Characterization of two plasma membrane protein 3 genes (PutPMP3) from the alkali grass, Puccinellia tenuiflora, and functional comparison of the rice homologues, OsLti6a/b from rice

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Two full-length cDNAs, PutPMP3-1 and PutPMP3-2, encoding PMP3 family proteins were isolated from Puccinellia tenuiflora, a monocotyledonous halophyte. Expression of both genes was induced by low temperature, salt stress, dehydration, ABA, and NaHCO₃. Transcripts of PutPMP3-2 were more strongly induced by these stresses relative to those of PutPMP3-1, particularly under low temperature and dehydration conditions. Expression of PutPMP3-1 and PutPMP3-2 in yeast mutants lacking the PMP3 gene can functionally complement the membrane hyperpolarization and salt sensitivity phenotypes resulting from PMP3 deletion. To compare the functions of PutPMP3-1 and PutPMP3-2, the orthologous genes in rice (OsLti6a and OsLti6b) were isolated. Both OsLti6a and OsLti6b could functionally complement the loss of PMP3 in yeast. PutPMP3-2 and OsLti6a were more effective in reversing membrane hyperpolarization than PutPMP3-1 and OsLti6b. However, the four yeast transformants each showed similar levels of salt tolerance. These results imply that these PMP3 family members don't function identically under different stress tolerance conditions.

INTRODUCTION

Plant growth and productivity are affected by various abiotic stresses, such as high salinity, drought, extreme temperature, chemical toxicity, and oxidative stress. Plants respond and adapt to these environmental stimuli at a physiological and biochemical level to initiate tolerance mechanisms (1-4). The cell membrane is often the direct target of injurious stress, which leads to physical and biochemical changes resulting in irreversible damage. Many researchers have directed their attention to the elucidation of changes in plasma membrane composition as well as its structure and function under stress. It has been shown that the membrane proteins that respond to stresses have putative roles in the repair and protection of membranes as well as signal transduction (5-10).

Many lower forms of eukaryotes and higher plants have a class of small molecular weight hydrophobic proteins that respond differently to stresses, such as cold temperature, salt, and dehydration (6, 11-14). These proteins belong to the yeast plasma membrane protein 3 (PMP3) family. The yeast pmp3 mutant, which lacks the PMP3 gene, accumulates excess Na⁺ ions within the cell and demonstrates an increased Na⁺ sensitivity (15, 16). Loss of PMP3 has been suggested to induce hyperpolarization of the membrane potential and promote a non-specific influx of monovalent cations. In Arabidopsis, RCI2A plays a role directly or indirectly in preventing accumulation of excess Na⁺ and K⁺ ions and contributes to salt tolerance (17). AcPMP3 in sheep grass has been shown to have a similar role (14). RCI2A may be involved in CBF/DREB1-independent signaling pathways during abiotic stress responses (18), but the OsLti6 proteins in rice are thought to be part of a battery of cold stress defense-related proteins regulated by DREB (13).

The exact role of the PMP3 proteins under stress remains unclear. In addition, little is known about the functional difference among the PMP3 homologous. Puccinellia tenuiflora (P. tenuiflora), a halophytic monocotyledonous species belonging to the genus Gramineae, is distributed in the saline-alkali soil of the Songnen plain in northeastern China, which is characterized by extreme temperatures and drought. P. tenuiflora appears to have developed an efficient stress tolerance system and is a potential source of genetic determinants for saline-alkali tolerance mechanisms that may be applied to rice. A com-
parison of the two species should provide useful clues for the molecular breeding of stress-tolerant crops.

In this study, we isolated two cDNAs from *P. tenuiflora* encoding small molecular weight proteins belonging to the PMP3 protein class. They are induced under different stress conditions. Yeast mutants show different phenotypes while over-expressing these two genes, even though they encode highly similar proteins at the amino acid level. The rice PMP3 homologous, OsLti6a and OsLti6b, were also isolated. The functional difference between the PMP3 genes from *P. tenuiflora* and their rice counterparts was also investigated.

RESULTS AND DISCUSSION

Cloning of PutPMP3 genes from a cDNA library

A cDNA library was constructed from total RNA extracted from *P. tenuiflora* seedlings subjected to 100 mM NaHCO₃ for 24 h, and nucleotide sequences of more than 3000 EST clones were determined (data not shown). Among these clones, we identified a full-length cDNA encoding a PMP3 homolog. Analyzing the data published by Wang et al. (20), a 91-bp fragment from one of the EST clones (Accession number, CN487549) was found to have high sequence similarity with the PMP3 gene. The full-length cDNA was obtained with 3' and 5' RACE. The 2 clones were designated PutPMP3-1 (GenBank accession number, AB363567) and PutPMP3-2 (GenBank accession number, AB363568), with a nucleotide identity of 46.1%. PutPMP3-1 and PutPMP3-2 encode small molecular weight polypeptides of 57 and 54 amino acid residues with calculated molecular masses of 6.4 and 5.9 kDa, respectively. Southern blot analysis detected a number of homologous to the PutPMP3 gene (data not shown).

A homology blast search of GenBank revealed that PutPMP3-1 and PutPMP3-2 were highly conserved in a number of plant species and lower eukaryotes. These include OsLti6a and OsLti6b from *Oryza sativa* (13), RC2A and RC2B from *Arabidopsis thaliana* (22), AcPMP3-1 and AcPMP3-2 from *Aneuropediium chinense* (14), BLT101 from *Hordeum vulgare* (11), WP16 from *Triticum aestivum* (8), ESI3 from *Lophopyrum elongatum* (X91499), and T23F2.3 as well as T23F2.5 from *Caenorhabditis elegans* (NM076533, NM076633) (Fig. 1A). BLT101, WP16, and ESI3 have the same amino acid sequences but differ in their nucleotide sequences (shown in Fig. 1A, C). Most organisms have at least 2 PMP3-family proteins. There are high similarities between AcPMP3-1 and AcPMP3-2, RC2A and RC2B, but not between OsLti6a and OsLti6b, or PutPMP3-1 and PutPMP3-2 (Fig. 1C). However, there is not enough information to classify them into different subfamilies.

Subcellular localization of PutPMP3::GFP fusion proteins

Both PutPMP3-1 and PutPMP3-2 were predicted to contain
two membrane-spanning domains, using the TMHMM Server v. 2.0 (23) (Fig. 1A, B). To determine the exact subcellular localization of PutPMP3 within plant cells, the sGFP gene was fused in-frame to the C-terminus of PutPMP3-1 and PutPMP3-2 and then transiently introduced into onion epidermal cells by particle bombardment (Fig. 2). Onion epidermal cells expressing GFP showed only cytoplasmic and nuclear localization (Fig. 2 GFP control). However, in the PutPMP3 fusion-expressing cells, GFP signals could be clearly detected in the plasma membrane following plasmolysis in 1.0 M sorbitol (Fig. 2 PutPMP3-1::GFP and PutPMP3-2::GFP). These observations confirmed that PutPMP3 was localized to the plasma membrane. Similar results were previously observed in sheep grass (14) and wheat (8).

Expression profiles of PutPMP3 genes under different stress conditions

Seedlings of *P. tenuiflora* were subjected to different environmental stresses to test for the possible involvement of PutPMP3 genes in tolerance mechanisms. PutPMP3-1 and PutPMP3-2 could be induced by low temperature (4°C), salt stress (150 mM NaCl), saline-alkali stress (100 mM NaHCO₃), exogenous ABA, and dehydration, in both leaves and roots (Fig. 3). PutPMP3-1 was quickly and transiently induced under either low temperature or salt stress. However, the transcript level of PutPMP3-2 showed a continuous increase over time in both leaves and roots under low temperature, and its transient induction under salt stress was more remarkable in the roots than in leaves. A slight and transient induction was observed in both leaves and roots after exogenous application of NaHCO₃ and ABA treatment. The expression of PutPMP3 was not obviously influenced by circadian rhythm (Fig. 3A, B). Under dehydration stress, both genes showed an early and quick induction at 5-10 % fresh weight loss, when no other obvious change in the plants could be observed. The transcript levels remained high up to a 30 % fresh weight loss, especially in the roots (Fig. 3C). Taken together, these results indicate that PutPMP3-1 and PutPMP3-2 were induced under different stresses, especially under low temperature, salt stress, and dehydration, where the transcript level of PutPMP3-2 demonstrated a greater increase relative to that of PutPMP3-1.

Similar results were obtained for AcPMP3-1 and AcPMP3-2 in sheep grass (14). RCI2A and RCI2B of Arabidopsis are also induced by low temperature, ABA, and dehydration, but not by salt stress (22). In rice, OsLt6a and OsLt6b exhibit a genotype-specific expression signature characterized by early and late stress-inducible expression in tolerant and intolerant genotypes, respectively. These genes also have an organ-specific expression pattern that wasn’t observed with the PutPMP3 genes (13). WPI6 was induced at low temperature (8); BLT101 was detected at low temperature but not under drought conditions or ABA treatment (11). However, ESI3 was induced by NaCl, osmotic stress, and ABA treatments (12). These results suggest that the responsiveness of PMP3 family proteins to
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Fig. 3. Northern blot analysis of PutPMP3-1 and PutPMP3-2 in leaves (A) and roots (B) of P. tenuiflora under low temperature (4°C), salt stress (150 mM NaCl), saline-alkali stress (100 mM NaHCO₃), 20 μM ABA, and dehydration (C). Thirty-five-day-old seedlings were used in all treatments. The seedlings were transferred to fresh Kimura solutions supplemented with 150 mM NaCl, 100 mM NaHCO₃, or 20 μM ABA. For low temperature stress analysis, the seedlings were placed in fresh Kimura solution pre-cooled to 4°C. For dehydration stress analysis, seedlings were blotted quickly with filter paper and placed horizontally on a paper towel at room temperature and the rate of seedling’s fresh weight loss was recorded, represented as % in (C). The leaves and roots were sampled at different times.

Fig. 4. Functional complementation of yeast mutants. (A) 10-fold dilutions of YR31-1 (Δpmr2Δnha1), YR93-31 (YR31-1Δpmp3), YR93-31-PutPMP3-1 (YR93-31 PutPMP3-1), and YR93-31-PutPMP3-2 (YR93-31 PutPMP3-2) were spotted onto solid YPD plates or YPD supplemented with 10 μg/ml Hygromycin B (Hyg. B), NaCl (25 or 50 mM), 25 mM NaCl + 10 mM CaCl₂ or MgCl₂. (B) 10-fold dilutions of four different YR93-31 (YR31-1Δpmp3) strains complemented with PutPMP3-1, PutPMP3-2, OsLti6a, and OsLti6b genes, were spotted onto solid YPD plates or YPD supplemented with NaCl (25 or 50 mM) and Hygromycin B (5, 10 or 15 μg/ml). The overnight culture was diluted to OD₆₀₀=0.1 for the starting concentration. Seven μl of solution was used for each dot. The plates were photographed after incubation at 30°C for 48 h except for 50 mM NaCl (Fig. 4A) was taken after 4 d of incubation.

stress may vary by plant genera.

Functional complementation of yeast mutants with the PutPMP3 genes
To characterize the functions of the PutPMP3 genes, the yeast mutant YR93-31 (YR93-1 Δpmp3), lacking the homologous PMP3 gene, was used to test the functional complementation of both PutPMP3 genes. YR93-1 (Δpmr2Δnha1) was used as a control. YR93-1 is highly sensitive to salt when compared to wild type (YR93), because the YR93-1 strain is disrupted for both the plasma membrane Na⁺-ATPase (PMR2) and Na⁺/H⁺ antiporter (NHA1), affecting the direct Na⁺ efflux system (15).
PutPMP3-1 and PutPMP3-2 under the ADH1 promoter were strongly expressed in YR93-31. A 10-fold dilution dot experiment demonstrated that expression of PutPMP3 genes could partially suppress the sensitivity to hygromycin B of the pmp3 mutant YR93-31 (Fig. 4A). As the electric potential of the plasma membrane has been reported to be a major determinant of toxic cation tolerance, such as hygromycin B, these results suggest that both PutPMP3 genes abolish the membrane hyperpolarization resulting from PMP3 disruption (15). The high NaCl sensitivity of the pmp3 mutant was also complemented by the expression of PutPMP3 genes, and the divalent cations Ca\(^{2+}\) or Mg\(^{2+}\) completely reversed the salt sensitive phenotype in wild type, the pmp3 mutant, and the PutPMP3 transformants. Ca\(^{2+}\) was reported to have a role in providing salt tolerance to plants (24). The NaCl sensitivity phenotype of these yeast strains decreased when the divalent cation concentrations ranged from 2, 5, to 10 mM (data not shown). Interestingly, PutPMP3-2 was more effective than PutPMP3-1 at reversing the membrane hyperpolarization (Fig. 4A), even though the two genes equally complemented the sensitivity of YR93-31 to NaCl. Other genes that could partially complement PMP3 function were Arabidopsis RC12A, sheep grass AcPMP-3-1, and wheat WP6 (8, 14, 15).

**Functional comparison among the PutPMP3 and OsLti6 genes**

*P. tenuiflora* is closely related to many monocotyledonous crop species, including rice (19). The PMP3 orthologues in rice (OsLti6a and OsLti6b) were isolated and introduced into the pmp3 mutant, YR93-31. The four yeast transformants had similar tolerance to 25 mM and 50 mM NaCl (Fig. 4B) and no obvious growth was observed after 48 h when the NaCl concentration reached 75 mM (data not shown). Growth of the transformants decreased with increasing hygromycin B concentration (Fig. 4B). The PutPMP3-2- and OsLti6a-transformed yeasts were clearly more tolerant towards hygromycin B, indicating that these two genes could effectively reverse the membrane hyperpolarization. Curiously, the OsLti6a-transformed yeast behaved more like the PutPMP3-2-transformed yeast, even though OsLti6a is more similar to PutPMP3-1 than PutPMP3-2 (Fig. 1C). These results imply that members of the PMP3 family of proteins don't function identically while regulating cell membrane potential and salt tolerance in spite of their high similarity in amino acid sequence. In plant cells, regulation of the membrane potential can modulate Na\(^{+}\) uptake (25). RC12A contributes to salt tolerance by preventing excess Na\(^{+}\) accumulation within the cell during high NaCl treatment in Arabidopsis (17).

In conclusion, both PutPMP3-1 and PutPMP3-2 were localized to the plasma membrane and their expression was induced under different stress conditions. Transcripts of PutPMP3-2 were more strongly induced under stress conditions relative to those of PutPMP3-1, particularly during low temperature and dehydration stress. Both PutPMP3 genes and their rice orthologues, OsLti6a and OsLti6b, can functionally comple-
were recovered on plates for 12 h at 28°C in the dark and then plasmolyzed in 1.0 M Sorbitol for 10 min to examine clearly localization at the plasma membrane. The fluorescence was observed by confocal microscopy (Nikon TE-200U Confocal CI). Filter sets were used to visualize and to discriminate between fluorophores.

**Northern blot analyses**

Total RNA was isolated from frozen samples using TRizol reagent (Invitrogen, U.S.A.). For RNA blot analyses, 8 μg of total RNA was denatured in formamide and formaldehyde, and then separated on 1 % formaldehyde agarose gels according to the standard protocol. The DIG-labeled cDNA probe was amplified by PCR with primers: *PutPMP3*-1 forward one 5'-GGTACCCAGAAGAATGGCGGA-3' and reverse one 5'-GAGCTCGGTCCGTCTACTTGG-3'; *PutPMP3*-2 forward one 5'-GAGCTCGGTCCGTCTACTTGG-3' and reverse one 5'-GAGCTCGGTCCGTCTACTTGG-3', using PCR DIG Labeling Mix (Roche Diagnostics, Switzerland). RNA blots were hybridized overnight at 50°C. The membranes were washed in 0.5 % SSC and 0.1 % SDS at 65°C. The signals were detected with CDP-Star detection reagent (GE Healthcare Life Sciences, UK) using a LAS-1000 plus image analyzer (Fuji Film, Japan).

**Functional complementation of *PutPMP3* genes in yeast**

The ORFs of *PutPMP3*-1 and *PutPMP3*-2 were amplified using primers with enzyme adapters: *PutPMP3*-1 forward primer, 5'-GGTACCCAGAAGAATGGCGGA-3' and reverse one, 5'-GAGCTCGGTCCGTCTACTTGG-3'; *PutPMP3*-2 forward primer, 5'-GAGCTCGGTCCGTCTACTTGG-3' and reverse one 5'-GAGCTCGGTCCGTCTACTTGG-3', respectively. The fragments were ligated into pBluescript cloning vectors. The positive plasmids and the yeast expression vector pAUR123 (Takara, Japan) were digested by KpnI and SacI simultaneously. Successfully ligated all of these products were transformed to the constructs pAUR123-*PutPMP3*-1 and pAUR123-*PutPMP3*-2. The constructs were confirmed by sequence analysis. *Saccharomyces cerevisiae* strains YR93-1 (MATa ade2 his3-Δ200 leu2-3,112 lys2-Δ201 ura3-52 gal2-Δpmr2-2::HIS3 Δrho1::LEU2) and YR93-31 (YR93-1 Δpmr3::URA3) were kindly donated by Dr. Goffeau (15). To test whether the *PutPMP3* genes can functionally replace yeast *PMP3* in the Δpmp3 yeast mutant (YR93-31), this strain was ligated with the above constructs, resulting in the strains YR93-31-*PutPMP3*-1 and YR93-31-*PutPMP3*-2.

For yeast growth experiments, YR93-1, YR93-31, YR93-31-*PutPMP3*-1 and YR93-31-*PutPMP3*-2 were cultured over night to an OD600-1.0 and then diluted to 0.1 with YPD medium (2 % polypeptone, 1 % w/v) yeast extract, 2 % (w/v) glucose). The diluted yeast strains were dotted onto solid YPD plates containing 2 % agar supplemented with NaCl, CaCl2, and MgCl2 before autoclaving or supplemented with hygromycin B after autoclaving, as indicated in Fig. 4A. The plates were photographed after incubation at 30°C for 48 h.

**Isolation of OsLti6 genes and introduction into yeast**

According to the GenBank sequence of OsLti6a (AY607689) and OsLti6b (AY607690), cDNA fragments of both genes were isolated via reverse transcription of total rice RNA from plants subjected to 100 mM NaHCO3. The ORFs were amplified using primers with enzyme adapters: OsLti6a forward primer 5'-GGTACCCAGAAGAATGGCGGA-3' and reverse one 5'-GAGCTCTCCTCTCTACTTGG-3'; OsLti6b forward primer 5'-GAGCTCTCCTCCTCTACTTGG-3' and reverse one 5'-GAGCTCTCCTCTCTACTTGG-3'. The plasmids were constructed and YR93-31 was transformed as described above. The four different YR93-31 yeast strains containing the OsLti6 and *PutPMP3* genes were dotted as described above onto solid YPD plates containing NaCl or Hygromycin B.

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