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Tuning growth cycles of *Brassica* crops via natural antisense transcripts of *BrFLC*

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Summary

Several oilseed and vegetable crops of *Brassica* are biennials that require a prolonged winter cold for flowering, a process called vernalization. *FLOWERING LOCUS C* (*FLC*) is a central repressor of flowering. Here, we report that the overexpression of natural antisense transcripts (NATs) of *Brassica rapa FLC* (*BrFLC*) greatly shortens plant growth cycles. In rapid-, medium- and slow-cycling crop types, there are four copies of the *BrFLC* genes, which show extensive variation in sequences and expression levels. In Bre, a biennial crop type that requires vernalization, five NATs derived from the *BrFLC2* locus are rapidly induced under cold conditions, while all four *BrFLC* genes are gradually down-regulated. The transgenic Bre lines overexpressing a long NAT of *BrFLC2* do not require vernalization, resulting in a gradient of shortened growth cycles. Among them, a subset of lines both flower and set seeds as early as Yellow sarson, an annual crop type in which all four *BrFLC* genes have non-sense mutations and are nonfunctional in flowering repression. Our results demonstrate that the growth cycles of biennial crops of *Brassica* can be altered by changing the expression levels of *BrFLC2* NATs. Thus, *BrFLC2* NATs and their transgenic lines are useful for the genetic manipulation of crop growth cycles.

Introduction

Brassica rapa subspecies include several oilseed and vegetable crops. *Brassica napus*, with its 19 chromosomes, originated ~7500 years ago as the result of a hybridization between *Brassica oleracea* and *B. rapa* (Chalhoub *et al.*, 2014). *Brassica juncea* originated from a hybridization between *Brassica nigra* and *B. rapa* (Nagaharu, 1935). Many *Brassica* species have been cultivated since prehistoric times for their seeds, edible roots, stems, leaves, buds and flowers. Many of these crops require a prolonged winter cold for flowering through a process called vernalization (Osborn *et al.*, 1997). For crops with higher seed yields (such as oilseed rape), the earliness of flowering is an important trait. For crops with high leafy head yields (such as Chinese cabbage), lateness of flowering is a favourable trait (Mao *et al.*, 2014; Wang *et al.*, 2014).

FLOWERING LOCUS C (*FLC*) is a central repressor of flowering. It encodes a MADS-box transcription factor that functions as a repressor of the floral transition in *Arabidopsis* (Michaels and Amasino, 1999; Sheldon *et al.*, 2000). Plants with high FLC activity are late-flowering because FLC directly represses the expression of the floral inducers FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1) (Helliwell *et al.*, 2006). Four copies of *FLC* have been reported in *B. rapa*, and five copies were identified in *B. napus* (Lin *et al.*, 2005; Kim *et al.*, 2006, 2007). A genetic–genomics approach revealed that *BrFLC2* is a major regulator of flowering time in *B. rapa* (Xiao *et al.*, 2013, 2014). The overexpression of *BrFLC* and *BnFLC* in *Arabidopsis* delayed flowering time from 4 weeks to more than 7 weeks (Kim *et al.*, 2007). However, the *FLC* cold-sensing mechanism is not clear in *B. rapa*, and the genetic manipulation of vernalization traits in crops has not been successful.

Recently, natural antisense transcripts (NATs) have been identified in the *FLC* locus. Among them, COOLAIR (COLD-INDUCED LONG ANTISENSE INTERGENIC RNA) plays an important role in the epigenetic silencing of *FLC* in *Arabidopsis* (Swiezewski *et al.*, 2009; Liu *et al.*, 2010). COOLAIR is multi-exonic and poly-adenylated, and its transcription covers the entire genomic DNA region of the gene. The COOLAIR antisense transcripts originate from a promoter adjacent to the *FLC* 3′ untranslated region and consist of two classes that terminate at proximal or distal sites (Swiezewski *et al.*, 2007; Hornyik *et al.*, 2010). COOLAIR has been proposed to be involved in vernalization-mediated *FLC* repression and may be part of the cold-sensing mechanism (Swiezewski *et al.*, 2009; Liu *et al.*, 2010; Csorba *et al.*, 2014; Marquardt *et al.*, 2014). Another work explains that COOLAIR plays a redundant role in regulating *FLC* expression and is not required for the vernalization response in *Arabidopsis* (Helliwell *et al.*, 2011). Therefore, the underlying mechanism of NATs *in vivo* is still obscure. In addition to COOLAIR, another long intronic noncoding RNA COLD-ASSISTED INTRONIC NONCODING RNA (COLDAIR) is required for the vernalization-mediated epigenetic repression of *FLC* in *Arabidopsis* (Heo and Sung, 2011).

Here, we report that several NATs of *BrFLC2* in *B. rapa* were induced during vernalization, concurrent with a rapid reduction in the activities of *BrFLC* genes. These NATs inhibit the activity of *BrFLC* genes and thereby shorten the vernalization duration and accelerate flowering. We identified *B. rapa* transgenic plants overexpressing *BrFLC2as816*, a NAT of *BrFLC2*, and selected 12 transgenic lines, all of which show annual properties. These data suggest that *BrFLC* NATs are potentially useful in the genetic manipulation of vernalization traits and growth cycles in high yield crops.

Results

BrFLC genes are triplicated and vary with crop types

Brassica rapa genomes contain four *FLC* copies (Lin *et al.*, 2005; Kim *et al.*, 2006, 2007). Using the specific primers for each copy, we isolated cDNAs of *BrFLC1*, *BrFLC2*, *BrFLC3* and *BrFLC5* genes from the inbred lines of Yellow sarson (YS-143) (*B. rapa* var. *trilocularis*, rapid-cycling crop type), Bre (*B. rapa* ssp. *pekinensis* var. *bre*, medium-cycling crop type) and Wantai (*B. rapa* ssp. *pekinensis* var. *wantai*, slow-cycling crop type) (Figure 1a). Interestingly, putative BrFLC1, BrFLC2, BrFLC3 and BrFLC5 in Yellow sarson show premature stop codons after the 5th, 106th, 127th and 10th AA, respectively (Figure 1b). In Bre, putative BrFLC1, BrFLC2 and BrFLC3 share a high identity (>85%) in amino acid sequences with the BrFLC reported in Chiifu-401 (http://brassicadb.org/brad/) (Figure S1), and the putative BrFLC5 lacks an IKC domain. In Wantai, putative BrFLC1, BrFLC2 and BrFLC3 proteins have a high identity (>98%) with those in Chiifu-401. Similar to BrFLC5 in Bre, the BrFLC5 of Wantai also lacks an IKC domain (Figure 1b).

In Bre, the *BrFLC1* has three alternative transcripts, all of which encode truncated proteins without intact C-terminal fragments (Figures 1b and S1). In Wantai, *BrFLC1* encodes a full-length protein and no alternative transcript was detected (Figure 1b). To examine whether the truncated proteins are functional in flowering repression, we transferred the *BrFLC2* and *BrFLC3* of Yellow sarson, two longer transcripts of *BrFLC1* and *BrFLC2* of Bre, and *BrFLC1*, *BrFLC2* and *BrFLC3* of Wantai into *Arabidopsis* ecotype C24 under the control of CaMV 35S promoter (Figure S2). The overexpression of the *BrFLC2* in Bre and *BrFLC1*, *BrFLC2* and *BrFLC3* in Wantai delayed the flowering time, whereas the overexpression of *BrFLC2* and *BrFLC3* in Yellow sarson and two longer transcripts of *BrFLC1* in Bre failed to change the flowering time. This result revealed that the four *BrFLC* genes in Yellow sarson and the two transcripts of *BrFLC1* in Bre are nonfunctional.

We wondered whether *FLC* controls the growth cycle in a dose-dependent manner *in planta*. If *FLC* quantitatively controls the growth cycle, then it could be possible to manipulate the growth cycle and to accelerate seed set by silencing *FLC* using a genetic engineering technique in *B. rapa*.

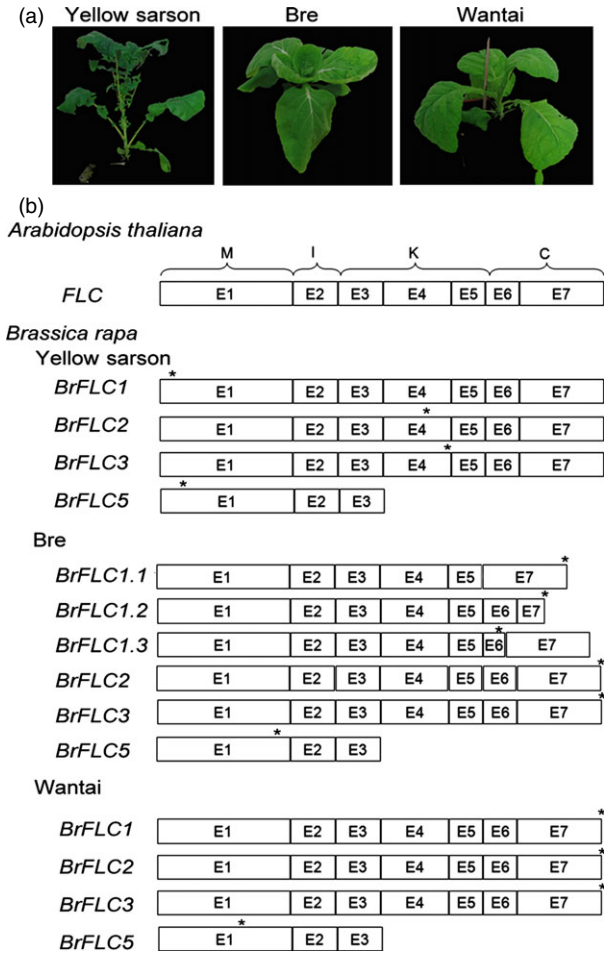


Figure 1 Members of *BrFLC* gene family in Yellow sarson (YS), Bre and Wantai. (a) Plant phenotypes of three crop types. (b) Four copies of *BrFLC* genes in YS, Bre and Wantai. *The stop codon. E, exon; M, MADS-box domain for DNA binding; I, the domain for dimerization; K, the domain for protein interaction; C, the domain for the diverse functions. Arabic numbers beneath the gene structures indicate the positions of primers used for real-time PCR.

Brassica rapa crops vary in vernalization requirements

The repression of *FLC* on bolting and flowering in *B. rapa* can be relieved by vernalization. To examine the time and duration of vernalization, we observed the bolting times of 121 varieties of *B. rapa* using 3-week-old seedlings under controlled conditions. The vernalization periods of these varieties varied with cold durations lasting from 0 to 11 weeks after germination. In the absence of the cold treatment, Yellow sarson took 110 days to set seeds, thus being a rapid-cycling cultivar without a vernalization requirement (Figure 2a,b). In the presence of the cold treatment, the genotype with the shortest cold duration threshold was Bre, while Wantai had the longest threshold. To define cold duration thresholds for vernalization, we incubated the 3-week-old plants of Bre and Wantai at 4 °C for various periods and then transferred them to the normal growth temperature (22 °C and 16 h light). A 20-day cold exposure period was enough to saturate the vernalization requirement of Bre and Bre plants started flowering on the 35th day of the following normal growth temperature period (Figure 2b). In contrast, 80 days of cold exposure were required for flowering in Wantai. The flowering time of Bre and Wantai was also affected by the normal growth temperature (22 °C) periods following the cold exposure. Bre plants that were exposed to 20 days of cold conditions did not flower unless they were grown for at least another 34 days at the normal growth temperature. Wantai plants that were exposed to 80 days of cold conditions required at least another 45 days at 22 °C for bolting. This result suggests that the vernalization duration in *B. rapa* varied in the two biennial crop types.

NATs of *BrFLC* in *B. rapa* are different from those of *Arabidopsis FLC*

COOLAIR plays an important role in epigenetic silencing of *FLC* in *Arabidopsis* (Swiezewski *et al.*, 2009; Liu *et al.*, 2010; Csorba *et al.*, 2014; Marquardt *et al.*, 2014). To examine NATs of *BrFLC* genes, *Arabidopsis* COOLAIR was aligned with the genomic sequence of four *BrFLC* genes in Bre. An 85-bp region of *BrFLC2* was homologous to a region of COOLAIR (Figure S3). To determine whether there were any NATs in *BrFLC2* intergenic

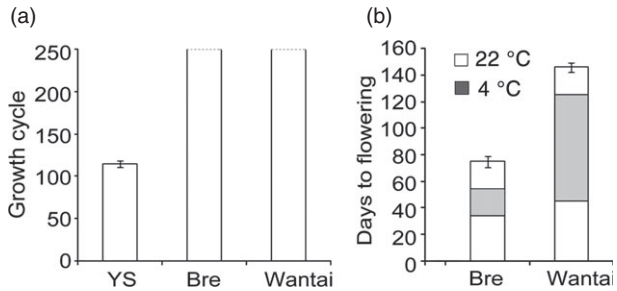


Figure 2 Growth cycles and flowering time of Yellow sarson (YS), Bre and Wantai. (a) Growth cycle of plants in the absence of cold treatment. The dashed lines indicate that the growth is not finished. (b) Flowering time of plants in the presence of cold treatment.

regions, we conducted reverse transcription with strand-specific primers. A primer downstream of the 85-bp intergenic region generated a transcript upon cold induction (Figure S4). To amplify the possible full-length NATs, extensive rapid amplification of cDNA ends (RACE)-PCR was performed using strand-specific primers. In total, five types of *BrFLC2* NATs were isolated (Figure 3a). Among them, three were cleaved and polyadenylated at the sequence antisense to the *BrFLC2* DNA located in intron 6, and two were cleaved and polyadenylated within the sequence antisense to the *BrFLC2* promoter. All five NATs were transcribed from the same sites in the intergenic regions antisense to DNA located 169 nt downstream of *BrFLC2*. Compared with the transcriptional start sites of *AtFLC* NATs, those of *BrFLC2* NATs were further away from the transcriptional stop sites of *BrFLC2*. According to polyadenylation sites, the three NATs polyadenylated within intron 6 were classified as class I NATs, while those polyadenylated within the promoter of *BrFLC2* were classified as class II NATs. The five NATs of *BrFLC2* were named as *BrFLC2as406*, *BrFLC2as599*, *BrFLC2as477*, *BrFLC2as816* and *BrFLC2as755* based on their lengths (Figure 3a). There was an overlapping fragment of 330 nt between the *BrFLC2* promoter and *BrFLC2as816*. Although extensive NAT-specific RT-PCR was applied, no NATs were isolated from *BrFLC1*, *BrFLC3* or *BrFLC5* genes, revealing that the NATs are specific to *BrFLC2*. As expected, the NATs of *BrFLC2* were conserved in crop types Bre, Yellow sarson and Wantai (Table S1).

In *Arabidopsis*, a long intronic noncoding RNA, COLDAIR, is required for the vernalization-mediated epigenetic repression of *FLC* (Heo and Sung, 2011). We wondered whether there was COLDAIR-like RNA in the *BrFLC* genes. The first introns of *BrFLC1*, *BrFLC2*, *BrFLC3* and *BrFLC5* were much shorter than those in *AtFLC* (3.5 kb) and did not contain any fragments homologous to *AtFLC* COLDAIR (Figure S5). Using several pairs of strand-specific primers, we performed tiling RT-PCR and RACE-PCR to detect COLDAIR homologs in the first introns of the four *BrFLC* genes, but failed to identify any COLDAIR transcripts. Moreover, the RNA-seq data of Bre and Wantai (data not shown) did not show any transcripts derived from these introns. Thus, we excluded the possibility of a COLDAIR homolog existing in the *BrFLC* genes.

BrFLC genes are down-regulated, while *BrFLC2* NATs are up-regulated under cold conditions

To define the relationship between *BrFLC2* and the *BrFLC2as*, the abundance of relative transcripts was analysed using real-time

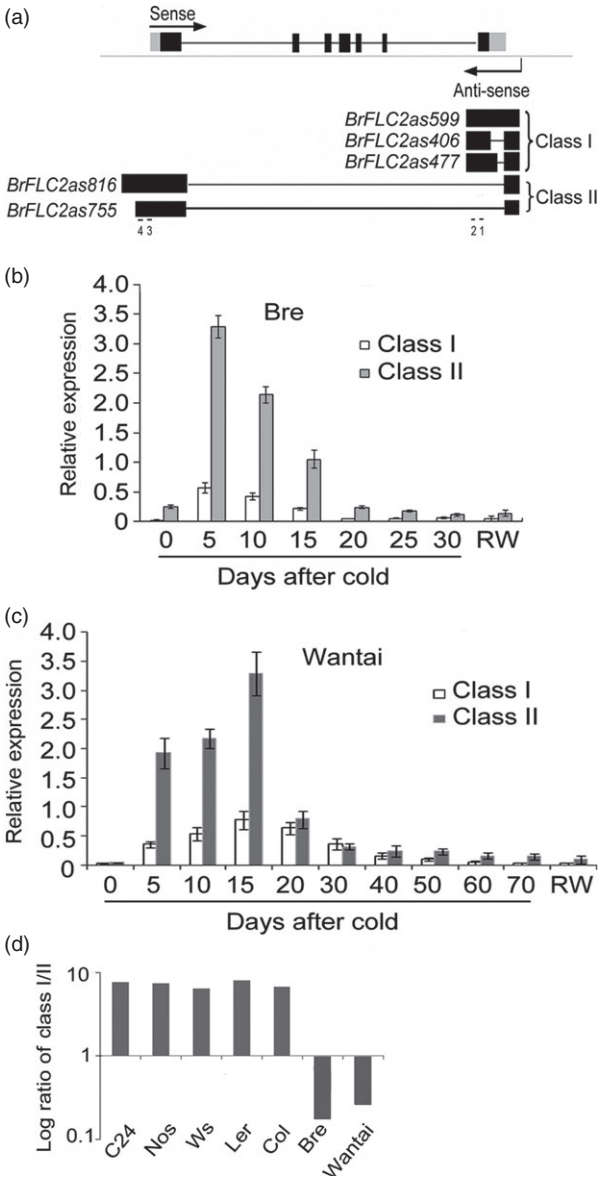


Figure 3 NATs (*BrFLC2as*) of *BrFLC* genes. (a) Schematical structures. Arrow heads indicate direction of transcription. Black boxes indicate exons, while grey boxes show the untranslated regions. (b) and (c) Relative expression levels of class I and class II NATs in the seedling leaves of Bre (b) and Wantai (c). (d) the COOLAIR ratios (Log) of class I to class II in *Arabidopsis thaliana* and *B. rapa*. Primers 1 and 2 were used to amplify class I NATs, while primers 3 and 4 to amplify class II NATs. RW, return to warm.

PCR. In Bre seedling leaves without a cold treatment, the transcripts of class I NATs were not detectable, while class II NATs were few (Figure 3b); after cold treatment, the transcript levels of class I and class II NATs sharply increased, reached the peaks at day 5 and then gradually declined. In Wantai, transcript levels of class I and II NATs were not detectable before cold treatment and were induced under cold conditions; however, these NATs under cold conditions were much lower than in Bre and reached the peaks much later at day 15 (Figure 3c). In both crop types, class I *BrFLC2as* transcript levels were much lower than class II *BrFLC2as* transcript levels during vernalization, which

is opposite in *Arabidopsis* (Figure 3d). This result indicates that *BrFLC2* NATs are cold-induced in two crop types, but their peaks and durations of expression are different.

In Bre leaves, *BrFLC2* expression was strong prior to cold treatment; however, *BrFLC3* expression was hardly detected (Figure 4a) and *BrFLC5* was not expressed, confirming that *BrFLC5* was a pseudogene (data not shown). In Wantai, *BrFLC2* expression prior to cold treatment was weaker than in Bre, but *BrFLC1* and *BrFLC3* expression levels were much stronger than in Bre (Figure 4b). During vernalization, the expression levels of *BrFLC1*, *BrFLC2* and *BrFLC3* in Bre and Wantai decreased progressively with time from 5 to 25 days, and the rate of decrease in Wantai was lower than in Bre (Figure 4a,b). This result reveals that the expression patterns of *BrFLC1*, *BrFLC2* and *BrFLC3* prior to and during cold treatment are quite different in Bre and Wantai.

We determined the BrFLC proteins in Bre leaves using Western blotting with a BrFLC antibody. During the course of vernalization, the total BrFLC protein content at day 5 after cold treatment was almost the same as before cold treatment, in contrast to the transcripts of *BrFLC2*, which decreased markedly. Then, the protein content decreased rapidly with time and eventually disappeared at day 20 (Figure 4c), concomitant with a decrease in *BrFLC* transcripts and an increase in *BrFLC2* NATs. This fact indicates that there was a time lapse between the decline in BrFLC proteins and the decrease in *BrFLC* transcripts at the cold treatment's early stages. After 5 days, however, the production and content of BrFLC proteins in plants of *B. rapa* under cold conditions were highly dependent on the expression of *BrFLC* genes. That is, both the transcriptional and translational products of the *BrFLC* genes are reduced.

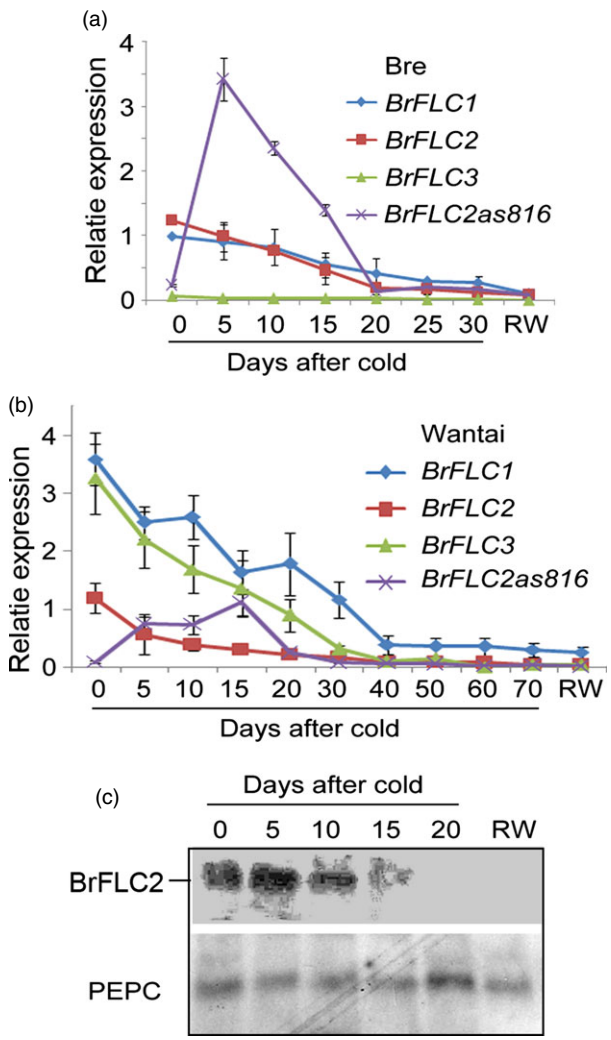


Figure 4 Relative expression and protein contents of *BrFLC* genes and *BrFLC2as816*. (a) Relative expression level of *BrFLC* genes and *BrFLC2as816* in the seedling leaves of Bre. (b) Relative expression levels of *BrFLC* and *BrFLC2as816* in the seedling leaves of Wantai. (c) BrFLC2 proteins detected by Western blotting in the seedling leaves of Bre after cold treatment. PEPC is the loading control. RW, return to warm.

Overexpression of *BrFLC2* NATs negates the requirement for vernalization in *B. rapa*

The genomic structure map showed that *BrFLC2as816* overlapped the promoter and exon 1 region of *BrFLC2* (Figure 3a). To examine whether *BrFLC2as* changes the growth cycles of *B. rapa*, we constructed *BrFLC2as816* under the control of AA6 (Patent WO 2007/069894) (Figure 5a) and transformed it into Bre plants using the vernalization–infiltration method (Bai *et al.*, 2013). In total, 12 homozygous transgenic lines were screened, named B2as (Figure 5b). Surprisingly, all of the transgenic lines in the growth room bolted prior to cold treatment, while the wild-type plants did not bolt (Figure 5c; Table 1, Figure S6), indicating the gradient among the transgenic lines at bolting time. Among them, five bolted after 6 weeks of normal growth temperature and were designated as super-early lines (Figures 5c and S6), with B2as-1 being a representative line; three bolted after 8 weeks and were designated as early lines, with B2as-4 being a representative line; and the rest bolted after 9 weeks and were designated as moderate lines, with B2as-7 being a representative line. Overall, these lines did not require cold conditions for vernalization.

To verify the transgenic lines, we performed PCR to detect the AA6 promoter in the transformed plants of the T3 generation and real-time PCR to quantify the transcripts of *BrFLC2as816* (Figures 5b,d, and S7). *BrFLC2as816* was overexpressed in the leaves of B2as-1, B2as-4 and B2as-7 plants, whereas expression levels of *BrFLC2*, *BrFLC1* and *BrFLC3* decreased markedly when compared with their levels in the wild type (Figure 5e), indicating that the *BrFLC* genes are down-regulated by *BrFLC2as816*. Among the down-regulated *BrFLC* genes, *BrFLC2* was the most down-regulated. Among the three types of transgenic lines, the super-early lines had the lowest levels of *BrFLC2* on average and the moderate ones had the highest levels. We hypothesize that the early bolting of the transgenic plants is negatively correlated with the expression of *BrFLC2*.

Normally, plant growth starts from seed germination and finishes with seed ripening. The length of a plant's growth cycle should be designated as the number of days from seed germination to seed ripening under ideal conditions. In the growth room under ideal growth conditions, Bre plants failed to flower and thus did not produce any seed. By contrast, super-early line B2as-1, early line B2as-4 and moderate line B2as-7 set seeds at days 103, 115 and 125, respectively, indicating a gradient in time of seed ripening. This result indicates that the transgenic lines overexpressing *BrFLC2* NATs are able to finish the growth cycles under ideal growth conditions without a period of cold and to provide the crop types with varying growth cycles.

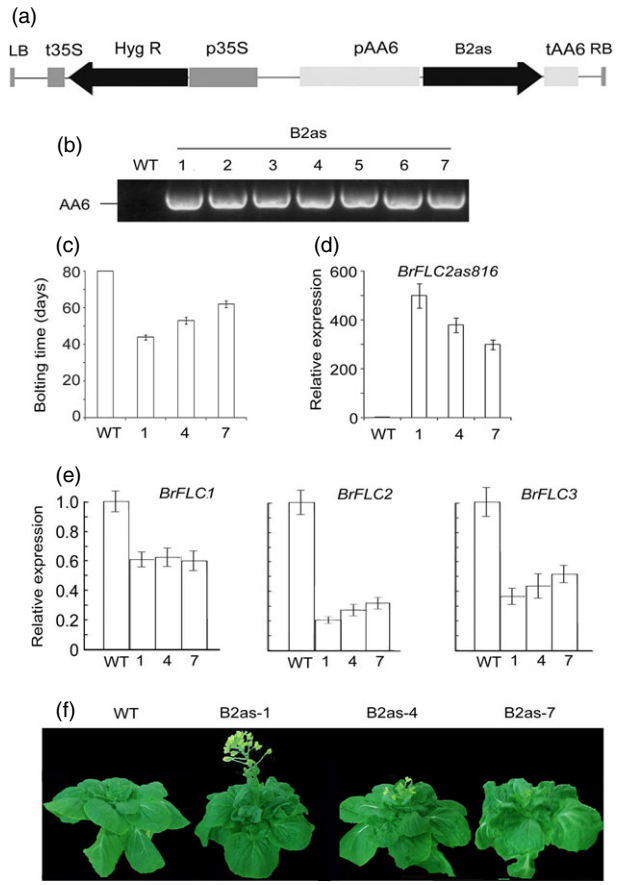


Figure 5 Transgenic Bre plants overexpressing *BrFLC2as816* grown in growth room. (a) Schematical structures of binary vectors of *BrFLC2as816*. (b) PCR of AA6 promoter for identification of the transgenic lines by PCR using the primers spanning AA6 promoter and *BrFLC2as816* fragment. (c) Days to bolting of *BrFLC2as816*-transgenic plants after germination. Error bars indicate the standard deviation. (d) and (e) Real-time PCR showing expression of *BrFLC2as816* and *BrFLC* genes in *BrFLC2as816*-transgenic plants. (f) Plants of the transgenic lines B2as in growth room. p35S, CaMV 35S promoter; Hyg R, hygromycin-resistant gene; pAA, AA6 promoter; LB, left border of T-DNA; t35S, CaMV35S terminator; tAA6, AA6 terminator.

To understand how *BrFLC* NATs affect other biological and economic traits, we examined the numbers of branches and siliques on the transgenic lines overexpressing *BrFLC2as816*. In the growth room, the number of rosette leaves, as well as branches and flowers in the super-early line, was much fewer than those of the moderate lines (Table S2). Among the three types of the transgenic lines, the super-early lines ripened earliest and the moderate lines ripened last. For seed yield per plant, the super-early lines were the lowest and the moderate lines were the highest (Table 1). These results revealed that the seed yield is different between the transgenic lines.

When the wild-type Bre plants were grown in the field in 2011, they underwent vegetative growth during the autumn, flowered normally and set seeds in the following spring (Figure S8a,c,e). Under these conditions, the super-early lines produced fewer rosette leaves and branches than both the Bre and the moderate lines, and flowered and set seeds during the same autumn (Table 2; Figure S8b,g,h). Their life cycles were finished before winter and thus became annual lines. However, the early lines flowered but stopped development after the commitment to the winter condition (Figure S8d,i,j), and they did not produce any seed. The moderate lines finished vegetative growth and bolted before winter (Figure S8f,i,j). Like Bre plants, the moderate lines flowered and set seeds during the following spring. Interestingly, they generated almost the same numbers of branches. Apparently, flowering and seed setting in the transgenic lines in the field were different from those in growth room. During 2012, super-early, early and moderate lines flowered approximately at the same time (data not shown).

BrFLC2 NATs shorten the growth cycle of *Arabidopsis* by repressing *BrFLC* genes

To demonstrate how *BrFLC2as* represses *BrFLC* genes to shorten growth cycles, we cloned three genomic fragments of *BrFLC2/BrFLC2as* to construct binary vectors (Figure 6a). First, a *BrFLC2* genomic fragment was transferred into *Arabidopsis* ecotype Col, in which the expression of *AtFLC* was repressed owing to the interference of the *fri* allele (Lee and Amasino, 1995; Johanson *et al.*, 2000). If *BrFLC2* and the *BrFLC2* NATs were expressed in the transgenic plants, it meant that the *fri* background could not block exogenous *BrFLC2* expression. Thus, we were able to investigate the roles of NATs in the activities of *BrFLC* genes. Each line of the transgenic plants was divided into two populations: one population was grown with a 3-week cold treatment and the other was grown without a cold treatment. The first genomic fragment, *BrFLC2/BrFLC2as* (2/2as), spanned the complete genomic sequence of *BrFLC2*, with a native promoter in the sense strand, and the DNA sequence of *BrFLC2as*, with a promoter in the opposite strand (Figure 6a). The resultant transgenic 2/2as plants flowered at the same time as wild-type plants that underwent a cold treatment (3 weeks of cold exposure). However, the plants flowered later than the wild-type plants that did not undergo a cold treatment, meaning that the cold treatment accelerated the bolting of the 2/2as plants. In the seedling leaves of 2/2as plants that were not subjected to a cold treatment, the *BrFLC2as*' expression levels were extremely low, but *BrFLC2* was expressed. In the seedling leaves that underwent a cold treatment, *BrFLC2as* were expressed, but the expression level on day 20 was much higher than on day 10. During the same period, *BrFLC2* expression on day 20 was lower than on day 10 (Figure 6c). This revealed that the *B. rapa BrFLC2as* were cold-induced in *Arabidopsis*, while *BrFLC2* expression was inhibited.

To verify whether *BrFLC2* was repressed by *BrFLC2as*, we chose the second genomic fragment *BrFLC2/BrFLC2asΔ* (2/2asΔ) that spanned *BrFLC2* with a native promoter on the sense strand and *BrFLC2as* without a native promoter on the opposite strand (Figure 6a). The 2/2asΔ transgenic plants flowered later than the wild type (Figure 6d). Under both conditions, *BrFLC2* expression levels were at almost the same levels, while *BrFLC2as* expression levels were undetectable (Figure 6c). This implied that *BrFLC2* expression was not inhibited when the *BrFLC2as* transcripts were absent.

The third genomic fragment, *BrFLC2Δ/BrFLC2as* (2Δ/2as), spanned *BrFLC2* without a promoter on the sense strand and *BrFLC2as* with a promoter on the opposite strand (Figure 6a). The 2Δ/2as transgenic plants flowered at the same time as the wild type.

Three *BrFLC2/BrFLC2as* constructs affect *BrFLC2* expression and flowering time differently, with and without the native promoter. The *BrFLC2as* act to repress *BrFLC2* and thereby to control flowering time and growth cycle. These results show that *BrFLC2*

Table 1 Flowering time and growth cycles of transgenic lines overexpressing *BrFLC2as816*. In growth room, the seeds were sown in plastic pots and grown at 22 °C in SIPPE Phytotron. For each parameter, more than 20 plants were measured. Means ± standard error. DAG, days after germination; NA, not available; NF, not flowering; S, super-early line; E, early line; M, moderate line

Lines	Bolting (DAG)	Flowering (DAG)	Seed ripening (DAG)	Siliques per plant	Seed yield per plant (gram)	Growth cycle
Bre	NA	NA	NA	NA	NA	NF
B2as-1	44 ± 1.3	51 ± 1.1	103 ± 2.6	189 ± 5.2	39.81 ± 4.35	S
B2as-3	47 ± 2.0	55 ± 2.1	105 ± 4.1	190 ± 6.7	43.08 ± 3.69	S
B2as-8	45 ± 2.2	54 ± 1.9	105 ± 2.9	200 ± 6.1	44.81 ± 4.65	S
B2as-9	42 ± 2.3	51 ± 1.5	103 ± 1.5	187 ± 7.7	43.08 ± 3.92	S
B2as-12	46 ± 1.3	55 ± 1.4	106 ± 2.1	197 ± 5.1	45.61 ± 4.63	S
B2as-4	55 ± 1.9	64 ± 1.8	115 ± 2.3	215 ± 8.1	49.63 ± 6.21	E
B2as-5	58 ± 1.2	69 ± 1.7	118 ± 1.6	211 ± 7.2	48.47 ± 5.92	E
B2as-11	58 ± 1.6	70 ± 1.8	118 ± 1.4	219 ± 5.9	49.01 ± 4.55	E
B2as-2	65 ± 1.9	76 ± 2.3	125 ± 2.1	248 ± 6.7	57.09 ± 4.96	M
B2as-6	69 ± 2.3	78 ± 1.0	129 ± 3.2	252 ± 8.2	55.33 ± 5.86	M
B2as-7	70 ± 1.4	80 ± 0.9	130 ± 2.8	260 ± 4.1	59.91 ± 4.01	M
B2as-10	66 ± 2.1	77 ± 1.2	130 ± 2.7	253 ± 6.9	59.19 ± 4.97	M

Table 2 Flowering time and growing cycles of the transgenic lines overexpressing *BrFLC2as816* in the field on 2011. The seedlings were transferred to the field in SIPPE Farm Station, Shanghai, China, on 9 September 2011 and grown under cultivation condition. For each parameter, more than 20 plants were measured. Means ± standard error. DAG, days after germination; NA, not available

Lines	Bolting (DAG)	Flowering (DAG)	Seed setting (DAG)	Growing cycle
Bre	123 ± 3.9	190 ± 3.6	242 ± 1.3	Normal
B2as-1	43 ± 1.1	53 ± 1.6	102 ± 2.0	Short
B2as-3	45 ± 0.9	53 ± 1.9	100 ± 1.2	Short
B2as-8	43 ± 2.1	55 ± 2.1	105 ± 2.1	Short
B2as-9	40 ± 1.2	52 ± 1.5	104 ± 1.2	Short
B2as-12	45 ± 1.7	54 ± 2.3	102 ± 0.9	Short
B2as-4	52 ± 0.7	68 ± 3.1	No seed	NA
B2as-5	54 ± 1.2	70 ± 1.8	No seed	NA
B2as-11	56 ± 1.9	71 ± 1.6	No seed	NA
B2as-2	64 ± 1.9	189 ± 3.2	230 ± 1.6	Normal
B2as-6	67 ± 3.2	193 ± 2.7	235 ± 1.4	Normal
B2as-7	68 ± 2.0	196 ± 3.5	240 ± 1.2	Normal
B2as-10	65 ± 1.3	192 ± 2.0	239 ± 1.5	Normal

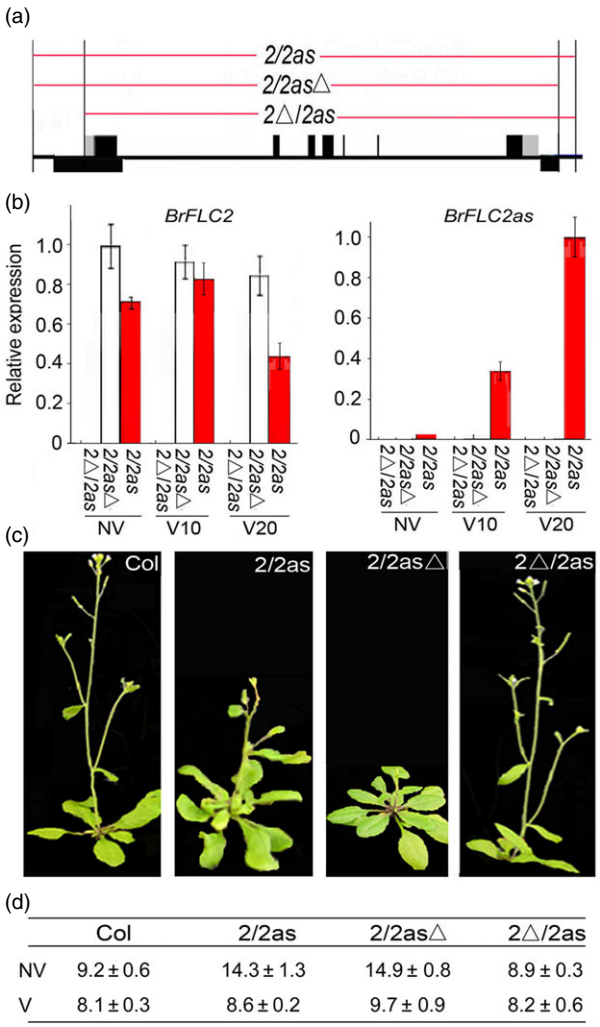
NATs shorten the growth cycle of *Arabidopsis* by repressing *BrFLC* genes.

Discussion

In *Arabidopsis*, FLC acts as an important flowering repressor and transcription factor responding to vernalization. Recently, long noncoding RNAs from the *FLC* locus, such as COOLAIR and COLDAIR, have been identified and isolated in *Arabidopsis* (Swiezewski *et al.*, 2009; Heo and Sung, 2011). We characterized a subset of the noncoding NATs of *BrFLC2* in three crop types of *B. rapa*, which had different vernalization requirements. The question arises whether *BrFLC2* NATs have the same roles in the three crop types. In the rapid-cycling crop type Yellow sarson, *BrFLC2* NATs did not affect the growth cycle because none of the *BrFLC* genes functioned in vernalization due to mutations in their genomic sequences. In the slow-cycling crop type Wantai, *BrFLC2* NAT expression levels are lower and declined more slowly during cold treatment compared with NATs in the medium-cycling crop type Bre. However, *BrFLC2* in the three crop types have NATs, while the other *BrFLC* genes do not. This means that the generation of the noncoding NATs is *BrFLC2* specific. These findings imply that the *BrFLC* NATs in the mesohexaploid *B. rapa* are more functionally complex than those in *Arabidopsis* (Wang *et al.*, 2011b). Nevertheless, it would be interesting to understand why *BrFLC1* and *BrFLC3* do not have any NATs even though they are highly homologous with *BrFLC2*.

In *Arabidopsis*, COLDAIR, derived from the first intron of *FLC*, physically interacts with the polycomb repressive complex, indicating its role in establishing and maintaining stable repressive chromatin at *FLC* (Heo and Sung, 2011). However, COLDAIR is not found in the first intron of *BrFLC* genes in *B. rapa*. This fact implies that the molecular mechanism underlying vernalization in *B. rapa* is different from that of *Arabidopsis*.

Generally, there are two phases in the vernalization response, the initial down-regulation of *FLC* by transcriptional repression and the subsequent phase of maintaining the repressed state (Sheldon *et al.*, 2002). Several studies indicate that the methylation of H3K37me3 inactivates the chromatin and blocks *FLC* expression in response to the cold (Sheldon *et al.*, 2002; Heo and Sung, 2011). COOLAIR plays a role in the epigenetic silencing of *FLC* expression during vernalization (Crevillen *et al.*, 2014; Csorba *et al.*, 2014; Marquardt *et al.*, 2014). We found that *BrFLC* NATs in *B. rapa* are induced by a cold treatment and highly expressed at the early stage of vernalization, concurrent with a decrease in the expression of the *BrFLC* genes. The transgenic lines overexpressing *BrFLC2as816* are able to flower without vernalization. When exogenous *BrFLC2as816* is expressed under the control of its own promoter in *Arabidopsis*, it inactivates the *BrFLC2* gene. These results suggest that *BrFLC* NATs suppress the activity of the *BrFLC2* gene in response to cold and accelerate vernalization. We could not estimate how much the NATs suppress the activities of *BrFLC1* and *BrFLC3*. However, we are certain that *BrFLC2* NATs contribute to the repression of all of the *BrFLC* genes in *B. rapa*. The *BrFLC2* fragments that are reversely complemented to the NATs show high homology (>95%) with *BrFLC1* and *BrFLC3*. It is



	Col	2/2as	2/2as△	2△/2as
NV	9.2 ± 0.6	14.3 ± 1.3	14.9 ± 0.8	8.9 ± 0.3
V	8.1 ± 0.3	8.6 ± 0.2	9.7 ± 0.9	8.2 ± 0.6

Figure 6 The transgenic plants of *Arabidopsis* (Col) overexpressing *BrFLC2as816*. (a) Diagrams of gene constructs of *2/2as*, *2/2asΔ* and *2Δ/2as*. (b) Real-time PCR showing expression of *BrFLC2* and *BrFLC2as* in the leaves of the transgenic *Arabidopsis* plants. NV, nonvernalization; V10–V20 indicate vernalization for different days. Arrows indicate the direction of transcription. (c) Phenotype of transgenic plants without cold treatment. (d) The flowering time in terms of number of rosette leaves was given. 2, BrFLC2; 2as, BrFLC2as; Δ, deletion of native promoter. Flowering time is the week(s) after germination.

reasonable to deduce that *BrFLC2* NAT suppresses *BrFLC1* and *BrFLC3* as well. The fact is that *BrFLC2as816* overexpression inhibits the expression of *BrFLC1* and *BrFLC3* in transgenic plants.

In *Arabidopsis*, the class I NATs of COOLAIR accumulate to a higher degree than class II NATs, which is consistent with proximal polyadenylation correlating with reduced levels of *FLC* transcription (Csorba *et al.*, 2014). Further, a core spliceosome mutant, *prp8-6*, was isolated and identified for its function in disturbing COOLAIR processing and reducing the proximal polyadenylation usage coupled with localized histone methylation reprogramming. In *B. rapa*, class II NATs accumulate to a higher degree than class I NATs and may be more important in vernalization. Considering the close evolutionary relationship between *Arabidopsis* and *B. rapa*, we assume that the class I NATs in *B. rapa* engage in the same pathway to silence *BrFLC* genes in localized histone methylation reprogramming as in *Arabidopsis*. *BrFLC2as816* belongs to the class II NATs, and its overexpression suppresses *BrFLC* genes in *B. rapa* and *Arabidopsis*. This reveals that long class II NATs play a role in the silencing of *BrFLC* genes using a post-transcriptional pathway. It remains unknown whether short class I NATs function in the repression of *BrFLC* genes. Anyway, class II NATs should contribute more to *BrFLC* repression than class I NATs because the former accumulates to a much higher degree than the latter in *B. rapa*. For further understanding of the function and interaction between *BrFLC* genes and the associated NATs, we would benefit from analysis of F_2, RIL or other segregating populations derived from hybrid crosses between the different crop types.

The growth cycle of seed plants begins with seed germination and culminates with seed ripening. In a growth room where the environment is optimized for the development of *B. rapa* crops, the transgenic lines overexpressing *BrFLC2as816* showed gradients in their growth cycles, which were closely associated with the expression levels of the *BrFLC* genes. Among the three types of transgenic lines, the super-early lines had the shortest growth cycles and the moderate lines had the longest ones. This suggests that *BrFLC2* NATs are involved in determining the growth cycles of *B. rapa* crops. Through the control of *BrFLC* NATs, the growth cycle of *B. rapa* could be adapted to different climates and regions. In this regard, the super-early lines are useful for the genetic manipulation of crop early flowering. In particular, they are valuable for those regions where the annual oilseed crops are not cultivated.

In the field, a long winter season interrupts the growth of biennial crops. The super-early lines finished the growth cycle before the winter season, and thus, their yields were not affected by the cold weather. The early lines flowered but failed to set seeds before winter, mainly because the reproductive organs were damaged by the cold weather. The moderate lines bolted but did not flower before winter. These lines underwent the winter season and set seeds in the same fashion as the wild type. For the yield in the field, the moderate lines need be compared with the wild type requiring vernalization. These additional data would be both desirable and of interest, in terms of better defining the roles of *BrFLC* genes in *Brassica* genus.

Thus, the three types of the transgenic lines showed the different growing cycles in the field. Growing cycles are different from growth cycles because they represent life cycles under growing conditions in the field and, as such, are greatly affected by climate and other factors in certain regions.

Both earliness of flowering and high yield are two important traits for oilseed crops. In growth rooms where the wild-type plants are not able to flower, the moderate lines generate more branches and seeds than the other transgenic lines. In the field, they produce the same numbers of branches than the wild type. The potential for seed yield in these lines is greater than the other transgenic lines. If earliness is the priority, then the super-early lines are the best choice. If earliness and high yield are equally important, then the moderate lines could be selected. Nevertheless, further optimization and modification of *BrFLC* NATs are possible for the genetic manipulation of crop flowering time and yield.

In *B. rapa*, NATs of *BrFLC2* show extremely strong gene silencing activities because they eliminate vernalization traits from this biennial crop species by inhibiting *BrFLC* genes. The conversion of a biennial to an annual crop type is important for oilseed crops of *B. rapa*, and growth cycle gradients are useful for selecting the proper crops for growing seasons in various regions.

Specifically, our transgenic lines overexpressing *BrFLC2as816* are valuable because they become annual and display growth cycle gradients. In cold and chilled regions, we can grow and harvest the seeds in 1 year. In warm areas, we could use early or moderate lines for high yields, because they have many branches and a higher potential yield. As long as the high yield vigour of biennial oilseed crops is combined with rapid-cycling traits, the productivity of *B. rapa* could be improved. In this way, new cultivars could be created having certain levels of early flowering using high yield cultivars. More importantly, technology that creates crops that do not require vernalization could be applied to other oilseed and grain crops, such as oilseed rape and wheat.

Materials and methods

Plant materials

The crop types of *B. rapa* used in this study include Yellow sarson (YS-143) (*B. rapa* var. *trilocularis*, rapid-cycling crop type), Bre (*B. rapa* ssp. *pekinensis* var. *bre*, medium-cycling crop type) and Wantai (*B. rapa* ssp. *pekinensis* var. *wantai*, rapid-cycling crop type). The seeds of the inbred lines of these crop types were germinated on moisture-absorbent papers in a plant growth chamber at 22 °C for 3 days and then transplanted to peat soil in plastic pots and moved into a growth room of SIPPE Phytotron in Shanghai. In this growth room, the plants were grown at 22 °C with 16 h of light per day under a light source of warm white fluorescent tubes (colour code 990), an irradiance of 150 μmol/m^2/s and a light intensity on the plant canopy of 75 μmol/m^2/s. The relative humidity was 65%–70%, and the air velocity was approximately 0.9 m/s. All of the seedlings were grouped randomly and grown under identical conditions for 3 weeks. For *B. rapa* vernalization, 3-week-old plants with three leaves were transferred to 4 °C for different time periods ranging from 20 to 80 days with an interval of 5 days. Afterwards, the seedling was returned to normal growth temperature conditions for flowering time statistics. For cultivation in the field, the 4-week-old seedlings were acclimated for 3 days and transferred to the field in SIPPE Farm Station on 24th August 2011 and 9th September 2012.

The seeds of *Arabidopsis* and transgenic lines were surface-sterilized in 70% ethanol for 1 min, followed by 20% bleaching water for 10 min, and then washed four times in sterile distilled water and plated on top of solid Murashige and Skoog medium and incubated at 22 °C for 7 days. For *Arabidopsis* vernalization, a 7-day-old plant was transferred to 4 °C for different time periods. Afterwards, the seedling was returned to normal growth temperature conditions for flowering time statistics.

Analysis of flowering time

The appearance of the first floral buds, the opening of the first flowers and the ripening of the first seeds were designated as bolting time, flowering time and ripening time, respectively. The days of bolting time, flowering time and ripening time were calculated from seed germination. More than 20 plants were measured for each cold treatment and each transgenic line. For the plants in the field, the number of rosette leaves was counted at flowering stage. In *Arabidopsis*, number of the rosette leaves was recorded at flowering stage, according to the method of Li *et al.* (2012). More than three independent transgenic lines were observed. More than 30 plants were measured for each transgenic line.

RNA extraction and real-time PCR

Total RNA was extracted using the TRIZOL reagent (Invitrogen, Carlsbad, CA) as directed by the manufacturer. Real-time PCR was performed as described (Wang *et al.*, 2011a,b; Yu *et al.*, 2012) with primers listed in Table S3. *BrActin* (accession code, Bra022356) was used as the internal control gene. For gene expression data analysis, we calculated differences between the C_t (Cycle Threshold) values for experimental and reference genes (*BrActin*) as $\Delta\Delta C_t$ and graphed the fold change of each RNA to the calibrator sample.

Rapid amplification of cDNA ends

Sequence information for *BrFLC* genes was retrieved from http://brassicadb.org/brad/. The RNA samples were isolated from Bre leaves using RACE ready cDNA (Takara, Tokyo, Japan), and the 5′ and 3′ ends of *BrFLC2* NATs were amplified by RACE-PCR using gene specific primers. One hundred picomoles of 5′ adaptor (rGrUrUrCrArGrArGrUrUrCrUrArCrArGrUrCrCrGrArCrGrArUrC) was directly ligated with 5 μg of total RNA. After ligation, the first-strand cDNAs were synthesized using Superscript III Reverse Transcriptase (Invitrogen, CA) and a specific primer. The cDNA was treated with RNase H to remove the RNA strand and amplified for two rounds using two sets of primers. The 3′ and 5′ PCR products were excised from the gel and cloned into the pMD18T vector (Takara). Twenty positive colonies were sequenced from each RNA sample.

Protein analysis

Total protein samples were isolated from the leaves upon cold treatment of plants. Western blotting analyses were performed to detect BrFLC proteins. Twenty micrograms of total protein were separated on 12% denaturing polyacrylamide gels by electrophoresis and transferred to HyBond-N+ membrane (Amersham, Buckinghamshire, UK) by electroblotting. Anti-FLC antibody and anti-PEPC antibody were used to hybridization.

Construction of binary vectors

The cDNA of *BrFLC2as816* was isolated from Bre seedlings by PCR using KOD TOYOBO DNA polymerase (Toyobo, Osaka, Japan) and inserted into pCAMBIA1300 binary vectors (http://www.cambia.org) under the control of the AA6 promoter. The fragments of *BrFLC2* genomic DNA were isolated from Bre seedlings and cloned into pCAMBIA1301 binary vectors (Table S3). The binary constructs were delivered into *Agrobacterium tumefaciens* strain GV3101 using a freeze–thaw method (Weigel and Glazebrook, 2006).

Genetic transformation

The Bre plants were transformed using vernalization–infiltration method (Bai *et al.*, 2013). This method is based on vacuum infiltration method (Bechtold and Pelletier, 1998). Briefly, a pot of germinated seeds was put in growth room and incubated at 4 °C for 25 days of vernalization treatment. The seedlings in the pots were transferred to growth room at 22 °C and grew for 2 weeks. Then, the plants with small flower buds at the early bolting stage were placed upside down in vacuum desiccator that contained infiltration medium and the engineered *Agrobacterium* for vacuum infiltration. The *Agrobacterium*-infected plants were transferred to the growth room. The seeds were harvested and screened by germination on agar medium containing hygromycin. The hygromycin-resistant seedlings were transplanted in

growth room. T2 and T3 seedlings were identified for the insertion of exogenous genes and analysed for the segregation of population.

Generation of *Arabidopsis thaliana* Columbia was transformed with the *BrFLC* genomic constructs. The inflorescences were dipped in the *Agrobacterium* solution containing sucrose and Silwet-77 for 1 min as described (Clough and Bent, 1998). The infected plants were cultured for 2 days in the dark and then moved in greenhouse for seed production.

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Author contributions

Y.H. designed the research. X.L. and S.Z. performed the research and analysed the data. J.B. contributed to genetic transformation of Chinese cabbage. X.L. and Y.H. wrote the article.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Alignment of putative amino acid sequences of *BrFLC* genes between Yellow sarson, Bre and Wantai.
Figure S2 Bolting time of the transgenic *Arabidopsis* lines overexpressing *BrFLC* genes from the crop types Yellow sarson, Bre and Wantai.
Figure S3 Homologous fragments between *BrFLC2as816* and one NAT of *AtFLC*.
Figure S4 RT-PCR showing *BrFLC2* NATs in *B. rapa*.
Figure S5 Diagrammatic representation of alignment of the intron 1 fragments of *BrFLC* genes with that of *AtFLC*.
Figure S6 Bolting time of Bre plants overexpressing *BrFLC2as816*.
Figure S7 Real-time PCR showing the expression of *BrFLC2* in Bre plants overexpressing *BrFLC2as816*.
Figure S8 Bre plants overexpressing *BrFLC2as816* at different stages in the field.
Table S1 Sequences of *BrFLC2* antisense transcripts.
Table S2 Growth parameters of *BrFLC2as816*-transgenic Bre plants in growth room and field.
Table S3 The primers used in this study.