Cloning of cDNA and expression analysis of a DnaJ-like gene under heavy metal stress in bean*

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Abstract A clone of PvSR6 encoding a new member of the DnaJ-like protein family was isolated from a mercuric-chloride-treated bean (*Phaseolus vulgaris* L.) cDNA library by differential screening using cDNAs derived from treated and untreated plants. The predicted protein contains the highly conserved J domain only, which is present in all DnaJ-like proteins and is considered to play a critical role in DnaJ protein-protein interactions. PvSR6 gene is constitutively expressed in roots but weakly expressed in stems and leaf tissue. Northern blot analysis revealed the transcripts of PvSR6 were at low levels in unstressed bean leaves, but the genes expression was strongly stimulated by heavy metals. These suggest that the PvSR6 might play an important role in resistance to the damage caused by heavy metals.

Keywords: DnaJ-like protein, heavy metals, bean.

HSP70 chaperones with their co-chaperones (DnaJ and GrpE), comprise the HSP70 molecular chaperone system, which performs a number of roles essential for cell survival, including initiating protein translation, facilitating protein folding, assembly and translocation. Moreover, chaperones can prevent of unfolded protein aggregation during stresses and promote renaturation of aggregated proteins after stresses[1—4]. DnaJ, a 41 kD HSP in *E. coli*, has been shown to modulate chaperone activity of HSC70 (DnaK)[2]. The genes encoding DnaJ-like proteins have been identified and cloned also from higher organisms[5—7], studies are mainly focused on its structure and function in yeast. A large number of eukaryotic DnaJ-like proteins have also been found to interact with the HSC70/ or HSP70, and the specificity of the DnaJ-like protein and HSC70 interaction has been proven[1,2]. The expression of HSP70 in maize could be induced by various stresses[8], such as heavy metals, elevated temperature, mechanical wounding and UV light. The levels of DnaJ-like transcripts increased also in response to elevated temperature, excess salt and oxidation stresses[5,6]. These results suggest that both HSP70 and DnaJ-like protein play important roles in increasing plant resistance to various stresses. Here, we report on the cloning and characterization of a cDNA of DnaJ-like protein from bean and its expression in response to heavy metals. Furthermore, the mechanism of heavy metal resistance in plants is discussed.

1 Materials and methods

1.1 Plant materials and stress conditions

Bean (*Phaseolus vulgaris* L. cv. Saxa) seeds were surface-sterilized with 2.5% calcium

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hypochloride solution for 10 min, rinsed several times with distilled water and then imbibed in sterile water for 16 h. The seeds were kept at 22℃ on the moistened filter paper for 6 days in the dark to allow them germination, after which the young plants were transferred onto a liquid culture medium containing KNO_3 2 mmol/L, $Ca(NO_3)_2$ 2.5 mmol/L, $MgSO_4$ 1 mmol/L, KH_2PO_4 1 mmol/L and Fe 2.8 mg/L, Mn 0.55 mg/L, Zn 0.65 mg/L, Cu 0.06 mg/L, B 0.32 mg/L, Mo 0.02 mg/L. Plant were grown in a growth chamber with a photoperiod of 16 h at 22 ℃ during the day and at 18℃ during the night and a photosynthetic photon flux density of 150 μmol m^2/s. When the two primary leaves were well expanded plant were stressed by adding one of the following salts of metals: $HgCl_2$, $CdCl_2$, $CuSO_4$, $ZnSO_4$ or Na_2AsO_3 at a final concentration of 100 μmol/L. Leaf tissue was harvested at various time points after stress treatment.

1.2 Construction and screening of a bean cDNA library

Total RNA was extracted using the phenol/chloroform/isoamylacohol technique[9]. Polyadenylated mRNAs were obtained by oligo-(dT) cellulose chromatography as described by Sambrook et al.[10]. Double-stranded cDNA were synthesized using the Pharmacia Biotech kit from polyadenylated RNA isolated from bean plants harvested 6 h after spraying with mercuric choloride solution. The cDNA library was constructed in the bacteriophage λgt 10 cloning vector.

By differential screening the bean cDNA library with [^{32}P]-labeled cDNA probes, a positive clone of PvSR6 (*Phaseolus vulgaris* stress-related) which gave a strong hybridization signal with the cDNA probe obtained from mercury-treated plants and a weak signal with the control probe was isolated. This clone was then sub-cloned into pBluescriptKS(+) plasmide vector and sequenced by the dideoxy method. DNA sequence analysis was performed on a VAX computer using the GCG package program[11].

1.3 Northern blot analysis

RNA samples were prepared and analyzed according to standard protocols[6]. After electrophoresis on 1.2% agarose-formaldehyde gels, RNA was transferred onto Hybond N membrane and hybridized for 24 h with ^{32}P-cDNA probes in a solution containing 6 × SSC (1 × SSC: NaCl 0.15 mol/L, $C_6H_5Na_3O_7$ 0.15 mol/L) and 50% formamide at 42℃. Hybridization was followed by three washes of 15 min each at 42℃ in 2 × SSC. Finally, the membrane was exposed to X-ray film for autoradiography at − 80℃.

1.4 Southern blot analysis

Bean genomic DNA was isolated from bean leaves. 20 μg bean genomic DNA were digested with appropriate restriction enzymes, separated by agarose gel elctrophoresis and blotted onto nylon transfer membranes. Hybridization was performed with ^{32}P-labeled PvSR6 probes in 50% formamide at 42℃ and membranes were washed at the same temperature, in turn with 6 × SSC-0.1% SDS, 2 × SSC-0.1% SDS and 2 × SSC.

2 Results

2.1 PvSR6 encodes a DnaJ-like protein

A heavy-metal-responsive gene clone of PvSR6 was isolated by differential screening of the bean

cDNA library. Sequence analysis showed that PvSR6 clone contains a 706 bp insert (fig.1). Northern blot analysis revealed the size of the mRNA is about 750 nucleotides (date not shown), indicating that PvSR6 is not a full-length cDNA clone. Indeed, the cDNA sequence of PvSR6 harbors an incomplete open reading frame, lacking the coding sequence at the 5′ end including ATG initiation codon; the stop codon is at position 485. The 3′ untranslated sequence includes a putative polyadenylation signal (underlined in figure 1).

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  1  GGCCTTTCAACCGTCTCTTCCCGCCGTAAACTTCTCCGGTAAAGTTCTCGCCTCGCCGCC
       A  F  Q  P  S  L  P  A  V  N  F  S  G  K  V  L  A  S  P  P
 61  CTGTCGCGTTAGATCCCGCCCAATAGTCGCCTTCGCCACCGCCACCGCCACCGCCACCGC
       C  R  V  R  S  R  P  I  V  A  F  A  T  A  T  A  T  A  T  A
121  CACCGCCACTTCAACCGAGGAAGCTCGCTCTTCCTGGACGGAGAAACCACGCCCTTCCTA
       T  A  T  S  T  E  E  A  R  S  S  W  T  E  K  P  R  P  S  Y
181  TCTCAACTCCTCTTGCTCTTCTCTCTACGATATCCTCGGCATCCCCGCCGGTGCCTCCAG
       L  N  S  S  C  S  S  L  Y  D  I  L  G  I  P  A  G  A  S  S
241  CCAGGAAATCAAGGCCGCGTACCGGCGACTGGCCAGAGTCTGCCACCCGGACGTGGCGGC
       Q  E  I  K  A  A  Y  R  R  L  A  R  V  C  H  P  D  V  A  A
301  GATCGACCGGAAAAACTCCTCCGCGGACGAATTTATGAAGATCCACGCCGCCTACTCCAC
       I  D  R  K  N  S  S  A  D  E  F  M  K  I  H  A  A  Y  S  T
361  TCTCTCGGATCCTGACAAACGCGCCAACTACGACCGGAGCCTGTTCAGGCGACAACGGCC
       E  S  D  P  D  K  R  A  N  Y  D  R  S  L  F  R  R  Q  R  P
421  GCTGTCTACTGCGGCGGTGTTCTCAGGCTACACGCGCCGGAACTGGGAAACGGATCAGTG
       L  S  T  A  A  V  F  S  G  Y  T  R  R  N  W  E  T  D  Q  C
481  CTGGTAGtgagtcactgagtcgactcggcgagcgaaagtgagaagacgcggttattgaag
       W  *
541  cgcctttccttgcttataaattattaatcactaatttgtagttcgtatatctaggtagag
601  tagaatagacggccaccagtactcagtgctacttgccacttgtaatataaacctttttca
661  tcaaatgacaaaaaaaaaacaaaaaaattgaaacggtaaaaaaaaa 706
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of PvSR6 cDNA. The conserved amino acids in the DnaJ motif are shaded. The asterisk indicates stop codon and the putative polyadenylation signal is underlined (GenBank Nucleotide Sequence Database under the accession number U77925).

Both nucleotide and deduced amino acid sequences of PvSR6 have no significant homology to any sequence deposited in GenBank. However, the predicted polypeptide was found to contain the highly conserved J domain present in DnaJ-like proteins[12—15], the conserved motif is: Y--LG----A----------HPD---- … ----F----A---L-D-----Y----- (fig.2). Therefore, PvSR6 is likely to encode a DnaJ-like protein. DnaJ proteins possess four domains: the N-terminal J domain, Gly-rich domain (G), Cys-rich domain (CRR), and a less conserved C-terminal domain. However, the structure of DnaJ-like proteins in eukaryotes varies greatly[7]. Some of the DnaJ-like proteins, such as the *Atriplex nummularia* ANJ1[5] and YDJ1[7], contain all three conserved domains (J, G and CRR). Others were found to contain only one or two of those domains. For example, human cytoplasmic HDJ1[7] and yeast SIS1[13] contain both J and G domains, while yeast Sec63[14] and the *Arabidopsis thaliana* D3[6] contain only the J domain which is present in seemingly random location in these pro-

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PvPR6-bean          65 CSSLYDILGIPAGASSQEIKAAYRRLARVCHPDV----AAIDRKNSSADEFMKIHAAYSTLSDPDKRANYDRSLFR
D3-A. thaliana      13 NRELYALLNLSPEASDEEIRKAYRQWAQVYHPDK---IQSPQMKEVATENFQRICEAYEILSDETKRLIYDLYGME
ANJ1-A. nummularia  11 STRYYEILGVPLDASPEDLKKAYKKAAIKNHPDK----------GGDPEKFKELAHAYEVLSDPEKREIYDQYGED
SIS1-yeast           4 ETKLYDLLGVSPSANEQELKKGYRKAALKYHPDK---------PTGDTEKFKEISEAFEILNDPQKREIYDQYGLE
HDJ1-human           2 GLDYYQTLGLA-AALGRGDQAGLPPPGLRYHPDL------NL-EPGAEELFLEIAEAYDVLSDPRLREIFDRYLEE
SEC63-yeast        123 LFDPYEILGISTSASDRDIKSAYRKLSVKFHPDKLAKGKTPDEKSVMEETYVQITKAYESLTDELVRQNYLKYGHP
DnaJ-E. coli         3 KQDYYEILGVSKTAEEREIRKAYKRLAMKYHPDR------NGGCENAAGRFKEINEAYSVLSDSQKRAAYDQYGHA
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Fig. 2. Alignments of amino acid sequence of bean PvSR6 with the conserved DnaJ motif of DnaJ-like proteins. Residues that are conserved in all sequence are shown in bold type.

teins. Finally, several DnaJ-like proteins, such as yeast TIM 44 and bovine auxilin, contain only small regions of limited homology to parts of the J domain[7]. Members of the DnaJ-like protein family are structurally diverse, containing different combination of these conserved domains. All DnaJ-like proteins described thus far, however, contain a J domain, which is proposed to mediate interactions with HSP70 that regulate ATPase activity. Regions in the C-terminus of the protein are proposed to mediate interactions with other polypeptides[15]. Thus, though the deduced polypeptide of PvSR6 containing the J domain only, it is a member of the DnaJ-like protein family.

2.2 Expression of PvSR6 gene in various tissues of young bean plants

Total RNA was extracted from different organs of bean seedlings and probed with PvSR6 cDNA. Northern blot analysis showed that PvSR6 gene was highly expressed in roots but weakly expressed in leaf tissue and in stems (fig.3), demonstrating that PvSR6 protein is constitutively expressed in bean seedlings.

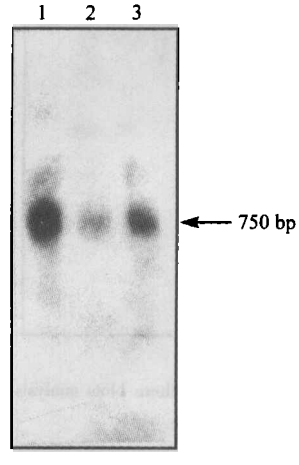

Fig. 3. PvSR6 gene expression in various tissues of *P. vulgaris* seedlings. 1, root; 2, stem; 3, primary leaves.

2.3 Effects of heavy metals on PvSR6 gene expression in primary leaves

The expression of PvSR6 was studied in response to stresses induced by several metals. As shown in fig.4, the amounts of PvSR6 transcripts increased rapidly after the onset of treatment, reached a maximum after 12—24 h and then decreased gradually in mercuric chloride treated plants. The level of PvSR6 mRNA continued to increase until a maximum was reached 48 h later after the treatment with cadmium. Arsenic, zinc or copper also stimulated the gene expression, the kinetics of induction was similar, the mRNA accumulated gradually after the treatment, and reached a maximum after 24—48 h and then declined.

2.4 Genomic southern blot analysis of PvSR6 gene

The genomic DNA was digested with the restriction enzymes *Eco*RI and *Bam* HI which do not cut within the PvSR6 insert, the resulting fragments were tested in hybridization using PvSR6 as a probe (fig.5). A single band was observed in digests from each restriction enzyme, two bands were

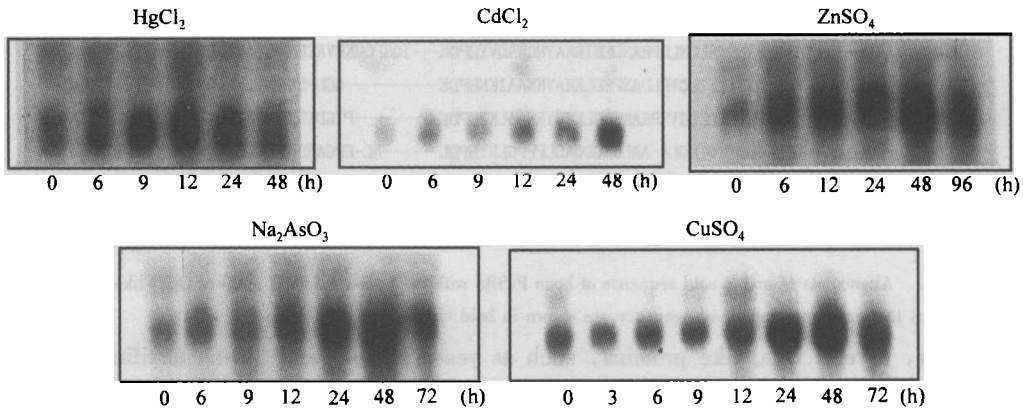

Fig. 4. Northern blots analysis showing the levels of PvSR6 mRNA in bean leaves upon treatment with various metals.

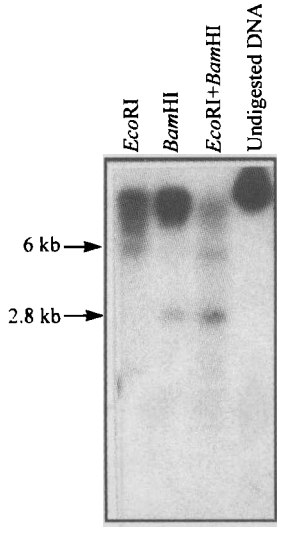

Fig. 5. Southern blots analysis of PvSR6 gene.

obtained in *Eco*RI/*Bam*HI digests, indicating that PvSR6 gene is present at low copy number or as a single copy in the bean genome.

3 Discussion

Various DnaJ-like proteins existing in eukaryotic cells have distinct physiological functions, because of the differences in their structure and localization in cell. Yeast contains at least eight different DnaJ-like proteins, which have been proved to participate in a number of vital processes[16]. For example, YDJ1 facilitates protein import into mitochondria. SIS1 is localized in the cytosol with YDJ1, it is involved in protein assembly and disassembly events essential for the initiation of translation. MDJ, which is localized to the matrix face of the inner mitochondrial membrane, assists in folding of newly imported proteins and assembly of respiration-competent mitochondria. Sec63 is an integral component of the membrane-bound translocation machinery that facilitates protein translocation into the endoplasmic reticulum. In bovine brain, auxilin is involved in uncoating of clathrin-coated vesicles during endocytosis[7]. In *A. nummularia*, ANJ1 has the highest sequence similarity to YDJ1, and can complement the temperature-sensitive growth phenotype of the ydj1 mutation[5]. PvSR6 protein encoded by a low copy number gene is constitutively expressed in bean seedling, suggesting that the protein plays important roles in maintaining metabolic processes of cells under normal growth conditions; its biological function needs to be studied further.

DnaK system can bind to the unfolding polypeptides induced by stress and prevent of aggregation *in vivo*, as well as promote renaturation of aggregated proteins after stress[2,4]. These results imply that HSP70 chaperone system protects the structure of protein from stresses. Heavy metals were shown to stimulate the expression of HSP70 genes in plants, such as maize[8], petunia[17] and tomato[18]. Moreover, the accumulation of HSP70 induced the cadmium tolerance[18], indicating that it plays an essential role in increasing plant tolerance to heavy metals. Heavy metal ions can bind to protein

sulfhydryl groups, resulting in alteration of protein structures and thereby deactivating enzymes. Oxidative damage by free radicals generated by metal redox cycling is another toxicity mode. Eventually, both processes may result in injury of macromolecules and destruction of membrane structure[19]. In tomato, a considerable amount of HSP70 was found to be associated with membranes after cadmium stress, especially to the plasmalemma, the ultrastructure of the cells appeared to be quite normal. These results suggested that HSP70 possibly prevent membrane protein denaturation from metal stresses and reintegrate them into the complex of the membrane proteins[18]. HSP70 has a strong affinity for misfolded proteins, however, it doesn't bind to polypeptides directly. DnaJ is considered to recognize and bind polypeptides firstly, and then targets it to HSP70. Furthermore, DnaJ regulates the stabilization of HSP70-substrate complex and accelerates the reaction cycle of HSP70. PvSR6 protein is a small DnaJ-like protein, its expression was strongly stimulated by heavy metals, implying that a great amount of DnaJ-like proteins are required for binding and refolding of the unfolded polypeptides or damaged proteins induced by metals.

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