J Korean Soc Appl Biol Chem (2014) 57(6), 775–782
DOI 10.1007/s13765-014-4238-2

Online ISSN 2234-344X
Print ISSN 1738-2203

ARTICLE

A Putative Cold Shock Protein-encoding Gene Isolated from *Arthrobacter* sp. A2-5 Confers Cold Stress Tolerance in Yeast and Plants

Seong-Kon Lee · Sung-Han Park · Jeong-Won Lee · Hae-Min Lim · Sun-Young Jung · In-Cheol Park · Soo-Chul Park

Received: 9 August 2014 / Accepted: 4 September 2014 / Published Online: 31 December 2014

Abstract A putative cold shock protein gene, designated as *ArCspA*, was isolated from *Arthrobacter* sp. A2-5 extracted from soil at the South Pole. The *ArCspA* gene is 873 nucleotide bp long and includes a 207-bp short open reading frame (ORF) with 49.3–92% amino acid identity to peptide sequences of other bacterial cold shock proteins. Northern blot analysis revealed that *ArCspA* was highly expressed at low temperatures. Bio-functional analysis using *ArCspA*-overexpressed transgenic *Saccharomyces cerevisiae* showed that *ArCspA* conferred cold tolerance on yeast at low temperatures (15°C). We then developed an *ArCspA*-overexpressed transgenic tobacco line to determine whether *ArCspA* is also functional in plants. After cold treatment at −25°C for 90 min followed by recovery for 4 weeks at 25°C, 17 transgenic lines survived at a high rate (60.0%), whereas under the same treatment conditions, wild-type plants did not survive. We also found that progeny of transgenic tobacco plants subjected to freezing stress at −20°C had significantly higher seed germination ability than wild-type plants. These results clearly indicate that the ArCspA protein plays an important role in cold tolerance in both yeast and plants.

Keywords ArCspA gene · ArCspA transgenic tobacco · ArCspA transgenic yeast

S.-K. Lee and S.-H. Park equally contributed to this study.

S.-K. Lee · S.-H. Park · J.-W. Lee · H.-M. Lim · S.-Y. Jung · I.-C. Park · S.-C. Park (✉)
National Academy of Agricultural Science, Rural Development Administration, Jeonju 560-500, Republic of Korea
E-mail: usdapark@korea.kr

Introduction

Living organisms are exposed to various biotic and abiotic stresses, such as disease, osmotic stress, oxidative damage, and nutrient depletion that influence their development, growth, and productivity. The major abiotic stresses affecting growth of all organic life are chilling and freezing stresses. The mechanisms responsible for stress response and adaptation to cold shock have been intensively investigated in many organisms, including yeast, bacteria, and plants. Previous studies have demonstrated that synthesis of several cold shock proteins (CSPs) is induced by cold temperatures in various microorganisms, including different species and genera of bacteria (Basnak'ian, 2001). The major cold shock protein in *Escherichia coli*, CspA, is dramatically induced immediately following temperature downshift, and accumulates to about 10% of total soluble protein (Phadtare et al., 2004). Nine members of the CSP gene family (*cspA* to *cspI*) have been identified in *E. coli*, four of which have been shown to be induced by cold shock (Brandi et al., 1996). All CSP family members possess RNA binding motifs RNP-1 (KGFGFI) and RNP-2 (VFVHF), which are identical to the domains of other bacterial cold shock proteins and conserved in cloned genes (Horn et al., 2007). RNP motifs act as ssDNA chaperones. Prokaryotes adapt to both high and low temperatures by adjusting membrane lipid composition and increasing production of "RNA chaperone" proteins required for transcription and translation as well as proteins that facilitate ribosomal assembly (Graumann and Marahiel, 1998; Inouye and Phadtare, 2004). Most CSPs are regulated sequentially in response to temperature downshift and have fundamental cell fate determination functions, such as DNA replication, transcription, translation, RNA stabilization, and ribosome assembly (Gualerzi et al., 2003). Cold-induced genes have been observed, and functions related to cold stress have been suggested for many genes. In particular, studies have recently

reported on microbial activity detected at various temperatures from 0 to −35°C in permafrost and similar frozen habits (Deming, 2002; Panikov and Sizova, 2007). A *CspA* has been isolated from the psychotropic bacterium *Arthrobacter globiformis* SI55, which grows between −5 and 32°C. This gene is regulated at low temperatures, and may be homologous to the *CspA* gene of *E. coli* (Berger et al., 1996; 1997). In addition, discovery of the mechanism determining CspA level has provided evidence that posttranscriptional regulation plays a crucial role in the cell (Brandi et al., 1996). In contrast, yeast cold-shock induced genes that are not essential for normal growth have been shown to be induced by a temperature shift from 30 to 10°C (Kowalski et al., 1995; Donzeau et al., 1996; Gualerzi et al., 2003; Homma et al., 2003; Inouye et al., 2004; Phadtare et al., 2004).

Cold stress is a major environmental stress that can delay plant growth and development, reduces productivity, and, in extreme cases, causes plants to die. During cold stress, many genes are induced in plants (Thomashow, 1999), such as CBF (Gilmour et al., 2004; Zhang et al., 2004) and WCSP1 (Karlson et al., 2002). In the present study, we isolated a putative cold shock protein gene (designated as *ArCspA*) from a South Pole soil bacterium, *Arthrobacter* sp. A2-5. The function of this gene during cold stress was tested by examining its ability to complement cold stress in *Saccharomyces cerevisiae* and tobacco plants.

Materials and Methods

Strains and culture conditions. Five bacterial strains isolated from South Pole soil, including *Arthrobacter* sp. A2-5, were obtained from the Korean Agricultural Culture Collection at the National Institute of Agricultural Biotechnology, Korea. For growth temperature tests, the strains were grown at 15, 20, 25, and 30°C on tryptic soy broth (TSB; 1.7% pancreatic casein digest, 0.3% soy peptone, 0.5% sodium chloride, 0.25% dipotassium phosphate, and 0.25% glucose).

Microbial identification and 16S rDNA analysis. The A2-5 strain was identified using a MIDI Sherlock Microbial Identification System (MIDI Inc., USA) and through 16S rDNA sequencing analysis. The MIS identifies microorganisms based on the unique fatty acid pattern of each strain. The cells were harvested and fatty acids were extracted from cellular membranes using a four-step procedure provided by the manufacturer. The extract containing fatty acid methyl esters was transferred to a sample vial for identification and quantification, which was carried out on an Agilent 5890II gas chromatograph (Agilent Technologies, USA) in conjunction with MIS software.

Primers, PCR amplification, and cloning of the putative cold-shock protein gene. The 16S rDNA region of A2-5 was PCR-amplified using universal primers fD1 (5'-AGAGTTTGATCCT GGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3'). Primers used for amplification of the cold shock protein gene were generated based on a conserved region of 20 bacterial cold shock protein gene sequences. PCR amplification was performed using sense (csp-S; 5'-GGNANNGTNAA[A,G]TGGTT[C,T]AA-3') and antisense (csp-AS; 5'-AC[A,G]TTNGCNGC[C,T]TGNG GNCC-3') primers.

Genomic library construction and plaque hybridization. An *Arthrobacter* sp. A2-5 genomic library was constructed using a Lambda ZAP-Express vector (Stratagene, USA). Genomic DNA was prepared and the ligated DNA was packaged with MaxPlax Lambda packaging extract (EPICentre, UK). Lambda phage DNA was isolated from the cDNA library according to the method described by Sambrook et al. (2001).

Northern and Southern blot analyses. After growing *Arthrobacter* sp. A2-5 in TSB liquid medium in a 25°C incubator for 2 days, a time-course gene expression experiment was performed by exposing the bacteria to cold shock at 10°C for 0.5, 1, 2, 4, 8, and 12 h. Total RNA was extracted from the stressed bacteria using Trizol reagent (Gibco BRL, USA) according to the manufacturer's instructions. Total genomic DNA (5 μg) was digested with *Eco*RI, *Hind*III, *Kpn*I, and *Pst*I. Total RNA (10 μg) and genomic DNA were blotted onto Hybond-N^+ nitrocellulose membrane (Amersham Pharmacia Biotech, USA), and Southern and Northern hybridizations were performed with a Gene Images system (Amersham Pharmacia Biotech, USA) using 207 bp of *ArCspA* cDNA fragment as the probe.

Introduction of the *ArCspA* gene into *S. cerevisiae*. An expression vector containing the complete *ArCspA* cDNA (pYES-DEST52/*ArCspA)* was constructed by inserting the 207 bp full length *ArCspA* cDNA under the control of a GAL promoter in the yeast expression vector, pYES-DEST52 (Invitrogen, USA*)* using Gateway TM Technology (name of country missing). pYES-DEST52/*ArCspA* was introduced into wild-type yeast strain INVSC2 (MATá his3Ä1 leu2 trp1-289 ura3-52, Invitrogen). Transfer and expression of *ArCspA* were confirmed by reverse transcription polymerase chain reaction (RT-PCR) analysis.

Induction of ArCspA protein in yeast and cold stress treatment. Yeast cells harboring the pYES-DEST52/*0ArCspA* were cultured for 2 days at 30°C in SC medium containing 2% raffinose. To induce expression of *ArCspA* (controlled by the *GAL1* promoter), raffinose was removed, and galactose was added as a carbohydrate source. For cold stress treatment, the cells were streaked and grown at 30 (control) 15 or 4°C in an incubator. Each of these cold stress experiments was repeated three times.

Western blot analysis. Transformed yeast cells were grown at 30°C in SC-ura containing 2% glucose medium until an OD_{600} of 0.4 was obtained, and expression was induced in SC-ura containing 2% galactose medium at 30°C. After 8 h induction, total proteins were extracted from the cells and separated on a 12% polyacrylamide SDS gel. Detection of ArCspA protein (fused to a C-terminus V5 epitope when expressed from the pYES-DEST52 vector) was analyzed by immunoblotting with anti-V5 antibody (Invitrogen). Antibody-bound proteins were detected by incubation with a horseradish peroxidase-conjugated secondary antibody using an enhanced chemiluminescence system

(GE Healthcare, UK).

Vector construction and transformation of the *ArCspA* gene into tobacco. An overexpression vector containing the complete *ArCspA* cDNA (pB7GW2D/*ArCspA*) was constructed by inserting the 207 bp full length *ArCspA* cDNA in the plant expression vector, pB7GW2D (Invitrogen*)* using Gateway TM Technology (Invitrogen) according to the method described by Lee et al. (2006). The recombinant plasmid, pB7WG2D/*ArCspA,* was introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation using a MicroPulser Electroporation System (Bio-Rad, USA). Tobacco (*Nicotiana tabacum* L. 'Xanthi') seeds were sterilized and grown aseptically on 0.6% agar-solidified medium containing Murashige and Skoog (MS) salt and 3% sucrose under culture conditions of 25°C and 16/8 h (day/night) photoperiod of white fluorescent light. *Agrobacterium*-mediated leaf disk transformation was carried out according to the method described by Maliga et al. (1995). Explants were subsequently transferred to the same medium used for seeds plus 250 mg l^{-1} cefotaxime and 10 mg l^{-1} DL-phosphinothricin (PPT). *ArCspA* mRNA Transfer and mRNA expression of *ArCspA* in transgenic tobacco plants were confirmed by RT-PCR analysis.

Cold tolerance analysis. Transgenic tobacco plants were grown in MS medium containing 10 mg l^{-1} PPT for 10 days at 25°C. Ten healthy young T_1-generation plants that survived in the PPT selection medium were transplanted to MS0 medium and grown for 7 days at 25°C. The plants were pre-treated for 1 day at 25°C under light conditions, and then subjected to –20°C-freezer for 90 min in the dark (wild-type plants died under these cold-shock conditions). After recovery for 14 days at 25°C, surviving tobacco plants were counted, and their phenotypes were recorded photographically. Each of these stress experiments was repeated three times.

Salt and drought stress assays. For germination assays, tobacco seeds were treated and grown as described by Wu et al. (2008). Briefly, seeds were plated in half-strength MS medium (0.6% agar) supplemented with 0.3 M mannitol or 0.2 M NaCl. About 60 seeds were used for each treatment, with three independent experiments carried out to ensure data reliability. For drought assays, both 3-week-old transgenic and wild-type seedlings in pots were grown in a growth chamber (FLI-301NH, EYELA, Japan) at 25°C with 70–80% humidity and water was then withheld for 11 days. Light intensity varied from 275 to 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Finally, the plants were allowed to recover by re-watering for 5 days.

Results

Selection and identification of cold tolerant *Arthrobacter* sp. A2-5. To determine the effect of temperature on microbial growth, strains A2-1, A2-2, A2-3, A2-5, and A2-6 were cultured for 2 days in TSB at 15, 20, 25, and 30°C (Fig. 1). All strains except A2-6 grew well at 20, 25, and 30°C, but they showed poor growth

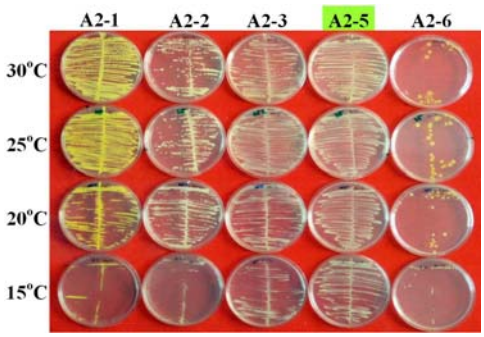

Fig. 1 Effect of low temperatures on microbial growth. Five bacterial strains including *Arthrobacter* sp. A2-5 isolated from South Pole soil, were grown at 15, 20, 25, and 30°C on tryptic soy broth (TSB).

at 15°C except A2-5. Only strain A2-5 exhibited good growth at 15°C. Phylogenetic analysis of 16S rDNA sequences confirmed the placement of these strains within the genus *Arthrobacter*. Relationships of isolate A2-5 and related *Arthrobacter* spp. are shown in Fig. 2. Fatty acid and 16S rDNA analyses generated the following MIDI fatty acid profile for A2-5 at 15:1 ISO (8.9%), 15:0 ANTEISO (57.7%), 17:1 ANTEISO (5.8%), and 17:0 ANTEISO (7.2%). The 16S rDNA sequence of A2-5 is 98% identical to that of *Arthrobacter sulfonivorans* strain ALL (GenBank accession no. AF235091). These findings imply that A2-5, like other known *Arthrobacter* species, may be cold-tolerant. The strain was thus selected and designated as *Arthrobacter* sp. A2-5.

Isolation of the *ArCsp* gene from *Arthrobacter* sp. A2-5. To isolate full-length cold shock protein cDNA from *Arthrobacter sp.* A2-5, CSP sequence-specific sense and antisense primers were designed by comparing conserved amino acid sequences of various CSP family genes from other microorganisms, *i.e. E. coli* CspA and CspG (GenBank accession nos. NP_756239 and BAB34568, respectively), *Bacillus subtilis* CspB (AAC80234), and *Arthrobacter globiformis* Csp (AAB81323). Using these primers, we generated a 180-bp cDNA fragment from genomic DNA by PCR amplification. Sequencing analysis showed that part of the deduced amino acid sequence of this cDNA fragment had high sequence similarity with CSP protein family members from other organisms. Based on the 180-bp nucleotide sequence, a nested primer set was designed for generating the full-length *csp* cDNA. Amplification of genomic DNA using the nested primer set generated products approximately 873 and 1,014 bp long. The 873-bp PCR product, expected to correspond to the full-length *csp* cDNA, was purified and ligated into *p*Bluescript KS-II (+) for nucleotide sequencing analysis.

Nucleotide and deduced amino acid sequences of the putative *CspA* gene. The cloned *CspA* gene was 873 bp long and contained a 207-bp open reading frame encoding a polypeptide of 68 amino acids with a molecular mass of approximately 7.1 kDa. This peptide sequence was compared with sequences in GenBank using the BLASTP algorithm (Fig. 3). The *CspA* sequence

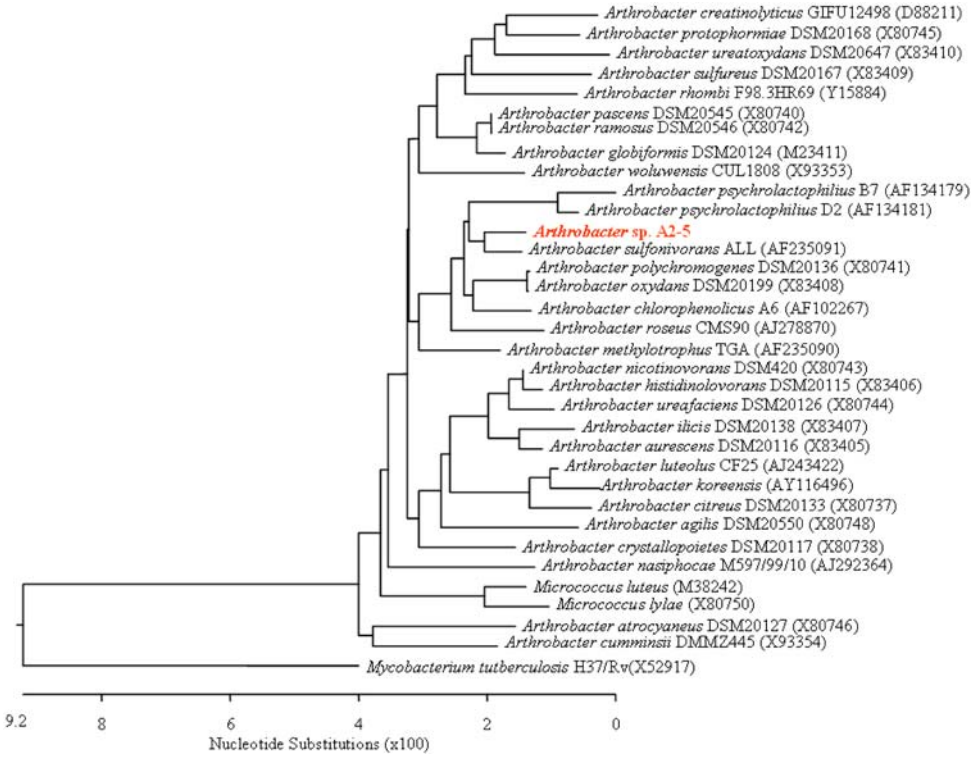

Fig. 2 Phylogenetic tree of the predicted amino acid sequence alignment between *Arthrobacter* sp. A2-5 CspA (ArCspA) and other bacterial Csps. Predicted peptide sequence of ArCspA protein isolated from *Arthrobacter* sp. A2-5 was aligned with deduced amino acid sequences of other bacterial CSP subfamily genes using the BLASTP algorithm.

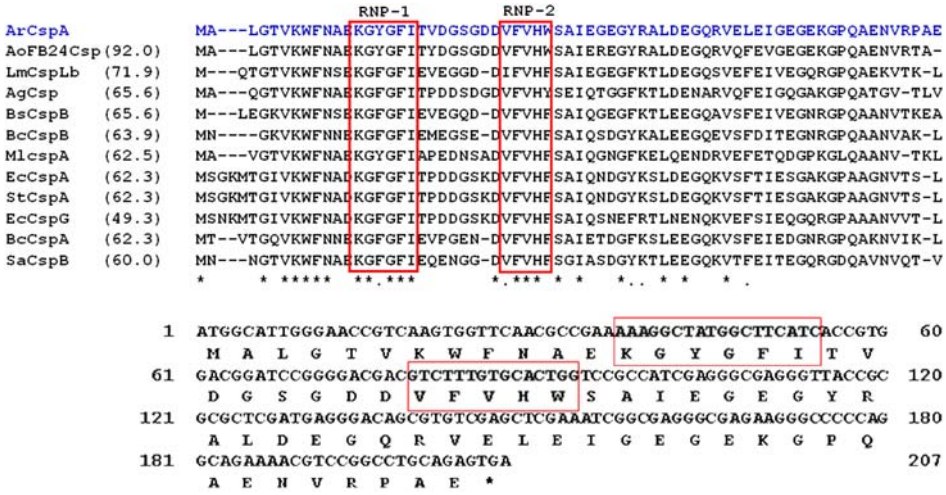

Fig. 3 Alignment of deduced amino acid sequences of *Arthrobacter* sp. A2-5 CspA (ArCspA) and other bacterial Csp subfamily proteins. RNA binding motifs, RNP-1 (KGFGFI) and RNP-2 (VFVHF), conserved in bacterial Csp family are outlined in red. Identical residues are indicated by asterisks. Csp proteins, source organisms, and GenBank accession numbers are as follows: AoFB24CSP, *Arthrobacter* FB24 (ZP_00412754); LmCspLb, *Listeria monocytogenes* (AAB48629); AgCsp, *Arthrobacter globiformis* (AAB81323); BsCspB, *Bacillus subtilis* (CAB12738); BcCspB, *B. cereus* (CAA63608); MlcspA, *Micrococcus luteus* (AAB70836); EcCspA, *Escherichia coli* (NP_756239); EcCspG, *E. coli* (BAB34568); BcCspA, *B. cereus* (CAA63607); Sa, *Staphylococcus aureus* (BAB96488). The *ArCspA* ORF consists of 207 bp encoding a putative peptide of 68 amino acid residues.

isolated from *Arthrobacter* sp. A2-5 was aligned with deduced amino acid sequences of other bacterial *CSP* subfamily genes. All CSP family members have RNA binding motifs RNP-1 (KGFGFI) and RNP-2 (VFVHF), which are identical to bacterial cold shock protein domains and conserved in cloned genes (Fig. 3). RNP motifs act as ssDNA chaperones. CspA, CspB, and CspG are all

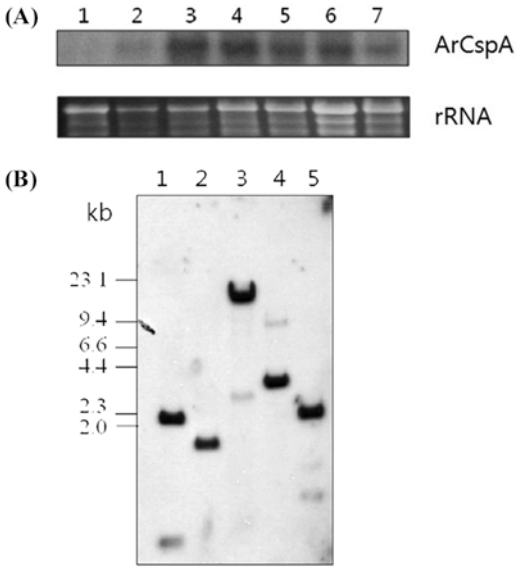

Fig. 4 Northern and Southern blot analyses of *ArCspA* in *Arthrobacter* sp. A2-5. (A) Northern blot analysis was carried out to investigate a time-course mRNA expression of the *ArCspA* gene. Total RNA was extracted from bacterial cells grown either at 25°C (lane 1, control) or after cold shock at 10°C for 30 min (lane 2), 1 h (lane 3), 2 h (lane 4), 4 h (lane 5), 8 h (lane 6), and 12 h (lane 7). RNA (10 μg per sample) for RNA gel blot analysis was denatured in formamide-formaldehyde and separated on 0.8% formaldehyde agarose gel. (B) Southern blot analysis was carried out to determine *ArCspA* copy number. Total genomic DNA digested with *BamH*I (lane 1), *Eco*RI (lane 2), *Hind*III (lane 3), *Kpn*I (lane 4), and *Pst*I (lane 5) were separated on a 0.7% agarose gel. Hybridization was carried out using a ^{32}P-labeled 207-bp *ArCspA* PCR fragment as a probe.

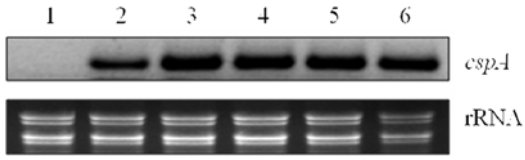

Fig. 5 mRNA expression of the *ArCspA* in the transgenic yeast cells. RT-PCR analysis was performed using total RNA from the *ArCspA* overexpressed transgenic yeast cells grown in galactose induction media for 3 h (lane 2), 6 h (lane 3), 9 h (lane 4), 12 h (lane 5), and 15 h (lane 6). *p*YES-DEST52/INVSc1 (lane 1) yeast strains were used as negative controls.

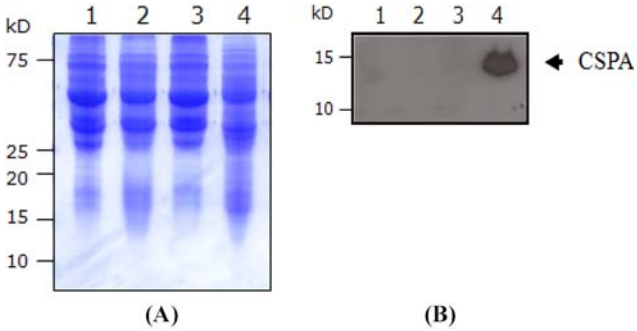

Fig. 6 Confirmation of ArCspA protein synthesis in the transgenic yeast cells. (A) Separation of total protein extracts from yeast strain W303-1A on a 12% polyacrylamide SDS gel. (B) Western blot analysis of ArCspA (fused to a C-terminus V5 epitope when expressed from the pYES-DEST52 vector). Lane 1, Non-induction (pYES-DEST52); lane 2, Induction (pYES-DEST52); 3, Non-induction (pYES-DEST52-*ArCspA*); 4, Induction (pYES-DEST52-*ArCspA*).

overexpressed following cold shock (Berger et al., 1996; Nakashima et al., 1996). The peptide sequence most similar to A2-5, sharing 92% identity, was that of the cold shock protein of *Arthrobacter* sp. FB24 (ZP_00412754). The lowest similarity (49.3%) was with *E. coli* CspG (BAB34568). These results confirm that the peptide obtained from *Arthrobacter* sp. A205 is a CspA protein.

Time-course expression of *AsCspA* under cold stress. Because *Arthrobacter* sp. A2-5 showed good growth at 15°C (Fig. 1), we investigated expression of this CSP subfamily gene. Time-course gene expression analysis of *ArCspA* was carried out under low temperature growth conditions (10°C), with Southern and Northern blot analyses performed using a full-length fragment as a probe. At 25°C, low levels of mRNA transcripts were observed, although *ArcspA* gene expression was not detected (Fig. 4A). After the temperature shifted to 10°C, mRNA levels increased over time. *ArcspA* transcription was doubled within 0.5 h and rapidly reached its maximum expression level (5-fold) within 1–2 h (Fig. 4A). Transcription levels then decreased gradually after 4 h. These results indicate that *ArcspA* may function during the early stage of the cold response process.

Determination of *ArCspA* gene copy number. To determine the number of *ArCspA* gene copies in the *Arthrobacter* sp. A2-5 genome, total genomic DNA was digested with five enzymes (*Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, and *Pst*I) and analyzed by DNA gel blot hybridization using a 207-bp *ArCspA* PCR fragment as the probe. Single hybridization band was obtained from genomic DNA each cut with different enzyme, indicating that the *ArCspA* gene is present as a single copy in the *Arthrobacter* sp. A2-5 genome (Fig. 4B).

Confirmation of *ArCspA* gene expression in eukaryotic cells. To analyze *ArCspA* gene expression when cells were grown in SC medium containing 2% galactose, we performed RT-PCR analysis. In SC-*ura*$^-$ medium containing galactose, *ArcspA* mRNA was hardly observed after 3 h, but heavily accumulated after 6 h (Fig. 5). The presence of the ArCspA protein was confirmed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis. Transgenic yeast cells readily produced ArCspA protein after culturing for 8 h in galactose containing inducible medium. Protein expression was reconfirmed by immunoblotting with anti-V5 antibody (Fig. 6).

Cold tolerance in transformed yeast cells. We next investigated functionality of the cold shock protein gene *ArCspA* in yeast. The results of this experiment are shown in Fig. 7. Although all yeast cells grew very well after 2 day-incubation at 30°C, no growth was observed when cells were grown at 4°C. At 15°C, the

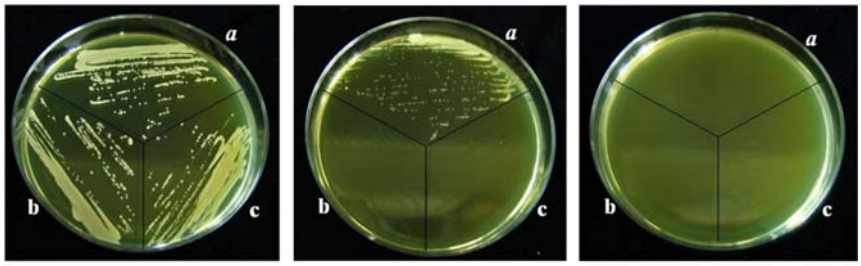

Fig. 7 Cold tolerance of the *ArCspA* overexpressed transgenic yeast. (A) Yeast cells grown for 2 days at 30°C (control). (B) Yeast cells grown for 7 days at 15°C. (C) Yeast cells grown for 2 weeks at 4°C. All cells were grown in YEPD medium. a: yeast cells transformed with *p*YES-DEST52/*ArCspA*/INVSc1, b: yeast cells transformed with *p*YES-DEST52/INVSc1, c: wild-type yeast cell INVSc1.

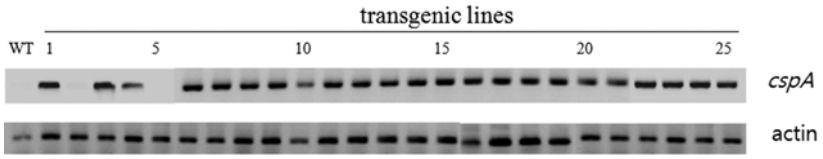

Fig. 8 Confirmation of *ArCspA* gene expression in transgenic tobacco plants. Expression of the *ArCspA* gene in transgenic tobacco plants was investigated by RT-PCR analysis. First-strand cDNA was synthesized from Total RNA isolated from wild-type and independent transgenic tobacco plants using M-MLV reverse transcriptase, and then ArCspA specific primer set was used in ensuing PCR analysis.

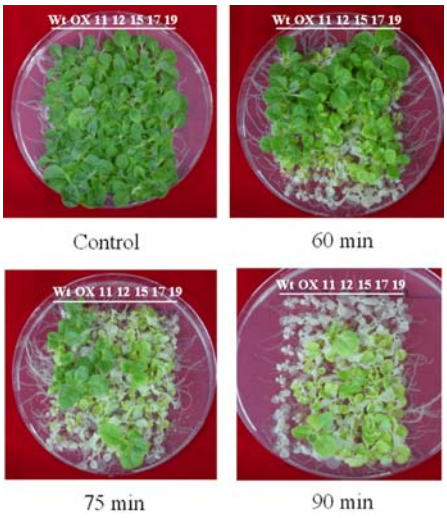

Fig. 9 Cold tolerance of *ArCspA*-transgenic tobacco plants. After cold stress at –20°C for (A) 0, (B) 60, (C) 75, and (D) 90 min, tobacco plants were recovered by growing at 25°C for 2 weeks. Each stress experiment was repeated three times.

transformants exhibited good growth, whereas wild-type cells and cells transformed with pYES-DEST52 showed no growth. A study by Berger et al. (1996) demonstrated that cold shock response in *Arthrobacter globiformis* SI55 is an adaptive process enabling restoration of physiological equilibrium at low temperatures. Our results demonstrate that *S. cerevisiae* cells growing at low temperature contained the *ArCspA* gene, suggesting that this gene can confer cold tolerance in living organisms.

Cold tolerance of ArCspA overexpressed tobacco plants. RT-PCR confirmed the expression of the *ArCspA* gene in T_0 generation tobacco plants (Fig. 8). PPT-selected T_1 shoots of transgenic lines, and wild-type plants were pretreated in a cold room (4°C) for 1 day under light conditions, and then placed under darkness in a –20°C-freezer for 30, 45, 60, 75, or 90 min. A pretreated plant not subjected to freezer treatment was used as a control. Plants were tested for 90 min at –20°C, because wild-type plants were unable to survive under those conditions (Fig. 9). In this fashion, cold resistance in progeny of transgenic tobacco was demonstrated (Table 1). After cold stress for 90 min, plants were allowed to recover and grow in a 25°C growth chamber. Although overexpression of CspA protein conferred enhanced cold tolerance compare to wild-type plant, not many transformants showed high survival recovery rate of their progeny under the cold condition. Transgenic lines 15 and 16 exhibited the lowest survival rate (3.3%), whereas 60.0% of line 17 plants survived following cold stress. This result clearly

Table 1 Cold resistance in progeny of transgenic tobacco subjected to temperatures of 20°C for 90 min

[1]Trans. line	[2]Germ. rate (%)	[3]Surv. rate (%)	Trans. line	Germ. rate (%)	Surv. rate (%)
[4]WT	97.2	0	8	7.6	14.0
1	21.6	0	11	84.0	33.3
2	35.3	26.7	12	28.4	16.7
3	56.4	42.5	15	73.2	3.3
4	29.2	42.0	16	55.2	3.3
6	34.8	50.0	17	60.6	60.0
7	20.1	22.5	19	66.9	26.7

[1]Transgenic tobacco lines (T_1), [2]germination rate, [3]survival recovery rate after cold stress treatment, [4]wild type (*Nicotiana tabacum* L. 'Xanthi').

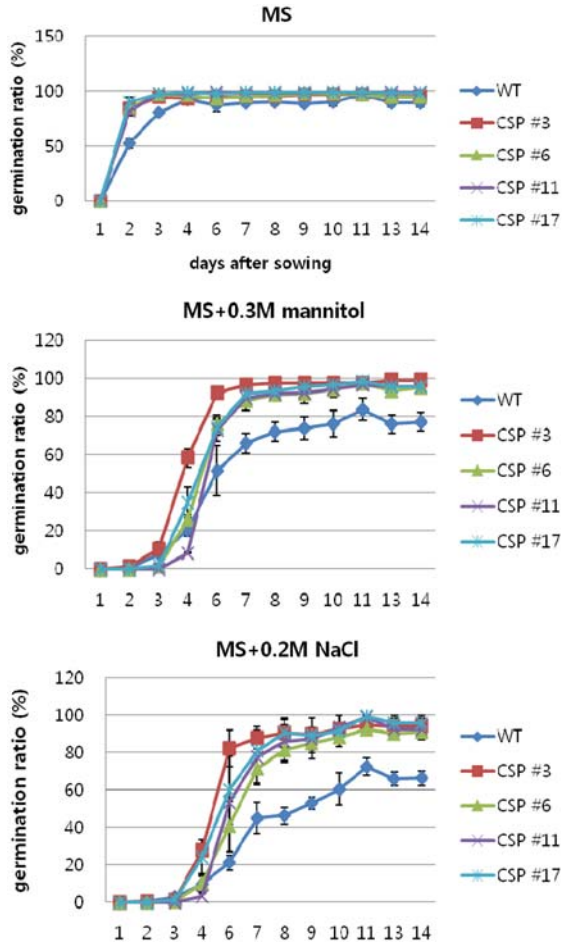

Fig. 10 Salt stress tolerance *of ArCspA* transgenic tobacco seeds as measured by germination under salt condition. Germination rates of four independent ArsCsp transgenic tobacco seeds, lanes 3, 6, 11, and 17, were measured under salt condition assays. Seeds were plated in half-strength MS medium (0.6% agar) supplemented with 0.3 M mannitol or 0.2 M NaCl. About 60 seeds were used for each treatment, with three independent experiments carried out to ensure data reliability.

indicates that ArCspA can act as a cold shock protein when introduced into other plants.

Salt and drought stress assays. To evaluate whether ArCspA may also introduce tolerance to other abiotic stresses to *ArCspA*-transgenic plants, we examined germination rates and plant height in *ArCspA*-transgenic plants growing under saline (0.3 or 0.2 M NaCl) and drought conditions. Transgenic plants exhibited significantly higher germination rates compared with wild-type plants under both saline treatments (Fig. 10). In addition, the results of drought stress experiments revealed that under drought stress, transgenic plants experienced little damage, and showed good recovery upon re-watering (Fig. 11).

Discussion

Arthrobacter sp. A2-5, a bacterium capable of growth between 15

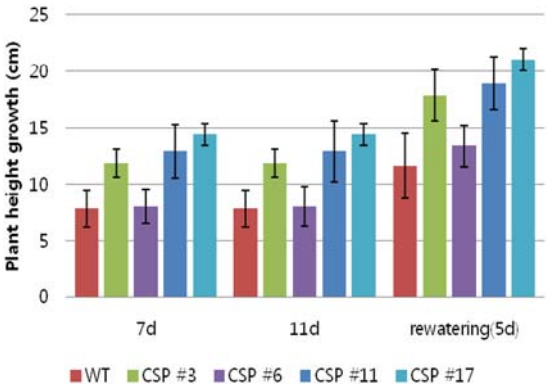

Fig. 11 Drought stress tolerance of *ArCspA* transgenic tobacco plants by measuring plant height growth under drought condition. *ArCspA* transgenic and wild-type tobacco seedlings were grown for 3 weeks under adequate water condition. Water was withheld for 11 days and then the plants were allowed to recover by re-watering for 5 days. Error bars indicate ± SD.

and 30°C, was isolated from soil collected at the South Pole (Fig. 1). We subsequently isolated a gene from *Arthrobacter* sp. A2-5. Because the gene encodes an overexpressed protein following cold shock and during prolonged growth at low temperatures (Fig. 1), we named this gene *ArCspA*. A variety of genes are induced by low temperatures, with their RNA levels subsequently reduced upon stress release. CspA was produced very rapidly following cold shock, and was still expressed during prolonged growth at low temperatures. CspA of *Arthrobacter* sp. A2-5 is highly similar to other CspA proteins from various microorganisms. CspAs have two functions; they act as RNA chaperones and as transcriptional enhancers of other cold shock genes (Horn et al., 2007). In addition, CspA family members have a molecular mass of approximately 7 kDa and contain two highly conserved internal consensus RNA-binding motifs (RNP-1 and RNP-2) (Landsman 1992; Xia et al. 2001). Striking sequence similarity has been found within the *E. coli* CspA cold shock protein family. The N-terminus of the *Arthrobacter* sp. A2-5 CspA contains these two consensus RNA-binding motifs (Fig. 3), which are among the most widely found and conserved RNP sequences, suggesting related functions for these proteins. CspA belongs to a redundant family of seven homologous proteins (CspA-CspG), in which only CspA, CspB, and CspG are transiently overexpressed following cold shock (Landsman 1992; Graumann and Marahiel 1998). Nakashima et al. (1996) suggested that CspA, CspB, and CspG constitute a subfamily of proteins with closely related functions that are distinct from other CspAs. The CspA protein of *Arthrobacter* sp. A2-5 is significantly similar (60% sequence identity) to other bacterial CspA and CspB proteins, but not to CspG (Fig. 3).

ArCspA is produced very rapidly (1 h) following temperature downshift from 25 to 10°C, demonstrating that *ArCspA* expression is an immediate response to low temperatures in *Arthrobacter* sp. A2-5 (Fig. 4). A similar expression pattern for CspA proteins has been reported for other Csp-containing microorganisms. Yeast

habitually survives at temperatures lower than 15°C. Soto et al. (2002) reported that *S. pombe* subjected to cold shock (downshift from 28 to 15°C) transiently activates the mitogen-activated protein kinase Sty1p by phosphorylation. In the present study, the function of the ArCspA protein was revealed when only *ArCspA*-transformed yeast cells were able to grow well at 15°C (Fig. 6). It is thought that cold shock response serves an adaptive function, and that CspA family proteins play a role in protecting cells from cold damage. Although cell growth is delayed after exposure to low temperatures, we believe that *Arthrobacter* sp. A2-5 and *ArCspA*-transformed yeast cell growth following low temperature damage is correlated with CspA synthesis. Because such effects were not observed in cells at 4°C, our results clearly demonstrate that cold response in *Arthrobacter* sp. A2-5 and yeast cells with *ArCspA* genes are adaptive processes that allow cells to return to physiological equilibrium at low temperatures. A number of cold-shock- or low-temperature-induced genes have been identified in yeast, including *Tip1* and *Tir1* (Homma et al., 2003). Yeast cells appear to initiate quite different responses to temperature reductions at around 10–8°C (cold-shock response) compared with those observed at temperatures of 10°C and lower (near-freezing response) (Kandror, 2004).

In our experiments, *ArCspA*-transformed tobacco plants also exhibited cold stress tolerance (Table 1). Because *ArCspA*-transformed yeast cells and tobacco plants were more robust than their wild-type counterparts at low temperatures, we suggest that ArCspA might be able to increase cold tolerance in eukaryotic organisms such as fungi and plants. In practice, many eukaryotes, including plants and animals, have proteins with a nucleic acid-binding domain that shows a strikingly high sequence identity and similar RNA-binding properties to bacterial CSPs (Graumann and Marahiel, 1998). Our experiments also revealed that ArCspA can confer salt and drought tolerance in transgenic tobacco plants. This indicates that information generated from focused studies of gene function in yeast and tobacco may be useful for improving abiotic stress tolerance in crop plants.

Acknowledgment This study was supported by a grant from the Next-Generation Biogreen 21 Program (No. PJ009008 & PJ008091), grant from Agenda Program and Post Doctoral Program of the National Academy of Agricultural Science (NAAS) in Rural Development Administration, Suwon, Korea.

References

Basnak'ian IA (2001) Cold shock in bacteria. *Vestn Ross Akad med Nauk* **5,** 18–21.

Berger F, Morellet N, Menu F, and Potier P (1996) Cold shock and cold acclimation proteins in the psychrotrophic bacterium Arthrobacter globiformis SI55. *J Bacteriol* **178,** 2999–3007.

Berger F, Normand P, and Potier P (1997) cspA, a cspA-like gene that encodes a cold acclimation protein in the psychrotophic bacterium *Arthrobacter golbiformis* SI55. *J Bacteriol* **179,** 5670–6.

Brandi F, Pietroni P, Gualerzi CO, and Pon CL (1996) Post-transcriptioinal regulation of CspA expression in *Escherichia coli*. *Mol Microbiol* **19,** 231–40.

Deming JW (2002) Psychrophiles and polar regions. *Curr Opin Microbiol* **5,** 301–9.

Donzeau M, Bourdineaud GJ-P, and Lauquin GJ-M (1996) Regulation by low temperatures and anaerobiosis of a yeast gene specifying a putative GPI-anchored plasma membrane. *Mol Microbiol* **20,** 449–59.

Gilmour SJ, Fowler SG, and Thomashow MF (2004) Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. *Plant Mol Biol* **54,** 767–81.

Graumann PL and Marahiel MA (1998) A superfamily of proteins that contain the cold-shock domain. *Trends Biochem Sci* **23,** 286–90.

Gualerzi CO, Giuliodori AM, and Pon CL (2003) Transcriptional and post-transcriptional control of cold-shock genes. *J Mol Biol* **331,** 527–39.

Homma T, Iwahashi H, and Komatsu Y (2003) Yeast gene expression during growth at low temperature. *Cryobiology* **46,** 230–7.

Horn G, Hofweber R, Kremer W, and Kalbitzer HR (2007) Structure and function of bacterial cold shock proteins. *Cell Mol Life Sci* **64,** 1457–70.

Inouye M and Phadtare S (2004) Cold shock response and adaptation at near-freezing temperature in microorganisms. *Sci STKE* **237,** pe26.

Kandror O, Bretschneider N, Kreydin E, Cavalieri D, and Goldberg AL (2004) Yeast adapt to near-freezing temperatures by STRE/Msn2,4-dependent induction of trehalose synthesis and certain molecular chaperones. *Mol Cell* **13,** 771–81.

Karlson D, Nakaminami K, Toyomasu T, and Imai R (2002) A Cold-regulated Nucleic Acid-binding Protein of Winter Wheat Shares a Domain with Bacterial Cold Shock Proteins. *J Biol Chem* **277,** 35248–56.

Kowalski LR, Kondo K, and Inouye M (1995) Cold-shock induction of a family of TIP1-related proteins associated with the membrane in *Saccharomyces cerevisiae*. *Mol Microbiol* **15,** 341–53.

Landsman D (1992) RNP-1, an RNA-binding motif, is conserved in the DNA-binding cold shock domain, *Nucleic Acids Res* **20,** 2861–4.

Lee JO, Jeong MJ, Kwon TR, Lee SK, Byun MO, Chung IM, and Park SC (2006) *Pleurotus sajor-caju* HSP100 complements a thermotolerance defect in *hsp104* mutant *Saccharomyces cerevisiae*. *J Biosci* **31,** 223–33.

Maliga P, Klessig DF, Cashmore AR, Gruissem W, and Varner JE (1995) In *Methods in plant molecular biology*, Cold Spring Harbor Laboratory Press, USA.

Nakashima K, Kanamaru K, Mizuno T, and Horikoshi K (1996) A novel member of the cspA family of genes that is induced by cold shock in Escherichia coli. *J Bacteriol* **178,** 2994–7.

Panikov NS and Sizova MV (2007) Growth kinetics of microorganisms isolated from Alaskan soil and permafrost in solid media frozen down to -35 degrees C. *FEMS Microbiol Ecol* **59,** 500–12.

Phadtare S (2004) Recent developments in bacterial cold-shock response. *Current Issues Mol Biol* **2,** 125–36.

Sambrook J, Fritsh EF, and Maniatis T (2001) In *Molecular cloning; A Laboratory Manual,* (3rd ed.), Cold Spring Harbor Laboratory Press, USA.

Soto T, Beltrán FF, Paredes V, Madrid M, Millar JBA, Vicente-Soler J, Cansado J, and Gacto M (2002) Cold induces stress-activated protein kinase-mediated response in the fission yeast *Schizosacchromyces pombe*. *Eur J Biochem* **269,** 5056–65.

Thomashow MF (1999) PLANT COLD ACCLIMATION: Freezing Tolerance Genes and Regulatory Mechanisms. *Annu Rev Plant Physiol Plant Mol Biol* **50,** 571–99.

Wu L, Zhang Z, Zhang H, Wang XC, and Huang R (2008) Transcriptional Modulation of Ethylene Response Factor Protein JERF3 in the Oxidative Stress Response Enhances Tolerance of Tobacco Seedlings to Salt, Drought, and Freezing. *Plant Physiology* **148,** 1953–63.

Xia B, Ke H, and Inouye M (2001) Acquirement of cold sensitivity by quadruple deletion of the cspA family and its suppression by PNPase S1 domain in *Escherichia coli*. *Mol Microbiol* **40,** 1827–30.

Zhang X, Fowler SG, Cheng H, Lou Y, Rhee SY, Stockinger EJ, and Thomashow MF (2004) Freezing-sensitive tomato has a functional CBF cold response pathway, but a CBF regulon that differs from that of freezing-tolerant Arabidopsis. *Plant J* **39,** 905–19.