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RESEARCH ARTICLE

Isolation and characterization of an *ERF-B3* gene associated with flower abnormalities in non-heading Chinese cabbage

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Abstract

BrcERF-B3 gene, a member of ethylene-responsive factor family, was screened from a mutant plant in non-heading Chinese cabbage (*Brassica rapa* ssp. *chinensis*) by cDNA-AFLP technology. We got full length cDNA of two *BrcERF-B3* genes by homology-based cloning from two materials and found that their nucleotide sequences were the same by sequencing. The BrcERF-B3 protein, belonging to the B3 subgroup of the ERF subfamily, shared a close relationship with *B. rapa*. RT-PCR result showed that *BrcERF-B3* expressed only in mutant stamen rather than maintainer stamen. qRT-PCR results indicated that *BrcERF-B3* expressed highly during reproductive growth development and in the early of mutant buds, suggesting *BrcERF-B3* might be involved in the formation of abnormal flower in mutant. What's more, the expression of *BrcERF-B3* was more significant to ABA, MeJA and cold stresses in mutant than in maintainer and was down-regulated in NaCl treatment in two lines, implying *BrcERF-B3* might be different roles in biotic and abiotic stresses.

Keywords: non-heading Chinese cabbage, stamen-petalody, ethylene-responsive factor, gene expression

1. Introduction

Controlling the fertility was an important goal in crop hybrid breeding, but it was difficult in some crops including non-heading Chinese cabbage. Most of phenotype of male sterile lines (CMS or GMS) was instable, whereas the stamen-dismissing material which was caused by stamen homologous transformation had a relatively stable sterility. Plant flower architecture was controlled by some key players (such as transcription factors), some other reports showed that homeotic conversion of stamens into petaloid structures in the basal Endicott *Eschscholzia californica* occurred due to the knocking down of *EScaAG*1 and 2 (Yellina *et al*. 2010). The third and fourth whorls in androecium and gynoecium had a homeotic transformation because of the down-regulation of both *PapsAG* homologs concurrently (Hands *et al*. 2011).

AP2/ERF transcription factor, one of the largest plant transcription factor families, played a crucial role in plant growing and in response to biotic and abiotic stresses (Riechmannn *et al*. 2000; Sakuma *et al*. 2002; Zhuang *et al*. 2008). The AP2/ERF superfamily was defined by the AP2/ERF domain, which consists about 60–70 amino acids and involved in DNA binding (Nakano *et al*. 2006). Based on differences in the binding domain sequences, the AP2/

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ERF superfamily can be grouped into five main subfamilies, AP2, CBF/DREB, ERF, RAV and one soloist, *At4g13040* (Sakuma *et al*. 2002; Nakano *et al*. 2006). They played a different role in the regulation of the plant, and there was a mutual regulation relation among them (Zhao *et al*. 2006). Ethylene-responsive transcription factors (ERFs) were firstly identified from tobacco as GCC box-binding proteins and could either induce or repress the expression of genes containing the GCC box and related elements in their promoters. Sequence analysis showed that ERFs contained a highly conserved, plant specific DNA-binding domain (DBD) consisting of 58–59 amino acids (Ohme-Takagi and Shinshi 1995). At present, the percentage of total *ERF* genes in all the AP2/ERF family has been reported in several plants, such as *Arabidopsis* (44.2%), *populus trichocarpa* (45.5%), Chinese cabbage (45.7%), *Vitis vinifera* (55%) and rice (48.2%) (Sakuma *et al*. 2002; Nakano *et al*. 2006; Zhuang *et al*. 2008, 2009; Li *et al*. 2013). Based on the sequence identities of their DBD, the ERF subfamily members can be classified into six small subgroups (B1 through B6) (Sakuma *et al*. 2002).

ERF family genes were reported mostly in responses to biotic and abiotic stresses (Gutterson and Reuber 2004; Kizis *et al*. 2001). The group IX *ERF* genes in cotton may be involved in jasmonate (JA), ethylene (ET) responses (Champion *et al*. 2009). *Arabidopsis* ERF family members B3 subgroup *AtERF98* regulated ABA synthesis and involved in salt stress (Zhang *et al*. 2012). It is reported that *AtERF13* and *AtERF15* regulate ABA response positively (McGrath *et al*. 2005), however, few researches associated with flower development have been found. Non-heading Chinese cabbage (*Brassica rapa* ssp. *chinensis*) is a cross-pollinated crop that is widely cultivated in East Asia. We created a stamen-petalody mutant line through chemical mutagenesis, in which stamens converted into petaloid and flower turning into unisexual female flower. We previously obtained an *ERF* gene fragment by cDNA-AFLP technique, and it was named as *BrcERF-B3* (not logged). However, the transcriptional regulatory function of *BrcERF-B3* and its expression levels remains unclear. In this study, we cloned the *BrcERF-B3* gene from the leaves and analyzed the expression levels of *BrcERF-B3* in different organs in two lines. In addition, we also analyzed the effects of *BrcERF-B3* responses to biotic and abiotic stresses. These results indicated that the expression level of *BrcERF-B3* expressed only in mutant stamen at the early bud stage. The *BrcERF-B3* existed different expression profiles in response to plant hormone, cold and NaCl treatments. These works will provide theoretical base for further studying stamen-petalody in non-heading Chinese cabbage.

2. Results

2.1. Cloning the *BrcERF-B3* cDNAs from cabbage

The *BrcERF-B3* genes were cloned from two different cabbage lines (Fig. 1), and both of their open reading frame (ORF) fragments were 807 bp in length. The sequencing results showed that nucleotide sequences of two *BrcERF-B3* genes were identical.

Based on the similarity of the amino acid sequences of their DNA-binding domains (DBD), a phylogenetic tree was created from the deduced amino acid sequences of BrcERF-B3 and other ERF proteins from *Arabidopsis*. The result revealed that BrcERF-B3 belonged to the B3 group of the ERF subfamily (Figs. 2 and 3). The alignment of the ERF-B3 showed that DBD of BrcERF-B3 shared a high degree of sequence homology with ERF-B3 from other species. The DBD of BrcERF-B3 consisted of 3 anti-parallel β-sheet and 1 α-helix, besides, the F (phenylalanine) in BrERF-B3 was replaced by T (threonine) in BrcERF-B3, N (asparagine) in BnERF-B3 was replaced by D (aspartic acid) in BrcERF-B3, respectively, these residues appeared to be responsible for binding specificity (Fig. 4). BrcERF-B3 exhibited much greater similarity (95%) to those of BrERF-B3 (Fig. 5).

2.2. Analysis of the expression of *BrcERF-B3* gene

BrcERF-B3 was completed at different leaf development stages by qRT-PCR. The gene expression profile presented in Fig. 6 showed *BrcERF-B3* notably increased during Rosette stage, then significantly decreased in maintainer line,

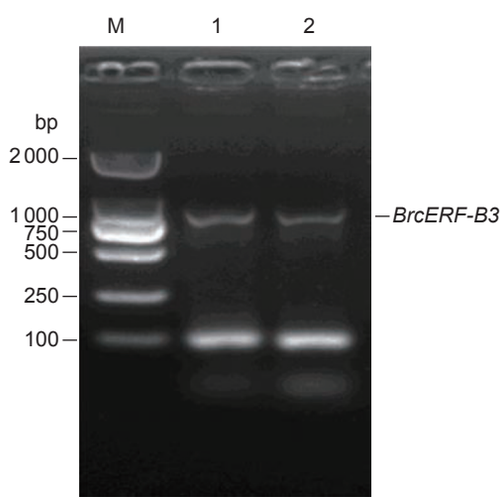

Fig. 1 The open reading frame of *BrcERF-B3* amplification products. M, DL2000 marker; 1, mutant; 2, maintainer.

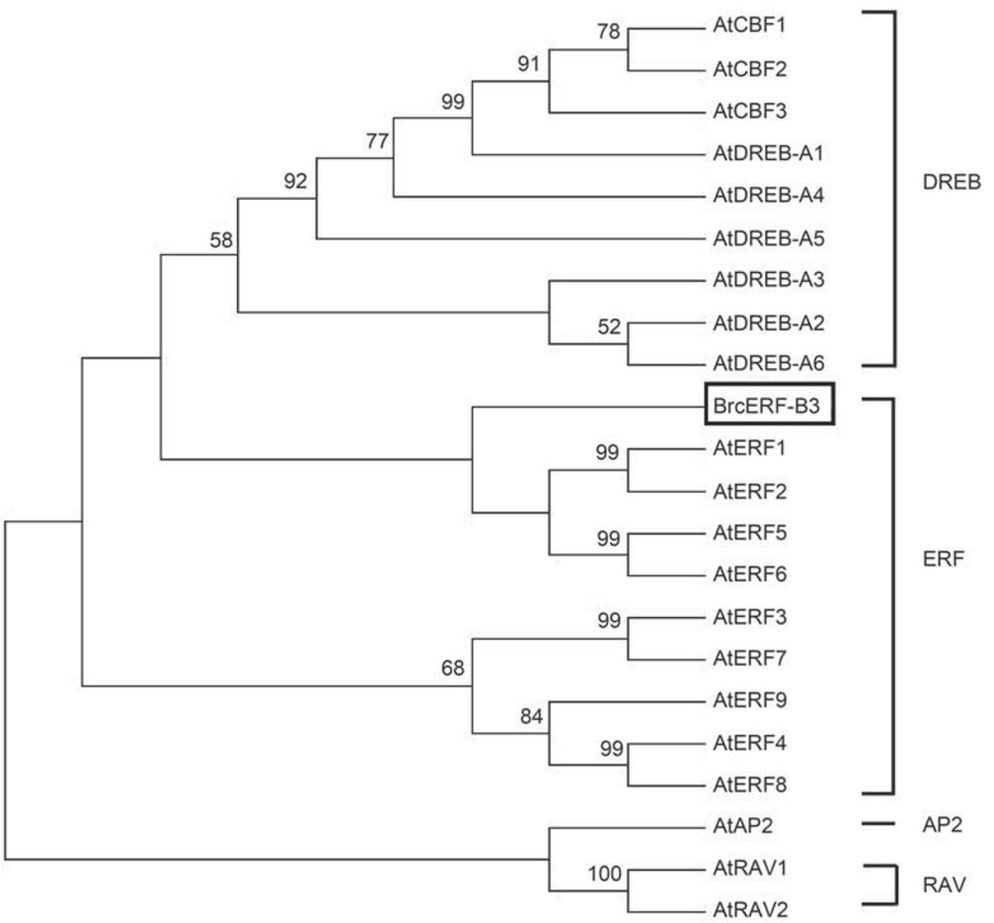

Fig. 2 The phylogenetic analysis between BrcERF-B3 and the *Arabidopsis thaliana* AP2/ERF superfamily transcription factors. The phylogenetic tree was produced with the neighbor-joining method by the MEGA 5 software. The numbers above the branches indicated the reliability percent of bootstrap values from 1 000 replicates. BrcERF-B3 was boxed. The same as below.

while the expression of *BrcERF-B3* in mutant firstly subtly decreased at rosette stage, then significantly increased, and keep a higher levels, suggested that *BrcERF-B3* enhanced reproductive growth development. The expression profile of *BrcERF-B3* gene showed unobvious expression difference during flower development stages in maintainer line, but the gene expressed significantly higher in mutant than in maintainer at the flower development (bud diameter <2 mm) stages indicated that *BrcERF-B3* might enhance abnormal flower development in mutant (Fig. 7).

RT-PCR was carried out on cDNA derived from floral organs to learn more about the *BrcERF-B3* expression profile. The results showed that *BrcERF-B3* expressed strongly in petal, stamen and carpal in mutant and slightly expressed in sepal, petal and carpel in maintainer. Particularly, *BrcERF-B3* expressed obviously higher in mutant stamen than in maintainer stamen, which meant that *BrcERF-B3* gene acted a positive role in the formation of mutant stamen (Fig. 8).

2.3. Investigating the responses of *BrcERF-B3* on different stresses

After ABA and MeJA treatments, similar trend were found in expression of *BrcERF-B3* in mutant and *BrcERF-B3* was up-regulated and then down-regulated as time goes on. In maintainer, the expression of *BrcERF-B3* was decreased compared to 0 h excepting at 1 h MeJA treatment. The expression of *BrcERF-B3* existed significant difference between maintainer line and mutant under ABA treatment and at 1, 2, and 12 h MeJA treatment. During the cold treatment, *BrcERF-B3* gene initially increased at 1 h and subsequently slowly reached peaks at the transcription level at 4 and 2 h in mutant and maintainer, respectively, then decreased slowly. The expression of the gene in mutant was significantly higher than in maintainer at 2–12 h. The transcription levels of *BrcERF-B3* in mutant and maintainer was obviously down-regulated in the NaCl treatment (Fig. 9).

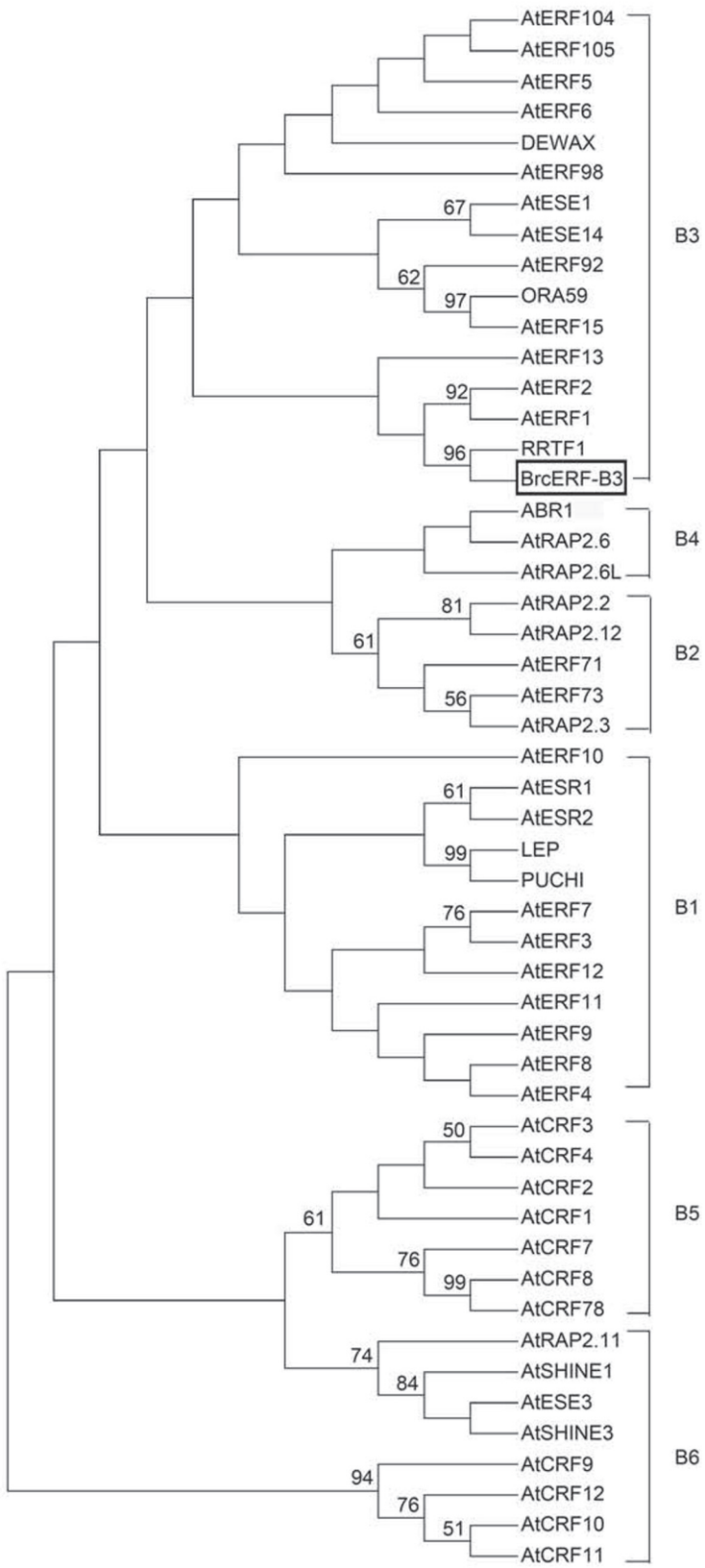

Fig. 3 The phylogenetic analysis of BrcERF-B3 with each subgroup of ERF superfamily transcription factors from *A. thaliana*. The phylogenetic tree was produced with the neighbor-joining method by the MEGA 5 software.

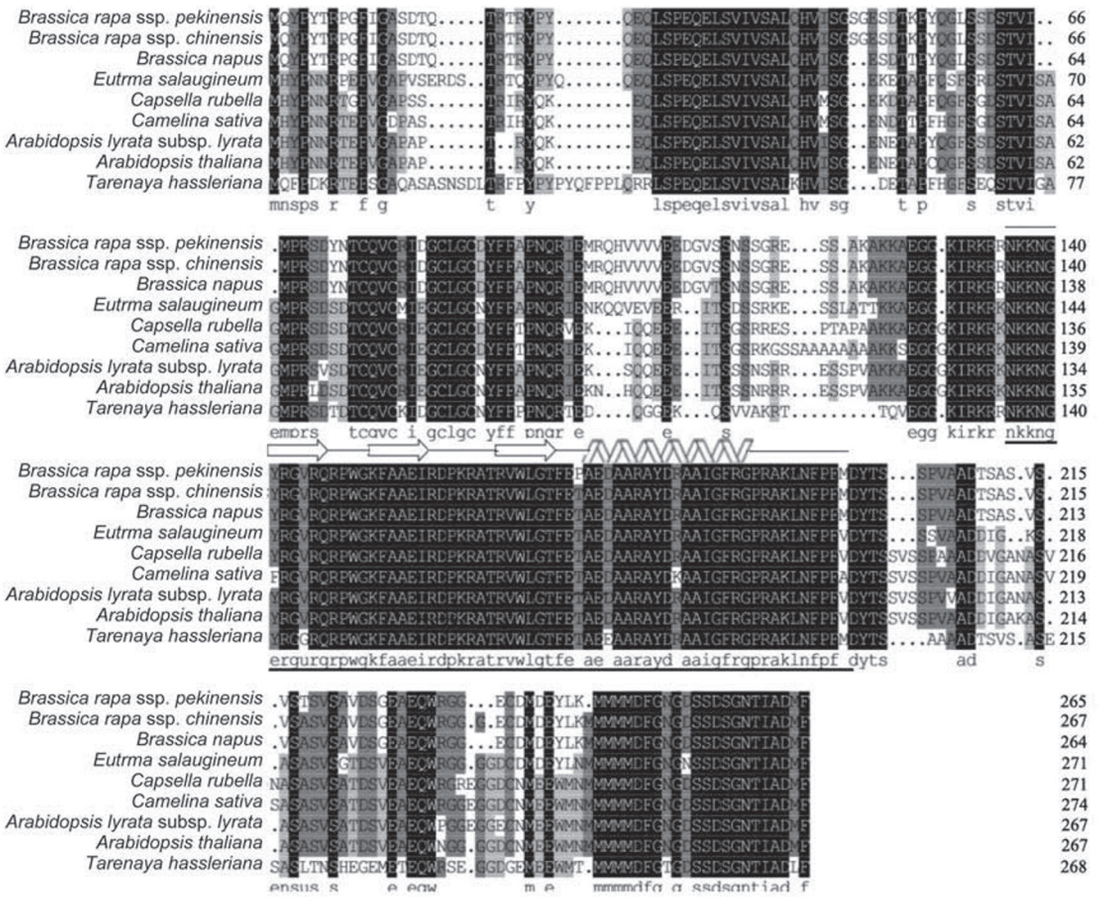

Fig. 4 Alignment of the conserved ERF signatures of BrcERF-B3 with other ERF-B3 from different plants. The identical amino acid residues were indicated with black background, while 75% conservation was marked as gray. The deduced motifs (3 β-sheets and 1 α-helix) were marked.

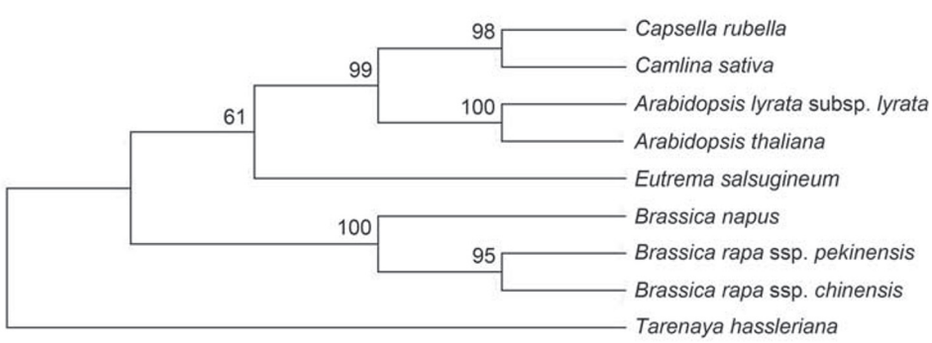

Fig. 5 The homology tree was conducted to show the relationships of ERF-B3 among different plants.

3. Discussion

Non-heading Chinese cabbage is an important crop in eastern Asia. Stamen-petalody is a novel type of male sterility in non-heading Chinese cabbage. In this study, we cloned the full-ORF cDNA sequences of *BrcERF-B3* from two non-heading Chinese cabbage lines and discovered that the nucleotide sequences of two *BrcERF-B3* genes were identical between these two lines. The phylogenetic tree revealed that BrcERF-B3 belonged to the ERF-B3 subfamily, which is similar to the result study that most of 77 encoding ERF-like proteins of ERF superfamily belonging to B3 in rice (Sharoni *et al*. 2011). The DBD of BrcERF-B3 protein, which contained 3 anti-parallel β-sheet and 1 α-helix, combined

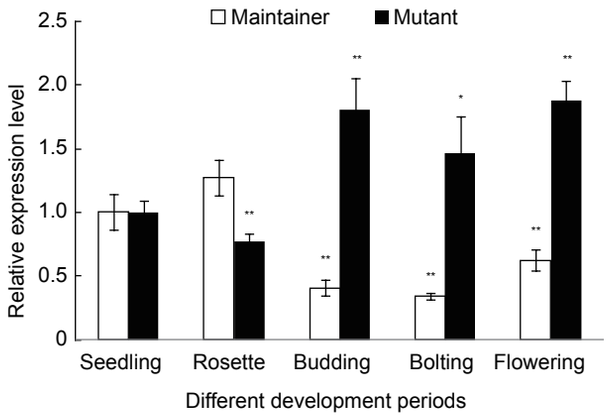

Fig. 6 Expression of *BrcERFB3* in different development periods. Seedling, five-leaf stage; rosette, fifteen-leaf stage; budding, squaring stage; bolting, bolting stage; flowering, blossom stage. *, $P<0.05$; **, $P<0.01$. The data of each column are means±SD ($n=3$). The same as below.

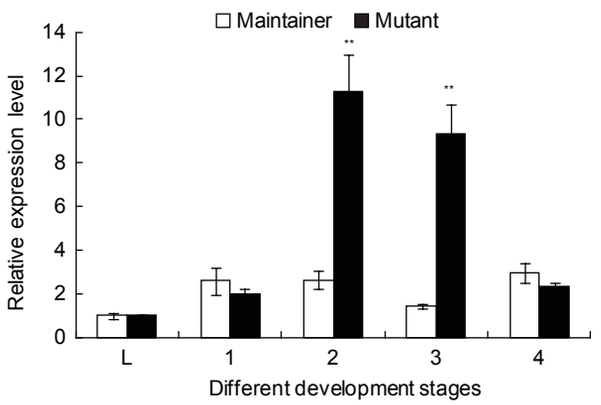

Fig. 7 Expression of *BrcERF-B3* gene at different flower development stages in mutant and maintainer lines. L, leaves; 1, 2, 3, 4, bud diameter (<0.5, 0.5–1, 1–2, and >2 mm, respectively).

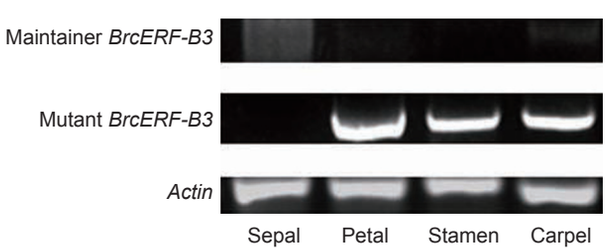

Fig. 8 Expression of *BrcERF-B3* gene in different flower organs of non-heading Chinese cabbage.

with the GCC box of the downstream gene promoter and activated the gene expression (Tang *et al*. 2006).

ERF genes had an important effect on the development of flower organs. *GmMADS28* expressed highly in stamen and petal in soybean mutant line 'NJS-10Hfs' which was stamen-petalody and constitutive expression of *GmMADS28* in tobacco caused early flowering and converted stamen-petalody and -sepalody (Huang *et al*. 2014). *SlERF52* gene specifically expressed in stalk and RNAi inhibited the gene expression, suggesting that *SlERF52* functioned in shedding process of stalk (Nakano *et al*. 2014). The expressions of *BrcERF-B3* were higher in mutant than in maintainer during reproductive growth development. The *BrcERF-B3* expression differences between maintainer and mutant in floral buds and floral organs hinted expression of *BrcERF-B3* might affect normal formation of petals and stamens by increasing the expression profile of nuclear gene in the bud development.

The transcriptional regulator with flowering, induced by environmental stress, would result in homeotic changes and produce flowers with an abnormal appearance (Ito *et al*. 2007). The ABA biosynthesis and signal transduction pathway occurred concomitantly with the transition of the apex to a closed bud structure (Ruttink *et al*. 2007). MeJA disrupted the balance of the gene expressions essential for cell differentiation in flower buds, being responsible for the different kinds of flower abnormalities in oilseed rape flowers (Pak *et al*. 2009). ERF proteins played an important role in response to salt stress. *ESE1–ESE3* were induced by high salt, *ESE1* regulated the expression of salt-related genes by combining with EIN3 (Zhang *et al*. 2011). Under cold treatment, *DREB1A*, *DREB1B*, and *DREB1C* expressed in *Arabidopsis* leaf and root, and it took part in cold-stress signal transduction pathways through the *cis*-element, DRE (Sakuma *et al*. 2001)

In our experiment, *BrcERF-B3* was more sensitive to plant hormone and cold temperature in mutant than that in maintainer. Plant hormone and cold treatments up-regulated obviously the level expression of *BrcERF-B3*, but the expression of *BrcERF-B3* was down-regulated after 4 h hormone stress. NaCl treatment strongly decreased the expression of *BrcERF-B3* in two lines. These results showed that *BrcERF-B3* might take part in the responses to stresses in mutant, however, the regulation mechanism needed the further study.

4. Conclusion

In conclusion, a *BrcERF-B3* gene was isolated from non-heading Chinese cabbage (*Brassica rapa* ssp. *chinensis*). This gene affects abnormal flower formation in mutant line and plays a special role in biotic and abiotic stresses. This work is likely to contribute to create new sterile materials that could be applied in hybrid breeding in non-heading Chinese cabbage.

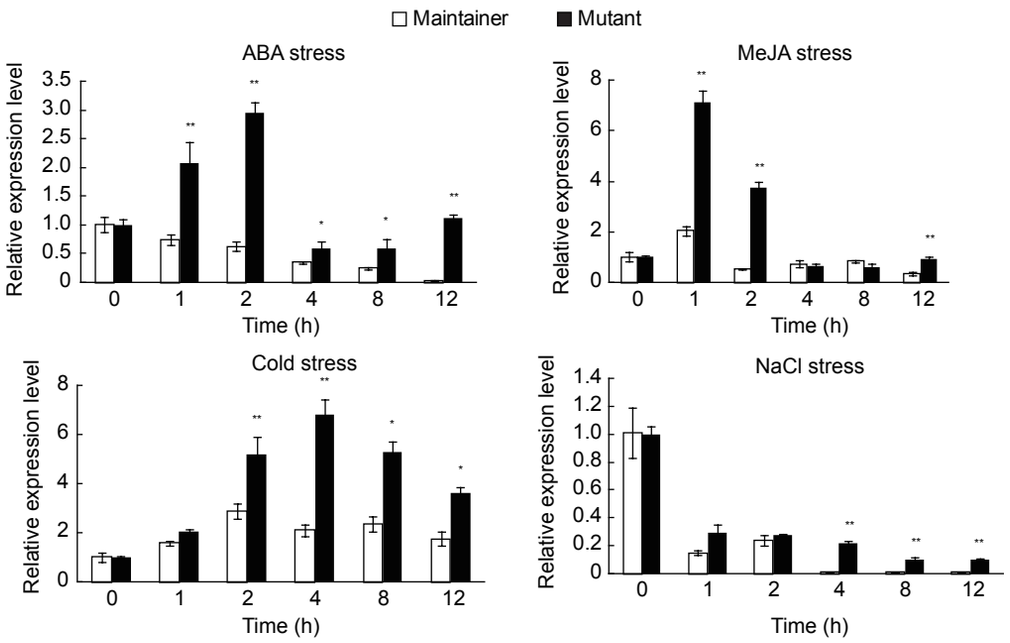

Fig. 9 Expression profiles of *BrcERF-B3* gene under abiotic stresses and exogenous regulators in non-heading Chinese cabbage.

5. Materials and methods

5.1. Plant materials

Seedlings of two non-heading Chinese cabbage lines (i.e., maintainer line, and mutant) were grown in plastic pots in vermiculite, peat moss (v/v=3:1) mixed substrate in a controlled-environment chamber. The temperature of the artificial climate chamber was set at 24/18°C day/night (16/8 h) with 400 μmol m^{-2} s^{-1} of light intensity and relative (65±5)% of humidity.

5.2. Stress treatments

The 2-mon-old seedlings of the two cabbage varieties were treated with abiotic stresses. In each case, the plants were all grown under the same day length and light intensity as previously noted. Four treatments were set, cold stress (4°C), salt stress (foliage spraying 200 mmol L^{-1} NaCl aqueous solution), ABA stress (foliage spraying 100 μmol L^{-1} ABA) and MeJA stress (foliage spraying 100 μmol L^{-1} MeJA). The samples were collected at 0, 1, 2, 4, 8, and 12 h after treatment, and frozen immediately in liquid nitrogen, and then stored at –80°C until further analysis.

5.3. RNA extraction, reversing and cDNA cloning, sequencing

The total poly (A+) RNA from the leaves of two non-heading Chinese cabbage lines was extracted in accordance with the manufacturer's instructions. The cDNA was synthesized by following the instructions of a PrimeScript RT Kit (TaKaRa, Dalian, China). The mutant leaves were used as templates to obtain the DNA fragment of the *BrcERF-B3* gene *via* PCR with the suitable primer *BrcERF-B3*-1. PCR was performed based on the following procedure, 94°C for 5 min; 35 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 1 min; with an extension of 10 min at 72°C. The amplified fragment was then recovered and subcloned into expression plasmid PMD-19 vector (TaKaRa, Dalian, China) and subjected to sequencing analysis.

5.4. qRT-PCR assays of *BrcERF-B3* gene

qRT-PCR was performed with leaves at five development stages (seedling, rosette, budding, bolting and flowering stages) and with buds at different flowering stages (bud diameter: <0.5, 0.5–1, 1–2, and >2 mm) in two lines. It depended on ABI7500 (Applied Biosystems) with SYBR Premix Ex-*Taq* (TaKaRa, Dalian, China). We used the *actin* gene of cabbage as an endogenous control to normalize the amplified levels of the target gene. The primer sequences of the *actin* and target *BrcERF-B3* genes were listed in Table 1. The conditions of qRT-PCR system were as follows, 95°C for 3 min; followed by 40 cycles of 95°C for 10 s, 58°C for 30 s. The dissolve curve analysis was then carried out at 60°C. The assays were performed with three technical replicates.

Table 1 Nucleotide sequences of primers used in polymerase chain reaction

Primer	Direction	Sequence (5′→3′)
BrcERFB3-1	F	ATGCAATATCTCTACACCAG for ORF cloning
BrcERFB3-1	R	CTGAAACAATTCAGACATAGTG for ORF cloning
BrcERFB3-2	F	GGTTGTCTGCTGCGACTATTGGT for qRT-PCR
BrcERFB3-2	R	ATCTTCCCGGGTTCCGCAAACTT for qRT-PCR
actin	F	CTCAGTCCAAAAGAGGTATTCT for qRT-PCR
actin	R	GTAGAATGTGTGATGCCAGATC for qRT-PCR

5.5. RT-PCR assays of *BrcERF-B3* gene

RT-PCR was carried out with different flower organs in two lines. *Actin* gene was used as an internal reference. The *BrcERF-B3* expression analysis was conducted and the amplification conditions were as follows, 94°C for 5 min; and 28 cycles of 94°C for 30 s, 58°C for 30 s; 72°C for 30 s; and finally 10 min at 72°C. The PCR products were detected by 1.2% agarose gel electrophoresis and visualized with ethidium bromide under UV light.

5.6. Data analysis

The amino acid sequences were carried out with the BLAST program of Plant Transcription Factor Database (PlantTFDB, http://planttfdb.cbi.pku.edu.cn/), the Clustal W program package (http://www.ebi.ac.uk/clustalw/). The related sequence alignment report was analyzed with DNAMAN6.0 (http://ishare.iask.sina.com.cn/f/34031431.html). The molecular phylogenetic analyses among the different *ERF* genes of *Arabidopsis* and different plants were conducted using MEGA 5 (Hall 2013) with the neighbor-joining (NJ) method. The bootstrap value was set at 1 000 replications to assess tree reliability. The qRT-PCR results were calculated by the $2^{-\Delta\Delta C_T}$ method, where C_T was the cycle threshold (Pfaffl 2001).

Acknowledgements

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