http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 17 (5): 619-629, 2014
ISSN 1028-8880 / DOI: 10.3923/pjbs.2014.619.629
© 2014 Asian Network for Scientific Information

Overexpression of *Arabidopsis* Dehydration-Responsive Element-Binding Protein 2A Confers Tolerance to Salinity Stress to Transgenic Canola

[1,2]Alireza Shafeinie, [1]Valiollah Mohammadi, [1]Houshang Alizadeh and [1]Abas Ali Zali
[1]Department of Agronomy and Plant Breeding, College of Agriculture and Natural Resources, University of Tehran, Karaj 31587-77871, Iran
[2]Department of Plant Breeding and Biotechnology, Ramin University of Agriculture and Natural Resources, Ahwaz 6341773637, Iran

Abstract: Stress responsive transcriptional regulation is an adaptive strategy of plants that alleviates the adverse effects of environmental stresses. The ectopic overexpression of Dehydration-Responsive Element Binding transcription factors (*DREBs*) either in homologous or in heterologous plants are the classical transcriptional regulators involved in plant responses to drought, salt and cold stresses. To elucidate the transcriptional mechanism associated with the *DREB2A* gene after removing PEST sequence, which acts as a signal peptide for protein degradation, 34 transgenic T_0 canola plants overexpressing *DREB2A* were developed. The quantitative Real time PCR of transgenic plants showed higher expression of downstream stress-responsive genes including *COR14*, *HSF*3, *HSP70*, *PEROX* and *RD20*. The transgenic plants exhibited enhanced tolerance to salt stress. At the high concentration of NaCl the growth of non-transformed plants had been clearly diminished, whereas transgenic line was survived. These results indicated that transformed *DREB2A* gene might improve the plant response to salinity in transgenic canola plants.

Key words: *DREB2A*, transcription factor, transgenics, overexpression, salinity stress, canola

INTRODUCTION

Various environmental stresses, such as salinity, drought, flooding and freezing cause metabolic toxicity, membrane disorganization, closure of stomata, decreased photosynthetic activity, generation of Reactive Oxygen Species (ROS) and altered nutrient acquisition (Mittler, 2006; Salavati *et al*., 2012a, b). These stresses, subsequently, have adverse effects on plant growth and crop yields. Plants have developed a complex and sophisticated signalling network that ensures their adaptation to continuously changing surroundings (Moller and Sweetlove, 2010). To meet the increasing demands for agricultural commodities it would be urgent to redesigning of crops to cope with environmental stresses. Accordingly, a great deal of effort has been invested in perception the molecular background of stress adaptation by which plants recognize and transfer adverse environmental stimuli to trigger adaptive responses (Xiong *et al*., 2002). In addition, great deal of effort has been invested in creating improved plants that show increased tolerance (Wang *et al*., 2003). Adaptation mechanisms such as re-establishment of cellular homeostasis and mitigating the effects of stress-induced toxic metabolites (Pastore *et al*., 2007), have polygenic nature. Breeding for stress tolerance using traditional methods has been limitedly successful (Cattivelli *et al*., 2008), which has prompted interest in transgenic strategies.

The activation of stress signalling pathways in plants begins with the perception of extra-cellular signals at molecular level, followed by the generation of secondary signalling molecules, and the induction or activation of transcription factors (TFs) (Bartels and Sunkar, 2005). TFs activation allows the plant to grow and propagate under these deteriorate conditions, especially, high-salt concentration, which cause to disturbance of cellular protein homeostasis (Rae *et al*., 2011). Regulation in transcription level is one of the most important steps for plant adaptation to stress conditions such as salinity. Transcription factors respond to stress signals, regulate the expression of many stress-related downstream genes, by specifically binding to cis-acting elements in the promoters of target genes. TFs which are related to stresses are categorized into several families, such as AP2/EREBP, bZIP, NAC, MYB, MYC, Cys2His2

Corresponding Author: Alireza Shafeinie, Department of Agronomy and Plant Breeding, College of Agriculture and Natural Resources, University of Tehran, Karaj 31587-77871, Iran
Tel: +98 916 612 4161 Fax: +98 612 322 4338

zinc-finger and WRKY (Umezawa *et al*., 2010). Likewise, many genes conferring stress tolerance are induced under stress conditions.

Plant stress responses have been classified to abscisic acid (ABA)-dependent and -independent regulatory pathways (Shinozaki and Yamaguchi-Shinozaki, 1997, 2000). The ABA-dependent TFs particularly bind to ABA-responsive (ABRE) core cis-acting sequences in the promoters and trigger the ABA-dependent plant stress response (Shinozaki and Yamaguchi-Shinozaki, 2000). In contrast, the AP2/EREBP super-family of TFs bind to Dehydration-responsive element/C-repeat (DRE/CRT) cis-element and trigger the expression of stress-related genes in an ABA-independent procedure (Riechmann *et al*., 2000). AP2/EREBP family is divided into several subfamilies such as ethylene responsive factor (ERF), APETLA2 (AP2), RAV and Dehydration responsive element binding factor (DREBs) confer stress endurance in plants and are the largest and most diverse family of proteins involved in the regulation of plant responses. These proteins, based on their involvement in signal transduction pathways under low temperature and dehydration, have been divided into two classes including DREB1 and DREB2, respectively (Agarwal *et al*., 2007). As DREBs induce several abiotic stress responsive genes and maintain water balance in plant system, they are appropriate candidates for generating transgenic plant with increased tolerance to drought, high salt, high temperate and cold stresses (Sakuma *et al*., 2006).

Dehydration-Responsive Element-Binding Protein 2a (DREB2A) initially was identified in a yeast one-hybrid screen using the *CaMV35s* promoter as bait (Liu *et al*., 1998). *DREB2* genes, then, were recognized as drought, heat and salinity response genes (Liu *et al*., 1998; Lim *et al*., 2007; Sakuma *et al*., 2006). Recent studies suggested that DREB2s may function as multi stimuli-response factor that interact with genes and/or transcription factors, including members of the ABA-responsive element binding protein (AREB)/ABRE binding factor (ABF) family, in downstream gene regulation, as well as proteins during different stress conditions (Lee *et al*., 2009). However, overexpression of a constitutively active form of DREB2A that lacks the negative regulatory domain led to increase stress tolerance in transgenic plants (Sakuma *et al*., 2006; Lee *et al*., 2010; Elfving *et al*., 2011). Domain analysis of DREB2A protein showed that its C-terminal region functioned a s a transcriptional activation domain and that its middle region functioned as a negative regulatory d omain in the regulation of DREB2A activity. Deletion of the negative regulatory domain resulted in the produce of a constitutive active domain of DREB2A (Sakuma *et al*., 2006).

In this study, transgenic canola plants overexpressing *DREB2A* under a *CaM35s* promoter from *Arabidopsis* were developed to reveal its role in stress adaptation. Genes downstream of *DREB2A* using transgenic plants overexpressing the constitutive active form of *DREB2A* were analuzed and many salt-inducible genes lying downstream of *DREB2A* were identified. In addition, the stress tolerance of the transgenic plants and found improvement of salt stress tolerance were analyzed. These results indicate that transformed *DREB2A* gene into canola might improve the plant response to salt stress.

MATERIALS AND METHODS

Deletion of PEST sequence and Vector construction: pBluescript plasmid carrying *DREB2A* gene with accession number AB007790 (Fig. 1) received from RIKEN bioresource centre (Japan) was introduced into *E.coli* JM109. The region related to complete coding DNA sequence (cds) between nucleotides 405 and 495 was deleted using SOEing PCR (Sakuma *et al*., 2006). The SOEing PCR method uses 2 pairs oligonucleotide primers (Table 1) with two rounds of PCR. In the first PCR, to delete negative regulatory domain, first and second primer pairs were used to delete the brackets of the upstream and downstream of region, respectively. Subsequently in the second PCR, forward primer from first pair and reveres primer from second pair conjunct these two segments to produce a longer segment without the Ser/Thr-rich region. The PCR rounds were performed for 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 1.5 min. The generated products were purified and ligated into pGEM-T easy vector (Promega, Madison, WI, USA) for sequencing to verify the deletion. The new segment of *DREB2A* without the Ser/Thr-rich coding region was named *DREB2A-CA*. This modified *DREB2A* was cloned into the pBI121 binary vector, therefore chimeric construct comprised of *DREB2A* open reading sequence fused in sense orientation downstream of *CaMV35s* promoter and upstream to *nos* terminator (Fig. 2). Finally, the place and direction of *DREB2A* gene in plasmid was confirmed by restriction cutting with *Eco*R I and *Hin*D III, *Xho* I.

Transformation procedure and plantlet formation: The recombinant vector pBI121(CaMV35s -*DREB2A*- *nos* terminator) was mobilized into *Agrobacterium tumefaciens* (LBA4404) by freeze-thawing. Transformation of canola was carried using 7-10 day-old cut surfaces hypocotyl of *B. napus* cv. PF7045/91.

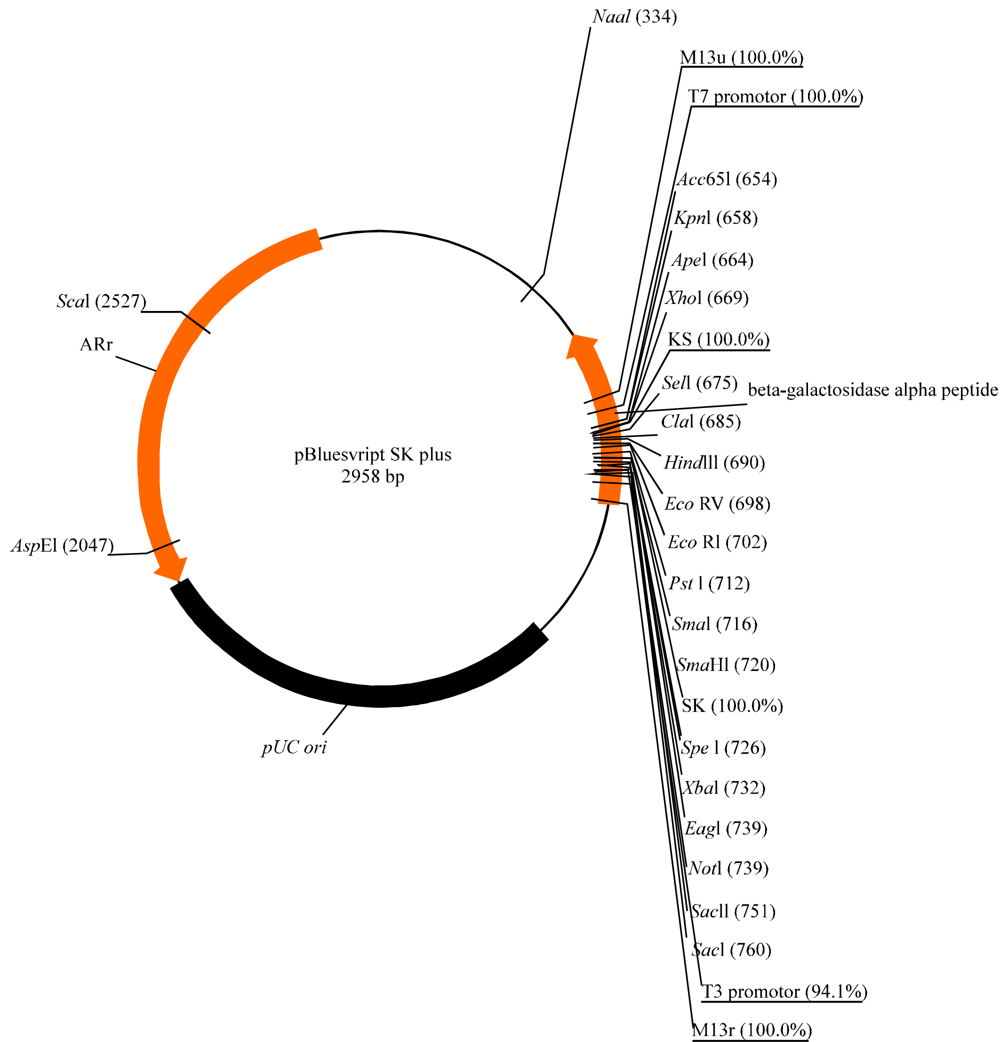

Fig. 1: Location of *DREB2A* gene in the pBluescript SK plasmid

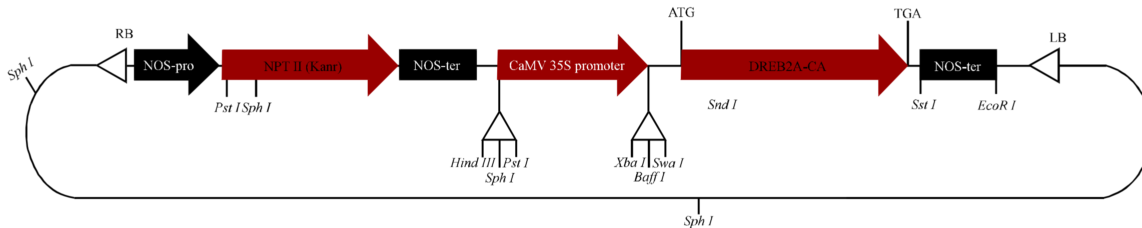

Fig. 2: Location of *DREB2A-CA* gene in the pBI121 plasmid

Hypocotyl explants were plated on pre-culture medium (Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 30 g L^{-1} sucrose supplemented with 100 mg L^{-1} myoinositol, 7-8 g L^{-1} 2,4-D) for 72 h at 25°C under a 16-h photoperiod. Hypocotyl explants were inoculated with the *A. tumefuciens* suspension supplemented with 0.2 mM acetosyringone, in 50 mL tubes for 30 sec with gentle shaking. The explants were then blotted dry on sterile filter paper for 3 min to remove excess solution before they were transferred to fortified co-cultivation medium (MS containing 20 g L^{-1} sucrose supplemented with

Table 1: Designed primers to this experiment

Sequences	Primers	Description
a. SOEing designed primers		
5′ AGA ATT CGT GTT TTT CTG GGA AGG GAG A 3′	AFF1	First Pair
EcoR I[a)]		
5′ AGA GCT CAG TCG TTG TGG GAT TAA GGC 3′	ARR1	
Sac I[b)]		
5′ TAA TTT CCC TGA TCC AGA TTG TGA ATC TAA ACC CTT C 3′	AFF2	Second Pair
c[d)] a[c)]		
5′ CAA TCT GGA TCA GGG AAA TTA AGA CGA GCC AAA GGA 3′	ARR2	
d[f)] b[e)]		
b. PCR designed primers		
5′ CGC ACA ATC CCA CTA TCC TT 3′	CaM-DRE	Forwards
5′ TCT CAG CAA CCC ATT TAC CC 3′	CaM-DRE	Reverses
c. RT-PCR designed primers		
5′ GGC TCT CGA CTA CGA GCA AG 3′	Forwards	*BnACT2*[g)]
5′ GGA ATC TCT CAG CTC CGA TG 3′	Reverses	
5′ AAA TGG CGA CGA TGT GTT TGC AGG 3′	Forwards	DREB2A
5′ AAA CCA CTT TGT TGA CTC TCG GGC 3′	Reverses	
5′ GTT GGA TGC GAG ACA GAT AGT G 3′	Forwards	HSF
5′ CCC TCA CCT TCA GGA GAA ATT G 3′	Reverses	
5′ TAC AAC ATG AGG AAC ACC ATC C 3′	Forwards	HSP70
5′ ACT GAA TCG CCT GCT CAA TAG 3′	Reverses	
5′ ACA GAC ACA ATC CCC TTG GTG 3′	Forwards	PROX
5′ AGG TGT AAT GCT GCC CAT CC 3′	Reverses	
5′ GAT TCG AGC ACC TAT GAC ACC GA 3′	Forwards	RD20
5′ ATT GCC ATT CGA TTT CCC TCG GT 3′	Reverses	
5′ GCC AGA AAA GGT GAT GGA AAC 3′	Forwards	Cor14
5′ ACG CCT CCT TTG TCT TGT CTG 3′	Reverses	

[a]cutting site to *EcoR I* enzyme. [b]cutting site to *Sac I* enzyme. [c]segment a is complement with segment d. [d]segment c is complement with segment b. [e]segment b is complement with segment c. [f]segment d is complement with segment a. [g]*BnACT2* was used as a standard control gene in RT-PCR experiment (Reference gene)

100 mg L^{-1} myoinositol, 7 g L^{-1} agar) and incubated for 2 days at 25°C under dark condition. After co-cultivation, the explants were transferred onto callus induction medium (MS containing 30 g L^{-1} sucrose augmented with 100 mg L^{-1} myoinositol, 6-8 g L^{-1} agar, 1.0 mg L^{-1} 2,4-D, 500 mg L^{-1} cefotaxime) for 7 days at 25°C under a 16-h photoperiod and then transferred to selective medium (MS containing 30 g L^{-1} sucrose augmented with 100 mg L^{-1} myoinositol, 6-8 g L^{-1} agar, 4.5 mg L^{-1} Benzlaminopurine (BAP), 0.3 mg L^{-1} Thidiazuron (TDZ), 250 mg L^{-1} cefotaxime, 10-20 mg L^{-1} kanamycin and 3 mg L^{-1} silver nitrate) for the induction of transgenic callus. As a control, uninfected explants were cultured on selective medium. After 3 weeks of culture, hypocotyl calli were transferred to shoot induction medium (MS containing 10 g L^{-1} sucrose supplemented with 100 mg L^{-1} myoinositol, 6-8 g L^{-1} agar, 200 mg L^{-1} cefotaxime and 25 mg L^{-1} kanamycin) for 4 weeks. Elongated shoots were transferred to rooting medium (MS media supplemented with 10 g L^{-1} sucrose, 100 mg L^{-1} myoinositol, 6-8 g L^{-1} agar, 150 mg L^{-1} cefotaxime and 25 mg L^{-1} kanamycin). Rooted plantlets were washed in sterile distilled water to remove traces of medium and were transferred to plastic pots (5 cm diameter) containing a mixture of sterile soil, perlite and vermiculite (3:1:1). After 3 weeks, the plants were transferred to pots containing soil and grown in a greenhouse. Putative transgenic lines that showed vigorous growth on kanamycin media and their self-pollinated heterozygous progenies (T_1) were used for the experiments described below.

PCR analysis: PCR was carried out in a total volume of 20 μL containing 200 ng genomic DNA, 200 μM dNTPs, 2.5 U *Taq* DNA polymerase, *Taq* buffer and 10 pmol each of forward and reverse primers, which were designed within the *CaMV35s* promoter and the *DREB2A* gene, respectively (Table 1). The PCR conditions were 94°C, 1 min; 58°C, 1 min and 72°C, 1.5 min for 30 cycles. Integration of T-DNA was analyzed by PCR amplification of the *DREB2A* transgenic cassette in the genome of both T_0 and T_1 canola plants.

Quantitative real-time PCR analysis: Total RNA was isolated and purified from canola leaves according to the protocol of the RNeasy plant mini kit (Qiagen, Valencia, CA, USA). cDNA was synthesized from total RNA using QuantiTect Revers Transcriptoin synthesis kit (Qiagen) and expression of the selected genes were analyzed by Real-Time PCR (RT-PCR) using the fluorescent intercalating dye SYBRGreen with a detection system (Step One Plus, Real-Time PCR System, Applied Biosystem, USA). Real-Time PCR reaction was performed using Real-Time PCR Master Mix (Applied Biosystem)

according to the manufacturer's instructions, with gene-specific primers (Table 1). The cycle threshold (Ct), as the PCR cycle at which a statistically significant increase of reporter fluorescence is first detected, was measured for two biological and two technical repeats for each sample. Relative quantity of the target gene expression level was performed using the comparative Ct method (Pfaffl, 2001). The relative value for the expression level of each target gene was calculated by the equation $R = ((E_{target})^{\Delta Ct}{}_{target})/((E_{ref})^{\Delta Ct}{}_{ref})$ which ΔCt_{target} is (Mean control–Mean sample) for target gene and ΔCt_{tef} is (Mean control–Mean sample) for *BnACT2*, as a standard control.

Evaluation of transgenic T_1 canola lines under salt stress: Control and transgenic T_1 lines were hardened and transferred to pots at 22°C with 70% humidity in a green house under white fluorescent light (600 μ mol m^{-2} s^{-1}, 12 h light period/day) supplemented with NaCl (150 mM) for 14 days. Biomass of control and transgenic plants was, then, measured under salt stress.

Statistical analyses: The statistical significance of the results was evaluated with the Student's t-test when only two means were compared or with two-way ANOVA followed by Duncan's multiple comparisons test otherwise. Data analyses and graphical representations were performed using Microsoft Office Excel 2007 or R, a language and environment for statistical computing and graphics (Ihaka and Gentleman, 1996).

RESULTS

Developing DREB2A transgenic canola plants: To analyze the function of *DREB2A* in canola plants, *DREB2A-CA*, *DREB2A* 1-135:166-335 which presented the maximum activity in the transgenic plants (Nakashima *et al*., 2009), were over expressed in canola plants. This constitutive active form of *DREB2A* was over expressed in canola under the control of the cauliflower mosaic virus *35S* promoter. The *CaMV35s*::*DREB2A* expression cassette was cloned into pBI121 binary vector for *Agrobacterium*-mediated transformation of *B. napus* cv. PF7045/91 using hypocotyl derived calli. Approx. 50 calli were transferred to the selection medium after co-cultivation with *Agrobacterium tumefaciens* the DREB2A cassette and about 30 kanamycin resistant calli were obtained after two rounds of selection on 25 mg kanamycin/l supplemented medium. Resistant calli were sequentially transferred to regeneration and rooting medium for formation of shoots and roots, respectively. Thirty four plants were regenerated and transferred to pots with soil and maintained under controlled greenhouse conditions. Out of 34 putative T_0 transgenic lines, which were hardened and transferred to greenhouse, 4 putative transgenic lines showing vigorous growth on kanamycin media and their self-pollinated heterozygous progenies (T_1) were selected for the experiments described below.

Molecular characterization of transgenic canola lines: To confirm the foreign gene integration in putative transgenic canola plants, PCR amplification was performed using forward primer from *CaMV35s* promoter and reverse primer from *DREB2A* gene, i.e., CaM-DRE (Table 1). Transformation experiments generated 34 independently regenerated T_0 plants. Thirty four putative T_0 transgenic lines have been regenerated from MS medium supplemented with kanamycin. These seedlings transferred to pots and were used for PCR analysis. Amongst these 34 transformed lines, 4 lines were showed the expected amplification of 518 bp DNA fragment from the integrated *CaMV35s*::*DREB2A* gene cassette (Fig. 3a). Therefore, these lines were chosen for future analysis and T_1 seed production. No such amplification was observed in non-transformed control. Meanwhile, to determine the expression of the *DREB2A-CA* in the transformed canola plants, six T_1 seedlings were randomly selected of each T_0 plants and PCR amplification was used on selected T_1 plants. Template DNA from all selected transgenic plants allowed the amplification of expected 518 bp fragment (Fig. 3b). Therefore it was concluded that the *CaMV35s*::*DREB2A* gene cassette was successfully located into the transgenic T_1 canola plants.

Morphological analysis of T_1 plants under salt stress: Overexpression of *DREB2A* under the *CaMV35s* promoter did not show any phenotypic abnormities either in stress or non-stress growth conditions in these transgenic lines. The growth performances of transgenic seedlings in terms of shoot were significantly higher for transgenic seedlings over the control seedlings during salt stresses (Fig. 4). Comparatively, transgenic plants grew healthily, whereas the surviving control seedlings were dwarf and developed pale green leaves during salt stress. The *DREB2A* transgenic T_1 canola plants exhibited superior growth performance in comparison to control and transgenic overexpression of *DREB2A* in canola conferred stress tolerance. Among transgenic lines studied, T1 plants originated from T0 KCA2 plant showed the highest performance and tolerance to salt stress. Therefore this line was used for further analysis. Furthermore, to assess the impact on biomass production the fresh weight of plants was determined two weeks after salt stress. As

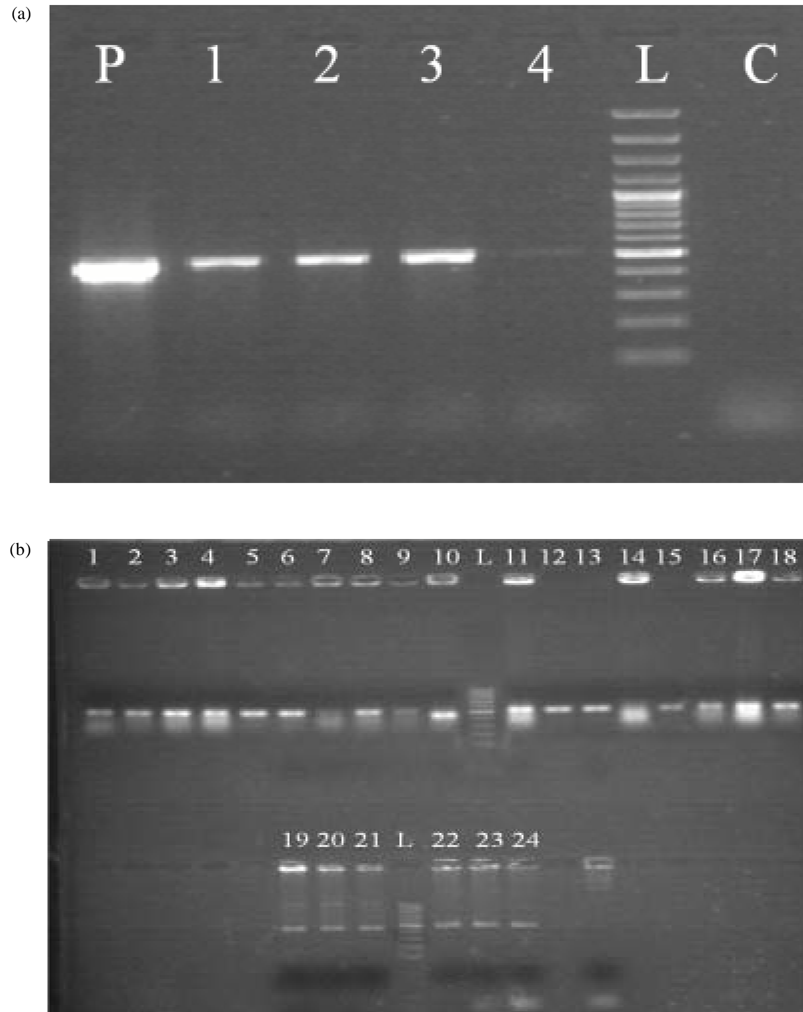

Fig. 3(a-b): PCR amplification results for T_0 generation of *DERB2A* transgenic canola. 1-4: transgenic T_0 plant with CaM-DRE pimers; P: Plasmid pBI121 with CaM-DRE pimers; L: GeneRuler™ 100 bp DNA Ladder (Fermentas); C: negative control with CaM-DRE pimers and (b) PCR amplification results of 24 randomly selected T_1 seedlings from four T_0 plants. 1-24: transgenic T_1 plant with CaM-DRE pimers; L: GeneRuler™ 100 bp DNA Ladder (Fermentas)

shown in Fig. 5 there were significant differences in biomass between non-transgenic and transgenic plants.

Functional analysis of DREB2A transgenic canola plants: To determine qualitatively whether or not the integrated and intact *DREB2A* transgenes were transcribed, and the expression of the transgene in the transformed plants, RT-PCR was used. Template cDNA from all of the transgenic plants examined allowed the expression of *DREB2A*, whereas RT-PCR of the untransformed control did not. Therefore, it was concluded that the *CaMV35s*::*DREB2A* gene cassette was successfully located into the transgenic canola plants. The constant expression of transgene in transformed canola plant, even in non stress condition, is due to locating the *DREB2A* gene in downstream of the constant promoter so RT-PCR was uses for additional confirmation and overexpression of *DREB2A* in T_1 generations, was recorded, more than 10000 times (Table 2, Fig. 6). To understand the entire transcriptional network of *DREB2A* in *B. napus* transgenic plants, some *DREB2A-CA* (*35S*::*DREB2A-CA*) downstream genes, which their remarkable expression ratios under abiotic stress had been previously described, were studied. These downstream genes were included heat shock factor (Zou *et al*., 1998), heat shock protein

Table 2: RT-PCR results

	Ct means		
Genes	Control	Sample	Expression ratios [f]
RD20[a]	23.785	22.951	1.63**
HSF3[b]	30.612	26.553	14.218**
HSP70[c]	27.896	25.434	4.669**
COR14[d]	27.258	35.920	2.254**
PEROX[e]	29.871	26.868	7.333**
DREB2A	37.295	23.622	11943.454**

[a]RD20 Ca^{2+}-binding protein, [b]Heat shock factor, [c]Heat shock protein, [d]Cyclic nucleotide-gated channel, [e]Peroxidase, [f]Ratio of mRNA in transgenic plant, to control plants; ** indicates significant at p = 0.01

Fig. 4(a-e): Transgenic lines and controls in early stage under salt stress. (a) KCA3 transgenic T_1 line, (b) KCA1 transgenic T_1 line, (c) Control line under stress treatment, (d) KCA2 transgenic T_1 line and (e) Control line in non stress situation

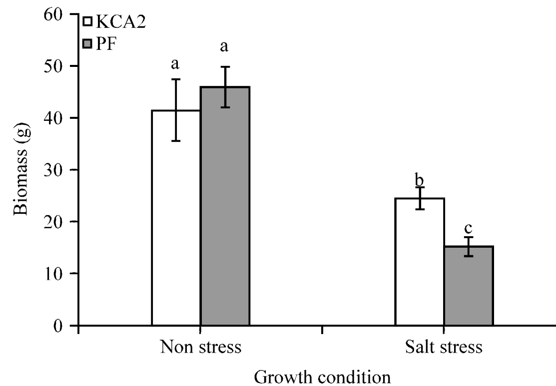

Fig. 5: Biomass of four-weeks-old transgenic and non-transgenic canola under stress and non stress conditions. Each value represents the mean±SE. The means were compared using the Duncan multiple range test. Letters above columns indicate a statistically significant difference ($p<0.05$). Standard errors are denoted by error bars

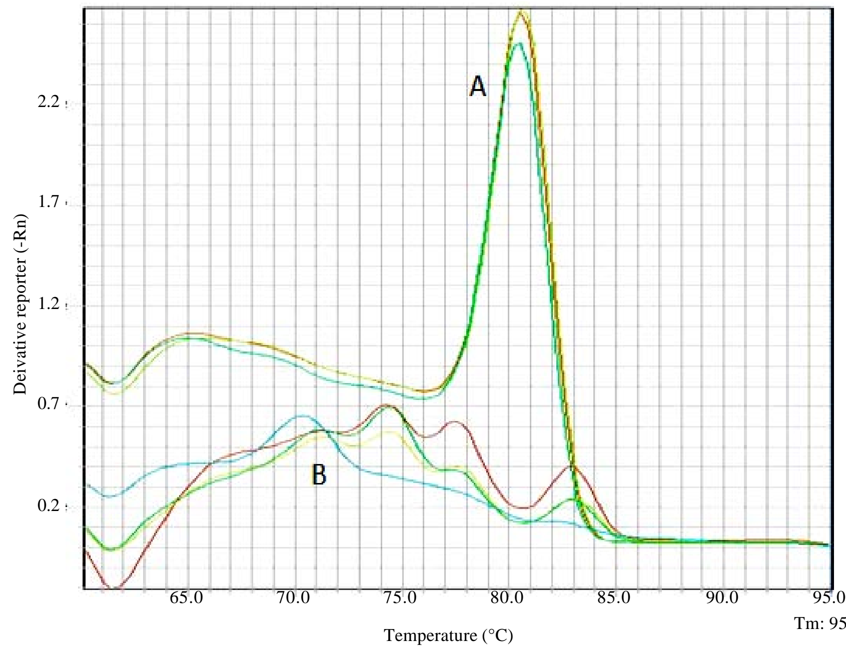

Fig. 6: Melt curve of amplified fragments in Real-Time PCR, A: Transgenic line and B: Non-transgenic line

(Wang *et al*., 2004), RD20 Ca^{2+}-binding protein (Takahashi *et al*., 2000), cold regulated protein 14, COR14, (Crosatti *et al*., 1995) and peroxidase (Zhu *et al*., 2001). The canola homologue of these genes from NCBI were selected and primers were designed (Table 1). Up-regulation of these genes was confirmed by quantitative RT-PCR (Table 2).

DISCUSSION

Regulatory effects of the cis-acting promoter element DRE, containing the core sequence A/GCCGAC, has been identified in response to drought, salt and cold stresses in *Arabidopsis* (Sakuma *et al*., 2006). Although, the function of *DREB2A* under these stresses especially in *Arabidopsis* has been studied (Liu *et al*., 1998; Desikan *et al*., 2001 and Schramm *et al*., 2008), the effect of *DREB2A* in abiotic stress in other plants such as canola has not been previously well evaluated. The overexpression of *DREB1A* in transgenic *Arabidopsis* induced expression of many downstream stress-responsive genes under unstressed conditions and led to enhancing freezing and dehydration tolerance (Liu *et al*., 1998). However, It is suggested that the presence of negative regulatory domain in the central region (136–165 aa) has an inhibitory effect in the normal condition and is modified under salt/drought stress. Deletion of this region transforms *DREB2A* to a constitutive active form (Sakuma *et al*., 2006), which results in more stable transcription factor than the full-length DREB2A protein in the nucleus of transgenic plants. Therefore, in current study, to investigate the role of *DREB2A* in transformed *B. napus* under salt stress, canola plants overexpressing *DREB2A-CA* transgene driven by a *CaMV35s* promoter were developed. Thirty four transgenic canola lines were regenerated under antibiotic supplemented medium, out of 34 regenerated seedlings. Four transgenic seedlings were confirmed by PCR analysis, survived and grew healthily during salt stress conditions (150 mM NaCl), in comparison to their corresponding control seedlings (Fig. 3). During salt stress, control plants showed wilting while transgenic lines grew without any visual wilting symptoms. Performance of *DREB2A* transgenic canola plants was relatively superior in terms of several phenotypic growth parameters in comparison to their respective non-transgenic control during stress condition (Fig. 4). Taken together, these results indicate that transgenic overexpression of *DREB2A* in canola positively correlates with stress tolerance.

The constant promoter in upstream of target gene caused to high-level persistent transgene expression even in non-stress condition, 10000 times higher than non transformed plants. On the other hand, the absence of *CaMV35s*::*DREB2A* gene cassette in genetic background of wild type plants naturally resulted in no expression in

non transformed plant. Therefore, the successful transformation of *DREB2A* gene into canola plant was confirmed by RT-PCR results as well as PCR results.

Several genes have been identified as functional components in the plant response to salt stress such as detoxifying enzymes like glutathione peroxidase (Roxas *et al*., 2000) and ion accumulation (Parida and Das, 2005). In this study, to analyze whether this transgene overexpression induces tolerance to salt stress in transgenic canola plants, the relative transcript levels of several downstream salt-stress responsive target genes were monitored. The downstream genes including peroxidise, heat shock proteins, heat shock factor, cold regulated protein 14 and RD20 Ca^{2+}-binding protein were analyzed by RT-PCR in both transgenic and corresponding non-transgenic control canola seedlings, in T_1 transgenic (Table 2).

Most tolerant genotypes to salinity have been to date reported the significantly increased abundance of peroxidase for example in tomato (Mittova *et al*., 2004), *Arabidopsis* (Jiang *et al*., 2007), potato (Aghaei *et al*., 2008) and rice (Yan *et al*., 2005). Most of the transgenic improvements in salt tolerance plants have been achieved through detoxification strategy (Zhu, 2001) and also regulation of the ROS by scavenging them with antioxidant enzymes (Askari *et al*., 2006; Bhushan *et al*., 2007). This is more obvious in the case of transgenic plants overexpressing enzymes involved in oxidative protection, such as glutathione peroxidase (Roxas *et al*., 2000), superoxide dismutase (Meloni *et al*., 2003), ascorbate peroxidases (Badawi *et al*., 2004) and glutathione reductases (Pilon-Smits *et al*., 2000). In this study, expression of peroxidase was highly upregulated in transgenics in comparison with non transformed plants, more than 7 times suggesting that the expression of peroxidase was due to expression of DREB2A transcription factor in transgenic canola.

Differential regulation for genes encoding carriers and channels involved in transporting ions and organic substrates were reported in tolerant crop genotypes. Cold-regulated genes had been reported to differentially express in freezing-sensitive and -tolerance plants under both laboratory and field conditions. Accumulation of encoded proteins by cold-regulated genes implies a direct interaction between the cold regulated gene expression coming from cold induced signaling pathway and redox situation of the chloroplast (Rapacz *et al*., 2008). The *RD20* gene, which encodes a Ca^{2+}-binding protein is induced by dehydration (Takahashi *et al*., 2000), salt stress (Liu *et al*., 2007), and ABA treatment (Lee and Chun, 1998). In current study, upregulation of Cold-regulated gene and *RD20* in transformed plant at transcript level were recorded in comparison to wild type plants, more than 3 times indicating that overexpression of *DREB2A* induces expression of the salt response downstream genes under even nonstressed conditions.

CONCLUSION

In order to investigate the effect of DREB2A-CA stress-inducible transcription factor on plant growth, its relationship to biomass production and expression of some down stream salt stress responses genes under salinity stress, T_1 generation originated from T_0 KCA2 plant that showed the highest performance and tolerance to salt stress were used for complementary analysis. Functional analysis showed that of *DREB2A*-CA gene enhances salt tolerance in canola transgenics and up-regulates downstream salt-responsive genes not only under osmotic stress but also under non stress condition, due to constant promoter in up-stream of gene. Thus, *DREB2A* could be considered as an important transcription factor that could be use to confer salt stress tolerance in canola plants.

ACKNOWLEDGMENTS

The authors are grateful to scholarship section of the Ministry of Science, Research and Technology of I. R. Iran and the Graduate Studies Office of University of Tehran.

REFERENCES

Agarwal, P., P.K. Agarwal, S. Nair, S.K. Sopory and M.K. Reddy, 2007. Stress-inducible DREB2A transcription factor from *Pennisetum glaucum* is a phosphoprotein and its phosphorylation negatively regulates its DNA-binding activity. Mol. Genet. Genomics, 277: 189-198.

Aghaei, K., A.A. Ehsanpour and S. Komatsu, 2008. Proteome analysis of potato under salt stress. J. Proteome Res., 7: 4858-4868.

Askari, H., J. Edqvist, M. Hajheidari, M. Kafi and G.H. Salekdeh, 2006. Effects of salinity levels on proteome of Suaeda aegyptiaca leaves. Proteomics, 6: 2542-2554.

Badawi, G.H., N. Kawano, Y. Yamauchi, E. Shimada, R. Sasaki, A. Kubo and K. Tanaka, 2004. Over-expression of ascorbate peroxidase in tobacco chloroplasts enhances the tolerance to salt stress and water deficit. Physiol. Plant., 12: 231-238.

Bartels, D. and R. Sunkar, 2005. Drought and salt tolerance in plants. Crit. Rev. Plant Sci., 24: 23-58.

Bhushan, D., A. Pandey, M.K. Choudhary, A. Datta, S. Chakraborty and N. Chakraborty, 2007. Comparative proteomics analysis of differentially expressed proteins in chickpea extracellular matrix during dehydration stress. Mol. Cell. Proteomics, 6: 1868-1884.

Cattivelli, L., F. Rizza, F.W. Badeck, E. Mazzucotelli and A.M. Mastrangelo *et al*., 2008. Drought tolerance improvement in crop plants: An integrated view from breeding to genomics. Field Crops Res., 105: 1-14.

Crosatti, C., C. Soncini, A.M. Stanca and L. Cattivelli, 1995. The accumulation of a cold-regulated chloroplastic protein is lighdependent. Planta, 196: 458-463.

Desikan, R., A.H. Mackerness, J.T. Hancock and S.J. Neill, 2001. Regulation of the *Arabidopsis* transcriptosome by oxidative stress. Plant Physiol., 127: 159-172.

Elfving, N., C. Davoine, R. Benlloch, J. Blomberg and K. Brannstrom *et al*., 2011. The *Arabidopsis thaliana* Med25 mediator subunit integrates environmental cues to control plant development. Proc. Natl. Acad. Sci. USA., 108: 8245-8250.

Ihaka, R. and R. Gentleman, 1996. R: A language for data analysis and graphics. J. Comput. Graphical Stat., 5: 299-314.

Jiang, Y., B. Yang, N.S. Harris and M.K. Deyholos, 2007. Comparative proteomic analysis of NaCl stress-responsive proteins in Arabidopsis roots. J. Exp. Bot., 58: 3591-3607.

Lee, K., K.S. Han, Y.S. Kwon, J.H. Lee and S.H. Kim *et al*., 2009. Identification of potential DREB2C targets in *Arabidopsis thaliana* plants overexpressing DREB2C using proteomic analysis. Mol. Cell., 28: 383-388.

Lee, S.J., J.Y. Kang, H.J. Park, M.D. Kim, M.S. Bae, H.I. Choi and S.Y. Kim, 2010. DREB2C interacts with ABF2, a bZIP protein regulating abscisic acid-responsive gene expression, and its overexpression affects abscisic acid sensitivity. Plant Physiol., 153: 716-727.

Lee, Y.H. and J.Y. Chun, 1998. A new homeodomain-leucine zipper gene from *Arabidopsis thaliana* induced by water stress and abscisic acid treatment. Plant Mol. Biol., 37: 377-384.

Lim, C.J., J.E. Hwang, H. Chen, J.K. Hong and K.A. Yang *et al*., 2007. Over-expression of the *Arabidopsis* DRE/CRT-binding transcription factor DREB2C enhances thermotolerance. Biochem. Biophys. Res. Commun., 362: 431-436.

Liu, J.X., R. Srivastava, P. Che and S.H. Howell, 2007. Salt stress responses in *Arabidopsis* utilize a signal transduction pathway related to endoplasmic reticulum stress signaling. Plant J., 51: 897-909.

Liu, Q., M. Kasuga, Y. Sakuma, H. Abe, S. Miura, K. Yamaguchi-Shinozaki and K. Shinozaki, 1998. Two transcription factors, *DREB1* and *DREB2*, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell, 10: 1391-1406.

Meloni, D.A., M.A. Oliva, C.A. Martinez and J. Cambraia, 2003. Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. Environ. Exp. Bot., 49: 69-76.

Mittler, R., 2006. Abiotic stress, the field environment and stress combination. Trends Plant Sci., 11: 15-19.

Mittova, V., M. Guy, M. Tal and M. Volokita, 2004. Salinity up-regulates the antioxidative system in root mitochondria and peroxisomes of the wild salt-tolerant tomato species *Lycopersicon pennellii*. J. Exp. Bot., 55: 1105-1113.

Moller, I.M. and L.J. Sweetlove, 2010. ROS signaling-specificity is required. Trends Plant Sci., 15: 370-374.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15: 473-497.

Nakashima, K., Y. Ito and K. Yamaguchi-Shinozaki, 2009. Transcriptional regulatory networks in response to abiotic stresses in *Arabidopsis* and grasses. Plant Physiol., 149: 88-95.

Parida, A.K. and A.B. Das, 2005. Salt tolerance and salinity effects on plants: A review. Ecotoxicol. Environ. Saf., 60: 324-349.

Pastore, D., D. Trono, M.N. Laus, N. Di Fonzo and Z. Flagella, 2007. Possible plant mitochondria involvement in cell adaptation to drought stress. A case study: Durum wheat mitochondria. J. Exp. Bot., 58: 195-210.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucl. Acids Res. 29: 2002-2007.

Pilon-Smits E.A.H., Y.L. Zhu, T. Sears and N. Terry, 2000. Overexpression of glutathione reductase in *Brassica juncea*: Effects on cadmium accumulation and tolerance. Physiol. Plant., 110: 455-460.

Rae, L., N.T. Lao and T.A. Kavanagh, 2011. Regulation of multiple aquaporin genes in *Arabidopsis* by a pair of recently duplicated DREB transcription factors. Planta, 234: 429-444.

Rapacz, M., B. Wolanin, K. Hura and M. Tyrka, 2008. The effects of cold acclimation on photosynthetic apparatus and the expression of COR14b in four genotypes of barley (Hordeum vulgare) contrasting in their tolerance to freezing and high-light treatment in cold conditions. Ann. Bot., 101: 689-699.

Riechmann, J.L., J. Heard, G. Martin, L. Reuber and C. Jiang *et al*., 2000. *Arabidopsis* transcription factors: Genome-wide comparative analysis among eukaryotes. Science, 290: 2105-2110.

Roxas, V.P., S.A. Lodhi, D.K. Garrett, J.R. Mahan and R.D. Allen, 2000. Stress tolerance in transgenic tobacco seedlings that overexpress glutathione S-transferase/glutathione peroxidase. Plant Cell Physiol., 41: 1229-1234.

Sakuma, Y., K. Maryyama, F. Qin, Y. Osakabe, K. Shinozaki, K. Yamaguchi-Shinozaki, 2006. Dual function of an Arabidopsis transcription factor DREB2A in water-stress-responsive and heat-stress-responsive gene expression. Proc. Natl. Acad. Sci., 103: 18822-18827.

Salavati, A., A. Khatoon, Y. Nanjo and S. Komatsu, 2012a. Analysis of proteomic changes in roots of soybean seedlings during recovery after flooding. J. Proteomics, 75: 878-893.

Salavati, A., A.A.S. Bushehri, A. Taleei, S. Hiraga and S. Komatsu, 2012b. A comparative proteomic analysis of the early response to compatible symbiotic bacteria in the roots of a supernodulating soybean variety. J. Proteomics, 75: 819-832.

Schramm, F., J. Larkindale, E. Kiehlmann, A. Ganguli, G. Englich, E. Vierling and P. von Koskull-Doring, 2008. A cascade of transcription factor DREB2A and heat stress transcription factor HsfA3 regulates the heat stress response of *Arabidopsis*. Plant J., 53: 264-274.

Shinozaki, K. and K. Yamaguchi-Shinozaki, 1997. Gene expression and signal transduction in water-stress response. Plant Physiol., 115: 327-334.

Shinozaki, K. and K. Yamaguchi-Shinozaki, 2000. Molecular responses to dehydration and low temperature: Differences and cross-talk between two stress signaling pathways. Curr. Opin. Plant Biol., 3: 217-223.

Takahashi, S., T. Katagiri, K. Yamaguchi-Shinozaki and K. Shinozaki, 2000. An *Arabidopsis* gene encoding a Ca^{2+}-binding protein is induced by abscisic acid during dehydration. Plant Cell Physiol., 41: 898-903.

Umezawa, T., K. Nakashima, T. Miyakawa, T. Kuromori, M. Tanokura, K. Shinozaki and K. Yamaguchi-Shinozaki, 2010. Molecular basis of the core regulatory network in ABA responses: Sensing, signaling and transport. Plant Cell Physiol., 51: 1821-1839.

Wang, W., B. Vinocur and A. Altman, 2003. Plant responses to drought, salinity and extreme temperatures: Towards genetic engineering for stress tolerance. Planta, 218: 1-14.

Wang, W.X., B. Vinocur, O. Shoseyov and A. Altman, 2004. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci., 9: 244-252.

Xiong, L., K.S. Schumaker and J.K. Zhu, 2002. Cell signaling during cold, drought and salt stress. Plant Cell, 14: S165-S183.

Yan, S.P., Z.C. Tang, W. Su and W. Sun, 2005. Proteomic analysis of salt stress-responsive proteins in rice root. Proteomics, 5: 235-244.

Zhu, J.K., 2001. Plant salt tolerance. Trends Plant Sci., 6: 66-71.

Zhu, X., R.J. Castellani, A. Takeda, A. Nunomura, C.S. Atwood, G. Perry and M.A. Smith, 2001. Differential activation of neuronal ERK, JNK/SAPK and p38 in Alzheimer disease the two hit hypothesis. Mech. Ageing Dev., 123: 39-46.

Zou, J., Y, Guo, T. Guettouche, D.F. Smith and R. Voellmy, 1998. Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. Cell, 94: 471-480.