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Full Length Research Paper

Arabidopsis thaliana thylakoid lumen 18.3 protein gene is up-regulated during dehydration

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Selaginella tarmariscina **is a resurrection plant and we have cloned several genes for dehydration in** ***S. tarmariscina*** **through differential display. One of the gene (DQ471954) which has 68% homology with** ***Arabidopsis thaliana*** **thylakoid lumen 18.3 kDa protein (TLP18.3) gene. This gene has a domain of unknown function (DUF477) which is a family of uncharacterized protein. The expression pattern of the** ***A. thaliana*** **TLP18.3 reveals that mRNA expression and protein accumulation up-regulated during dehydration. The protein coding region of** ***A. thaliana*** **TLP18.3 gene consist of 3 exons and 4 introns encoding 285 amino acid with putative chloroplast transit peptide (cTP) target sequence at N-terminal. GFP fusion protein with N-terminal 80 amino acid residue reveals that this fusion protein is localized into chloroplast. Our data strongly suggest that** ***A. thaliana*** **TLP18.3 gene has important role in dehydration process.**

Key words: *Arabidopsis thaliana*, dehydration, gene regulation, TLP18.3, thylakoid lumen.

INTRODUCTION

Plants have the capability to adopt adverse environmental conditions such as drought, cold and high salt. Plant growth is affected adversely by different environmental abiotic stresses such as cold, salinity, drought stress and fluctuations in incident light (Partelli et al., 2009; Partelli et al., 2010). Drought is the major environmental threat to the agricultural production through out the world. 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. Adaptation by plants to dehydration stress is a complex biological process that involves the changes in gene expression. Among the external stresses, drought is a major limitation for the crop productivity (Bartels and Sunkar, 2005; Umezawa et al., 2006). Tolerance of drought is a complex phenomenon, because it changes according to drought intensity and duration, and the plant's developmental stage during which drought occurs. Also, more than one stress may affect the plant simultaneously, activating many genes in the stress response. Plant acclimation to stresses in general, including drought, induces, at least to some extent, common reactions, such as signaling pathways, target-gene expression, and bio-chemical/metabolic changes. As a result, differences in water-stress tolerance among cultivars, or within a cultivar at various developmental stages, may result from differences in the expression of genes in signal-perception and transduction mechanisms (Chinnusamy et al., 2004). A number of genes have been described that respond to the dehydration at 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 are also reported (Ramanulu and Bartels, 2002; Shinozaki and Yamaguchi-Shinozaki, 2000). So it is critical to study the function of stress induced genes to know the molecular mechanism of stress tolerance. *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

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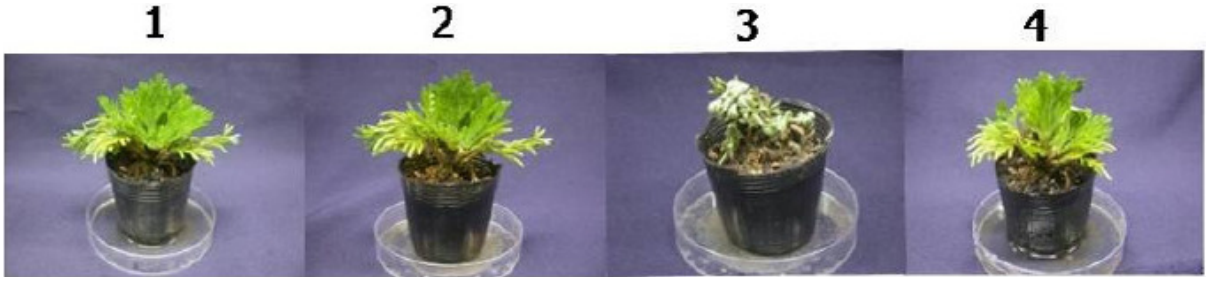

Figure 1. Resurrection of *S. tarmariscina* during dehydration and re-watering cycle. 1-Control; 2-dehydration for 2 days; 3-dehydration for 5 days; 4-after re-watering for 7 h.

genes for dehydration in *S.tarmariscina* through differential display. One of the gene (GenBank Accession No. DQ471954) which is up-regulated during dehydration has 68% homology with *Arabidopsis thaliana* thylakoid lumen 18.3 kDa protein gene (TLP18.3 gene, At1g54780). *A. thaliana* TLP18.3 gene has domain of unknown function (DUF477) which is family of uncharacterized proteins. It is hard to work on *S. tarmariscina* as less information are available regarding molecular biology of this so, decided to work on *A. thaliana* as this genome is sequenced.

In this study we have characterized the *A. thaliana* thylakoid lumen 18.3 protein gene (TLP18.3). *A. thaliana* TLP18.3 gene is up-regulated during dehydration and localizes into chloroplast.

MATERIALS AND METHODS

Cloning of *S. tarmariscina* gene for dehydration

S. tarmariscina is a resurrection plant, our group has cloned several genes for dehydration in *S. tarmariscina* through differential display during dehydration treatment (Liu et al., 2008). Resurrection of *S. tarmariscina* during dehydration and re-watering cycle is shown in Figure 1.

Plant material and drought treatment

A. thaliana ecotype Columbia wild type, were used in this study. 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. MS medium was supplemented with 2% sucrose. For drought treatment, filter paper was removed from the plate and placed to dehydrate in growth chamber and RNA was isolated.

RNA isolation, reverse transcriptional PCR and RNA gel blot analysis

Total RNA for RT-PCR was isolated from 3 weeks old leaf of *A. thaliana* by REzol reagents kit (PROtech Technology, Taiwan) according to the manufacturer's instructions. cDNA synthesis (using SuperScript III Reverse Transcriptase) was done as described by the supplier (Invitrogen). The cDNA was diluted 25 times and used to amplify the TLP18.3 gene using gene specific primers, Th-1 (5'-ATGGAGCCCTTCTCTCCCCTCGTGC-3') and Th-2 (5'-TTACTTCCTGGAGACATAAGCAAAGT-3'). For RNA gel blot analysis RNA was analyzed on 1.2% formaldehyde agarose gel. After electrophoresis, the RNA was transferred from 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 DIG Luminescent Detection Kit (Roche, Germany). Signals were captured on a LXA3000 Image System (Fiji) after 2 h of exposure.

Expression of TLP18.3 protein in *Escherichia coli* and antibody production

The coding region of *A. thaliana* TLP18.3 gene was cloned into the expression vector pET-30 Ek/LIC (Novagen, Madison, WI, USA), resulting in a fusion protein of TLP18.3 with N-terminal His-tag sequences. 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. 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.

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).

Protein extraction from *Arabidopsis thaliana* leaf tissues and western blot analysis

Leaves of *A. thaliana* were grounded 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 20 to 30 mg of fresh tissue were separated by SDS-PAGE (14% polyacrylamide). The gels were either stained with Coomassie brilliant blue (R-250) or electroblotted on to a Protran BA85 nitrocellulose membrane (Schleicher and 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.

Transformation of green fluorescent protein fusion constructs into plant cells

A. thaliana TLP18.3 complete coding region and coding region with deletion of N-terminal first 80 amino acid without termination codon were cloned into 326 GFP vector (Kindly provided by Dr. Inhwang Hwang, POSTECH, Pohang, Korea). Plasmids were purified using Qiagen DNA Maxi Purification kit according to the manufacturer's protocol. The fusion constructs were introduced into *A. thaliana* protoplast by polyethylene glycol-mediated transformation with some modification in Kang et al. (1998) method. *A. thaliana* protoplast prepared from leaf tissue (2 to 3 g) of 5 week old plants grown in soil 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 to 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 to 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. The protoplasts were incubated on ice for 30 min.

To transform plasmid DNA into protoplasts, protoplasts were pelleted again at 100 g for 5 min and resuspended in MaMg solution (400 mM Mannitol, 15 mM $MgCl_2$ and 5 mM MES-KOH, pH 5.6) at a density of 3-5×10^6 protoplasts/ml. 10 µg Plasmid DNA was added to 100 µl of protoplasts suspension followed by 100 µl of PEG solution (400 mM mannitol, 100 mM $Ca(NO_3)_2$, and 40% polyethylene glycol 4000). The mixture was mixed gently and incubated for 30 min at room temperature. After incubation, mixture was diluted with 10 ml of W5 solution. Protoplasts were recovered by centrifugation at 100 g for 5 min, resuspended in 1 ml W5 solution, and incubated at 22°C in dark. Fluorescent signals were analyzed after 12 h using a Leica TCS SPII, confocal laser scan microscope (Leica Microsystems AG, Wetzlar, Germany).

RESULTS

Resurrection phenomenon of *S. tamariscina*

S. tamariscina is one of the resurrection plants, which like many other resurrection plants can remain alive in a dry state and then resume growing when water again becomes available. In this study, the resurrection phenomenon of this species was observed (Figure 1). The aerial parts were fully opened in well-irrigated plants which contained about 74% water content. After water deprivation for 2 days, the detritus matrix had almost completely dried out. The shortage of water caused the aerial parts of the plant to curl slightly, while the water content was about 43% at that point. On the fifth day of water deprivation, the aerial parts of the plant had curled tightly, and the water content had dropped to 8%. When water was again provided, the plant resurrected, and the aerial parts of the plant fully opened within 7 h under a saturated humidity condition (Liu et al., 2008).

Isolation of *Arabidopsis thaliana* TLP18.3 gene

A. thaliana TLP18.3 cDNA was cloned through RT-PCR using gene specific primers, Th-1 (5'-ATGGAGCCCTTCTCTCCCCTCGTGC-3') and Th-2 (5'-TTACTTCCTGGAGACATAAGCAAAGT-3') and cloned into PGEMT-Easy vector. *A. thaliana* TLP18.3 full length cDNA (GeneBank Accession No. NM_104353) was 1163 bp with open reading frame of 858 bp, 5' untranslated region of 99 bp and 3' untranslated region of 206 bp. Open reading frame encodes 285 amino acid residue with calculated molecular mass of 31.0 kDa. 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. This gene composed of 3 exons and 4 introns and located at Chromosome1. *A. thaliana* TLP18.3 has domain of unknown function (DUF477) which is family of uncharacterized proteins.

Expression analysis of *A. thaliana* TLP18.3 gene

To study the role of *A. thaliana* TLP18.3 gene in plants during dehydration, aseptically grown seedlings (12 days old) on filter paper were dehydrated for 2, 4, 6 and 8 h. mRNA level goes up with dehydration time course and transcript level goes down after 6 h dehydration (Figure 2A). To confirm that the up-regulation of expression of TLP18.3 transcript is relevant to the dehydration, the amount of TLP18.3 protein was also measured. This was performed by the western blot analysis of *A. thaliana* leaves as described before in materials and methods with polyclonal antibodies raised against the TLP18.3 protein. The protein expression patter was the same as in the mRNA transcript. The protein expression level reached maximum in 6 h dehydration treatment and goes down at 8 h dehydration. This indicated that TLP18.3 accumulation increase at early dehydration later this decreased.

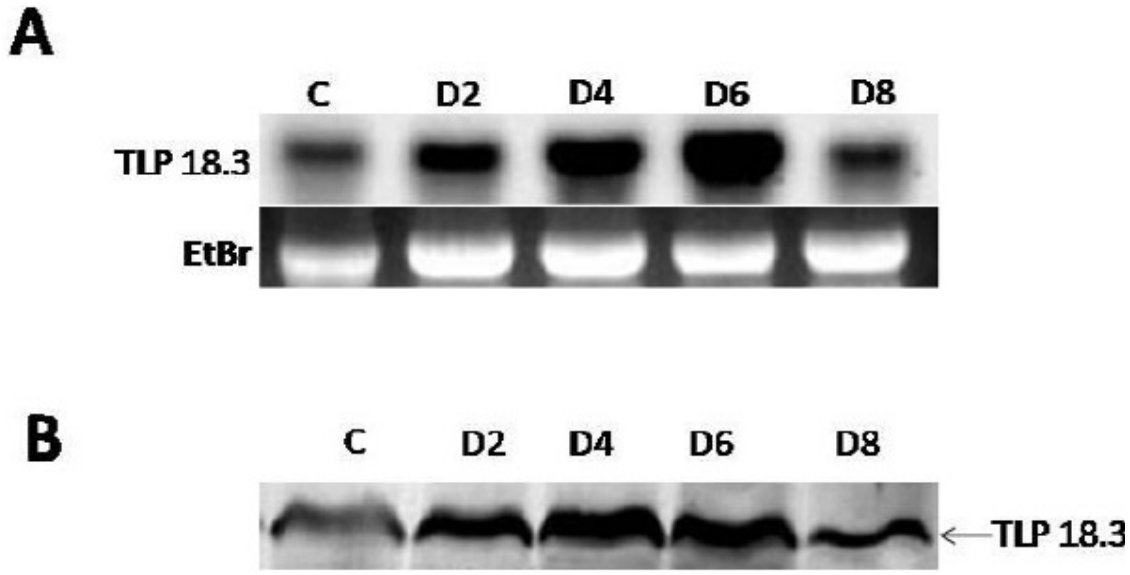

Figure 2. Changes in *A. thaliana* TLP18.3 mRNA and *A. thaliana* TLP18.3 protein levels in response to dehydration. For Northern blot analysis (Panel A), each lane was loaded 12 µg RNA from 12 days old aseptically grown plants of filter papers containing MS media and samples were separated on a 1% agarose gel. Membrane was hybridized with full-length TLP18.3 cDNA. For western immunoblot analysis (Panel B), the protein extract equivalent to 30 mg of fresh leave of 12 days old aseptically grown on filter paper containing MS media were separated by SDS-PAGE (14% polyacrylamide). The membrane was incubated with anti-TLP18.3 antibodies. C refers to control plant of 12 days old. D2 refers dehydration for 2 h, D4 refers to dehydration for 4 h, D6 refers to dehydration for 6 h and D8 refers to dehydration for 8 h. Ethidium bromide (EtBr) staining is shown as loading control.

Expression of TLP18.3 protein in *E. coli*

To express TLP18.3 protein in *E. coli,* IPTG induction of recombinant pET-30 Ek/LIC containing TLP18.3 gene was carried out. Very strong protein band became visible by SDS-PAGE after IPTG induction (Figure 3). Before IPTG induction, no fusion protein could be detected. After 3 h of IPTG induction very intense band was visible (Figure 3). The detected protein has apparent molecular weight of around 36 kDa which corresponds to the predicted molecular mass of TLP18.3 protein (31.0 kDa) plus the fused His tag (5.0 kDa).

Localization of *A. thaliana* TLP18.3

In order to investigate the localization of *A. thaliana* TLP18.3 gene, we first analyzed the peptide sequence by Aramemnon database (Schwacke et al., 2003). According to the prediction, the chloroplast transit peptide (cTP) targeting sequence was located at the N-terminal ends of the *A. thaliana* TLP18.3 peptide sequence. To confirm this prediction the complete coding region of TLP18.3 fused with 326 GFP vector and localization of fusion protein was observed under confocal microscope. The fluorescent signals were analyzed, after 12 h incubation at 22°C in dark using a Leica TCS SPII, confocal laser scan microscope (Leica Microsystems AG, Wetzlar, Germany). As shown in Figure 4, the fusion protein is localized in the chloroplast of *A. thaliana* cells where as in the control with deletion of 80 amino acid at N-terminal, the fusion protein was not present in chloroplast (data not shown). So the fusion construct revealed that the fusion protein is localized in the chloroplast of *A. thaliana* cells.

DISCUSSION

During the past few years, hundreds of *A. thaliana* chloroplast proteins have been reported and these are localized in the inner envelop of chloroplast membrane. Involvement of thylakoid lumen proteins in regulation of photosynthesis has been reported by several scientists (Ishihara et al., 2007; Sirpio et al., 2007; Ifuku et al., 2005; Yi et al., 2006; Lima et al., 2006; Plucken et al., 2002). 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,).

The temporal profile of TLP18.3 mRNA expression and protein accumulation (Figures 2A and B) demonstrated the up-regulation of TLP 18.3 gene during dehydration.

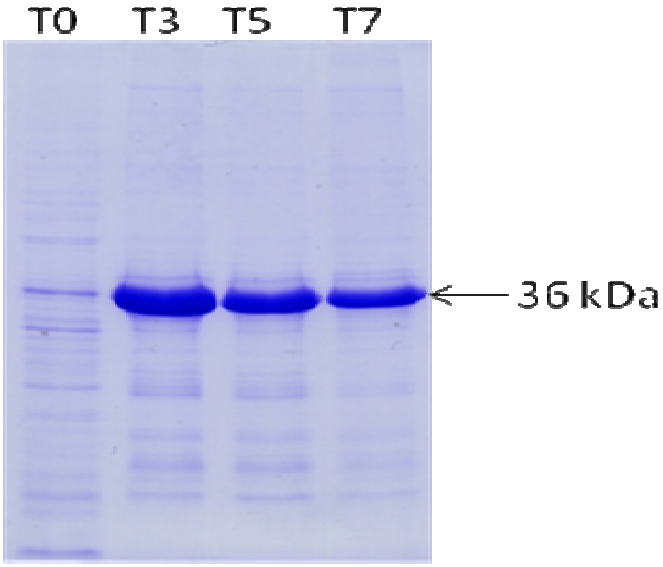

Figure 3. Time course of TLP 18.3 protein expression in total cell protein of *E. coli* strain BL-21 (DE-3) containing recombinant pET-30 Ek/LIC construct. T0 corresponds to the time before induction of IPTG and T-3, T-5 and T7 corresponds to the period of induction (in hours). Arrow indicates the expressed protein.

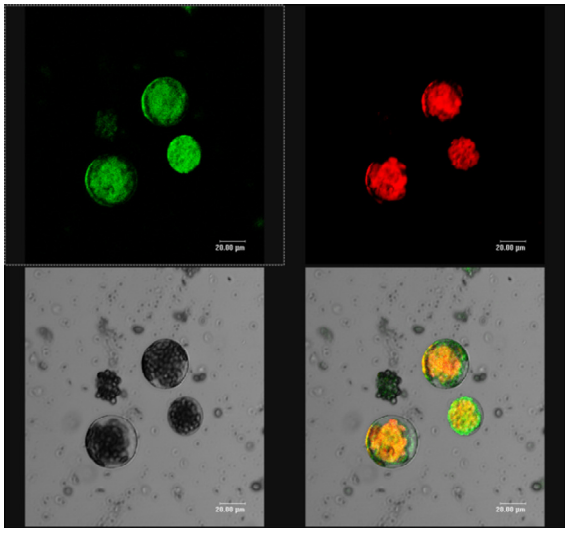

Figure 4. Subcellular Localization of *A. thaliana* TLP18.3: Expression of the *A. thaliana* TLP18.3-326GFP in *Arabidopsis* protoplast after 12 h incubation. The fusion protein transported into chloroplast. Green and red images show GFP fluorescence and chlorophyll autofluorescence, respectively (upper panel). The right-most photo of yellow color (lower panel) showing the merged image of green and red.

Exogenous application of ABA (100 µM) on aseptically grown seedlings (12 days old) demonstrated that mRNA expression level goes up with ABA treatment. The plant hormone ABA is renowned for being a stress hormone, as ABA level rise in response to abiotic stress (Nambara and Marion-Poll, 2005), it is obvious that ABA level

increases with the dehydration reason TLP18.3 mRNA expression up-regulated during the dehydration as well as with ABA treatment. This gene transcript level disappear with mannitol treatment (data in press) demonstrated that this gene is down regulated with osmotic stress. It has been reported that mannitol play the role of both up-regulation as well as down regulation of genes. Jung et al. (2010) has reported in microarray data that with 200 mM mannitol versus without treatment in *A. thaliana* a total 879 genes are up-regulated and 568 genes are down regulated. Further, mannitol creats osmotic stress and known to induce increase in plant hormone, cytokinin (Thomas et al., 1992). The cytokinin biosynthesis pathway involve in osmotic stress, so increase in cytokinin content in *A. thaliana* has been reported (Jung et al., 2010). ABA level increase in vegetative tissue and ABA induces the stomata closure to minimize the water loss through transpiration. In plants the increased ABA levels under water-deficit conditions results mainly from increased de nono biosynthesis through the transcriptional activation of ABA biosynthesis gene (Milborrow, 2001). So mannitol and ABA had two different biosynthesis regulation.

The predicted the chloroplast transit peptide (cTP) targeting sequence (Schwacke et al., 2003) was confirmed by subcellular localization of *A. thaliana* TLP18.3-326GFP vector expression in *Arabidopsis* protoplast. GFP green fluorescence image, which was completely overlapped with red autoflorescence of chloroplast image clearly showed the chloroplast localization of TLP18.3 (Figure 4).

Taken together the present study shows that the *A. thaliana* TLP18.3 gene is up-regulated during dehydration, localizes into chloroplast as this gene has been reported as diurnal expression changes, having up-regulation during light period (Sirpio et al., 2007) so need to study the mutant of this gene to know the proper regulation of *A. thaliana* TLP18.3 gene.

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