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Characterization of a chloroplast localized wheat membrane protein (*TaRCI*) and its role in heat, drought and salinity stress tolerance in *Arabidopsis thaliana.*

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ABSTRACT

Drought and heat are the two major abiotic stresses that are detrimental to the yield and quality of crop plants such as wheat. In the present study, we cloned and characterize a membrane protein gene from wheat, previously identified through cDNA subtractive hybridization. BLAST analysis revealed that the newly identified gene belongs to *Arabidopsis* and rice RCI (*R*are *C*old *I*nducible) genes and hence named as *TaRCI*. In the present investigation, *Arabidopsis* transgenics were raised expressing *TaRCI* for functional analysis. The subcellular localization by translational fusion of TaRCI with GFP revealed the localization of GFP:TaRCI into the chloroplast. *Arabidopsis* transgenics expressing *TaRCI* performed better than the wild-type under simulated heat, drought and salinity stress conditions. Under heat stress conditions, *TaRCI* expressing transgenic seedlings showed faster recovery post heat stress and were healthy and greener than wild-type plants. Further, the *TaRCI* expressing plants accumulated more biomass in terms of size, rosette diameter and root length under simulated drought stress conditions also. Increased leaf size, and rosette diameter were also observed in the presence of ABA and SA. Transgenic plants also showed increase in physiological parameters such as maximum photosynthetic efficiency (F_v/F_m), proline and chlorophyll contents. Our study thus provides insight into a new wheat gene that could be an important regulator involved in multiple abiotic stresses and could be a potential candidate gene manipulation for improving stress tolerance in crop plants in general and wheat in particular.

1. Introduction

Plants are exposed to various stresses in their environment, including both biotic and abiotic stresses and being sessile they cannot escape these stresses. Nevertheless, plants have developed molecular, physiological and biochemical mechanisms and adaptations to cope with biotic and abiotic stresses provided by resistance/tolerance genes. The *RCI* (*Rare Cold Inducible*) gene is one such gene that plays an important role in the stress response of plants (Morsy et al., 2005).

In our previous study, a detailed transcriptome analysis in wheat was carried out at three different developmental stages viz. young seedling, pre-pollinated flower and developing grains and a total of 3516 ESTs were generated and submitted to NCBI GenBank (Chauhan et al., 2011a). Some of these EST were cloned as full-length cDNAs and functionally validated in detail. One of them (accession number GD189885) was found up-regulated under heat and other abiotic stress conditions and was found to contain one uncharacterized trans-membrane domain UPF0057. BLAST analysis revealed that this newly cloned cDNA belongs to *Arabidopsis RCI* genes (Capel et al., 1997) which are responsive to multiple abiotic stresses (Medina et al., 2007).The small hydrophobic protein PMP3 (Plasma membrane protein 3) is homologue of *Arabidopsis* RCI (Navarre and Goffeau, 2000). Pmp3/RCI-like genes are highly conserved and encode hydrophobic proteins with two putative transmembrane domains belonging to yeast PMP3 family (Wang and Shiozaki, 2006; Medina et al., 2007; Chang-Qing et al., 2008). Genes from the *RCI* family have been described in many plant species and have been found to contribute towards various abiotic stresses, such as low-temperature inducible *OsLti6a* and *OsLti6b* from rice, low-temperature inducible *BLT101.1* and *BLT101.2* from barley, and cold-induced *wpi6* from wheat and salt-induced *ESI3* from wheat grass (Morsy et al., 2005; Goddard et al., 1993; Galvez et al., 1993). In Arabidopsis, the expression of *AtRCI2A* and *AtRCI2B* is induced by cold,other abiotic stresses such as dehydrationand salt, and by the hormone ABA (Medina et al., 2001). The expression of these genes was also found to be differentially regulated in different plant organs as revealed by promoter-GUS analysis (Medina et al., 2001). Maintenance of cellular homeostasis has emerged

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as an important characteristic of Pmp3 genes and it was proposed that they could interact with other membrane proteins to control the hydric balance of the cells (Capel et al., 1997). Deletion of Pmp3 gene in yeast increased the plasma membrane potential and conferred sensitivity to cytotoxic cations such as Na^+ (Navarre and Goffeau, 2000; Nylander et al., 2001, Mitsuya et al., 2005). In *Arabidopsis* overexpression of AtRCI2A provided enhanced salt tolerance by restricting Na^+ uptake and it was also recently reported that expression of maize *ZmPMP3-1* enhanced growth of transgenic *Arabidopsis* under salt stress condition, while, On the other hand, the disruption of AtRCI2A led to over accumulation of Na^+ and increased Na^+ sensitivity (Mitsuya et al., 2005, 2006; Fu et al., 2012). It has also been found that RCI2 from plants can complement the defects of ΔPMP3 mutant in yeast (Medina et al., 2007).

In this study, we characterize a wheat RCI protein gene by transgenic expression in *Arabidopsis*. The transgenics were analysed phenotypically, physiologically and morphologically under different abiotic stress conditions. Further sub cellular localization of this gene has been determined through translational fusion protein with GFP (Green Fluorescent Protein).

2. Materials and methods

2.1. Cloning of TaRCI and phylogenetic analyses

Full length cDNA sequence named as *TaRCI* on the basis of homology was retrieved from already reported plasmid with accession number GD189885. Predicted amino-acid sequences of *RCI* genes from Rice and *Arabidopsis* gene family that showed maximum homology with *TaRCI* were obtained from GenBank as well as the sequences were confirmed from Medina et al. (2007). The two accession numbers of rice RCI genes viz. Os01g18375 and Os09g15365.1 were retrieved from TIGR (The Institute for Genomic Research). For phylogenetic analysis, amino acid sequences were used for multiple sequence alignment, employing ClustalX v2.0. Subsequently, phylogenetic analysis was performed using MEGA5 program. The un-rooted phylogenetic tree was generated by the neighbour-joining (NJ) algorithm with p-distance method and pair wise deletion of gaps, using default parameters and with a bootstrap statistical analysis for 1000 replicates (Tamura et al., 2011).

2.2. Plasmids construction and plant transformation

A full-length cDNA (GD189885) of *TaRCI* having an ORF of 222 bp along with 5′ and 3′ UTR was amplified by PCR using gene-specific primers. For over-expression studies, *TaRCI* was cloned into plant transformation GATEWAY vector pMDC32 mediated by pENTR/D-Topo cloning system. For subcellular localization studies, ORF of *TaRCI* was translationally fused with green fluorescent protein (GFP) in pCAMBIA1302 vector. Seeds of *Arabidopsis thaliana* (Col0) were used for raising transgenics in the present study. For raising transgenics, wild-type seeds were spread in pots containing soilrite for the generation of full grown plants and were kept in the culture room maintained at 22 ± 1 °C with 16:8 h light and dark regime with a light intensity of 100–125 μmol m^{-2} s^{-1}. Both the vectors were then used to transform *Arabidopsis* plants by floral dip method (Clough and Bent, 1998). Stable transgenics of T4 generation with pMDC32:*TaRCI* were used for various abiotic stress assays and pCAMBIA1302: *TaRCI Arabidopsis* transgenics were used to isolate protoplast and confocal microscopy for the localization of fusion protein., The presence of transgene in putative homozygous transgenic plants of T_4 generation was confirmed by Semi-quantitative RT-PCR expression analyses by using *TaRCI* (RT-TaRCI-F 5′-ATGGCGTC CCGGAGCTGC-3′ and RT-TaRCI-R 5′- TCAACCAAGGGCGTCGTAGTC-3′)and *Hygromyin phosphotransferase* (*HPT*) (RT-HPT-F 5′-ATGAAAAA GCCTGAACTCACCG-3′ and RT-HPT-R 5′-GCGACGGACGCACTGACG-3′) specific primers.

2.3. Expression of GFP-TaRCI in protoplast of transgenic Arabidopsis plants and confocal imaging

From the transgenic *Arabidopsis* plants, protoplasts were isolated in order to confirm the localization of TaRCI-GFP fusion protein. Fresh CPW salt solution (KNO_3, KH_2PO_4, $MgSO_4$, $CuSO_4$ and $CaCl_2$) was used. Washing solution contained mannitol and MES hydrate and was prepared in CPW salt solution. To 10 ml of washing solution, 200 mg of cellulose RS and 200 mg of macerozyme R10 were added and without any stirring, they were allowed to dissolve for 30–40 min. Two-week-old *Arabidopsis* transgenic seedlings grown on MS plates were chopped. Approximately 1 g of tissue was dipped in the beaker with enzyme solution. The beaker was covered and kept on a rocker for 30 min in the dark. After 30 min, crude extract of chopped tissue was examined using Leica confocal microscope (Leica, Germany). Software used was Leica Application Suite (Version 2.1.0.R1). The GFP fluorescence was imaged under UV light and the cellular architecture was imaged using DIC (Differential Interference Contrast). An overlay image was prepared by merging both the images by using the same software.

2.4. Stress treatments, phenotypic and morphometric analysis of transgenic Arabidopsis plants

Homozygous transgenic lines were used to assess the tolerance of pMDC32:*TaRCI* expressing transgenic lines towards heat stress, drought stress, salt stress and hormonal treatments. For heat stress, 8 day old wild type and transgenic seedlings were exposed to 42 °C for 2 h in an incubation chamber and plants were allowed to recover for 6 days and then photographed. For drought tolerance stress, seeds were plated onto MS medium supplemented with 400 mM Mannitol and 2% poly ethylene glycol (PEG) for PEG or mannitol induced osmotic stress. For salinity stress, seeds were plated onto MS medium supplemented with 150 mM NaCl and observed for two weeks. Similarly for hormonal treatments, wild type and transgenic seedlings were grown on MS medium supplemented with 2.5 μM abscisic acid (ABA) and 50 mM salicylic acid (SA). Three transgenic lines were analysed, the phenotype was noted down for two-week-old seedlings and one of the transgenic line was photographed. For morphometric analysis, three transgenic lines were plated onto MS medium supplemented with Mannitol, PEG, NaCl, ABA and SA. Two-week-old wild type and transgenic lines were analysed for different parameters viz. root length, plant height, fresh weight, leaf length, leaf width and rosette diameter. Standard deviation and significance of difference (p-value) were calculated by t-test function in MS-Excel.

2.5. Physiological analysis of transgenic Arabidopsis plants

pMDC32:*TaRCI* expressing transgenic lines were also analysed physiologically by analysing different parameters on three-week-old plants. Three different transgenic lines were grown on MS medium supplemented with different stress inducers analysed in the study. After abiotic stress treatments, the wild-type and transgenic seedlings were analysed. The different parameters were: PS II activity, Proline and Chlorophyll content.

2.5.1. Photosynthetic yield

PSII activity was measured according to Krause and Weis (1991). Measurements of modulated chlorophyll fluorescence emission from the upper surface of the leaf were made using a pulse amplitude modulation fluorometer (PAM-210, H. Waltz, Germany). Leaves were dark-adapted for 20 min before measuring the induction of fluorescence. The measuring beam was used to induce the minimum fluorescence (F_0). Saturating flashes were provided to completely reduce the PSII acceptor site Q_A- and to measure the maximum fluorescence yield (F_m). The variable fluorescence (F_v) was calculated as $F_m - F_0$.

Maximum photosynthetic efficiency was measured using the following formula:

F_v/F_m where $F_v = F_m - F_o$

2.5.2. Proline content

It was estimated in control and stressed plants as described by Bates et al. (1973). 100 mg of sample tissue was weighed and was grinded in 1 ml of 3% sulphosalicylic acid. It was centrifuged for 15 min at 10,000 rpm. Pellet was discarded and supernatant was transferred into fresh 2 ml micro centrifuge tube (MCT). This supernatant was divided into 2 tubes each containing 500 μl of the supernatant. To each MCT, 400 μl of glacial acetic acid and 400 μl of ninhydrin was added and reaction was allowed to proceed at 100 °C was 1 h. After 1 h the reaction was stopped by placing the tubes in ice. To the tubes, 800 μl of toluene was added and it was vigorously vortexed. Supernatant was transferred in a fresh MCT. The absorbance was recorded at 520 nm in a UV–visible spectrophotometer (U-2810 spectrophotometer, Hitachi, Japan) against a toluene blank. Total proline content was calculated as:

$$\mu \text{ moles of proline/gm fresh wt} = \frac{(\mu g \text{ protein/mL} \times \text{mL toluene} \times 5)}{115.5\ \mu g/\mu moles \times \text{g sample}}.$$

2.5.3. Chlorophyll estimation

5 ml of di methyl sulphoxide (DMSO) was taken in each tube and 100 mg of leaf discs was added for both WT and transgenic lines. The tubes were than kept in dark for overnight for chlorophyll leaching. Readings were taken next day at two wavelengths i.e. 663, 645 with the help of a UV–visible spectrophotometer (U-2810 spectrophotometer, Hitachi, Japan). Chlorophyll content was then calculated using the formula given by Deshmuukh et al. (1991). Observations were plotted on the graph and compared for different samples.

$$\text{Total chlorophyll} = [(20.2 \times A645) + (8.02 \times A663)] \times 1000/\text{grams of tissue}$$

3. Results

3.1. Phylogenetic analyses and subcellular localization of TaRCI

The EST GD189885 harboured a full length cDNA having an ORF of 222 bp, coding for 73 amino acids residues and its protein is suggested to be highly hydrophobic (Chauhan et al., 2011a). Using online software SOSUI, TaRCI is predicted to have two transmembrane helices and a pictorial representation of both transmembrane helices is also shown in Fig. 1. The deduced protein sequence of TaRCI was submitted to SMART (Smart Modular Architecture Research Tool) for its domain analysis. It was found to contain one uncharacterized domain named UPF0057, which belonged to Pmp3 superfamily (Proteolipid membrane potential modulator) as revealed by BLAST search (Fig. 1B). Next, a multiple alignment of full-length MP protein sequences was done by ClustalW. For this purpose many protein sequences were retrieved from NCBI belonging to monocot plants such as *Hordeum vulgare* (BAJ90014.1), *Sorghum bicolor* (XP_002460652.1), *Zea mays* (ACG26755.1), *Oryza sativa* (NP_001063909.1), *Brachypodium distachyon* (XP_003578630.1), and *Populus trichocarpa* (XP_002316342.1), and dicots such as *Vitis vinifera* (XP_002277561.1) and *Arabidopsis thaliana* (NP_565897.1). Multiple sequence alignment showed that TaRCI shared high sequence similarity throughout the N-terminal region with this predicted hydrophobic protein homologues from other plant species (Fig. 1C). TaRCI showed maximum protein alignment of 82% with a similar predicted protein from *H. vulgare* (BAJ90014.1). To get the phylogenetic relevance of the newly cloned wheat *TaRCI* gene, we made a tree with MEGA5 by using a deduced amino acid sequence of TaRCI along with *Arabidopsis* and rice RCI proteins. It revealed that *TaRCI* is related to OsRCI2-11, which makes a separate clade along with AtRCI2C and AtRCI2H in the tree (Fig. 2).

For subcellular localization of TaRCI, we translationally fused it with GFP and transformation of Arabidopsis was done by floral dip method. Stable *Arabidopsis* transgenics were used to isolate protoplast and confocal microscopy was used to observe the green florescent signals. As can be seen in Fig. 3, the green fluorescent signals of TaRCI:GFP fusion protein co-localized with the auto-fluorescent signals of chlorophylls in the chloroplasts.The merged picture shows a bright orange colour specifically in the chloroplastssuggesting that TaRCI is localized to the chloroplast.

3.2. Analysis of Arabidopsis transgenics expressing TaRCI

3.2.1. Phenotypic analysis under heat and other abiotic stresses

As we observed that *TaRCI* is induced by heat stress and other abiotic stresses as well such as drought, cold and salt stress (Chauhan et al., 2011a), we wanted to know whether it can be used as a transgene to enhance plant tolerance for abiotic stresses. Arabidopsis transformation was performed and three transgenic lines were selected at T4 generation for further characterization. The expression of transgenes viz., *HPT* and *TaRCI* was confirmed by RT-PCR (Supplementary Fig. S1). Various abiotic stress assays were performed to assess the tolerance of *TaRCI* expressing transgenics in *Arabidopsis*. *TaRCI* expressing transgenics showed a phenotype better than wild-type plants. Under high temperature stress condition the transgenics did not show any visible difference as compared to wild type when heat stress was given at 37 °C for 2 h and for 4 h (data not shown). However, when plants were exposed to a more severe heat stress at 42 °C for 2 h, all the plants showed visible injury. Nevertheless, upon one week of recovery, transgenic plants showed better recovery and growth in terms of increase in plant height, rosette diameter, greener leaves and also an increase in root length. This was shown in all the three transgenic lines viz. T1.7, T2.2, T6.3 expressing *TaRCI* (Fig. 4A). Phenotypic analysis was undertaken after one week of recovery for plants given heat stress and showed that overexpression of transgenic lines produced an increase in shoot length, plant height, fresh weight and rosette diameter. (Fig. 4B).

Effect of drought stress was also checked on wild-type and transgenic plants grown on MS medium supplemented with varied concentrations of mannitol. The observations were taken after 15 days of germination. We observed that under 400 mM mannitol, transgenic plants showed more tolerance than wild-type plants. While wild-type plants had smaller rosette diameter and both shoot and root length were inhibited, *TaRCI* expressing transgenic seedlings showed larger leaves and rosette diameter, more plant height and root length and also accumulated more fresh weight. Also in overall appearance the transgenics look healthier and greener than wild-type plants (Fig. 5Aa). Similarly, the drought response of *TaRCI* expressing transgenics was further confirmed when wild-type and transgenics were grown on 2% PEG (polyethylene glycol). PEG (MW:9000) is a high molecular weight osmotic substance and is one of the most common approach for simulating drought stress response (Turkan et al., 2005). Transgenic seedlings survived better in response to 2% PEG in comparison to wild type plants. The transgenic seedlings showed remarkable increase in the plant height as compared to the wild-type plants (Fig. 5Ab). In transgenics, rosette size was increased and the roots were, longer and well-developed while in wild-type seedlings, plants were much smaller in size with a single root that was short in length. Thus, demonstrating increased tolerance of transgenics towards drought stress.

For assessing *salinity stress* tolerance, wild-type and transgenics were grown on 150 mM NaCl for 15 days and observations were noted down. As observed in Fig. 5Ac, transgenic seedlings retained their green colour while wild-type plants were severely bleached and turned white in colour. When root-length was compared between the two, the root length was comparatively longer and growing in transgenics as compared to

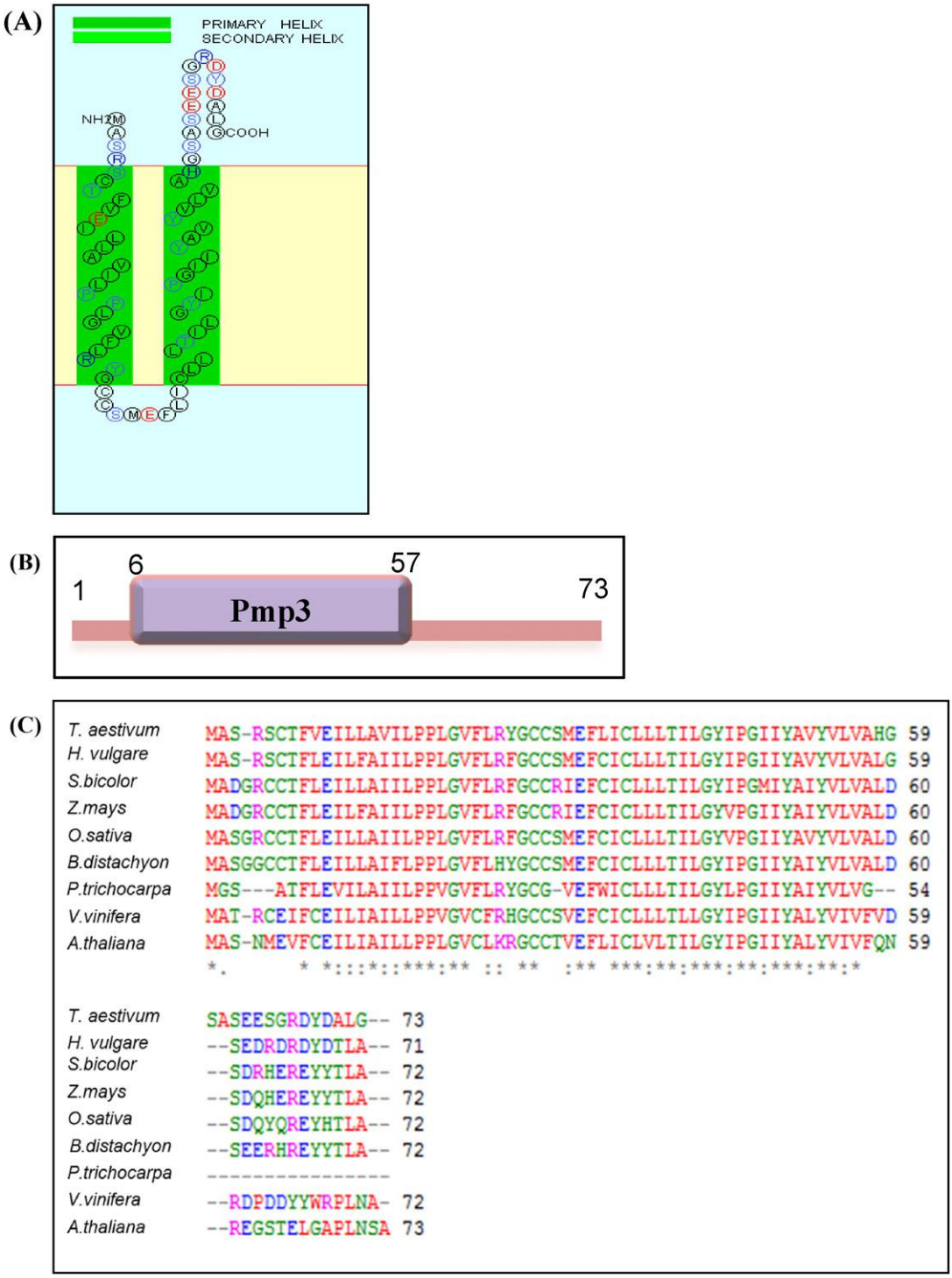

Fig. 1. Amino acid sequence and transmembrane domains of TaRCI (A). The sequence of transmembrane domains of TaRCI. Two transmembrane helices of TaRCI protein as predicted by SOSUI. (B) Domain structure organization of TaRCI protein as obtained by SMART (Simple Modular Architecture Research Tool). (C) Multiple alignments of the full-length RCI proteins obtained by Clustal W. Plant RCI protein Sequences are *Triticum aestivum*, *Hordeum vulgare* (BAJ90014.1), *Sorghum bicolor* (XP_002460652.1), *Zea mays* (ACG26755.1), *Oryza sativa* (NP_001063909.1), *Brachypodium distachyon* (XP_003578630.1), *Populus trichocarpa* (XP_002316342.1), *Vitis vinifera* (XP_002277561.1), *Arabiopsis thaliana* (NP_565897.1).

wild-type plants. The rosette size of transgenics was also comparatively increased than wild-type plants.

Effect of *hormones* on the phenotype of transgenics was also observed. Wild-type and transgenics were grown on MS medium supplemented with 2.5 μM ABA and seedlings were observed after 15 days. It was observed that transgenic seedlings survive better in response to 2.5 μM ABA (Fig. 5Ba). The root length of transgenics was comparatively longer than wild-type plants. The leaf size and rosette diameter were also higher in transgenics. When wild-type and transgenic plants were grown on MS medium supplemented with SA for 15 days, transgenic plants showed better seedling growth (Fig. 5Bb). We find that under SA, both wild type and *TaRCI* expressing transgenic has similar root length, nevertheless there were more adventitious roots observed in case of transgenics (Fig. 5Bb).

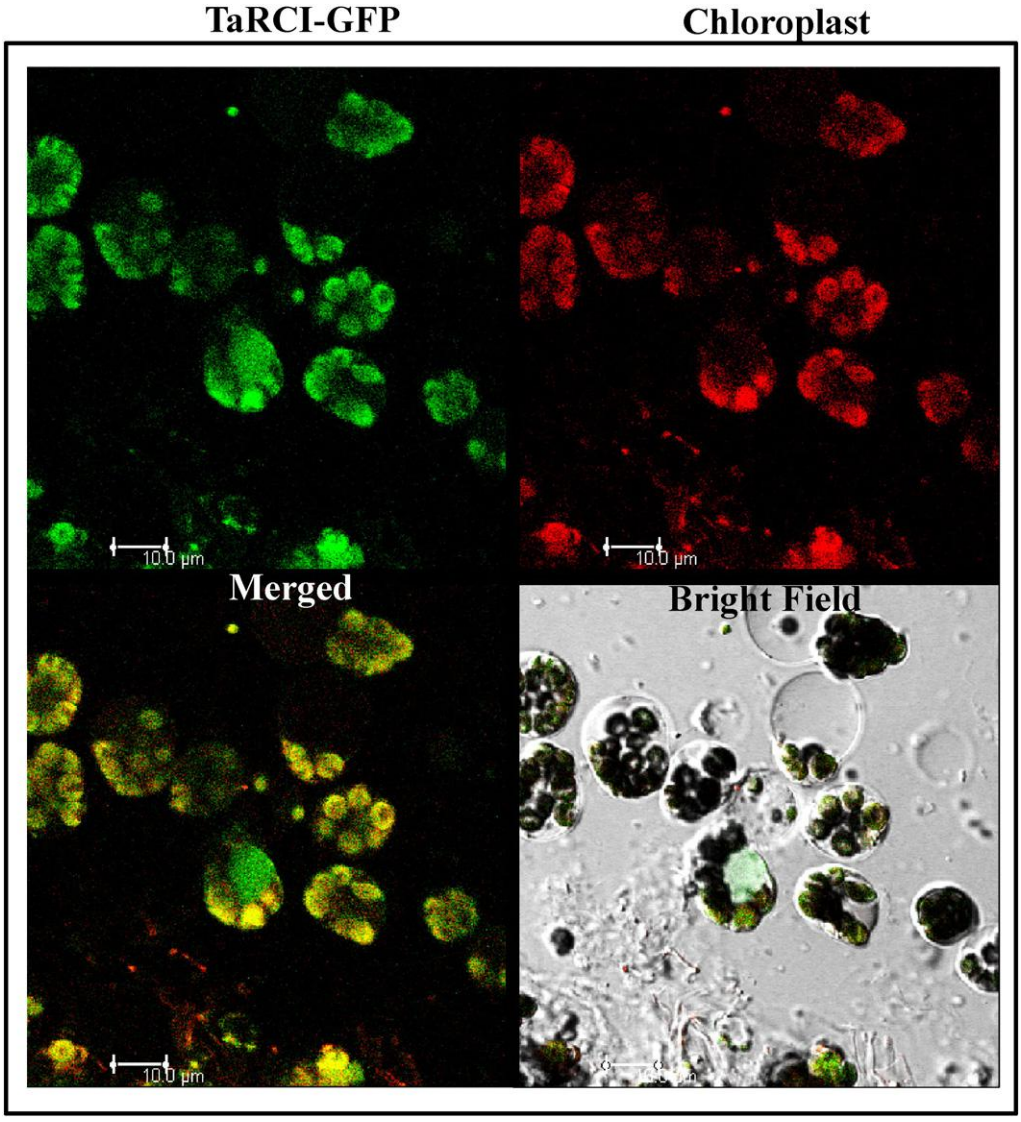

Fig. 2. Subcellular localization of TaRCI. *TaRCI*:GFP fusion construct was transformed into *Arabidopsis* and at T4 homozygous stage, *Arabidopsis* protoplasts were isolated and expression of GFP fusion protein was monitored by confocal microscope. Auto-fluorescence of chloroplasts were shown in red colour and merged picture shows a bright orange colour in the chloroplasts.

For a comprehensive comparative analysis, all the three transgenic lines 1.7, 2.2 and 6.3 were analysed for some selected parameters under the abiotic stresses and hormone treatments (ABA, NaCl, SA, PEG, and mannitol). Root lengths of 15 seedlings were measured in each of the abiotic stress. It was observed that root length was almost comparable in transgenics in SA and salt stress except in transgenic line 1.7 (Fig. 6A). However, root length showed significant increase in case of transgenics in ABA, PEG and mannitol. When total plant height was measured, transgenics showed better response when exposed to drought stress viz. PEG and mannitol (Fig. 6B). Transgenic plants of all the three lines showed significantly higher plant height in PEG and mannitol stress as compared to wild types plants. Also, rosette diameter showed an increase in the transgenic lines in all the abiotic stresses, increase being more in PEG and mannitol (Fig. 6C). It was also observed that transgenics fared better than wild-type in all abiotic stresses and accumulated almost double biomass under induced drought stress (Fig. 6D). Although transgenic plants grown on SA and NaCl showed only a slight increase in height as compared to wild-type plants, fresh weight of transgenics was significantly higher in these two abiotic stresses. Overall it is observed that in comparison to wild type plants, transgenic plants expressing *TaRCI* showed better biomass accumulation and plant growth under various abiotic stresses.

3.2.2. Physiological characterization of pMDC32:TaRCI transgenics under abiotic stress responses

pMDC32:*TaRCI* transgenics were also analysed on the basis of some physiological parameters. Since membranes are the first targets for many stresses such as heat and drought, we measured the membrane functions of chloroplast PSII in terms of maximum photochemical efficiency (F_v/F_m). Our analysis showed that there is a significant increase in the F_v/F_m ratio (maximum photosynthetic efficiency), in all the abiotic stresses studied and the increase in maximum photosynthetic efficiency was significantly higher in simulated draught stress by PEG and mannitol (Fig. 7A). Proline levels were also analysed and it was observed that transgenic plants accumulated significantly more proline in case of PEG-mediated drought stress. The increase in proline levels was observed however, in all the abiotic stresses (Fig. 7B). Similarly chlorophyll content was also measured and was found to be significantly higher in the transgenics plants than wild-type plants in all the abiotic stresses (Fig. 7C).

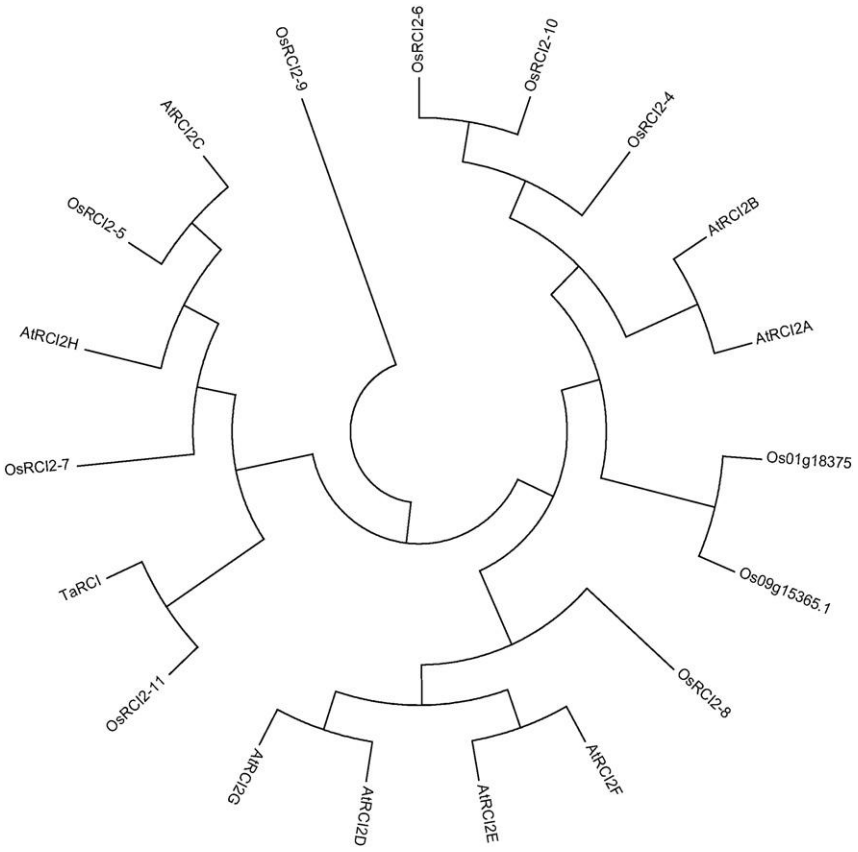

Fig. 3. Phylogenetic analysis of TaRCI with other members of *RCI* gene family from rice and *Arabidopsis*. The un-rooted phylogenetic tree was constructed by MEGA5 by using neighbour-joining (NJ) method with default parameters and with a bootstrap statistical analysis for 1000 replicates.

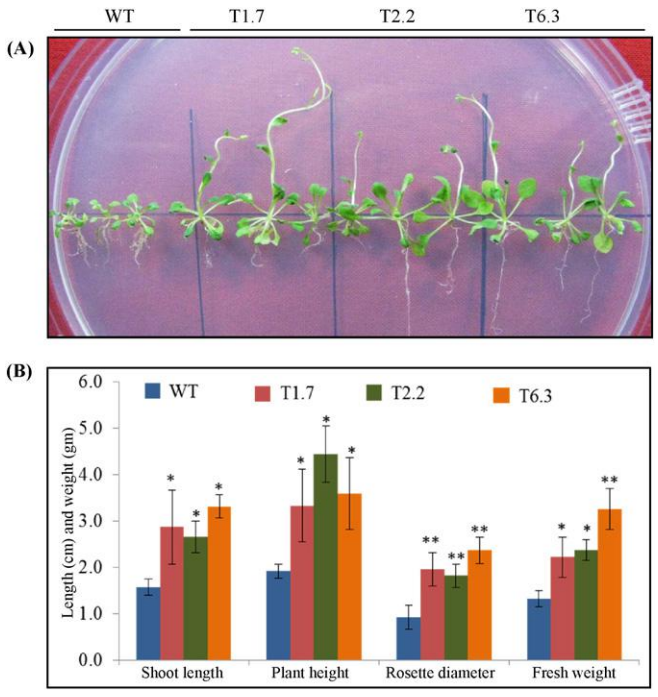

Fig. 4. Effect of heat stress on seedling growth of wild type and *TaRCI*-expressing transgenic *Arabidopsis* plants. Heat stress was given at 42 °C for 2 h on 8-days-old WT and transgenic lines of *Arabidopsis* expressing *TaRCI*. The photographs were taken after 1 week of recovery. (A) Phenotype of wild type and three *TaRCI* expressing transgenic lines (viz. T1.7, T2.2 and T6.3) seedlings grown on MS medium. (B) Transgenic lines analysed physiologically after heat stress exposure for four different parameters: shoot length, plant height, fresh weight and rosette diameter.

4. Discussion

Tolerance to abiotic stresses is a complex phenomenon provided by stress resistance genes and it has been observed that sometimes genes responding to abiotic stresses occur as gene families (Yokoi et al., 2002; Shigaki et al., 2006; Chauhan et al., 2011a, 2011b), arising from gene duplication and providing plants greater tolerance to unfavourable environments (Paterson et al., 2006). A comparative analysis of RCI gene family has been done in *Arabidopsis*, rice and *Caenorhabditis elegans* by Medina et al. (2007). There are eight, twelve and thirteen members in this gene family in *Arabidopsis*, rice and *C.elegans* respectively. These can be broadly divided in to two groups, containing around 52–55 amino acid and 72–77 amino acid residues, and all of them are predicted to contain two transmembrane domains. We found that TaRCI also possess two transmembrane domains as predicted by multiple membrane topology prediction programmes (Fig. 1) and belong to the larger group having 73 amino acid residues.

Although, all the members of *Arabidopsis* RCI gene family predicted to contain transmembrane domain and no predicted signal peptide, not all the members were localized to plasma membrane. AtRCI2D was found to localize in ER and Golgi organelles (Medina et al., 2007). We have also found that TaRCI:GFP fusion protein localize to chloroplast (Fig. 2) though there is no predicted chloroplast transit peptide in TaRCI. This is not a rare phenomenon as far as chloroplast localization is concerned (Armbruster et al., 2009). There are non-canonical chloroplast proteins such as ceQORH (chloroplast envelope Quinone Oxidoreductase Homologue), Tic32/IEP32, (Miras et al., 2007; Nada and Soll, 2004), which do not have N-terminal transit peptide but localized to chloroplasts. Previously, Navarre et al. (2000) showed that deletion of Pmp3 increases the plasma membrane potential and confers sensitivity to Na^+ ions and thus confers salt sensitivity in yeasts. Lack of *RCI2A* gene (homologous to *Pmp3* gene) also caused a salt sensitive phenotype in

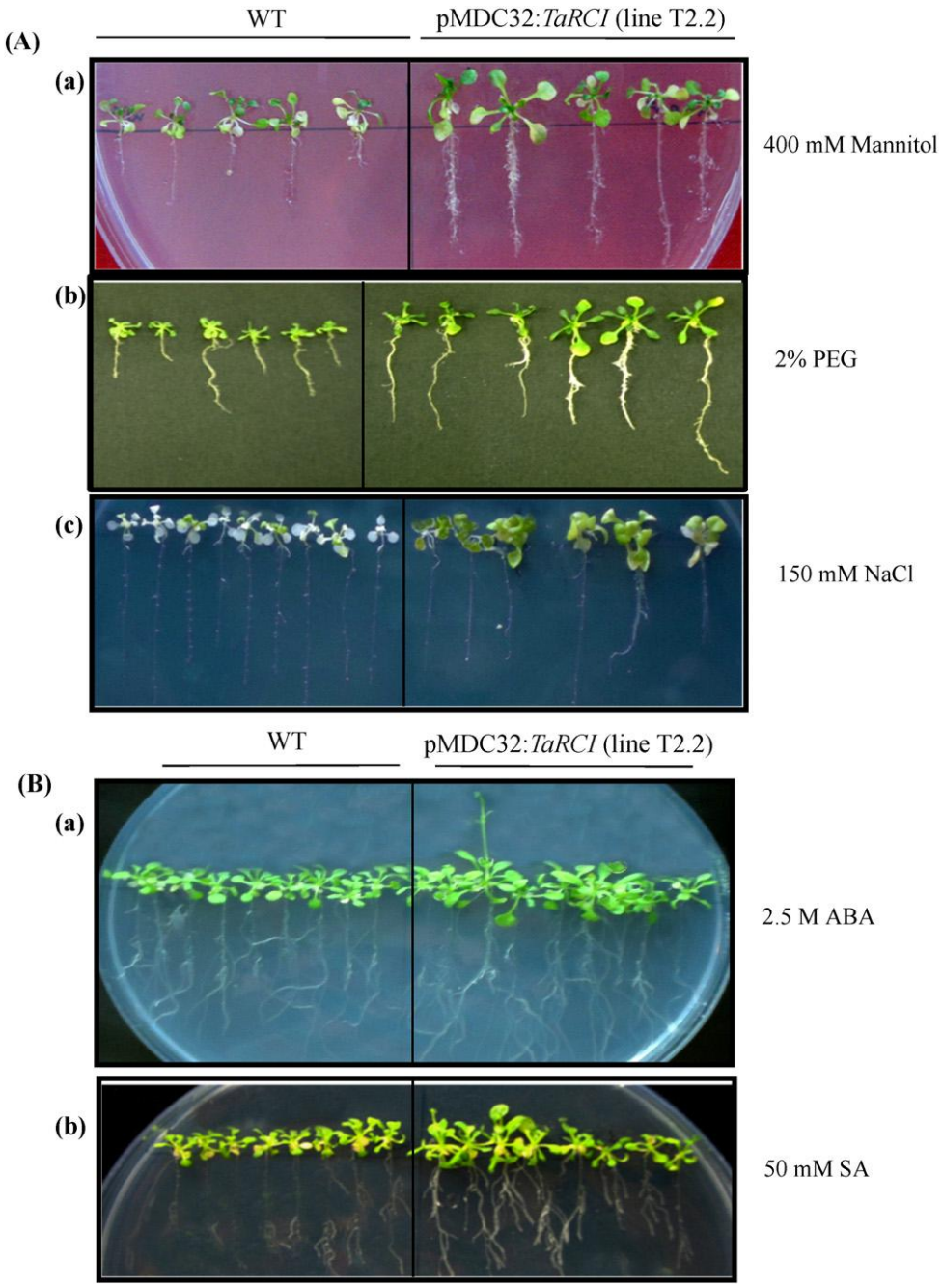

Fig. 5. Effect of different abiotic stresses on seedling growth of wild type and *TaRCI*-expressing transgenic *Arabidopsis* plants (A) Effect of drought stress on 15-day-old wild type and transgenic seedlings grown on MS medium with 400 mM mannitol and 2% PEG respectively (a & b) Effect of salt stress on 15-day-old wild type and transgenic seedlings grown on MS medium with 150 mM NaCl (c). (B) Effect of hormonal stress on 15-days-old WT and *TaRCI* transgenic lines of *Arabidopsis*. Phenotype of wild type and transgenic seedlings grown on MS medium with 2.5 μM ABA and 50 mM SA (a and b).

Arabidopsis (Mitsuya et al., 2005). Further, *rci2a* plants along with wild-type were treated with various sodium salt derivatives the mutants of rci2a showed growth retardation and a significant decrease in root-shoot fresh weight than wild-type. Since, *TaRCI* gene is also found to be a member of Pmp3 superfamily, to evaluate *Arabidopsis* transgenics expressing *TaRCI* gene for their ability to stress tolerance, various stress assays were performed.

PSII is long known to be highly sensitive to elevated temperatures (Berry and Bjorkman, 1980; Tanaka et al., 2000; Aminaka et al., 2006). Under salt and drought stress conditions, transgenic plants expressing *TaRCI* showed significantly higher PSII activity in terms of maximum photochemical efficiency than the wild type plants, which may be attributed to the higher chlorophyll content in the transgenic plants (Fig. 7). Osmoprotectants such as proline and glycine betaine are known to help plants to overcome the effects of water stress and salt stress (Bartels and Salamini, 2001). We found that TaRCI expressing transgenics showed more tolerance for abiotic stresses in these physiological and biochemical parameters (Figs. 5–7). Together these results, we hypothesise that RCI participate in stress amelioration however, more studies are required to find the exact manner by which different RCI protein functions.

Homologue of Pmp3 genes are also shown to be induced by ABA (Medina et al., 2001; Morsy et al., 2005) and H_2O_2 (Inada et al., 2005). Their homologues also show response to low temperature stress like barley BLT101.1 and BLT101.2 (Goddard et al., 1993) and rice OsLti6a and OsLti6b (Morsy et al., 2005). SA (Salicylic acid), a potent signalling

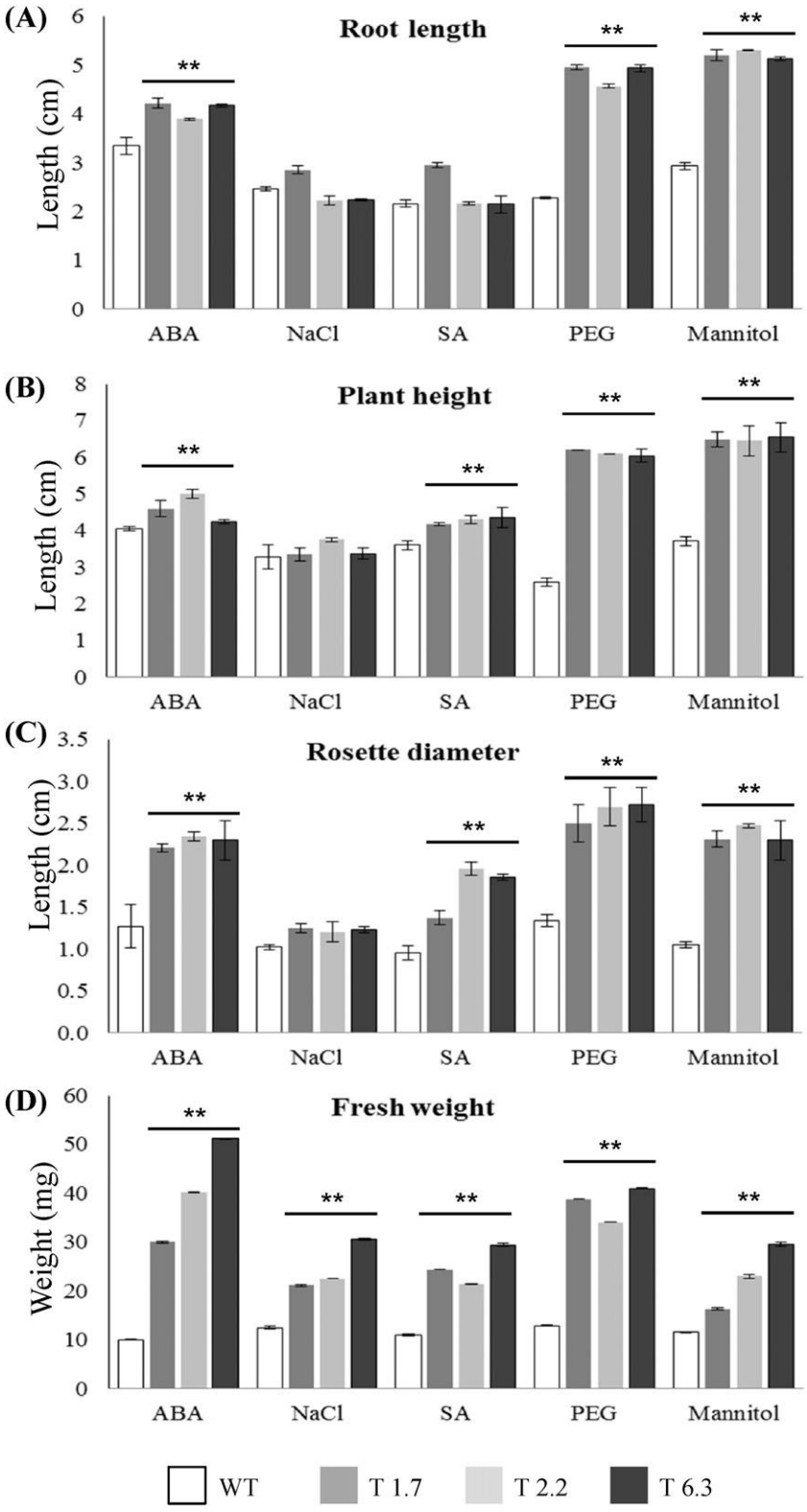

Fig. 6. Effects of different environmental factors on the growth of the wild-type and TaRCI-expressing transgenic plants. Effect of abiotic stresses on wild type and *TaRCI* expressing transgenic lines on the different parameters viz. (A) Root length (B) Plant height (C) rosette diameter (D) Fresh weight. Three transgenic lines were analysed and error bars showed standard deviation, ** represents p-value = 0.05.

molecule in plants is known to be involved in biotic and abiotic stress signalling (Shah, 2003) viz. heat (Larkindale and Knight, 2002), cold (Janda et al., 1999), NaCl (Tari et al., 2002) and water deficit (Bezrukova et al., 2001). In a halophytic monocotyledonous plant species, *Puccinellia tenuiflora*, expression of genes encoding Pmp3 were induced by low temperature, salt stress, dehydration, ABA, and $NaHCO_3$ (Chang-Qing et al., 2008). Expression of *TaRCI* is also induced by ABA and SA in roots and shoots of two-week-old wheat seedlings (Chauhan et al., 2011a). Thus, it has been inferred that Pmp3 gene family may play an important role in stress-responsive mechanisms in

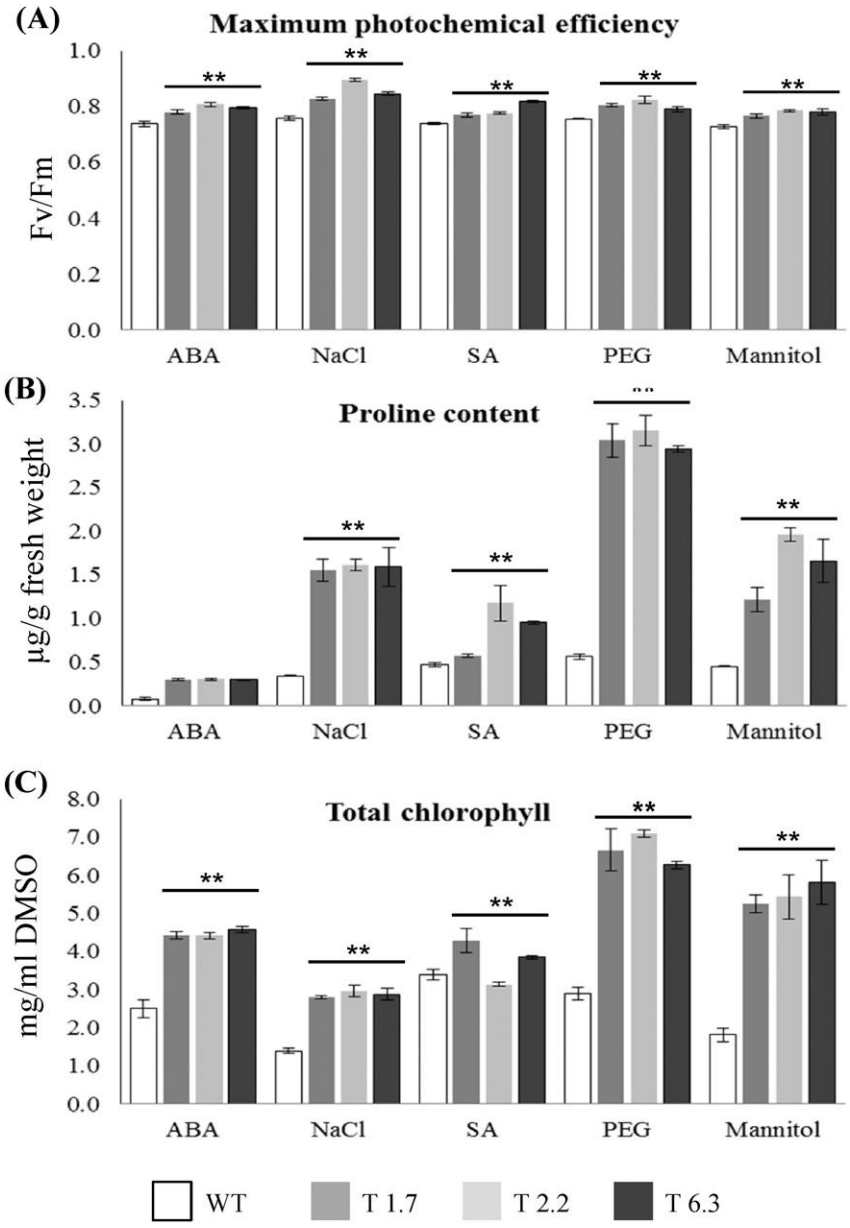

Fig. 7. Effects of different environmental factors on the growth of the wild-type and TaRCI-expressing transgenic plants. Effect of different abiotic stresses on (A) Maximum photochemical efficiency (F_v/F_m) (B) Proline content (C) Total chlorophyll content. Three transgenic lines were analysed and error bars showed standard deviation, ** represents p-value = 0.05.

plants. In our study, characterization of the membrane protein, *TaRCI* by expression in transgenic *Arabidopsis* plants also results in increased tolerance towards abiotic stresses. The *TaRCI* expressing transgenics resulted in enhanced rosette size, increased root length, plant height and were healthier than wild-type plants.

5. Conclusions

As mentioned above that membranes are sensitive for various types of stresses, plants have developed stress related membrane proteins to cope with extreme environmental conditions such as extreme temperatures. Since TaRCI is found to localize to chloroplast, we hypothesise that it functions in protecting chloroplast membrane architecture during heat and other abiotic stresses. Our study provides insight into a novel wheat gene that could be an important regulator involved in heat, drought and salt stress and could be a potential candidate gene manipulation of improving stress tolerance in crop plants in general and wheat in particular. We are now currently working towards the over-expression of this gene in monocots such as wheat and rice and to ascertain the exact function and agronomic benefits.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.plgene.2015.09.005.

Competing interests

The authors declare that they have no conflict of interest.

Author contributions

Conceived and designed the experiments: NK HC PK. Performed the experiments: NK HC. Analysed the data: NK HC PK. Contributed reagents/materials/analysis tools: PK. Wrote the paper: NK PK.

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