

**EXPRESSION PATTERNS OF AN ABIOTIC STRESS-INDUCIBLE
DEHYDRATION RESPONSIVE ELEMENT BINDING PROTEIN-2 (DREB2)
GENE IN TOMATO**

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ABSTRACT

A novel DREB (dehydration-responsive element binding) gene, designated as *LeDREB2*, was isolated from tomato. Based on multiple sequence alignment and phylogenetic characterization, *LeDREB2* gene was classified into an A-2 group member of the DREBs family. We examined the expression pattern of *LeDREB2* gene in tomato under different abiotic stresses. Southern blot analysis showed that *LeDREB2* is a single copy gene in tomato genome. The organ specific expression profiling indicated that *LeDREB2* gene was strongly expressed in young leaves and roots but weak expression in mature leaves and shoots. Northern blot analysis revealed that various kinds of environmental stress such as salt, drought and cold were significantly induced by *LeDREB2* gene after application of time courses treatments except little increase in ABA. Our findings showed that the expression of *LeDREB2* gene was induced by high salinity, drought and cold but not by the abscisic acid (ABA) treatment. These results indicated that the *LeDREB2* gene is a member of the DREB transcription factors, which may play a role in both abiotic and oxidative stress responses.

Key words: Abiotic stresses, *LeDREB2*, expression analysis, tomato

INTRODUCTION

Plants are always facing adverse environmental conditions such as salt, drought, cold, and so on which have seriously affected the geographical distribution and limited the growth and yield of plants. Plant responses to environmental stresses involve a complex variety of tolerance mechanisms that are activated and integrated by the expression of thousands of genes (Seki *et al.*, 2002). To cope with these stresses, plants execute a number of physiological and metabolic responses (Knight and Knight, 2002). The importance of crop resistance to water stress, extremes of salinity and harsh temperature are likely to increase a vast range of environments (Ramonell and Somerville, 2002). The DREB transcription factors play an important role in regulating abiotic stress-related genes and thereby imparting tolerance to stresses such as high-salt, drought and cold to the plant system (Liu *et al.*, 1998). The products of these genes are thought to function not only in stress tolerance but also in the regulation of gene expression and signal transduction in stress response (Xiong *et al.*, 2002).

Differential expression of genes involved in defense mechanisms against cell dehy-

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dration plays a key role in water-deficit tolerance. Expression analyses of *ZmDBF1*, *DREB2A*, *OsDREB2A* and *CBF4* showed that these genes are induced by dehydration and high-salt stresses (Yamaguchi-Shinozaki and Shinozaki, 1994). DREB1b is thought to be functional in cold-responsive gene expression, whereas DREB2s are involved in the drought-response (Wang et al., 2008). DREB transcription factors play key roles in plant stress signaling transduction pathway and they can specifically bind to the DRE element (G/ACCGAC) and activate the expression of many stress inducible genes (Kim et al., 2004). The C-repeat/dehydration responsive element (C/DRE) stress-inducible genes RD29A and COR15a were identified from the *Arabidopsis thaliana* which is responsive to cold and drought, and found in other cold inducible genes in various plants (Huang et al., 2008). It was found that the *GhDBP2* transcripts were greatly induced by drought, NaCl, low temperature and ABA treatments in cotton cotyledons (Schramm et al., 2008). DREB2A is induced by heat stress and is a regulator of the heat stress response in *Arabidopsis thaliana* (Cong et al., 2008).

A novel DREB gene was isolated from *Brassica juncea* and found that *BjDREB1B* gene was induced by abiotic stresses and exogenous phytohormones, such as drought, salt, low temperature, heavy metals, abscisic acid, and salicylic acid by RT-PCR (Islam and Wang, 2009). It was demonstrated that the expression of *OsDREB1A* and *OsDREB1B* gene was induced by cold, whereas expression of *OsDREB2A* gene was induced by dehydration and high salt stresses (Yamaguchi-Shinozaki and Shinozaki, 1994). It was found that *LeDREB3* gene expression was significantly induced by NaCl, drought, low temperature but slightly regulated by treatment with ABA in tomato (Shen et al., 2003). *DREB2*-type genes were investigated in other species of the grass family, such as wheat *TaDREB1* (Xue and Loveridge, 2004), barley *HvDRF1* (Qin et al., 2007), and maize *ZmDREB2A* (Okamuro et al., 1997). These results suggest that DREB is an important transcription factor that regulates stress responsive gene expression through DRE *cis*-elements and it might play a vital role in providing tolerance to multiple stresses.

Characterization of *DREB2* genes from tomato would help us understand its surprising resistances to environmental stresses. Hence, we report a novel DREB2 homolog, *LeDREB2* gene, from various environmental stresses treated a tomato leaf. Its expression patterns were investigated under different stress conditions. The implication of this investigation is that the *DREB2* genes in tomato will become another target for engineering biotech crops with enhanced tolerance to abiotic stress.

MATERIALS AND METHODS

Plant materials, growth conditions, and stress treatments

Seeds of tomato (*Lycopersicon esculentum* L.) were surface-sterilized for 5 min in 1% (w/v) sodium hypochloride and finally washed with distilled water. Then seeds were cultured in Murashige and Skoog (MS) medium (pH 5.8) including 3% sucrose and 0.8% agar. The germinated plants were transferred to pots and kept in culture room at 25°C for 4 weeks. Drought was induced by removing plants from the pots and placing them on filter paper at 25°C under dim light for 48 h. Tomato leaves were collected after 0 (untreated, control), 3, 6, 12, 24 and 48 h after drought treatment. For cold treatment, the leaves were placed in distilled water and kept in a 4°C cold chamber under dim light and photoperiodic conditions described above upto 48 h. For oxidative stresses such as salinity, ABA treatments were applied by submerging the whole seedlings continuously in a water solution of 250 mM NaCl (salt), 100 µM ABA (abscisic acid) until 48 h respectively. Treated samples were collected after indicated time periods. Sterile water

was used as a control for all treatments. All stress treated plant materials were immediately frozen in liquid nitrogen and stored at -80°C until further use.

Isolation of LeDREB2 cDNA from tomato and sequence analysis

RT-PCR procedures were applied to clone the full-length cDNA. First, we searched the tomato EST database (<http://www.ncbi.nlm.nih.gov/>) using the tblastn program with the amino acid sequences of *LeDREB2* gene (AF500012), which is an A-6 group member of the *Arabidopsis* DREB subfamily (Sakuma *et al.*, 2002) and found a tentative consensus sequence in tomato with a deduced protein sequence that is highly homologous with that of RAP2.4. This sequence extends from a putative ATG initiation site to a stop codon at the 3' end. To obtain a cDNA encoding the coding region of this putative protein, a normal PCR reaction was then performed by using a tomato leaf cDNA library. The two primers are DREB2-Forward (5'-ATGATAATAATGTCTACAGAGCAA-3') and DREB2-Reverse (5'-CTAATGTTGCCATAAAAACTCTC-3'). The resulting product, which is confirmed to have the predicted length, was cloned into the pGEM T-Easy vector (TakaRa) and sequenced to confirm the presence of an open reading frame (ORF) related to the tentative consensus sequence. DNA sequence data were assembled and analyzed by the DNAMAN analysis program (Lynnon Biosoft, USA). Database searches were performed with the NCBI BLAST search program (Bethesda, MD). Multiple alignments were generated according to the program <http://www.ebi.ac.uk/tools/t-coffee>, from the proteomics server of the European Bioinformatics Institute (EBI). Alignment of the LeDBEB2 protein with other structurally related AP2/ERF proteins was performed by the ClustalW software. Phylogenetic tree were generated from Genebee Molecular Biology Server which is maintained by <http://www.genebee.msu.su/genebee>.

DNA isolation and Southern blot analysis

Genomic DNA was isolated from mature tomato leaves. Genomic DNA samples (10 μg) were completely digested with *EcoRI* and *HindIII*. Digested genomic DNA was separated by electrophoresis on a 1% agarose gel, denatured, and blotted onto a nylon membrane (Amersham Pharmacia, Uppsala). Membranes were then hybridized with the full-length of *LeDREB2* cDNA probe labeled with [α - ^{32}P] dCTP. Hybridization was performed overnight at 65°C in 5% dextran sulfate, 0.25 M disodium phosphate (pH 7.2), 7% (w/v) SDS, and 1 mM EDTA. After hybridization, the blot was washed twice with $2 \times \text{SSC}$ and 0.1% SDS for 10 min each at room temperature and twice with $0.1 \times \text{SSC}$ and 0.1% SDS for 5 min each at 65°C . The blots were then dried and developed on X-ray film incubated at -80°C for 1 week.

RNA isolation and Northern blot analysis

Total RNA was isolated from stress treated and control tomato plants using Trizol-reagent according to the manufacturer's instructions (MRC, USA). To ensure approximately equal loading of RNA, 20 μg of total RNA for salt and drought while 10 μg for cold and ABA were loaded onto 1.2% (w/v) denaturing formaldehyde agarose gels and transferred to Hybond-N⁺ membranes (Amersham Pharmacia, UK) with $20 \times \text{SSC}$ (3 M NaCl, 0.3 M sodium citrate, pH 7.0) as transfer solution. It was cross-linked to the membranes by baking at 2 h in 80°C . RNA blots were pre-hybridized overnight at 65°C in hybridization buffer: 50% formamide, $5 \times \text{SSPE}$, $5 \times \text{Denhardt's}$, 0.1% SDS. PCR products corresponding to *LeDREB2* cDNAs were labeled with [α - ^{32}P] dCTP by random priming (Promega, USA). All membranes were hybridized at 65°C with

hybridization buffer containing labeled DNA probe. Hybridization was performed 3-4 days at 65°C in 5% dextran sulfate, 0.25 M disodium phosphate (pH 7.2), 7% (w/v) SDS, and 1 mM EDTA. Following hybridization, the filter was washed twice with $2 \times$ SSC and 0.1% SDS for 10 min each at room temperature, and twice with $0.1 \times$ SSC and 0.1% SDS for 5 min each at 65°C. After the washing steps, the blots were exposed to X-ray film (Kodak) at -80°C for a week and developed.

For tissue-specific expression of *LeDREB2* gene, total RNA was prepared from tomato seedling mature leaves, young leaves, roots, and stems. Northern blot was performed according to the above mentioned procedures.

RESULTS AND DISCUSSION

We successfully isolated dehydration responsive element binding-2 gene from tomato by RT-PCR method and it is designated as *LeDREB2* (GenBank accession no. AF500012). Its cDNA is 900 bp in length and it encodes a deduced protein of 300 amino acid residues. Genomic PCR products amplified by primers designed from the 5' and 3' untranslated region revealed that *LeDREB2* gene had no intron interruption of its coding region.

Multiple alignments showed that *LeDREB2* gene shared high sequence similarity with other DREB related proteins throughout the ERF/AP2 domain (Fig. 1). It revealed high sequence similarity of *LeDREB2* cDNA to other putative dehydration responsive element binding protein (Fig. 1). *LeDREB2* gene shared 73% identity with *Glycine max* (ABQ42205), 76% similarity with *Triticum monococcum* (AAX28965), 65% homology with *HvCBF7* protein in *Hordeum vulgare* subsp. *Vulgare* (AAX23704), 49% homology with dehydration responsive element-binding protein 1C protein in *Zea mays* (NP_001151300), 74% similarity with *Arabidopsis thaliana* putative AP2 domain transcription factor (AAM64613), and 48% identities with *Glycine max* TINY (ACP40513). Phylogenetic analysis revealed high sequence similarity of the deduced amino acid sequence of *LeDREB2* cDNA to other dehydration proteins (Fig. 2) and which are classified to the A-2 group of DREB transcription factors. Phylogenetic analysis indicated that *LeDREB2* gene belongs to the A-2 group, according to the classification of ERF/AP2 transcription factors in *Arabidopsis* (Kim *et al.*, 2004). The DREB2 exists in the N-terminal and is conserved among *LeDREB2* gene. The dehydration responsive element binding proteins those have been determined from other plants, suggesting that this domain might have a significant biological function.

To detect the copy number of the *LeDREB2* gene in tomato, Southern blot analysis was performed on tomato genomic DNA digested with *EcoRI* and *HindIII* using a full-length *LeDREB2* cDNA probe. Hybridization of the genomic DNA blot resulted in a single band in DNA samples digested by *EcoRI* and *HindIII* (Fig. 3a). These results are indicating the presence of a single copy *LeDREB2* gene in tomato genome.

Tissue specific expression analysis showed that *LeDREB2* gene was expressed in all tested organs, with the highest expression levels observed in roots and young leaves. A comparatively strong signal was observed in roots and young leaves, but weak signal was detected in the mature leaves and stem (Fig. 3b). DREB2 transcripts differentially accumulated in various organs of the mature tomato plant, and the developmental regulation of the *DREB2* gene expression is likely to vary among different organs. Expression of *LeDREB2* gene can be detected in roots, stems and leaves under normal conditions. To determine whether the *LeDREB2* gene in tomato mediates plant responses to different stress conditions, its expression was investigated by northern blot analysis after exposure to various abiotic and oxidative stresses such as treatment with 250 mM

NaCl, drought, cold, 100 μ M ABA (Fig. 4a-d). These genes were reportedly expressed under various stress conditions such as dehydration, cold, and high salinity (Nakashima *et al.*, 2000).

LeDREB2	MIIMSTEQPNCESTESSCNSSSSSSPSSVLLQPLPQINSKNRLKRCRGEVEVEEE	:60
GmDREB6	---MVMEESNPLDNEVQPTSSSSFTITTPSTSSSSIEEATNTTKE---KTKK-----	:48
TmCBF7	---NEADASHAPTTSSS-SVSSSTLSTSSSSSLATSALELPKSPKPKHPKRRKRAASDR	:56
HvCBF7	-----MGATEDSSSGSETTTSSVEALASPPSPATTASSK----	:36
ZmDREB1C	-----MDDAQEPSPSPSSQSS-----SSSSSSSVAPNP----	:30
AtAP2	---MARQINIESSVSQVTFISSAIPAVSSSSSITASALSSSPTSSSSSSSTNSNFIE	:56
GmTINY	---MRVSTQNNSE-TESS--SSTSLPPSPS-----PSP-----	:27
LeDREB2	DVVVNNPNPKMN-----KNNNGSSTSVVSVVGVRRKANGKVVSEIR	:103
GmDREB6	-----ANSN--EG-----KHPTVYRGVRYRQNGKVVSEIR	:75
TmCBF7	EQDAANTNGVVRGDESSCCSTDEDNAAASVKAAPKSGFKHPSYRGVRRRSWGRKVVSEIR	:116
HvCBF7	-----KKRARNDG-RHPTVYRGVRRRSWGRKVVSEIR	:65
ZmDREB1C	-----KKRARKDGRHPTVYRGVRRRSWGRKVVSEIR	:60
AtAP2	EDNSKRKASRRSLSSLSVVEDDDQNGGGKRRKTNGGDKHPTVYRGVRRRSWGRKVVSEIR	:116
GmTINY	---SSPPSSEFK-----TEKRVRESNKHVYRGVRRRSWGRKVVSEIR	:66
LeDREB2	EPKKSRIWLGTFATPEMAARAHDAVAAVMSIKG-TSAILNFRQFSHLLPRPVTCSPRDIDV	:162
GmDREB6	EPRKKSRIWLGTFPTPEMAARAHDAVAAALTIKQ-SSAYLNFPPELADELPRPASTSPKDIDQ	:134
TmCBF7	EPRKKSRIWLGTFPTPEMAARAHDAVAAALAIKQ-RSARLNFPPELAHELPRPDSTSPADIDQ	:175
HvCBF7	EPRKKSRIWLGTFATAEMAARAHDAVAAALAIKQ-RAAHLNFRDLAHELPRPATAARKDVQA	:124
ZmDREB1C	EPRKKSRIWLGTFATAEMAARAHDAVAAALAIKGARDAHNFRPGLAHLPRPATAARKDVQA	:120
AtAP2	EPRKKSRIWLGTYPTAEMAARAHDAVAAALAIKQ-TTAYLNFRKLAGELRPVVNSPKDIDQ	:175
GmTINY	EPRKKSRIWLGTFPTPEMAARAHDAVAAALSIKQ-NSAIIISFPPELAASLPRPVSLAPRDVQA	:125
LeDREB2	AAVKAAMHDLNPKFSLPETSAAATMTSSSSLSLVSQVTSSSSSFQDDEESRPSPEPEI	:222
GmDREB6	AAAKAAALDF--GHRSHDA-----ESELRAVSSS-----ILTQA-----SSSSSN	:173
TmCBF7	AAAKAAATAA--VQCEAEF-----EHEPETPSSSSGAASETPEPA-----PCAEAA	:219
HvCBF7	AAALAA-----ADFF-----ASATANAGAKNPDPGPEPN-----AASAST	:161
ZmDREB1C	AAALAAA-----ADFF-----LPPSPGG-ANAKSPSSSSSG-----SASASA	:156
AtAP2	AAALAAVNHQ--DSVNDVSNSEVAEIVEAEPRTVVAQLFSSDSTTT-----TTQSQE	:227
GmTINY	AAAKAAHMD-----LPST-----SSSISSSLVPTTQASSSSSS--LSSLTSADLL	:169
LeDREB2	PEATGQLSEIVELPKLG-SSYELVESTQSLFESDEMNDNNYGNCYFFGQDNYISSNMEF	:281
GmDREB6	SSSLK-----GVDDTFLDLPDLSLDLS-----HGADDFHYSSANLVAGAHEH	:214
TmCBF7	PADSG-----EVDNALFDLPDLDLDR-----DG---LFWSPVMPAALAAE	:257
HvCBF7	PPDT-----AEDALFDLPDLDLDR--HG---PPSCQVSCASTMDDVAF	:202
ZmDREB1C	SDDASASPAAPQDAEPNPDALFDLPDLDLDR--CC--GP-----SSKAVDDOVA	:204
AtAP2	YSEASCSTACTDKD-SEEEKLFDLPDLFTDENENMI---RNDAFCYYSSTWQLCGADA	:283
GmTINY	PTASEELSEIILPRLETTSYELGN-EFVFMDSQDAW--YQPPIPWLHTTYDGSDADEF	:226
LeDREB2	TGLENVSTSFESFLWQH-	:299
GmDREB6	IELG--FRLEDPFLWESY	:230
TmCBF7	EYDGGCCVGLSEPLWAE-	:275
HvCBF7	GPGAGVFR-LEEPQWEY-	:219
ZmDREB1C	G--GGAFRIIEEPLWMEY-	:220
AtAP2	G----FR-LEEPFLSE-	:295
GmTINY	VVPESGVVTSFESFLWDY-	:244

Fig. 1. Deduced amino acid sequences of *LeDREB2* gene and its alignment with *Glycine max* DREB6 (ABQ42205), *Triticum monococcum* (AAX28965), *Hordeum vulgare* subsp. *Vulgare* (AAX23704), *Zea mays* (NP_001151300), *Arabidopsis thaliana* (AAM64613), and *Glycine max* TINY (ACP40513). Number of the amino acid residues are shown to the right of each sequence. Fully conserved residues among the different sequences are shown with a black background. Chemically similar residues are denoted with a gray background. Dashes represent gaps introduced to maximize similarities.

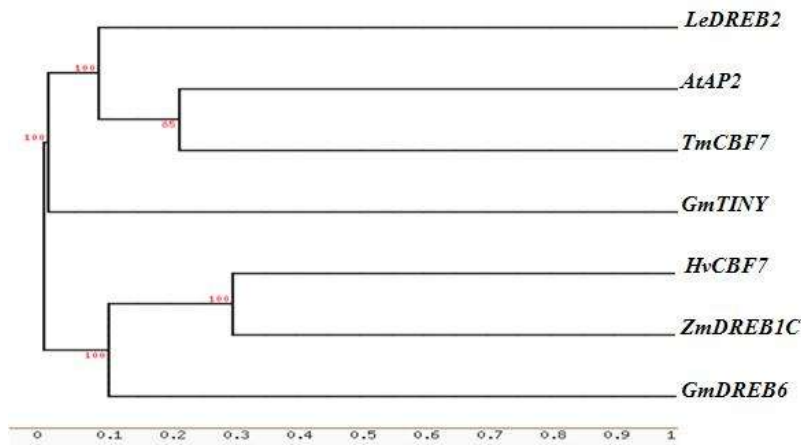


Fig. 2. Phylogenetic relationship among DREBs proteins. Dendrogram based on the amino acid sequence alignment of the following proteins: *Arabidopsis AtAP2* (AAM64613), *triticum monococcum TmCBF7* (AAX28965), Soybean *GmTINY* (ACP40513), Barley *HvCBF7* (AAX23704), Maize *ZmDREB1C* (NP_001151300), and Soybean *GmDREB6* (ABQ42205). The tree was constructed using the ClustalWx methods. Bootstrap values are indicated for each branch divergence.

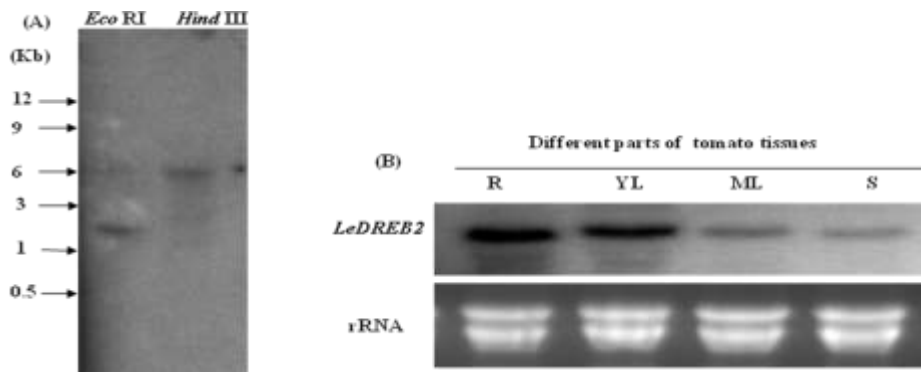


Fig. 3. (a) Genomic DNA blot analysis of tomato *LeDREB2* gene. Each lane was loaded with 10 μ g tomato genomic DNA digested with *EcoRI* and *HindIII*. The membrane was hybridized with 32 P-dCTP-labelled *LeDREB2* cDNA. The sizes of the molecular weight markers are indicated in kilobases to the left. (b) Expression of the *LeDREB2* gene in a variety of organs from normally grown tomato plant. Each lane was loaded with 20 μ g of total RNA prepared from roots (R), young leaf (YL), mature leaf (ML), and stems (S). rRNAs blotted on the membrane were visualized by staining with ethidium bromide.

The expression of *LeDREB2* gene could be induced with salinity treatment. When treated with 250 mM NaCl, the accumulation of *LeDREB2* gene transcripts slowly declined until 24 h thereafter increased at 48 h (Fig. 4a). The *LeDREB2* gene transcripts level gradually declined until 48 h in case of drought treatment (Fig. 4b). In cold-treated tomato leaves, *LeDREB2* gene expression was increased after 3 h of cold treatment and maintained a comparatively more or less similar trends until 48 h (Fig. 4c). DREB1 transcription factors were identified in *B. napus*, wheat, rye, and rice, and all of them showed a rapid response to cold stress (Abe *et al.*, 1997). When exposed to cold,

PeDREB2 is highly expressed, unlike *DREB2A* gene in *Arabidopsis* (Liu *et al.*, 1998). In hot pepper plants, expression of *CaCBF1B* gene was found to be induced by cold, dehydration, and heat-shock treatment, whereas *CaDREBLB1* gene is induced by dehydration, NaCl, and wounding treatment (Nakashima *et al.*, 2000). The function of DREB1 in cold-responsive gene expression has been extensively analyzed, but that of DREB2A has not yet been elucidated (Yamaguchi-Shinozaki and Shinozaki., 1994). The DREB genes that are induced by cold and water stress, encode transcription factors that bind to the DRE promoter element of stress related genes, and turn on their expression (Yamaguchi-Shinozaki and Shinozaki., 1994)

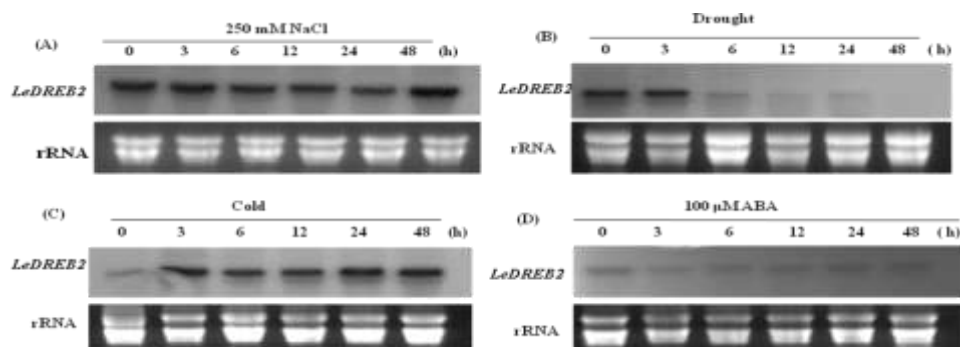


Fig. 4. Northern blot analysis of *LeDREB2*. 20 μ g total RNA for salt and drought and 10 μ g total RNA for cold and ABA extracted from the leaves of tomato plants. A fragment from the 900 bp region of the EST cDNA clone of *LeDREB2* was used as the hybridization probe (a-d). Time-course analysis of expression under salinity, drought, cold and ABA, treatment of plants, respectively. Plants were harvested at 0, 3, 6, 12, 24 and 48 h after treatments. Control plants (0) were not treated. rRNA bands are shown in lower part of each panel.

In the ABA-treated tomato leaves, the *LeDREB2* gene transcripts were remained similar until 48 h (Fig. 4d). It indicated that ABA does not influence the expression of *LeDREB2* gene. Their expression is not induced by ABA treatment therefore they must functions in ABA-independent stress-inducible gene expression. DREB proteins are well known for playing important roles in ABA-independent gene expression (Liu *et al.*, 1998).

These findings demonstrate that *LeDREB2* gene, a homolog of the transcription factor gene that is induced after application of stress treatments in tomato which is an abiotic stress related gene in tomato. The implication of this investigation is that, this *LeDREB2* gene will become another target for engineering biotech crops with enhanced tolerance to abiotic stress. We intend to test whether overexpression of *LeDREB2* gene in transgenic tomato plants enhances their resistance to abiotic stresses. In addition, an *in vivo* analysis of different gene-silencing phenotypes will help to clarify the role of *LeDREB2* gene in its responses to abiotic stresses.

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