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Genome-wide analysis of heptahelical protein (*HHP*) gene family and expression of *BcHHP1* in response to stresses in *Brassica rapa*

J. WANG[1], F.Y. HUANG[1], X.L. HOU[1]*, and X. YOU[2]*

State Key Laboratory of Crop Genetics and Germplasm Enhancement, Ministry of Agriculture, Nanjing 210095, P.R. China[1]
College of Sciences, Nanjing Agricultural University, Nanjing 210095, P.R.China[2]

Abstract

Heptahelical protein (HHP) signalling pathway is involved in cold acclimation responses to low temperature and other stresses. The *HHP* transcription factor family is the key component regulating this signalling pathway. In this study, five *HHP-like* genes, *BcHHP1*, *BcHHP2*, *BcHHP3*, *BcHHP4*, and *BcHHP5*, were isolated from non-heading Chinese cabbage (*Brassica rapa* ssp. *chinensis* cv. Suzhouqing). Multiple sequence alignment and phylogenetic analysis showed that BcHHP proteins are highly homologous to HHP proteins from *Arabidopsis thaliana*, *Glycine max*, *Oryza sativa*, and *Zea mays*. Some of these HHP proteins might share similar functions in some aspects, which might be further proved by interaction network of *BcHHP* genes. Furthermore, real-time quantitative PCR showed that BcHHP1 was induced under cold and salt treatments. Besides, BcHHP1 was also accumulated in response to abscisic acid and salicylic acid, indicating that *BcHHP1* gene might participate in response to hormone treatments. In addition, a BcHHP1-YFP fusion protein was localized to the nucleus and cytoplasm. These results indicated that five *BcHHP* genes might play important roles in a functional HHP signalling pathway responding to cold treatment. This work might be useful for future functional analysis of other *HHP-like* genes.

Additional key words: abscisic acid, cold stress, expression analysis, non-heading Chinese cabbage, salicylic acid, salinity, subcellular localization.

Introduction

Cold stress, is an important environmental factor which affects plant production and quality worldwide by limiting plant growth, development, and geographical distribution. Some plants can acquire higher freezing tolerance after a period of low but not-freezing temperature, this process is named cold acclimation (Graham and Patterson 1982). Cold acclimation is a very complex process involving a series of changes in gene expression and in protein metabolism (Chinnusamy *et al*. 2007). So plants use an interconnected signalling network in order to respond to multiple abiotic stresses such as cold, freezing, drought, salinity, and so on (Chinnusamy *et al*. 2005, Yamaguchi-Shinozaki and Shinozaki 2006). *Heptahelical-like* (*HHP1*) is an important member of *HHP* gene family that consists of at least five members *HHP1*, *HHP2*, *HHP3*, *HHP4*, and *HHP5* in *Arabidopsis thaliana* (Hsieh and Goodman 2005). These genes are homologous to membrane progestin receptors (mPRs) and human adiponectin receptors (AdipoRs) (Hsieh and Goodman 2005). One of five HHP proteins in *Arabidopsis*, HHP1 has been characterized as a reliable molecular link to ABA regulation of cold signal transduction components (Chen *et al*. 2009, 2010). *HHP1* is induced by ABA and by cold, and activated HHP1

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Abbreviations: ABA - abscisic acid; AdipoR - adiponectin receptor; bHLH - basic helix-loop-helix; CAMTA - calmodulin-binding transcription activator; CBF - C-repeat binding factor; HHP - heptahelical protein; ICE - inducer of cbf expression; mPR - membrane progestin receptor; MS - Murashige and Skoog; ORF - open reading frame; pI - isoelectric point; qPCR - quantitative PCR; SA - salicylic acid; Mr - relative molecular mass.
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* Corresponding authors; fax: (+86) 025 8439-5917, e-mails: hxl@njau.edu.cn; youx@njau.edu.cn

protein interacts with basic helix-loop-helix (bHLH) inducer of C-repeat binding factor (*CBF*) expression 1 (*ICE1*; Chen *et al*. 2010), an upstream regulator of *CBF3* (Chinnusamy *et al*. 2003, Lee *et al*. 2005, Miura *et al*. 2007), to trigger cold acclimation responses. The mRNA expression patterns of five *HHP* genes have been described in response to extreme temperature, salt, and phytohormones (Hsieh and Goodman 2005), however, further experiments are needed to elucidate the functions of *HHP* gene family. It is worth noting that by binding to their promoters MYB96 regulates *HHP* genes. Further, HHP proteins in turn interact with *CBF* upstream regulators, such as *ICE1* and calmodulin-binding transcription activator 3 (*CAMTA3*). They form specific interaction networks of *HHPs* with *CBF* upstream regulators, which are necessary to facilitate transcriptional activation of *CBF* regulon under stressful conditions (Lee and Seo 2015).

Non-heading Chinese cabbage (*Brassica rapa* ssp. *chinensis*) has a long history of cultivation in the middle and lower reaches of the Yangtze river. It is related to *Arabidopsis thaliana* and has a cold acclimation ability. Therefore, we speculate that there is a HHP cold signalling pathway similar to that in *Arabidopsis thaliana*. Some of cold-related genes, such as *BcICE1*, *BcCBF3*, *BcCOR14* and *BcWRKY46*, were successfully cloned and their functions were analyzed. However, *BcHHP* gene family remains to be studied. Therefore, five *BcHHP* genes from *Brassica rapa* ssp. *chinensis* cv. Suzhouqing were cloned. Five *BcHHP* genes were divided into *BcHHP1*, *BcHHP2*, *BcHHP3*, *BcHHP4*, and *BcHHP5* by homologous comparison. We analyzed the sequence structure and studied their evolutionary position through phylogenetic trees. We analyzed the phylogenetic relationships, conserved motifs, and subcellular localization of *BcHHP1,* to demonstrate whether *BcHHP1* and *AtHHP1* have similar functions in response to cold stress. In addition, the expression patterns of BcHHP1 under multiple abiotic stresses were examined by real-time quantitative PCR (qPCR). This work might help to reveal the biological function of BcHHP1, which could be a candidate gene for genetic improvement of this species.

Materials and methods

Plant culture and stress treatments: Non-heading Chinese cabbage (*Brassica rapa* L. ssp. *chinensis* cv. Suzhouqing), obtained from the Nanjing Agricultural University (Nanjing, China), was used in the following experiments. Healthy seeds were harvested from plants grown in a climate chamber at 20 - 24 °C under continuous irradiance of of 100 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps (Gilmour *et al*. 1998). Healthy seeds were soaked and germinated in a soil:sand mixture (3:1) in a growth chamber (a 16-h photoperiod, day/night temperatures of 24/20 °C, irradiance of 100 μmol m^{-2} s^{-1}, and an air humidity of 40 %). with other conditions remained unchanged. Stress treatments were performed using the seedlings at five-leaf stage with other conditions remained unchanged. For cold treatment, plants were incubated at 4 °C under continuous irradiance of 100 μmol m^{-2} s^{-1}. For salt, ABA, and SA treatments, plants were watered with 100 mM NaCl, 100 μM ABA, and 0.1 mM SA, respectively (Tang *et al*. 2013). Samples were harvested after 0, 0.5, 1, 2, 4, 8, and 24 h, then frozen in liquid nitrogen and stored at -70 °C for total RNA extraction.

Isolation and identification of cDNAs encoding five BcHHP proteins: Taking into account the feature that nucleotide sequences are relatively conserved evolutionarily in different plant species, we extracted the *Arabidopsis thaliana* gene sequences of HHP1, HHP2, HHP3, HHP4, and HHP5 from *NCBI* GeneBank (http://www.ncbi.nlm.nih.gov/genbank/). Using protein sequences as probes to search the *Brassica EST* database (http://www.Brassica.nbi.ac.uk), we successfully obtained several homologous fragments, which were further spliced by *DNAstar's Seqman* tool, using the encoding product sequences of *AtHHP* genes as the controls. Then we inspected the integrity of the open reading frame (ORF) of the obtained sequences through *BLAST* (http://brassicadb.org/brad/). Subsequently, the corresponding forward primers and reverse primers were designed by *Primer 5.0* software with sequences of these five homologous genes (Table 2 Suppl.).

The cDNA of *BcHHP1* was amplified from a cDNA library of *B. rapa* treated with diverse abiotic stresses by real-time quantitative PCR (qPCR) analysis. Total RNA was extracted from leaves with the *RNAeasy* mini kit (*Tiangen*, Beijing, China), following the manufacturer's instructions. The first-strand cDNA was synthesized with 1 μg of mixed total RNA by using a *Superscript II* kit (*Takara*, Dalian, China), to construct a stress-induced cDNA library. Conserved regions were identified based on sequence information from *Arabidopsis HHP* gene family (http://arabidopsis.org/index.jsp) and the Chinese cabbage *chiifu* genome (http://brassicadb.org/brad/). We designed the qBcHHP1 and qBcACTIN primers according to sequences of the conserved regions to amplify the core fragment by real-time qPCR (Table 1). The ORF of *BcHHP1* cDNA was amplified with the primers gBcHHP1 (Table 2 Suppl.). For molecular cloning of genes, we designed primers BcHHP1, BcHHP2, BcHHP3, BcHHP4 and BcHHP5 (Table 2 Suppl.). The amplification program was: 94 °C for 5 min, 35 cycles of 94 °C for 3 s, 52 - 63 °C for 30 s, and 72 °C

for 1 min, and extension at 72 °C for 10 min. Then PCR products were cloned into pMD19-T vector (*Takara*, Dalian, China). The ORF sizes of *BcHHP* gene family were confirmed by sequencing. The molecular mass, isoelectric point, half life, and instability index were predicted using the online *ExPasy* program (http://www.expasy.org/tools/) (Table 1).

Table 1. The overall information on *BcHHP* genes.

Gene name	ORF [bp]	Amino acidc	Mr [kDa]	pI	Half life [h]	Instability index
BcHHP1	972	323	36.95	8.70	30	44.81
BcHHP2	1074	357	40.88	9.26	30	54.00
BcHHP3	1044	347	39.35	9.08	30	48.35
BcHHP4	1164	387	44.49	8.73	30	51.75
BcHHP5	1488	495	56.61	8.17	30	53.73

Molecular cloning of *BcHHP* gene family: The reverse transcription and synthesis of first strand cDNA were carried out from the leaves of *B. rapa* (at the five-leaf stage) and primers BcHHP1, BcHHP2, BcHHP3, BcHHP4, and BcHHP5 (Table 2 Suppl) were used to perform PCR amplification. The amplification program was 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 52 - 63 °C for 30 s and 72 °C for 1 min, and extension at 72 °C for 10 min. To detect PCR products, 1 % (m/v) agarose gel electro-phoresis was used. The target fragments were recovered and ligated with the cloning vector pMD19-T at 16 °C for 12 h and then were transformed into DH5α competent cells, extracted the plasmid. The plasmid was extracted and named *BcHHPs*-pMD19-T and sent to *Invitrogen* for sequencing. Then PCR amplification was performed and PCR products were assayed with 1 % agarose gel electrophoresis.

Multiple sequence alignment and phylogenetic analysis: By comparing *NCBI* homologous sequences online (https://www.ncbi.nlm.nih.gov/pubmed/), we obtained HHP protein sequences from some different crops (Table 1 Suppl.). In this study, to analyze the deduced amino acid sequence of HHP, we used *DNAMAN* software (*Lynnon Biosoft*, USA). To perform multiple sequence alignment of HHP, we used *MEGA 5.0* software with default parameters and manual correction (Tamura *et al*. 2014). *MEGA 5.0* software was also used for phylogenetic tree drawings. Bootstrap values were estimated at 1 000 replicates. The conserved motifs were analyzed by using *MEME Suite* (http://meme.nbcr.net/meme/) with the default settings except the maximum width was set to 200, and the minimum and maximum numbers of motifs were defined as 2 and 10, respectively.

Identification of orthologous and paralogous genes: *GeneMANIA* was used to search for orthologous and paralogous genes between *B. rapa* and *Arabidopsis* using the entire HHP protein sequences. By setting the related parameters, we found out physical interactions, predicted co-expression, shared protein domains, genetic interactions, and co-localization. An interaction network of *BcHHP* genes was constructed to understand genome-wide regulation network. By replacing *Arabidopsis* proteins with corresponding orthologous and co-orthologous *B. rapa* proteins, an interaction network of BcHHP proteins was constructed and displayed by using *GeneMANIA* software (https://genemania.org/#/opennewwindow).

Real-time qPCR analysis of *BcHHP1* under stress treatments: To investigate the change of *BcHHP1* expression under diverse stress treatments, we performed real-time qPCR. The primers were designed by the online primer design tool from *Genscript Biotechnology Company* (https://www.genscript.com/ssl-bin/app/primer) and listed in Table 2 Suppl. The five-leaf stage seedlings were utilized after cold, salt, ABA, and SA treatments for 0, 0.5, 1, 2, 4, 8, and 24 h. Total RNA was extracted from the frozen plant tissues with an RNA extraction kit (*Tiangen*, Beijing, China). Genomic DNA contamination was removed using *DNase I* (*Takara*). The first-strand cDNA was synthesized from 1 μg DNase I-treated total RNA with the *PrimeScript*™ RT reagent kit (*Takara*). The cDNA reaction mix was diluted (1:10) by using the *EASY* solution for qPCR (*Takara*). The qPCR assay was carried out with 2 mm^3 of cDNA and 10 mm^3 of *SYBR® Premix Ex Taq* (*Takara*) by using the *7500 Fast qPCR System* (*Applied Biosystems*, Foster City, CA, USA). The programs were as follows: initial denaturation at 95 °C for 4 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and polymerization at 72 °C for 30 s. In order to confirm that amplified and detected product was specific, a melting curve was generated after each qPCR. Relative expression of *BcHHP1* was calculated by the $2^{-\Delta\Delta CT}$ method using *BcACTIN* gene as an internal standard (Livak and Schmittgen 2001).

Statistical analysis: Data from the real-time qPCR

experiments were tested using one-way *ANOVA*). The standard errors of the mean from three replicates were calculated. Statistical analyses were performed by using *SPSS v.19.0* software. Differences between control and while treated plants were analyzed by two-way *ANOVA*, according to Duncan's test.

Subcellular localization of BcHHP1 protein: To predict the subcellular localization of BcHHP1 protein, we used *WOLF PSORT* and *Nuc-PLoc*. To determine the subcellular localization of BcHHP1 protein, we constructed two vectors. The full-length coding region of BcHHP1 amplified with *Gateway*-specific primers (Table 2 Suppl.), which was cloned into an entry vector, then further introduced into *pEarleyGate101* vector by using the *Gateway* system (*Invitrogen*, Carlsbad, CA, USA) to generate a 35S::BcHHP1-YFP fusion construct. About 20 bp from both ends of the ORFs were added to *attB* joint sequences, respectively, to obtain *Gateway* primers (Table 1). The PCR reaction was performed as described above. The pDONR221 vector and pEarleyGate101 vector were conserved in our laboratory, and *BP* enzyme mix and *LR* enzyme mix were purchased from *Invitrogen* and used in accordance to instructions. To transiently express *BcHHP1*, *35S::BcHHP1-YFP* was bombarded into onion epidermal cells with a *PDS-100/He* particle delivery system (*Bio-Rad*, Hercules, CA, USA). The bombarded onion epidermal cells were incubated in the dark at 22 °C for 12 h and then analysed by confocal microscopy (*TCS SP2*, *Leica*, Wetzlar, Germany).

Results

The full-length *BcHHP1* gene was isolated. The clone included a 972 bp open reading frame (ORF) encoding 323 deduced amino acids, with an estimated molecular mass (Mr) of 36.95 kDa, a theoretical isoelectric point (pI) of 8.70 and an instability index of 44.81. The information on other *BcHHP* genes was also based on this method (Table 1). The ORF was confirmed by sequencing three times. The predicted amino acid sequence lacked secretory and nuclear localization signal sequences.

The total RNA was extracted from *B. rapa*, then reverse transcribed into cDNA, it was ligated with pMD19-T cloning vector. The corrected *BcHHPs*-pMD19-T plasmid was used as a template (the relevant primers were listed in Table 2 Suppl). The coding sequence of *BcHHP1* gene was amplified, and an amplified band of 972 bp was obtained, which matched the expected result. The coding sequences of the other *BcHHP* genes were also amplified, and amplified bands were obtained, which were consistent with the expected results.

In order to show the evolution of BcHHP protein, we collected HHP proteins coding sequence of *B. rapa* and other species. Then phylogenetic tree was constructed by *MEGA 5.0* (Fig. 1*A*). The phylogenetic tree was divided into three groups, named Group I, Group II, and Group III. In Group I, HHP1 homologous protein from *Arabidopsis thaliana*, *Brassica oleracea*, and *Brassica rapa* were highly homologous to BcHHP1. In Group II, the homology of BcHHP4 with HHP4 proteins from *B. rapa* was higher than that of HPP proteins from *Oryza sativa* and *Zea mays*. Group III contains BcHHP2, BcHHP3, and BcHHP5 homologous proteins, BcHHP2 and BcHHP5 on the same branch. In general, BcHHP proteins from *B. rapa* were more homologous to other *Brassica* HHP proteins than to HHP members from *Arabidopsis thaliana* and other dicotyledonous plants and were highly conservative. The HHP proteins from *Oryza sativa* were homologous to HHP proteins from *Zea mays*. The homology of HHP proteins from *Daucus carota* and HHP proteins from *Solanum lycopersicum* were higher than with HHP proteins from *Glycine max*. The location of some different HHP protein motif sites is shown in Fig. 1*B*. Each block shows the position and strength of a motif site. There were at least six motif sites for each HHP protein, motif location of AtHHP2 protein was very similar to motif location of AtHHP3 protein, motif location of AtHHP4 protein was similar to motif location of AtHHP5 protein. Interestingly, motif location of OsHHP1 protein was similar to motif location of ZmHHP1 protein. More importantly, motif location of BcHHP1 protein was very similar to motif location of AtHHP1 protein. Therefore, we hypothesized that BcHHP1 and AtHHP1 had similar functions in response to abiotic stresses.

The deduced amino acid sequence of BcHHP1 was aligned with sequences from several representative monocots and dicots by using *DNAMAN* (Fig. 2). The sequence of BcHHP1 was similar to the other plant HHPs sequences, high sequence similarities were detected in the N-terminus. The sequence alignment showed that BcHHP1 proteins had approximately 55.34 % amino acid sequence homology (Fig. 2*A*). The results revealed that BcHHP1 protein sequence was more homologous to *Arabidopsis thaliana* protein sequences than to those of *Glycine max*, *Oryza sativa*, and *Zea mays* (Fig. 2*A*), suggesting that HHP proteins in dicot plants are conserved. More importantly, sequence alignment showed that BcHHP1 protein had about 87.65 % amino acid sequence homology with AtHHP1 protein (Fig. 2*B*). Among five HHP proteins, HHP1 has been characterized as a reasonable molecular chain of ABA regulated cold signal components (Chen *et al*. 2009, 2010). Therefore, we speculated that BcHHP1 might have a similar low temperature response function to AtHHP1.

To further investigate the interaction of *BcHHP* genes with other genes, an interaction network associated with *AtHHPs Arabidopsis* orthologs was built (Fig. 1 Suppl.). The purple, red, green, blue, cyan, magenta and, yellow lines standing for physical interactions, co-expression, predicted with shared protein domains, genetic interactions, integrative protein signature database (http://www.ebi.ac.uk/interpro/), and co-localization, respectively. In addition, the interaction network of *BcHHP* genes showed a very complicated correlation with other genes in *B. rapa*.

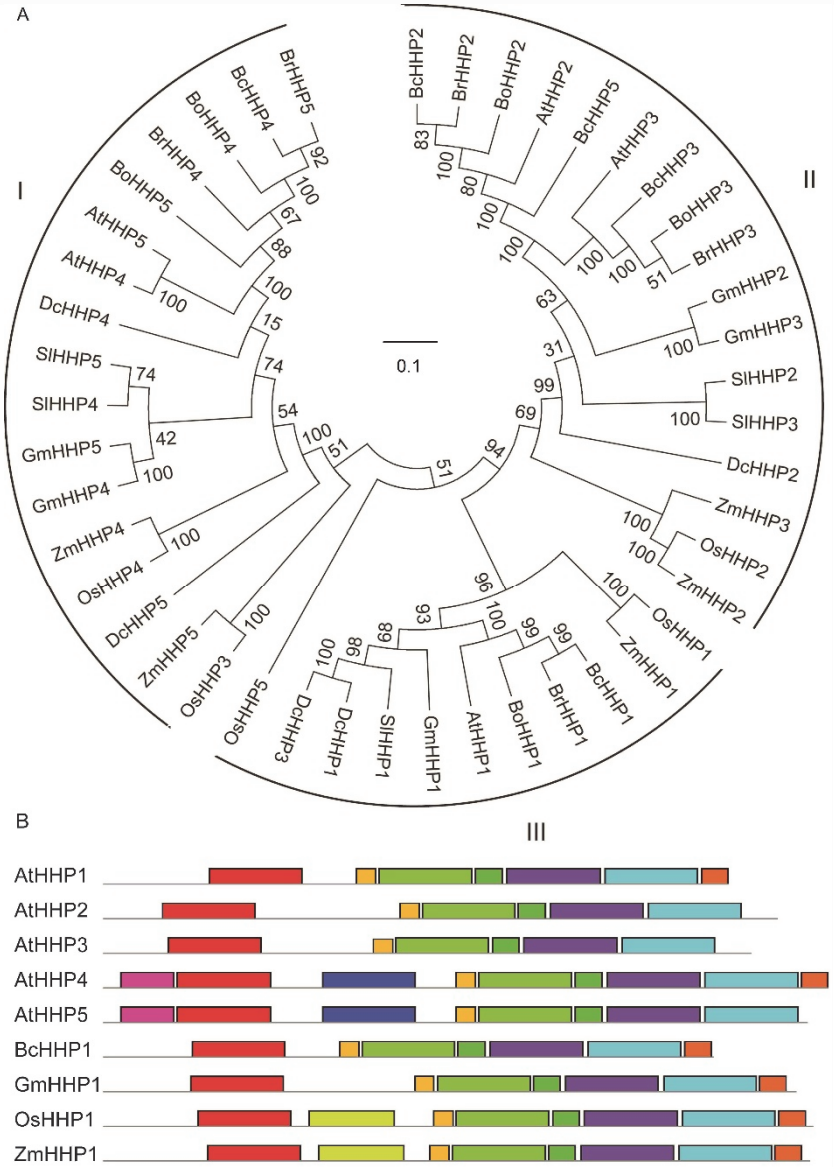

Fig. 1. Phylogenetic and structural analysis of putative BcHHP1 protein and other HHP proteins available from GenBank and other databases were carried out. *A* - The unrooted tree is based on an alignment of full-length protein sequences and was constructed by the maximum likelihood method. *B* - The schematic structure of some of HHP proteins in the selected plants. Different types of patterns are represented by boxes of different colour. The same box in different proteins indicates the same motif. The *scale bar* represents 0.1 substitutions per amino acid position. Species are as follows: At - *Arabidopsis thaliana;* Bc - *Brassica rapa* ssp. *chinensis*; Bo - *Brassica oleracea*; Br - *Brassica campestris*; Dc - *Daucus carota*; Gm - *Glycine max*; Os - *Oryza sativa*; Sl - *Solanum lycopersicum*; Zm - *Zea mays*.

To investigate the expression patterns of *BcHHP1* under different abiotic stresses, we performed real-time qPCR analysis for *BcHHP1* gene. Under cold treatment, *BcHHP1* expression was marginally down-regulated after 0.5 h and then markedly up-regulated after 2 h, reaching a 1.6-fold increase as compared with the control (Fig. 3*A*). Under NaCl treatment, the *BcHHP1* expression was rapidly increased, reached a maximum rate at 8 h after treatment, and then slowly decreased (Fig. 3*B*). Under ABA or SA treatments, the *BcHHP1*

expression increased upto 1 h after treatments and then slowly decreased (Fig. 3*C*). Thus, these data suggested that *BcHHP1* expression was influenced by cold, salt, ABA, and SA treatments.

Plant expression vectors were constructed for determination of subcellular localization. We chose BcHHP1, one of five BcHHP proteins for this experiment. The nuclear localization score of BcHHP1 was 9.0 (KNN = 14) according to *WOLF PSORT* and subcellular location predicted by *Nuc-PLoc* was also nucleus. Onion epidermal cells transiently expressing the *BcHHP1::YFP* fusion construct were also tested for BcHHP1 subcellular localization (Fig. 4). YFP alone was used as a control and was distributed in the cytoplasm and nucleus. Confocal images showed that BcHHP1-YFP was also localized to the cytoplasm and nucleus. These results confirmed that BcHHP1 was present in both cytoplasm and nucleus, which is consistent with its predicted function as an RNA chaperone.

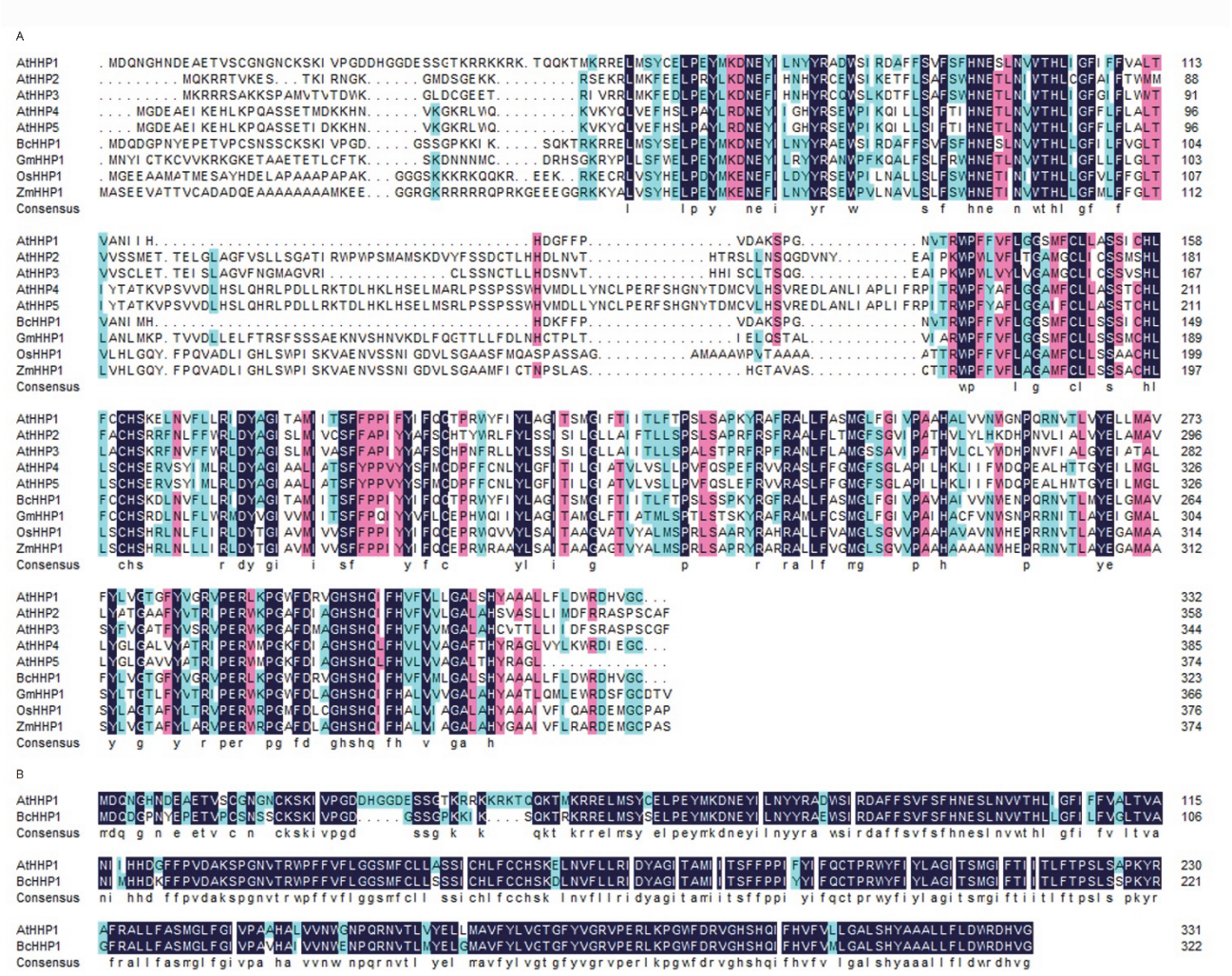

Fig. 2. Amino acid sequence alignments of HHPs from *B. rapa* and other crops. BcHHP1s were isolated in this study and the other proteins were selected from *GenBank*. Less conserved residues, highly conserved residues and perfectly matched residues are represented by *light blue*, *pink*, and *dark blue boxes*, respectively.

Discussion

As one of the most popular fresh vegetables, *B. rapa* ssp. *chinensis* is often subjected to a variety of biotic and abiotic stresses throughout all development stages (Wei *et al*. 2016). Low temperature limits the growth by the inhibition of metabolic reactions, oxidative stress, and inhibition of water uptake (Chinnusamy *et al*. 2007). Under low but not-freezing temperatures, plants can acquire cold tolerance by a process named cold acclimation (Chinnusamy *et al*. 2007). Nowadays, *HHP1* has been shown to be involved in ABA-mediated

osmotic stress signalling and to act as a negative regulator of early growth (Chen *et al*. 2009). This supports the idea that *HHP1* can regulate the function of *ICE1* through protein-protein interaction, because *ICE1* is found to bind directly to the promoter region of *CBF3* (Chinnusamy *et al*. 2003). It is known that expression of *CBF1* is not regulated through the expression of *ICE1* (Yamaguchi-Shinozaki and Shinozaki 2006). We found at least four other protein homologues to HHP1, HHP2, HHP3, HHP4, and HHP5, which are present in *Arabidopsis* (Hsieh and Goodman 2005). In this study, *BcHHP1* gene was isolated from *B. rapa* based on amino acid sequence alignment. The results revealed that BcHHP1 protein sequence was highly similar to HHP protein sequences of *Arabidopsis* and was also homologous to HHP protein sequences of *Glycine max*, *Oryza sativa*, and *Zea mays* (Fig. 1*A*). Phylogenetic and structural analysis of the putative BcHHP1 protein and other HHP proteins available from GenBank and other databases was carried out. The results suggested that these orthologous genes might be derived from a common ancestor and tend to be conserved during evolution.

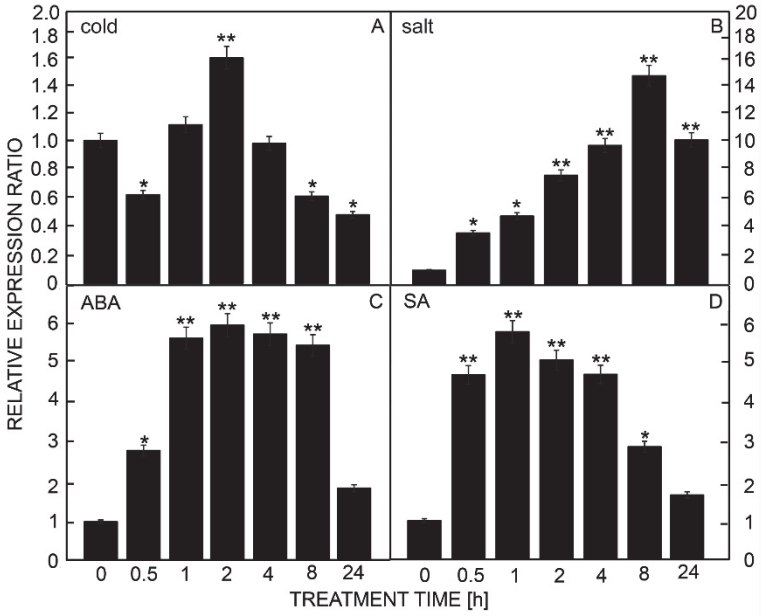

Fig. 3. Expression patterns of *BcHHP1* under multiple abiotic stresses. *A* - *BcHHP1* expression under cold treatment (4 °C); *B* - *BcHHP1* expression under 100 mM NaCl; *C* - *BcHHP1* expression under 100 μM ABA; *D* - *BcHHP1* expression under 0.1 mM SA. The relative transcription to that of *BcACTIN* were quantified using $2^{-\Delta\Delta CT}$;method. Means ± SEs, n = 3, * and ** - significant differences between treatments at $P < 0.05$ and $P < 0.01$, respectively (Duncan's test).

HHP1 is likely to be an upstream positive regulator of cold, drought, and other abiotic stresses signalling pathways, as we speculated. Interestingly, it plays a negative regulatory role in seed germination and early growth (Chen *et al*. 2009). The HHP proteins family is homologous to the PAQR proteins family, which include the membrane progestin receptor (mPR) and adiponectin receptor (AdipoR) (Yamauchi *et al*. 2003, Zhu *et al*. 2003), Lyons *et al*. 2004, Tang *et al*. 2005). The mRNA expression patterns of five *HHP* genes in response to phytohormones, low temperature, and salt treatments have been described (Hsieh and Goodman 2005). However, further experiments are needed to elucidate the functions of *HHP* gene family. In this study, BcHHP1 and AtHHP1 have a high degree of homology but they differ in some functions. In *Arabidopsis*, *AtHHP1* gene, in response to low temperature regulation, while *BcHHP1* and *AtHHP1* have a high degree of homology, so we speculated that *BcHHP1* might also respond to cold stress (Fig. 2*B*). Thus, we speculated that *BcHHP1* might also be involved in the regulation of low temperature response. The genes positively or negatively regulated by BcHHPs were more or less involved in abiotic stress tolerance (Fig. 1 Suppl.).

Real-time qPCR analysis revealed that *BcHHP1* played an important regulatory role in response to cold, NaCl, ABA, and SA treatments. However, the expression patterns of *BcHHP1* differ from expression patterns of *AtHHP1* in some aspects. Expressions of *HHP1* increase in response to low temperature, ABA, and NaCl treatments (Chen *et al*. 2009). The expression of *BcHHP1* showed similar expression patterns under ABA and SA treatments (Fig. 3). The expression and regulation of *Arabidopsis HHP* gene family have been studied; *e.g.*, the expression of *HHP* gene family is positively regulated by the presence of sucrose in the medium. In addition, low temperature stress and salt stress differently affect expression patterns of *HHP*

genes (Hsieh and Goodman 2005). These results suggest that physiological functions of *BcHHP1* might differ from those of *AtHHP1*. In addition to this, we speculated that *BcHHP1* might cross-respond to multiple stresses which was meaningful for plant growth and development.

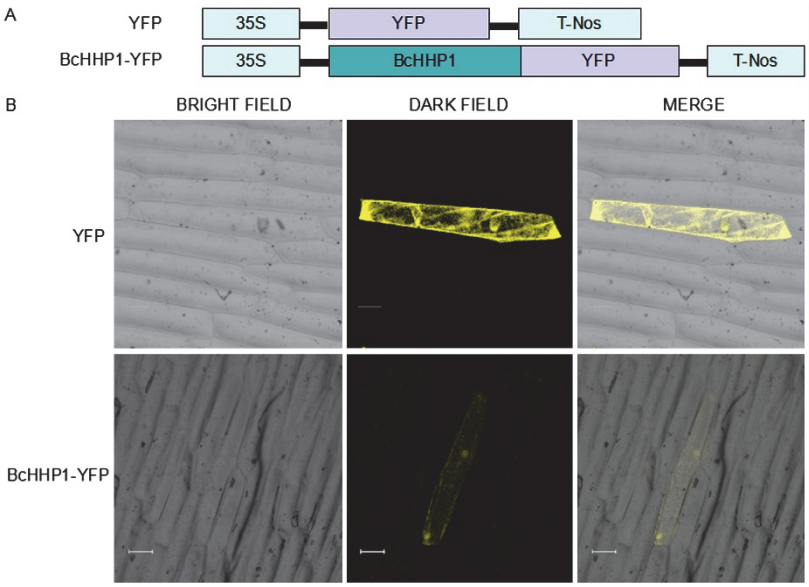

Fig. 4. Subcellular localization of BcHHP1 in onion epidermal cells. *A* - Constructs used in the experiments. *B* - BcHHP1-YFP was transferred to onion epidermal cells through particle bombardment. The *upper panel* shows bright field, dark field and merged images of the YFP control; the *lower panel* shows bright field, dark field and merged images of BcHHP1-YFP (*bar* 20 μm).

Transient expression of BcHHP1-YFP fusion protein showed that BcHHP1 localized to the cytoplasm and nucleus in onion epidermal cells (Fig. 4). In the reproductive organs, significant expression of HHP1 was showed by Hsieh and Goodman (2005). It is possible that HHP1 plays different roles in different organs (or cells) and at different growth stages in *Arabidopsis* (Chen *et al*. 2009). Considering the conserved structure of BcHHP1 and AtHHP1, similar expression patterns under low temperature and localization in the cytoplasm and nucleus, we hypothesized that BcHHP1 might act as an RNA chaperone during cold adaptation.

A comparative study of *HHP* gene family factors between *B. rapa* and other plant species provided valuable information for further functional studies. Our study contributes to understanding how *BcHHP1* transcription factors respond to multiple abiotic stresses. The functions of other *HHP-like* genes need to be further explored.

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