Expression profiles of hot pepper (*Capsicum annuum*) genes under cold stress conditions

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In an attempt to determine a cold defense mechanism in plants, we have attempted to characterize changes occurring in the expression of cold-regulated transcript levels in the hot pepper (*Capsicum annuum*), using cDNA microarray analysis, combined with Northern blot analysis. After analysing a 3·1 K hot pepper cDNA microarray, we isolated a total of 317 cold inducible genes. We selected 42 genes which were up-regulated and three genes which were down-regulated due to cold treatment, for further analysis. Among the 45 genes which appeared to be up-regulated by cold, 19 genes appeared to be simultaneously regulated by salt stress. Among the up-regulated cold-stress genes, we identified a variety of transcription factors, including: a family of 4 ethylene-responsive element binding protein (EREBP, designated CaEREBP-C1 to C4) genes, a bZIP protein (CaBZ1), RVA1, Ring domain protein, HSF1, and the WRKY (CaWRKY1) protein. As mentioned earlier, several genes appeared to be induced not only by cold stress, but also simultaneously by salt stress. These genes included: CaEREBP-C3, CaBZ1, putative *trans*-activator factor, NtPRp27, malate dehydrogenase, putative auxin-repressed protein, protein phosphatase (CaTPP1), SAR8·2 protein precursor, late-embryogenesis abundant protein 5 (LEA5), DNAJ protein homologue, xyloglucanendo-1,4-*b*-D-gucanase precursor, PR10, and the putative non-specific lipid transfer protein StnsLTP.

[Hwang E-W, Kim K-A, Park S-C, Jeong M-J, Byun M-O and Kwon H-B 2005 Expression profiles of hot pepper (*Capsicum annuum*) genes under cold stress conditions; *J. Biosci.* **30** 657–667]

1. Introduction

Plants have, over time, evolved a variety of protective mechanisms to overcome diverse abiotic environmental stresses, including severe temperature changes, drought, and salinity (Thomashow 1999; Hasegawa *et al* 2002; Zhu 2001a,b; Xiong *et al* 2002; Xiong and Zhu 2002). Stresses including cold, drought, high salinity, and freezing damage have been shown to be induced by similar mechanisms, most notably, dehydration or water stress (Thomashow 1998; Van Breusegem *et al* 1999). Under conditions of water stress, plant cells lose their water and reduce their turgor pressure (Holmberg and Bulow 1998). Levels of the plant hormone, abscisic acid (ABA), tend to increase when the plant is subjected to water stress, and ABA has been established to play an important role in the tolerance of plants to such stresses. The results of many studies have indicated that these protective mechanisms are regulated by alterations in the expression levels of stress genes.

Many of these stress genes are known to be regulated at the transcriptional level. The mRNAs of the abiotic stress-inducible genes appear to be alterable, upon the exposure of the plants to stress conditions (Shinozaki and Yamaguchi-Shinozaki 1997; Jaglo-Ottosen *et al* 1998; Cheong *et al* 2002; Zhu and Provart 2003; Shinozaki *et al* 2003). Genes which are induced as the result of these abiotic stresses are believed to play roles not only in the protection of cells, via the generation of important metabolic and cellular protection proteins, but also in the

Keywords. Abiotic stresses; *Capsicum annuum*; cold-stress regulated genes; microarray

regulation of genes which are involved in the transduction of stress response signals (Shinozaki and Yamaguchi-Shinozaki 1997; Kasuga *et al* 1999; Seki *et al* 2002; Xiong *et al* 2002).

Studies of abiotic stress signal transduction have identified a pathway which leads to a response to both drought and cold stresses, and appears to function via members of the ethylene responsive element binding factor (ERF, known as also EREBP) transcription factor family. As these factors appear to recognize the drought-responsive element (DRE) in the target promoters, they are commonly referred to as the DRE-binding proteins (Yamaguchi-Shinozaki and Shinozaki 1994; Kizis *et al* 2001). DREB1A-C, DREB2A-B (Liu *et al* 1998) and DBF1-2 (Qiang *et al* 2000) are the most thoroughly studied DRE-binding proteins to date. The DRE-binding factors appear to be involved in the regulation of many drought and cold inducible genes, including *rd29A*, *kin1*, *cor6*‵*6*, *rd17*, and *edr10* (Qiang *et al* 2000). Like the ERF transcription factors, the bZIP transcription factors have been strongly implicated in cellular defense against a varity of abiotic and biotic stresses, including those associated with UV, ABA, and wounding and pathogenic attacks (Kusano *et al* 1995; Rushton and Somssich 1998; Kim *et al* 2001; Robatztek and Somssich 2001, 2002). Soybean zinc-finger protein, SCOF-1, has reportedly been induced by both low temperature and ABA, but not by high salinity nor by dehydration (Kim *et al* 2001). They also demonstrated that transgenic tobacco and *Arabidopsis* plants which overexpressed SCOF-1 also exhibited enhanced cold tolerance properties, which were attributed to the increased expression of cold-regulated genes. Another transcription factor, WRKY, has been implicated in cellular defense against a variety of abiotic and biotic stresses, including drought, pathogenic attack, wounding, and heat (Rushton and Somssich 1998; Eulgem *et al* 2000; Chen *et al* 2002; Robatztek and Somssich 2001, 2002; Rizhsky *et al* 2002).

There have been several reports trying to produce abiotic stress-tolerant crop plants, by overexpressing the abiotic stress-regulated genes. Some of these genes, like the late embryogenesis abundant (LEA) protein of HVA1 from barley have been shown to be effective in increasing cold tolerance when introduced to rice plants (Xu *et al* 1996). In these trials, transcription factors were the primary genes to introduce into the plants. Overexpression of DREB1B (CBF1) or DREB1A (CBF3) under the control of the 35S promoter or the RD29A stress-inducible promoter in transgenic *Arabidopsis* plants, not only resulted in the activation of down-stream cold-responsive genes, but also conferred heightened tolerance to freezing, salt, and drought stresses (Liu *et al* 1998; Jaglo-Ottosen *et al* 1998; Kasuga *et al* 1999; Sakamoto and Murata 2001).

In the studies described here, we have identified and characterized cold-regulated genes in the hot pepper plant, via cDNA microarray and RNA blot analyses. Another objective of our studies was to determine if gene expression was also modulated by stress other than cold stresses. Several transcription factors were characterized further in this study, including a family of cold-induced EREBP genes.

2. Materials and methods

2.1 *Plant materials and stress treatment*

The variety of hot pepper used for the construction of the cDNA library was *Capsicum annum*. cv Chung-Ryong Cho. The seeds of this variant were acquired from the seed bank at the Rural Development Administration of Korea (RDA). The plants were grown either in a controlled growth chamber or in a temperature-controlled green house at 25°C. We conducted cold treatment at 4°C, for various durations. For salt treatment, 250 mM NaCl was applied by the soil-drenching method. Drought conditions were simulated by air-drying for various durations. ABA treatment was conducted via the spraying of 100 mM ABA solution.

2.2 *RNA preparation and the construction of the hot pepper cDNA library*

The cDNA library of cold stress-regulated genes was constructed using mRNA isolated from the arial portions (leaves and stems) of hot pepper plants exposed to 4°C cold stress for 1, 2, 4, 8, 12, 24 and 48 h, as well as from the control plants.

Total RNA was extracted with Tri-Reagent (MRC, USA), according to the manufacturer's instructions. Poly(A^+) RNA was purified from total RNA using a Poly(A) Quick mRNA isolation kit (Stratagene, USA). We constructed a cDNA library using a Uni-ZAP XR cDNA synthesis kit, and a Gigapack III kit (Stratagene, USA). The plasmid clones generated by the mass excision of bacteriophage plaques resulted in the generation of 300 bp to 3 kbp of DNA inserts, with an average length of approximately 1·2 kbp (data not shown). In order to analyse the population of cDNA in the library, 200 randomly-selected clones were sequenced, beginning from their 5′ ends. About 20% of the clones were inferred to harbour the full-length cDNA, based on the putative amino acid sequence of the genes and the results of the homology searches. In order to remove the most redundant housekeeping genes, such as those coding for ubiquitin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and rDNA, we conducted colony hybridization using a probe which had been prepared from several housekeeping genes.

2.3 *Microarray preparation and analysis*

In order to isolate the genes specifically regulated by cold stress conditions, we first prepared a cDNA chip using the polymerase chain reaction (PCR) products from the 2,800 plasmid DNA clones randomly isolated from the cold-specific hot pepper library, and the 300 clones randomly isolated from the control hot pepper library. In the PCR amplification these clones, we employed sense (T3) and antisense (T7) primers, and the plasmid DNA obtained by the *in vivo* excision clones from the hot pepper cDNA library as templates. The PCR products were then precipitated with ethanol, and resuspended in 10 μl of 3 x SSC buffer. The PCR fragments were then spotted onto a DNA chip. For the microarray analysis, the mRNAs were isolated from 100 μg of total RNA with a Qiagen mRNA purification kit, according to the manufacturer's instructions, then reverse-transcribed using Superscript II reverse transcriptase (Life Technologies, USA) and oligo (dT) 23 mer. The resultant cDNA was treated for 30 min with one unit of RNase H at 37°C, then purified with a Centricon-30 spin column (Amicon, USA). The cDNA sample was labelled with Cy3-dUTP for both the control and unstressed mRNA samples and labelled with Cy5-dUTP for the experimentally stressed samples, via randomly primed polymerization reactions. We also conducted dye-swapping experiments. The sample was then dissolved in 15 μl of hybridization buffer (5x SSC, 0·2% SDS, 0·2 mg/ml BSA). The probe was denatured for 5 min at 95°C, and applied to the microarray. Hybridization was then conducted using a hybridization chamber, at 65°C for 12–16 h. After hybridization, the slides were washed four times with 2x SSC and 0·2% SDS for 5 min at room temperature, followed by a washing with 0·2x SSC and another with 0·1x SSC. After hybridization, the slides were scanned with an Axon 4000 B scanner and the GenePix Pro 4·0 software. The scanned images were then analysed using GeneSpring 6·0.

2.4 *DNA sequencing and data analysis*

The clones were sequenced for gene characterization, using a Perkin-Elmer ABI377 automatic sequencer. The sequences were then analysed using the National Center for Biotechnology Information (NCBI) database. The DNASTAR program was employed for sequence analysis.

2.5 *Northern blot analysis*

To examine the expression of cold-regulated genes, we isolated total RNA using Tri-Reagent solution (MRC, USA) from stress-treated and control hot pepper plants. In the Northern blot analysis, 15 μg of total RNA was separated on 1·2% denaturing formaldehyde/MOPS agarose gel, then transferred to a nylon membrane. The membranes were subsequently hybridized with a probe DNA labelled with ^{32}P-dCTP (Hwang *et al* 2004).

3. Results

3.1 *Isolation of cold-regulated genes using microarray analysis*

In order to isolate genes regulated by cold conditions, we screened a microarray which had been spotted with 3,100 genes obtained from the hot pepper cDNA library, which had been constructed using both control and cold-treated mRNAs. The genes expressed at a rate in the stressed samples greater than two-fold that of the control were defined as stress-inducible genes. Similarly, the genes expressed in the stressed samples at less than half of the control values were regarded as stress-repressible genes (data not shown). Analysis of these data showed that 317 of the genes spotted on the microarray exhibited a significant degree of differential expression, in response to cold treatment; 42 genes were up-regulated while three genes were down-regulated. These were selected for further analysis, including expression pattern analysis and plant transformation. We also attempted to identify salt-regulated genes via microarray analysis. Among the 42 genes which appeared to be up-regulated as the result of cold treatment, 19 of them appeared to simultaneously be regulated by salt stress.

When the cold-regulated genes were classified on the basis of functional categories using the MATDB (MIPS *Arabidopsis thaliana* data base), and on the basis of gene functions (table 1), the majority of genes were observed

Table 1. Homology comparison of cold-inducible CaEREBP cDNAs.

DNA \ Protein	CaEREBP1	CaEREBP2	CaEREBP3	CaEREBP4
CaEREBP1	–	25·3 (14·4)	20·4 (14·3)	25·7 (14·0)
CaEREBP2	29·8 (21·8)	–	24·2 (14·9)	50·0 (36·9)
CaEREBP3	20·8 (21·7)	20·4 (25·9)	–	25·1 (14·3)
CaEREBP4	26·1 (21·6)	48·2 (41·5)	24·6 (23·4)	–

The numbers in parentheses (%) show homology comparisons among CaEREBP cDNAs outside the AP2 domain.

to be related to either metabolism or cellular defense. Among these, many of the pathogenically-induced genes, including PR-1 (clone No. CaCo33, microarray ratios of all clones are shown in figure 1), PR-10 (CaCo256), SAR8·2 (CaCo145), STH-2 (CaCo241), DC1·2 homologue (CaCo283), and dehydrin (CaCo400) were included. We also included genes believed to be involved with the signal transduction pathway. Several genes encoding for transcription factors were included in this category, including four ethylene-responsive element-binding proteins (EREBP) from *Capsicum annuum* [clone numbers CaCo262, (designated as CaEREBP-C1), 90 (CaEREBP-C2), 231 (CaEREBP-C3), c104 (CaEREBP-C4)], bZIP protein (CaCo81, designated as CaBZ1) RVA1, Ring domain protein, HSF1, and WRKY (CaCo236, designated as CaWRKY1) proteins. Tyrosine-specific protein phosphatase (designated as CaTPP1) was also included (figure 2).

Among the genes which appeared to be down-regulated in response to cold stress were the *a*-L-arabinofuranosidase/*b*-D-xylosidase isoenzyme (clone CaCo24), the aspartic proteinase precursor (CaCo352), and the disulfide-isomerase protein (CaCo398).

3.2 *Northern blot analysis of cold induced genes*

We conducted Northern blot analysis in order to confirm the gene expression patterns as the result of cold treatment, using 45 genes which had been up- or down-regulated by cold stress, using microarray analysis. Results indicated that 41 genes were up-regulated by cold stress, and three were down-regulated. The former category included the ADR11-2 protein (CaCo10), cold-inducible proline-rich protein (CaC0133), late embryogenesis-abundant protein (lea5, CaCo177), SRC (CaCo242), WRKY transcription factor (CaCo236), DNAJ protein homologue (CaCo240) and the bZIP protein (CaCo81). The genes which were down-regulated included wound-inducible aspartic proteinase (CaCo352), and disulfide-isomerase (CaCo398).

However, it should also be noted that the results of microarray analysis were not completely consistent with those of the Northern blot analyses. For example, PR4 (CaCo5), PR6 (CaCo11), cell wall protein (CaCo194) and Prf protein (CaCo282), all of which exhibited up-regulation as the result of cold stress according to the microarray analysis, were not induced by cold stress according to Northern blot analysis. This inconsistency may be attributable to the weak gene expression level, as well as high backgrounds or different growth stages in the preparation of the probes.

The Northern blot analysis of salt sensitive genes showed that several genes were also induced simultaneously, by both cold and salt stress. These genes included the putative *trans*-activator factor (CaCo14), NtPRp27 (CaCo25), malate dehydrogenase (CaCo61), putative auxin-repressed protein (CaCo67), bZIP transcription factor (CaCo81), SAR8·2 protein precursor (CaCo145), Late-embryogenesis protein lea5 (CaCo177), DNAJ protein homologue (CaCo240), xyloglucanendo-1,4-*b*-D-glucanase precursor (CaCo254), PR10 (CaCo256), and the putative non-specific lipid transfer protein, StnsLTP (CaCo309).

3.3 *Expression profiles of cold-inducible transcription factors in response to cold, salt, ABA, and drought stresses*

The fact that a number of genes in several plants are simultaneously induced by cold, drought and/or salt indicates that some cross-talk is inherent to the mechanisms underlying plant defense against different abiotic stresses. In addition, ABA has long been recognized to be involved in the environmental stress responses in plants (Kizis *et al* 2001; Shinozaki *et al* 2003). Therefore, the expression of several genes has been monitored by RNA blot analysis, according to the effects of various stresses, including those associated with cold, salt, ABA, and drought.

The gene which encodes for CaEREBP-C1 (Acc. No. AY789635) was induced by both cold stress and drought conditions. It was not induced by either salt or by ABA treatment (figure 2). However, CaEREBP-C2 (Acc. No. AY789636) was induced by cold stress and, to a slight degree, by salt treatment. CaEREBP-C3 (Acc. No. AY789637) was induced by cold, salt, and ABA stresses. CaEREBP-C4 (Acc. No. AY789638), RAV1 (Acc. No. AAQ05799), Zn finger (Acc. No. CX912393) and CaWRKY1 (Acc. No. AY789641) appeared to be induced primarily by cold stress. CaBZ1 (Acc. No. AY789639) appeared to be induced by both salt and drought stress, as well as by cold stress. CaTPP1 (Acc. No. AY789640) was induced to a slight degree by salt, ABA, and drought, as well as by cold stress.

We identified a family of four EREBP genes which clearly responded to cold stresses. Each of these genes, however, exhibited a different expression pattern response to cold. Three of the EREBP genes (CaEREBP-C1, C2 and C4) exhibited maximal expression at 12 h of cold treatment, whereas CaEREBP-C3 achieved maximum expression at 6 h of cold treatment. Upon the application of other abiotic stresses, CaEREBP-C2 was shown to exhibit a slight response to ABA and salt stresses, whereas CaEREBP-C4 exhibited only nominal responses to salt, ABA, and drought conditions. CaEREBP-C1 responded only to salt and cold stress. However, CaEREBP-C2 exhibited a profound response to 12 h of salt and ABA treatment, but did not respond to drought treatment.

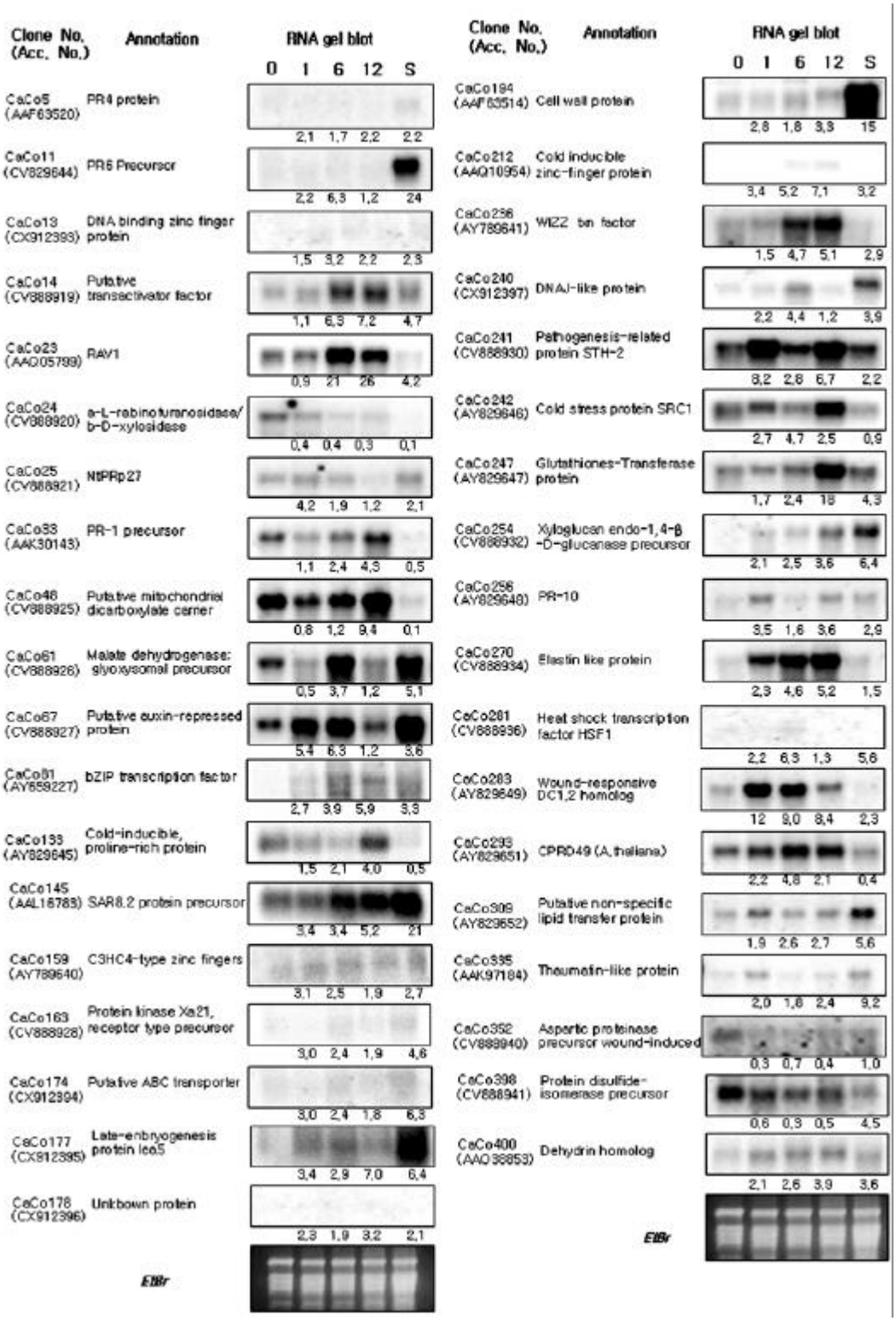

Figure 1. Expression pattern of genes in response to cold and salt treatment. 15 μg of total RNA was used for RAN gel blot analysis. Northern blots and microarrays of cold stress-responsive genes were compared. Expression ratios are expressed as means, normalized after hybridization and scanning using cold-response probe in cDNA microarray analyses. 0, 1, 6 and 12 indicate the duration (hour) of cold stress treatments. S indicates 12 h of salt treatment. EtBr indicates ethidium bromide stained RNA gel.

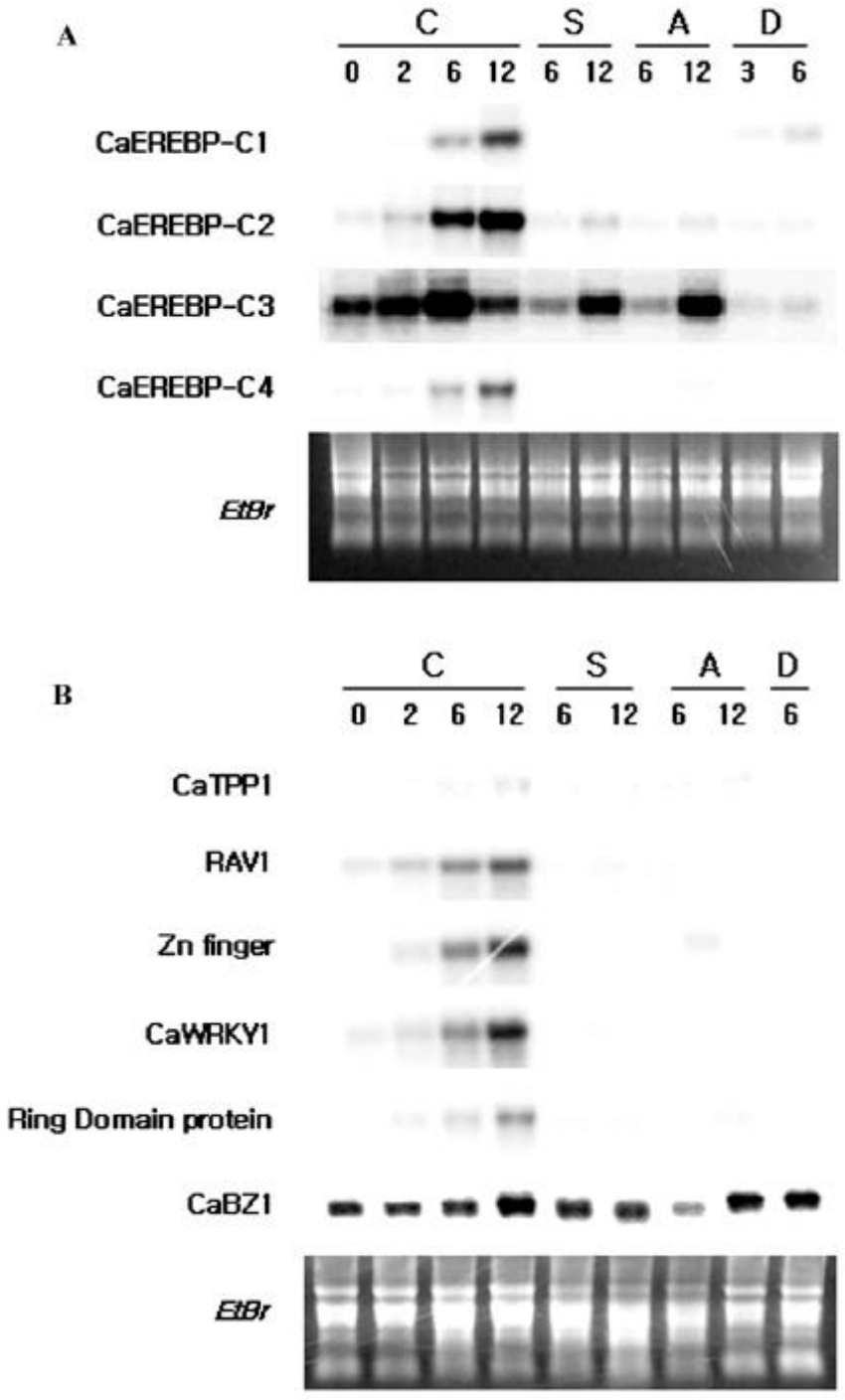

Figure 2. Expreession pattern of some transcription factors in response to various abiotic stresses. 15 μg of total RNA were used for RNA gel blot analysis. 0, 2, 6, 12 indicate durations of stress treatment. C indicates 4°C cold treatment. S, A and D indicate 400 mM NaCl treatment, 100 mM ABA spraying and drought treatment. EtBr indicates ethidium bromide-stained RNA gel. The accession number of CaEREBP-C1 is AY789635; CaEREBP-C2, AY789636; CaEREBP-C3, AY789637; CaEREBP-C4, AY789638; RAV1, AAQ05799; Zn finger, CX912393; CaWRKY1, AY789641; CaBZ1, AY789639; CaTPP1, AY789640.

3.4 *Sequence analyses of cold-inducible transcription factors*

We also analysed the sequences of a variety of cold-induced transcription factors, including CaBZ1 and CaWRKY1 cDNAs, as well as members of the CaEREBP cDNA family (CaEREBP-C1 to C4). Sequence analysis of CaBZ1 cDNA revealed it to be 868 bp long, and that its cDNA encodes for a 170-amino acid protein with a molecular weight of 10·4 kDa. CaBZ1 also was determined to harbour a basic region, as well as a leucine zipper region for DNA binding, which consists of 65 amino acids and conserved in the other bZip proteins (figure 3). CaWRKY1 cDNA is 1,646 bp long, and its cDNA product is 361 amino acids long, with a 39·7 kDa molecular weight. It possesses one WRKYGQK (179–182) sequence and a C2H2-type zinc finger motif (C-X5-C-X22-H-N-H). CaTPP1 cDNA is composed of 1,319 bp, and its cDNA product is 225 amino acids, with a molecular weight of 25·7 kDa. cDNA sequence analysis revealed that the cDNAs of CaEREBP-C1 to CaEREBP-C4 were 976 bp, 1,025 bp, 1,166 bp, and 1,163 bp long, respectively. These cDNAs were found to encode for proteins of 211, 276, 273 and 272 amino acids, with predicted molecular masses of 22·7, 31·4, 30·6 and 30·1 kDa, respectively.

Database searches revealed that the deduced amino acid sequences of CaEREBPs, with the exception of CaEREBP-C3, harboured a conserved DNA binding domain of 58 amino acids, which is present in a large family of plant DNA binding proteins, including the EREBP genes in tobacco and the AP2 of *Arabidopsis* (figure 4). CaEREBP-C3 contains a 47-amino acid of the AP2 domain. The putative CaEREBP proteins include the essential amino acid residues which interact with nucleotides in the GCC box. A cluster of serine-rich residues has been located in several of the proteins of the AP2/ERF family, and these residues may be involved in the activation of transcription.

In order to determine the primary sequence relationship between the CaEREBPs and the other AP2/EREBP-related proteins, we conducted phylogenetic analyses with the DNASTAR program. As shown in figure 5, the CaEREBPs exhibited a high degree of homology with the AP2/EREBP proteins from a variety of plants, including Arabidopsis, tobacco, and tomato. CaEREBP-C2, in particular, exhibited a high degree of homology with *Arabidopsis* CBF2 (40·4% identity), whereas CaEREBP-C3 exhibited homology with tobacco EREBP5 (44·6%), and *Arabidopsis* CBF1 (44·0%) and CBF2 (40·4%). These results indicate that the functions of CaEREBPs may be similar to those of other structural homologues of CaEREBP. However, the CaEREBPs family do not exhibit any pronounced amino acid sequence homology, outside of the AP2 domain (table 1).

The AP2 domain of CaEREBP-C1 is located proximally to the N-terminal regions of the proteins, whereas the AP2 domain of CaEREBP-C3 is located proximally to the C-terminal regions of the proteins. The AP2 domains of CaEREBP-C2 and CaEREBP-C3 are located near to the central regions of the proteins (figure 6).

4. Discussion

In this study, we have isolated and characterized, via cDNA microarray and Northern blot analyses, the genes in *C. annuum* which are regulated in response to cold stress. The results of the microarray analyses were remarkably consistent with the results of Northern blot analysis. This shows that microarray analysis using cDNAs provides a primary means and a powerful and accurate tool for the mass identification of genes involved plant responses to environmental stress conditions (Wendy *et al* 2000; Schenk *et al* 2000; Seki *et al* 2001; Kim *et al* 2003).

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CaBZ1        26   lmdqRKRKRMISNRESARRSRMRKQKHLNDLMAQVSTLRKENDQILTSMNVTTQHYLNVEAENSI  90
Gcn4          4   ESSDPAALKRARNTEAARRSRARKLQRMKQLEDKVEELLSKN-------YHLENEVARLKKLVGE  61
TAF-1       192   ERELKREKRKQSNRESARRSRLRKQAEAEELAIRVQSLTAENatlkseiNKLMENSEKLKLENAA  256
CPRF-1      267   DRDLKRERRKQSNRESARRSRLRKQAEAEELAIKVDSLTAENaalkaeiNRLTLTAEKLTNDNSR  331
AtbZIP54    247   EKEVKREKRKQSNRESARRSRLRKQAETEQLSVKVDALVAENaslrsklGQLNNESEKLRLENEA  311
EMBP-1      248   ERELKRERRKQSNRESARRSRLRKQQECEELAQKVSELTAANgtlrselDQLKKDCKTMETENKK  312
MBP-1a      250   ERELKKQKRKLSNRESARRSRLRKQAECEELGQRAEALKSENsslrielDRIKKEYEELLSKNTS  314
AtbZIP41    220   ERELKRQKRKQSNRESARRSRLRKQAECEQLQQRVESLSNENqslrdelQRLSSECDKLKSENNS  284
CPRF-3      194   ERELKRQRRKQSNRESARRSRLRKQAKSDELQERLDNLSKENrilrknlQRISEACAEVTSENHS  258

consensus     1   ekelKREKRRQKNRESARRSRLRKQAY.EEL..KV..L.AENkalkselERL..EC.KL..EN..  65
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Figure 3. Comparison of the deduced amino acid sequences of the DNA-binding domains of CaBZ1 with those of the other bZIP proteins. The numbers indicate the positions of the amino acid sequences of the basic and leucine zipper regions. CaBZl (Accession No. AY789636, *Capsicum annuum*), Gcn4 (1DGC_A, *Saccharomyces cerevisiae*), TAF-1 (Q99142, *Nicotiana tabacum*), CPRF-1 (Q99089, *Petroselinum crispum*), AtbZIP54 (P42775, *Arabidopsis*), EMBP-1 (P25032, *Triticum aestivum*), HBP-1a (P23922, *Triticum aestivum*), AtbZIP41 (P42774, *Arabidopsis*), CPRF-3 (Q99091, *Petroselinum crispum*).

The cold-regulated genes isolated in this study were primarily cold-inducible genes, although a very few cold-repressed genes were also isolated. Out of the 3,100 cDNA clones spotted on the microarray, 2,800 cDNA clones were prepared from a cold-specific library, and only 300 genes from the non-cold treated control cDNA library.

When the 317 genes which exhibited significant differential expression patterns in response to cold treatment were classified into categories on the basis of their functions, the majority of genes were determined to be associated with cellular rescue and defense (22%), followed by genes which associated with metabolic functions (18%). Genes involved with energy (10%), transcription (9%), and cellular biogenesis (9%) were also represented.

Among the cellular rescue and defense genes, many were also determined to be induced by pathogenic attack. Many PR genes shared this attribute, including the PR-1 (clone No. CaCo33), PR-4 (CaCo5), PR-6 (CaCo256), PR-10 (CaCo258), SAR8·2 (CaCo145), and STH-2 (CaCo241) genes (figure 1). Also, many genes appeared to be induced by salt stress as well as by cold stress. The genes which exhibited this attribute included the putative auxin-repressed protein (CaCo67), bZIP transcription factor (CaCo81), SAR8·2 (CaCo145), C3H4-type zinc fingers (CaCo159), DNAJ-like protein (CaCo245), and the putative non-specific lipid transfer protein (CaCo309). This finding constitutes another indicator of cross-talk occurring between the signal transduction pathways induced by both biotic and abiotic stresses. In a related result, Kasuga *et al* (1999) demonstrated that the overexpression of DREB1A (CBF3) induced by the RD29A stress-inducible promoter enhances tolerance to drought, salt, and freezing stresses.

We also isolated several transcription factors which were induced by cold-treatment. Among these were included a family of 4 EREBP (CaEREBP-C1 to C4) genes, as well as the WRKY (CaWRKY1) and bZIP (CaBZ1) genes. All WRKY proteins harbour either one or two WRKY domains. The WRKY domain is defined by the possession of the conserved amino acid sequence, WRKYGOK, at its N-terminal end, coupled with a novel zinc-finger-like motif. WRKY proteins are generally classified into 3 groups, on the basis of the number of

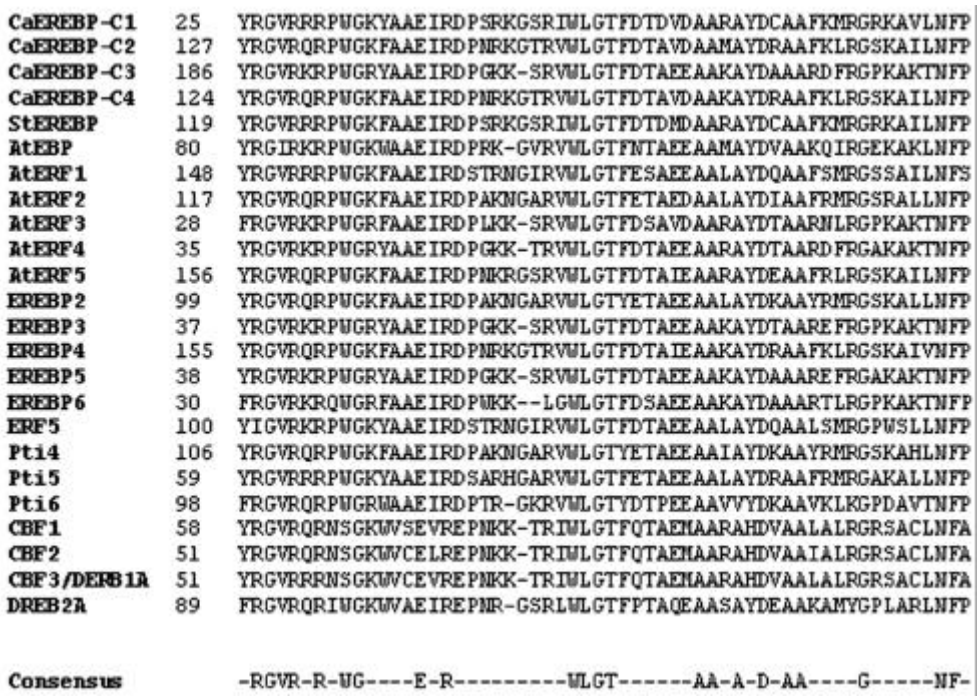

Figure 4. Comparison of the deduced amino acid sequences of the DNA-binding domains of the CaEREBPs with those of the other EREBP/AP2-related proteins. CaEREBP-C1 (Accession No. AY789635, *Capsicum annuum*). CaEREBP-C2 (AY789636), CaEREBP-C3 (AY789637), CaEREBP-C4 (AY789638). StEREBP (AY301558, potato), AtEBP (AJ001911, *Arabidopsis*), AtERFlA (O80337, *Arabibopsis*), AtERF2 (O80338), ATERF3 (O80339), AtERF4 (O80340), AtERF5 (O80341), EREBP2 (D38126, tobacco), EREBP3 (D38124), EREBP4 (D38125), EREBP5 (AY627865), EREBP6 (AY627866), ERF5 (AY655738, tobacco), Pti4 (U89255, tomato), Pti5 (U89256), Pti6 (U89257), CBF1 (=DREB1B, P93835, *Arabidopsis*), CBF2 (=DREB1C, AF074601), CBF3/DREB1A (AF074602), DREB2A (AY691903).

WRKY domains harboured, and the features of the zinc-finger-like motif in each protein. The first of these groups, Group I, exhibits two WRKY domains and a C2H2 type zinc finger motif. The second group, group II, possesses one WRKY domain (WRKYGQK at the N-terminal end) and one C2H2-type zinc finger motif, The 3rd group, group III, exhibits either one or two WRKY domains and a C2HC-type zinc finger motifs (Eulgem *et al* 2000). CaWRKY1 exhibits one WRKYGQK domain at 179–182, as well as a C2H2-type zinc finger motif. Thus, the CaWRKY1 we have isolated is considered belong to group II WRKY gene (Eulgem *et al* 2000).

Jakoby *et al* (2002) classified bZIP transcription factors in *Arabidopsis* into 10 groups (group A to I and S) on the basis of the structure and function. The group A has 3–4 leucine repeats. CaBZ1 contain 3 leucine repeats. While Group A bZIP proteins contain 234–454 amino acids, CaBZ1 has only 170 amino acids (figure 3).

The AP2/EREBP family of plant transcription factors all harbour either one (EREBP subfamily) or two (AP2 subfamily) copies of an approximately 60 amino acid domain, which is normally referred to as the AP2/ERF repeats (Riechmann and Meyerowitz 1998). All of the cold-regulated CaEREBP genes have been shown to harbour only one copy of the AP2/ERF domain, and, with the exception of CaEREBP-C3, which harbours 57 amino acids, all CaEREBP genes contain 58 amino acids in their AP2/ERF domains (figure 4).

Based on amino acid sequence identifies within the ERF domain, the AtERF of *Arabidopsis* has been categorized into three classes (Fujimoto *et al* 2000). Class I includes AtERF1 and ATERF2, and exhibits a high degree of amino acid identity with the ERF from tobacco and with the Pti4 from tomato (Ohme-Takagi and Shinshi 1995; Zhou *et al* 1997). AtERF3 and AtERF4 comprise class II. This class is characterized by an ERF domain consisting of 58 amino acids, one residue shorter than that of the class I ERFs. The third class includes AtERF5. According to the results of a sequence homology comparison between CaEREBPs and the other AP2/EREBP-related proteins, CaEREBP-C3 from *C. annuum* is considered to belong to class II, in that it exhibits a high degree of homology with AtERF3 and ATERF4. However, CaEREBP-C1, C2 and C4 appear to belong more appropriately to class III (figure 4). However, if the CaEREBPs are categorized on the basis of the position of the AP2/ERF domain, CaEREBP-C2 and C4 can be grouped into one class, in that their AP2/ERF domains are located

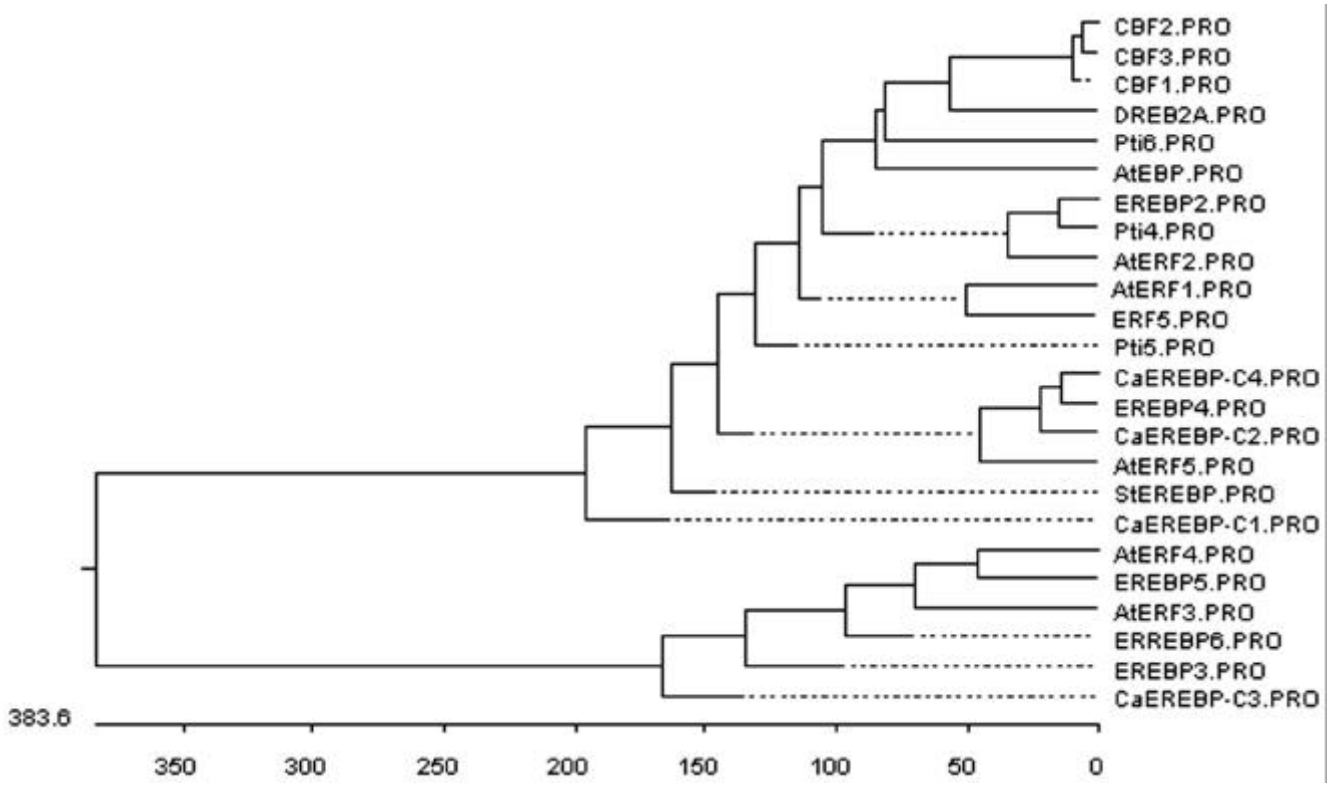

Figure 5. Phylogenetic tree of relationship between StEREBP and other AP2/EREBP-related proteins. The phylogenetic trees were generated with the DNASTAR program. Sequence sources of the genes and accession numbers are shown in the legend to figure 4.

Figure 6. A schematic representation of the CaEREBP-C1 to CaEREBP-C4 cDNA is shown. Boxes indicate open reading frames, starting from the ATG codon, and the lines indicate the putative 5′ and 3′ UTR (untranslated region). Black boxes represent the AP2 domain. Number above the line indicate the positions of the amino acid residues, and the numbers below the line refer to the nucleotide positions.

within the central regions of the proteins. CaEREBP-C1 and CaEREBP-C3 can be categorized into a group of their own (figure 6).

All four of the CaEREBP genes exhibited very distinct expression patterns in response to a variety of abiotic stresses (figure 2). CaEREBP-C1 was induced by cold and drought, C2 only by cold, C3 by cold, salt, and ABA but not drought, and C4 only by cold. This result suggests that, although all four CaEREBP genes may be associated with cold-stress, their method of function and underlying mechanisms may differ substantially.

Currently, studies involving a yeast two-hybrid system are being conducted in order to isolate the genes which interact with cold-inducible transcription factors. Moreover, another study, designed to facilitate the characterization of cold-inducible transcription factors using transgenic plants is underway, and it is hoped that this study will allow us to elucidate the cold signal transduction pathway.

Acknowledgments

This study was financially supported by the Crop Functional Genomics Project of the 21C Frontier Program governed by the Ministry of Science and Technology and Rural Development Administration (RDA), and by the Biogreen21 Program of the RDA, Korea.

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MS received 20 May 2005; accepted 18 August 2005

*e*Publication: 18 October 2005

Corresponding editor: MAN MOHAN JOHRI