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ORIGINAL PAPER

cDNA Clones and Expression Analysis of cpHSC70 and mtHSC70 in Non-Heading Chinese Cabbage

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Abstract The 70-kDa heat shock proteins (HSP70s) are required for thermotolerance and development in plants and other organisms. We applied the techniques of homology-based cloning and real-time quantitative reverse transcription PCR to identify the functions of BccpHSC70s and BcmtHSC70s—heat shock cognates (HSCs) located in chloroplasts and mitochondria, respectively—in non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis*). cDNA clones of BccpHSC70s and BcmtHSC70s were highly homologous to each other and to those from other individual plants. Unlike other HSP70s, *BccpHSC70-1* has only two signature motifs (three classical motifs were found in the predicted amino acid sequence of HSP70s, but not all HSP70s contain all three domains), and was expressed constitutively in three detected cultivars; the expression of *BccpHSC70-1* in a thermotolerant cultivar (NHCC001) was greater than in other cultivars (NHCC002 and NHCC004) during development. High expression level of *BccpHSC70-2* in leaves might be explained by the fact that chloroplasts are sensitive to temperature stress, and this gene might be a candidate gene for developing thermotolerant cultivars of non-heading Chinese cabbage. Expression of *BcmtHSC70-2* was inducible under temperature stress. Expression of BcHSC70s in the sterile line showed it played an important role in the process of apoptosis during development of reproductive organs.

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Keywords Heat shock protein (HSP) · Non-heading Chinese cabbage · Real-time PCR · Sterile line (pol CMS)

Abbreviations

CMS	Cytoplasmic male sterility
EST	Expressed sequence tag
HSP	Heat shock protein
ROS	Reactive oxygen species

Introduction

Elevated temperatures activate a cellular heat stress response (HSR) in plants. The HSR causes enhanced expression of heat stress genes, which are multi-gene families encoding molecular chaperones called heat shock proteins (HSPs) (Boston et al. 1996; Hartl and Hayer-Hart 2002; Bukau et al. 2006; Rampelt et al. 2012). Generally, HSPs are classified into five major families on the basis of their molecular weights: HSP110, HSP90, HSP70, HSP60, and low molecular weight HSPs (Cowan and Lewis 2002; Petrucelli et al. 2004). Expression of HSPs is regulated by a large family of heat shock factors (HSFs) that bind to the heat shock elements in the promoters of HSPs (Von Koskull-Doring et al. 2007). HSPs are not only present after stress stimulation but they are also essential components of cells and developmental processes under normal physiological conditions (Rutherford 2003).

The 70-kDa heat shock proteins (HSP70s) are a class of chaperone proteins that are highly conserved in all organisms (Lin et al. 2001). Several HSP70 family genes have been isolated from *Arabidopsis*. The subgroups of *Arabidopsis* HSP70s can be distinguished by their C-terminal motifs and

differences in intron/exon structure (Marchesi and Ngo 1993; Sung et al. 2001). HSP70s assist in the folding of nascent polypeptides released from ribosomes (Hartl and Hayer-Hart 2002; Meimaridou et al. 2009), in the sorting of proteins to cell organelles by interaction with mitochondrial and chloroplast protein import complexes (Zhang and Glaser 2002; Mirus and Schleiff 2009), and form a link to the ubiquitin-mediated proteasomal degradation pathway (Ballinger et al. 1999; Dou et al. 2003; Lee et al. 2008; Rampelt et al. 2012).

HSP70s are predicted to be located within different cellular compartments, including chloroplasts, mitochondria, endoplasmic reticulum (ER), and cytoplasm. Cytoplasm HSP70s are clearly induced by thermal stress (Rochester et al. 1986), but the function of cpHSC70 and mtHSC70 in non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis*) under stressful environmental conditions are less understood. *cpHSC70-1* and *cpHSC70-2* are both located in chloroplasts, and both harbor ATPase, which is essential for plant development. *mtHSC70-1* and *mtHSC70-2* are both located in mitochondria and are essential for the maintenance of mitochondrial membranes (Moczko et al. 1995). Mitochondria are involved in the control of cell metabolism and signal transduction (Nisoli and Carruba 2006), and are susceptible to reactive oxygen species (ROS) generated by stress (Belyaeva et al. 2008).

Plant development is a tightly regulated process. Changes in a cell's ability to react to environmental conditions are dependent upon intracellular signal transduction pathways that coordinate cellular responses (Manceau et al. 1999; Nordhues et al. 2010). Some reports suggest that the process of development is accompanied by an accumulation of HSP70s (Dafny-Yelin et al. 2005; Wang et al. 2010). *B. campestris* ssp. *chinensis* is not a thermotolerant plant; high temperatures impact annual production. So, this subspecies could be used as a model for studying HSPs and their effects on chloroplasts and mitochondria in plants susceptible to high temperatures. However, the functional significance of the accumulation of *BccpHSC70* and *BcmtHSC70* in *B. campestris* ssp. *chinensis* is not fully understood.

The goal of this study was to elucidate the roles of HSP70s in adaptation and tolerance mechanisms to environmental stresses. In addition, we set out to identify which gene may be the best candidate gene for distinguishing different thermotolerant cultivars with *B. campestris* ssp. *chinensis* as a model. To this end, we characterized and cloned the *BccpHSC70* and *BcmtHSC70* genes, which were specifically and highly expressed in cultivars with *B. campestris* ssp. *chinensis* with differing levels of thermotolerance. In addition, we used maintainer and sterile plants to perform a quantitative study of BcHSP70 expression in leaves and flowers during different stages of development and under different stress treatments using real-time PCR (RT-PCR).

Materials and Methods

Plant Material and Treatments

Plant Culture

Three distinct inbred lines of *B. campestris* ssp. *chinensis*, with differing heat tolerances, were used in this study: NHCC001 (tolerant of high temperatures), NHCC002 (sensitive to high temperatures), and NHCC004 (tolerant of moderate temperatures). In addition, a fertile line (FL) and a sterile line (SL) were used for some experiments. *B. campestris* ssp.

Table 1 Nucleotide sequences of primers used in cDNA clone and polymerase chain reaction

Primer	Direction	Sequence (5′ to 3′)
BccpHSC70-1f	F	TGGCTTCGTCCGCCGCT, for OFR cloning
BccpHSC70-1r	R	TCATTTGCTGTCAGTGAAGTC, for OFR cloning
BccpHSC70-2f	F	GGCTTCCTCCACCGCCCA, for OFR cloning
BccpHSC70-2r	R	ACCAGCATCTTTAGTAGCTGTC, for OFR cloning
BcmtHSC70-1f	F	GGCCTCCGTCGCGCTTCTA, for OFR cloning
BcmtHSC70-1r	R	TCACTTCTTTGAACCGCTGGC, for OFR cloning
BcmtHSC70-2f	F	TGGCCTCCGTCGCGATTC, for OFR cloning
BcmtHSC70-2r	R	TCACTTCTTTGAACCGCTCG, for OFR cloning
BccpHSC70-1f	F	ATGTCGGGTAAAGGAGAAGGT, for RT-PCR
BccpHSC70-1r	R	TTCTTCGATAGTCAAAAGCGA, for RT-PCR
BccpHSC70-2f	F	AGTGATATTGATGAAGTGAT, for RT-PCR
BccpHSC70-2r	R	CTGTGACATTAGGTTCTT, for RT-PCR
BccpHSC70-1f	F	GAAGAACTATGCTCTGATT, for RT-PCR
BccpHSC70-1r	R	CACCAACAAGGATTACTT, for RT-PCR
BcmtHSC70-2f	F	ATGGCCTCCGTCGCGATTCT, for RT-PCR
BcmtHSC70-2r	R	TCACTTCTTTGAACCGCTCGC, for RT-PCR
BcGAPDHf	F	CCACTAACTGCCTTGCTCCAC, for RT-PCR
BcGAPDHr	R	GCTTGCCCTCAGATTCCTCCT, for RT-PCR

Table 2 Positions and signatures of BccpHSC70s and BcmtHSC70s

Name	Position	Signature 1	Position	Signature 2	Position	Signature 3
BccpHSC70-1	–	–	258–271	+	399–413	+
BccpHSC70-2	77–84	+	261–274	+	402–416	+
BcmtHSC70-1	52–59	+	235–248	+	376–390	+
BcmtHSC70-2	57–64	+	240–253	+	381–395	+

Signature 1 is "IDLGTTNS", *Signature 2* is "VFDLGGGTF ><DVS[VI]L", *Signature 3* is "V[IL]LVGG[MS] TR[VI]P [KA]VQ[ED]"(http://motif.genome.jp/) ."+",existent; "-", nonexistent

chinensis seeds from each line were placed on water-soaked filter paper for 3 days to germinate. Seedlings were then transferred to a commercial soil mix containing sphagnum peat, perlite, and vermiculite (1:1:1, respectively), watered every other day, and fertilized once a week with a commercial fertilizer. Plants were grown under 70 % humidity with a light/dark cycle of 16/8 h in growth cabinets.

Plant Treatments

Plants (NHCC001, NHCC002, NHCC004, FL, and SL) were grown in pots at a constant temperature of 25 °C. To induce heat stress, plants were transferred to 38 °C (heat stress); for cold treatment, plants were transferred to 4 °C. Plants were maintained at these different temperatures for a range of time periods (0, 2, 4, 6, and 8 h). Samples of roots, leaves, stems, and flowers were collected from each NHCC001, NHCC002, and NHCC004 plant; leaves and flowers (<0.6, 1.8, 3.0, and >4.5 mm) were collected from sterile and maintainer plants. Samples were immediately frozen in liquid nitrogen and stored at −70 °C for the extraction of RNA at a later date.

Cloning of BcHSP70 Genes

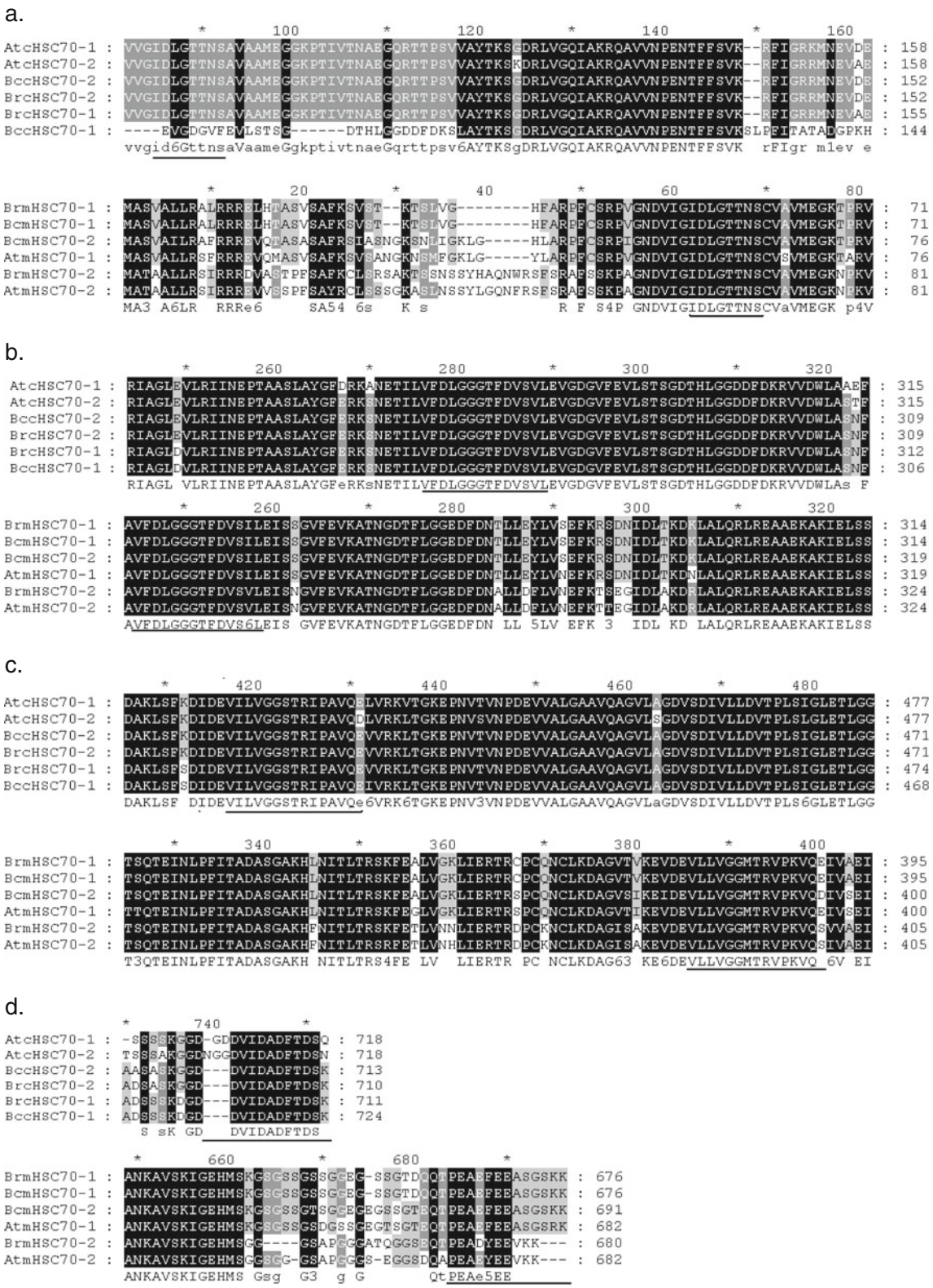
The full length of the *BcHSP70* gene was cloned by comparative cloning and rapid amplification of cDNA ends. A gene-specific forward primer for *BcHSP70* was designed based on the HSP70 homologous sequences in *B. napus* and *Arabidopsis thaliana*. The reverse primer for *BcHSP70* was designed according to the EST sequence. To amplify *BcHSC70*, an initial denaturation (94 °C, 2 min) was followed by 35 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s), extension (72 °C, 1.2 min), and a final extension (72 °C, 10 min) step. The sequences of primers mentioned above are listed in Table 1. All primers were synthesized by Invitrogen (Nanjing, China). All PCR reactions above were carried out in a 20-μl reaction system using a reverse primer (0.2 mM), a cDNA template (1 μl), Takara Ex Taq (1 U), 1× Ex Taq buffer (plus Mg^{2+}), a dNTP mixture (0.2 mM), and a forward primer (0.2 mM). PCR products were analyzed on 1 % agarose gels, extracted with a QIA quick gel extraction kit (QIAGEN), and cloned into a pMD18-T vector (TaKaRa). The resultant recombinant plasmid was then transformed into an *Escherichia coli* host DH5 alpha. The positive transformers were screened by PCR and then sequenced by Genscript Biocompany (Nanjing, China).

BcHSC70s mRNA Expression by Real-Time PCR

NHCC001 was grown to the 5-leaf stage at room temperature for experiments, and RT-PCR was used to extract RNA from roots, stems, leaves and flowers of NHCC001. Total RNA was isolated from plant tissues with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After extraction of total RNA, the isolated RNAs were treated with RNase-free DNase to ensure that the target gene was derived from mRNA. The quality of total RNA sample was evaluated by electrophoresis on 1 % agarose gels stained with ethidium bromide and concentrations were measured with a spectrophotometer ND-1000 (Nano-Drop, USA). Only RNA without detectable

Table 3 Annotation reference for BcHSC70 genes and their deduced polypeptides and predicted subcellular localization

Gene name	Gene ID	Protein (aa)	MW (Da)	Theoretical pI	Chromosome location	Length (bp)	Subcellular localization
BccpHSC70-1	CabbageG_a_f_g001889	724	77,282.7	4.88	A01	3332	Chloroplast
BccpHSC70-2	CabbageG_a_f_g017335	713	76,019.7	5.27	A03	2936	Chloroplast
BcmtHSC70-1	CabbageG_a_f_g039291	676	72,489.3	5.79	A08	2433	Mitochondrion
BcmtHSC70-2	CabbageG_a_f_g000145	691	73,965.8	5.56	A01	2516	Mitochondrion



degradation of 26S rRNA was used for subsequent preparation of poly (A)+—mRNA. A subsample (100 ng) was converted into cDNA using anchored Oligo dT-primer and PrimeScriptTM RT Enzyme MixIfrom PrimeScriptTM RT Reagent Kit (Takara).

◀ **Fig. 1** Sequence alignment of chloroplast and mitochondrial HSP70s. Non-heading Chinese cabbage plastid HSP70s (*BccpHSC70-1, BccpHSC70-2*) and mitochondrial HSP70s *(BcmtHSC70-1, BcmtHSC70-2*) were aligned with chloroplast HSP70s and mitochondrial HSP70s from *Brassica* and *Arabidopsis. Black shading* indicates consensus residues common to both chloroplast and mitochondrial HSP70s. *Gray shading* indicates consensus residues specific in either chloroplast or mitochondrial HSP70s. *Underlined* residues indicate the beginning of the N-terminal (**a**), highly conserved ATP-binding motif "IDLGTTNS". *Underlined* residues (**b**) are the conserved signature "VFDLGGGTFDVS[VI]L", *Underlined* residues (**c**) are the signature "V[IL]LVGG[MS]TR[VI]P [KA]VQ[ED]", Underlined residues (**d**) are C-terminal signature motifs for organelle localization. *Dots* indicate at the point that all residues are nonexistent

The amount of poly (A)+—mRNA/cDNA double stranded products obtained after reverse transcription was used as a template preparation for RT-PCR.

To quantify the mRNA levels of genes of interest, we evaluated the potential of "house-keeping genes" as expression standards. *BcGAPDH* was taken as an internal standard for RT-PCR. Each gene was replicated three times for RT-PCR. RT-PCR was performed in a reaction mixture (20 μl) composed of cDNA and gene forward primer (1 μl; 10 μM), gene reverse primer (1 μl; 10 μM), and master mix (10 μl SYBR Premix Ex Taq (2×), 2 μl cDNA, and ddH2O up to 20 μl) using an iCycler iQ system (Bio-Rad, Hercules, CA, USA). Amplification of PCR products was monitored via intercalation of SYBR-Green (Takara). The following program was applied: initial polymerase activation 94 °C, 30 s; 40 cycles at 94 °C, 20 s; 52 °C, 20 s; 72 °C, 20 s; and final extension at 72 °C for 10 min. In order to confirm that only one specific PCR product was amplified, a melt cycle, in which PCR product was denatured from 65 to 94 °C, was added to each thermal profile to produce melt curves (Tables 1, 2). Relative gene expression was calculated according to Pfaffl (2001).

DNA Sequence Database Analysis

The theoretical isoelectric point (pI) and molecular weight of polypeptides were determined by prot-Param (http://web.expasy.org/protparam/). The HSP70 gene family signatures were detected by scanning sequences against PROSITE patterns and profiles, through the ExPASy proteomics server (http://au.expasy.org/prosite/). Subcellular localization of the deduced polypeptides was predicted using WoLPPSORT (http://wolfpsort.org/). The TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) program makes a prediction of membrane-spanning regions and their orientation. Introns/exons were detected using the Gene Structure Display Server (GSDS) (http://gsds.cbi.pku.edu.cn/). The amino acid sequences of different species were obtained from the GenBank database (http://www.ncbi.nih.gov), and a multiple sequence alignment was created using DNAMAN (v.5.2.2). Subsequently, the homologous tree was also generated using DNAMAN. Characteristics of the HSP70 family were determined by searching the BLOCKS database, v.14.2 (http://bioinformatics.weizmann.ac.il/blocks/) (Jiang et al. 2005).

Results

cDNA Cloning and Sequence Analysis of *BcHSP70s*

The full open reading frame (ORF) for the BcHSC70 cDNA sequence was obtained by PCR amplification. *BccpHSC70-1* contained an ORF encoding 724 amino acids, which was the longest sequence of the four genes analyzed. *BcmtHSC70-1 encoded* 676 amino acids and was the shortest of all the genes. The predicted molecular weight of *BccpHSP70-1* was 77.38 kDa using prot-Param analysis compared to the theoretical pI of 4.88, which was lower than those of other three (Table 3). Three conserved signature sequences of HSP70s were detected by scanning the sequences against PROSITE patterns and profiles through the ExPASy proteomics server (Fig. 1). All the BcHSP70s except *BccpHSC70-1* had signature for the motif 'IDLGTTNS', which was closed in the N-terminal region (Fig. 1a). Only two conserved signature motifs 'VFDLGGGTFDVS[VI]L' and 'V[IL]LVGG[MS] TR [VI] P[KA]VQ[ED]' were detected in *BccpHSC70-1* by the ExPASy proteomics server (Fig. 1b) (Karlin and Brocchieri 1998). *BccpHSC70s* and *BcmtHSC70s* were identified in chloroplasts and mitochondria, respectively, using WoLPPSORT and TargetP software packages (Meimaridou et al. 2009). *BccpHSC70-1* protein had a high degree of sequence homology with HSC70s from other species (Fig. 3). *BccpHSC70-2* showed a much greater similarity (92 and 86 %, respectively) to those of *B. rapa HSC70-2* and *A. thaliana HSC70-2*; *BcmtHSC70-*

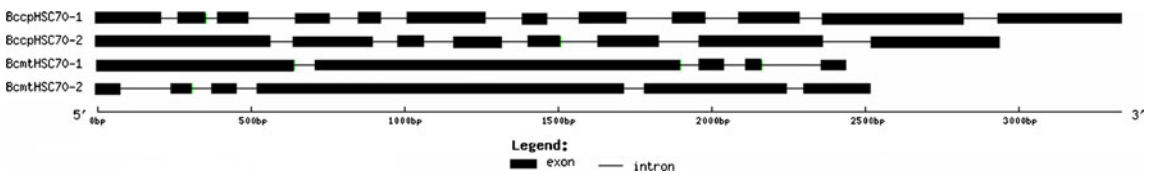


Fig. 2 Exon-intron structures for *BccpHSC70* and *BcmtHSC70* genes. The exons are represented as *green boxes*, and the introns are represented as *lines*. Total gene lengths, from the predicted translation initiation colon (left exon) until the stop colon (right exon), are indicated for each gene. (http://gsds.cbi.pku.edu.cn/)

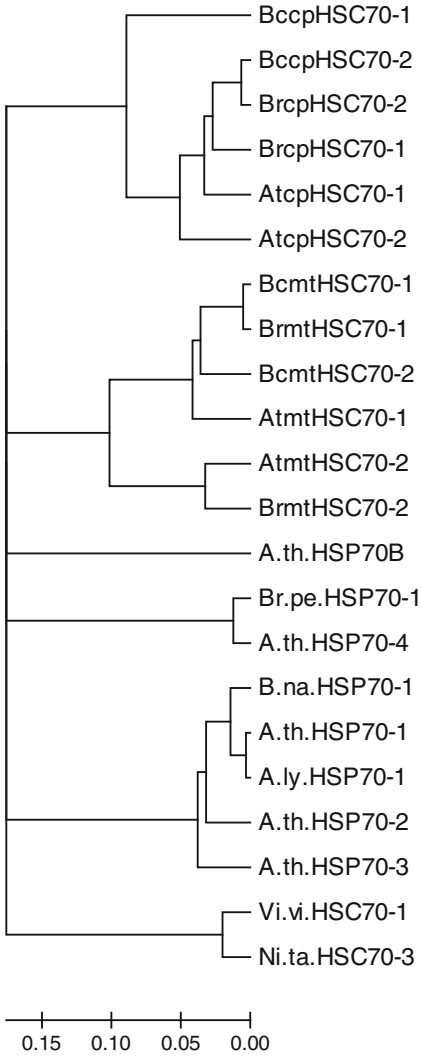


Fig. 3 Phylogenetic relationships among non-heading Chinese cabbage (*BcHSC70*), Chinese cabbage (*BrHSC70*) (http://brassicadb.org/brad/searchGene.php), *Arabidopsis thaliana* (*AtHSC70*) and the other species HSP70 isoforms (http://www.ncbi.nlm.nih.gov/). The NCBI/GenBank accession Nos for the proteins are shown as follows: *B.na. HSP70-1, B. napus HSP70-1,* AAB88009.1; *A.ly.HSP70-1, A.lyrata HSP70-1,* XP_002873056.1*; Vi.vi.HSP70-1, Vitis vinifera HSP70-1,* XP_002284008; *Ni.taHSP70-3, Nicotiana tabacum HSP70-3,* AAR17080.1; *A.th.HSP70-1, A.thalina HSP70-1,* AAM53305.1; *A.th. HSP70-2, A.thalina HSP70-2*, AAP37770.1; *A.th.HSP70-3, A.thalina HSP70-3,* AEE74767.1; *A.th.HSP70-4, A.thalina HSP70-4,* AEE75218.1; *A.th.HSP70B, A. thalina HSP70B,* AAP37760.1. The tree was constructed according to the neighbor-joining algorithm, and the bootstrap value was set as 1,000

2 shared 95 and 92 % identity with its counterparts in *B. rapa* and *A. thaliana*, respectively (Merchant et al. 2007). TMpred analysis showed the protein contained a distinguishing transmembrane helix corresponding with the hydrophobic region. Based on this analysis for *BccpHSC70-1, BccpHSC70-2, BcmtHSC70-1*, and *BcmtHSC70-2* there were 4, 5, 6, and 6 transmembrane helixes, respectively. There were 12, 8, 5, and 6 exons in *BccpHSC70-1, BccpHSC70-2, BcmtHSC70-1*, and *BcmtHSC70-2*, respectively. (Figs. 2, 3) The

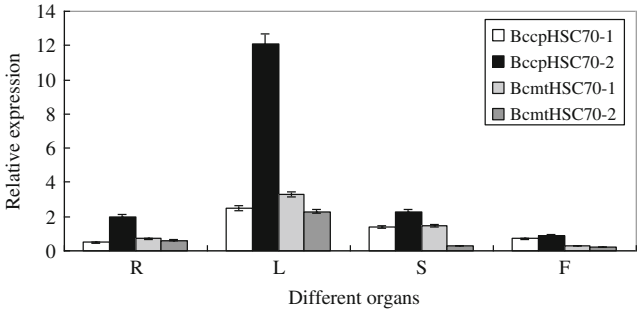


Fig. 4 Expression of organelle *BcHSC70s* in different organs (*R* root, *L* leaf, *S* stem, *F* flower)

corresponding homologous genes to these BcHSP70 genes were located on chromosomes 1, 3, 8, and 1, respectively (Table 3).

Expression Analysis of *BcHSC70* in Different Tissues

BccpHSC70-2 was at relatively high levels in all organs tested; highest level of expression (12.05) was observed in the leaves. The four organelle-localized genes (especially *BccpHSC70-2* and *BcmtHSC70-1*) were expressed in tissues of roots and flowers at mostly comparable levels. However, *BccpHSC70-1* and *BcmtHSC70-2* were expressed in leaves and stems, where transcripts were at relatively low levels. This revealed that *BccpHSC70-2* might play an important role in chloroplast (Fig. 4).

Expression Analysis of BcHSC70 During Development

RT-PCR analysis of *BccpHSC70s* was completed at five development stages: Fl, line with 5 leaves; Ff, line with 15 leaves; Bp, bolting period; Sp, squaring period; and Bs, blossom stage. The RNAs for RT-PCR were from NHCC001, NHCC002, and NHCC004. The gene was expressed during the development period; expression of *BccpHSC70-1* was highest in NHCC001 at each point during development. The same gene was expressed at very low levels in NHCC002. Expression of *BccpHSC70-1* in NHCC004 was intermediate. In addition, different tissues expressed varying levels of the *BccpHSC70-1* gene (Kristensen et al. 2002).

The four gene expression levels presented in Fig. 5 showed higher expression of *BccpHSC70-2* than the other three genes in the three cultivars during different growth periods. Expression of *BccpHSC70-2* in NHCC001 was highest during all vegetative growth stages. Interestingly, expression of *BccpHSC70-2, BcmtHSC70-1*, and *BcmtHSC70-2* in NHCC002 was higher than in NHCC001 and NHCC004 during the reproductive growth stage. Otherwise, *BccpHSC70-2*, *BcmtHSC70-1*, and *BcmtHSC70-2* in NHCC004 were expressed at their highest levels during the Ff growth stage.

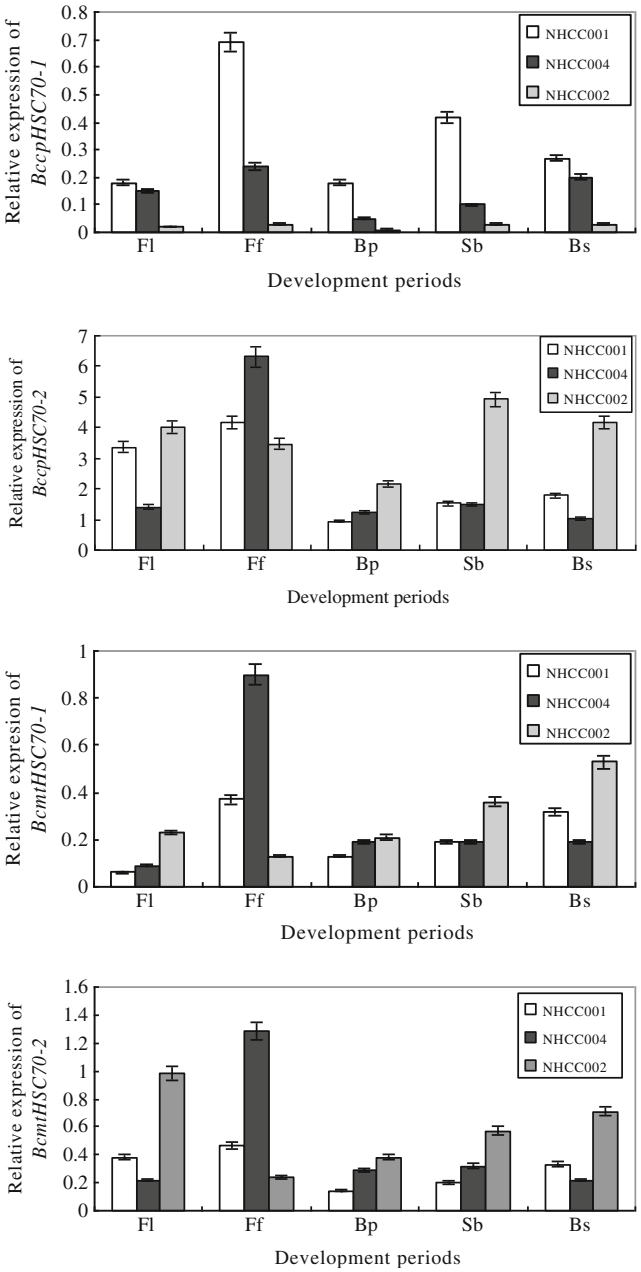


Fig. 5 Expression analysis of *BccpHSC70s* and *BcmtHSC70s* in different development stages (*Fl* line with 5 leaves, *Ff* line with 15 leaves, *Bp* bolting period, *Sp* squaring period, *Bs* blossom stage)

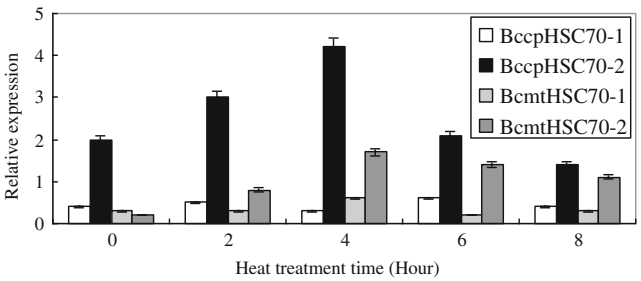


Fig. 6 Expression of different genes under 38 °C treatment

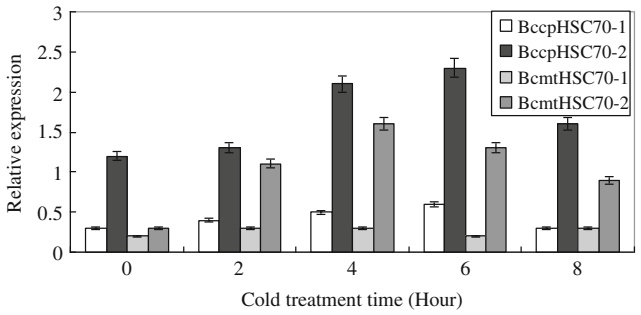


Fig. 7 Expression profile of *BcHSC70* mRNA under 4 °C cold shock conditions

The four genes were expressed at different levels in the three cultivars during different development periods.

Expression of BcHSC70 Genes in Heat Stressed Plants

In *B. campestris* ssp. *chinensis* under heat shock (38 °C), the highest level of *BccpHSC70-2* occurred after 4 h. Chloroplasts were likely sensitive to temperature stress. The transcripts of *BcmtHSC70-2* also increased significantly. The fact that *BcmtHSC70-2* expression was inducible suggested that it was more associated with heat stress. *BccpHSC70-1* and *BcmtHSC70-1* were not up-regulated after heat shock in the organs tested. In fact, the expression of *BcmtHSC70-1* was

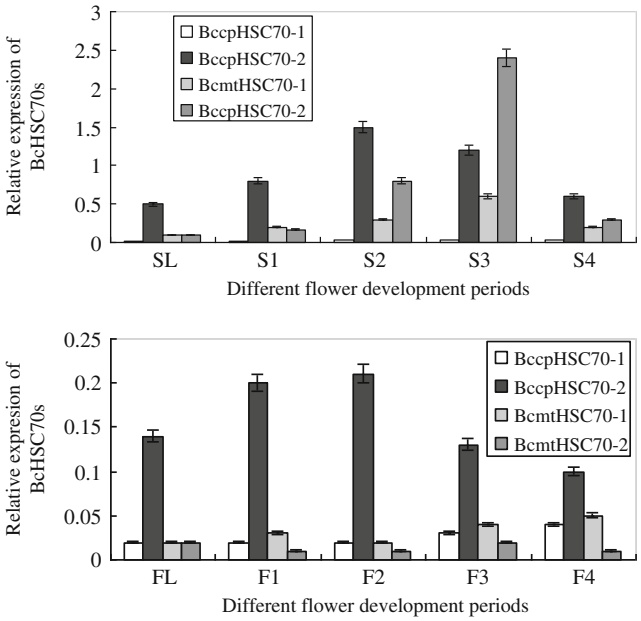


Fig. 8 Alterations of mRNA levels for *BcHSC70* genes during different development stages in non-heading Chinese cabbage. *S1*, *S2*, *S3*, *S4* sterility flower in different sizes (<0.6, 1.8, 3.0, >4.5 mm); *SL* sterile leaves; *F1*, *F2*, *F3*, *F4* maintainer flower in different sizes (<0.5, 1.5, 2.5, >3.5 mm); *FL* maintainer leaves. Poly (A)-mRNA was isolated from flowers at different stages of development, converted to cDNA, and subjected to comparative RT-PCR quantification. Each *data point* represents mean ± SE ($n=3$)

very low and there was no change of its expression; expression of *BcmtHSC70-1* could be regarded as constitutive (Fig. 6).

Expression of *BcHSC70* mRNA in Cold Shocked Plants

The mRNA levels of *BccpHSC70-2* genes in NHCC001 were higher than other cultivars when plants were cold shocked (4 °C). *BcmtHSC70-2* was 9-fold higher than that of the control at the starting point and remained higher. The mRNA levels of *BccpHSC70-2* in NHCC001 slowly increased during the period of cold shock. There was no detectable change in transcript level of *BcmtHSC70-1* during the cold treatment. The expression level of *BcmtHSC70-2* mRNA greatly increased relative to the other genes (Fig. 7).

Tissue Development Distribution of *BcHSC70* mRNA Between Sterile Line and Maintainer Line During Flower Development

Different genes have their expression in the organelles during different development periods (Fig. 8). *BccpHSC70-2* and *BcmtHSC70-2* were both expressed; *BccpHSC70-2* was relatively highly expressed in two lines. Compared to leaves, the mRNA level of *BcmtHSC70-2* was increased to a greater extent in flowers during the third stage of development. The expression of the *BcmtHSC70-2* gene in the sterile line was highest of all at S3 (1.8 mm < flower size < 3.0 mm) and S2 (0.6 mm < flower size < 1.8 mm)—about 8- to 30-fold that of buds at S1, but the *BcmtHSC70-2* gene transcript was low in maintainer line. The expression of *BcmtHSC70-2* mRNA was similar between the two lines except at S2 and S3.

Discussion

*BcHSC70*s Share Highly Conserved Structure

HSP70 is often co-chaperoned with other proteins, such as DnaJ family proteins (Preisig-Muller et al. 1994; Pratt and Toft 2003; Arakawa et al. 2011), and also combined with proteins such as HSP90 (Becker et al. 1996; Chapple et al. 2004), HSP70 is a highly conserved and abundant protein that provides functions involved in protein folding, cytoprotection, proteosomal degradation, and a number of cellular regulatory pathways (Morshauser et al. 1999; Zhang and Burrows 2004; Craig et al. 2006; Brown et al. 2007). In this study, the full ORF of the cDNA sequences of HSP70s (BccpHSC70s and BcmtHSC70s), which are located in chloroplast and mitochondria, were cloned from non-heading Chinese cabbage (*B. campestris* ssp. *chinensis*). Three conserved family signatures were identified in the BcHSC70 amino acid sequences, although *BccpHSC70-1* had only two signature motifs. Highly conserved domains of BcHSC70 guarantee the conserved functions of BcHSC70s (Hartl and Hayer-Hart 2002; Brown et al. 2007).

In the N-terminal of HSP70s, the conserved motif "IDLGT TNS" was detected (Fig. 1a). In the M-domain, the conserved motifs "VFDLGGGTFDVSVL" and "VILVGGSTRIPA VQE" were found in HSP70s of chloroplasts and mitochondria (Fig. 1b). In the C-domain of HSP70, about 20 residues were highly variable among different cultivars. The conserved "DVIDADFTDSK" and "PEAEYEEV" motifs located at the C-terminus of HSP70 indicated that HSP70s had motifs for organelle localization (Fig. 1d). Other HSP70s in the cytoplasm all had the C-terminal polypeptide "IMEEVD" (Bloch and Johnson 1995; Lin et al. 2001; Qiu et al. 2006). The four HSP70s belonged to the *Brassica* genus and were located in the Cruciferae family. Both the alignment result and the phylogeny tree analysis revealed that orthologous BcHSC70s had closer genetic relationships than the paralogs (Sung et al. 2001).

Levels of *BccpHSC70-1* mRNA May be Associated with Heat Tolerance in Various Cultivars

Many HSPs protect plants from heat stress by either preventing irreversible protein denaturation (e.g., small HSPs) or rescuing heat-denatured proteins (e.g., Hsp70s and Hsp100s) (Rampelt et al. 2012). Other mechanisms, besides enhanced protein quality controlled by HSPs, may contribute to thermotolerance (Gromoff et al. 1989; Oka et al. 1998; Larkindale et al. 2005). *BcHSC70* combined with chaperone DnaJ and *BcHSP90* increased to some degree, and higher *HSP70* expression can increase heat tolerance (Morejohn et al. 1987; Frugis et al. 1999; Jiang et al. 2005; Kampinga and Craig 2010). In this experiment, the lack of obvious change in *BccpHSC70-1* gene expression in response to heat shock showed *BccpHSC70-1* expressed constitutively. The expression of *BccpHSC70-1* stayed at a lower level in NHCC002 during different development periods (Fig. 5). However, the level of *BccpHSC70-1* expression in the NHCC001 cultivar was higher at the same time point, suggesting the level of *BccpHSC70-1* mRNA accumulation was associated with the heat stress tolerance (Fig. 6). Thus, *BccpHSC70-1* may be the best candidate gene for distinguishing different thermotolerant cultivars.

Expression of *BccpHSC70s* in Developmental Stages and in Response to Extreme Temperature

In normal development temperature conditions (25 °C), the expression of *BccpHSC70-2* in three cultivars was high in leaves and low elsewhere. Transcripts of *BccpHSC70-2* were higher in all organs compared with those of the other genes. The developmental signals suggested *BccpHSC70-2* played a specific role in chloroplasts. Morphological changes were

observed such as damage to chloroplast envelopes and thylakoid structures. There were obvious differences in heat resistance in the different cultivars (Renner and Waters 2007). Chloroplasts can be used as an important indicator to evaluate the heat tolerance (Ehlers and Hall 1998; Camejo et al. 2005). In our experiment, *BccpHSC70-2* indicated chloroplasts were sensitive to heat stress. As in previous research (Muller et al. 1992), our results at the molecular level were consistent with changes at the cellular level.

The Expression of *BcmtHSC70-2* Was Involved in the Process of Morphogenesis

The complicated regulatory mechanisms of HSP70 in the cell were involved in the process of morphogenesis. In *B. campestris* ssp. *chinensis*, the development of the pol CMS anthers was inhibited at the stage of archesporial cells. In this stage, the size of the flower was less than 0.5 mm. The *BcmtHSC70-2* gene in the maintainer line may play a regular role in flower development, and has a strategic role in the developmental dynamics that govern early morphogenesis. Thus, we concluded that the *BcmtHSC70-2* gene in pol CMS is likely to be an important factor in signaling or protecting flower development in sterile males (Fig. 8).

Expression of *BcmtHSC70-2* May Be Associated with Oxidative Stress in Mitochondria

In many plant species, it has been demonstrated that HSPs increase membrane stability. Injuries associated with heat stress ultimately lead to production of ROS (Ehlers and Hall 1998; Ireland et al. 2004; Camejo et al. 2005). Mitochondria multiply when the energy needs of a cell increase. In corn, for example, the numbers of mitochondria increase by 40 times during the period of pollen grain formation compared with seedlings to meet energy needs (Deng et al. 2007). The pol CMS line caused the oxidative stress, which was proved by the higher activities of POD, SOD, and CAT in pol CMS lines than those in its maintainer line. Microspores in cytoplasm of sterile males suffered from severe oxidative stress during pollen development (Li et al. 2004). HSP70 may protect the cells against apoptosis and/or necrosis (Ishioka et al. 2006). This suggests that BcHSP70 is responsive to oxidative stress in mitochondria and will result in progressed cell death during the flowering stage. In this study, we examined differences in HSP70 expression between the sterile line and the maintainer line, and its temporal relationship with ROS produced in leaves exposed to extreme heat. *BcHSP70* may be associated with enhanced mitochondria proliferation and cell survival, and eventually suppresses apoptosis, or down-regulation of hsp70 may permit apoptosis (Zhu et al. 2006).

Like many crop plants, *B. campestris* ssp. *chinensis* is not a thermotolerant species, and annual production could be impacted in the context of global climate change. In using this plant species as a model, we were able to create cDNA clones and perform molecular analyses of cpHSC70 and mtHSC70. We found that *BccpHSC70-1* may be the best candidate gene for distinguishing thermotolerance of plant cultivars. In addition, we believe *BccpHSC70-2* plays a critical role in protecting chloroplasts from heat stress, and might be a candidate gene for developing thermotolerant cultivars of *B. campestris* ssp. *chinensis*. These data should be used to further study the physiological and molecular functions in plants susceptible to high temperatures. To further explore the function of the HSC70 genes, we plan to create transgenic plants (i.e. *Arabidopsis* and tobacco plants) that overexpress BccpHSC70s or BcmtHSC70s.

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