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Molecular Analysis of the TLP18.3 Gene in Response to Abiotic Stresses in *Arabidopsis thaliana*

Mohammad Israil Ansari[1*] • Jaime A. Teixeira da Silva[2]

[1] Amity Institute of Biotechnology, Amity University Uttar Pradesh, Lucknow Campus, Lucknow, India
[2] Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Kagawa, 761-0795, Japan

Corresponding author: * ansari_mi@hotmail.com, miansari@amity.edu

ABSTRACT

The *Arabidopsis thaliana* thylakoid lumen 18.3 kDa protein (TLP18.3) gene has a domain of unknown function (DUF477), which is a family of uncharacterized proteins. We have investigated the expression pattern of the TLP18.3 gene in response to different abiotic stresses. The TLP18.3 gene was exclusively up-regulated when plants were exposed to high salt, drought, strong light and methyl viologen while, with treatment with cold and osmotic stress, it was down-regulated. Furthermore, a *A. thaliana* TLP18.3 T-DNA insertion mutant (SALK_109618) and wild type plants were examined for water loss. In post-germination, root length was measured during stress condition both in wild and mutant plants. As a result, in the TLP18.3 T-DNA insertion mutant plants, root length was much more affected than wild type, so water loss was greater than in wild type plants. Taken together, these results suggest that the TLP18.3 gene is involved in the response of *A. thaliana* plants to abiotic stresses.

Keywords: *Arabidopsis thaliana*, gene regulation, thylakoid lumen protein

INTRODUCTION

Abiotic stresses are a major environmental threat to agricultural production throughout the world. Environmental abiotic stresses such as drought, salinity and cold cause adverse effects on plant growth and development. During the response and adaptation to adverse abiotic stresses, many stress-related genes are up-regulated, and a variety of stress-resistant proteins are accumulated (Suzuki *et al.* 2005; Partelli *et al.* 2009, 2010). A complex network of signaling is responsible for a plant's adaptation to such adverse environmental conditions. Plants respond and adapt to these stress in order to survive. These stresses induce various biochemical and physiological changes, including growth inhibition, to acquire stress tolerance. The adaptation by plants to abiotic stresses is a complex biological process that involves changes in gene expression (Bartels and Sunkar 2005; Umezawa *et al.* 2006). A number of genes have been described that respond to abiotic stresses at the transcriptional level (Skriver and Mundy 1990; Bray 1991; Yamaguchi-Shinozaki and Shinozaki 2006; Sunkar *et al.* 2007; Jung *et al.* 2010). Although various genes are induced by these stresses, many stress-down-regulated genes have also been reported (Shinozaki and Yamaguchi-Shinozaki 2000; Ramanulu and Bartels 2002). It is critical to study the function of abiotic stress-induced genes to know their molecular mechanism(s). *Selaginella tarmariscina* is a primitive vascular plant that can remain alive in a desiccated state and is resurrected when water becomes available (Liu *et al*. 2008). Our group has cloned several genes for abiotic stress (dehydration) in *S. tarmariscina* through differential display (Liu *et al.* 2008). One of the genes (GenBank Accession No. DQ471954) has 68% homology with *A. thaliana* thylakoid lumen 18.3 kDa protein gene (TLP18.3 gene, At1g54780). The *A. thaliana* TLP18.3 gene has a domain of unknown function (DUF477), which is in a family of uncharacterized proteins. It is difficult to work on *S. tarmariscina* as little information is available on this plant. *A. thaliana* is frequently used as a model organism in plant biology as its genome is completely sequenced. In this study we have analyzed the mRNA expression patter of the TLP18.3 gene in response to abiotic stresses as well as the effect of germination and water loss in an *A. thaliana* TLP18.3 T-DNA insertion mutant and wild type (WT) plants to understand the function of the TLP18.3 gene.

MATERIALS AND METHODS

Plant material and growth conditions

A. thaliana ecotype Columbia WT and *A. thaliana* TLP18.3 T-DNA insertion mutant plants (SALK_109618 obtained from Arabidopsis Biological Resource Centre, Ohio State University but donated by Dr. Tsan-Piao Lin, Institute of Plant Biology, National Taiwan University, Taipei, Taiwan) were used in this study. *A. thaliana* TLP18.3 homozygous T-DNA insertion mutant plants were discovered using PCR with primers from the left and right borders of T-DNA and with a primer from the flanking region. Plants were grown at 22°C under long-day conditions (16-h photoperiod at 300 µmole photon/m^2/s) aseptically or on soil. For soil growth, seeds were sown in Bio-Mix Potting Substratum (Tref group, Netherlands) and placed at 4°C for 4 days in the dark to break residual dormancy and later transferred to normal growth conditions. For aseptic growth condition, seeds were treated with 70% ethanol for 5 min and then with 30% household bleach (sodium hypochloride) for 15 min, washed 10 times with sterile double distilled water and plated on MS medium (Murashige and Skoog 1962) solidified with 0.8% agar (Gelidium).

Application of abiotic stresses

To monitor the effect of stresses, plants were grown under sterile conditions in liquid MS medium on filter paper. For drought treatment, filter paper was removed from the plate and dehydrated in a growth chamber and for the stress treatment, filter paper was removed from the plate and placed in a solution containing 75 mM NaCl, 100 µM methyl viologen, or 75 mM mannitol. For the cold treatment (4°C), plants were placed at 4°C for a designated period

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(0, 6, 12 and 24 h). Strong light stress was accompanied by exposure to illumination at 300 µmol photon/m^2/s. The 15-days-old plants were exposed to various stress treatments for various time periods (0, 6, 12 and 24 h) and the treated leaves were cut from the plants, frozen in liquid nitrogen and stored at -80°C until further use.

RNA isolation, reverse transcriptional PCR and RNA gel blot analysis in response to various abiotic stresses

Total RNA for RT-PCR was isolated from 3-weeks-old leaves of *A. thaliana* by the REzol reagent kit (PROtech Technology, Taiwan) according to the manufacturer's instructions. cDNA synthesis (using SuperScript III Reverse Transcriptase) was performed as described by the supplier (Invitrogen). The cDNA was diluted 25 times and used to amplify the TLP18.3 gene using gene-specific primers (GeneBank Acession No. NM_104353), Th-1 (5′-ATG GAGCCCTTCTCTCCCCTCGTGC-3′) and Th-2 (5′-TTACTT CCTGGAGACATAAGCAAAGT-3′). For RNA gel blot analysis of different stress conditions, RNA was analyzed on a freshly prepared 1.2% formaldehyde agarose gel. After electrophoresis, the RNA was transferred from an agarose gel to a positively charged nylon membrane (Boehringer Mannheim GmbH, Mannheim, Germany). Hybridization was performed at 65°C in the FastHyb-hybridization solution (BioChain Institute, USA) with DIG-labeled *A. thaliana* TLP18.3 full-length cDNA using the DIG Luminescent Detection Kit (Roche, Germany). Signals were captured on a LXA3000 Image System (Fiji) after 2 h of exposure.

A thaliana TLP18.3 T-DNA insertion mutant verification

A. thaliana TLP18.3 T-DNA insertion mutant lines were obtained from the Arabidopsis Biological Resource Centre, Ohio State University (SALK_109618). The *A. thaliana* TLP18.3 homozygous T-DNA insertion mutant plants were discovered using PCR with primers from left (5′-TACCAACCGGTTCCGGATCA-3′) and right border (5′-GAAGCTTTGTCTGATCTTGA-3′) of T-DNA and a primer from the flanking region (5′CACTGTCCGAAAGCT CACC-3′). The PCR products were further analyzed by DNA sequencing to confirm T-DNA insertion in the gene.

Water loss measurement in *A. thaliana* TLP18.3 T-DNA insertion mutant and in WT plants

To evaluate water loss, rosette leaves of the TLP18.3 mutant of *A. thaliana* and WT plants that were grown under normal conditions for 25 days, were excised, weighed immediately and incubated on a bench at 22°C and at 60% humidity. Losses in fresh weight were monitored at 6-h intervals until 24 h.

Root length measurement under different abiotic stress conditions in *A. thaliana* TLP18.3 T-DNA insertion mutant and WT plants

Root elongation under different abiotic stress condition (75 mM NaCl, 100 µM GA_3, 75 mM mannitol, cold at 4°C, strong light of 300 µmol photon/m^2/s with MS media) of the TLP18.3 T-DNA insertion mutant of *A. thaliana* and WT of the plants were analyzed.

DNA sequencing and computational analysis

DNA sequencing was performed by the Applied Biosystems 3730 XL DNA Analyzer. A homology search against the sequence database was performed using the BLAST program at the National Center for the Biotechnology Information, Bethesda, MD. Amino acid and nucleotide sequence were analyzed with Vector-NTI Suit 5.5 (Informax Inc., Bethesda, MD).

RESULTS

Isolation of *A. thaliana* TLP18.3 gene

A. thaliana TLP18.3 cDNA was cloned through RT-PCR using gene-specific primers and cloned into PGEMT-Easy vector. The full length cDNA (GeneBank Accession No. NM_104353) was 1163 bp long with an open reading frame of 858 bp, a 5′ untranslated region of 99 bp and a 3′ untranslated region of 206 bp. The open reading frame encodes a 285 amino acid residue with a calculated molecular mass of 31.0. *A. thaliana* TLP18.3 has a domain of unknown function (DUF477) which belongs to a family of uncharacterized proteins.

Expression analysis of *A. thaliana* TLP18.3 gene in response to various abiotic stresses

Expression of the TLP18.3 gene in response to various abiotic stresses was analyzed in rosette leaves from 2-3-week old plants by northern blot analysis (**Fig. 1**). Expression of the TLP18.3 gene gradually increased until 12 h, thereafter declined in 24 h following the 75 mM salt (NaCl) treatment (**Fig. 1A**). The expression level of TLP18.3 was elevated until 12 h and declined slightly in 24 h with drought treatment (**Fig. 1B**). The transcript level with 75 mM mannitol treatment drastically decreased in 6 h and almost disappeared in 12 h (**Fig. 1C**). The TLP18.3 transcript level remarkably increased with strong light (300 µmole photon/m^2/s) by 6 h and increased continuously until 24 h (**Fig. 1D**). Expression levels during the cold treatment were down-regulated in 6 h and there was a further decrease in transcript level until 24 h (**Fig. 1E**). The mRNA expression level gradually increased by methyl viologen treatment (**Fig. 1F**). These results show differences in the expression profile of the TLP18.3 gene under different abiotic stress conditions.

Water loss and abiotic stresses response of TLP18.3 T-DNA insertion mutant of *A. thaliana* and WT plants during post-germination growth

Water loss in the TLP18.3 T-DNA insertion mutant and WT plants were studied in detached leaves from 25-days-old plants grown in soil. The loss in fresh weight in the first 6 h was 28% in WT but was 39% in mutant plants and this difference of almost 10% was maintained until 24 h after which the loss was 70% in WT while in the mutant it was 80% in 24 h (**Fig. 2**). We examined the post-germination growth stage (root length) on MS medium containing 75 mM NaCl, 100 µM GA_3, 75 mM mannitol, strong light and cold conditions. To quantify the differences between WT and the TLP18.3 T-DNA insertion mutant in different abiotic stress conditions, the root length of plants was measured (**Fig. 3**). Under normal conditions, the root length of TLP18.3 T-DNA insertion mutant decreased almost 30%. With salt and osmotic stress, root length was greatly affected in mutant plants. However, in cold and strong light conditions, the root length was affected in both WT and in the mutant. GA_3 increased root length by 30% in WT while in the mutant the increase was 35% (**Fig. 3**).

DISCUSSION

The involvement of the TLP18.3 gene in regulation of abiotic stresses indicates that this gene plays a very important role under different stress conditions. Over the past few years, hundreds of *A. thaliana* chloroplast genes have been reported. The involvement of thylakoid lumen proteins genes in the regulation of photosynthesis has been reported by several scientists (Ifuku *et al.* 2005; Lima *et al.* 2006; Yi *et al.* 2006; Ishihara *et al.* 2007; Sirpio *et al.* 2007). A study of publically available microarray data indicated that the majority of genes encoding for the lumen protein undergo changes in diurnal expression, with the expressing peaking

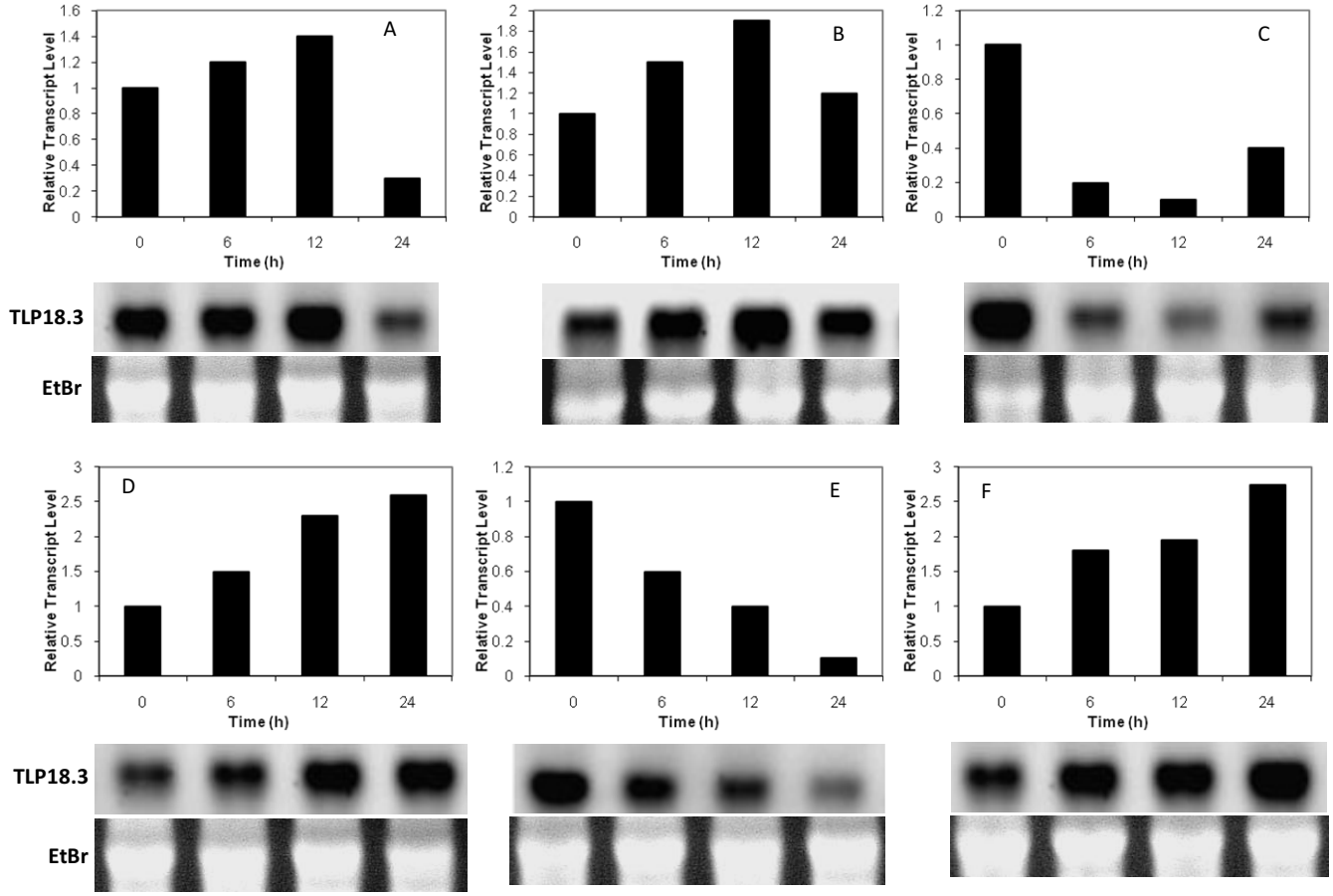

Fig. 1 Expression of *Arabidopsis thaliana* TLP18.3 mRNA level in response to the several abiotic stresses. (**A**) 75 mM NaCl, (**B**) drought, (**C**) 75 mM mannitol, (**D**) strong light (300 µmol photon/m^2/s), (**E**) cold (4°C), (**F**) 100 µM methyl viologen. 12 µg of total RNA from 15-day-old aseptically grown plants was loaded into each lane. For the dehydration treatment, the filter paper (Whatman) was removed from plate and dehydrated in the growth chamber. Plants in the strong light (300 µmol photon/m^2/s) and cold condition (4°C) treatments were simply transferred to these conditions. In the NaCl, mannitol, and methyl viologen treatments, the filter paper was transferred from the plate onto MS medium containing 75 mM NaCl, 75 mM mannitol and 100 µM methyl viologen, respectively. Samples were taken every 6 h for each treatment until 24 h. RNA gel blots were hybridized with the *A. thaliana* TLP18.3 cDNA. Ethidium bromide (EtBr) staining is shown as the loading control (0.5 µg/ml).

during the light period (Arabidopsis eFP browser; http://bar.utoronto.ca).

In this study, we report on the expression pattern of the TLP18.3 gene obtained from *A. thaliana* plants treated with various abiotic stresses such as salinity, drought, osmotic stress, cold, methyl viologen and strong light. Northern blot analysis for the expression of the TLP18.3 gene demonstrated that this gene is induced by salt, drought, strong light and methyl viologen but down regulated by osmotic stress and cold (**Fig. 1**). The response of the TLP18.3 gene to strong light and methyl viologen increased until 24 h; however, with salt and drought the increase was only until 12 h after which the transcript level decreased. In a microarray data analysis employing a stress treatment of 200 mM of mannitol, a total of 879 genes were up-regulated in *A. thaliana* while 568 genes were down regulated (Jung *et al*. 2010). In *A. thaliana*, rice, wheat, and barley several genes are rapidly induced in response to dehydration, cold and high salt (Kim *et al.* 2002; Dubouzet *et al.* 2003; Ansari *et al*. 2011). In contrast, other genes from *A. thaliana*, wheat and tomato responded poorly to drought, salinity, strong light down regulated with cold condition (Nakashima *et al.* 2000; Shen *et al.* 2003; Takenaka *et al.* 2009; Ma *et al.* 2010). Similarly, our result showed that TLP18.3 is induced by salt, drought, strong light and methyl viologen but not by cold. Treatment with methyl viologen results in the formation of reactive oxygen species in chloroplasts and is used to study general stress responses in plants (Fujibe *et al.* 2003). The TLP18.3 gene is chloroplast related so the TLP18.3 mRNA expression level was highly up-regulated by treatment with methyl viogen.

A comparative study on WT plants and TLP18.3 T-DNA insertion mutant plants for water loss in detached leaves from 25-day-old plants grown in soil showed that the loss in fresh weight in the first 6 h was 28% in WT but 39% in mutant plants; moreover, there was a 70% loss in fresh weight in 24 h in WT while in mutant the loss was 80% (**Fig. 2**). Post-germination growth on MS medium showed longer roots in WT plants than in TLP18.3 T-DNA insertion mutant plants (**Fig. 3**). As the WT plants roots were longer, so the rate of water loss was slower. A similar observation was reported in the *A. thaliana* mutant *sc17-1* (Ma *et al.* 2010). In that study, when plants both WT and mutant were exposed to abiotic stresses, the root tips were usually the first to encounter the environmental stress. Cell divisions and differentiation at the root tips were better able to respond to environmental stress conditions (Ma *et al.* 2010). The primary roots of *A. thaliana* under salt, osmotic and cold stress reduce the growth rate by reducing the number of dividing cells in the meristems, thus producing smaller mature cells without changing the duration of the cell cycle (West *et al.* 2004). To quantify the differences between WT and TLP18.3 T-DNA insertion mutants under different stress conditions, the root length of plants was measured (**Fig. 3**). Under normal conditions, the root length of TLP18.3 T-DNA insertion mutant decreased almost 30%. With salt and osmotic stress, root length was much more affected in the mutant plant. However, in response to cold and strong light the root length was effect in both WT and mutant. GA_3 increased root length by 30% in WT and by 35% in the mutant (**Fig. 3**). Exogenous application of GA_3 in mutant seedlings has been observed in *A. thaliana* with different T-DNA

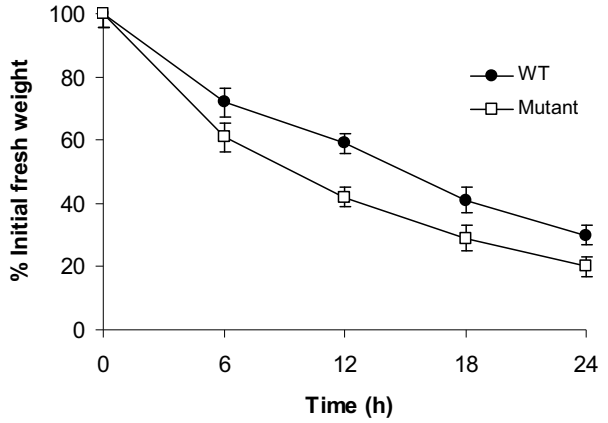

Fig. 2 Water loss in wild-type and *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant plants. Detached leaves from 25-day-old plants grown on soil were incubated on a bench and the fresh weight was measured at 6-h intervals until 24 h. Water loss is expressed as a percentage of initial fresh weight of detached leaves. Values indicate the mean ± SE of 3 experiments.

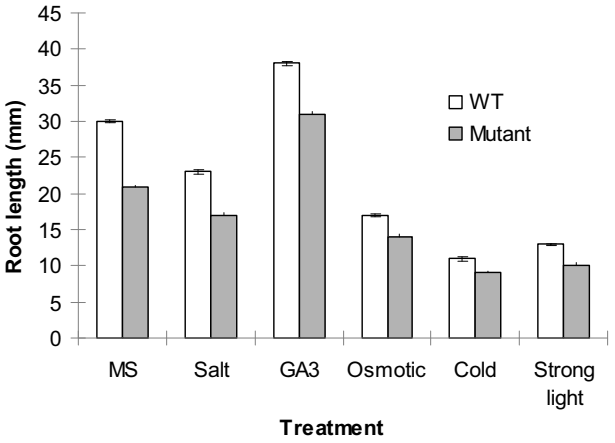

Fig. 3 Root elongation of wild type and *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant plants under normal and stressed conditions (MS, 75 mM NaCl, 100 µM GA_3, 75 mM mannitol, cold (4°C), strong light (300 µmol photon/m^2/s). Values indicate the mean ± SE of 3 experiments.

insertion mutants (Groot and Karssen 1987; Nonogaki *et al.* 2000; Yamaguchi and Kamiya 2002) All studies reported an increase in root length growth with the application of 100 µM GA_3.

In the present study, we investigated the expression of the TLP18.3 gene following the application of abiotic stresses by northern blot analysis. TLP18.3 gene expression was significantly induced by salt, drought, strong light and methyl viologen, suggesting that this gene is related to the abiotic stress response. Furthermore, in the *A. thaliana* TLP18.3 T-DNA insertion mutant, root length was strongly affected compared to the WT; consequently, water loss was higher than in WT plants. Taken together, these results suggest that the TLP18.3 gene is strongly involved in the response to abiotic stresses in *A. thaliana*.

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