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 / Published online: 14 May 2018
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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  PDS  Phytoene desaturase
FLC  FLOWERING LOCUS C  SPL15  SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 15
FT  FLOWERING LOCUS T  SOC1  SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1
MAF  MADS AFFECTING FLOWERING  TEM1  TEMPRANILLO 1
NC  Negative control

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...
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 (Fujisawa 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* functions as a major repressor of flowering. *FLC* represses flowering time mainly results from the down-regulation of floral meristem identity genes to determine the exact flowering time (Fujisawa et al. 2003). These two integrators are antagonistically regulated by two upstream regulators: *FLC* and *FRI* have a late-flowering phenotype, while silencing of *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:soil, 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/).

**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 T0 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 T3 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 the yeast strain EBY100-1b and the transformants were separately plated on SD medium lacking leucine supplemented with 300 ng/mL AbA at 30 °C for 3 days.

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.
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 T₃ 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
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)GG) 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
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.
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
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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