

Full Length Research Paper

Identification of genes induced by salt stress from *Medicago truncatula* L. seedlings

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In order to identify genes induced during the salt stress response in barrel medic (*Medicago truncatula* L.) seedlings, a cDNA library by salt stress was constructed by suppression subtractive hybridization (SSH). Total RNA from 15-day-old seedlings was used as a 'driver', and total RNA from seedlings induced by salt was used as a 'tester'. One hundred and sixty nine clones identified as positive clones by reverse northern dot-blotting resulted in 75 uni-ESTs that comprised of 13 contigs and 62 singletons. Basic Local Alignment Search Tool (BLAST) analysis of deduced protein sequences revealed that 35 expressed sequence tags (ESTs) had identity similar to proteins with known function, while 27 could not be annotated at all. Most of the known function sequences were homologous to genes involved in abiotic stress in plants. Among these protein, citrate synthase, ribulose- 1,5-bisphosphate carboxylase, chloroplast protein, phosphoenolpyruvate carboxylase and chloroplast outer envelope protein are related to photosynthesis; DNA binding/transcription factor, putative AP2/EREBP transcription factor, Cab9 gene, photosystem II polypeptide and calcium-dependent protein kinase play a significant role in signal transduction and transcription regulation; and aldolase and sucrose synthase are interrelated to osmolyte synthesis. Moreover, 5 of the ESTs, similar to genes from other plant species and closely involved in salt stress were isolated from *M. truncatula* L. They are superoxide dimutase (SOD)-1, gene for copper/zinc superoxide dismutase, cysteine protease, Na⁺/H⁺ antiporter and salt overly sensitive 2 (SOS2). To further assess the expression level of salt-induced ESTs, real-time polymerase chain reaction (PCR) analysis was employed, and the result showed that these genes have significantly increased expression and probably play an important role in the response of plants to salt stress.

Key words: Barrel medic (*Medicago truncatula* L.), suppression subtraction hybridization (SSH), reverse northern dot-blotting, salt stress, real-time polymerase chain reaction (PCR).

INTRODUCTION

Salt stress is a major abiotic stress in agriculture world-wide. It is estimated that about 20% of the earth's land mass and nearly half of all irrigated land are affected by

salinity. Increased salinization of arable land is expected to have devastating global effects, with predictions of 30% land loss within the next 25 years, and up to 50% by the year 2050 (Wang et al., 2003). This has led to the research of salt stress with the aim of improving crop salt tolerance by genetic engineering, identifying novel genes and determining their expression patterns in response to salt stress. Understanding their functions in stress adaptation will provide us with the basis for effective engineering strategies to improve crop stress tolerance (Kawasaki et al., 2001).

Generally, a high concentration of salt causes ion imbalance, hyperosmotic stress and oxidative damage (Zhu, 2002). Plants can perceive the stress signals and

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Abbreviations: SSH, Suppression subtractive hybridization; ESTs, expressed sequence tags; SOD, superoxide dimutase; SOS2, salt overly sensitive 2; PCR, polymerase chain reaction; MS, Murashige and Skoon; BLAST, basic local alignment search tool; NCBI, national center for biotechnology information; BADH, betaine aldehyde dehydrogenase.

transmit them to the cellular machinery to activate adaptive responses. The adaptation is completed in part by regulating gene expression. One important way to study the mechanism of salt-stress response is to survey the expression of genes regulated by salt stress. Plants respond to stress, in part, by modulating gene expression which eventually leads to the restoration of cellular homeostasis, detoxification of toxins and recovery of growth in tolerant plants or when the stress is removed. For instance, cysteine proteases are widely distributed in the plant kingdom and are involved in defense systems against salt-stress, drought-stress and some other abiotic stresses (Hassan et al., 2001). Superoxide dismutase (SOD) is an important biological antioxidant and can remove the superoxide radical anion produced during the bio-oxidation process. It is called the first line of antioxidant defense (Bowler et al., 1992). Calcium-dependent protein kinase is involved in regulating cell growth, cell cycle and maintenance of normal chromosome structure (Urao and Katagiri, 1994; Hwang and Goodman, 1995). The Na^+/H^+ antiporter plays an important role in the regulation of cell water potential and intracellular distribution of salt signal conduction (Yang et al., 2005).

Polymerase chain reaction (PCR)-selected cDNA subtraction, often referred to as suppression subtractive hybridization (SSH), is an efficient method for studying differential gene expression (Diatchenko et al., 1996; Gao et al., 2003). In recent years, this method has gained extensive application in higher plants in studying the molecular regulation in adverse environmental conditions, such as drought, high-salt and cold stress etc. SSH technology can be used to develop cDNA libraries and characterize genes of widely diverse plant species in responses to salt stress

Medicago truncatula is a model or reference species for legume genetics, genomics and breeding. It is a relative of alfalfa with a short generation time and relatively small genome (520 Mb), and an important leguminous forage crop worldwide. Legumes such as alfalfa and soybeans are particularly important because of their symbiotic relationship with nitrogen-fixing bacteria. But many leguminous crops are sensitive to high salt conditions. High salt stress significantly limits their productivity. Our current work is focused on construction of a cDNA library by SSH to acquire gene expression profiles of *M. truncatula* influenced by salt stress. In the future, these data will be supplemented with transcriptome data from plants subjected to salt stresses. We anticipate that these data will aid gene function determination and molecular breeding efforts.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of *M. truncatula* L. obtained from the U.S. Department of

Agriculture (Natural Resources Conservation Service Bridger Plant Materials Center) were germinated in MS (Murashige and Skoog, 1962) agar plates under dark for 1 day, and then grown for 15 days under a 16-h photoperiod supplemented with fluorescent lamps (28/26°C day/night and 60 - 80% relative humidity). Fifteen days seedlings were then transferred to a filter paper soaked with MS salts solution containing 200 mM NaCl for 6 h (Yang et al., 2005).

Isolation of total RNA and mRNA

Total RNA was isolated from the leaves of the samples according to the method of Cathala et al. (1983). Any contaminated genomic DNA was removed by incubating the total RNA with RNase free DNase (Promega) at 37°C for 30 min. For PCR-select DNA subtraction, mRNA was purified from total RNA using a PolyA Tract® mRNA isolation system III kit (Promega).

Construction and amplification of subtracted cDNA library

PCR-select cDNA subtraction was performed using a PCR-Select™ cDNA Subtraction Kit (Clontech) according to established protocols. A mixture of cDNAs that were reverse-transcribed from 2 µg mRNA of *M. truncatula* L. seedlings treated with salt for 6 h was used as a tester and the cDNA from untreated seedlings was used as a driver. The cDNAs were digested with RsaI and then ligated to different adapters. Two rounds of hybridization and PCR amplification were processed to normalize and enrich differentially expressed cDNAs. Products of the secondary PCR were directly inserted into a pGEM®T-easy Vector (Promega) and transformed into *Escherichia coli* DH5α cells. All recombinant clones were picked up to establish the subtracted cDNA library.

The clones were grown overnight in 400 µl LB-Amp medium in a 96-well plate at 37°C. The cDNA inserts were amplified by PCR (P×2Thermal Cycler, Thermo Electron, USA) using nested PCR primer 1 and 2R provided in the PCR-selected cDNA subtraction kit, which were complementary to sequences flanking both sides of the cDNA insert. The PCR reactions contained 18.1 µl sterile water, 2.5 µl × 10 buffer, 0.5 µl of each primer (10 µM each), 0.5 µl dNTP mix (2.5 mM each), 2 µl MgCl_2 (25 mM), 0.5 units of Tag DNA polymerase (promega) and 1 µl of bacterial culture.

Thermo-cycling conditions were as follows: an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 66°C for 30 s, 72°C for 90 s and a final extension at 72°C for 5 min. The PCR products were electrophoresed on a 0.8% agarose gel to confirm the amplification quality and quantity.

Reverse northern dot-blotting and sequencing of the subtracted cDNA library

In order to further confirm positive clones, reverse northern dot-blotting was performed according to a DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Germany) protocol. Two microliters of the PCR products were dot-blotted onto Hybond-N⁺ nylon membranes (MagnaProbe Nylon Membranes, USA Osmonics). Two identical membranes with cDNA arrays were prepared for two differential screening probes, an unstressed cDNA probe and a salt stressed cDNA probe which were labeled using a DIG High Prime DNA Labeling System (Roche, Germany). Hybridization solution was used as negative controls and RT-PCR reaction production of house-keeping gene 18S 3' and 5' primers were used as positive controls. Hybridization and washing were carried out following the method described in the kit. Analysis of results and classification were also performed according to criteria described in the kit. The positive clones selected by dot-blotting

Table 1. Sequence of the primers for real time PCR.

Number of target EST	Primer name	Sequence(5'-3')
Mt61	61-1	AGATGTAATGCGCCATAC
	61-2	GCAGAGTTGAAGGTGAAA
Mt94	94-1	GAACAAAGGCAACTTCCACATT
	94-2	TGACATTACCTCCATCACAAAGC
Mt47	47-1	TTCACCTCAGCCTCACAT
	47-2	GGATTGCAGCCAAGACAA
Mt163	163-1	TAGTAATAAAGGGTGGTGC
	163-2	ATTGGCTAAGGAAGTAGAAT

were sequenced by Autolab Co., Ltd (Beijing, China). EST sequences were analyzed for homology by Basic Local Alignment Search Tool (BLASTX) in the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/BLAST>) and classified according to the MIPS database (http://mips.gsf.de/proj/funcatDB/search_main_frame.html) and AMIGO (<http://amigo.geneontology.org/cgi-bin/amigo/go.cgi>).

Measuring temporal expression of selected clones by real-time PCR analysis

The real-time PCR was used to characterize 4 salt-induced ESTs with unknown or putative functions using primers listed in Table 1. Total RNA was isolated from 15-day-old barrel medic seedlings induced by 200 mmol/l NaCl for 0, 30 min, 1, 3, 6, 12 and 24 h, and reverse transcribed by M-MLV RTase. A SYBR green reporter assay kit (Qiagen) was used for detection in the real-time PCR assay with the 18s rRNA as an endogenous control sequence on the ABI PRISM 7700 instrument. The program for amplification included 10-min polymerase activation at 95°C followed by 40 cycles of 95°C for 15 s and 72°C for 1 min.

RESULTS

Construction of the subtracted cDNA library

Two micrograms of mRNAs from tester (salt-stressed) and driver (unstressed) seedlings were reverse-transcribed prior to PCR-selected cDNA subtraction. The subtractive PCR products were ligated into T-easy vector prior to transfer into *E. coli*. 855 white colonies were picked from the plates and one microliter of bacterium culture from each clone was used to amplify the inserts with nested primers. The resulting insert fragments ranged from ~250 to ~750 bp (Figure 1). Thus, a putative salt stress specific subtracted cDNA library of barrel medic seedlings was constructed.

Reverse northern dot-blotting of subtracted cDNA library

cDNA clones for differentially expressed genes were further identified by screening with unsubtracted tester and driver cDNA pools as probes (Figure 2). In the screen

identified, there were many differentially expressed genes which were induced by salt stress. The results for the two hybridizations was recorded for each clone, and those showing obvious differential expression were selected as positive clones according to criteria described in the kit. As a result, 169 cDNA clones of differential expression were selected from the 855 clones in the subtracted cDNA library.

Sequence analysis of selected cDNA clones

169 cDNA clones of differential expression were sequenced and clustered analysis using DNAMAN, 75 unique ESTs (including 13 contigs and 62 singleton sequences) were obtained out of 108 available ESTs. This uni-EST set include 35 with high amino acid sequence homology to proteins with known function or putative function in the NCBI, MIPS or AMIGO databases. Five additional uni-ESTs had homology to non-annotated genes from other plant species, while 27 had no homologues at all. The 27 uni-ESTs may be either new genes or segments unique to *M. truncatula* L. and located in 3' or 5' non-translated regions.

The cDNAs with significant protein homology were sorted into 7 groups according to the functional categories of the *Arabidopsis* proteins (Figure 3). These functions include signal transduction mechanism/cellular communication, cell growth/rescue and defense, resistance/rescue, matter transport, transcription regulation and metabolism. The induction of more than one group of genes suggests that these unique ESTs may play roles in different biological processes. While the largest number of genes was assigned to the resistance/defense category, the unclassified or unknown proteins still represent a significant group.

Temporal expression of selected genes by real-time PCR analysis

Real-time PCR was conducted to verify expression of four salt-induced genes using independently prepared RNA that was extracted from barrel medic seedlings treated and

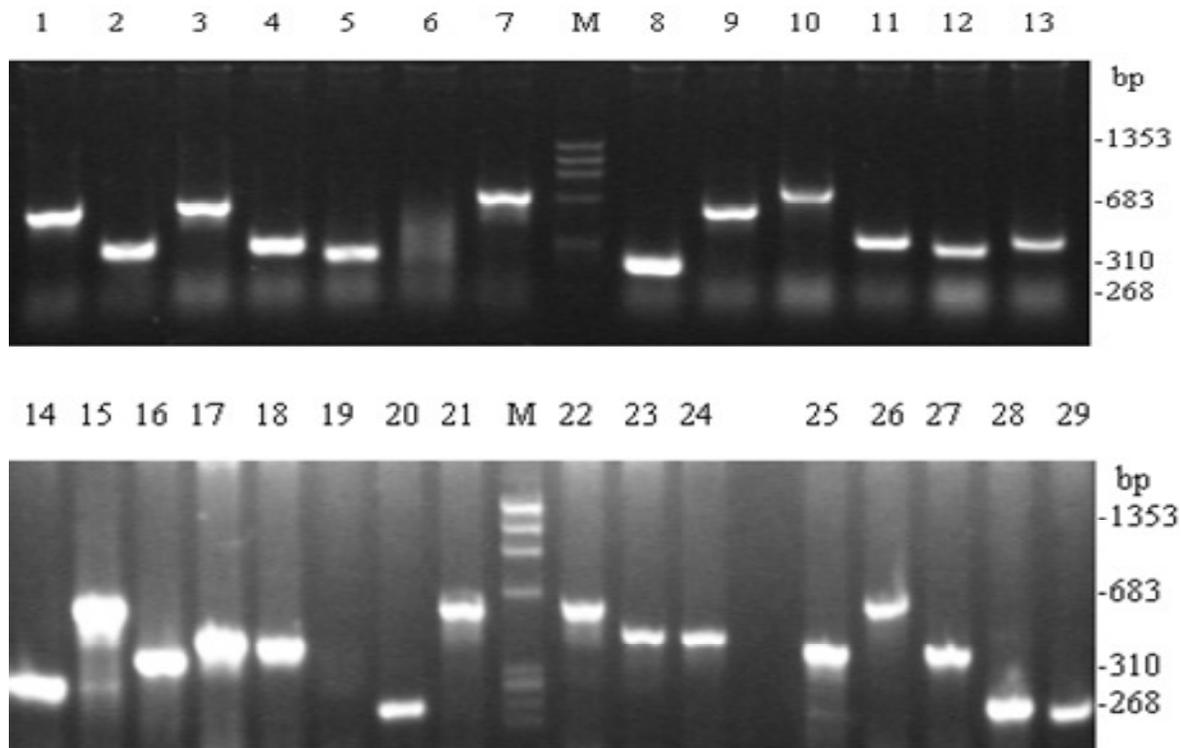


Figure 1. PCR analysis of partial clones from the subtracted library. M, Size markers (ϕ X174-*Hae* III digest); Lanes 1 to 29, PCR products from different clones. The twenty-nine clones are Mt62, Mt31, Mt64, Mt113, Mt121, Mt539, Mt29, Mt85, Mt33, Mt201, Mt46, Mt35, Mt20, Mt133, Mt38, Mt26, Mt20, Mt94, Mt596, Mt47, Mt53, Mt197, Mt11, Mt143, Mt63, Mt7, Mt75, Mt5 and Mt163, respectively from lanes 1 - 29.

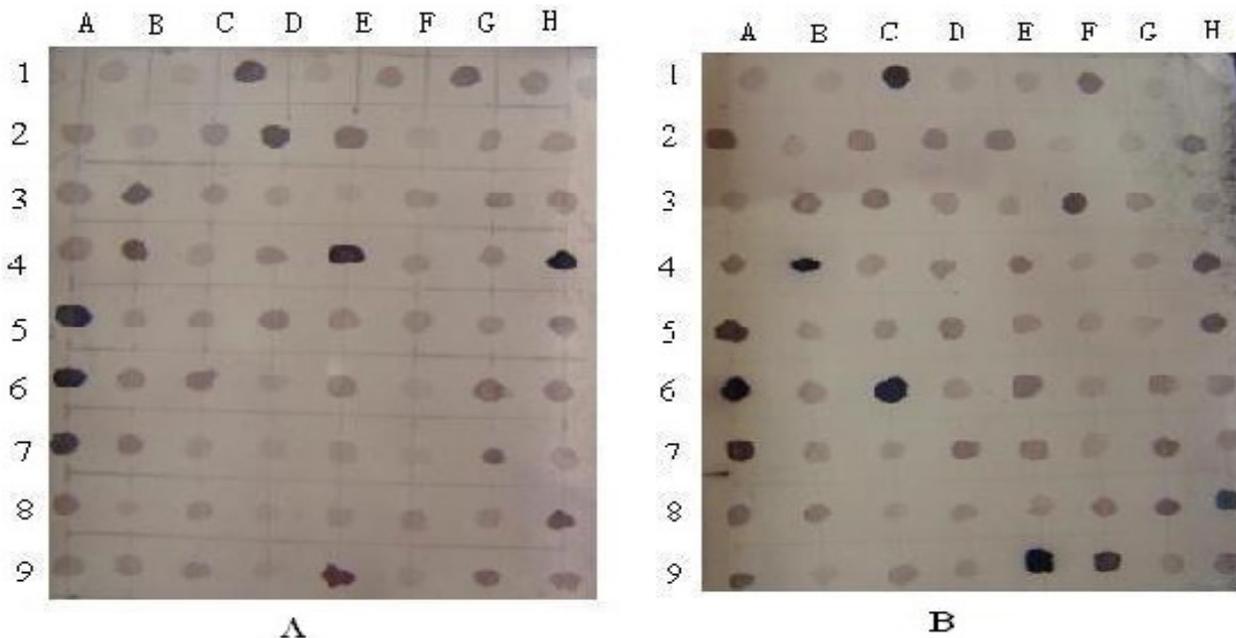


Figure 2. Differential screening of partial positive clones from the subtractive library. A total of 855 clones of subtracted cDNA were arrayed on positively charged nylon membranes and hybridized to different probes labeled by DIG. Clones which displayed a stronger signal in panel B than in panel A were selected as candidate clones for sequencing and further characterization (e.g. C6, F3, C6, E9 and E10). (A) Clones hybridized to control probe. Spots marked with open squares were identified as positive clones. (B) Clones hybridized to salt-induced tester probe.

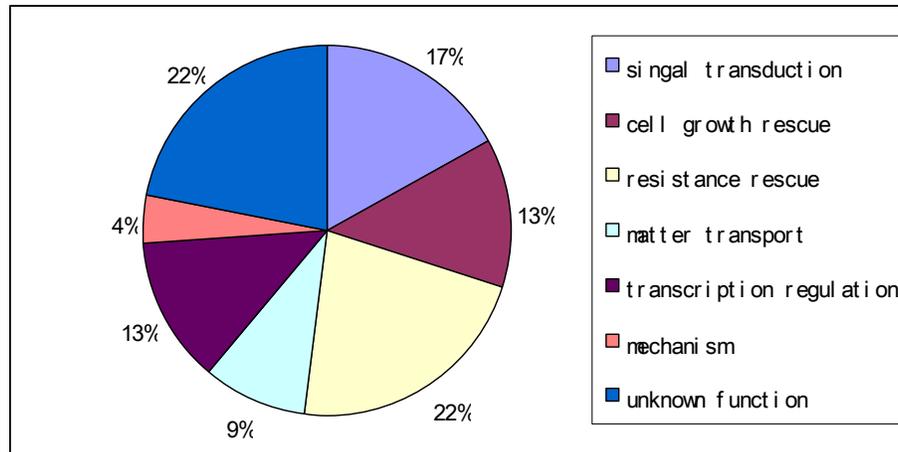


Figure 3. Functional classification of the ESTs with significant protein homologies.

untreated. The result showed that the expression level of four ESTs tended to increase after salt stress, though there were some individual fluctuations (Figure 4). It also justified the reverse northern dot-blot pattern of saline induction. The transcription of two of these sequences (Mt163, Mt61) reached the highest level when plants were induced for 30 min and then declined. The expression pattern implies that the two genes might play a role in the response of plants to salt stress. The expression of gene Mt17 could be detected at different times induced by salt, and its expression reached maximum at 1 h after treatment. It was inferred from the result that the gene may encode a protein that act in signal transduction after salt stress. One of these ESTs (Mt47), which reaches maximum at 6 h after salt stress, might encode a protein that terminates signal transduction by salt stress.

DISCUSSION

Resistance to different stresses is accomplished by cell rescue, defense and signal transduction, and via enhanced metabolic pathways (Scheideler et al., 2002). Categorization of the inferred function shows that many different kinds of genes are involved in the response to salt stress in *M. truncatula* L. A variety of genes are affected by abiotic stresses in other plant systems and some enzymes encoded by them include betaine aldehyde dehydrogenase (BADH) (Rhodes and Hanson, 1993; Hideki and Masumi, 2005), SOD (Bowler et al., 1992) and cysteine protease (Hassan et al., 2001; Grudkowska and Zagdanska, 2004). These stress enzymes play a part in the synthesis of osmotic molecules, removal of reactive oxygen species, protein turnover and recycling of amino acids (Shin et al., 2000; Grudkowska and Zagdanska, 2004; Kingston et al., 2005), respectively. We also encountered several previously-reported defense-related genes such as cysteine protease (Yang et al., 2005),

copper/zinc superoxide dismutase (Bowler et al., 1992), calcium-dependent protein kinase (Subbaiah et al., 2000), Na^+/H^+ antiporter (Yang et al., 2005) and SOS2 (salt overly sensitive 2) (Zhu, 2000). These genes are known to be involved in the signal network and defense system that copes with environmental stresses.

Our reverse northern blotting analyses using salt-stressed *M. truncatula* cDNAs showed differences in expression e.g. Mt94 (ribulose-1,5-bisphosphate carboxylase), Mt146 (chloroplast outer envelope protein translocator Toc1), Mt33 (GSLTPGH30ZA04 of hormone treated), Mt18 (Lox gene), Mt133 (glyoxysomal citrate synthase), Mt29 (*Vigna unguiculata* CPRD49) and Mt11 (*Pisum sativum* lipoxygenase), suggesting that the enhanced resistance of *M. truncatula* L. to environmental stresses stemmed not only from the expression of novel genes, but also from altered expression of genes implicated in defense. We proposed to test the efficacy of these genes at protecting *M. truncatula* or alfalfa against salt stress when we have completed a study of over-expression and promoter analysis. Some of the undefined clones, that is, those without a matching sequence in the NCBI database, may be unknown defense-related genes present under salt-stress conditions but not under normal conditions. These unknown transcripts offer an opportunity to study the unique defense mechanisms of *M. truncatula* L., and consequently, develop new strategies for alfalfa molecular breeding.

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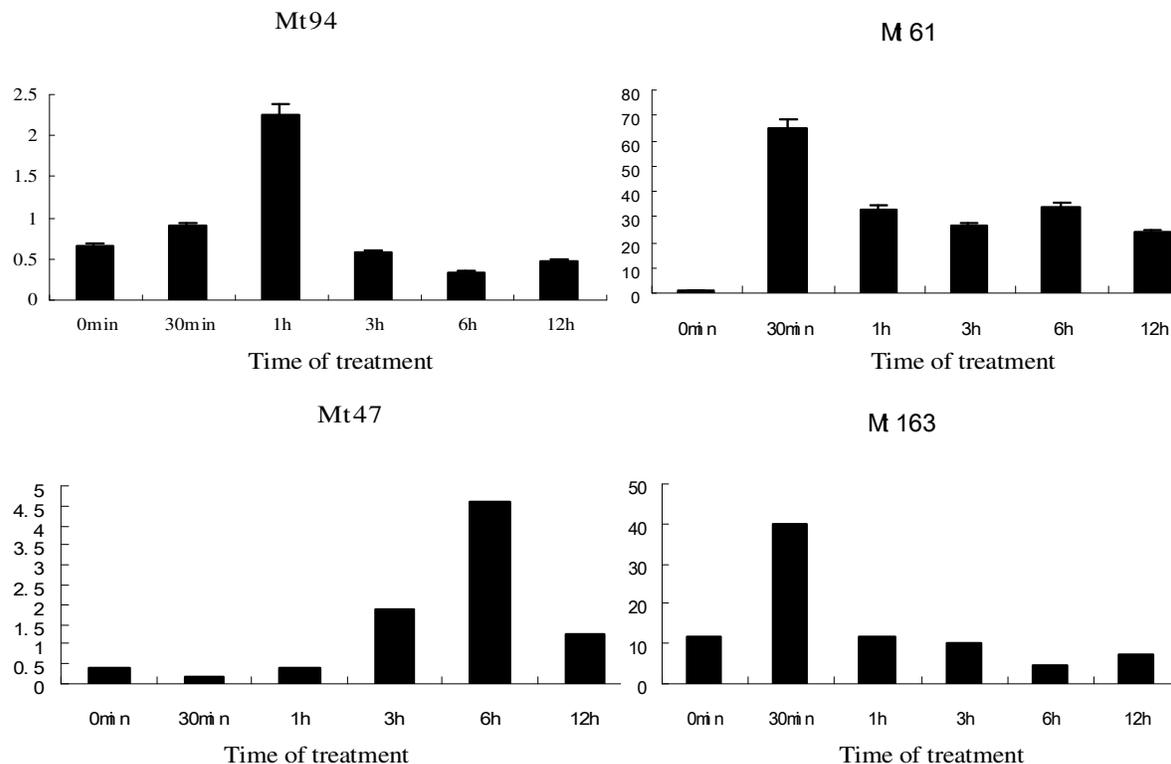


Figure 4. Real-time PCR analysis of expression patterns of four ESTs substantially induced in the salt-induced library. The four ESTs were designated Mt94, Mt61, Mt47 and Mt163. Fifteen-day-old *M. truncatula* seedlings were induced by salt for 0, 30 min, 1, 3, 6 and 12 h. The relative expression level was related to tendency of gene expression. The relative expression was calculated as: $2^{-\Delta Ct} = 2^{-(Ct_t - Ct_r)}$. Ct: cycle threshold; Ct,t: cycle threshold of target gene; Ct,r: cycle threshold of 18S rRNA.

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