

Full Length Research Paper

Over-expression of Arabidopsis DnaJ (Hsp40) contributes to NaCl-stress tolerance

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DnaJ (Hsp40), a heat shock protein, is a molecular chaperones responsive to various environmental stress. To analyze the protective role of DnaJ, we obtained sense transgenic Arabidopsis plants that constitutively expressed elevated levels of DnaJ. In this study, sense transgenic plants show large thinner, fade color and malformed leaves, as well as less floss of back leaves. Plants with enhanced levels of DnaJ in their transgenic sense lines exhibited tolerance to NaCl stress. Under 120 mM NaCl, root length was higher in transgenic sense plants than wild-type plants. *In vitro* expression system, DnaJ protein shows tolerance to high NaCl. These results suggest that over-expression of DnaJ can confer NaCl-stress tolerance.

Key words: DnaJ (Hsp40), NaCl, sense-transgenic plants, *in vitro* expression.

INTRODUCTION

Heat shock proteins are synthesized by all the cells in response to the various type of environmental stress. It is well documented that under stressful conditions like infection, heat, NaCl, ABA, etc, heat shock proteins are induced to help cells to tide over these assaults. Heat shock proteins are molecular chaperones involved in a variety of cellular processes including protein folding, protein transport across membranes, regulation of protein degradation, modulation of protein activity and prevention of irreversible protein aggregation (Sun et al., 2001; Lee et al., 1995; Mohd et al., 2006). Plant BiPs, a member of the HSP70 protein family, were shown to be induced by a variety of environmental stresses, including water stress (Cascardo et al., 2000). Over-expression of BiP- enhanced water stress tolerance in transgenic tobacco plants, where

as the antisense transgenic plants showed hypersensitivity to water stress. Over-expression of tobacco NtHsp 70-1 contributes to drought-stress tolerance in plants (Eun and choo, 2006).

A typical DnaJ protein is composed of four modules including a highly conserved 70 amino acid region at the N-terminal end (J -domain), a glycine -rich region (G-domain) of about 30 residues, a domain containing four Cys-X-X-Cys-X-Gly-X-Gly motifs, where X can be any amino acid residue and a low similarity region at the C-terminal region of 120 - 170 residues (Bork et al., 1992). DnaJ and GrpE proteins can function as cochaperones and together stimulate the ATPase activity of *Escherichia coli* stress 70 (Liberek et al., 1991). DnaJ can increase the avidity of interactions between DnaK and target proteins (Hendrick et al., 1993) and there have been reports that GrpE can either stabilize (Straus et al. 1990) or destabilize interactions between DnaK and target proteins. DnaJ also can itself bind to nascent polypeptides and maintain them in an unfolded or loosely conformation (Hendrick et al., 1993). To reactivate proteins *in vitro*, Hsp40 and Hsp70 can assist Hsp104; Hsp104 performs the first step in dissociating protein aggregates so that Hsp70 and Hsp40 can recognize the denatured substrate and complete the refolding process (Glover and Lindquist, 1998). Bacterial Hsp70 and Hsp40 homologues have same capability (Mogk et al., 1999;

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Abbreviations: DNA, Deoxyribonucleic acid; cDNA, complementary DNA; Hsp, heat shock protein; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase – PCR; CTAB, cetyl trimethyl ammonium bromide; dCTP, deoxycytidine 5'- triphosphates; SDS, sodium dodesyl sulfate; RE, relative enrichment; EDTA, ethylene diamine tetraacetic acid; PAGE, polyacrylamide gel electrophoresis

Motohashi et al., 1999). DnaJ might function independently as a molecular chaperone. Several DnaJ homologues have been identified in organisms ranging from yeast to mouse and human (Lindquist and Craig, 1998; Cheetham et al., 1992; Berruti et al., 1998). It is not known whether DnaJ transgenic plants have any similar function in several stressful conditions. In this report, we showed function of DnaJ was tolerance to NaCl.

MATERIALS AND METHODS

Plant material and grown conditions

Arabidopsis seeds (ecotype RLD) were washed in 75% ethanol, treated for 8 min in 0.1% Hg₂Cl₂, rinsed three times with sterile water and spread on MS medium plates. The plates were vernalized for 2 days in darkness at 4°C and then grown under standard conditions at 22°C under 70% humidity with a light/dark cycle of 16/8 h.

Constructs and transgenic plants

Total RNA was prepared from 30-day-old *Arabidopsis* plants. The samples were homogenized in 1 ml of Trizol reagent (Invitrogen) in a prechilled mortar. Total RNA was extracted and converted to cDNA by using M-MLV reverse transcriptase with oligo-d (T)₁₈ primers, according to the manufacturer's instructions (TIANGEN). The primers were as follows for:

DnaJ-sense 5' CTAATATGGCTATAATACTTGAAG 3'
DnaJ-antisense 5' GTGTTTCTAATCATCTACTGGTGCTAT 3'.

The sequence was subcloned using the pMD18-T vector system (TaKaRa). DnaJ gene was subcloned into the polylinker cloning site of the binary vector in between the 35S promoter of *Cauliflower mosaic virus* and the NOS terminator. *Arabidopsis* plants were transformed with *Agrobacterium tumefaciens* strain EHA105 as described by Clough and Bent (1998). After transformation, seeds from individual transgenic plants were spread on MS medium plates containing 50 mg l⁻¹ Kanamycin and then under the same growth conditions. Transgenic plants were transferred to soil for further analysis.

Analysis of mRNA expression by RT-PCR

For the analysis of different expression, RT-PCR was performed for 27 cycles to determine the linearity of the PCR. The thermal cycling parameters used for the RT-PCR for all genes were as follows: 94°C for 30 s, 56°C for 30 s, 72°C for 120 s; followed by 72°C for 10 min. The cDNA was amplified from 30 ng of total RNA, using specific primers sets for DnaJ-S 5'ATTACTATTCGGTACTCGGAGTTTCG'3, DnaJ-A 5' CTATAACAACAACAGATCGCCTGG'3. As a positive control, act 7 fragment was amplified under the same RT-PCR conditions, using the primer pair:

Forward 5'GGTGAGGATATTCAGCCACTTGTCTG'3.
Reverse 5'TGTGAGATCCCGACCCGCAAGATC'3.

PCR and southern blot for detection of the transgenic plants

Genomic DNA was obtained from 1 g of fresh leaf of transgenic

plants which were selected by Kanamycin. Plant tissues were ground in liquid nitrogen by using a mortar and pestle and then the genomic DNA was isolated by accordance with the CTAB methods. PCR for the qualitative detection of the transgenic plants was conducted using a Bio-RAD DNA Engine systems. Each reaction mixture harbored 50 ng of genomic DNA. The primers were as follows for: 35S-F 5' CGCCACAGATGGTTAGAGAG 3', DnaJ-antisense 5' GTGTTTCTAATCATCTACTGGTGCTAT 3', in accordance with the following program : 94°C for 1 min, 35 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 120 s; followed by 72°C for 5 min. The products were separated on 1.0% agarose gel containing 0.1% ug/ml ethidium bromide. Genomic DNA of transgenic plants was digested overnight at 37°C with HindIII, separated by electrophoresis on 0.8% agarose gel and then transferred onto a positively charged nylon membrane Hybond N⁺ (GE Healthcare, Piscataway). The full of DnaJ gene was labeled with P³²-dCTP by 35S-F and DnaJ-antisense primers and was used as a hybridization probe. The membrane was hybridized with the probe and the final washing was performed in 2×SSC, 0.1% SDS at 65°C for 15 min and twice in 0.5×SSC, 0.1% SDS at 65°C for 10 min. The filters were exposed to Kodak BioMax MR film for 3d at - 80°C with intensifying screens.

Protein extraction and immunoblotting analysis

For protein blot analysis, 1 g of plant material was ground in liquid nitrogen and resuspended in 1 ml extraction buffer (50 mM sodium phosphate pH7.0, 10 mM β-mercaptoethanol, 10 mM EDTA pH8.0, 0.1% N-lauroyl sarosine and 0.1% Triton X-100). After 5 min of incubation at 4°C, cell debris was removed by centrifugation for 20 min at 12,000 rpm with a microcentrifuge. 100 μg of soluble protein from each sample was separated on a 12% SDS-PAGE electrophoresis. Proteins on the polyacrylamide were electroblotted onto a nitrocellulose membrane in glycine electrode buffer. After preincubation with 5% non-fat milk in TBS (10 mM Tris pH7.5, 150 mM NaCl), the membrane was incubated with a commercial anti-rabbit DnaJ diluted 1:1000 in 5% non-fat milk in TBS for 1 h at 25°C on an orbital shaker. After washing for three periods of 10 min in TBS containing 0.1% Tween-20, the membrane was incubated for 1 h in goat anti-rabbit IgG conjugated with horseradish peroxidase diluted 1:4,000 in 5% non-fat milk in TBS. Membrane was washed for three periods of 15 min in TBS containing 0.1% Tween 20 and developed with an enhanced chemiluminescence kit as recommended by the manufacture (Amersham). To ensure reproducibility of the results obtained from immunoblots, multiple experiments with varying exposure times were performed.

NaCl-stress treatments

Seeds from transgenic lines and wild-type were plated together on MS medium with 120 mM NaCl, under long day condition (16 h light/8 h dark), in controlled temperature growth chamber. After NaCl treatments, plants growth was monitored and photographed after 10 days.

In vitro expression assays

In order to produce fragment for *in vitro* expression reactions, the cDNA fragment was amplified with common primer 5'GGAATTCCTAAATATGGCTATAATACTTGG'3, 5'GCGTCGACGTGTTTCTAATCATCTACTGGTGCT'3, restriction sites *EcoRI/SalI* were added to the cDNA fragment, respectively, and inserted into the *EcoRI/SalI* sites of the vector pET32a (Novagen). The proteins were synthesized according to the manufacture's instructions. Proteins were analyzed by sodium dodesyl sulfate (SDS) poly-

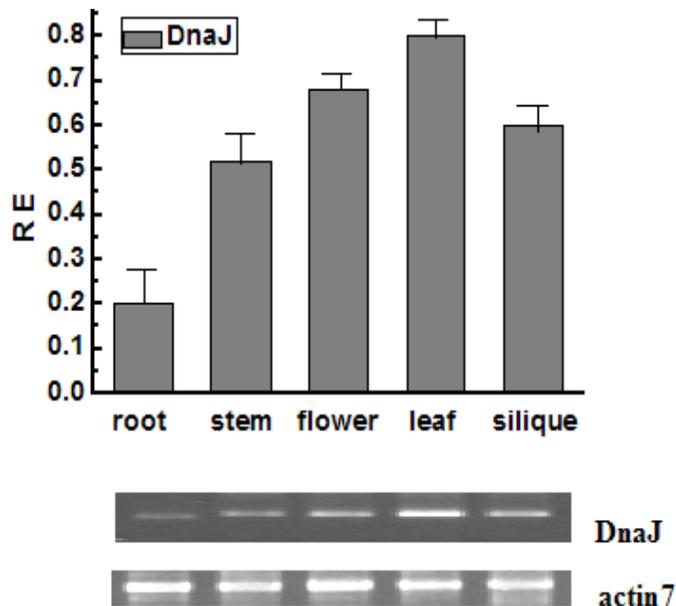


Figure 1. Expression of organellar, DnaJ in different organs by RT-PCR analysis. The ethidium bromide-stained

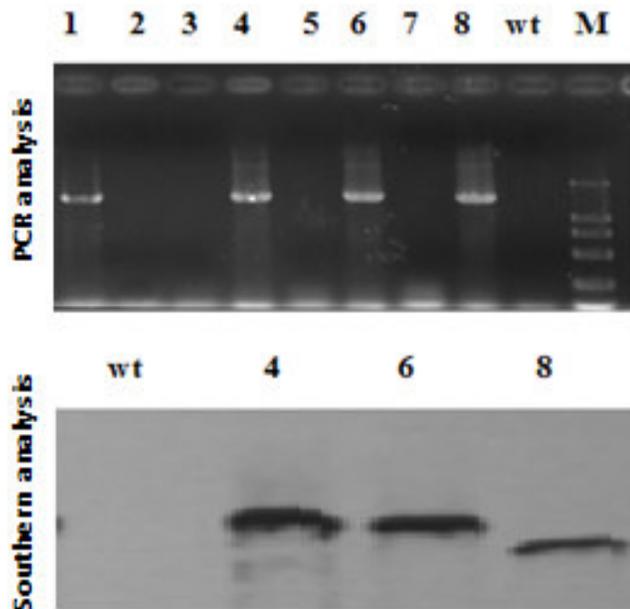


Figure 3. PCR analysis of transgenic plants. Bands corresponding to the DNA were detectable in the transgenic plants (lanes 1 - 8), but were absent from the wild-type plants (wt). Southern blot analysis of transgenic plants. Genomic DNA digested with HindIII, which has no enzyme site in the plasmid. Lanes 4, 6, 8, transgenic plants have DnaJ gene; wt, wild-type plants; M, DNA marker (DL2000). The radiolabelled DnaJ fragments was used as a probe in the hybridization experiments.

acrylamide gel electrophoresis on 12% gel and were transferred to a polyvinylidene difluoride membrane. Histidine (His)-tagged anti-

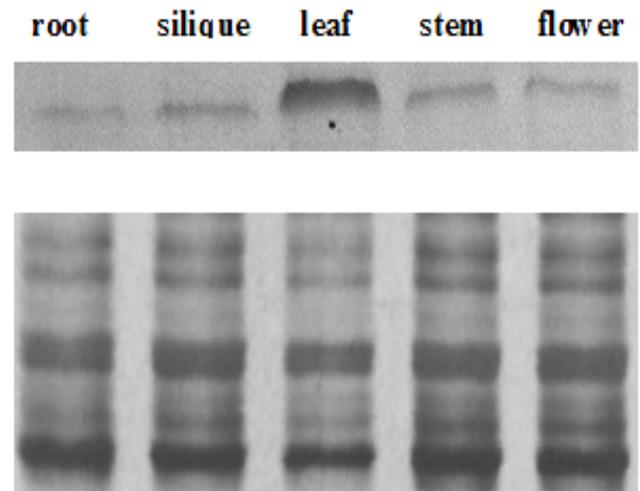


Figure 2. Protein blot analysis for DnaJ in different organs. Wild-type Arabidopsis was grown under normal conditions. Protein from each sample was separated on a 12% SDS-PAGE electrophoresis

bodies at 1:500 dilutions were used for gel blots with chemiluminescent detection to verify the presence of His-DnaJ in the samples. Polypeptide was purified by B-PER™ 6×His Fusion Protein Column Purification Kit (Pierce), after over-expression in bacterial cells. Growth of *E. coli* containing plasmid was carried out using standard procedures. Spot assays were performed by spotting 5-fold serial dilutions of a saturated overnight culture onto the LB media containing 0.5 M NaCl. Plates were put in 37°C and photographed after 12 - 15 h.

RESULTS

DnaJ was differentially expressed in the organs of Arabidopsis. To identify the expression of Arabidopsis, DnaJ was analyzed to determine in particular organs. Independent wild-type lines were screened to evaluate DnaJ levels by RNA and immunoblotting. DnaJ transcripts were abundant in leaf, but small amount detectable in root (Figure 1). DnaJ protein levels of the Arabidopsis leaves were significantly higher than that of other organs (Figure 2). The results showed that expression of DnaJ was higher in the leaves and lower in the roots.

Transgenic plants detection and phenotype analysis

To confirm the putative transgenic plants, PCR and southern blot analysis were performed to investigate the stable integration and number of insertion loci of the DnaJ gene. DnaJ fragments were amplified in these transgenic plants (Figure 3). No amplification of DNA was detected in non-transgenic plants. Genomic DNA was digested with HindIII and the restriction products were loaded onto an agarose gel. The electrophoresis showed that the restriction DNA fragments size ranged from 5 - 10 kb

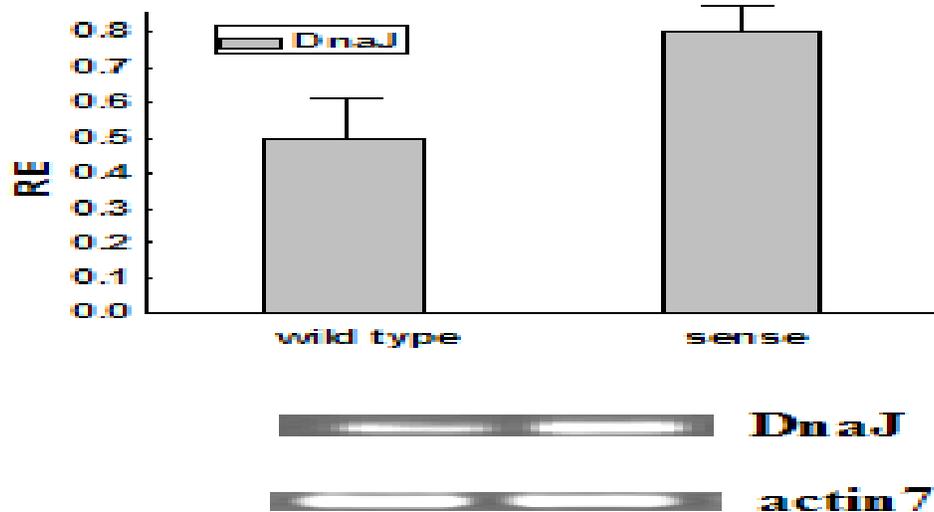


Figure 4. RT-PCR of transgenic and wild-type plants. Bands corresponding to cDNA were detectable in sense transgenic and wild-type plants. Amplification of actin cDNA was used as an internal standard and relative enrichment (RE) values were shown as histogram.



Figure 5. Phenotype of sense transgenic plants. Sense transgenic lines exhibited the more tillers, large size leaves, serrated leaves obviously, less floss of back leaves.

(data not shown). No hybridizing band was detected in non-transgenic plants, a single hybridizing band was detected in line 4, 6, 8 (Figure 3). The results indicated that DnaJ gene was present in the genomic DNA and steady state expression in transgenic plants.

The full-length DnaJ protein of the *Arabidopsis thaliana* is reported in the NCBI database (NP-565533.1); it contains 442 amino acid residues. To create transgenic *Arabidopsis* plants with altered levels of DnaJ expression,

the full-length cDNA sequence derived from the wild-type plants, was placed under the control of the constitutive *cauliflower mosaic virus* 35S promoter. Expression of DnaJ in the sense orientation was higher than that of wild-type (Figure 4). On the same growth conditions, sense transgenic plants had more tillers and less floss of back leaves than wild-type. Leaves show bigger size, faded color, and crenate shape, significantly different from the wild-type plants (Figure 5).



Figure 6. Effects of transgenic and wild-type plants respond to salt. **a)** Plants were grown vertically on a MS plate containing 120 mM NaCl for 7 days. **b)** Average root length of plate grown seedlings was measured. Values were the average \pm SD of three independent experiments. For each experiment, the root length of more than 10 seedlings was measured.

DnaJ was associated with NaCl-stress tolerance in plants

Transgenic and wild-type plants were grown on MS medium containing 120 mM NaCl (Figure 6A). Root growth was measured on vertical plates, root length of sense plants was about 125% of wild-type plants (Figure 6B). Transgenic Arabidopsis plants that over-expressed DnaJ maintained their stress-tolerance phenotype under conditions of NaCl-stress. Under normal, non-stress condition, transgenic and wild-type plants were grown on MS medium and we found that growth of transgenic and wild-type plants had no difference (results not showed). The results given above confirmed that expression of DnaJ had effect on NaCl-stress tolerance.

Effects of NaCl concentrations on growth of *E. coli* and expression of bacterial fusion proteins

The bacterial cells were incubated at different concentrations of NaCl (0, 0.01, 0.1 and 1 M) for 6 h. The bacterial cells were harvested by centrifugation at 6000 g and the pellets were resuspended in the PBS buffer, added 5 volumes loading buffer, boiled for 5 min. Soluble and insoluble fractions were separated by centrifugation at 12000 g, and analyzed by 12% SDS-PAGE. As the concentrations of NaCl increased, the expression of pET32a decreased sharply, while pET32a-DnaJ decreased slowly (Figure 7). The same results were obtained by western blot. The *E. coli* with the plasmids was grown overnight and dilutions speckled onto 0.5 M NaCl plates. The cells containing pET32a-DnaJ could grow on LB containing NaCl, but cells containing pET32a plasmid could not grow. The results indicated that *E. coli* containing DnaJ gene had tolerance of NaCl stress (Figure 8).

DISCUSSION

Heat shock proteins had been testified to play important roles in helping cells to cope with environmental stress. The cytosolic Hsp100 plays major roles in acquired thermo-tolerance (Hong and Vierling, 2000, Hong and Vierling, 2001, Hong et al., 2003; Katiyar et al., 2003). Heat shock protein 101 plays a crucial role in thermo-tolerance in *Arabidopsis* (Christine et al. 2000). In *Saccharomyces cerevisiae*, Hsp42 expression was up-regulated by increases in salt concentration, as well as by conditions of limiting growth and overgrowth of cell cultures. Thus, Hsp42 may play a more important role in the response to increased salt concentration (David et al., 1996). In this study, the expression of DnaJ was examined in different organs. Transcript and protein of DnaJ were abundant in leaves, but small amount detectable in root. Under severe NaCl stress conditions, transgenic sense plants showed better growth than wild-type plants. Transgenic Arabidopsis plants that constitutively over-expressed DnaJ showed increased NaCl tolerance compared with wild-type genotype. It was concluded that DnaJ played an important role in NaCl tolerance in Arabidopsis. The complexity of the plant response to salt stress can be partially explained by the fact that salinity imposes salt toxicity in addition to osmotic stress (Niu et al., 1995; Hasegawa et al., 2000). In plants, exclusion of excess Na^+ from the cytoplasm and the accumulation in the vacuole represents one of the adaptive mechanisms during salt stress. High salt concentrations inhibited the activities of most enzymes because of perturbation of the electrostatic balance between the forces maintaining protein structure (Serrano, 1996). Extracellular enzymes from glycophytes and halophytes had been shown in vitro to be remarkably salt-insensitive tolerating concentrations up to 500 mM

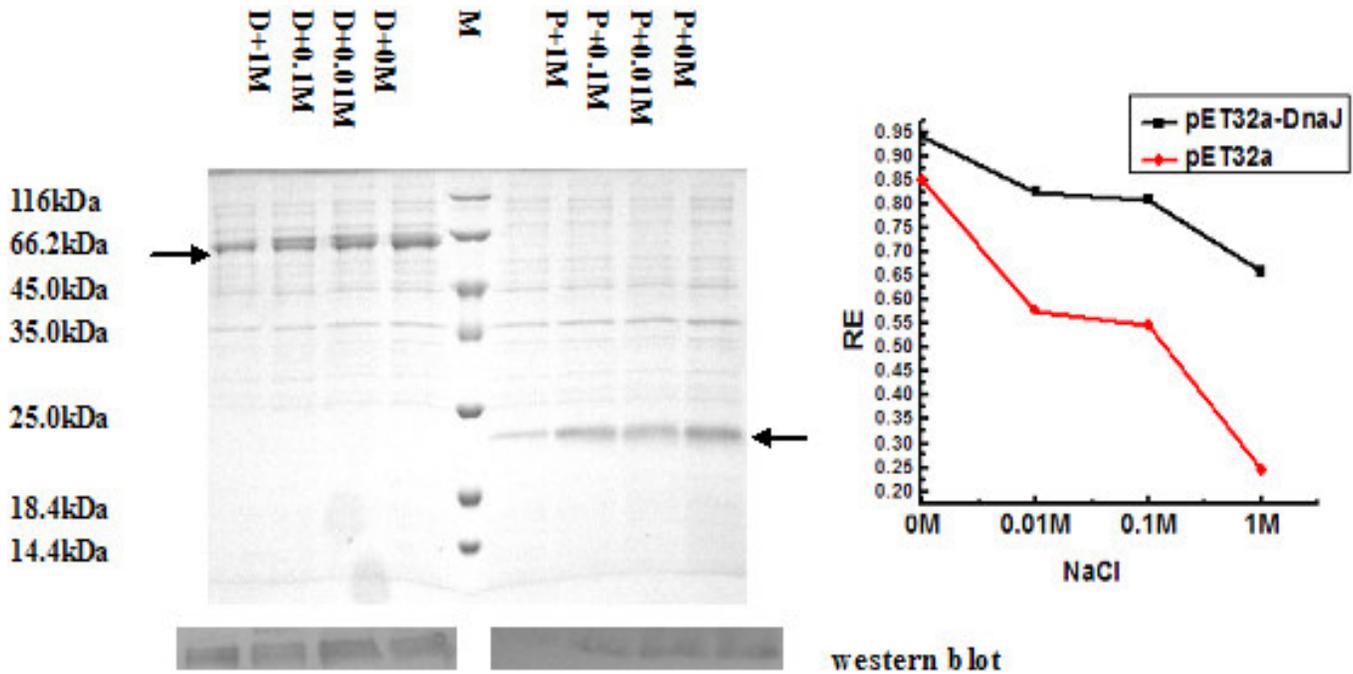


Figure 7. Expression of pET32a-DnaJ (D) and pET32a (P) at the different concentrations of NaCl. Protein gel analysis with 12% SDS-PAGE, total protein from the *E. coli* treated with NaCl. His-tagged antibodies were used for gel blots, relative enrichment values were shown at right, protein molecular weight marker was standard.

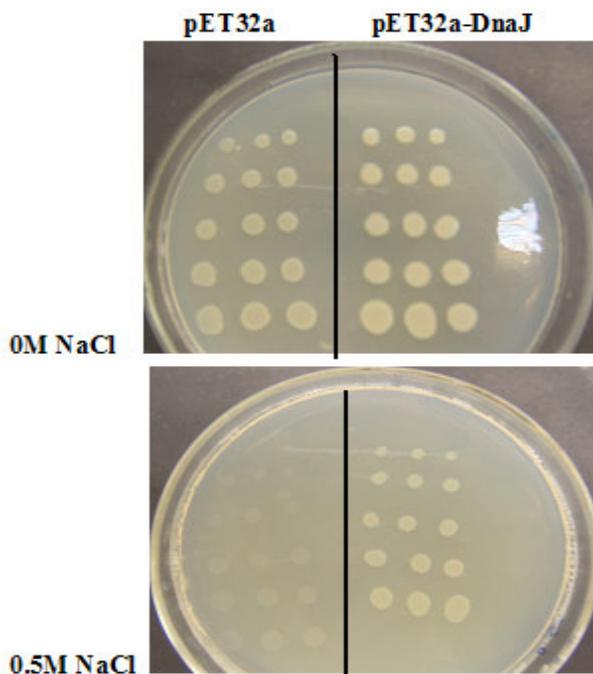


Figure 8. The *E. coli* containing plasmids was grown overnight on LB or LB containing 0.5M NaCl. Plates were photographed after 12 - 15 h.

toxic effects of Na⁺ in the cytosol, considerable progress had been made in understanding ion homeostasis (Hasegawa et al., 2000; Blumwald et al., 2000; Apse and Blumwald, 2002; Zhu, 2003). Ion transporters were considered to play an important role in salt tolerance. The *E. coli* with pET32a-DnaJ plasmid showed tolerance to NaCl. It was possible that DnaJ play a role in the tolerance of salt concentration, under stress condition being due to protein folding, modulation of protein activity and overlapping response mechanisms. In summary, we had shown that over-expression of DnaJ in plants and *E. coli* were tolerance to NaCl. During stress conditions, DnaJ may be some other mechanisms in plants and *E. coli*.

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