Planta
https://doi.org/10.1007/s00425-018-2891-0

ORIGINAL ARTICLE

Isolation and functional characterization of a floral repressor, *BcFLC2*, from Pak-choi (*Brassica rapa* ssp. *chinensis*)

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Received: 8 November 2017 / Accepted: 5 April 2018

Abstract

Main conclusion ***BcFLC2*** **functioned as a repressor of flowering by directly regulating** ***BcTEM1***, ***BcMAF2***, ***BcSOC1*** **and** ***BcSPL15*** **in Pak-choi.**

FLOWERING LOCUS C (*FLC*) plays an important role in regulating flowering time. Here, we functionally described an *FLC* homologous gene, *BcFLC2*, that negatively regulated flowering in Pak-choi (*Brassica rapa* ssp. *chinensis*). The sequence comparison to *Arabidopsis* FLC showed that BcFLC2 also had a MADS-box domain at the N terminus. *BcFLC2* was highly expressed in the leaves, roots, stems and stamens, and its expression was repressed by vernalization in Pak-choi. Interestingly, *BcFLC2* expression exhibited a small peak at 2 weeks of vernalization treatment, suggesting that *BcFLC2* may be involved in preventing premature flowering under short-term cold exposure in Pak-choi, which is different from the *AtFLC* expression pattern. Overexpression of *BcFLC2* in *Arabidopsis* caused late flowering, while silencing of *BcFLC2* in Pak-choi caused early flowering. BcFLC2 localized to the cell nucleus and functioned as a transcription factor. Yeast one-hybrid analysis revealed that BcFLC2 could bind to the promoters of Pak-choi *Tempranillo 1* (*BcTEM1*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*BcSOC1*), *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 15* (*BcSPL15*) and *MADS AFFECTING FLOWERING 2* (*BcMAF2*). Taken together, the present results suggested that *BcFLC2* played a key role in flowering regulation as a negative regulator by controlling *BcTEM1*, *BcMAF2*, *BcSOC1* and *BcSPL15* expression.

Keywords *FLOWERING LOCUS C 2* · Flowering time · Late flowering · *MADS AFFECTING FLOWERING 2* · Short-term cold exposure · *TEMPRANILLO 1* · Vernalization

Abbreviations

AbA	Aureobasidin A
FLC	*FLOWERING LOCUS C*
FT	*FLOWERING LOCUS T*
MAF	*MADS AFFECTING FLOWERING*
NC	Negative control
PDS	Phytoene desaturase
SPL15	*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 15*
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*
TEM1	*TEMPRANILLO 1*

Feiyi Huang and Tongkun Liu contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00425-018-2891-0) contains supplementary material, which is available to authorized users.

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Introduction

Flowering is an essential process in the life cycle of higher plants so that plants can switch from the vegetative to reproductive phase, which is vital to agricultural production. It is a complex process determined by multiple environmental and developmental signals, such as temperature, light and phytohormonal levels, which all ensure that flowering occurs at the appropriate time (Boss et al. 2004). In *Arabidopsis*, floral induction is mainly regulated by four pathways, namely the photoperiod-, vernalization-, gibberellin- and

Published online: 14 May 2018

autonomous-dependent pathways. The four pathways regulate two flowering pathway integrators, *FLOWERING LOCUS T* (*FT*) and *SOC1*, which act prior to the activation of floral meristem identity genes to determine the exact flowering time (Fujiwara et al. 2003). These two integrators are antagonistically regulated by two upstream regulators: *FLC* acts as a negative regulator, and *CO* acts as a positive regulator of flowering (Lee et al. 2000; Samach et al. 2000).

Exposure to cold winter conditions renders biennial and winter-annual plants responsive to the inductive photoperiod, resulting in flowering in the spring, which is known as vernalization (Reeves and Coupland 2000). The vernalization requirement mainly results from *FLC* and *FRIGIDA* (*FRI*) (Michaels and Amasino 1999; Johanson and Dean 2000). *FLC* encodes a MADS-box transcription factor and functions as a major repressor of flowering. FLC represses *SOC1*, *SPL15* and *FT* by directly binding to the promoters of *SOC1* and *SPL15* or the first intron of *FT* (Helliwell et al. 2006; Searle et al. 2006). *Arabidopsis* ecotypes with high-abundance *FLC* are usually late flowering, whereas ecotypes with low-abundance *FLC* are early flowering (Lister et al. 2005). Overexpression of *FLC* shows a late-flowering phenotype in *Arabidopsis* and *Brassica rapa* (Kim et al. 2007). *FLC* expression is activated by FRI and suppressed by the vernalization and autonomous pathways (Choi et al. 2011). Repression of *FLC* by vernalization is initially regulated by *VERNALIZATION INSENSITIVE 3 (VIN3)*, encoding a PHD-domain protein that interacts with the member of the polycomb repressive complex 2 (PRC2) (De Lucia et al. 2008). PRC2 complexes catalyze the methylation of H3K27 at the *FLC* locus to promote an inactive chromatin state (Sung and Amasino 2004). In addition to VIN3, VERNALIZATION1 (VRN1) and VRN2 are required to maintain the silenced state of *FLC* (Bastow et al. 2004). Recent studies revealed that long non-coding RNAs are also involved in the epigenetic repression of *FLC*. COOLAIR enhances the down-regulation of *FLC* expression (Swiezewski et al. 2009), and COLDAIR helps to recruit PRC2 to the FLC locus (Heo and Sung 2011). This epigenetic repression can be maintained through cell division after vernalization treatment, which can only be "forgotten" in the next generation (Finnegan and Dennis 2007). MADS-box transcription factors typically act as multimers with other MADS-domain proteins (de Folter et al. 2005). SVP, another MADS-domain protein, interacts directly with FLC. Research has shown that the FLC–SVP complex directly binds to the *FT* and *SOC1* to repress their expression (Li et al. 2008). The loss of *SVP* partially suppresses *FLC*-mediated flowering inhibition and does not influence flowering as greatly as the loss of FLC, and thus, there may be some redundancy in SVP function (Lee et al. 2007).

In addition to *Arabidopsis*, other members of Brassicaceae can also regulate flowering through vernalization (Halevy 1989). In *Brassica* species, two major QTLs related to vernalization-responsive flowering time are collinear with *Arabidopsis FRI* and *FLC*, suggesting that the homologs of *FLC* and *FRI* may play vital roles in flowering time during the process of vernalization in other *Brassica* species (Osborn et al. 1997). Although numerous studies have reported the roles of *FLC* in some *Brassica* species, such as *Brassica napus* (Tadege et al. 2001) and *Brassica oleracea* (Schranz et al. 2002), the regulation mechanism of *FLC* in Pak-choi (*Brassica rapa* ssp. *chinensis*) is still not well understood. Pak-choi, belonging to the *B. rapa* family, is one of the most important vegetable crops and is widely cultivated in Asia (Huang et al. 2016). *Wuyueman* was used in this work, which normally requires 5 weeks of vernalization treatment (4 °C) and which flowers later than other cultivars. Here, we performed a series of studies, including sequence, expression, subcellular localization, ectopic expression, silencing and downstream gene analysis of *BcFLC2* in Pak-choi. The results showed that *BcFLC2* was repressed by vernalization and highly expressed in the leaves, roots, stems and stamens. *BcFLC2* encoded a MADS-box transcription factor and regulated the expression of *BcSOC1*, *BcSPL15*, *BcMAF2*, and *BcTEM1* by directly binding to their promoters. Overexpression of *BcFLC2* in *Arabidopsis* caused a late-flowering phenotype, while silencing of *BcFLC2* in Pak-choi caused an early flowering phenotype. Our results indicated that *BcFLC2* functioned as a flowering repressor by directly inhibiting *BcSOC1* and *BcSPL15* and activating *BcTEM1* and *BcMAF2* in Pak-choi.

Materials and methods

Plant materials

Arabidopsis wild type (WT) (obtained from Prof. Isabelle Jupin, University Paris 7, France) and *BcFLC2*-overexpressing seedlings were Col-0 ecotype background. Pak-choi (*Brassica rapa* ssp. *chinensis*) cultivars *wuyueman* and *49caixin*, kept at Nanjing Agricultural University, and all *Arabidopsis* plants were grown in plastic trays with a growth medium (vermiculite:loam, 1:2, v:v) in a culture room under long-day conditions (16 h light at 22 °C/8 h dark at 18 °C). To extract mesophyll protoplasts, WT *Arabidopsis* seedlings were grown under short-day conditions with an 8/16 h light/dark cycle for 1 month.

Cloning and sequence analysis

RNA extraction and cDNA synthesis were performed according to our previous report (Huang et al. 2016). *BcFLC1*, *BcFLC2* and *BcFLC3* (*CabbageG_a_f_g052019*, *CabbageG_a_f_g006153* and *CabbageG_a_f_g011915*)

were isolated from the leaf cDNA of the Pak-choi cultivar *wuyueman* with three pairs of primers—BcFLC1-S and BcFLC1-A, BcFLC2-S and BcFLC2-A, and BcFLC3-S and BcFLC3-A—based on homology cloning. The primers were designed based on the *BcFLC* homologue genes *Bra009055* (*BrFLC1*), *Bra028599* (*BrFLC2*) and *Bra006051* (*BrFLC3*). Then, the PCR products were cloned into the pMD18-T vector before sequencing. The amino acid sequences of BcFLCs and the other FLCs from *Arabidopsis* and *Brassica rapa* were used for phylogenetic analysis. The protein sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Phylogenetic analysis and multiple sequence alignment were performed according to our previous report (Huang et al. 2016).

The open reading frame (ORF) sequences of *BcSOC1*, *BcSPL15*, *BcTEM1* and *BcMAF2* were also obtained according to the above methods. The genomic DNA of the Pak-choi cultivar *wuyueman* was isolated with the Plant Genomic DNA Kit (Tiangen, Beijing, China). The genomic sequences of *BcSOC1*, *BcSPL15*, *BcTEM1* and *BcMAF2* were cloned using four pairs of primers—BcSOC1-S and BcSOC1-A, BcSPL15-S and BcSPL15-A, BcTEM1-S and BcTEM1-A, and BcMAF2-S and BcMAF2-A—from genomic DNA. Based on the genomic sequences, the predicted promoter regions were amplified using Self-Formed Adaptor (SEFA) PCR using a KX Genome Walking Kit (Zoman Biotechnology, Beijing, China). The predicted promoter region of *BcCO* was amplified using the same method. The primers used in the study are listed in Table S1. The CArG boxes in the promoters of *BcSOC1*, *BcSPL15*, *BcTEM1* and *BcMAF2* were analyzed using Softberry (http://www.softberry.com/).

Subcellular localization

The full-length ORF of *BcFLC2* without the termination codon was obtained by PCR using the primers O1 and O2. The target fragment and linear pCambia 1302 vector (*35S:GFP*) with *Xba*I and *BamH*I were purified using a double enzyme digestion reaction. The recombinant fusion vector 1302-*BcFLC2* (*35S:BcFLC2-GFP*) was generated by ligation using a DNA ligation kit (Takara, Beijing, China). *35S:GFP* was used as a control. The *35S:BcFLC2-GFP* and *35S:GFP* plasmids were separately transformed into *Agrobacterium tumefaciens* (strain GV3101) by electroporation for transformation of the tobacco leaves (Zhang et al. 2012). DAPI (nucleus specific dye) was used to stain the nuclei. After incubation for 48 h at 25 °C, GFP in tobacco leaves was detected using confocal microscopy (Leica, TCS SP2, Wetzlar, Germany).

Generation of *BcFLC2*-overexpressing *Arabidopsis* lines

Arabidopsis (Col-0) was transformed with *Agrobacterium tumefaciens* (strain GV3101) harboring *35S:BcFLC2-GFP* or *35S:GFP* (negative control, NC) using the floral dip method (Clough and Bent 1998). The seeds of the T_0 transgenic *Arabidopsis* were sowed on 1/2 MS medium containing 35 mg/L hygromycin for selection. Four transgenic *Arabidopsis* lines were obtained (#1, #2, #3 and #4). To confirm the presence of *BcFLC2* in the four transgenic *Arabidopsis* lines, we isolated cDNA from the seedlings of the NC and *BcFLC2*-overexpressing lines. The *35S:BcFLC2-GFP* plasmid was used as the positive control (PC). Then, PCR was performed using a pair of specific primers (O1 and O2). However, seeds were only obtained from two positive lines (#1 and #3), and thus, two T_3 homozygous transgenic lines were used for subsequent experiments. The days from sowing to opening of the first flower were counted. The number of rosette leaves was counted at the time of bolting. Each experiment was calculated from 30 plants. Values are expressed as the means ± standard deviation. The differences between the lines were separated using the least significant difference (LSD) test at $P < 0.01$.

Virus-induced gene silencing (VIGS) in Pak-choi for silencing *BcFLC2*

A specific 40-bp fragment of the *BcFLC2* coding region and its antisense sequence were synthesized and inserted into the *pTY-S* (*pTY*) vector of the turnip yellow mosaic virus-induced gene silencing (TYMV-VIGS) system to form a *BcFLC2*-silencing construct by the company (GenScript, Nanjing, China) (Pflieger et al. 2008). *pTY-BcPDS* was constructed to examine the efficiency of the silencing protocol in the Pak-choi seedlings. The empty *pTY* plasmid was used as the NC. The sequences of oligonucleotides used for VIGS are listed in Table S2. The 2-week-old Pak-choi cultivar *49caixin* plants, which usually bolt at 8 weeks and do not require vernalization, were used for VIGS. The *pTY*, *pTY-BcPDS* and *pTY-BcFLC2* plasmids (5 μg) coated on gold particles were bombarded into 4–5 Pak-choi plants using particle gun bombardment (Bio-Rad, PDS1000/He) based on the previous protocol with some modification (Hamada et al. 2017). Three biological replicates were performed. Three weeks later, leaves showing virus symptoms were sampled for detection. Two *BcFLC2*-silencing Pak-choi plants, *pTY-BcFLC2-3* and *pTY-BcFLC2-4*, were confirmed by qPCR and used for the following experiments. The days from sowing to the time of bolting were counted.

Expression analysis in Pak-choi and *Arabidopsis*

For cold treatment, 1-month-old Pak-choi cultivar *wuyueman* plants were transferred to a novel growth chamber, exposed to 4 °C for 0, 1, 2, 3, 4 and 5 weeks and harvested at the same time point. Plants grown in the culture room without vernalization treatment were used as a control. Three biological replications were performed in each sample. For organ-specific expression analysis, the root, stem, leaf, style, stamen, petal and sepal tissues of the flowering Pak-choi cultivar *wuyueman* were sampled. To investigate the changes in downstream gene expression, the seeds of the transgenic and NC plants were grown on MS medium with 35 mg/L hygromycin and harvested after 15 days. Total RNA was extracted, reverse-transcribed, and used for qPCR as described in our previous report (Huang et al. 2016). The Pak-choi and *Arabidopsis actin* genes were used as the internal control. Primers for qPCR were designed using Primer 5 and are listed in Table S1.

Yeast one-hybrid assay

For the yeast one-hybrid assay, the Matchmaker® Gold Yeast One-Hybrid System was used. The 1000-, 1243-, 2000- and 1507-bp promoter sequences of *BcTEM1*, *BcSOC1*, *BcMAF2* and *BcSPL15* were inserted into the pAbAi reporter vector to form the bait vectors. The information for the promoters of *BcTEM1*, *BcSOC1*, *BcMAF2* and *BcSPL15* is shown in Table S3. To detect whether BcFLC2 could bind to the CArG box in the *BcMAF2* promoter, we mutated the CArG box in the *BcMAF2* promoter using the Fast Mutagenesis System (Transgen Biotechnology, Beijing, China). The bait vectors were then integrated into the yeast genome (strain Y1H Gold), separately. The recombinant yeast cells were separately plated on SD medium lacking uracil supplemented with different concentrations of Aureobasidin A (AbA) to select the minimal inhibitory concentration. The full-length ORF of *BcFLC2* without the termination codon was constructed in the pGADT7 vector. The pGADT7-BcFLC2 plasmid was then transformed into bait strains. Transformants were screened by growing them on SD medium lacking leucine supplemented with 300 ng/mL AbA at 30 °C for 3 days.

Results

Molecular characterization of *BcFLC2*

We identified three *FLC* homologous genes, *CabbageG_a_f_g052019*, *CabbageG_a_f_g006153* and *CabbageG_a_f_g011915*, in Pak-choi. Alignment of protein sequences revealed that these three FLC homologous proteins all had a conserved MADS-box domain in the N terminus (Fig. 1a). Based on our previous transcriptome sequencing data of five developmental stages in three Pak-choi cultivars (NHCC001-*suzhouqing*, 002-*aijiaohuang* and 004-*wuyueman*) (Song et al. 2014), we only found two *FLC* homologous genes, *CabbageG_a_f_g006153* and *CabbageG_a_f_g011915*. The expression of the two genes was higher in the seedling stage and almost undetectable in the flowering stage in the three Pak-choi cultivars (Table S4). Phylogenetic analysis suggested that CabbageG_a_f_g006153 and CabbageG_a_f_g011915 showed high homology to BrFLC2 and BrFLC3 and may have similar roles to those of BrFLC2 and BrFLC3, respectively (Fig. 1b). In addition, *BrFLC2* was reported as the key *FLC* gene based on the previous report (Xiao et al. 2013). Thus, we further investigated *CabbageG_a_f_g006153* and designated it as *BcFLC2*. The full-length cDNA sequence of *BcFLC2* is 591-bp long, encoding a putative protein of 196 amino acids with a molecular mass of 21.9 kDa and a p*I* of 8.84.

Expression pattern of *BcFLC2* in Pak-choi

To investigate whether the *BcFLC2* transcript was affected by vernalization, we performed qPCR to analyze its expression pattern in the leaves of the Pak-choi cultivar *wuyueman*. The expression level of *BcFLC2* declined during the process of vernalization, suggesting that *BcFLC2* was repressed by vernalization (Fig. 2a). We found that its expression had a small peak at 2 weeks of treatment. The results indicated that *BcFLC2* responded to vernalization and may play a role in preventing premature flowering under short-term cold exposure. We further detected the tissue-specific expression of *BcFLC2*. *BcFLC2* was expressed in all detected tissues, including the root, stem, leaf, style, stamen, petal and sepal tissues. *BcFLC2* expression was higher in the roots, stems, leaves and stamens than in other tissues (Fig. 2b).

Subcellular localization of BcFLC2 protein

The subcellular localization of a protein will help us to understand its possible functions. To examine the subcellular localization of BcFLC2, the *35S:BcFLC2-GFP* and *35S:GFP* constructs were transiently introduced into tobacco leaves, separately. The GFP fluorescence of the cells transformed with *35S:GFP* was detected in both, the nucleus and the cytoplasm (Fig. 3). The GFP fluorescence of the cells transformed with *35S:BcFLC2-GFP* was co-observed with DAPI in the nucleus, indicating that BcFLC2 is a nuclear protein similar to other transcription factors.

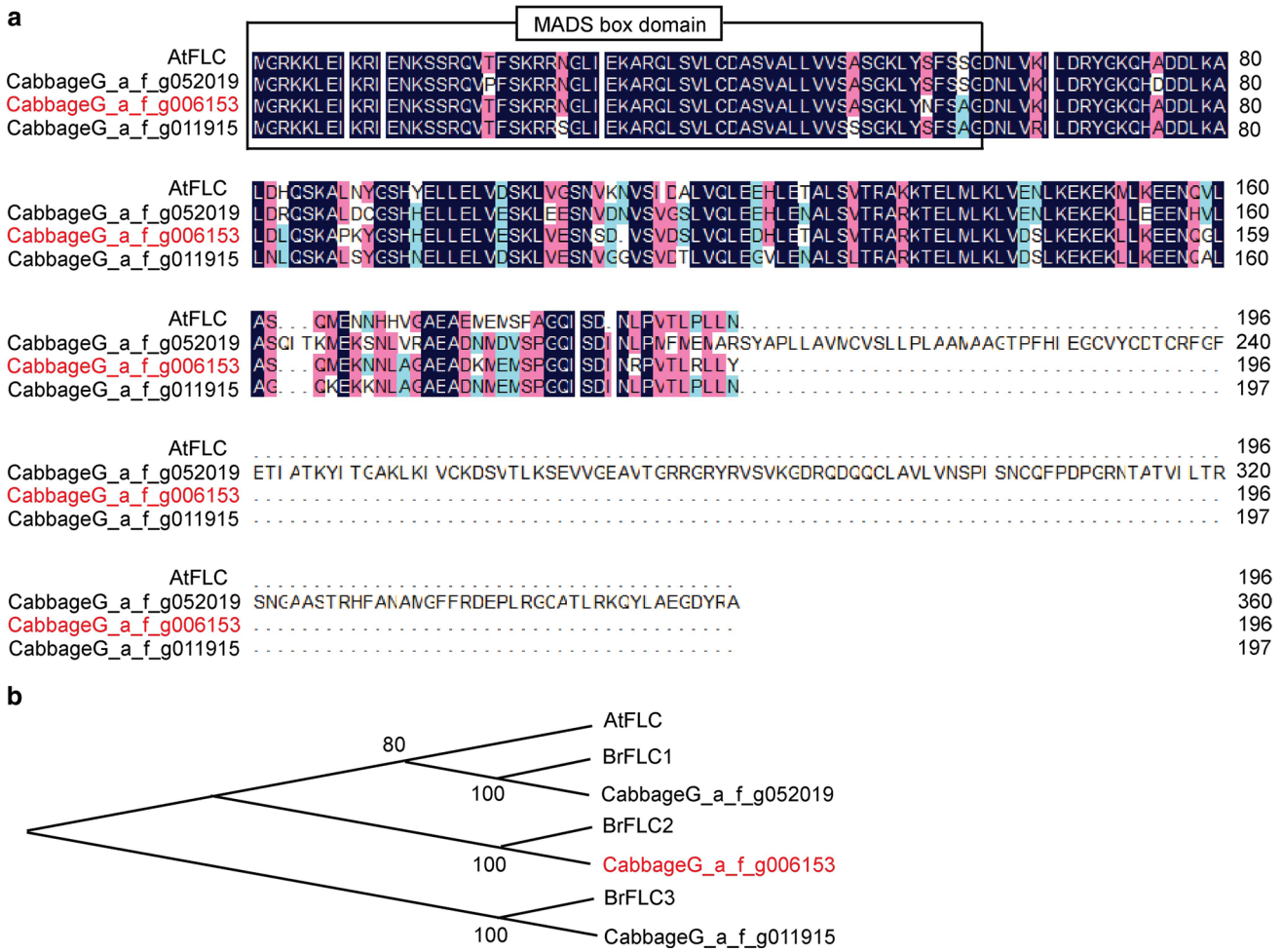

Fig. 1 Multiple sequence alignment and phylogenetic analysis. **a** Alignment of AtFLC with three Pak-choi FLC homologues (CabbageG_a_f_g052019, CabbageG_a_f_g006153 and CabbageG_a_f_g011915). Perfectly matched residues, highly conserved residues and less conserved residues are marked in dark blue, pink and sky blue, respectively. **b** Phylogenetic tree of three Pak-choi FLCs and FLC homologues from *Arabidopsis* and *Brassica rapa*. The tree was constructed using the maximum likelihood method in MEGA6. The gene accession numbers were as follows: AtFLC (AT5G10140), BrFLC1 (Bra009055), BrFLC2 (Bra028599) and BrFLC3 (Bra006051)

Overexpression of *BcFLC2* caused late flowering and affected expression levels of flowering-related genes in *Arabidopsis*

Based on the above findings, we predicted that *BcFLC2* may be a repressor of flowering. To test this hypothesis, we first overexpressed *BcFLC2* in *Arabidopsis* to investigate its function. Two homozygous T_3 transgenic *Arabidopsis* lines (#1 and #3) confirmed by PCR (Fig. S1) were selected for further analyses. Compared to the NC, *BcFLC2*-overexpressing lines presented obvious late flowering (Fig. 4a). In addition, the rosette leaves number at the time of bolting for #1 and #3 plants was higher than that for the NC plants, with an average of 46 and 51 leaves, respectively (Fig. 4b). The opening time of the first flower of the #1 and #3 plants was later by approximately 27 and 31 days compared to the NC plants, respectively (Fig. 4c). These results suggested that *BcFLC2* may function as a floral repressor.

To identify the targets regulated by BcFLC2, the transcripts of some flowering-related genes were analyzed in the NC and transgenic *Arabidopsis* lines. Of the genes examined, *AtSOC1* and *AtSPL15* were down-regulated, while *AtTEM1*, *AtMAF2* and *AtFLC* were up-regulated in the transgenic lines compared to the NC plants (Fig. 4d). These results indicated that *BcFLC2* may delay flowering by regulating the expression of *AtSOC1*, *AtSPL15*, *AtTEM1* and *AtMAF2*.

Silencing of *BcFLC2* in Pak-choi resulted in early flowering

To further clarify the function of *BcFLC2* in Pak-choi flowering regulation, we used the TYMV-VIGS approach to

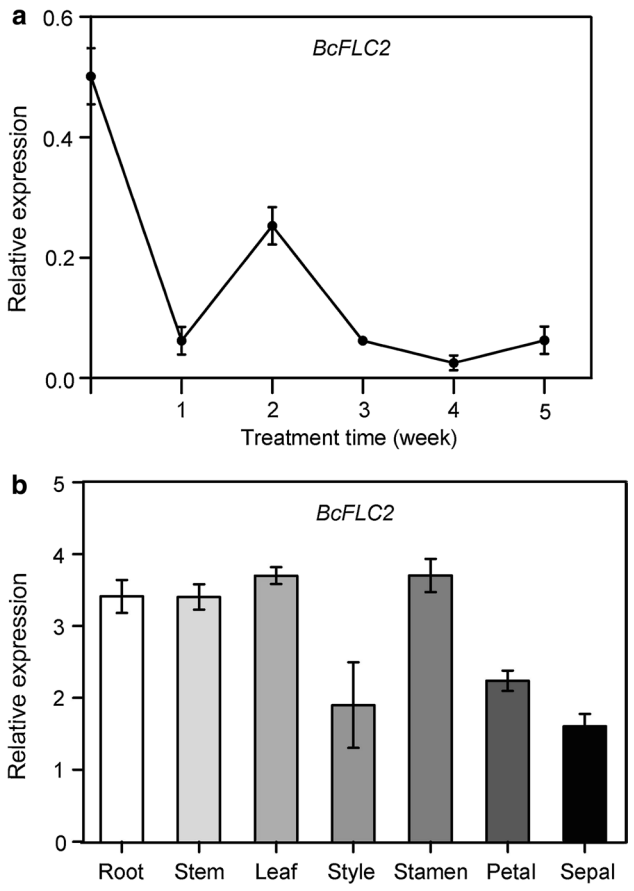

Fig. 2 qPCR analysis of *BcFLC2* transcript level in vernalization treatment (**a**) and different tissues (**b**) of Pak-choi. Data shown are the means ± SE of three biological replications

generate *BcFLC2*-silenced Pak-choi plants. Three weeks after the '*49caixin*' plants underwent particle gun bombardment, the photobleaching or mosaic leaf phenotype that is typical of PDS deficiency or TYMV was visible on the upper leaves of *pTY-BcPDS*, *pTY-BcFLC2* or NC plants, suggesting that TYMV-mediated gene silencing was effective in Pak-choi. The silencing efficiency was examined using qPCR by analyzing the abundance of *BcPDS* or *BcFLC2* in the *BcPDS*-silenced or *BcFLC2*-silenced plants. Overall, the silencing efficiency of *BcPDS* or *BcFLC2* was approximately 50% (Fig. 5b, c). As expected, the *BcFLC2*-silenced plants exhibited an early flowering phenotype compared to the NC plants (Fig. 5a). The bolting time in *pTY-BcFLC2* Pak-choi was approximately 15–17 days earlier than that in the NC plants (Table S5). The transcript levels of the predicted downstream genes were also detected by qPCR. Compared to the NC plants, the transcript levels of *BcSOC1* and *BcSPL15* were higher and the transcript levels of *BcTEM1* and *BcMAF2* were lower in the *BcFLC2*-silenced plants. In addition, the expression of *BcFLC1* and *BcFLC3* in the *BcFLC2*-silenced plants was detected, which exhibited no almost change. These results suggested that the early flowering phenotype might be specifically caused by the reduction of *BcFLC2* (Fig. 5d).

BcFLC2 directly bound to the promoters of *BcMAF2*, *BcTEM1*, *BcSOC1* and *BcSPL15*

FLC can bind to the CArG box ($CC(A/T)_6GG$) in the promoters of its targets (Deng et al. 2011). We found that the CArG box was present in the promoters of *BcSOC1*, *BcSPL15*, *BcTEM1* and *BcMAF2* (Table S3). The transcripts of *AtSOC1*, *AtSPL15*, *AtTEM1* and *AtMAF2* were significantly altered in *BcFLC2*-overexpressing *Arabidopsis* seedlings. In addition, the transcripts of *BcSOC1*, *BcSPL15*, *BcTEM1* and *BcMAF2* were also significantly altered in *BcFLC2*-silenced Pak-choi seedlings. To determine whether BcFLC2 could directly bind to the promoters of *BcSOC1*, *BcTEM1*, *BcSPL15* and *BcMAF2*, the yeast one-hybrid assay was performed. Based on these results, the promoter fragments of *BcSOC1*, *BcSPL15*, *BcTEM1* and *BcMAF2* that contained the CArG box were selected. As shown in Figs. 6 and 7a, the yeast cells, containing *BcSOC1*, *BcSPL15*, *BcTEM1* or *BcMAF2* promoter fragments, transformed with pGADT7-BcFLC2 could grow on SD/-Leu/AbA*. The results indicated that BcFLC2 might directly bind to the promoters of *BcSOC1*, *BcSPL15*, *BcTEM1* and *BcMAF2*. As we know, FLC cannot bind to the *CO* promoter, we used the *BcCO* promoter as the negative control promoter. The yeast cells, containing the *BcCO* promoter fragment, transformed with pGADT7-BcFLC2, could not grow on SD/-Leu/AbA* (Fig. 7a), suggesting that BcFLC2 does not bind indiscriminately.

Since the relationships among *SOC1*, *SPL15*, *TEM1* and FLC have been studied (Deng et al. 2011), we further analyzed the relationship between *BcMAF2* and BcFLC2. To investigate whether BcFLC2 could bind to the CArG box in the *BcMAF2* promoter, we mutated the CArG box (Fig. 7b). When the CArG box was mutated, there was no binding (Fig. 7a). Together, our results indicated that BcFLC2 could

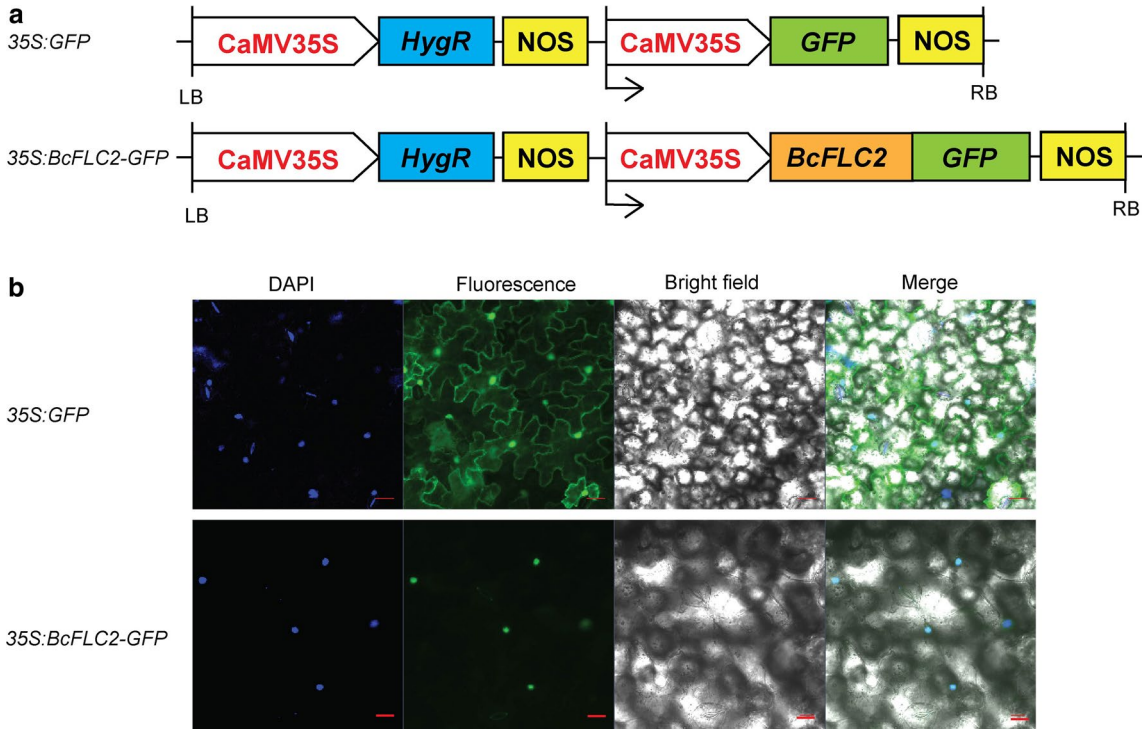

Fig. 3 Subcellular localization of BcFLC2 protein. **a** *35S:GFP* and *35S:BcFLC2-GFP* constructs. **b** Transient expression of *35S:GFP* and *35S:BcFLC2-GFP* fusion protein in tobacco leaves. Scale bars = 50 μm

not bind to the *BcMAF2* promoter when its CArG box was mutated.

Discussion

The elucidation of the underlying mechanism of flowering regulation is important for Pak-choi. In the present study, a new *FLC* homologous gene, *BcFLC2*, was isolated and functionally characterized in Pak-choi. *Arabidopsis* only has a single *FLC* gene (Deng et al. 2011), but Pak-choi has three *FLC* genes, of which *BcFLC2* functioned as a key player according to our previous transcriptome data and reports (Xiao et al. 2013; Song et al. 2014). BcFLC2 was a nuclear protein and functioned as a transcription factor (Fig. 3). To investigate the regulatory roles of *BcFLC2* in Pak-choi, we analyzed its expression pattern with vernalization treatment and in different tissues. We observed that *BcFLC2* expression was relatively higher in the roots, stems, leaves and stamens (Fig. 2b), similar to the expression pattern displayed by *AtFLC*. *AtFLC* was primarily expressed in the shoot apical meristem (SAM) and leaves to control flowering time in *Arabidopsis* (Searle et al. 2006). Interestingly, although *BcFLC2* was also negatively regulated by vernalization, its transcript slightly increased at 2 weeks of vernalization treatment, which was different from the expression pattern of *AtFLC*, suggesting that *BcFLC2* may prevent premature flowering under short-term cold in Pak-choi.

The ectopic and constitutive expression of *BcFLC2* in *Arabidopsis* exhibited a higher expression level of *AtTEM1* and lower expression levels of *AtSOC1* and *AtSPL15*, causing significantly late flowering (Fig. 4d). The silencing of *BcFLC2* in Pak-choi led to the up-regulation of *BcSOC1* and *BcSPL15* and down-regulation of *BcTEM1*, resulting in early flowering (Fig. 5c). These findings suggested that *BcTEM1*, *BcSOC1* and *BcSPL15* acted downstream of BcFLC2. In *Arabidopsis*, *TEM1*, *SOC1* and *SPL15* are controlled by FLC via direct binding to their promoters (Deng et al. 2011; Tao et al. 2012). In *Arabidopsis*, direct up-regulation of *TEM1* by both FLC and SVP contributes to the eventual

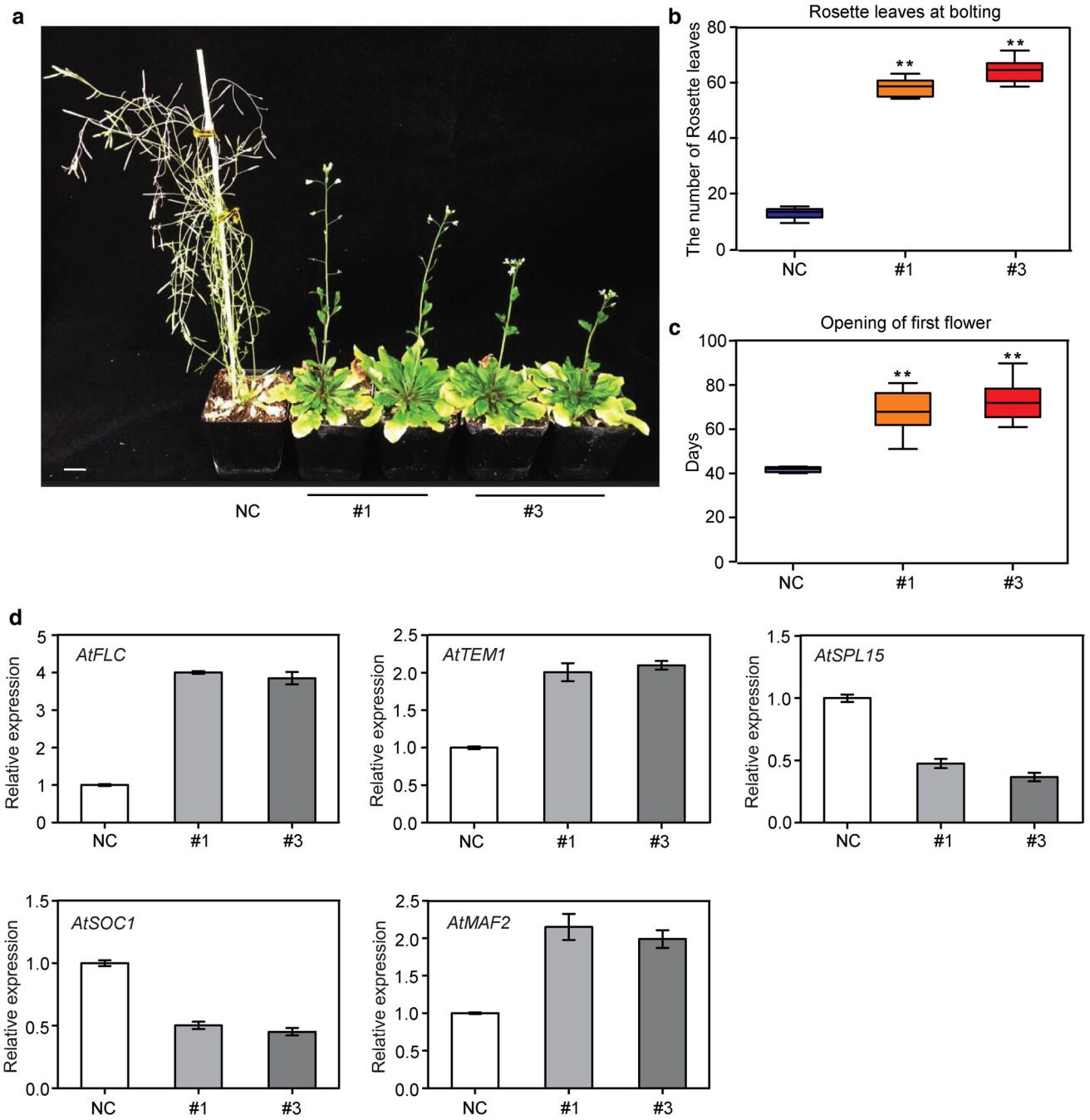

Fig. 4 Overexpression of *BcFLC2* in *Arabidopsis*. **a** The NC and *35S:BcFLC2-GFP #1* and *#3* plants grown in a chamber (16 h light/8 h dark photoperiod at 22 °C/18 °C). Scale bars = 1.5 cm. Rosette leaf number at bolting (**b**) and opening of first flower (**c**) in the NC and *35S:BcFLC2-GFP #1* and *#3* plants. Error bars represent the standard deviation of the mean number of 30 plants for each line. ** indicates significant differences from control ($P < 0.01$). **d** Expression analysis of predicted downstream genes in the NC and *35S:BcFLC2-GFP #1* and *#3* plants

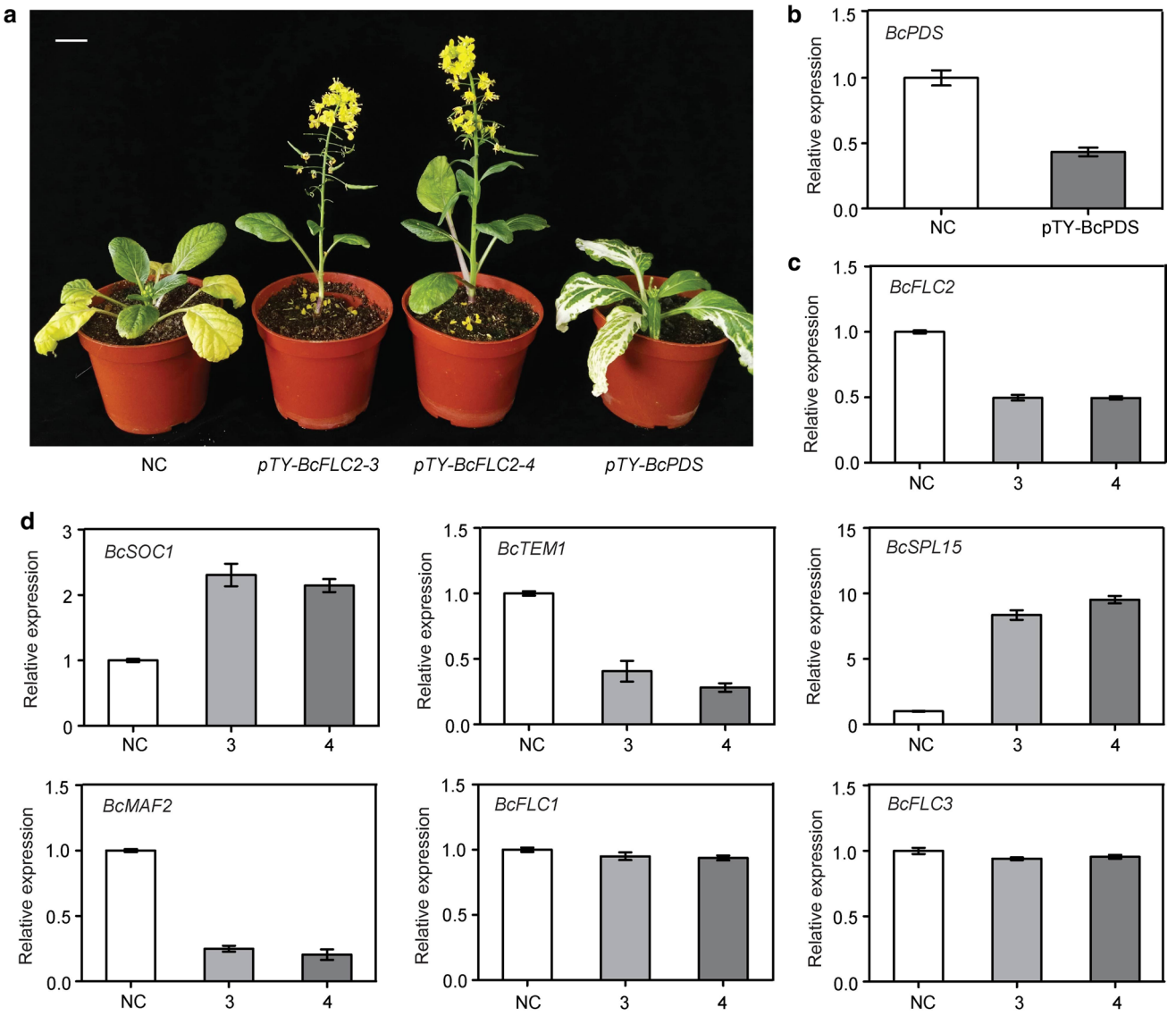

Fig. 5 **a** Early flowering phenotype of the *BcFLC2*-silencing Pak-choi plants. Pak-choi plants bombarded with *pTY* (NC), *pTY-BcFLC2* (*pTY-BcFLC2-3* and *pTY-BcFLC2-4*) and *pTY-BcPDS* plasmids. Scale bars = 2.5 cm. **b** Expression analysis of *BcPDS* in the NC and *pTY-BcPDS* plants. **c** Expression analysis of *BcFLC2* in the NC and *pTY-BcFLC2* plants. **d** QPCR analysis of predicted downstream genes in the NC and *pTY-BcFLC2* plants

suppression of both, *FT* and *SOC1* (Tao et al. 2012). *TEM1* functions as a direct *FT* repressor and acts upstream of *FT* (Ikeda and Ohme-Takagi 2009). Overexpression of *TEM1* in *Arabidopsis* results in a late-flowering phenotype, while the *tem1-1* mutation exhibits early flowering (Castillejo and Pelaz 2008). *SOC1* is a positive regulator of flowering and has been proven as the common target in multiple flowering pathways (Moon et al. 2003). *SPL15* promotes flowering and is involved in the transition from the vegetative to the reproductive phase (Hyun et al. 2016). Here, a yeast one-hybrid analysis showed that BcFLC2 could also bind to the promoters of *BcTEM1*, *BcSOC1* and *BcSPL15* (Fig. 6). *BcFLC2*, *BcSOC1* and *BcSPL15* were predominantly expressed in the leaves, while *BcFLC2* and *BcTEM1* were predominantly

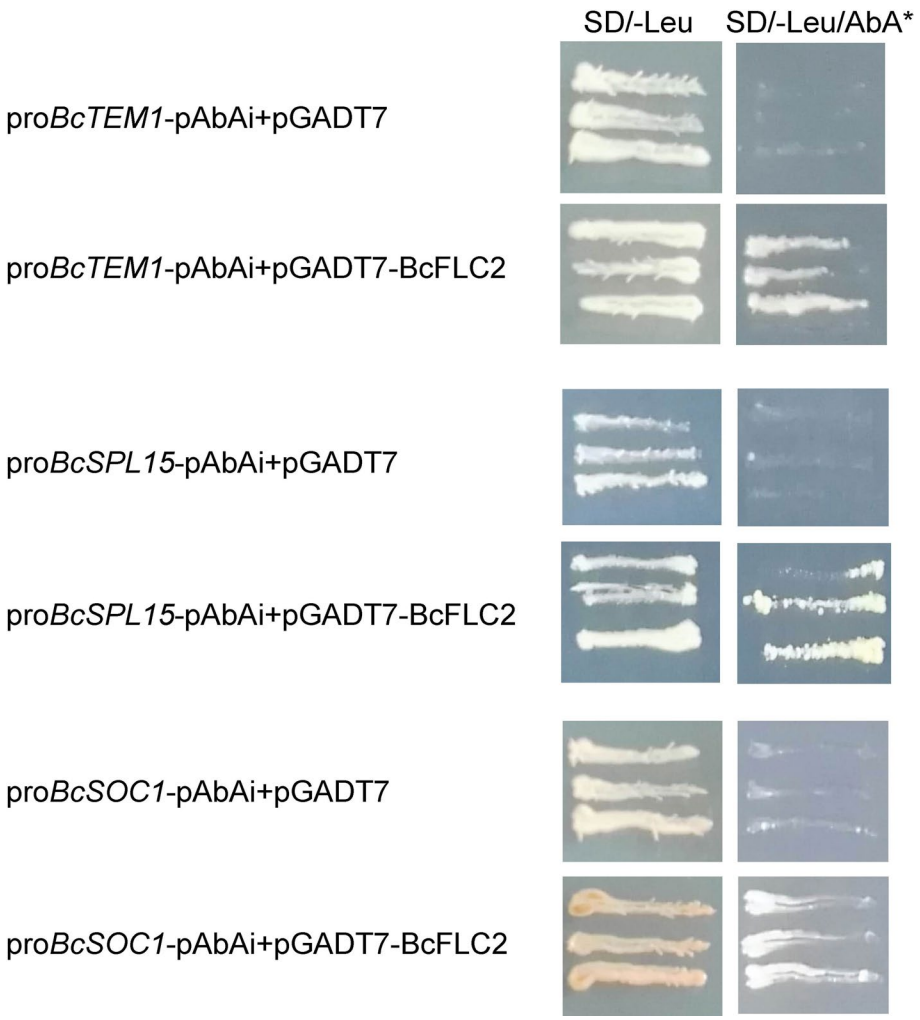

Fig. 6 Binding activities of BcFLC2 protein with the promoters of *BcTEM1*, *BcSOC1* and *BcSPL15* detected by yeast one-hybrid assays. Yeast cells were grown on an SD/-Leu medium plate supplemented with or without 300 ng/mL AbA

expressed in the stamens of Pak-choi (Figs. 2, S2). Thus, we suggested that BcFLC2 might directly repress *BcSOC1* and *BcSPL15* and activate *BcTEM1* expression in Pak-choi.

MAF2 encodes a floral repressor and can prevent premature vernalization under short periods of cold exposure. The *maf2* mutants flower earlier than the wild type after short periods of cold exposure but retain a normal vernalization response (Ratcliffe et al. 2003). The expression level of *AtMAF2* was up-regulated in the *BcFLC2*-overexpressing *Arabidopsis* plants, while the expression level of *BcMAF2* was down-regulated in *BcFLC2*-silenced Pak-choi (Figs. 4d, 5c), which suggested that BcFLC2 might positively regulate the expression of *BcMAF2*. In addition, the yeast one-hybrid assay showed that BcFLC2 directly bound to the *BcMAF2* promoter. In addition, *BcFLC2* and *BcMAF2* were all predominantly expressed in the leaves (Figs. 2, S2). Thus, we hypothesized that BcFLC2 might directly activate *BcMAF2* expression in Pak-choi. Although *FLC* predominantly acts as a repressor, it can also positively regulate genes. For example, FLC activates the expression of *SMZ* and *TOE3* to repress flowering (Deng et al. 2011).

In conclusion, the present work provides evidence that *BcFLC2* is a transcription factor and plays a crucial role in repressing flowering in Pak-choi (Fig. 8). BcFLC2 could directly bind to the promoters of *BcSOC1*, *BcSPL15*, *BcTEM1* and *BcMAF2* to regulate their expression. This study provides an important clue to the *BcFLC2*-mediated regulatory mechanism of flowering time in Pak-choi.

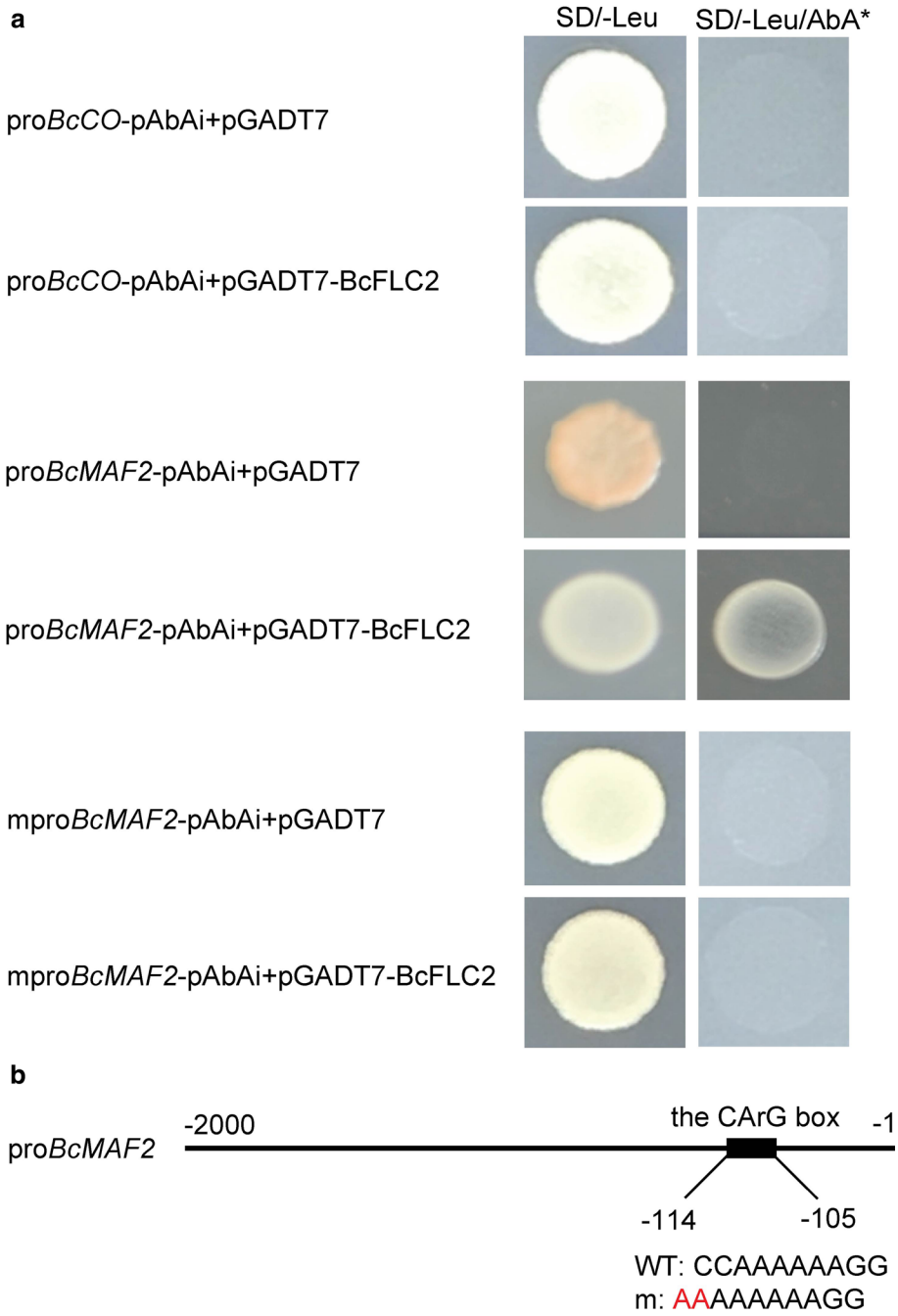

Fig. 7 **a** Binding activities of BcFLC2 protein with *BcMAF2* and *BcCO* promoters detected by yeast one-hybrid assays. Yeast cells were grown on an SD/-Leu medium plate supplemented with or without 300 ng/mL AbA. **b** Diagram of *BcMAF2* promoter. CArG box is represented by a black box. Red letters indicate positions of mutations

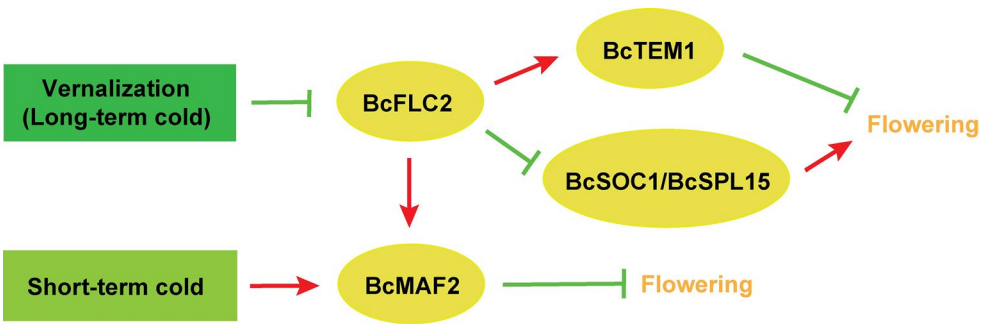

Fig. 8 Hypothetical model for the regulation of flowering time controlled by BcFLC2 in Pak-choi. Arrows and "T" bars represent positive and negative regulations, respectively

Author contribution statement FH, TL and JW conceived and designed the research. FH conducted experiments and wrote the manuscript. XH contributed to the interpretation of the results and coordinated the study. All authors read and approved the manuscript.

Acknowledgements We would like to thank Prof. Isabelle Jupin for providing the plasmid *pTY-S*. This work was supported by grants from the Integrated Innovation Center of Industrial Technology System of Modern Agriculture (vegetables) of Jiangsu (SXGC[2017]273), the Fundamental Research Funds for the Central Universities (Y0201700179) and the Major Program of National Key Research and Development of China (2017YFD0101803).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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