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Molecular characterization and expression analysis of pearl millet *plasma membrane proteolipid 3* (*Pmp3*) genes in response to abiotic stress conditions

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ABSTRACT

Plasma membrane proteolipid 3 (*Pmp3*) is a gene family involved in abiotic stress response and cellular protection. Here we report cloning of two genes *PgPmp3-1* and *PgPmp3-2* from *Pennisetum glaucum*, and characterization with respect to their functions and responsiveness to various abiotic stresses. Both *PgPmp3-1* and *PgPmp3-2* genes are 171 bp long and encode for 56 amino acid long peptides. *PgPmp3* sequences share 70–99% sequence identity with their homologs. Protein secondary structure prediction revealed membrane-spanning regions containing a membrane potential modulator domain in both PgPmp3 proteins. *In silico* network analyses revealed *Pmp3* co-expression and association with proteins conferring abiotic stress tolerance in plants. Expression profiles of *PgPmp3-1* and *PgPmp3-2* revealed their up-regulation in *P. glaucum* under cold and salt stresses, but showed reduced expression in response to heat stress. These findings provide insight into the role of *P. glaucum Pmp3* in abiotic stress amelioration.

1. Introduction

Crop productivity is adversely affected primarily by abiotic stress conditions such as drought, salinity and extreme high or low temperatures. To withstand abiotic stress, plants alter their physiological, biochemical and molecular processes and successfully adapt to stressful environments (Islam and Tuteja, 2012). *Pennisetum glaucum*, commonly known as pearl millet, is a hardy and robust crop found in arid and semi-arid regions of India and Africa. It often encounters abiotic stress conditions such as drought, extreme temperatures, high salinity and low pH of soil. High salinity and low temperature stresses during seed germination and seed setting prove detrimental to the overall yield of *P. glaucum*. Several studies have elucidated the effect of environmental stresses on expression of various genes and proteins in this dry land cereal crop (Reddy et al., 2012, 2014, 2015). Nevertheless, studies regarding response of various genes and their respective roles in alleviating the stress conditions, especially extreme temperatures and high salinity, might provide additional insights into *P. glaucum* stress adaptation.

The initial effect of abiotic stresses is perceived on the cell wall and plasma membrane that act as an interface between the cell and external environment (Panjabi-Sabharwal et al., 2010). Plasma membrane plays an important role in keeping the cell intact, maintaining cellular osmosis and signal transduction. Exposure to abiotic stresses increases the membrane permeability thereby allowing loss of electrolytes through the cell (Lyons, 2012). Thus, protection of plasma membrane is important during exposure to abiotic stress conditions. Some integral membrane proteins play important roles in cell-cell interaction, ion transport and signal transduction (Marmagne et al., 2004). Proteins such as gated aquaporins (plasma membrane intrinsic proteins), H^+ ATPase, receptor protein kinases and calmodulin, which are present on plasma membrane, contribute to stress tolerance in plants (Arazi et al., 1999; Li et al., 2015; Roy et al., 2005; Osakabe et al., 2010).

Plasma membrane proteolipid 3 (*Pmp3*), first reported in *Saccharomyces cerevisiae*, is a gene involved in combating low temperature and salt stress induced membrane instability (Navarre and Goffeau, 2000). The homologs of Pmp3, also known as *rare cold inducible (RCI)* or *low temperature inducible (LTI)* genes (Medina et al., 2001; Chang-Qing et al., 2008) have been shown to express under abiotic stress conditions in several plant species such as *Arabidopsis thaliana*, *Triticum aestivum*, *Oryza sativa*, *Zea mays* and *Hordeum vulgare* (Medina et al., 2001; Khurana et al., 2015; Chang-Qing et al., 2008; Goddard et al.,

Abbreviations: Pmp3, plasma membrane proteolipid 3; qPCR, quantitative real-time PCR; RCI, rare cold induced; LTI, low temperature induced

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1993). *Pmp3* encode for highly hydrophobic proteins that are embedded in plasma membrane with two putative transmembrane domains and small extracellular and cytoplasmic regions. Pmp3 protein is known to modulate plasma membrane potential to maintain cellular ion homeostasis and helps in survival during salt stress (De Block et al., 2015; Serrano and Rodriguez-Navarro, 2001). It is also suggested that *Pmp3* regulates a depolarizing cation and proton leak, reducing sensitivity to salinity and low pH conditions. Individual *Pmp3* genes belonging to the same organism could be differentially expressed under various stress conditions, as reported in *A. thaliana* and the alkali grass, *Puccinellia tenuiflora* (Medina et al., 2007; Chang-Qing et al., 2008). Although *Pmp3* has been reportedly induced in response to salt or cold stress, its exact role is still unclear. Thus, we considered it important to study the function of *Pmp3* genes from *P. glaucum*, a highly resilient crop plant grown in dry lands.

In the present study, two genes, *PgPmp3-1* and *PgPmp3-2* from *P. glaucum*, were cloned and characterized with respect to abiotic stress responsiveness. The quantitative expression of these genes was monitored in response to different abiotic stress conditions such as drought, high salinity, high and low temperatures. To shed some light on the role of *Pmp3* genes in stress alleviation, their interaction and co-expression with other proteins was predicted using computational methods.

2. Materials and methods

2.1. Plant material and abiotic stress treatments

Fourteen days-old seedlings of *P. glaucum* were subjected to different abiotic stress conditions for variable periods, as described earlier (Reddy et al., 2015). Briefly, the seedlings were subjected to drought stress by withholding water for 12 to 72 h. Low and high temperature stress conditions were simulated by incubating the plants at 4 °C and 45 °C, respectively, for different time intervals, ranging between 0.5 and 10 h. Salinity stress was administered by dipping the seedlings in 250 mM solution of sodium chloride for 1 h, 4 h, 8 h, 12 h, 24 h and 36 h. Control conditions were maintained in greenhouse for each stress treatment. The tissue samples were collected from plants subjected to stress conditions and respective control plants, at different time intervals. The samples were collected in three biological replicates, flash frozen in liquid nitrogen and stored at − 80 °C for subsequent RNA isolation and transcript analysis.

2.2. Cloning of the PgPmp3 cDNA and genomic clones

The stress responsive EST database of *P. glaucum* was searched to find clones showing maximum identity with the *Pmp3* genes (*PgPmp3-1* GenBank accession no. CD725134 and *PgPmp3-2* GenBank accession no. CD724750) (Mishra et al., 2007). The *PgPmp3-1 and 3-2* genes were PCR amplified by using cDNA and genomic DNA as templates. 150 ng of *PgPmp3-1* forward (5′-ATGTCGGACGGCACGGCGAACT-3) and reverse (5′-CTACTTGGTGATGGCCCAGAC) and *PgPmp3-2* forward (5′-ATGTCAGAGGGGACGGCCAACT-3′) and reverse (5′-CTACTTGGTGATGGCGTAGACG-3′) gene specific primers along with 200 μM of dNTPs, 2.5 units of Taq DNA polymerase (Invitrogen) and genomic DNA/cDNA template in a 50 μl reaction volume. The PCR cycling conditions include 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min for 30 cycles. Amplified PCR products were cloned into the Topo-TA 4.0 vector (Invitrogen) according to the manufacturer's protocol and sequenced.

2.3. Sequence analysis of PgPmp3 genes

The BLASTP and BLASTN programmes from National Centre for Biotechnology Information (NCBI) were used to perform sequence similarity searches for identification of genes encoding *Pmp3* from other plant species. Multiple sequence alignments were performed by using ClustalW of MacVector. The *PgPmp3* genes were characterized by determining the open reading frame (ORF) length and intron numbers. This was confirmed by comparing the sequences of cDNA and respective genomic clones using the EMBL sequence alignment and MacVector ClustalW programmes. Translated cDNA sequences from other plant species were used to construct a neighbor-joining tree. Theoretical isoelectric point (pI), molecular weight, hydropathy analysis, aliphatic index and estimated half-life of the PgPmp3-1 and PgPmp3-2 proteins were determined using the Expert protein analysis system (EXPASY) tools (http://www.ebi.ac.uk/Tools).

2.4. Structural and network analysis of Pmp3 proteins

The secondary structure of Pmp3 proteins was predicted by using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) (Buchan et al., 2013). The interaction of *Pmp-3* with other genes and proteins, its derived functions and conserved co-expression was predicted using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (Szklarczyk et al., 2015).

2.5. RNA isolation, cDNA synthesis and qPCR analysis

Total RNA was isolated from *P. glaucum* seedlings exposed to different abiotic stress conditions and their corresponding controls using the TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany). cDNA was synthesized using first strand cDNA synthesis kit (Invitrogen GmbH, Karlsruhe, Germany) and used for qPCR amplification using specific primers (*PgPmp3-1* [F: 5′-CGAACTGCATCGACATCATC-3′ and R: 5′-GGCAGATCCAGAACTCAACC-3′], *PgPmp3-2* [F: 5′-AACTGCGTGGACATCCTGA-3′ and R: 5′-GCGTAGACGGCGTAGATGAT-3′] and *PgMDH* [F: 5′-AGAAGGCGCTTGCTTACTCAT-3′ and R: 5′-CAGTTCTGGGTGAGGGAATCT-3′]). qPCR reactions were performed in optical 96-well plates with an iCycler (BioRad, USA) using SYBR® Green. The reaction conditions were programmed to 2 min at 95 °C (polymerase activation), 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplicon dissociation curves were recorded after cycle 40 by heating from 60 °C to 95 °C with a ramp speed of 1.9 °C min^{-1}. Experiments were performed independently three times, and the average data was considered for further analysis. The relative change in expression levels of *Pmp3* transcripts in different tissues of the plant or in response to abiotic stress conditions was predicted using REST software (Pfaffl et al., 2002) using *PgMDH* as the reference gene (Reddy et al., 2015). Statistical analyses were performed using the CoStat version 6.204 (Cohort Software, Monterey, CA, USA), applying the one-way ANOVA test. Means were compared using the Tukey-Kramer; difference regarded statistically significant at $p < 0.05$.

3. Results

3.1. Cloning, sequence analysis and genomic organization of the PgPmp3 genes

The sequence similarity searches for identifying *Pmp3* genes in *P. glaucum* revealed 3 putative *Pmp3* homologs based on sequences from other plant species. In addition to *PgPmp3-1* and *PgPmp3-2*, an EST CD725521.1 was identified. Upon alignment with *Pmp3* gene homologs from other organisms, 20 to 83% homology was observed with a maximum query cover of 25% which was much lower than what was achieved with *PgPmp3-1* and *PgPmp3-2* (70 to 99% identity with a query cover of 30 to 41%). The sequence similarity of EST CD725521.1 with yeast *Pmp3*, *PgPmp3-1* and *PgPmp3-2* at nucleotide level was lower (66%, 49% and 42% respectively). Considering that these were not significant, we chose to restrict the scope of this study with *PgPmp3-1* and *PgPmp3-2* only.

The 171 bp long cDNA fragments corresponded to *PgPmp3-1* (GenBank accession no. CD725134) and *PgPmp3-2* (GenBank accession no. CD724750) respectively. BLASTN indicated that the two genes shared 87% identity with each other, while exhibiting 70 to 99% identity with homologous genes from monocots and dicots. The structural organization of *PgPmp3-1* and *PgPmp3-2* genes was studied by comparative analysis of the genomic and CDS sequences. The

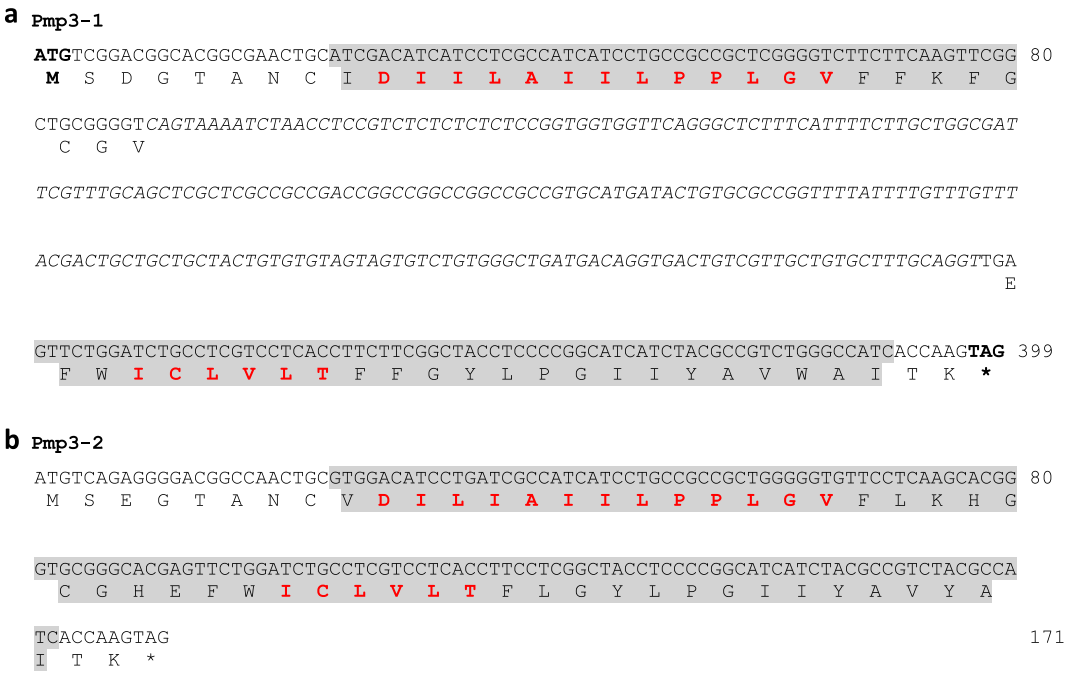

Fig. 1. Sequence analysis of *PgPmp3* genes. (a) *PgPmp3-1* shows presence of two exons, interrupted by an intron, while (b) *PgPmp3-2* contains a single exon. The highlighted region corresponds to the conserved domain encoding plasma membrane potential modulator.

genomic organization indicated presence of two exons of 89 bp and 82 bp length, interrupted by a single 229 bp intron in *PgPmp3-1* gene (Fig. 1a), corresponding to a 171 bp coding region. In contrast, *PgPmp3-2* gene contained only a single 171 bp long exon (Fig. 1b).

3.2. Characteristics of PgPmp3 proteins and phylogenetic analysis

The two proteins, *viz.*, PgPmp3-1 and PgPmp3-2 were found to be comprised of 56 amino acids each, and their molecular weights were predicted as 6.172 kDa and 6.109 kDa respectively, using the ProtParam tool (Expasy). The theoretical isoelectric points were estimated to be 4.56 and 5.97 respectively. The aliphatic index, a measure of volume occupied by the side chains of the protein was 132.32 for PgPmp3-1, while it was 139.29 for PgPmp3-2. BLASTP indicated that PgPmp3-1 and PgPmp3-2 proteins shared 83% identity with each other. Proteins exhibiting > 70% sequence identity with PgPmp3-1 and PgPmp3-2 were identified by using BLASTP analysis. The amino acid sequence alignment showed two conserved domains, IILAIILPPLGV and EFWICL (Fig. 2), specific for the Pmp3 genes (Pfam01679), as confirmed by using NCBI's conserved domain database (Marchler-Bauer et al., 2011). Additionally, a 46 amino acid long proteolipid membrane potential modulator motif was predicted to be present between the 8th and 54th position of the Pmp3 protein.

The sequences of proteins homologous to *P. glaucum* PgPmp3-1 and

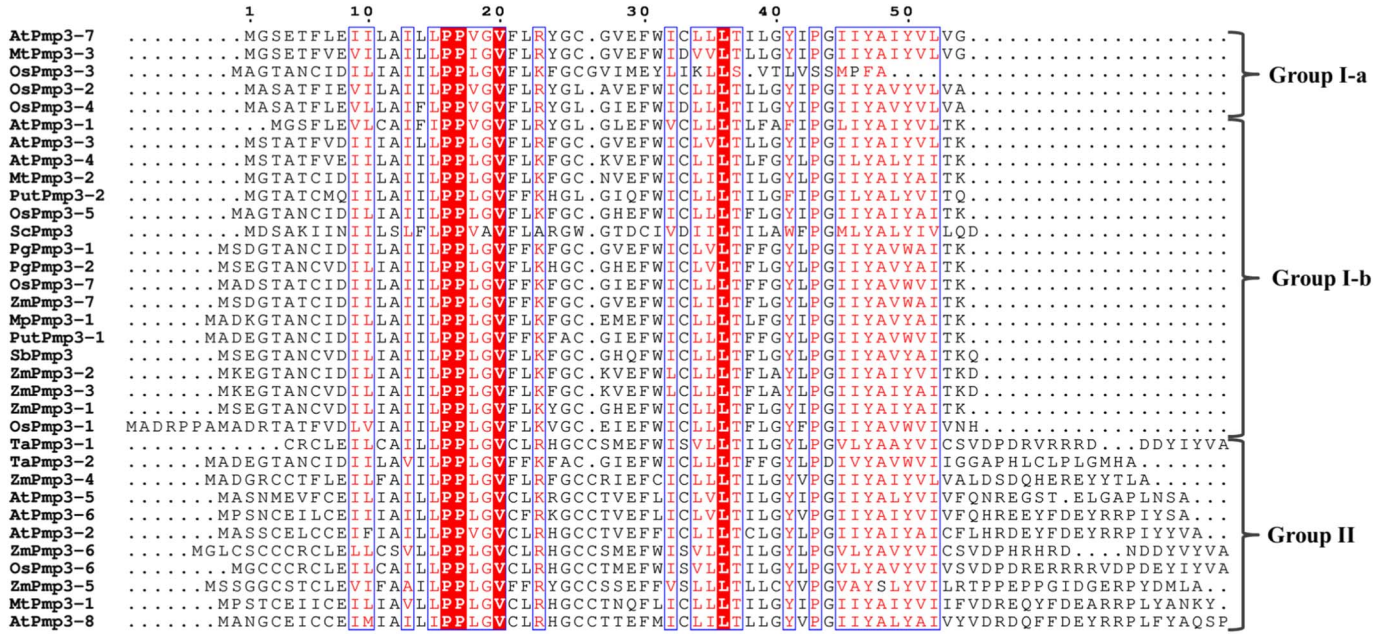

Fig. 2. Sequence analysis of Pmp3 family proteins: Multiple sequence alignment of Pmp3 proteins belonging to monocots, dicots and yeast, showing conserved domains LPPLGV and LVLT (displayed in boxes).

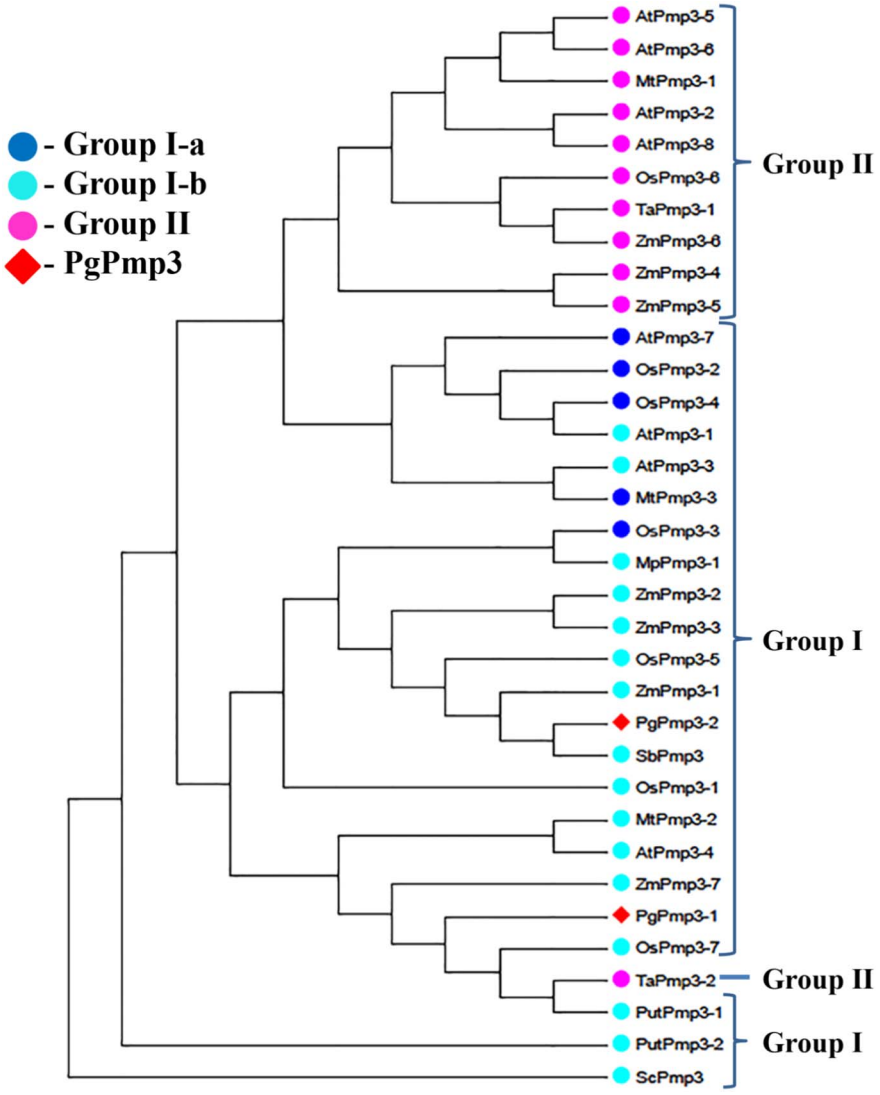

Fig. 3. Phylogenetic analysis of Pmp3 family proteins: Neighbor Joining tree of Pmp3 protein sequences belonging to *P. glaucum*, *Z. mays*, *O. sativa*, *S. bicolor*, *T. aestivum*, *M. paradisica*, *A. thaliana*, *M. trunculata*, *P. tenuiflora*, *S. cerevisiae*. The proteins are classified as Group Ia (dark blue), Group Ib (light blue) and Group II (pink). PgPmp3-1 and PgPmp3-2 are depicted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

PgPmp3-2, were selected from the following plants: *Z. mays* (NP_001107634.1, NP_001147403.2, NP_001147508.1, NP_001151727.2, NP_001151840.1, NP_001151922.1, NP_001152565.1, NP_001307385.1), *O. sativa* (AAG46140.1, AAT77365.1, XP_015633211.1, XP_015640253.1, XP_015643303.1, XP_015647973.1), *Sorghum bicolor* (XP_002440557.1), *T. aestivum* (AAN06944.1, CDM82662.1), *Brachypodium distachyon* (XP_0035-68974.1), *Musa paradisica* (ACA66247.1), *A. thaliana* (NP_001323801.1, NP_176067.1, NP_179982.1, NP_187239.1, NP_187240.1, NP_194794.1, NP_194795.1, NP_565897.1, NP_974629.1), *Medicago truncatula* (XP_003610298.1, XP_003626132.1, XP_013451651.1, XP_013458588.1), *P. tenuiflora* (BAG54793.1, BAG54794.1) and *S. cerevisiae* (NP_010562.1) and were used to conduct multiple sequence alignment analysis. All Pmp3 sequences were observed to be highly conserved and could be classified into two groups, I and II (Figs. 2, 3). In general, Group I Pmp3s have been reported to be 49 to 58 amino acids long; while the group II was 64 to 76 amino acids in length. Group I Pmp3s were further divided into sub-groups Ia and Ib on the basis of their C-termini hydropathicity (Group Ia proteins present a hydrophobic C-termini while Group Ib proteins have hydrophilic C-termini ends. Group II proteins have an extra 20 to 30 highly charged residues at the C-terminus tail).

For a systematic study on the evolutionary relationship between Pmp3 proteins from *P. glaucum* and other organisms, a NJ phylogenetic tree was constructed with 1000 bootstrap iterations, by aligning the sequences. The phylogenetic tree showed distinct clades representing group I and group II Pmp3 proteins. While, both PgPmp3-1 and PgPmp3-2 broadly fall in group I, PgPmp3-1 was found to be evolutionarily close to Pmp3 from maize (ZmPmp3-7) while PgPmp3-2 showed a close relationship with SbPmp3 (Fig. 3).

3.3. Structure prediction and network analysis of PgPmp3 proteins

The secondary structure predicted using PSIPRED suggested a presence of five helices with a β-strand between the second and third helix in PgPmp3-1 (Fig. S1a). The TMHMM profile indicated transmembrane localization between the 5th and 27th positions, followed by an intracellular region (28 to 31 amino acids), tailed by another transmembrane region (32nd to 54th positions), as shown in Fig. 4a. The secondary structure of PgPmp3-2 showed four helices and a β-sheet (Fig. S1b). PgPmp3-2 was partially localized in the plasma membrane, separating two transmembrane regions, the first between 5th and 22nd positions and the second between 32nd and 54th positions. PgPmp3-2 showed a longer extracellular region between 23rd to 31st residues, as described in Fig. 4b.

To gain some insight regarding the interactions of Pmp3 proteins with other functional partners, network analysis was performed using the

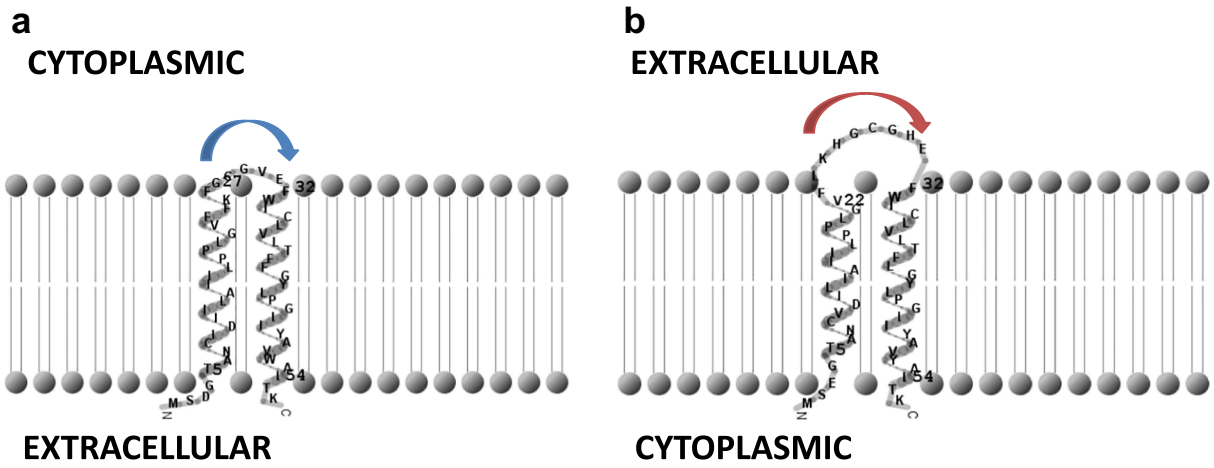

Fig. 4. Transmembrane orientation of PgPmp3 proteins. (a) PgPmp3-1, showing extracellular N- and C-terminals, two membrane spanning domains, 4 residue long cytoplasmic region and (b) PgPmp3-2, showing cytoplasmic N- and C-terminals, two membrane spanning domains and a 9 residue long extracellular region.

STRING database. For this, LTI6A and LTI6B, homologs of PgPmp3-1 and PgPmp3-2 from *Z. mays* were used. Association of these two proteins with 8 functional partners with confidence score exceeding 0.4 were selected (Table 1). Proteins LTI6A and LTI6B exhibited co-occurrence across genomes. Proteins LTI6B and cl37957_1, homologs of PgPmp3-2 were predicted to be co-expressed along with gst30, dhn-2, gpm592 and GRMZM2G037452_P01 (Fig. 4b). Similarly, LTI6A and LTI6B were involved in protein binding and post-translational modifications with other proteins such as GRMZM2G181378_P01 (E3 ubiquitin-protein ligase) and GRMZM2G080439_P01 (uncharacterized protein) (Fig. 5a). The putative role of each of these proteins based on Gene Ontology and KEGG analyses could be linked to abiotic stress tolerance (Table 1) (Gene Ontology Consortium, 2015; Kanehisa et al., 2016).

3.4. Expression profiling of PgPmp3 genes in response to abiotic stress treatments

The expression of the *Pmp3* genes from *P. glaucum* was analyzed in response to abiotic stress treatments such as low and high temperatures, salinity and drought. It was found that the expression of *PgPmp3-1* and *PgPmp3-2* genes was induced in response to cold stress showing maximum expression at 6 h and 8 h, showing 5-fold and 18-fold upregulation respectively (Fig. 6a). High temperature stress caused a rapid 4-fold and 48-fold higher upregulation up to 2 h, followed by gradual downregulation in both *PgPmp3-1* and *PgPmp3-2*, respectively (Fig. 6b). Furthermore, the relative expression of both the genes showed gradual upregulation when subjected to salt stress and positively correlated with the duration of the stress (Fig. 6d). However, in plants subjected to drought stress, the relative transcript abundance of *PgPmp3-1* decreased from 1.2 to 0.2 fold, over a period of 72 h, whereas, *PgPmp3-2* was steadily upregulated from 2 to 22 fold with time (Fig. 6c). Tukey-Kramer test revealed significant relative expression ($p < 0.05$) of *PgPmp3-1* and *PgPmp3-2* genes, at different time points in response to abiotic stress conditions (Fig. 6).

4. Discussion

P. glaucum (L.), commonly known as pearl millet belongs to the *Poaceae* family. It is mostly cultivated in the semi-arid regions, and is well adapted to heat, salinity and drought stress. Besides these stresses, *P. glaucum* also thrives in low temperatures (Desai et al., 2006) being treated as a post monsoon cool season crop in certain parts of India, growing in temperatures as low as 10 to 15 °C (Mula et al., 2009). Though the crop thrives under low temperatures, poor seed set clubbed with low yields are main concern in certain genotypes. To counter such losses, it is important to elucidate the factors contributing to low temperature tolerance in *P. glaucum* genotypes. Abiotic stress conditions, particularly low temperature stress, affect the plasma membrane (Osakabe et al., 2013). Freezing temperatures are detrimental for the integrity of the membrane and may induce electrolyte leakages (Lyons,

Table 1
Predicted functional partners of Pmp3 proteins and interacting proteins as predicted using the search tool for the Retrieval of Interacting Genes/Proteins (STRING).

String ID	Protein	Family	Length (amino acids)	Function
umc1359	LTI6B	Pmp3	57	Membrane potential modulator and resistance to ionic stress
cl37957_1	uncharacterized protein	Pmp3	58	Membrane potential modulator and resistance to ionic stress
pmpm3	LTI6B	Pmp3	75	Membrane potential modulator and resistance to ionic stress
pmpm5	LTI6A	Pmp3	56	Membrane potential modulator and resistance to ionic stress
gst30	Glutathione-S-transferase 30	Glutathione-S-transferase 30	257	Detoxification of electrophilic compounds
GRMZM2G181378_P01	uncharacterized protein	E3 ubiquitin-protein ligase	871	Regulation of cell processes
GRMZM2G080439_P01	uncharacterized protein	E3 ubiquitin-protein ligase	880	Regulation of cell processes
dhn-2	dehydrin	dehydrin	289	Survival during drought stress
GRMZM2G037452_P01	uncharacterized protein	CYSTM (cysteine-rich membrane proteins)	69	Cell signaling

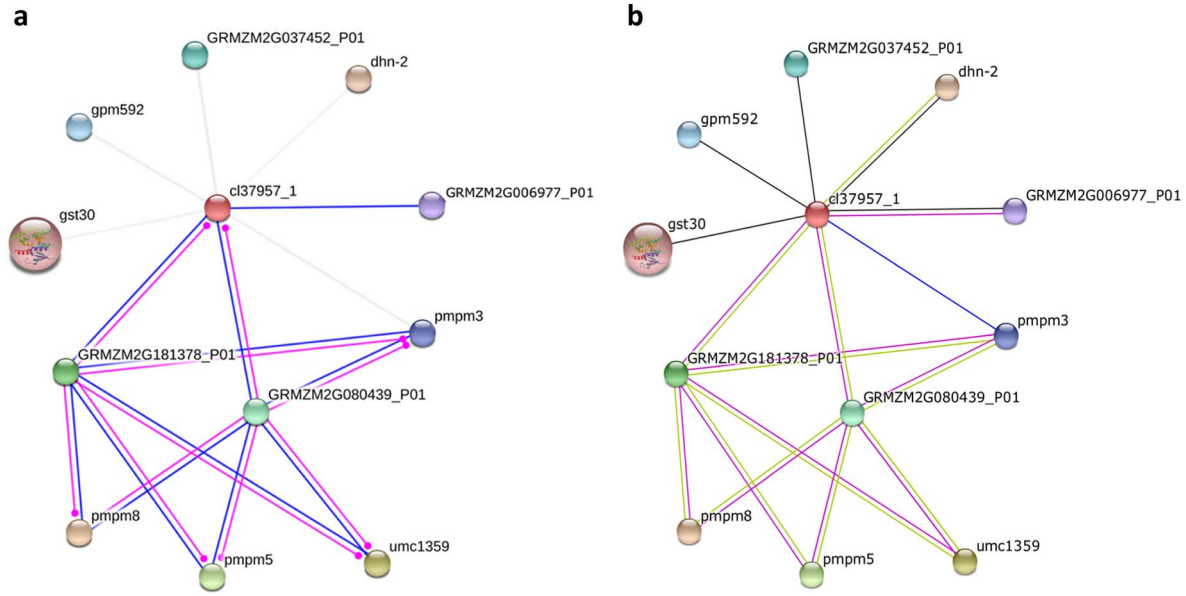

Fig. 5. STRING network analysis of Pmp3 family proteins. Homologs of PgPmp3-1 and 642 PgPmp3-2 from *Z. mays* showing (a) functional associations [pink: protein binding; blue: post-translational modifications; grey: text mining] and (b) co-occurrence and co-expression across genomes [blue: co-occurrence across genomes; green: co-expression; magenta: used in same experiments]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2012). Temperatures as low as 4 °C affect the ion transport at the membrane. Many integral and surface proteins are known to contribute towards maintaining membrane integrity during stress conditions. For example, *RCI2A* and *RCI2B* gene homologs of *Pmp3* from *A. thaliana* showed expression in response to cold, salt and dehydration stresses (Medina et al., 2001). Similarly, the expression of *RCI2* gene, another homolog *of Pmp3* from *M. paradisiaca*, conferred enhanced tolerance to low temperatures when expressed in tobacco (Feng et al., 2009). Likewise, *Pmp3* genes from various organisms are reported to be expressing under various abiotic stress conditions, indicating that these might play a crucial role in stress tolerance.

In the present study, two genes *PgPmp3-1* and *PgPmp3-2* from *P.*

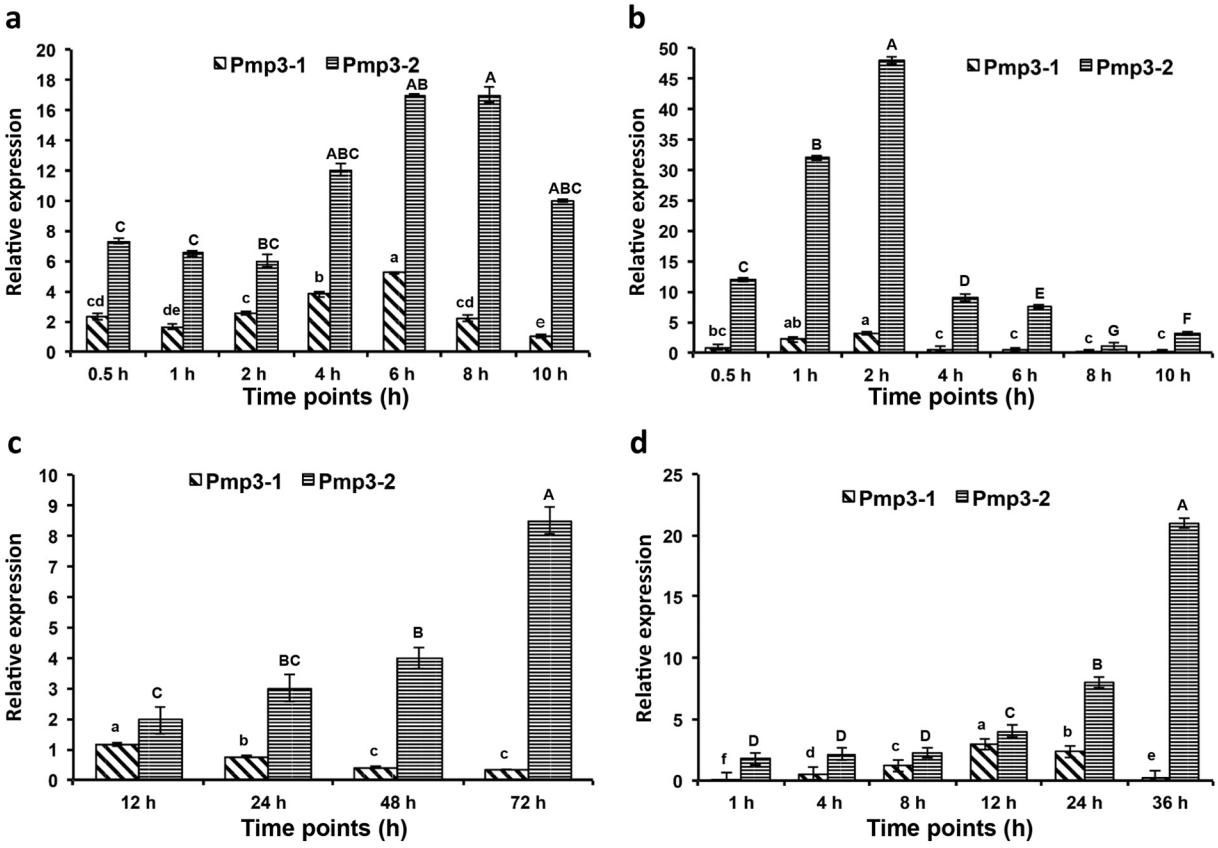

Fig. 6. Real time expression profile of *PgPmp3-1* and *PgPmp3-2* genes in tissue collected from 14 days old plants subjected to abiotic stress conditions. (a) low temperature, (b) high temperature, (c) drought and (d) salt stress conditions. Data represent means ± SEM ($n = 3$). Statistical relationships between groups are indicated by small letters (*PgPmp3-1*) and capital letters (*PgPmp3-2*) where significant differences were detected ($p < 0.05$).

glaucum were identified based on their high similarity with the yeast *Pmp3* genes. These genes were isolated and cloned from the subtracted stress responsive cDNA library, prepared in our earlier efforts (Mishra et al., 2007). Our current study reveals that *PgPmp3-1* had one intron while *PgPmp3-2* gene has none (Fig. 1). These observations are in agreement with previous report on *Pmp3/RCI* genes that have less number of introns, and are expressed under stress conditions (Zhao et al., 2014). Low intron number has also been reported in other stress responsive genes such as *trehalose-6-phosphate synthase* and *late embryogenesis abundant* (*LEA*) (Liang et al., 2016), possibly associated with enhanced gene expression by improving the efficiency of transcript production.

PgPmp3-1 and *PgPmp3-2* showed homology with genes encoding Pmp3/RCI2/LTI and hydrophobic proteins from other organisms. *PgPmp3-1* showed 98% identity with its closest homolog from *Z. mays* and 96% identity with its homolog from *S. bicolor*, while *PgPmp3-2* showed 94% identity with *S. bicolor Pmp3* (Fig. 2), indicating evolutionarily conservation in genes of major cereal crop grasses. The structural diversity of Pmp3 proteins that is reflected from the multiple sequence alignment study is in agreement with previous reports by Medina et al. (2007) and Rocha (2016). Comparative analyses indicated that both genes belonged to the sub-group Ib, as these were predicted to be of 56 amino acids length with hydrophilic C-termini. This structural diversity was also evident from the phylogenetic analysis (Fig. 3). The NJ tree exhibited different clades corresponding to the Pmp3 groups - Ia, Ib and II. The phylogenetic groupings closely matched the hydropathicity of their C-termini.

The PgPmp3-1 and PgPmp3-2 proteins were predicted to contain two hydrophobic stretches corresponding to two transmembrane domains. The orientation of PgPmp3-1 and PgPmp3-2 in the plasma membrane varied with PgPmp3-1 showing extracellular C- and N-terminals as opposed to PgPmp3-2, which had cytoplasmic ends (Fig. 4a and b). The amino acids Phe, Phe and Val at the 24th, 26th and 30th positions in PgPmp3-1 were replaced by Leu, His and His respectively in PgPmp3-2 (Fig. 2a). It is believed that the orientation of the membrane proteins depends on the amino acid composition of the cytoplasmic and extracellular sides (Nakashima and Nishikawa, 1992). Thus, the histidine residues in PgPmp3-2 might be responsible for extracellular orientation of the region. Furthermore, according to ProtLoc (http://bioinf.uab.es/cgi-bin/trsdb/protloc.cgi), the algorithm described by Cedano et al. (1997), the peptide fragment FLKHGCGHE from PgPmp3-2 showed a likeliness of extracellular localization, whereas the fragment CGVEF from PgPmp3-1 was predicted to have intracellular localization. Although the use of structure prediction tools shed a light upon the structure and localization of PgPmp3 proteins, little is known about the exact topology of these proteins through experimental studies. Earlier, Rocha (2016) obtained conflicting topology results using various prediction tools and hence highlighted limitations in such studies. Considering this, in our study we chose to use multiple prediction tools such as TMHMM, MEMSTAT, Phobius, Philius and Scampi to avoid such conflicts. The topology of PgPmp3-1 predicted by TMHMM was in agreement with results of MEMSTAT, while the TMHMM topology of PgPmp3-2 matched the output of tools such as Phobius, Philius and Scampi. The presence of conserved domains related to the proteolipid membrane potential modulator, reflects the role of Pmp3 in abiotic stress tolerance, especially under cold stress (Shabala et al., 2016). Moreover, the Gene Ontology server suggested that Pmp3 proteins might mediate a proton leak suggesting that Pmp3 plays a role in ion homeostasis, an important step in countering salt stress (Zhu, 2001).

Proteins homologous to the Pmp3 family, that is, LTI6A and LTI6B from *Z. mays*, were analyzed *in silico* for presence of functional interactions with other orthologs by using STRING database. This study revealed the synergistic interaction of proteins contributing towards abiotic stress tolerance. The proteins were co-expressed with other Pmp3 proteins, responsible for membrane potential modulation, particularly to resist ionic stress (Fig. 5a and b; Table 1). Other proteins such as dehydrin, glutathione-s-transferase 30 (gst30), ubiquitin-protein transferase and cysteine-rich membrane proteins (CYSTM) were found to be associated with LTI6A and LTI6B. Dehydrin, a protein aiding survival during drought stress (Graether and Boddington, 2014) is highly hydrophilic, and binds to the membrane periphery playing an important role in protecting the membrane from freezing (Drira et al., 2013). Likewise, glutathione-s-transferase involved in detoxification of electrophilic compounds, has been shown to bestow salt tolerance to the cell (Chen et al., 2012). In a similar fashion, ubiquitin-protein transferase, a protein involved in ubiquitination has been associated with Pmp3 proteins in stress tolerance. Ubiquitination mediated by E3 ubiquitin ligases regulate numerous cell processes and facilitate tolerance towards stress conditions (Lyzenga and Stone, 2012). Similarly, cysteine rich membrane proteins, particularly receptor-like kinases, activate signaling pathways in response to environmental stimuli (Kanehisa et al., 2016). As a whole, the combined effects of all these associations potentially aid the plant in building tolerance to adverse environmental conditions.

It is a well-known fact that abiotic stresses such as salt, low temperature and drought conditions result in ionic imbalance leading to secondary stresses (Mahajan and Tuteja, 2005). The immediate defense response of plants involves stress alleviation, followed by maintaining homeostasis and regaining growth. This requires complex molecular responses (Zhu et al., 1997). In our study, the *P. glaucum* genes belonging to the *Pmp3* family were found to show increased expression under simulated low temperature and high salt stress (Fig. 6a and d). In addition to cold and salinity, *PgPmp3-2* was also induced by drought conditions (Fig. 6c). This could be attributed to presence of different *cis*-regulatory elements in the promoter region of *PgPmp3-1* and *PgPmp3-2* genes. However, this specifically could not be studied in detail due to non-availability of *P. glaucum* genome sequence. In several studies, *Pmp3* genes from various sources have demonstrated an increase in transcript levels in response to cold and salinity. For instance, expression of a number of *Pmp3* genes from *A. thaliana* and *Z. mays* has been reported to be induced in response to cold, drought and salinity stress (Rocha, 2016). Furthermore, maize *Pmp3* genes were also reported to be involved in salt stress tolerance (Fu et al., 2012). Similarly, *Pmp3* from the yeast (*S. cerevisiae)* has not only shown to be expressed in response to abiotic stress conditions such as low temperature and salt stress (Bari et al., 2015), but also deletions in *Pmp3* have led to membrane hyperpolarization and salt sensitivity (Navarre and Goffeau, 2000). In another study, *P. tenuiflora Pmp3* genes, along with their rice homologs functioned in reversing stress induced membrane hyperpolarization and countering salt stress (Chang-Qing et al., 2008).

5. Conclusion

In this study, we have cloned and characterized two *P. glaucum* genes belonging to the Pmp3 family, encoding 56 amino acid long transmembrane proteins. These genes were found to share major identity with stress inducible genes belonging to other cereals such as maize and sorghum. Conserved domains corresponding to a plasma membrane potential modulator were identified in these proteins, supporting their role in abiotic stress response. *PgPmp3-1* and *PgPmp3-2* were induced under abiotic stress conditions such as high salt and low temperature. *PgPmp3-2* also showed enhanced expression under drought stress. Moreover, Pmp3 proteins were predicted to interact along with other proteins contributing to stress tolerance in plants. Further studies pertaining to the mechanism of Pmp3 in stress alleviation may lead to greater insight in plant stress tolerance.

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Author contribution statement

Conceived and designed the experiments: PSR and MKR. Performed the experiments: RY, RN and PSR Analyzed the data: RY, TC and PBM. Wrote the paper: RY, RN and PSR.

Conflict of interest

The authors declare no conflict of interest.

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