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Cucumber BAX inhibitor-1, a conserved cell death suppressor and a negative programmed cell death regulator under cold stress

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Abstract

Programmed cell death (PCD) is a genetically controlled and conserved process in eukaryotes during development as well as in response to pathogens and other stresses. BAX inhibitor-1 (BI-1) has been implicated as an anti-PCD factor which is highly conserved in plants. Sequence of putative cucumber BI-1 protein exhibited 77.7 % identity and 91.2 % positive value with the homologue *Blast* BI-1 protein of *Arabidopsis thaliana* (AtBI-1). This highly homologous protein to the AtBI-1 protein was named CsBI-1. This protein contains an open reading frame (ORF) of 250 amino acids with a BAX inhibitor domain and five transmembrane regions conserved among members of the BI-1 family. Primers designed by the cDNA of *CsBI-1*gene were used for further sequencing. Cell death in cold-stored cucumber developed concomitantly with increased expression of the *CsBI-1* gene and reached maximum at day 6. However, cell death accelerated significantly after 9 d when sharp decrease of the *CsBI-1* expression occurred. After warming to 20 °C, expression of the *CsBI-1* gene was the highest at day 3, decreased afterwards, and the lowest expression was detected at day 9 when PCD obviously appeared. The overall results indicate that *CsBI-1* is cucumber homologue of *Arabidopsis thaliana AtBI-1* gene. *CsBI-1* is a conserved cell death suppressor induced by cold stress and a negative regulator of PCD.

Additional key words: *Arabidopsis thaliana*, *Cucumis sativus*, RT-PCR.

Introduction

Programmed cell death (PCD) is a genetically controlled and conserved process in eukaryotes (Lam 2004). Many abiotic stresses like heat stress (Qu *et al.* 2009), cold stress (Ning *et al.* 2002), salt stress (Lin *et al.* 2006), exogenous hormones (Yun and Chen 2011, Lu *et al.* 2012), and ozone (Pasqualini 2003) can trigger PCD in plants. The distinguishing morphological and biochemical features of PCD are cytoplasmic shrinkage, chromatin condensation, cytochrome *c* leakage from the mitochondria, altered nuclear morphology, protease activation, and DNA fragmentation (Reape and McCabe 2010). The specific pattern of genomic DNA cleavage, DNA ladder due to internucleosomal excision of chromatin fragments (Wyllie 1980), is still currently used to distinguish PCD from necrosis (Danon *et al.* 2000). Features of PCD were detected in cucumber exposed to continuous ethylene (Hurr *et al.* 2010). Cucumber fruit is usually stored at low temperature to prolong postharvest life. However, cucumber fruit is sensitive to cold temperature and exhibit injury (water soaking) which culminates in cell death. Members of the diverse BCL-2 family of proteins in animals have emerged as important activators or suppressors of PCD (Fadeel *et al.* 1999). Among the important regulators of PCD, much interest has been centered on BCL2-associated X protein (BAX) as the pro-PCD factor. One of the well-characterized mechanisms that can drive apoptosis in metazoans is triggered by BAX that interferes with mitochondrial function by forming pores in the outer membrane (Danial and Kormeyers 2004). One candidate regulator for such a mechanism is BAX inhibitor-1 (BI-1) that balances out the activity of BAX in the developmental

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Abbreviations: BI-1 - BAX inhibitor 1; PCD - programmed cell death; RT-PCR - reverse transcription-polymerase chain reaction.
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processes and responses to environment. BI-1 is an evolutionary conserved endoplasmic reticulum (ER)-resident protein that represents an ancient cell death suppressor conserved in plants and mammals (Watanabe and Lam 2009, Ishikawa *et al.* 2011). BI-1 was originally isolated from a human cDNA library by functional screening in yeast which can suppress BAX-induced cell death in yeast (Xu and Reed 1998). Plant BI-1 genes from rice and *Arabidopsis* were isolated and shown to encode an evolutionary conserved protein that, when over-expressed in yeast and plant cells, suppresses cell death induced by mammalian BAX (Kawai *et al.* 1999). Two *Arabidopsis thaliana* BI-1 (*AtBI-1*) mutants exhibit accelerated progression of cell death using a PCD-inducing fungal toxin fumonisin B1 (FB1) and increased sensitivity to heat shock-induced cell death. However, over-expression of *AtBI-1* mRNA in wild type shows suppression of cell death progress (Watanabe and Lam 2006). In hot pepper, the transcription of *CaBI-1* is induced in response to high or low temperatures, drought, high salinity, flooding, heavy metal stresses, and abscisic acid (ABA). The transgenic plants display markedly improved tolerance to high temperature, water deficit, and high salinity in comparison to the control plants (Isbat *et al.* 2009, Watanabe and Lam 2009). Over-expression of *Triticum aestivum* BI-1 (*TaBI-1*) in tobacco blocks BAX-induced cell death. TaBI-1 exhibits positive transcriptional responses to *Puccinia striiformis* infection and abiotic stresses. Silencing *TaBI-1* in plants of a resistant wheat genotype converts a resistant reaction to a relatively susceptible reaction when inoculated with an avirulent pathotype of the pathogen (Wang *et al.* 2012).

BI-1 functions as an ancient suppressor of programmed cell death. BI-1 is one of the few cell death suppressors conserved in animals and plants (Hückelhoven 2004). The purpose of this study was an identification of a BAX inhibitor-1 homologous gene from cucumber by homologue *BLAST*. The relative transcription expression of *CsBI-1* gene was detected under cold stress.

Materials and methods

All cucumber (*Cucumis sativus* L. cv. Zhexiu-1) fruits used in this study were harvested from the farm of Zhejiang University in Hangzhou. Fruits were harvested at their commercial maturity, about 8 - 12 d after full bloom, and transported to the laboratory within 2 h after harvest. Fruits were selected for uniform size and color without mechanical damage and stored in the dark at temperature of 2 ± 1 °C and relative humidity of 85 - 95 % for 3, 6, or 9 d, respectively, and subsequently being warmed at 20 °C for 2 d.

Multiple sequences of BI-1 protein were collected in the National Center for Biotechnology Information (*NCBI*) database. Potential orthologous BI-1 amino acid sequence of cucumber was collected based on *BLAST* homology search in the *Phytozome* database (http://www.phytozome.net/search.php?how=blast&method=Org_Csativus) using the *AtBI-1*(AED95473.1) protein sequence. Conserved domains were deduced using *NCBI* conserved domain searches (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Transmembrane analysis was performed using *TMHMM Server v.2.0* (http://www.cbs.dtu.dk/services/TMHMM/). Alignments were performed with the *MEGA* (*v. 5.05*) and viewed using *GeneDOC* software (*v. 2.5*). A phylogenetic tree was constructed using full-length amino acid sequences of CsBI-1 and other members by the *MEGA*, applying the neighbour-joining (NJ) method. Bootstrap values over 50 %, based on 500 replicates, were indicated at the nodes of the phylogenetic tree. The *HsBI-1* protein of *Homo sapiens* (AAU29521.1) was used as an outgroup for constructing phylogenetic trees. Conserved domains were deduced using *NCBI* conserved domain searches (http:// www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Multiple alignments were created using the program *ClustalW* (Thompson *et al.* 1994) with minor manual adjustment to optimize the alignment as necessary.

Primers (Table 1) designed by the cDNA of the putative *CsBI-1*were used for further sequencing. PCR amplification was performed with 20 ng cDNA under the following conditions: 94 °C for 3 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and 72 °C for 10 min. The PCR products from four independent tubes were sent to sequencing (*Shanghai Sunny Biotechnology Co.*, Shanghai, China).

Slices (1.5 mm thick) comprising epidermis were taken with a vegetable peeler from five fruits and stored at -80 °C for later DNA and RNA extraction. About 4 g tissue samples were ground to a fine powder in liquid N_2 using a mortar and a pestle. The isolation of intact DNA was based on hexadecyl trimethyl ammonium bromide (CTAB) method (Hurr *et al.* 2010). Each sample was incubated for 90 min at 65 °C in 20 cm^3 of 2 % (m/v) CTAB buffer (1.4 mM NaCl, 20 mM sodium ethylene-diaminetetraacetate (Na_2EDTA), 100 mM Tris-HCl, pH 8.0, and 0.2 %, v/v, β-mercaptoethanol), then mixed with an equal volume of chloroform + isoamylalcohol (24:1, v/v). After gently shaking for 5 min, the mixture was centrifuged at 12 000 *g* for 20 min. DNA was precipitated by the addition of isopropanol to the supernatant (1:1, v/v). After an incubation at -20 °C for 1 h, the DNA was pelletted at 12 000 *g* and 4 °C for 20 min. The supernatant was decanted and the pellet was washed in 75 % (v/v) ethanol, air dried at room temperature, dissolved in 0.05 cm^3 of Tris-EDTA (TE) buffer (10 mM Tris-HCL, pH 8.0 , and 1 mM Na_2EDTA supplemented with 0.1 mg of RNase per 1 cm^3 of the mixture. DNA

content was determined by a *Thermo NanoDrop 2000* spectrophotometer (*Thermo Fisher Scientific*, Waltham, MA, USA). DNA (100 μg) samples were run on a 2 % (m/v) agarose gel at 30 V for 9 h and stained with 0.5 g m^{-3} ethidium bromide.

For the spectrophotometric estimation of trypan blue uptake, a modified method of Qu *et al.* (2009) was used. Slices (1.5 mm thick) comprising epidermis were taken with a vegetable peeler from three fruits. Epidermal pericarp discs (∅ 1 cm) were excised from the middle region of the slices with a cork borer. The epidermal discs were submerged in 10 cm^3 of 0.25 % (m/v) trypan blue in Petri dishes and incubated on a platform shaker for 5 min. The discs were rinsed well with deionized water until no more blue stain was eluted and then dried by filter paper. The dry discs (0.5 g) were ground with 20 cm^3 of deionized water prior to centrifugation at 1 000 *g* for 5 min. The supernatant was collected and the absorbance at 585 nm was determined spectrophotometrically.

Total RNA was extracted from cucumber epidermis using the *RNAiso Plus* (*Takara Biotechnology*, Dalian, China). The integrity and quality of total RNA was verified by 1.2 % (m/v) denaturing formaldehyde-agarose gel electrophoresis and RNA content was determined using *Thermo NanoDrop 2000* spectrophotometer. Total RNA was treated with RNase-free *DNase I* (*Promega*, Madison, WI, USA) to digest the residual DNA. Reverse transcription polymerase chain reactions (RT-PCR) were performed using 500 ng total RNA and a *PrimeScript RT* reagent kit *Perfect Real Time* (*Takara Biotechnology*) according to the manufacturer's protocol.

The specific RT-PCR primers (Table 1) were used for the quantification of *CsBI-1* gene expression. Housekeeping gene of 18S rRNA cDNA (Qian *et al.* 2012) was used to normalize any differences in the amounts of cDNA in each reaction. RT-PCR primers for 18S rRNA are shown in Table 1. Each cDNA sample was run in triplicate and repeated in three independent sets of treatments. The mean ratio of *CsBI-1* gene transcripts (cDNA) to the corresponding 18S rRNA cDNA was used to compare levels of *CsBI-1* gene expression between treatments.

Real-time PCR was performed in 0.02 cm^3 of reaction volume with 0.01 cm^3 of *SYBR Premix Ex Taq*™ (*Takara Biotechnology*), 0.002 cm^3 of each primer, 0.007 cm^3 of distilled water, and 0.0004 cm^3 of sense and antisense primers, respectively. Real-time PCR reactions were run in triplicate on a *LightCyber* real time PCR sequence detection instrument (*Light Cycler® 480II*, *Roche Diagnostics*, Switzerland) with cycling conditions: 95 °C for 10 s, followed by 40 cycles at 95 °C for 5 s and at 60 °C for 20 s. The melting curves were obtained at 65 °C for 15 s. The data analysis by fold-change method was carried out according to Livak and Schmittgen (2001) as follows: fold-change = $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT$ = (CTCsBI-1[a] - CT18S rRNA[a]) - (CTCsBI-1[b] - CT18s rRNA[b]), a - cucumber fruit stored at 2 °C for 3, 6, and 9 d and subsequently at 20 °C for 2 d, respectively, and b - fresh cucumber fruit.

All experiments were done using completely randomized design. Each experiment was repeated three times and the data were analyzed by analysis of variance (*ANOVA*) with *SPSS v. 11.0* statistical software. Significances of differences were performed by a least significant difference method (LSD test) and Student's *t*-test at $P < 0.05$.

Table 1. Primers used for *CsBI-1* sequencing and RT-PCR assay. Housekeeping gene 18S rRNA sequences were according to Qian *et al*. (2012).

	Gene	Sense	Antisense
Sequencing	*CsBI-1*	TGGACGCATTCTCTTCTTTC	CACCATCATTGCTACTTCAGTC
RT-PCR	*CsBI-1*	CTTCAGCGGGTTTATCTCACTCTT	CGAGAAGAGCAGCCCCAAG
	18S rRNA	GGCGGATGTTGCTTTAAGGA	GTGGTGCCCTTCCGTCAAT

Results

The highly homologous sequence to the *AtBI-1* protein sequence was named *CsBI-1*. The open reading frame (ORF) of the *CsBI-1* gene had 1 142 bp. *CsBI-1* contained 42-bp 5'-untranslated region and 347-bp 3'-untranslated region flanking the putative ORF of 250 amino acids (Fig. 1). The deduced *CsBI-1* protein had 77.7 % identity and 91.2 % positive value with *AtBI-1* protein. Alignment analysis of *CsBI-1* with other orthologous genes showed high identity values: *SlBI-1* (77 %), *CaBI-1* (76 %), *BoBI-1* (76 %), *AtBI-1* (78 %), *NtBI-1* (75 %), *OsBI-1* (70 %), *TaBI-1* (68 %), and *ZmBI-1* (69 %). The protein conserved domain analysis indicates that the primary structure of *CsBI-1* was composed of five transmembrane regions (57-79, 91-113, 118-140, 147-166, and 214-231) (Fig. 2*A*) and a BAX inhibitor domain. Alignment of the *CsBI-1* amino acid sequence with BI-1 proteins from several other species shows considerable similarity (Fig. 2*A*). The phylogenetic analysis using the full-length amino acid sequences revealed that CsBI-1 and AtBI-1 were mostly closely

related (Fig. 2*B*). A 768 bp sequence was amplified and the sequencing result was the same as the coding sequence (CDS) of putative *CsBI-1* gene.

The occurrence of DNA laddering is considered as a symptom of inter-nucleosomal fragmentation. Typically, the ladder 'rungs' are multiples of 180 bp due to inter-nucleosomal excision of chromatin fragments by an endonuclease (Wyllie 1980). The slight DNA laddering first appeared when warming after 6 d of cold storage and obvious laddering was observed after 9 d of the cold storage. Dispersion of fluorescence signal aggravated gradually from day 3 to 9 at 2 °C, which indicated random degradation of genomic DNA (Fig. 3).

Epidermis cell death was evaluated by spectrophotometric estimates of trypan blue uptake (Baker and Mock 1994). Cell death increased slowly before 6 d at 2 °C. However, a significantly higher absorbance at 585 nm was observed at day 9, indicating that cell death was significantly triggered after 9 d of cold storage (Fig. 4*A*). At recovery, even higher absorbance was detected and the highest was observed after 9 d of the cold stress.

The relative expression of the *CsBI-1* gene detected by quantitative real-time PCR was triggered by cold stress and reached a maximum at day 6 followed by a decline at day 9 (Fig. 4*B*). However, upon warming, expression of the *CsBI-1* gene was the highest after 3 d of cold storage followed by a significant decrease after longer cold storage.

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1     ACA GTT TGT TTA ACG TAC TCG CTA AGA AAT TTG TTC AAC GCC ATG GAC GCA TTC TCT TCT
1                                                             M   D   A   F   S   S
61    TTC TTC GAT TCT CAA TCT GGA TCC AGA ACC CGC TGG AGT CAT GAA TCT CTC AAG AAC TTC
7      F   F   D   S   Q   S   G   S   R   T   R   W   S   H   E   S   L   K   N   F
121   CGG CAG ATT TCG CCC GCC GTT CAA TCT CAT CTT CAG CGG GTT TAT CTC ACT CTT GGT TGT
27     R   Q   I   S   P   A   V   Q   S   H   L   Q   R   V   Y   L   T   L   G   C
181   GCT TTG GTT GCA TCT GCT GCT GGA GCT TAT CTG CAT ATA CTT TGG AAT ATT GGT GGT TTT
47     A   L   V   A   S   A   A   G   A   Y   L   H   I   L   W   N   I   G   G   F
241   CTT ACA ACA CTT GCA ACT ATC GGA TGT ATT ACA TGG CTA ATG GCC ACT CCT CCT TAT GAA
67     L   T   T   L   A   T   I   G   C   I   T   W   L   M   A   T   P   P   Y   E
300   GAG AAA AAG AGG GCC TCT ATT TTA CTT GGG GCT GCT CTT CTC GAA GGG GCT TCC ATT GGT
87     E   K   K   R   A   S   I   L   L   G   A   A   L   L   E   G   A   S   I   G
361   CCT TTG ATC AGT CTG GCT ATT GAT TTT GAC CCA AGT GTT CTG GTG AGC GCT TTC GTG GGA
107    P   L   I   S   L   A   I   D   F   D   P   S   V   L   V   S   A   F   V   G
421   ACT GCG GTT GCC TTT TGT TGT TTC TCA GGA GCA GCC TTG TTG GCA AGA CGT AGA GAA TTC
127    T   A   V   A   F   C   C   F   S   G   A   A   L   L   A   R   R   R   E   F
481   CTT TAT CTC GGT GGC TTA CTT TCT TCC GGT GTA TCC ATG TTA CTC TGG TTA CAT TTC GCC
147    L   Y   L   G   G   L   L   S   S   G   V   S   M   L   L   W   L   H   F   A
541   TCC TCT TTA TTC GGT GGT TCT ACT GCC CTT TTC AAG TTT GAG TTG TAC TTT GGG CTG TTG
167    S   S   L   F   G   G   S   T   A   L   F   K   F   E   L   Y   F   G   L   L
601   GTT TTT GTT GGC TAC ATG GTA GTT GAT ACT CAG GAA ATA ATT GAG ATG GCA CAT ATG GGT
187    V   F   V   G   Y   M   V   V   D   T   Q   E   I   I   E   M   A   H   M   G
661   GAT ATG GAT TAT GTG AAA CAT GCA TTA ACT CTC TTC ACT GAT TTC ATT GCG GTG TTT GTC
207    D   M   D   Y   V   K   H   A   L   T   L   F   T   D   F   I   A   V   F   V
721   CGA ATT CTC ATT ATA ATG CTA AAG AAC TCT GCT GAG AAG AAC GAG AGG GAG AGG AAG AAG
227    R   I   L   I   I   M   L   K   N   S   A   E   K   N   E   R   E   R   K   K
781   AAG AGG AGG GAC TGA AGT AGC AAT GAT GGT GGA GGG ATA TGA TTG ATT GTG AAG TGA AAT
247    K   R   R   D   -
841   GAA TGT TGA GTG AAG AAT CTT ATG GAT TGT GTA TAA CTT CTG TTT CTT TGT GCC ATT TTG
901   TCT TTT CTC ACA CTT ATT GTT AAC CCC GAT GAT ACT TGT AGA TTG CAA TGG ATA GGC TTA
961   TAA TAA TGG CCT TGT AAT ATG TAT TTC TGA TTG GAC TAA GGG AAT ATT TTG GTG TTT TAG
1021  GAT TTT AAC CTT TTG ATT TTT TCG CAT AAA ATC TGA AAC AAG AGT TGG CTT TGG GTT TAC
1081  CAA GGC TGA TTT GAA AAC GTA GTT GGT TTA ATT ATT TGA ATC TTC AAT TAA CAA ATG CTA
1141  AA
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Fig. 1. Sequence analysis of cDNA of *CsBI-1* nucleotides and deduced amino acid sequences. Putative open reading frame is shown below the nucleotide sequence in one-letter symbols of amino acids. Amino acid numbers are below the nucleotide numbers. The putative termination codon is marked by a short horizontal line (-).

Discussion

This study identified and characterized *CsBI-1* as a BAX inhibitor gene in cucumber for the first time. *CsBI-1* protein contains an ORF of 250 amino acids, a BAX inhibitor domain, and five transmembrane regions conserved among members of the BI-1 family. This is consistent with all known BI-1-related proteins containing 5 - 7 transmembrane α-helices (Lam 2004). The high sequence identity confirmed that cucumber *CsBI-1* gene is related to *AtBI-1* gene of *Arabidopsis thaliana*.

The relative expression of the *CsBI-1* gene was detected by real-time quantitative PCR analysis in cucumber fruit exposed to cold stress. Cell death developed slowly with the prolonged cold stress concomitantly with an increased expression of the *CsBI-1* gene which reached maximum at day 6. However, cell death increased significantly after 9 d at 2 °C when a sharp decrease of the *CsBI-1* expression occurred.

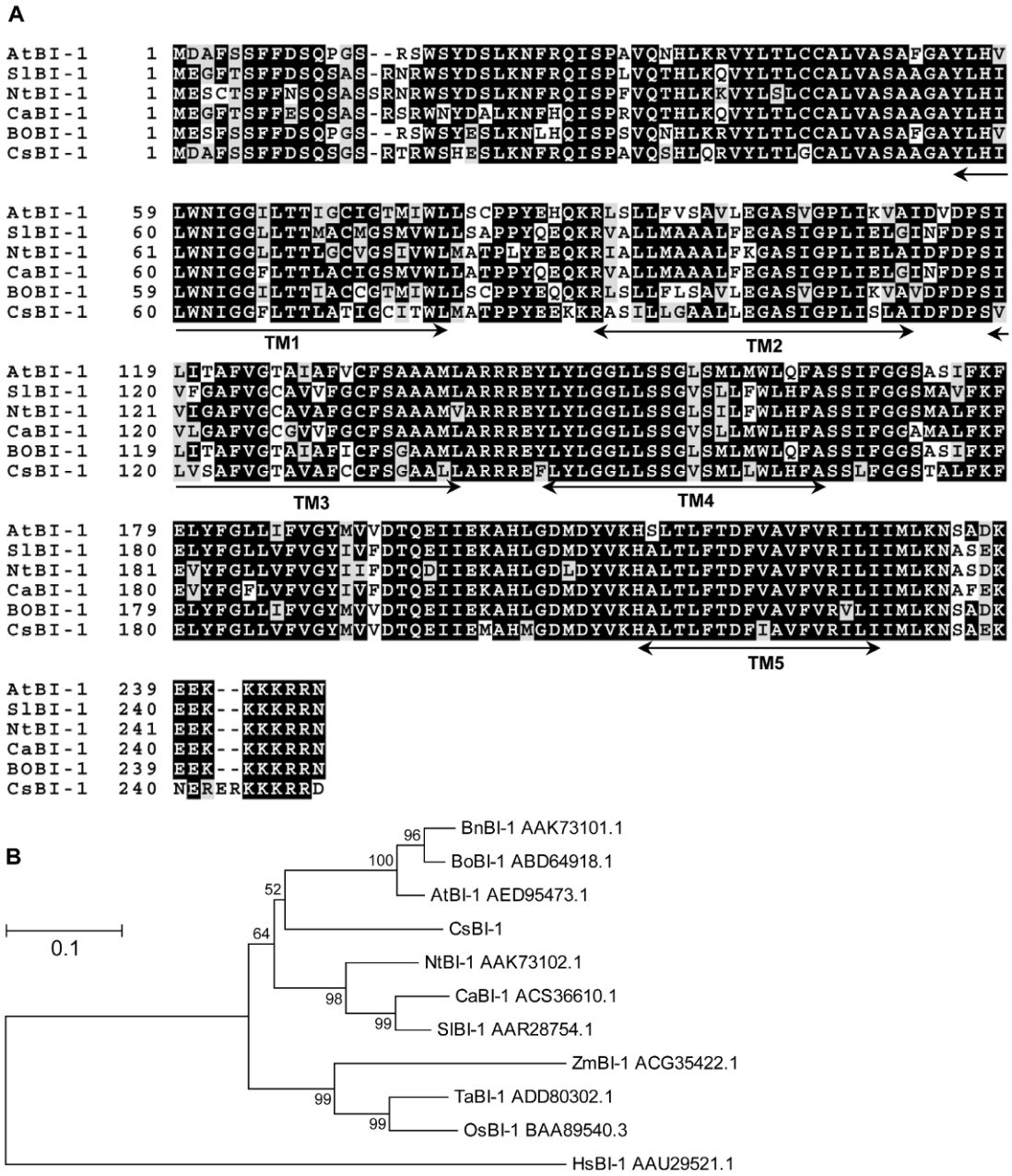

Fig. 2. Multiple alignment and phylogenetic analysis of the deduced amino acid sequences of *CsBI-1* and other BI-1 family member proteins. *A* - Amino acid sequence comparisons: amino acid residues conserved in all five sequences *in black*; residues conserved among the four sequences in *light grey*; TM - transmembrane domains. *B* - A representative phylogenetic tree of CsBI-1 and other BI-1 protein family members constructed using full-length amino acid sequences and the neighbour-joining method. At - *Arabidopsis thaliana*, Bn - *Brassica napus*, Bo - *Brassica oleracea*, Ca - *Capsicum annuum*, Cs - *Cucumis sativus*, Hs - *Homo sapiens*, Nt - *Nicotiana tabacum*, Os - *Oryza sativa*, Sl - *Solanum lycopersicum*, Ta - *Triticum aestivum*, Zm - *Zea mays*. Bootstrap values (> 50 %) based on 500 replications are shown at branch nodes. HsBI-1 was used as an outgroup. *Bar* - 1 substitution per 10 nucleotide positions.

Transcription of *CaBI-1* in hot pepper is induced in response to high or low temperatures, drought, salinity, flooding, heavy metals, and abscisic acid (ABA) (Isbat *et al.* 2009). The *TaBI-1* transcription is also upregulated significantly in wheat seedling treated by high salinity, drought, ABA, ethylene (ET), methyl jasmonate (JA), and salicylic acid (SA) (Wang *et al.* 2012). Over-expression of BI-1 suppresses cell death in rice treated with fungal elicitor (Matsumura *et al.* 2003), in yeast during wounding and pathogen challenge (Sanchez *et al.* 2000), and in barley after powdery mildew inoculation (Hückelhoven *et al.* 2001). It could be concluded that

BI-1 expression was ubiquitous and that it functioned as a basal regulator for various responses to biotic and abiotic stresses in plants.

BI-1 has been proved as a key molecular switch downstream from a variety of biotic and abiotic stress signals and a negative regulator of plant PCD (Watanabe and Lam 2009). Upon warming after 3-d cold stress, expression of the *CsBI-1* gene was up-regulated. Afterwards, a decrease of transcription of *CsBI-1* followed and the lowest one was detected at day 9 when PCD occurred. Significant increase of epidermal cell death when warming from 6-d cold storage was also identified synchronously with PCD occurring. Therefore, increased PCD may be attributed to the downregulation of transcription of *CsBI-1* gene. This result is consistent with Zhang *et al.* (2010) who observed that inhibition of *BI-1* expression by siRNA cause a significant increase in spontaneous apoptosis. Two *AtBI-1* mutants (*AtBI-1-1*and *AtBI-1-2*) exhibit hypersensitivity to tunicamycin (TM) with accelerated PCD progress. Conversely, *Arabidopsis* mutant over-expressing *AtBI-1* has markedly reduced sensitivity to TM (Watanabe and Lam 2008).

The overall results indicate that *CsBI-1* gene was a cucumber homologue of *AtBI-1* gene. *CsBI-1* gene was a conserved cell death suppressor induced by cold stress and a negative regulator of PCD.

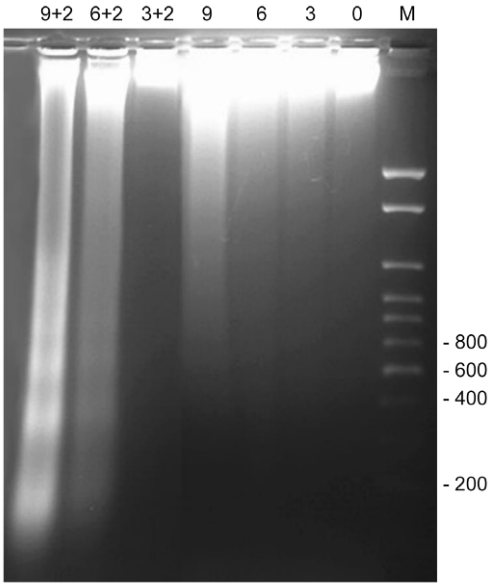

Fig. 3. Result of electrophoresis of total DNA isolated from cucumber fruit epidermis after 0, 3, 6, and 9 d of cold storage (2 ± 1 °C) and subsequent warming at 20 °C for 2 d as showed 3+2, 6+2, and 9+2. DNA samples were run on a 2 % agarose gel and stained with ethidium bromide. A molecular marker of 200-bp ladder was applied.

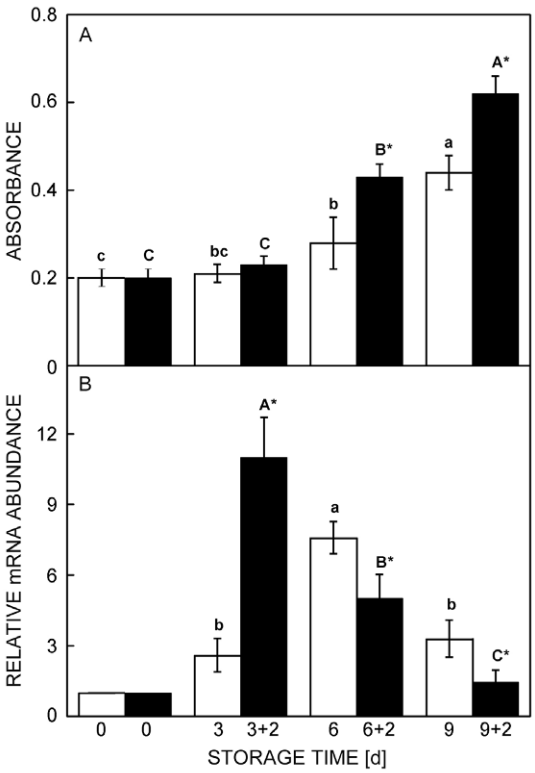

Fig. 4. Cell death (*A*) and quantitative real-time PCR (*B*) analyses of *CsBI-1* gene expression in cucumber epidermis during cold stress 2 ± 1 °C (*open bars*) and subsequent warming at 20 °C for 2 d (*black bars*). Means ± SD, $n = 4$. Different lower case and capital letters indicate statistically significant differences induced by cold stress and cold stress + warming, respectively, according to LSD ($P < 0.05$). *Asterisks* mean significant differences between the respective values after cold stress and warming according to *t*-test ($P < 0.05$). Cell death was estimated by monitoring trypan blue uptake by spectrophotometry at 585 nm (*A*). The relative level of *CsBI-1* gene expression in cucumber fruit at day 0 was set as 1 (*B*) and all other samples were quantified relative to this. 18S rRNA was chosen as an endogenous control.

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