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Arabidopsis thaliana thylakoid lumen 18.3 protein (TLP 18.3) gene regulate developmental process

Mohammad Israil Ansari[1] and Tsan-Piao Lin[2]

1 Amity Institute of Biotechnology, Amity University Uttar Pradesh, Lucknow Campus, Lucknow-226 010, India

2 Institute of Plant Biology, National Taiwan University, Taipei, Taiwan

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CORRESPONDENCE

Dr. Mohammad Israil Ansari, Ph.D., Amity Institute of Biotechnology, Amity University Uttar Pradesh, Lucknow Campus, Lucknow-226 010, India

E-mail: ansari_mi@hotmail.com, miansari@amity.edu, Tel.: ++91-983-954-1698, Fax: ++91-522-2721-934

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ABSTRACT

Arabidopsis thaliana thylakoid lumen 18.3 kDa protein (TLP18.3) gene (At1g54780) has a domain of unknown function, which is a family of uncharacterized protein. To examine the developmental regulation of this gene, *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant (SALK_109618) and wild type plant were observed for the effect of ABA, NaCl and mannitol on the germination of seeds. 50 mM NaCl inhibit more germination rate in mutant plants than wild type plants followed by 0.1 μM ABA and 20 mM mannitol. But in control condition also mutant plants have less (54%) germination rate than the wild type plant which having germination (77%). *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant plants (SALK_109618) have shown 6-9 days of flowering delay but after *Agrobacterium* mediated transformation of *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant with pPZP200GB-TLP18.3 construct rescued the flowering delay and germination. The translational analysis have shown that the TLP18.3 protein accumulation wild type plant of *Arabidopsis thaliana* and it was not detected in western blot analysis of *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant demonstrated that this protein was absent in mutant plants. For the subcellular localization of *Arabidopsis thaliana* TLP18.3, the protoplast of transformed *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant after *Agrobacterium* mediated transformation with pPZP200GB-TLP18.3 construct which is having GFP green fluorescence image, which was completely overlapped with red autoflorescence of chloroplast image clearly showed the chloroplast localization of TLP18.3.

Introduction

Plants have a number of mechanisms for respond to the changeable environmental condition. For many years the thylakoid lumen was believed to be the deprived of proteins, apart from those involved in photosynthetic reaction such as oxygen-evolving complex protein, plastocyanin and vilaxanthin de-epoxidase (Sirpio *et al,* 2008). Recently it has been reported that major portion of the lumen proteome exhibits increased protein expression in light-adapted as opposed to dark-adapted (Granlund *et al.* 2009). The chloroplast genomes of plants have retained fewer than 100 protein-coding genes, many of which are necessary for expression of an even smaller subset of chloroplast encoded photosynthesis genes. A number of nuclear genes are also involved in expression of the chloroplast genome at multiple levels, including transcription, mRNA maturation, translation, targeting, and assembly of complexes that comprise a functional photosynthetic electron transport system (Jung *et al.* 2010).The chloroplast is indispensable for the growth and development of plants. Essential biochemical reactions are carried out in the chloroplast, ranging from photosynthesis and carbon fixation to nitrogen assimilation and amino acid biosynthesis (Givan and Leech 1971). Most of chloroplast proteins are encoded by the nucleus and synthesized in the cytosol before being transported into the chloroplast (Inaba and Schnell 2008), so it is unsurprising that many mutants specifically disrupted in chloroplast biogenesis had mutations on nuclear-encoded chloroplast genes with diverse molecular functions, including chlorophyll biosynthesis, thylakoid biogenesis and lipid biosynthesis, protein import, photosystem assembly, protein maturation and degradation, plastid gene expression, among others (Gutie´rrez-Nava *et al.* 2004, Waters and Langdale 2009). Therefore, identification and characterization of mutants have been helpful in elucidating the mechanism of photosynthetic development and in understanding nucleus–plastid interactions. In our study we have used *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant (SALK_109618).

Selaginella tarmariscina is primitive vascular plant, can remain alive in a desiccated state and resurrect when water become available (Liu *et al.* 2008). Our group has cloned several genes for dehydration in *Selaginella tarmariscina* through differential display. One of the gene (GenBank Accession No. DQ471954) which has 68% homology with *Arabidopsis thaliana* thylakoid lumen 18.3 kDa protein gene (At1g54780). *Arabidopsis thaliana* TLP18.3 gene has domain of unknown function (DUF477) which is family of uncharacterized proteins. It is hard to work on *Selaginella tarmariscina* as less information are available regarding molecular biology of this so, decided to work on *Arabidopsis thaliana* as this genome is sequenced. We have already reported that this protein is localized into chloroplast (Ansari et al. 2011).

With the accomplishment of the *Arabidopsis* genome sequencing project and multiple proteomic studies localizing unknown proteins to different chloroplast compartments have established a basis for identification of novel proteins possibly associated with the dynamics of the PSII complex (Friso *et al.* 2004, Peltier *et al.* 2002, Schubert *et al.* 2002). Microarray analysis has shown that the mRNA-expression of genes encoding several subunits of both photosystems are under circadian control (Harmer *et al.* 2000) and that, 23% of photosynthesis genes exhibit 1.75 fold diurnal expression changes (Blasing *et al.* 2005). Plants are found to exhibit increased photosynthesis, growth, survival and competitive advantages when there is synchronization between the circadian clock and light-dark cycles (Dodd *et al.* 2005).

In this study we have observed that germination rate was inhibited by 50 mM NaCl more followed by 0.1 µM ABA and 20 mM mannitol in mutant plants. Further mutant plants have shown 6-9 days of flowering delay but after *Agrobacterium* mediated transformation of *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant with pPZP200GB-TLP18.3 construct rescued the flowering delay and germination. For the localization of *Arabidopsis thaliana* TLP18.3, the protoplast of transformed *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant clearly showed the chloroplast localization of TLP18.3.

Materials and Methods

Plant material and growth conditions: *Arabidopsis thaliana* ecotype Columbia wild type and *Arabidopsis thaliana* TLP18.3 homozygous T-DNA insertion mutant plants (SALK_109618 obtained from ABRC, Ohio State University) were used in this study. The homozygous *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant plants were find out using PCR with primer from left and right border of T-DNA and primer from flanking region. Plants were grown at 22° C for long day condition (16 h light / 8 h dark cycle) 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 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 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. For seed germination solid MS medium was used.

Plasmid construction and *Arabidopsis* transformation: Coding region of *Arabidopsis thaliana* TLP18.3 gene (GeneBank Accession No. NM_104353) was cloned into binary vector pPZP200GB using *XbaI* and *BamHI* restriction enzymes. This pPZP200GB with *β-glucuronidase* and BAR (BASTA resistance gene) cassettes was derived from pBI221 (Clontech Laboratories, Palo Alto, CA) and pSK-35S-BAR (Chu *et al.* 2005) (Fig.1). The obtained plasmid construct was named pPZP200GB-TLP18.3. This binary vector has spectinomycin resistance for *E. coli* and glufosinate resistance for plant. The pPZP200GB-TLP18.3 construct was transformed into *Agrobacterium tumefaciens* strain C-58 by electroporation. *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant (SALK_109618) plants were transformed through *Agrobacterium* mediated transformation by floral dipping method (Clough and Bent 1998). Transgenic plants were selected by spraying seedlings at 7, 9 and 11 days after germination with a solution of 0.4% of BASTA herbicide (McDowell *et al.* 1998). T2 generations were selected for isolating homozygous lines.

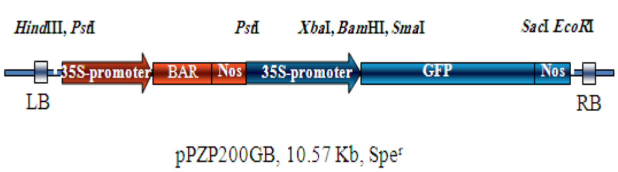

Fig. 1. Schematic representation of the pPZP200GB vector showing double 35S promoter, multiple cloning site, BAR (BASTA resistance gene), GFP (green fluorescent protein) and Nos terminator. This binary vector has spectinomycin resistance for *E. coli* and gulfosinate resistance for plants

Plasmid construction for fusion protein purification: The coding region of *Arabidopsis thaliana* TLP18.3 gene (GeneBank Accession No. NM_104353) was cloned into the expression vector pET-15b (Novagen). Over-expression of the recombinant TLP18.3 protein in *E. coli* BL21(DE3) was induced by the addition of 1 mM IPTG. To prepare TLP18.3 protein in large scale for antibody induction in rabbit, the cells were grown at 37 °C and the recombinant protein was produced as inclusion bodies. The (His)6-tagged protein was then purified by a nickel affinity column (His-bind kit, Novagen, Madison, WI, USA) and eluted with the buffer containing 400 mM imidazole under the denaturing condition.

Production and purification of anti TLP18.3 antibody: The partially purified protein was further subjected to preparative gel electrophoresis on an SDS-polyacrylamide gel (14%). The recombinant TLP18.3 protein band was excised and electroeluted, and the resultant protein solution was concentrated by a Centricon -30 concentrator. Polyclonal antibodies specific to TLP18.3 were raised in a New Zealand White rabbit with 500 µg of the purified TLP18.3 protein. The antiserum was subjected to ammonium sulfate precipitation at 30% saturation. The resultant antibodies were dissolved in phosphate-buffered saline (pH 7.0) and stored at -70 °C.

Protein extraction from *Arabidopsis thaliana* leaf tissues and western blot analysis:
Leaves of *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant and wild type plants (14, 21 and 28 days old) were ground in liquid nitrogen and two volumes of the extraction buffer containing 150 mM Tris-HCl (pH 8.0), 5 mM EDTA, 2% β-mercaptoethanol, 0.3 M NaCl, 100 µM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, and 25% (v/v) glycerol were added as described by Ansari *et al.* 2005. The mixture was shaken for 1 h at 4 °C and centrifuged at 20,000 x *g* for 30 min. The supernatant was collected for protein quantification and western blot analysis as described by To *et al.* (1996). The protein extracts equivalent to 30 mg of fresh tissue were separated by SDS-PAGE (14% polyacrylamide). The gels were either stained with Coomassie brilliant blue (R-250) and electroblotted on to a Protran BA85 nitrocellulose membrane (Schleicher & Schuell Inc, Keene, NH, USA). The blot was incubated with anti-TLP18.3 antibodies and later incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG followed by color development with 4-chloro-1-naphthol.

DNA sequencing and computational analysis: DNA sequencing was performed by the Applied Biosystems 3730 xl DNA Analyzer. 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).

Isolation of *Arabidopsis thaliana* protoplast for GFP fusion protein localization:
Arabidopsis thaliana protoplast was isolated as described by Kang *et al.,* 1998 with some modifications. 2-3 g leaves of *Arabidopsis thaliana* 5 week old plants grown in soil were taken and lower epidermis of the leaf was removed and incubated in 20 ml enzyme solution (1% Macerozyme R-10, 1% Cellulase R-10, 400 mM Mannitol, 8 mM CaCl2 and 5 mM MES-KOH pH 5.6) at 22° C for 5 h with gentle shaking only 3-4 times. After incubation, the protoplast suspension was filtered through Miracloth and protoplasts were collected by centrifugation at 100 g for 5 min. The pelleted protoplasts were suspended in 5-10 ml of W5 solution (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 5 mM Glucose and 1.5 mM MES-KOH, pH 5.6), overlaid on the top of 20 ml of 21% sucrose, and centrifuged for 10 min at 100 g. The intact protoplasts at the interface were transferred to tube containing 20 ml of W5 solution. The protoplasts were pelleted again by centrifugation at 100 g for 5 min and resuspended in 20 ml of W5 solution at a density of 3-5x10^6 protoplasts/ml. The protoplasts were incubated on ice for 30 min. Fluorescent signals were analyzed using a Leica TCS SPII, confocal laser scan microscope (Leica Microsystems, Germany).

Results

Germination and flowering of *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant and wild type plants: In order to investigate the functions of *Arabidopsis thaliana* TLP18.3 gene, we have homozygous *Arabidopsis thaliana* TLP18.3 mutant

(SALK_109618), caused by T-DNA insertion in the second exon. We have examined the seed germination on MS media containing 0.1 µM ABA, 50 mM NaCl, 20 mM mannitol and control (water) separately has shown 38, 20, 38 and 54% germination in *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant plants whereas in wild type plants retained 65, 55, 65 and 77% germination respectively in first 24 hours (Fig. 2). Later with time course the germination was almost maximum after 30 hours treatment, and it reaches maximum germination 48, 25, 48 and 71% with 0.1 µM ABA, 50 mM NaCl, 20 mM mannitol and control (water) in *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant plants whereas in wild type plants retained 86, 68, 86 and 92% germination respectively in first 48 hours (Fig. 2). Mutant plants were hypersensitive to ABA, NaCl and mannitol at germination stage to reduce the germination but it was lowest with 50 mM NaCl treatment (Fig. 2). Further for the evaluation of visible phenotypes, we have taken 100 plants from each wild type and *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant and were grown in the same pot. Compare to the wild type *Arabidopsis thaliana* TLP18.3 mutant showed a delay in flowering for 6-9 days under normal growth conditions (Fig. 3). Probably delayed flowering in mutant was defect in developmental regulation. *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant after transformation with pPZP200GB-TLP18.3 construct revert back to the normal flowering (Fig. 4). This confirmed the role of TLP18.3 in delayed flowering.

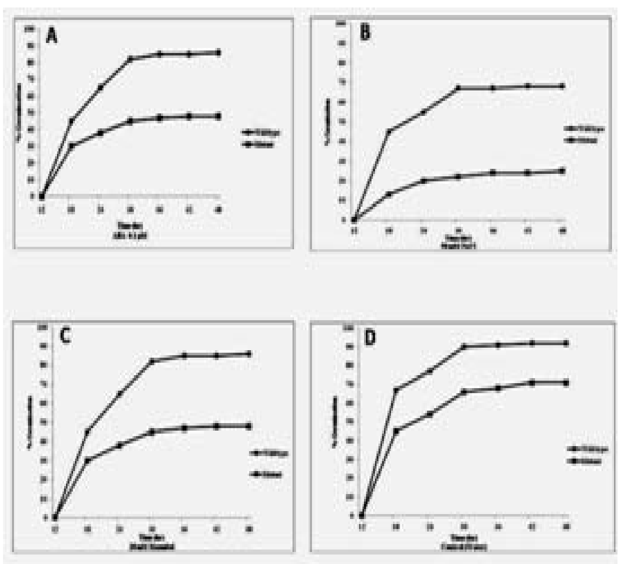

Fig. 2. Effect of ABA, NaCl, Mannitol and Water (control) on seed germination of *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant (SALK_109618) and wilt type plants

Fig. 3. *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant (SALK_109618) plant showing delayed flowering compare to the wild type plant in one month old plant

Fig. 4. *Agrobacterium* mediated transformation of *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant (SALK_109618) with pPZP200GB-TLP18.3 construct rescued the flowering delay of SALK_109618 mutant (one month old plant). Mutant - *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant, Wild type - *Arabidopsis thaliana* wild type, Mutant transformed - TLP18.3 T-DNA insertion mutant transformed with pPZP200GB-TLP18.3 construct

Expression analysis of *Arabidopsis thaliana* TLP18.3 protein in *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant and wild type plants: To study the role of *Arabidopsis thaliana* TLP18.3 protein expression, 14, 21 and 28 days old plants leaves samples were used. To confirm presence of TLP18.3 protein in wild type plants, western blot analysis was performed in both wild and TLP18.3 T-DNA insertion mutant. The protein express very well in 14, 21 and 28 days old wild plants of *Arabidopsis thaliana* but in TLP18.3 T-DNA insertion mutant there was no protein expression (Fig.5). In wild type plants the protein expression was almost same in 14, 21 and 28 days of leaf sample but in 7 days old plant the protein expression level was at basal level (data not shown).

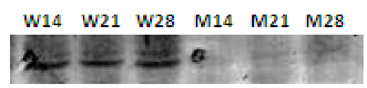

Fig. 5. Changes in *Arabidopsis thaliana* TLP18.3 protein levels in *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant and wild type plants. For western immunoblot analysis, the protein extract equivalent to 30 mg of fresh leave of 14, 21 and 28 days were separated by SDS-PAGE (14% polyacrylamide). The membrane was incubated with anti-TLP18.3 antibodies. W14 refers to wild type plant of 14 days old, W21 refers wild type plant of 21 days W28 refers wild plant of 28 days, M14 refers to T-DNA insertion mutant plant of 14 days old, M21 refers T-DNA insertion mutant of 21 days, M28 refers T-DNA insertion mutant of 28 days

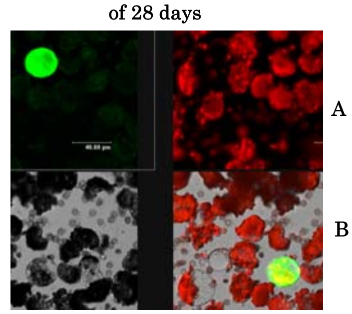

Fig. 6. Localization of *Arabidopsis thaliana* TLP18.3: *Agrobacterium* mediated transformation of *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant (SALK_109618) with pPZP200GB-TLP18.3 construct protoplast was isolated and analysed under confocal microscope, using a Leica TCS SPII, confocal laser scan microscope (Leica Microsystems AG, Wetzlar, Germany), *Arabidopsis thaliana* TLP18.3-GFP fusion protein transported into chloroplast. A- Green- GFP florescence, Red- chlorophyll autofluorescence, respectively (upper panel). B- The right-most photo of yellow color (lower panel) showing the merged image of green and red.

Localization of TLP18.3 in transformed *Arabidopsis thaliana* T-DNA insertion mutant

According to the prediction of Aramemnon database (Schwacke *et al.,* 2003) the chloroplast transit peptide (cTP) targeting sequence are located at the N-terminal of TLP18.3 protein sequence. To investigate the localization of *Arabidopsis thaliana* TLP18.3 gene, the pPZP200GB-TLP18.3 construct transformed *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant (SALK_109618) protoplast was isolated and analysed under confocal microscope, using a Leica TCS SPII, confocal laser scan microscope (Leica Microsystems AG, Wetzlar, Germany). As shown in Fig. 6 the expression of the *Arabidopsis thaliana* TLP18.3-GFP fusion protein transported into chloroplast. The green- GFP florescence and autofluorescence of chlorophyll of red color, merged image of both gives yellow color confirm its localization into chloroplast.

Discussion

Past few years, hundreds of *Arabidopsis thaliana* chloroplast proteins have been reported and these are localized in the inner envelop of chloroplast membrane. Thylakoid lumen proteins have been reported in regulation of photosynthesis by several scientists (Ishihara *et al.* 2007, Sirpio *et al.* 2007, Yi *et al.* 2007, Lima *et al.* 2006). A study of public available microarray data indicated that majority of the genes encoding for the lumen protein under go diurnal expression changes, with the express peaking during the light period (Arabidopsis eFP browser, bar.utoronto.ca,). We have examined the effect of ABA, NaCl and mannitol on the germination of seeds for the developmental regulation of TLP18.3 gene. 0.1 uM ABA inhibit the germination rate more in *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant plant than the wild type (38% and 65% respectively in 24hrs), 50 mM NaCl inhibit more germination rate in mutant plants than wild type (20% and 55% respectively in 24 hrs) with 20 mM mannitol the germination rate was same as ABA treatment. But in control condition also mutant plants have 54% germination rate than the wild type plant which having germination 77% (Fig. 2). The germination rate goes down with ABA and NaCl treatment because ABA, whose level increases with high concentration of NaCl and several studies have shown that ABA plays a role in the seed germination inhibition process (Werner and Finkelstein 1995). Probably TLP18.3 have role in germination, this is reason mutant have low germination rate than the wild type plants. Further we have done the evaluation of the visible phenotype under the standard growth conditions in *Arabidopsis thaliana* TLP18.3 mutant (SALK_109618) and we have the same finding regarding the visual phenotypes as reported by Siprio *et al.* 2007, except 6-9 days delayed in flowering in TLP18.3 T-DNA insertion mutant (Fig. 3). Probably delayed flowering in *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant (SALK_109618) was defect in the developmental processes regulation. In complementation analyses, *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant (SALK_109618) after *Agrobacterium* mediated transformation with pPZP200GB-TLP18.3 construct, the mutant plant rescued the flowering delay (Fig. 4). The germination rate was also recovered in mutant plants (data not shown). It proved that the delay in flowering and germination was due to the TLP18.3 gene of *Arabidopsis thaliana.*

The temporal profile of TLP18.3 protein accumulation of wild type plant of *Arabidopsis thaliana* and it was not detected in western blot analysis of *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant (Fig. 5) demonstrated that this protein was absent in mutant plants. Though we have taken the leaf sample of both wild type and mutant types at 14, 21 and at 28 days. For the localization of *Arabidopsis thaliana* TLP18.3, the protoplast of transformed *Arabidopsis thaliana* TLP18.3 T-DNA insertion mutant (SALK_109618) after *Agrobacterium* mediated transformation with pPZP200GB-TLP18.3 construct which is having GFP green fluorescence image, which was completely overlapped with red autoflorescence of chloroplast image clearly showed the chloroplast localization of TLP18.3 (Fig. 6). Present study shows that the *Arabidopsis thaliana* TLP18.3 gene has important role in germination, flowering and is localizes into chloroplast.

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