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Identification and abiotic stress analysis of calmodulin-binding transcription activator/signal responsive genes in non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis* Makino)

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Abstract

In cells, calmodulin (CaM) is the most remarkable Ca^{2+} transducer. BcCAMTA gene family members are calmodulin-binding transcription activators, which contain new type of sequence-specific DNA-binding domain (CG-1), an ankyrin repeats and tow IQ calmodulin-binding motifs. In our study, 8 calmodulin-binding transcription activator (*CAMTA*) genes were identified from non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis* Makino, NHCC), and named as BcCAMTA1, BcCAMTA2.1, BcCAMTA2.2, BcCAMTA3.1, BcCAMTA3.2, BcCAMTA4, BcCAMTA5 and BcCAMTA6 through *BcCAMTA* genes cloning and according *AtCAMTAs*. Compared with the classification between *Arabidopsis* and Chinese cabbage, BcCAMTA family was divided into six subgroups (respectively named as BcCAMTA1-6). Subcellular localization prediction showed that most of the BcCAMTAs were located in the nucleus, except BcCAMTA2.2 and BcCAMTA6 that were located in the cytosol, indicating the different function among BcCAMTAs. The evolution and phylogenetic analysis of BcCAMTAs together with their orthologs from other species showed that CAMTA transcription factor family members duplicated in evolution of species, as well as BcCAMTAs, which showed closer evolutionary relationship with *Arabidopsis* and Chinese cabbage. Seedlings were exposed to four abiotic stresses including cold, drought, copper ion and nitrate stress to explore the transcriptional levels of *BcCAMTA* genes. The result exhibited that *BcCAMTAs*, except for *BcCAMTA2*, were up-regulated under cold stress in 4 h, among which showed the positive regulation to resist cold stress of NHCC. The expression of *BcCAMTA2.1* and *BcCAMTA3.2* were found significantly differential expression in five development stages of NHCC, and expressed highest in flowering stage.

Keywords: non-heading Chinese cabbage; CAMTA/SR; transcription factor; abiotic stress; expression profile.
Abbreviations: NHCC_non-heading Chinese cabbage, CaM_calmodulin, CAMTA_Calmodulin-binding transcription activator CG-1- a DNA-binding domain, ANK_ankyrin, IQ_a calmodulin-binding motifs, TPM_transcripts per million.

Introduction

Transcription factors play an important role in regulating every appearance of the organism's life cycle and are adapt to respond to signals arising from within and without the organism (Finkler et al. 2007). Ca^{2+} plays a pivotal role in regulating gene transcription (Ikura et al. 2002). In eukaryotic cells, calmodulin (CaM) is the most remarkable Ca^{2+} sensor, that regulating the activity of plentiful proteins with multitudinous cellular actions. CaM is an immanent Ca^{2+} transducer protein which does not have catalytic activity but can upon binding Ca^{2+}, activate target proteins refer to various cellular processes (Bouché et al. 2005). Calmodulin-binding transcription activator (CAMTA) gene family is a novel protein family and was first identified in tobacco (Yang and Poovaiah, 2000), then CAMTAs were reported existing in various eukaryotes, including *Arabidopsis thaliana*, rice and many other plants such as grapevine (Choi et al., 2005; Finkler et al., 2007; Shangguan et al., 2014). Almost all CAMTAs share one similar domain organization which was a new type of sequence-specific DNA-binding domain (named CG-1), which could straight bind DNA and activate transcription, or interact with other transcription factors, not through DNA binding (Finkler et al. 2007). CAMTA genes contain one transcription factor immunoglobulin domain, ankyrin repeats (Doherty et al. 2009), and a varying number of IQ calmodulin-binding motifs (Bouché et al. 2002), which IQ motif-containing proteins are known to bind calmodulin (CaM) show a wide diverseness of biological functions that parallel the Ca^{2+}-dependent targets according to the examination (Bähler and Rhoads, 2002). There are six *CAMAT* genes in *Ababidopsis thaliana* (*AtCAMTA1*–*AtCAMTA6*) (Bouché et al. 2002).In *Arabidopsis*, AtCAMTA3 is a negative regulator of plant immunity (Du et al. 2009). Moreover, six *Arabidopsis CAMTA* genes (*AtCAMTA1-6*) are responded to multiple environmental signals such as extreme temperature, salt, and wounding quickly and distinctively, and they could be induced by hormones such as ethylene and abscisic acid (ABA), signal molecules such as methyl jasmonate, H_2O_2, and salicylic acid. Under cold stress (4°C), except *AtCAMTA2*, *AtCAMTAs* showed effect on the expression (Yang and Poovaiah, 2002). AtCAMTA3 is a positive regulator of *CBF2*, which plays an important role in cold acclimation, and that double *camta1 camta3* mutant plants are impaired in freezing tolerance (Kim et al. 2013). CAMTA3/SR1 regulating

Table 1. The overall information of *BcCAMTA* genes in non-heading Chinese cabbage (NHCC).

Gene name	Length (bp)	Amino acids	Theoretical pI	Molecular weight (kDa)	Sub(WoLF)	Tertiary structural template	Instability index
BcCAMTA1	11758	1824	5.08	207	Nuclear/ Chloroplast	d2cxka1	44.83
BcCAMTA2.1	6026	831	6.25	93	Nuclear/ Cytosol	d2cxka1	43.11
BcCAMTA2.2	10357	981	6.46	110	Cytosol/ Nuclear	C2ix7C	40.88
BcCAMTA3.1	5011	1031	5.42	115	Nuclear	c4oauC	47.36
BcCAMTA3.2	4837	884	5.98	99	Nuclear/ Cytosol	c4oauC	46.93
BcCAMTA4	7629	1297	6.43	147	Nuclear/ Cytosol	d2cxka1	47.18
BcCAMTA5	4330	909	7.44	103	Nuclear/Endoplasmic Reticulum	c4oauC	43.17
BcCAMTA6	4573	863	6.64	98	Cytosol/ Nuclear	c4oauC	43.73

the expression of genes involved in biological defense reaction (Galon et al. 2008). CAMTA proteins in cold acclimation provide a possible point of integrating low-temperature calcium and calmodulin signaling with cold-regulated gene expression. CAMTA1 was reported that it can regulate drought response and mediates auxin signaling and responds to stresses in *Arabidopsis* (Galon et al., 2010; Pandey et al., 2013). Non-heading Chinese cabbage *(Brassica campestris* ssp. *chinensis L. Makino*, NHCC) is native to China. In recent years, east South Asia, Japan, the United States and some European countries are introduced the variety (Xi-lin 2003). For the past few years, there were some researches about NHCC involving abiotic stress such as cold, salt and dehydration stress (Jiang et al., 2011; Wang et al., 2012). However, until now, the research of expression pattern of *BcCAMTAs* in the stress was not clear. We planned to explore the expression levels of *BcCAMTAs* in several stress conditions to probe the response of *BcCAMTAs* in stress ambiences. In experiment, 8 *BcCAMTA* genes from NHCC will be cloned and named respectively. Bioinformatics analysis will be used to compare *BcCAMTA* genes with corresponding genes from other species.

Result

CAMTA/SRs in non-heading Chinese cabbage

To discover NHCC *CAMTA* gene models, primers of *BcCAMTA1* to *6* were designed according to *Arabidopsis* (*Arabidopsis thaliana*) *CAMTA* genes. After amplified by PCR, 8 candidate *BcCAMTA* genes were obtained. The total information about the putative BcCAMTA proteins sequences was listed in Table 1. According to the sequence similarity, conserved domain analysis and phylogenetic analyses among AtCAMTAs and BcCAMTAs (Fig. 1), the 8 putative BcCAMTA proteins were named as *BcCAMTA1*, *BcCAMTA2.1*, *BcCAMTA2.2*, *BcCAMTA3.1*, *BcCAMTA3.2*, *BcCAMTA4*, *BcCAMTA5* and *BcCAMTA6*, respectively. The protein sequences of ten *Arabidopsis thaliana* CAMTAs (AtCAMTA1.1, AtCAMTA1.2, AtCAMTA1.3, AtCAMTA2.1, AtCAMTA2.2, AtCAMTA3.1, AtCAMTA3.2, AtCAMTA4, AtCAMTA5 and AtCAMTA6) were downloaded from the PlantTFDBv3.0. Multiple sequences alignment of 18 CAMTA protein sequences (ten *Arabidopsis* CAMTA and eight NHCC CAMTA proteins) showed that BcCAMTAs have high similarity with AtCAMTAs, especially in the A, B and C regions (Fig.S1). BcCAMTAs were found contained one CG-1 (A regions), one ANK (B regions) and tow IQ domain (C regions) at the N-terminus, center and C-terminus, respectively (Fig.S1), representing the specific domains of the CAMTA protein family. On the basis of studies of predecessor, CG-1 is a sequence-specific DNA-binding domain; ANK (ankyrin) repeats is a tandemly repeated module of 33 amino acids

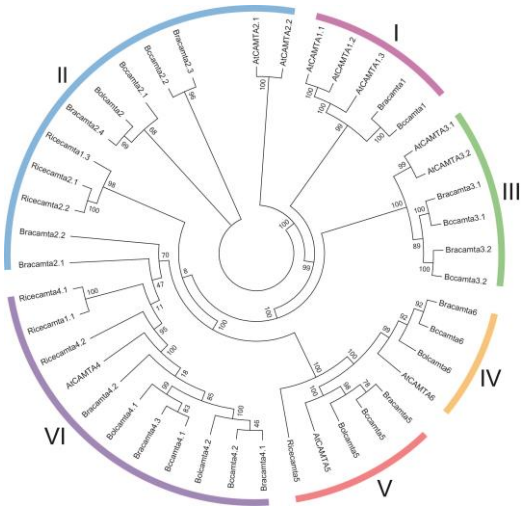


Fig 1. Phylogenetic tree analysis of CAMTA proteins in NHCC and *Arabidopsis thaliana*. The neighbor-joining (NJ) phylogenetic tree was constructed with MEGA5 software bootstrap 1000. The tree was classified into six groups represented by CAMTA1, CAMTA2, CAMTA3, CAMTA4, CAMTA5 and CAMTA6.

existence in eukaryotic proteins and viruses, and involved in the protein–protein interaction (Bennett, 1992; Sedgwick and Smerdon, 1999; Song et al., 2006); And two IQ calmodulin-binding motifs which are composed of low complexity regions with the repetitive motif IQXXXRGXXXand are known to be associated with the binding of CaM and CaM-like proteins (Bähler and Rhoads, 2002; Rhoads and Friedberg, 1997). The number of amino acids, the molecular weights and the theoretical PIs are important arguments used in the characterization of protein primary structure. The number of amino acids of BcCAMTA1 (1824) was the largest of BcCAMTA protein sequences, and its theoretical PIs was 5.08, indicated that this protein was weak acidic protein. BcCAMTA2.2 (981 aa, 110 kDa) was larger than BcCAMTA2.1 (831aa, 93 kDa). Their theoretical PIs (6.46 and 6.25) inferred that they might be acidic proteins. The number of amino acids, theoretical PI and molecular weight of BcCAMTA3.1 and BcCAMTA3.2 were (1031, 5.42 and 115 kDa), (884, 5.98 and 99 kDa), respectively. BcCAMTA5 (909aa, 103 kDa) was predicted as an alkaline protein (7.44), meanwhile BcCAMTA4 (1297, 147 kDa) and BcCAMTA6 (863, 98 kDa) were predicted as acidic proteins (6.43, 6.64) (Table 1). In term of protein tertiary structure, BcCAMTA3.1

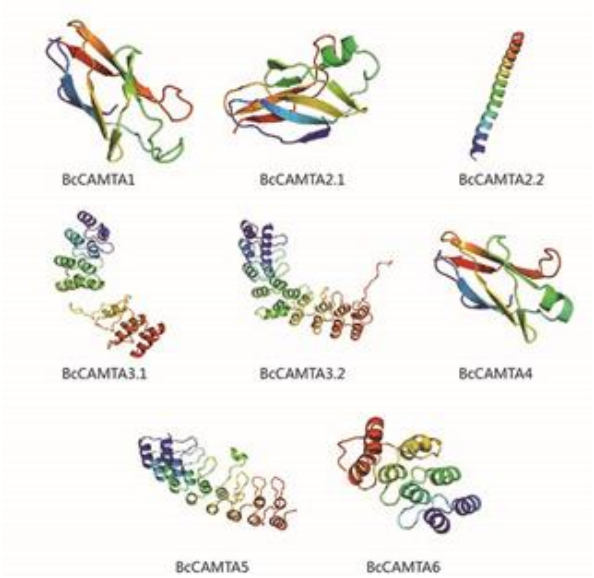
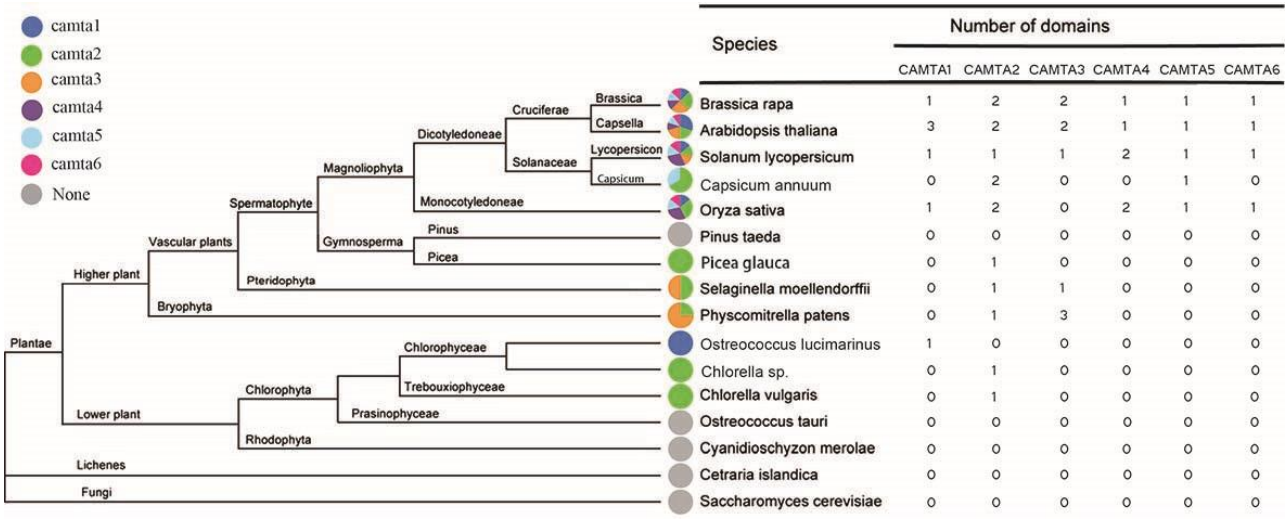


Fig 2. Protein tertiary structural analysis of NHCC *CAMTA* gene models.



Species	Number of domains					
	CAMTA1	CAMTA2	CAMTA3	CAMTA4	CAMTA5	CAMTA6
Brassica rapa	1	2	2	1	1	1
Arabidopsis thaliana	3	2	2	1	1	1
Solanum lycopersicum	1	1	1	2	1	1
Capsicum annuum	0	2	0	0	1	0
Oryza sativa	1	2	0	2	1	1
Pinus taeda	0	0	0	0	0	0
Picea glauca	0	1	0	0	0	0
Selaginella moellendorffii	0	1	1	0	0	0
Physcomitrella patens	0	1	3	0	0	0
Ostreococcus lucimarinus	1	0	0	0	0	0
Chlorella sp.	0	1	0	0	0	0
Chlorella vulgaris	0	1	0	0	0	0
Ostreococcus tauri	0	0	0	0	0	0
Cyanidioschyzon merolae	0	0	0	0	0	0
Cetraria islandica	0	0	0	0	0	0
Saccharomyces cerevisiae	0	0	0	0	0	0

Fig 3. *CAMTA* transcription factors comparison among different species. Different colors represent each family member in the *CAMTAs*. The colored sections represent the number of transcription factor members identified in a species. Gray represents the absence of a member.

and BcCAMTA3.2 were similar with each other (Fig.2).

Evolution and phylogenetic analysis of CAMTAs from NHCC

We subdivided the BcCAMTA transcription factors into 6 groups, based on conserved domain similarities to AtCAMTA transcription factors. Cumulatively, NHCC, *Arabidopsis* and tomato were found transcripts of all *CAMTA1-6* genes. CAMTA gene family was also identified in Rice, in which five *CAMTA* orthologs were found, except *CAMTA3*. Transcription products of *CAMTA1* and *CAMTA2* were revealed both in lower and higher plants. However, the rest CAMTA members were discerned in higher organisms. CAMTA transcription factors were not identified in lichens, fungi, and other lower plants in our analysis (Fig.3). The evolutionary relationship between NHCC and other plants (*Arabidopsis thaliana, Brassica rapa, Brassica oleracea* and Rice) based on CAMTA transcription factors was assessed by MEGA 5.0. The results were shown in Fig. 5 and the phylogenetic tree could be divided into 6 clades. BcCAMTA1 and BrCAMTA1 were all clustered into Group Ⅰ, in which BcCAMTA1 and BrCAMTA1 constituted a subgroup and behaved consistently with the performance of their orthologs from *Arabidopsis*. Furthermore, BcCAMTA3 to 6 represented uniform homology with BrCAMTAs. However, BcCAMTA2.2 displayed high similar with BrCAMTA2.3, in addition, BcCAMTA2.1 constituted a group and behaved consistently with a subgroup consist of BolCAMTA2 and BrCAMTA2.4. In summary, the consequence of the evolutionary relationship displayed BcCAMTA transcription factors shared the highest homology with BrCAMTAs and AtCAMTAs (Fig. 5).

Predicted subcellular localization of CAMTA transcription factors in non-heading Chinese cabbage

WoLF PSORT protein subcellular localization tool was used to predict the subcellular localization of BcCAMTA members. BcCAMTA2.2 and BcCAMTA6 were found located in the

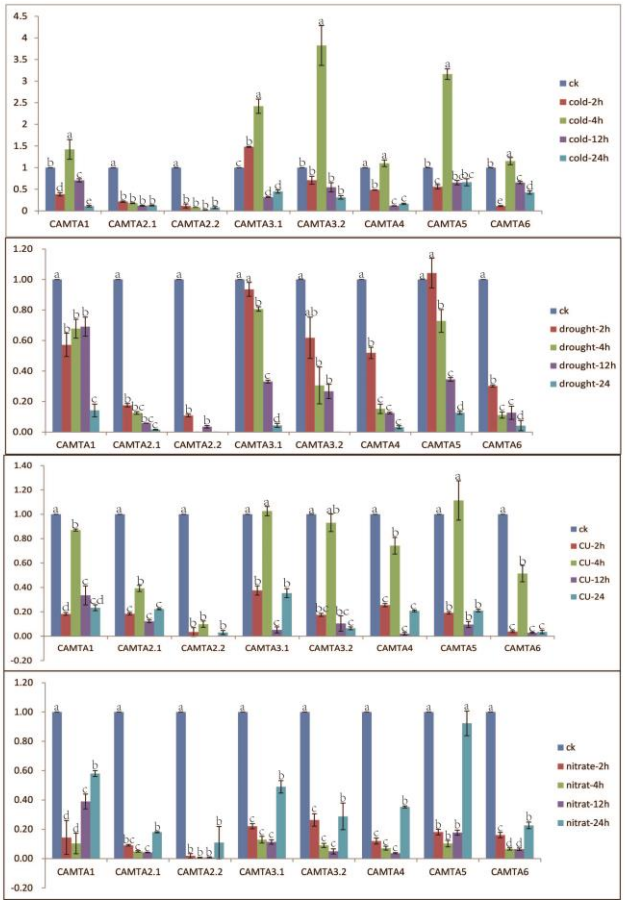


Fig 4. Changes in *BcCAMTA* transcript levels in response to abiotic stress. Seedlings at 22 ^{o}C were subjected to low temperature (4 ^{o}C), drought treatment, copper ion stress and nitrate stress. The transcript levels of *CAMTA1* to *6* and several other related up/down-stream genes were determined by qPCR analysis. The expressions of the genes are represented using fold change compared to control (0h). Error bars of standard deviation (SD) were calculated based on three replicates. X axis: gene members of BcCAMTA family; Y axis: Log_2 (fold-change value) of the relative expression levels. Different coulours mean different treatments separately representing different time of treatments.

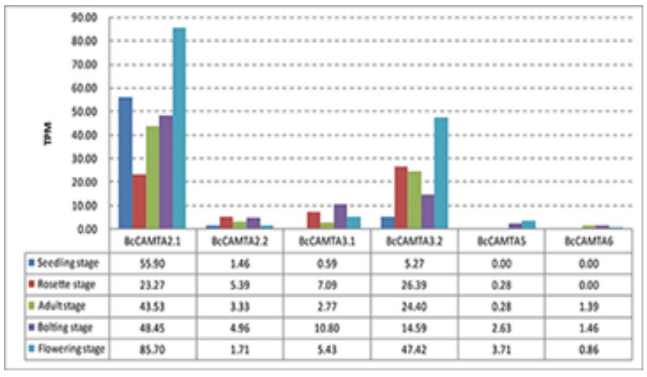


Fig 5. Expression profile analysis of NHCC *CAMTAs.* X axis: five periods of seedling, rosette, adult, bolting and flowering stage. Y axis: the expression profile of *BcCAMTAs.*

cytosol. Except them, others BcCAMTA family members were all located in the nucleus (Table 1). BcCAMTA2.2 and BcCAMTA6 were slightly different in protein sequences from all the other members in the BcCAMTA family. For instance, proline was replaced by valine and asparagines at 573 and 377 sites of BcCAMTA2.2 and BcCAMTA6 protein sequences, respectively (Fig.S1).

The expression of CAMTAs responding to abiotic stresses

To explore the expression pattern of *BcCAMTAs*, we performed qPCR analysis for the eight *BcCAMTA* genes. Our result showed that expression patterns under cold, drought, nitrate and copper ion stresses, *BcCAMTAs* were mostly down-regulated (Fig. 6). Under cold, the expressions of *BcCAMTA2.1* and *BcCAMTA2.2* were gradually decreased over time and down-regulated. The rest of *CAMTA* family genes were up-regulated and the expression level reached peak at 4 h. Under drought, almost all genes exhibited a similar trend of transcription level along with the time, excepted *BcCAMTA1*. Under drought, *BcCAMTA2* to *6* showed the higher expression levels in 2 h, afterwards gradually reduced. Nevertheless, highest expression level of *BcCAMTA1* was at 12 h, which was slight higher than expression at 4 h. Refer to expression of BcCAMTA gene family under copper ion stresses, barring *BcCAMTA1* and *BcCAMTA3.2*, *BcCAMTA* transcriptions levels all reached peak at 4 h and then reduced to valley in 12 h, finally increased in 24 h. The transcription levels of *BcCAMTA1* and *BcCAMTA3.2* reached peak at 4 h and then gradually reduced until 24 h. Under nitrate treatment, the expression of *BcCAMTAs* were gradually decreased until 12 h and then increased to 24 h, except *BcCAMTA1* and *BcCAMTA5*, among which expression levels in 12 h were higher than in 4 h (Fig.4).

Expression profile analysis of non-heading Chinese cabbage CAMTAs

Song et al. (2014) had published the expression profile of non-heading Chinese cabbage inbred line, '001', at 2014, which included the expression of parts of BcCAMTA gene family (no contains of *BcCAMTA1* and *BcCAMTA4* genes) at five development stage (seedling stage, rosette stage, adult stage, bolting stage and flowering stage) (Song et al. 2014). Only *BcCAMTA2.1* and *BcCAMTA3.2* were detected significant difference expressions in five development stages. Both *BcCAMTA2.1* (transcripts per million [TPM] > 80) and *BcCAMTA3.2* (TPM > 40) showed higher expression at the flowering stage than other four stages. Transcription levels of *BcCAMTA2.1* in five development stages showed valley in rosette stage and gradually increased behind, until flowering stage reached peak. On the contrary, expression of *BcCAMTA3.2* exhibited opposite trend, among which expression index increased form seedling to rosette stage and decreased in adult and bolting stages, until reached peak in flowering stage. *BcCAMTA5* showed expression levels at bolting and flowering stages and *BcCAMTA6* showed expression levels at adult, bolting and flowering stages (Fig.5).

Discussion

CAMTA gene family is an active transcription factor family in most plants (Finkler et al. 2007). Acquisition of CAMTA gene family of non-heading Chinese cabbage was according to the same gene family of *Arabidopsis thaliana*. The phylogenetic analysis showed the systematization of BcCAMTA transcription factor family.

Then BcCAMTAs were named as BcCAMTA1, BcCAMTA2.1, BcCAMTA2.2, BcCAMTA3.1, BcCAMTA3.2, BcCAMTA4, BcCAMTA5 and BcCAMTA6. Multiple sequence alignment between NHCC and *Arabidopsis* indicated that the same motifs (CG-1, ANK and IQ) existed in both BcCAMTA and AtCAMTA protein sequences, CG-1, ANK and IQ motifs were used to identify CAMTA transcription factor family in other multicellular organisms (Bouché et al. 2002). According to the phylogenetic analyses among NHCC and other plants, we found that NHCC demonstrated great high homologies with Chinese cabbage and *Arabidopsis*. NHCC and *Brassica rapa* both belong to Cruciferae Brassica means close relationship in species evolutionarily. In addition, *Arabidopsis*, NHCC and *Brassica rapa* all belong to Cruciferae. Except CAMTA2.2 and CAMTA6 (located in the cytosol), other CAMTAs were located in the nucleus, as well as *Arabidopsis*, which subcellular fractionation of *Arabidopsis* tissues revealed the presence of CAMTAs predominantly in the nucleus (Bouché et al. 2002).

The CAMTA transcription factor family is important in the plant response to multiple abiotic and biotic stresses, including cold, wounding, drought and pathogens (Reddy et al. 2011). Under abiotic stress, most *BcCAMTAs* genes were down-regulated. Except *BcCAMTA2*, *BcCAMTA*1 to 6 were all had an increasing expression under cold. Previous studies had proved that CG-1 sequence act as a significant role for *Arabidopsis* CAMTA proteins in the early response to low temperature (Doherty et al. 2009), which was consistent with our results. Under drought, the expression pattern of *BcCAMTA1* was different with other *BcCAMTAs*, among which transcription level gradually increased in front 12 h and reduced in 24 h. Neha Pandey et al. studied *CAMTA1* regulated drought response in *Arabidopsis* and revealed a result that *CAMTA1* was an important role to regulate drought response in *Arabidopsis* (Pandey et al. 2013). We conjectures *BcCAMTA1* possibly has similar role with *AtCAMTA1* in responses to drought. Under copper ion stress, the expression trend of *BcCAMTAs* was similar with transcription level under cold. In addition, a Cu-dependent increase in the proline level of detached rice leaves was detected within 4 h (Chen et al. 2001). Further, cold-induced genes regulated the expression of genes involved in osmolyte biosynthesis, which including proline (Chinnusamy et al. 2007). Therefore, we inferred that the expression pattern of *BcCAMTAs* under cold and copper stress possible was parallelism. The expression of *BcCAMTAs* under nitrate stress almost followed a similar trend, among which the expression reduced in 12 h and reach highest level in 24 h. Nitrate was absorbed by plants and used to synthetize glutamic acid (Solomonson and Barber, 1990). We surmised that the expression decreased in 12 h treatment because of the response to nitrate stress and reached highest expression level in 24 h because of utilize of superfluous nitrate. In a word, *BcCAMTA1*, *3*, *5* were up-regulated under cold and *BcCAMTA2* was gradually down-regulated. *BcCAMTA1* possible related to drought stress response. In a certain range of concentration, NHCC absorb nitrate to defense nitrate stress. In the result of putative protein structure analysis, it was showed that the protein tertiary structure of BcCAMTA3.2, BcCAMTA3.1 and BcCAMTA5 were similar (Fig.2), whose structure models were same to be 'c4oauC' (Table 1). Therefore we speculated that the tolerance applied to cold stress relative to CG-1 sequence and protein tertiary structure. CAMTA proteins were reported to be involved mainly in calmodulin-binding transcription and our expression profile indicated that *BcCAMTA2.1* and *BcCAMTA3.2* expressed significant than other *BcCAMTA* genes in five stages (Bouché et al. 2002). *CAMTAs* could play an important role in the process of Ca^{2+} signal transduction in various tissues (Yang and Poovaiah, 2002), for instance, all *AtCAMTA*s were found expressed in flower and some members were found in leaf, root, seed and silique, and so on (according to data at PlantFTDB v3.0). The expression level of *BcCAMTA2.1* and *BcCAMTA3.2* reached highest level in flowering stage revealed homogeneity between *Arabidopsis* and NHCC on *CAMTA2.1* and *CAMTA3.2*. Moreover, *BcCAMTA6* and *BcCAMTA5* showed expression levels in bolting and flowering stages. *AtCAMTA5* was found possibly enhance one gene expression in pollen (Mitsuda et al. 2003).

Materials and Methods

Plant materials

A non-heading Chinese cabbage inbred line, '001', was used in this research, which obtained from the non-heading Chinese cabbage project team in Nanjing Agricultural University, Nanjing, China. Healthy seeds were harvested from the same batch which grown in an artificial climate chamber at 20 °C to 22 °C, and under continuous cool-white fluorescent illumination at an intensity of 100 $\mu molm^{-2}s^{-1}$ (Gilmour et al. 1998).

Stress treatments

Stress treatments include four stress conditions, such as cold, drought, copper ion and nitrate stress. For the low temperature treatment, plants were incubated at 4 °C. In order to simulate drought stress, 20 % PEG (polyethylene glycol 8000) was used to treat the plants. 200 μm/L $CuSO_4 \cdot 5H_2O$ was used to created a copper ion stress condition. For the sake of nitrate stress, in the research 20 mg/L KNO_3 was putted to use. The leaf samples were collected at 0, 2, 4, 12 and 24 h after four stress treatments. Stress treatments were performed using the seedlings at five-leaf stage and all treatments were processed under hydroponic conditions, the Murashige and Skoog (MS) culture medium was used for nutrient supply. Except seedlings growing in cold treatment, the rest seedlings grown in an artificial climate chamber at 20 °C to 22 °C. Relative humidity was 55-60%. Continuous cool-white fluorescent illumination at intensity of 100 $\mu molm^{-2}s^{-1}$ was provided.

Isolation of cDNAs encoding the eight BcCAMTA proteins

Taking into consideration the feature that the protein sequences are relatively conserved evolutionarily in different plant species, we extracted the *Arabidopsis* protein sequences of *AtCAMTA1.1* (AT5G09410.1), *AtCAMTA1.2* (AT5G09410.2), *AtCAMTA1.3* (AT5G09410.3), *AtCAMTA2.1* (AT5G64220.1), *AtCAMTA2.2* (AT5G64220.2), *AtCAMTA3.1* (AT2G22300.1), *AtCAMTA3.2* (AT2G22300.2), *AtCAMTA4* (AT1G67310.1), *AtCAMTA5* (AT4G16150.1), *AtCAMTA6* (AT3G16940.1) from the Plant TFBD website (http://planttfdb.cbi.pku.edu.cn/). Then according to *AtCAMTAs* we designed forward and reverse primers, in older to clone the homologous *BcCAMTAs* in NHCC. Through preliminary bioinformational analyses, the sequences without CAMTA characteristic motifs were excluded, and then 8 sequences were selected as candidates of putative non-heading Chinese cabbage CAMTA transcription factor members. In order to research the expression pattern of *BcCAMTAs* in abiotic stress, total RNA was isolated from seedlings cultivated at low temperature 4 °C, 20% PEG (polyethylene glycol 8000) , 200 μm/L $CuSO_4 \cdot 5H_2O$ and 20 mg/L KNO_3 treatments for 0 hours (h), 2 h, 4 h, 12 h and 24 h, respectively. RNA extraction was achieved using the TaKaRa RNAiso Reagent (Takara, Dalian, China). The first strand cDNA was reversed transcribed using TaKaRa RNA PCR Kit (AMV) Ver.2.1 (Takara, Dalian, China). The PCR mixture

contained 2 μL buffer (10×PCR), 2 μL $MgCl_2$ (25 mM), 1 μL dNTPs (2.5 mM for each), 0.2 μL rTaq DNA polymerase (5 $UmL.L^{-1}$), 10-pmol-specific primers each, 50 mg cDNA, and ddH_2O up to 20 μL. Amplification profile was 94 °C for 10 minutes (min), 35 cycles of 94 °C for 30 seconds (s), 55 °C for 30 s, 72 °C for 90 s, and a final extension of 72 °C for 10 min. Each of the eight PCR products was resolved in 1.5 % (w/v) agarose gel and cloned into the pMD-18 vector (Takara, Dalian, China) after purifying the gel. Sequencing was performed by the Genscript Biotechnology Company (Nanjing, China).

Bioinformatics analyses

In this study, *Brassica* Genome Gateway (http://www.Brassica Genome Gateway.htm), and PlantTFDB (http://planttfdb.cbi.pku.edu.cn/) were used to search protein sequences of Chinese cabbage and *Arabidopsis* (Finkler et al. 2007). Primer 5.0 was used to conduct primers design; DNAman 4.0 was used to analyze protein sequences of CAMTAs; Mega 5.0 was used to obtain evolutionary tree drawings by Neighbor-joining method. PROSITE (http://www.Expasy.org/prosite/), InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) and ProtParam analyses (http://web.expasy.org/protparam/) were used to obtain the number of amino acids, molecular weight, and theoretical PI of putative BcCAMTA proteins on the basis of their sequence. $Phyre^2$ Gateway (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) was used to assist in the analyses of the high structures of these proteins (Kelley and Sternberg, 2009). NLS Mapper website (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) was used to determine the prediction of NLSs (Kosugi et al., 2008; Kosugi et al., 2009a; Kosugi et al., 2009b). Wolf Psort was used to predict the subcellular localization of CAMTA proteins (http://psort.hgc.jp/form.html) (Klee and Sosa, 2007; Qian and Zhang, 2009).

Real-time quantitative PCR (qPCR) analysis and statistics analysis

We performed qPCR to explore the expression patterns of the 8 *BcCAMTA* genes. The qPCR primers were designed by Beacon Designer 7.0 and were listed in the Table S1. Plants undergoing different treatments were prepared separately. For different treatments, seedlings were transferred to four treatments (low temperature, 20% PEG 8000 treatment, $CuSo_4 \cdot 5H_2O$, KNO_3) for varying lengths of time (0 h, 2 h, 4 h, 12 h and 24 h). After reverse transcription, the cDNA samples were diluted with water (1:19). PCR reactions were performed according to the manufacturer's instructions (TaKaRa Real-time PCR SYBR Premix EX Taq ^{TM} (TaKaRa, Dalian, China). The hot-start procedure was 45 cycles of 95 ^oC, 2 min, 95 ^oC, 20 s, 50.9 ^oC, 20 s, 72 ^oC,20 s; 75 ^oC, 1 s; 78 ^oC, 1 s; 72 ^oC, 10 min. The internal reference control was actin gene of NHCC. Each gene in each treatment was repeated three times in qPCR and the experimental data were analyzed by the Rotor-Gene 6 software (QIAGEN, Germantown, USA). In all cases, relative quantitation (RQ) was performed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). At the same time, the standard errors of the mean among replicates were calculated. LSD test for multiple-comparisons was used to determine significant differences of data.

Digital gene expression tag profiling

The expression levels of *BcCAMTA* genes at the five development stages were obtained using digital gene expression-tag profiling methods, as in a previous report (Wang et al. 2010). The quantitative expression of the corresponding transcript in tissues was represented by the number of times that a unique tag sequence was detected. Transcripts Per Million reads (TPM) was used to express the BcCAMTA gene expression amount after standardization. High quality clean tags were compared with the BcCAMTA gene sequences of non-heading Chinese cabbage and the expression level of BcCAMTAs were quantified as TPM (Chen et al. 2006).

Conclusion

Taken together, in this study, 8 *CAMTA* genes in non-heading Chinese cabbage were successfully identified. Subcellular localization of some of these CAMTA proteins further proved that most of them were nucleus localized. Through the above-mentioned bioinformatics analysis and expression pattern assays, it is highly presumed that a *BcCAMTA* gene family in NHCC response to cold stress probably. *BcCAMTA3.1*, *BcCAMTA3.2* and *BcCAMTA5* were up-regulated in the cold stress. In the study, we can hypothesize that *BcCAMTA3.2* and *BcCAMTA2.1* highly expressed than other family members in the common surroundings.

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