed by use of the Detection Starter Kit II, following the manufacturer’s instructions (Roche). The signal was analyzed with use of a luminescent image analyzer (LAS-1000 plus; Fujifilm, Tokyo) combined with Image Gauge version 3.12 software (Fujifilm).

Construction of Transgene and Arabidopsis Transformation

The sequence containing the 2.0-kb promoter and complete 5’ UTR, exons, and introns of BoFLC-1 (5.5 kb in total) was placed upstream of the GUS reporter gene in the pCambia 1381Z vector (Cambia, Canberra, Australia). The stop codon of BoFLC-1 was deleted and immediately fused to the start codon of the GUS gene in the vector. A dominant-negative clone was generated by overexpressing N-terminal or C-terminal truncated BoFLC-1 in the pCambia 2300 (Camiba) driven by a cauliflower mosaic virus 35S promoter. The N-terminal truncated protein lacked the N-terminal MADS-box domain of 59 amino acids (DN1), whereas the C-terminal truncated protein lacked the C domain of 49 amino acids (DN2). These constructs were sequence verified to rule out any mistakes during cloning. They were subsequently transformed into Agrobacterium tumefaciens GV3101pMP90. Arabidopsis transformation was carried out by use of the floral dip method (Clough and Bent, 1998).

GUS Staining

GUS staining was performed according to Jefferson et al. (1987), with minor modifications. Briefly, transgenic plants were incubated in 100 mM NaPO₄, pH 7.0, 0.5 mM K-ferricyanide, 0.5 mM K-ferrocyanide, and 1.9 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide). Plant tissues were vacuum infiltrated briefly, then incubated at 37°C overnight. After staining, chlorophyll was cleared from the sample by 75% ethanol. Photos were taken under an Olympus SZX12 microscope (Tokyo).

ACKNOWLEDGMENTS

We thank Drs. Kenrick Jaichard and Ning-Sun Yang and Miss Laura Heraty for critically reading the manuscript, Dr. Pei-Ing Hwang and Miss Ho-Ming Chen for sequence analysis, and Dr. Yee-Yung Charing for valuable discussion during the process of this study.

Received December 20, 2004; returned for revision December 27, 2004; accepted December 27, 2004.

LITERATURE CITED


Koornneef M, Blanketijn-de Vries H, Hanhart C, Soppe W, Peeters T (1994) The phenotype of some late-flowering mutants is enhanced by a locus on chromosome 5 that is not effective in the Landsberg erecta wild-type. Plant J 6: 911–919


Michaels SD, Amasino RM (1999) FLOWERING LOCUS C encodes a novel
MADS domain protein that acts as a repressor of flowering. Plant Cell 11: 949–956


