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Isolation and Characterization of Drought-Induced cDNA Clones from Hot Pepper (*Capsicum annuum*)

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Drought is a major environmental factor that limits plant growth and productivity. After constructing a cDNA library from progressively drought-stressed hot pepper plants (*Capsicum annuum*), we differentially screened it to isolate cDNA clones whose expressions were drought-inducible. We named these clones *CaDSi-1, 5, 7, 11,* and *12*. RNA blot analyses revealed that all transcripts of these genes were induced strongly or slightly during progressive drought stress. Their deduced amino-acid sequences showed high homologies with such proteins as thioredoxin *m*, glycine-rich protein, nonspecific lipid transfer protein, oxygen-evolving enhancer protein 3, and auxin-repressed protein. In other plant species, those proteins are induced by pathogens and water or salt stresses, and are predicted to be involved in drought tolerance. The data suggest that these five *CaDSi* gene products have possible protective roles against drought stress in hot pepper.

Keywords: *Capsicum annuum*, cDNA clones, differential screening, drought tolerance, drought-induced

Plants are constantly exposed to abiotic stresses, such as drought, high salinity, and low temperatures. Among these, drought is a major limiting factor for growth and crop production worldwide (Boyer, 1982) because it can elicit various biochemical and physiological reactions. However, depending on the efficiency of their response, some plant species can withstand periods of drought by inducing the expression of certain genes (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Bray, 1997). Their products include osmolyte biosynthetic enzymes, a class of late embryogenesis abundant (LEA) proteins, chaperones, detoxification enzymes, and water-channel proteins. Many regulatory genes that code for transcription factors, protein kinases, and phospholipases are also induced by drought stress, and play adaptive or protective roles (Hirayama et al., 1995; Jonak et al., 1996; Frank et al., 2000; Romeis et al., 2001).

Some drought-induced genes have been ectopically expressed in plants to examine their in-vivo functions, the results of which may eventually lead to the engineering of drought-tolerant plants (McKersie et al., 1996; Kasuga et al., 1999; Yamaguchi-Shinozaki and Shinozaki, 2001; Kang et al., 2002). The objective of our current study was to analyze the dehydration-stress response in hot pepper (*Capsicum annuum*), a major crop in Korea. To do so, we used differential screening and RNA blot analyses to characterize five induced cDNA clones from progressively water-stressed plants with regard to their expression patterns and tentative functions under droughty conditions.

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MATERIALS AND METHODS

Plant Material

Seeds of hot pepper (*C. annuum* cv. Bu Gang) were purchased from the Heung-Nong Seed Co., Korea. Twenty seeds were germinated in 3-L pots containing vermiculite. Plants were raised in a growth chamber (16-h photoperiod; 25°C; 60% humidity; and 200 μE/m^2·s, from white fluorescent lamps). The control plants were irrigated daily. For our stress treatments, 10-d-old plants were subjected to progressive drought by withholding water for up to 20 d.

Water-Potential Measurements

Both irrigated and drought-stressed plants were sampled daily. Their water potentials were determined from 0.5-cm^2 leaf discs, using a C-52 thermocouple chamber connected to a HR 33T Dew point microvoltmeter (Wescor, USA).

Isolation of RNA

Total RNA was isolated according to the method of Sambrook et al. (1989). Briefly, whole plants were frozen in liquid nitrogen after water potential was measured, then the tissues were ground in an electric mixer in five volumes of extraction buffer (50% guanidine thiocyanate, 0.5% N-lauroyl sarcosine, 0.1% β-mercaptoethanol, and 25 mM EDTA; pH 7.5). The samples were centrifuged at 6000*g* for 10 min at 4°C, then the upper phase was collected and layered on a cushion of 5.7 M CsCl and 0.05 M EDTA in 11- × 60-mm centrifuge tubes (Beckman, USA). After re-centrifugation in a SW60 rotor (40,000 rpm, 12 h, 20°C), the pellets were re-suspended in 10 mM Tris·HCl (pH 7.5) with 0.1% SDS. The suspensions were then extracted twice with a 4:1 chloroform:1-butanol mixture. RNA was collected by ethanol precipitation, and poly(A)+ RNA was purified via the Poly(A) Tract mRNA Isolation System (Promega, USA).

Construction and Differential Screening of a Drought Stressed-Hot Pepper cDNA Library

A unidirectional EcoRI/XhoI cDNA library was constructed in a Uni-λZAP XR vector (Stratagene, USA) using poly(A)+ RNA from whole plants that had not been watered for 14 to 20 d. The cDNA and vector recombinants were treated with GigapackIII gold packaging extracts (Stratagene, USA), according to the manufacturer's directions. Phage plaques were analyzed by differential screening with ^{32}P-labeled single-stranded cDNA probes, which were synthesized from poly(A)$^+$ RNA isolated by reverse transcriptase from irrigated or drought-stressed plants using the random primer-labeling method. Plaque blotting and hybridization were performed using nylon membranes, following the supplier's instructions (Stratagene, USA). The phage plaques that hybridized preferentially to the cDNA probe from drought-stressed plants were then selected and conversed to pBluescript SK(-) by in vivo excision, using the ExAssist helper phage (M13; Stratagene).

RNA Blot Analysis

RNA samples of 15 μg were fractionated on a 1.2% agarose formaldehyde gel. The quality and equal loading of RNA in each lane were verified by ethidium-bromide staining. Fractioned RNAs were blotted onto Hybond-N (Amersham, UK), using the capillary procedure of Sambrook et al. (1989). The transferred RNAs were cross-linked to the membranes by UV radiation. Probe DNA was prepared by PCR amplification of the cDNA insert, using T7 and SK. PCR was carried out for 30 cycles (94°C, 30 s; 52°C, 30 s; 72°C, 2 min) followed by 5 min at 72°C, all in a DNA thermal cycler (Perkin-Elmer, USA). The amplified cDNA inserts were ^{32}P-labeled by random priming with a Prime-a-Gene Labeling System (Promega), and hybridization was carried out at 68°C for 12 to 16 h. Afterward, the membrane was washed once in 1× SSPE and 0.1% (w/v) SDS at room temperature for 10 min, then twice in 0.5× SSPE and 0.1% (w/v) SDS at 68°C for 15 min. This washed membrane was exposed to X-ray film (Fuji, Japan) at −75°C for 12 to 48 h, using two intensifying screens (DuPont, USA).

Nucleotide Sequencing and Analysis

Nucleotide sequences of the double-stranded cDNA inserts in pBluescript SK(-) were determined by the dideoxy chain-termination method and an automated sequencer (ABI 3100 Genetic Analyzer, USA). We translated the amino-acid sequences and aligned the nucleotide- and amino-acid sequences with DNASTAR-MegAlign (Genetics Computer Group, USA) and ClustalW (1.81) (http://biowb.sdsc.edu/CGI/BW.cgi). All sequences were compared with the databases at the US NCBI, using the BLAST network services.

RESULTS AND DISCUSSION

Leaf water potential in progressively drought-stressed plants began to decrease 5 d after the last watering (Fig. 1). The leaves started to wilt at −1.5 MPa; when water potential was <-2.0 MPa, the plants were considered severely stressed and most did not recover after re-watering. Total RNA for our cDNA library construction and blot analysis was isolated from plants whose leaf water potential was between −1.1 and −2.0 MPa (Fig. 2). Following the differential screening, we selected five putative cDNA clones for further analysis because their expression had been strongly induced by dehydration stress. These clones were named *CaDSi* - *Capsicum annuum* drought stress-induced.

To determine the effects of progressive drought on mRNA accumulation for each *CaDSi*, we performed blot analyses on total RNA extracted from plants showing water potentials of −0.8, −1.3, −1.5, −1.7, or −2.0 MPa. Although transcript levels of all five clones increased in progressively drought-stressed tissues, the time course and degree of induction differed among the clones. For example, *CaDSi-1* and *CaDSi-11* tran-

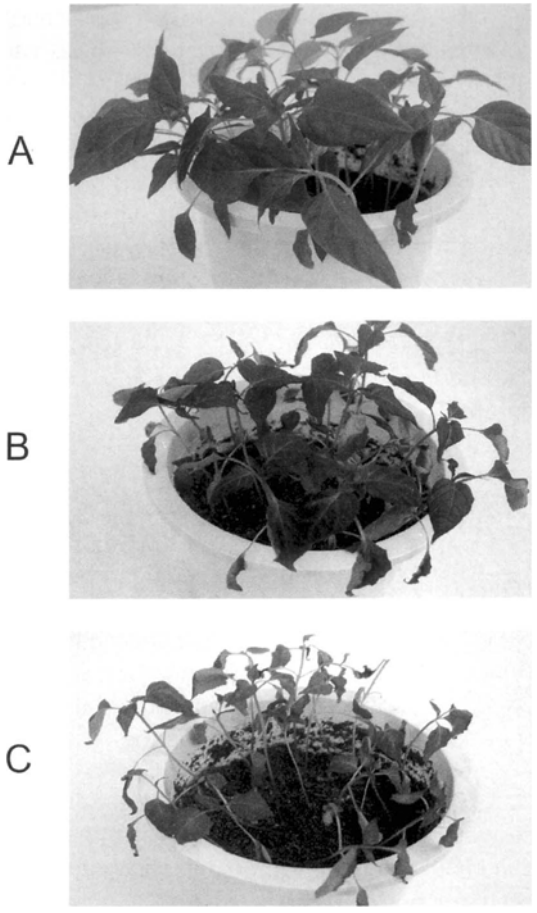


Figure 1. Young hot pepper plants during progressive drought stress. Irrigation was withheld from 10-d-old seedlings for 16 to 30 d. Control and stressed plants were sampled daily for determination of water potential and RNA extraction. **A,** irrigated plants; **B,** plants without water for 15 d; **C,** plants without water for 20 d.

scripts were present at basal levels to −1.5 MPa, then accumulated to high levels at −1.7 MPa followed by a decrease at −2.0 MPa. In contrast, mRNA levels from *CaDSi-5* and *CaDSi-12* were strongly detected under normal conditions, and were further increased after dehydration stress. Finally, the transcript of *CaDSi-7* was barely detected until plants attained a water potential of −1.5 MPa, and the level then increased at −2.0 MPa (Fig. 3).

Nucleotide sequences were determined in both directions of the cDNA insert. For each clone, amino-acid sequences were deduced from the longest open reading frame (ORF), and sequence homology was searched in the database using the BLAST program (translated query-protein database). *CaDSi-1* was 620 bp long, with an ORF of 258 nucleotides that encoded 86 amino acids. This clone presented 40 to 70% iden-

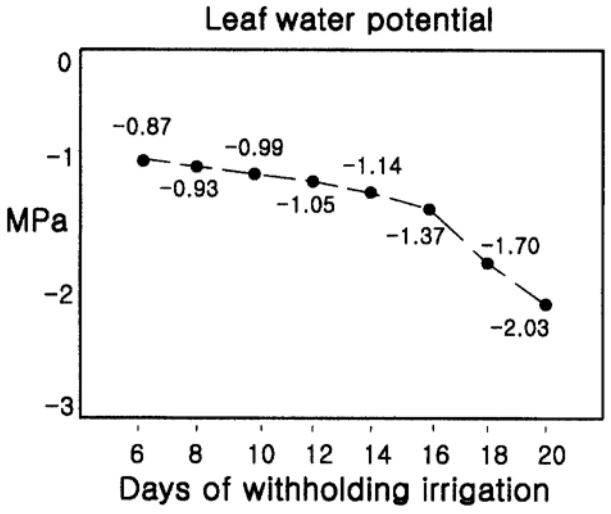


Figure 2. Changes in water potential in dehydration-stressed hot pepper plants. Leaf water potential was determined three times and averaged during the progressive drought period.

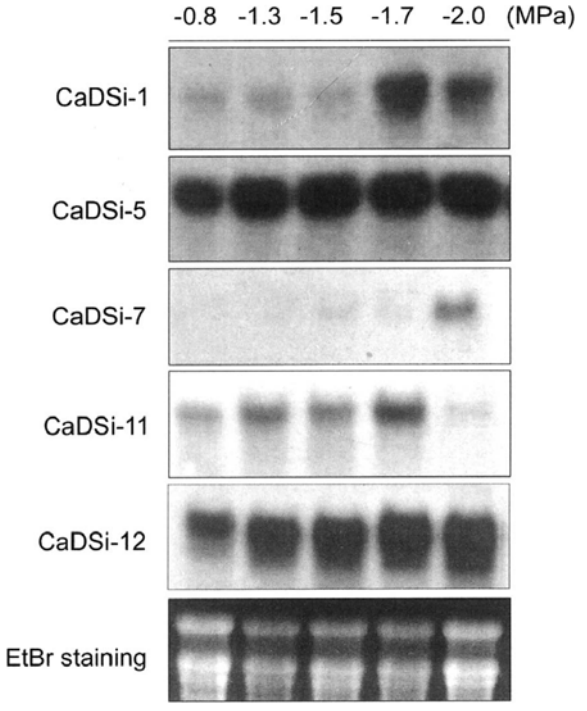
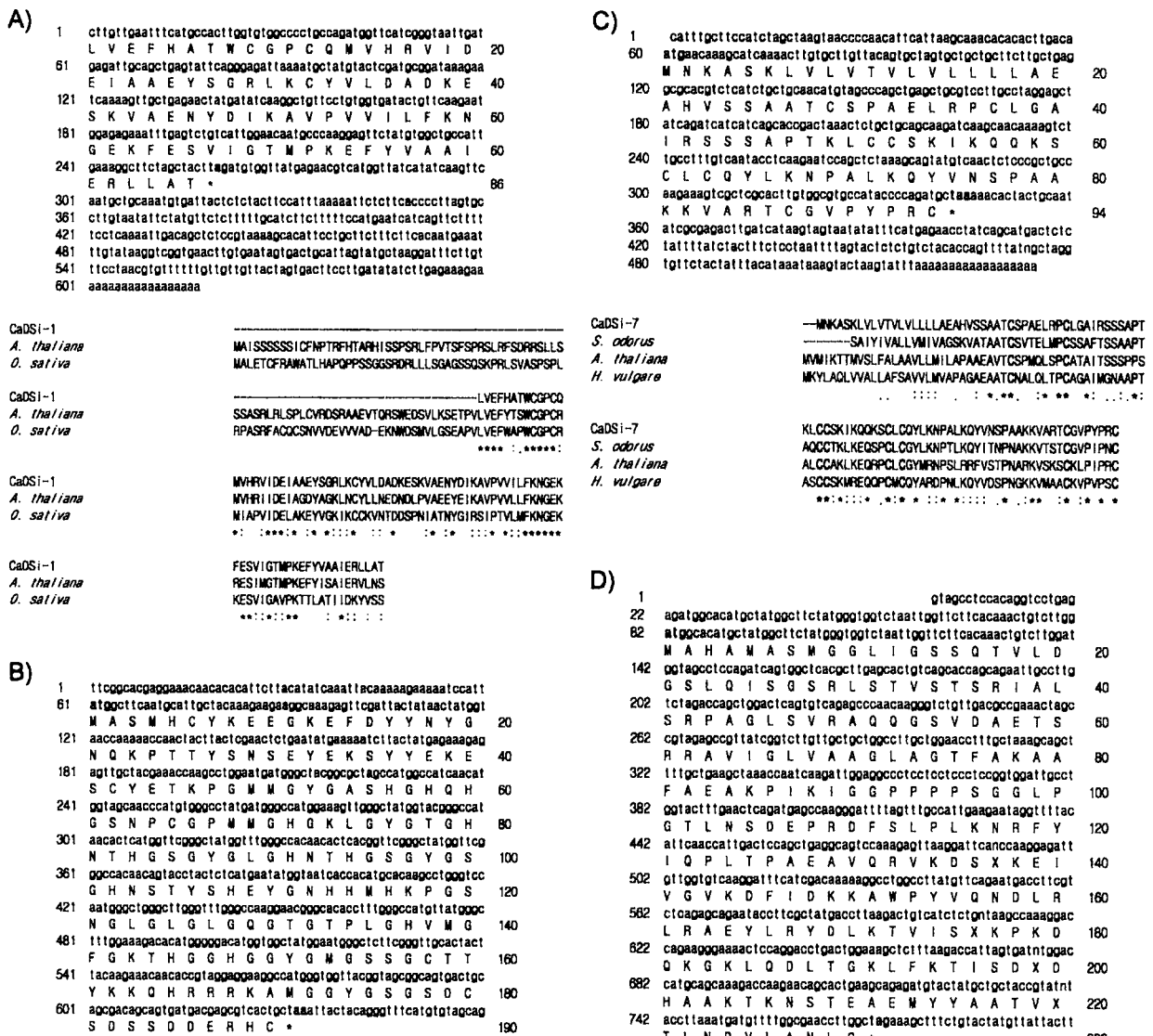


Figure 3. RNA blot analyses of five *CaDSi* clones under progressive drought stress. Each lane was loaded with 15 μg of total RNA extracted from hot pepper plants at five dehydration stages. RNAs were fractionated on a formaldehyde-agarose gel, blotted onto a membrane, and probed with each cDNA insert. Ethidium-bromide staining of RNA showed equal amounts of loading on each lane. *CaDSi-1*, putative thioredoxin *m*; *CaDSi-5*, putative glycine-rich protein; *CaDSi-7*, putative nonspecific lipid transfer protein; *CaDSi-11*, putative oxygen evolving enhancer 3; *CaDSi-12*, putative auxin-repressed protein.



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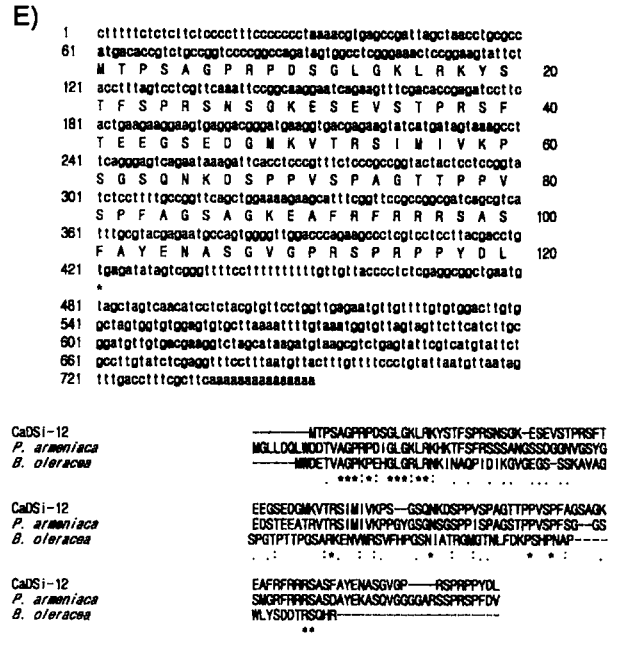

Figure 4. Nucleotide and deduced amino-acid sequences of *CaDSi-1* (**A**), *CaDSi-5* (**B**), *CaDSi-7* (**C**), *CaDSi-11* (**D**), and *CaDSi-12* (**E**); and alignments of the predicted sequences with those from the database. Putative translation start codon is shown in bold face; putative translation stop codon, indicated by an asterisk. Asterisks are also used to identify fully conserved residues among the different sequences, and chemically similar residues are denoted by one or two dots that present conservation at the weak or strong level, respectively. **A.** Deduced sequence of *CaDSi-1* is aligned with thioredoxin *m* proteins of *Arabidopsis* (NCBI accession number AAF15950) and rice (*O. sativa*; NCBI accession number CAA06736). **B.** Deduced sequence of *CaDSi-5* is aligned with glycine-rich proteins of rice (NCBI accession number CAA38315) and buffalograss (*P. ciliare*; NCBI accession number AAK15500). **C.** Deduced sequence of *CaDSi-7* is aligned with nonspecific transfer proteins of *S. odorus* (NCBI accession number AAA33933), *Arabidopsis* (NCBI accession number AAK64007), and barley (*H. vulgare*; NCBI accession number AAB47967). Eight Cys and four Pro residues at the highly conserved positions in plant nsLTPs are indicated in bold face. **D.** Deduced sequence of *CaDSi-11* is aligned with oxygen evolving enhancer 3 proteins of *Arabidopsis* (NCBI accession number Q41932) and spinach (NCBI accession number CAA29056). **E.** Deduced sequence of *CaDSi-12* is aligned with auxin-repressed proteins of apricot (*P. armeniaca*; NCBI accession number AAB88876) and *Brassica oleracea* (NCBI accession number AAL67436).

tity with chloroplast m-type thioredoxin from *Arabidopsis thaliana* and rice (*Oryza sativa*). Comparison of the amino-acid sequence with reported sequences indicated that *CaDSi-1* is a partial cDNA clone lacking the 5' region (Fig. 4A). *CaDSi-5* was 904 bp long, with an ORF of 570 nucleotides. Its deduced amino-acid sequence displayed 35 to 40% identity with glycine-rich proteins or dehydrins from *Pennisetum ciliare* and rice (Fig. 4B). *CaDSi-7* (534 bp, ORF encoding 94 amino acids) showed 60% identity with a nonspecific lipid transfer protein (nsLTP) from *Senecio odorus* and barley (*Hordeum vulgare*) (Fig. 4C). *CaDSi-11* (809 bp, ORF of 230 amino acids) shared 55% amino-acid identity with OEE 3, the oxygen-evolving enhancer protein 3 from spinach (Fig. 4D). Finally, *CaDSi-12* was 751 bp long, with an ORF of 133 amino acids and 51% identity with an auxin-repressed protein from *Prunus armeniaca* (Fig. 4E).

Possible roles in drought tolerance can be suggested for the gene products of these five *CaDSi* clones, based on reports for their homologs. For example, thioredoxins, which show significant identity with *CaDSi-1*, are ubiquitous, small proteins (100 to 120 amino-acid residues) with a highly conserved and reactive active-site sequence (Trp-Cys-Gly-Pro-Cys) that catalyzes thiodisulphide oxido-reduction. Three main types of thioredoxins have been identified in plants. Thioredoxin *h* is located in the cytosol, while thioredoxins *m* and *f* (TRX*m*, TRX*f*) are chloroplastic. TRX*m* is highly similar to prokaryotic thioredoxins, and may possibly act as an anti-oxidant in the chloroplast (Broin et al., 2000; Issakidis-Bourguet et al., 2001). Because drought elicits substantial changes in the chloroplastic redox state, leading to oxidative stress (Issakidis-Bourguet et al., 2001), we suggest that the induction of *CaDSi-1* by drought demonstrates that this clone participates in the chloroplast response to oxidative stress in hot peppers.

CaDSi-5 had high levels of Gly (21%), His (10%), and Tyr (10%), and was highly hydrophilic. Its predicted amino-acid sequence is not typical of any known class of glycine-rich proteins. This clone showed 39% identity with the glycine-rich protein from rice and 37% identity with the dehydrin RAB18-like protein from *A. thaliana*. However, the absence of glycine-rich repeats, cell-wall sorting-signal peptides, and conserved motifs suggests that CaDSi-5 is part of a subfamily of RAB dehydrins or a noble glycine-rich protein. Because of its extreme hydrophilic characteristics, we propose that the CaDSi-5 protein likely is involved in protecting cellular constituents during drought stress, a role that was previously proposed for LEA proteins and dehydrins (Close, 1996).

The predicted CaDSi-7 protein displayed high sequence homology with the nsLTPs from *S. odorus* and barley. Because these are mainly epidermal cell-wall proteins, it has been proposed that they are involved in the biosynthesis of epicuticular wax or cuticles (Sterk et al., 1991; Segura et al., 1993; Lemieux, 1996). Based

on these reports, therefore, we suggest that expression of *CaDSi-7* was induced in our pepper plants as an adaptive response to drought stress, thereby reducing water loss by increasing cuticle thickness.

The predicted amino-acid sequence of *CaDSi-11* showed 50 to 53% identity with OEE3 from *A. thaliana* and spinach. The OEE3 protein is a component of the oxygen evolving enhancer complex (OEC) of photosystem II (Mayfield et al., 1989). Treatment with NaCl can increase the mRNA level of OEE3 in mangrove, a result that suggests that this protein may play a role in maintaining PSII activity under salt stress (Sugihara et al., 2000). Likewise, the increase in *CaDSi-11* transcript in our study indicates that this may be one mechanism for sustaining the activity and stability of PSII in drought-stressed hot pepper plants.

An auxin-repressed protein has been isolated from auxin-deprived strawberry receptacles in research showing that the mRNA level of that protein was lower during normal fruit development (Poovaiah and Reddy, 1990). Although homologs of an auxin-repressed protein have been isolated from other plant species, little is known about its physiological function. The high level of *CaDSi-12* mRNA during our drought study suggests that CaDSi-12, a putative auxin-repressed protein, may be involved in the drought response in hot peppers.

The ability of plants to tolerate drought is probably determined by multiple biochemical pathways that facilitate water retention while protecting proteins and cellular structures. All five cDNA clones isolated in our study showed stress-induced transcripts, and each shared a high level of homology with genes that code the proteins implicated in drought tolerance in other species. Identifying five such clones demonstrates that multiple pathways for dehydration-stress responses exist in *C. annuum*. Therefore, this discovery is a good starting point toward resolving those presumably complicated pathways in that species.

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LITERATURE CITED

Boyer JS (1982) Plant productivity and environment. Science 218: 443-448

Bray EA (1997) Plant responses to water deficit. Trends Plant Sci 2: 45-54

Broin M, Cuine S, Peltier G, Rey P (2000) Involvement of CDSP 32, a drought-induced thioredoxin, in the response to oxidative stress in potato plants. FEBS Lett 467: 245-248

Close TJ (1996) Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins. Physiol Plant 97: 795-803

Frank W, Munnik T, Kerkmann K, Salamini F, Barkels D (2000) Water deficit triggers phospholipase D activity in the resurrection plant *Craterostigma plantagineum*. Plant Cell 12: 111-123

Hirayama T, Ohto C, Mizoguchi T, Shinozaki K (1995) A gene encoding a phosphatidylinositol-specific phospholipase C is induced by dehydration salt stress in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 92: 3903-3907

Ingram J, Bartels D (1996) The molecular basis of dehydration tolerance in plants. Annu Rev Plant Physiol Plant Mol Biol 47: 377-403

Issakidis-Bourguet E, Mouaheb N, Meyer Y, Miginiac-Maslow M (2001) Heterologous complementation of yeast reveals a new putative function for chloroplast m-type thioredoxin. Plant J 25: 127-135

Jonak C, Kiegerl M, Ligterink W, Barker PJ, Huskisson NS, Hirt H (1996) Stress signalling in plants: A MAP kinase pathway is activated by cold drought. Proc Natl Acad Sci USA 93: 11274-11279

Kang JY, Choi HI, Im MY, Kim SY (2002) *Arabidopsis* basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell 14: 343-357

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature Biotech 17: 287-291

Lemieux B (1996) Molecular genetics of epicuticular wax biosynthesis. Trends Plant Sci 1: 312-318

Mayfield SP, Bennoun P, Rochaix JD (1989) Analysis of the genes of the OEE1 OEE3 proteins of the photosystem II complex from *Chlamydomonas reinhardtii*. Plant Mol Biol 12: 683-693

McKersie BD, Bowley SR, Harjanto E, Leprince O (1996) Water-deficit tolerance field performance of transgenic alfalfa overexpressing superoxide dismutase. Plant Physiol 111: 1177-1181

Poovaiah BW, Reddy ASN (1990) Molecular cloning sequencing of a cDNA for an auxin-repressed mRNA: Correlation between fruit growth repression of the auxin-regulated gene. Plant Mol Biol 14: 127-136

Romeis T, Ludwig AA, Martin R, Jones JDG (2001) Calcium-dependent protein kinases play an essential role in a plant defense response. EMBO J 20: 5556-5567

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. 2nd ed, Cold Spring Harbor Laboratory, New York

Segura A, Moreno M, Garcia-Olmedo, F (1993) Purification antipathogenic activity of lipid transfer proteins (LTPs) from the leaves of *Arabidopsis* and spinach. FEBS Lett 332: 243-246

Shinozaki K, Yamaguchi-Shinozaki K (1996) Molecular responses to drought and cold stress. Curr Opin Biotechnol 7: 161-167

Sterk P, Booji H, Schellekens GA, van Kammen A, de Vries SC (1991) Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell 3: 907-921

Sugihara K, Hanagata N, Dubinsky Z, Baba S, Karube I (2000) Molecular characterization of cDNA encoding oxygen evolving enhancer protein1 increased by salt treatment in the mangrove *Bruguiera gymnorrhiza*. Plant Cell Physiol 41: 1279-1286

Yamaguchi-Shinozaki K, Shinozaki K (2001) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Novartis Found Symp 236: 176-186; discussion, 186-189