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Over-expression of *BcFLC1* from non-heading Chinese cabbage enhances cold tolerance in *Arabidopsis*

T. LIU[1,2,3], Y. LI[1,2,3], J. REN[1,2,3], C. ZHANG[3], M. KONG[1,2,3], X. SONG[1,2,3], J. ZHOU[1,2,3], and X. HOU[1,2,3]*

State Key Laboratory of Crop Genetics and Germplasm Enhancement[1], Key Laboratory of Biology and Germplasm Enhancement of Horticultural Crops in East China[2], College of Horticulture[3], Nanjing Agricultural University, Nanjing 210095, P.R. China

Abstract

A gene (named *BcFLC1*) homologous to the *AtFLC* gene, which encodes a floral repressor, was isolated from the non-heading Chinese cabbage (*Brassica campestris* L. ssp. *chinensis*) cv. NJ074. The gene showed high similarity to *AtFLC*. For studying the gene function, we designed to introduce the *BcFLC1* gene into *Arabidopsis thaliana*. The results showed that *BcFLC1* had effects on flowering time similar to *AtFLC*. We also found that *Arabidopsis* cold-tolerance was enhanced by *BcFLC1* overexpression. Under low temperature stress, the *BcFLC1* transgenic plants exhibited stronger growth than wild-type plants. The elevated cold tolerance of the *BcFLC1* over-expressing plants was also confirmed by the changes of electrolyte leakage and malonyldialdehyde and proline content.

Additional key words: *Brassica campestris*, electrolyte leakage, flowering, low temperature, malondialdehyde, proline.

Introduction

In flowering plants, the switch from vegetative to reproductive growth is controlled by a number of developmental and environmental signals. Early genetic analyses have shown that the allelic variation in *FLOWERING LOCUS C* (*FLC*) is the main reason for the difference of flowering time between early and late flowering ecotypes (Michaels and Amasino 1999, Sheldon *et al*. 1999, 2000). *FLC*, which encodes a MADS-box transcription factor, functions as a repressor of flowering (Sheldon *et al*. 1999, 2000, He and Amasino 2005). Two flowering-time genes, *FT* and *SOC1*, have been reported to be targeted to *FLC* (Searle *et al*. 2006). *FLC* binds to the promoter of *SOC1* as well as to the first intron of *FT*. This binding prevents the transcriptional activation of these genes (Helliwell *et al*. 2006). Constitutive expression of *AtFLC* (Michaels *et al*. 1999, Sheldon *et al*. 1999) and the *Brassica rapa* ssp. *pekinesis* *FLC* homologs (Kim *et al*. 2007) in *Arabidopsis thaliana* could delay flowering, most likely through the direct repression of *FT* and *SOC1*.

Non-heading Chinese cabbage plants remain in the vegetative growth phase until they have experienced prolonged exposure to cold temperature known as vernalization. This inhibition of flowering is caused by the high *FLC* expression. To increase the product value by inhibiting the floral transition, *BcFLC1*, homologous to the *AtFLC* gene, was isolated from the non-heading Chinese cabbage cv. NJ074 (Rong *et al*. 2010). Through sequence and expression analysis, Rong *et al*. (2010) showed that *BcFLC1* likely acts similarly to *AtFLC* and plays a central role as repressors of flowering. To gain more information about the function of *BcFLC1*, further investigation was done in the current paper.

Cold induces the expression of many genes encoding a diverse array of proteins that enhance the tolerance of plants to low temperature. Such CRT/DRE (C-repeat/dehydration response elements) binding factors (CBFs) act as the key regulator of cold response pathway in *Arabidopsis* and COR genes share CRT/DRE in their promoters (Movahedi *et al*. 2012, Thomashow 1999). It was reported that overexpression of *CBF1*, *CBF2*, and *CBF3* causes late flowering and dwarf phenotypes as well

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Abbreviations: bp - base pair; CBF - C-binding factor; CRT/DRE - C-repeat/dehydration responsive element; *FLC* - *FLOWERING LOCUS C*; GAPDH - glyceraldehyde-3-phosphate dehydrogenase; MDA - malondialdehyde; RT-PCR - reverse transcriptase-polymerase chain reaction.

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* Corresponding author; fax: (+86) 025 8439-5266, e-mail: hxl@njau.edu.cn

as properties associated with cold tolerance, such as increases of proline and sugar content and transcriptional activation of COR genes (Gilmour *et al*. 2004). Seo *et al*. (2009) suggested that *SOC1* and *FLC* are the key regulators of cross talk between cold response and flowering time regulation. It was shown *SOC1* functions as a negative regulator of the cold response pathway through the direct repression of CBFs. However, whether *FLC* affects the expression of the *CBF* was not analyzed. Therefore, we discussed the cold tolerance in *35S::BcFLC1*.

In this paper, we transformed *BcFLC1* into *Arabidopsis thaliana* which is the most popular model plant nowadays. The gene was expressed at high steady-state mRNA levels in transformed plants leaves. We found *BcFLC1* can delay flowering in *Arabidopsis*. In *35S::BcFLC1* line, *CBF1,2,3* and *COR15a* expression were increased in comparison to that in the wild type. Through cold-tolerance assays, *BcFLC1* transgenic plants revealed high cold tolerance.

Materials and methods

Seeds of wild-type *Arabidopsis thaliana* L. cv. Columbia (Col) were germinated on moist filter paper at 4 °C for 4 or 5 d. Then, plants were grown in a 1:1 mixture of soil and *Vermiculite* in growth chambers at temperature of 22 °C, relative humidity of 60 %, 16-h photoperiod, and irradiance of 100 μmol m^{-2} s^{-1} (cool white fluorescent tubes).

According to Rong *et al*. (2010), specific primers BcFLC1-F (5'-ATGGGGAGGAAGAAACTTGA-3') and BcFLC1-R (5'-CTATAAAAATTCCACTCCCA-3') were designed to amplify *BcFLC1* gene using the cDNA from leaves of non-heading Chinese cabbage cv. NJ074 seedlings (with five leaves) grown in the same growth chamber. To express the *BcFLC1* genes in *Arabidopsis*, *BcFLC1* gene was inserted into the pEarleyGate103 binary vector along with an herbicide-resistance gene (*bar*; Interuniversity Institute for Biotechnology, http://www.psb.rug.ac.be/gateway) as a selectable maker using *Gateway* technology (*Invitrogen*, https://www.invitrogen.com) to construct pGate-BcFLC1. The final binary vector, pGate-BcFLC1, was introduced into *Agrobacterium tumefaciens* strain GV3101.

Arabidopsis thaliana flowers were sprayed with *A. tumefaciens* GV3101 suspended in 5 % (m/v) sucrose, and then the plants were incubated in a growth chamber at 25 °C and 100 % humidity for 1 d and finally allowed to grow in a growth chamber under a 16-h photoperiod and 23 °C. Transformants were selected using two applications of herbicide (0.3 % *Basta*) and PCR assay.

Total RNA was isolated from plant leaves by the RNeasy plant mini kit (*Qiagen*, Germany) according to the manufacturer's instructions. For cDNA production, 4 μg of total RNA was reverse transcribed with *Oligo* $(dT)_{18}$ primer (*Fermentas*) in a 0.02 cm^3 reaction mixture using *RevertAid M-MLV* reverse transcriptase (*Fermentas*). After heat inactivation, total volume of the reaction mixture was diluted to 0.58 cm^3 with sterilized water, and 0.004 cm^3 was used for the real-time quantitative RT-PCR. All quantitative RT-PCR analyses were performed by *iQ5* multicolor real-time PCR detection system (*Bio-Rad*, Hercules, USA) using 2× *SYBR Green SuperMix* (*Bio-Rad*). We adopted the guidelines for the experimental design and statistical analysis of quantitative RT-PCR data (Rieu and Powers 2009). The PCR conditions were as follows: 40 cycles of after the initial 2-min denaturation step at 95 °C. Data were collected at 72 °C in each cycle and the expression levels of genes were calculated by *iQ5* optical system software *v. 2.0* using *TUB2* as the reference gene. The quantitative RT-PCR analysis was biologically repeated three times and each consisted of three technical replicates. Real-time RT-PCR primer sequences are shown in Table 1.

Table 1. The primers used in this work. Sequence data can be found in the GenBank data library under the accession numbers: *Bar* (AJ251014.1); *GFP* (U28417.1); *BcFLC1* (not logged); *LFY* (NM_125579.1); *FT* (NM_105222.2); *SOC1* (NM_130128.3); *CBF1* (AF076155.1); *CBF2* (AF074601.1); *CBF3* (AF074602.1); *COR15a* (NM_129815.4); and *TUB2* (NM_125664.3).

Gene name	Direction	Sequence (5'-3')
Bar	F	TGGAAGGCACGCAACGCCTA
	R	TATCCGAGCGCCTCGTGCAT
GFP	F	GCTGAAGTCAAGTTTGAAGG
	R	AACTCAAGAAGGACCATGTG
BcFLC1	F	AGTGTGGGTTCCCTGGTTCAGCT
	R	TCAGCTTCGGCTCGCACAAGATT
LFY	F	GTGGAACCCAACGAGAGCA
	R	ACCAAGTCGCATCCCAAAAG
FT	F	CAGGTTCAAAACAAGCCAAGAG
	R	ATTCTCGGAGGTGAGGGTTG
SOC1	F	ATCGAGTCAGCACCAAACCGGT
	R	CTGTTGCAGCTCCTCGATTGAGCA
CBF1	F	GAGAGAGCCAAACAAGAAAACCA
	R	CCGAGTCAGCGAAGTTGAGA
CBF2	F	ACGAGCTGCCCCAAGAAAC
	R	AAAGTCCCGAGCCAAATCCT
CBF3	F	GGCTATTTACACGGCGGAAC
	R	AGCCAACAAACTCGGCATCT
COR15a	F	GAAAGCTGCGGCGTATGTG
	R	GCATCCTTAGCCTCTCCTGCT
TUB2	F	TCGTCCTACTTTGTGGAGTGG
	R	CTCGCCTGAACATCTCTTGG

Flowering time was determined by the number of rosette leaves formed when the inflorescent stem reached approximately 1 to 3 cm in length. The flowering time of 20 independent T_2 lines were assessed.

Three-week-old plants were placed at -4°C for 3 h and

then they were incubated at 23 °C for 3 d for recovery. The percentage of plants that survived after this cold stress and recovery was calculated. Electrolyte leakage was determined in excised leaves immersed in 1.5 mM 5-chloro-3-methyl-4-nitro-1H-pyrazole (CMNP) solution or water and measured as described by Alferez *et al*. (2006). The extent of lipid peroxidation in leaves was estimated by measuring the amount of MDA, a decomposition product of the oxidation of polyunsaturated fatty acids, as described Havaux *et al*. (2003). Proline content was measured as described by Bates *et al*. (1973) and Gupta *et al.* (2012).

Results and discussion

In this paper, we studied *BcFLC1* gene function through transferring this gene into *A. thaliana*. Constitutive expression of *BcFLC1* in *A. thaliana* delayed flowering. We obtained transgenic plants that were identified by RT-PCR (Fig. 1). We selected two transgenic lines (called OE11 and OE15, respectively) that demonstrated relatively high expression of *BcFLC1*. Primary transformants T_2 of *BcFLC1* (OE11) flowered when they had 19 ± 0.4 rosette leaves, whereas wild-type only 10 ± 0.7 indicating that overexpression of *BcFLC1* led to late flowering. Thus, transgenic *Arabidopsis* plants overexpressing the *BcFLC1* gene flowered approximately 10 d later than nontransgenic plants. These results indicates that *BcFLC1* has an effect on flowering time similar to *AtFLC*.

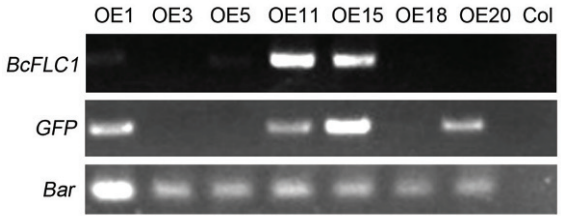

Fig. 1. Identification of *BcFLC1* gene in T_1 generation of transgenic *Arabidopsis* plants with PCR. Col - Columbia, OE1, 3, 5, 11, 15, 18, and 20 - transgenic plants.

FLC is a repressor of *SOC1* (Lee *et al*. 2000, Helliwell *et al*. 2006). *Soc1-2* mutant increases the induction of *CBFs* and *COR* genes improving the cold tolerance of *Arabidopsis* (Seo *et al*. 2009). Therefore, we examined the *CBF1, 2, 3,* and *COR15a* transcript levels in *35S::BcFLC1* overexpression line. Compared with wild-type, these genes showed increased expression in seedlings (Table 3). It was interesting to determine whether *35S::BcFLC1* overexpression line exhibit differences in cold resistance. To address this question, plants were exposed to -4 °C for 3 h and transferred to room temperature to check the survival rate. As expected, more *35S::BcFLC1* plants survived than the wild-type ones (Fig. 2). Therefore, we could consider that *BcFLC1* may induce cold-tolerance in transgenic lines.

Increased electrolyte leakage is used to monitor loss of membrane integrity, and hence, indicates cellular damage caused by stress (Lafuente *et al*. 1991). We examined the effects of low temperature on the electrolyte leakage in the leaves of transgenic lines (OE11 and OE15) and wild-type seedlings. The electrolyte leakage in wild-type and transgenic plants increased after cold treatment. However, the increase in the transgenic plants was significantly lower than that in wild-type plants (Table 2). This result showed that wild-type seedlings were more damaged than transgenic plants at low temperature.

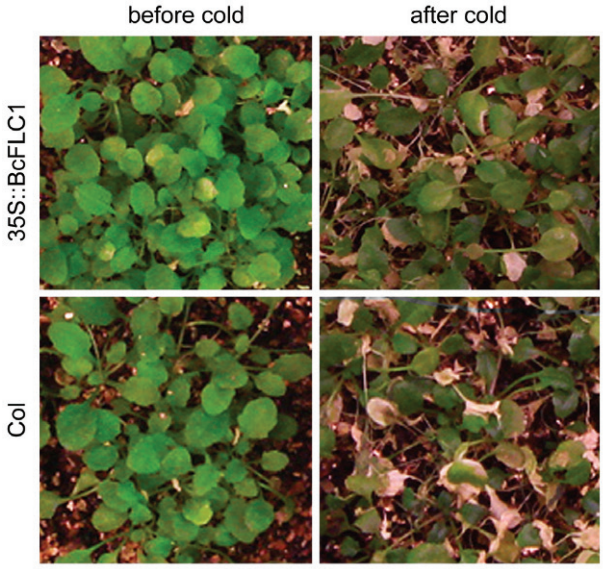

Fig. 2. Effect of *BcFLC1* on cold-tolerance in *Arabidopsis*. The cold-tolerance of 35S::BcFLC1 transgenic plants compared with wild type Col plants.

The level of lipid peroxidation for wild-type and transgenic plants was measured on the basis of the accumulation of malondialdehyde (MDA). MDA content was low under normal conditions but increased after cold treatment. This increase was higher in wild type than in transgenic lines (Table 2).

Proline appears to be the most widely distributed osmolyte accumulated under stress conditions (Chen *et al*. 2007). The proline content at cold temperature stress was higher than under normal conditions. The increase of proline content in the transgenic plants was significantly higher than in wild-type plants (Table 2).

As important integrators of floral signaling pathways, the *FT*, *SOC1*, and *LFY* genes respond to the light, cold, gibberellins, and the developmental state of the plant (Helliwell *et al*. 2006). In this paper, we examined the *SOC1*, *LFY*, and *FT* transcript level in both *35S::BcFLC1*

Table 2. Effects of low temperature stress (4 °C for 2 d) on eletrolyte leakage, proline content, and MDA content in the wild-type (WT) plants and transgenic lines (OE11 and OE15). Means ± SE of three replicates. The same letters represent no significant differences between means at $P < 0.05$ determined by Tukey's test.

Parameter		Control	Cold stress
Leakage	WT	5.37 ± 0.21^{d}	21.30 ± 0.44^{a}
[%]	OE11	5.17 ± 0.31^{d}	13.03 ± 0.15^{c}
	OE15	5.03 ± 0.10^{d}	15.07 ± 0.25^{b}
Proline	WT	0.03 ± 0.01^{e}	0.12 ± 0.015^{c}
[μmol g^{-1}(f.m.)]	OE11	0.05 ± 0.005^{de}	0.18 ± 0.011^{b}
	OE15	0.07 ± 0.015^{d}	0.22 ± 0.026^{a}
MDA	WT	0.13 ± 0.005^{d}	0.28 ± 0.015^{a}
[nmol g^{-1}(f.m.)]	OE11	0.10 ± 0.015^{d}	0.21 ± 0.01^{b}
	OE15	0.12 ± 0.01^{d}	0.18 ± 0.02^{c}

Table 3. Effect of *BcFLC1* on gene expressions in *35S::BcFLC1* line. Expression levels of *CBF1*, *CBF2*, *CBF3*, *COR15a*, *SOC1*, *LFY*, and *FT* were determined by quantitative RT-PCR in wild type (Col) and 35S::BcFLC1-11 line. Means ± SE of three replicates. The same letters represent no significant differences between means at $P < 0.05$ determined by Tukey's test.

Gene	Col	35S::BcFLC1-11
CBF1	1.7282 ± 0.1727^{b}	2.8618 ± 0.2427^{a}
CBF2	0.1867 ± 0.0125^{b}	0.3675 ± 0.0357^{a}
CBF3	0.1458 ± 0.0168^{b}	0.4856 ± 0.0330^{a}
COR15a	2.7018 ± 0.2925^{b}	6.4809 ± 0.0353^{a}
SOC1	4.0338 ± 0.1812^{a}	0.6379 ± 0.0210^{b}
LFY	0.1841 ± 0.0176^{b}	2.1020 ± 0.2977^{a}
FT	0.9842 ± 0.1684^{b}	5.7554 ± 0.2814^{a}

overexpression line and wild-type when flowering. The results showed that the *SOC1* expression decreased but the level of *LFY* and *FT* mRNA expression increased compared with wild-type (Table 3). The analysis revealed that the *35S::BcFLC1* overexpression line in *Arabidopsis* requires a greater dose of *LFY* and *FT* to flower than the wild-type. Onouchi *et al*. (2000) found *soc1 35S::CO* mutants not only come into bloom but also having similar number of rosette leaves like wild type when flowering. So we suggested the high expression of *LFY* and *FT* could overcome the deficiency of *SOC1*.

Seo *et al*. (2009) reported overexpression of CBFs increased the expression of *FLC*. In our paper, we checked if CBFs expression is affected by overexpression of *BcFLC1* which has function similar to *AtFLC*. Indeed, the CBFs expression was increased about 2-fold in *35S::BcFLC1* (Table 3). These results suggest *FLC* may show feedback regulation in the expression of CBFs. From cold tolerance assays, we find almost all of the wild-type plants died whereas many of the transgenic plants survived. This also supports an idea that *BcFLC1* increases the induction of CBFs and COR genes improving the cold tolerance of *Arabidopsis*.

Low temperature stress caused a significant increase in electrolyte leakage. The accumulation of malon-dialdehyde (MDA) is often used as an indicator of lipid peroxidation (Smirnoff *et al*. 1995, Belintani *et al.* 2012). Membrane lipid peroxidation occurs from a malfunction of the scavenging system which can lead to the damage of main cellular components (Monk *et al*. 1989). The degree of cold injury in transgenic plants was estimated from the MDA content indicating the extent of lipid peroxidation after exposing the plants to cold stress. The MDA content was also increased after low temperature stress, but the degree of increase in the transgenic plant was lower than that in the wild-type (Table 2). Low electrolyte leakage and less MDA accumulated in transgenic lines than in wild-type plants (Table 2) suggested enhanced cold-tolerance because the *BcFLC1* gene was over-expressed in transgenic plants at low temperature stress. *FLC* is a repressor of *SOC1* (Lee *et al*. 2000, Helliwell *et al*. 2006). In *35S::BcFLC1* over-expression line, the expression of *SOC1* was repressed (Table 3). So we suggested the induction of cold-inducible genes may occur through the *SOC1*-dependent pathway.

Taken together, our results indicate that over-expression of the *BcFLC1* gene in *Arabidopsis* may contri-bute to cold-tolerance by regulating expression of cold-related genes in *Arabidopsis* under low temperature stress. Further investigation should be done to get more information about the function of *BcFLC1*.

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