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Transgenic rice expressing a cassava (*Manihot esculenta* Crantz) plasma membrane gene *MePMP3-2* exhibits enhanced tolerance to salt and drought stresses

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ABSTRACT. Plasma membrane proteolipid 3 (PMP3) is a class of small hydrophobic proteins found in many organisms including higher plants. Some plant *PMP3* genes have been shown to respond to abiotic stresses and to participate in the processes of plant stress tolerance. In this study, we isolated the cassava (*Manihot esculenta* Crantz) *MePMP3-2* gene and functionally characterized its role in tolerance to abiotic stress by expressing it in rice (*Oryza sativa* L.). *MePMP3-2* encodes a 77-amino acid protein belonging to a subgroup of plant PMP3s that have long hydrophylic C-terminal tails of unknown function. *In silico* analysis and co-localization

studies indicated that MePMP3-2 is a plasma membrane protein with two transmembrane domains, similar to other PMP3s. In cassava leaves, *MePMP3-2* expression was up-regulated by salt and drought stresses. Heterologous constitutive expression of *MePMP3-2* in rice did not alter plant growth and development but increased tolerance to salt and drought stresses. In addition, under stress conditions *MePMP3-2* transgenic plants accumulated less malondialdehyde, had increased levels of proline, and exhibited greater up-regulation of the stress-related genes *OsProT* and *OsP5CS*, but led to only minor changes in *OsDREB2A* and *OsLEA3* expression. These findings indicate that *MePMP3-2* may play an important role in salt and drought stress tolerance in transgenic rice.

Key words: Drought; Salt; Cassava; *MePMP3-2*; Rice

INTRODUCTION

Abiotic stresses are the primary cause of crop failure, resulting in average yield losses of >60% in major crop production worldwide. Drought and salinity, which cause osmolarity stress, are the two most crucial abiotic stresses that limit the production of cereal crops. In order to survive under these environmental stress conditions, plants have evolved sophisticated mechanisms of adaptation including the regulating of multiple genes involved in stress-tolerance (Sahi et al., 2006). Genes related to stress-tolerance in rice include *OsP5CS* encoding D^1-pyrroline-5-carboxylate (P5C) synthetase, which is thought to control the accumulation of proline (Pro) under high salt conditions (Igarashi et al., 1997), the Pro transporter gene *OsProT* (Igarashi et al., 2000), the dehydration-responsive element-binding transcription factor gene *OsDREB2A*, which is induced by high temperature, drought, and high salinity stresses in an ABA-independent manner, and the late embryogenesis abundant protein gene *OsLEA3*, which encodes an osmoprotective hydrophilin and depends on ABA for activation by abiotic stresses (Xiao et al., 2007). The activation of these genes, which protect plants from oxidative damage caused by salt, drought, and oxidative stresses, is closely linked to the production of malondialdehyde (MDA) and Pro, and these are frequently used as markers of stress responses. MDA is commonly used to determine the extent of lipid peroxidation caused by abiotic stresses. Accumulation of free Pro is an important mechanism that occurs in response to abiotic stress, and Pro stabilizes subcellular structures from damage by adjusting the intracellular osmotic potential (Liu and Zhu, 1997).

Plant *PMP3* genes have also been shown to respond to various stresses including salt, drought, or cold, and participate in plant tolerance to abiotic challenges. PMP3 represents a class of small hydrophobic membrane proteins that are highly conserved among species, including yeast (Navarre and Goffeau, 2000), simple animals (Medina et al., 2007), moss (Reski et al., 2004), and higher plants (Medina et al., 2007; Fu et al., 2012). In plants, multiple structural variants of PMP3 proteins exist, which are encoded by multigene families. Among the abiotic stress-induced plant *PMP3* genes are *LeESI3* from wheatgrass (*Lophopyrum elongatum*), *OsRCI2-5*, *OsLti6a/b*, and *R1G1B* from rice (Li et al., 2014), *AcPMP3-1* from sheepgrass (*Aneurolepidium chinense*), *AtRCI2A/B/D/F* from *Arabidopsis* (*Arabidopsis thaliana*) (Medina et al., 2007), *PutPMP3-1/-2* from alkali grass (*Puccinellia tenuiflora*), and *ZmPMP3-2/3/4* from corn (*Zea mays*) (Fu et al., 2012). There is some evidence for functional specialization of plant *PMP3*s and of their participation in stress responses being effected in different ways. *AtRCI2A*, for example, may be involved in CBF/

DREB1-independent signaling pathways during abiotic stress responses in *Arabidopsis*, whereas OsLti6a proteins in rice are thought to be part of many cold stress defense-related proteins regulated by DREB. Plant *PMP3*s can also be induced by signaling molecules such as ABA and H_2O_2, indicating that some of these proteins may have important roles in ABA-dependent stress-responses in plants (Shinozaki and Yamaguchi-Shinozaki, 2000).

In yeast, PMP3, which is involved in the maintenance of plasma membrane potential, is involved in preventing Na^+ entry (Navarre and Goffeau, 2000). Plant PMP3s may have similar roles, because the heterologous expression of some plant *PMP3*s can functionally complement yeast *pmp3* mutants (Medina et al., 2007; Fu et al., 2012). Disruption of *AtRCI2A* increases *Arabidopsis* sensitivity to salt and Na^+ uptake while AtRCI2A over-expression has the reverse effects (Mitsuya et al., 2006); In sheepgrass, AcPMP3-1 is localized in the root cap where it regulates cytoplasmic Na^+ and K^+ accumulation. While it is generally agreed that PMP3s are indirectly involved in cation uptake, the precise mechanism of action is currently unknown (Navarre and Goffeau, 2000). Due to their small size, PMP3s are unlikely to be ion transporters but their roles in stress responses remain unclear.

Cassava is one of the world's most important food crops and is grown in regions at high risk of climate change. Its root is a major source of calories for people on low-income due to its high productivity and resistance to abiotic and biotic factors (dos Reis et al., 2012). Although in a recent study, *MeLti6a*, a homolog of the stress-responsive *OsLti6a*, was isolated from cassava (Li et al., 2012), its function remains unknown. In this study, we identified *MePMP3-2* by searching sequences with similarity to the rice gene *OsRCI2-5*. *OsRCI2-5* is a stress-responsive gene of rice and, when over-expressed, greatly enhances drought tolerance of transgenic rice (Li et al., 2014). The results of our studies show that *MePMP3-2* is induced by salt and drought stresses. To further explore how *MePMP3-2* is involved in stress tolerance, transgenic rice plants constitutively expressing this cassava gene were generated. The resulting transgenic rice plants exhibited enhanced salt and drought tolerance, with higher accumulation of Pro, reduced MDA content, and altered expression of some relevant stress-related genes under stress conditions relative to wild-type (WT) plants.

MATERIAL AND METHODS

Plant materials, growth conditions, and stress treatment

Cassava seedlings (*Manihot esculenta* Crantz) SC124 were obtained from the Chinese Academy of Tropical Agricultural Sciences (Haikou, Hainan, China) and grown in a growth room maintained at 28°C and 60-80% relative humidity under a 12-h light (flux density of 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$) photoperiod. For salt and drought (PEG) stress experiments, 28-day-old cassava plants were treated with 200 mM NaCl or 20% PEG for 0, 1, 3, 6, or 12 h, respectively. About 3 g leaves were collected from plants subjected to each treatment and were immediately frozen in liquid nitrogen until RNA isolation. The sample taken at 0 h corresponds to leaves harvested prior to treatment.

The rice variety Taipei309 (ssp *japonica*) was used for transformation. Rice plants were grown in a growth room at 28°C. The T3 generations of two selected transgenic rice *MePMP3-2* over-expression lines (L-3 and L-5) were used in stress assays together with WT plants. To evaluate their tolerance to salt or drought stress, seeds were germinated and grown in half-strength Murashige and Skoog (½MS) medium plus 0 mM, 140 mM NaCl or 400 mM mannitol for 7 days. The shoot and root lengths of the seedlings were then measured.

For drought treatments, at least 15 plants from the *MePMP3-2* over-expression lines were grown in square pots filled with a mixture of sand and soil (1:1) together with WT control plants. Twenty-four-day-old seedlings were kept out of water for 6 days until their leaves began to roll. After drought treatment, the seedlings were allowed to recover for 7 days in the presence of water, after which the survival rates of plants were calculated.

For salt treatments, the same number of 24-day-old seedlings was irrigated with 140 mM NaCl for 6 days. After salt treatment, the seedlings were allowed to recover for 7 days in the presence of water and the survival rates were then determined.

For qRT-PCR analysis of known stress-responsive genes, 3-week-old WT and transgenic seedlings were treated for 48 h with nutrient solution (Ni, 1985), or nutrient solution containing either 140 mM NaCl or 20% PEG, respectively. Total RNAs were extracted and qRT-PCR analyses were performed.

Construction of the plant expression vector and generation of transgenic plants

To construct the plant expression vector, a cDNA fragment including the whole open reading frame (ORF) of *MePMP3-2* was amplified from the total RNA of cassava SC124 with primers *MePMP3-2*-F, 5'-**TCTAGA**GTTTTAAAGAAAAATGCCTTCTCG-3' (*Xba*I site in bold) and *MePMP3-2*-R, 5'-**CGTCAG**CATAAAGAGTAAGAACAAGGC-3' (*Pst*I site in bold). The PCR fragment was ligated as an *Xba*I-*Pst*I fragment into the binary expression vector pHB, thus allowing the gene to be driven by the 2X CaMV35S promoter. The resulting pHB::*MePMP3-2* construct was introduced into the *Agrobacterium tumefaciens* strain EHA105 via a freeze-thaw method, which was then used for rice transformation (Hiei et al., 1997). The presence of the transgene in T0 seedlings was tested by PCR with the same primers that were used to amplify the *MePMP3-2* gene. Selected transgenic plants were transplanted to soil and their seeds were harvested for further use.

RNA extraction and quantitative real-time PCR analysis

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract RNA from cassava leaves and rice seedlings according to the manufacturer instructions, and RNA samples were further treated with DNase (Promega, Madison, WI, USA). Total RNA was used for first-strand cDNA synthesis with an M-MLV First-Strand Kit (Invitrogen) according to the manufacturer instructions. qRT-PCR analysis was carried out using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). Ubiquitin 5 (*UBQ5*) and cassava tubuline (*MeTub*) were used as internal controls for the rice and cassava qRT-PCR experiments, respectively. The first-strand cDNA reaction products were used as templates in qRT-PCR with primers for the cassava genes *MePMP3-2* and *MeTub* and for the rice genes *OsProT*, *OsP5CS*, *OsDREB2A*, *OsLEA3,* and *UBQ5* (Table 1). PCRs were performed according to the manufacturer instructions in an ABI 7900HT (Applied Biosystems, USA) at 95°C for 10 min followed by 45 cycles at 95°C for 15 s, 60°C for 30 s. The data were analyzed using the comparative Ct method.

Subcellular localization of protein MePMP3-2

To construct the vector for use in the subcellular localization assay, the full-length ORF of *MePMP3-2* was PCR amplified using the primers *MePMP3-2-GFP*-F, 5'-**AAGCT T**CCGTTTTAAAGAAAAATGCCTTCTCG-3' (*Hin*dIII site in bold) and *MePMP3-2-GFP*-R,

5'-**GGATCC**ACTGTAGATCGGGGAGTAGAG-3' (*Bam*HI site in bold). The ORF was then ligated as a *Hin*dIII-*Bam*HI fragment into the intermediate vector pJIT163-GFP, thus allowing the resulting *MePMP3-2*::*GFP* fusion gene to be driven by the 2X CaMV 35S promoter. Next, the 2X CaMV35S::*MePMP3-2*::*GFP* cassette was ligated into the pCAMBIA1300 vector as a *Kpn*I-*Xho*I fragment. Vector p2X CaMV35S::*GFP* was used as the control in the co-localization experiments. The constructs were delivered into onion epidermal cells by *Agrobacterium*-mediated transient transformation (Sun et al., 2007). After 1-2 days co-cultivation, GFP fluorescence was observed under a Leica MZ16FA fluorescent stereomicroscope (Leica Microsystems, Wetzlar, Germany).

Table 1. Primer sequences used in qRT-PCR analysis.

Gene	Sense primer (5'-3')	Antisense primer (5'-3')
MePMP3-2	TCTAGAGTTTTAAAGAAAAATGCCTTCTCG	CGTCAGCATAAAGAGTAAGAACAAGGC
MePMP3-2-GFP	AAGCTTCCGTTTTAAAGAAAAATGCCTTCTCG	GGATCCACTGTAGATCGGGGAGTAGAG
MeTub	ATCCTTCTCAAGGGCAGCAAGAT	ACATGGAAAGTACATGGCCTGCTG
OsProT	GTGAAGCAGAACAAGATGTCCA	CACTTTCCAGCCTCACATATCA
OsP5CS	GCTGACATGGATATGGCAAAAC	GTAAGGTCTCCATTGCATTGCA
OsDREB2A	GGTAAGTGGGTGGCTGAG	TCTTCCGCTCCTGACAAA
OsLEA3	AAGGAGGCGACGAAGGAGAA	ACCTGCTCACTCGCCTGTTG
UBQ5	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT

Measurement of Pro and MDA

Three-week-old WT and transgenic rice plants (lines L-3 and L-5) were treated with 140 mM NaCl or 20% PEG for 48 h, following which the leaves were harvested. For quantification of MDA, 0.5 g leaf tissue was ground in 5% TCA and centrifuged at 4000 rpm for 10 min. Two milliliters of supernatant was mixed with 0.7% TBA, was then boiled for 30 min and the absorbance at 600 nm was measured with a Bio-Photometer (Eppendorf). The final quantity of MDA (nmol/g) was calculated as previously described (Kramer et al., 1991, Du et al., 2010). The measurement of Pro content was performed according to the method described by Troll and Lindsley (1955).

RESULTS

MePMP3-2 encodes a PMP3 family protein

Results from our previous research showed that rice *OsRCI2-5* (AK070872) is a drought resistance gene (Li et al., 2014). To identify homologous genes in cassava that could have a similar role, we performed a BLAST of the OsRCI2-5 sequence against the Phytozome database (http://phytozome.jgi.doe.gov/pz/portal.html#). *MePMP3-2* (cassava4.1_020550m.g) encoded a protein that showed high-sequence similarity (75.0%) with that of OsRCI2-5. The ORF of *MePMP3-2* encoded a polypeptide of 77 amino acids. Comparison between genomic and coding sequences revealed that *MePMP3-2* contains two exons separated by an intron of 130 bp. MePMP3-2 was predicted to be 8.65 kDa in size with a pI of 4.94 (http://web.expasy.org/compute pi/), and was highly hydrophobic with a Grand Average of Hydropathicity (GRAVY) value of 1.135 (http://web.expasy.org/protparam/). The BLASTx revealed MePMP3-2 to be similar to numerous plant PMP3s including several encoded by stress-inducible genes such as LeESI3, rice OsRCI2-5, OsLti6a/b, R1G1B) (Li et al., 2014), AcPMP3-1/2, AtRCI2A/B/D/F (Medina et al., 2007), PutPMP3-1/2, and ZmPMP3-2/3/4 (Fu et al., 2012) (Figure 1A and B). In particular, these polypeptides share the

PROSITE PMP3 family signature pattern PS01309, ([LIVF]-x-[STAC]-[LIVF](3)-P-[PF]-[LIVA]-[GAV]-[IV]-x(4)-[GKN]). This pattern was also conserved in MePMP3-2 (Figure 1A). Like other PMP3 proteins (Medina et al., 2007; Fu et al., 2012), analysis of MePMP3-2 using the PHD algorithm (https://www.predictprotein.org/) (Rost et al., 2004) predicted that the two well-conserved regions of MePMP3-2 are membrane spanning domains linked by a putative hydrophilic turn, which orient both the N- and C-termini towards the apoplastic side of the membrane (Figure 1A).

To obtain information on the relationship of MePMP3-2 with MeLti6a and other higher plant PMP3s, a phylogenetic analysis was performed that included PMP3s from *Arabidopsis*, rice, corn, and others encoded by stress-responsive genes. The analysis revealed that MePMP3-2 consistently clustered with AtRCI2D-G (Figure 1B) irrespective of the method or the substitution model used (data not shown). Sequence similarity between MePMP3-2 and AtRCI2D-H ranged from 72 to 81%. Interestingly, these PMP3s belong to a subgroup containing longer, charged hydrophylic C-terminal regions (73-77 amino acids). However, the clustering was independent of their C-terminal extensions.

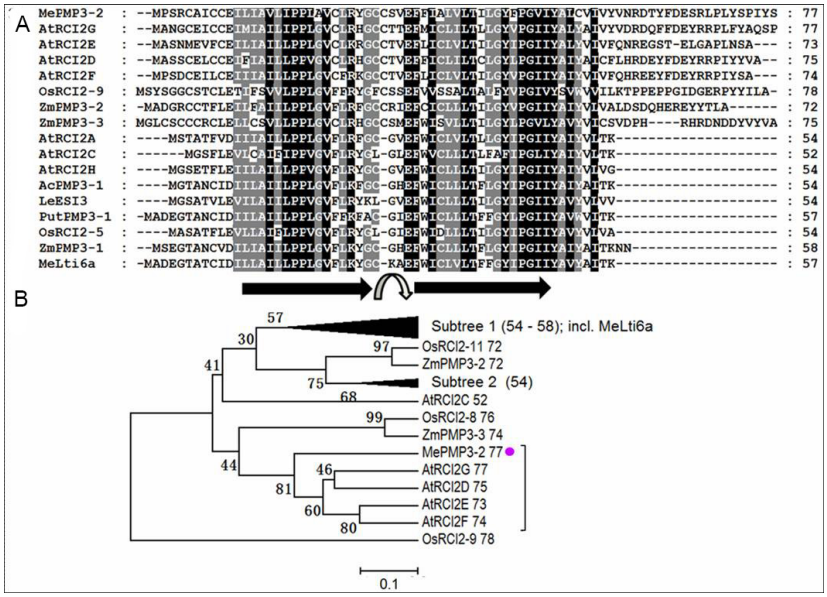

Figure 1. Comparison of MePMP3-2 and other plant PMP3 proteins. **A.** Alignment of predicted amino acid sequences of MePMP3-2 with other PMP3s. Shown is a subset of 32 aligned sequences including the cassava MeLti6a (cassava4.1_020620m) (Li et al., 2012), *Arabidopsis* AtRCI2A/C-H (Capel et al., 1997) and rice OsRCI2-5/9 (Medina et al., 2007), corn ZmPMP3-1/2/3 (Fu et al., 2012), wheatgrass LeESI3 (P68178.1) (Gulick et al. 1994), sheepgrass AcPMP3-1 (BAD34658.1) (Inada et al., 2005), and alkali grass PutPMP3-1 (BAG54793.1) (Chang-Qing et al., 2008). For the complete alignment see **Figure S1A**. The alignment was produced with Clustal Omega (Sievers et al., 2014) and illustrated with GeneDoc (Nicholas and Nicholas, 1997). Sequence identity is shown by shading in dark gray (>75%) and light gray (>50%). The numbers on the right indicate sequence length. Straight arrows mark predicted membrane spanning domains. The curved arrow indicates a putative hydrophilic turn. **B.** Phylogenetic tree of plant PMP3s inferred using the UPGMA method in MEGA6 (Tamura et al., 2013). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown (Felsenstein, 1985). The evolutionary distances were computed using the JTT matrix-based method (Jones et al., 1992). All positions containing gaps and missing data were eliminated. For simplicity several branches are shown collapsed. The complete tree is provided in **Figure S1B**. The robust branch containing MePMP3-2 is indicated. The sizes (in aa) of the PMP3s are shown after their names.

Expression profile of *MePMP3-2* under various stresses

To ascertain whether *MePMP3-2* responds to stresses typical of tropical regions, its pattern of expression in cassava plants subjected to salt or drought stresses was monitored by qRT-PCR (Figure 2). *MePMP3-2* responded in a similar pattern to both salt and drought treatments. Relative to the non-treated sample at 0 h, expression of *MePMP3-2* increased 5.85-fold at 1 h after salt treatment, reaching peak expression (8.7-folds) at 3 h, decreasing considerably to 3.2-fold at 6 h, and reaching a second peak expression (6.55-fold) at 12 h. Drought treatment resulted in a 6.05-fold increase in expression after 1 h relative to the expression of *MePMP3-2* at 0 h, peaking at 8.75-fold at 3 h, decreasing to 5.3-fold at 6 h, and peaking again (7.55-fold) at 12 h.

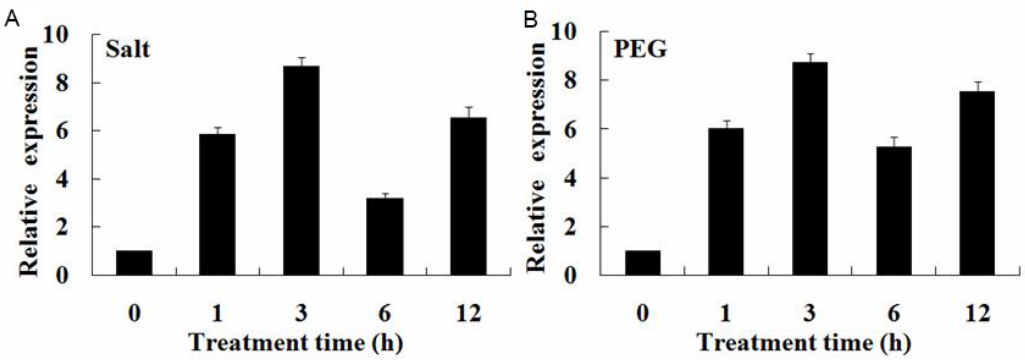

Figure 2. Expression profile of *MePMP3-2* in cassava seedlings under different stresses. Cassava seedlings were respectively treated with 200 mM NaCl (**A**) or 20% PEG (**B**) at the time intervals indicated. Quantitative RT-PCR was performed to determine the relative expression levels of *MePMP3-2* under different treatments. The cassava *MeTub* gene was used as the qRT-PCR internal control. Error bars represent ± SD for three independent experiments.

Subcellular localization of MePMP3-2

To gain insight into the cellular distribution of MePMP3-2, bioinformatics-based prediction was performed. The results indicated that MePMP3-2 may be an integral membrane protein, with TargetP (http://www.cbs.dtu.dk/services/TargetP/) predicting with high probability (P = 0.987) that is targeted to the secretory pathway. Neither TargetP nor NucPred (http://www.sbc.su.se/~maccallr/nucpred/) (Brameier et al., 2007) detected any organelle-targeting signal in MePMP3-2. To investigate the subcellular localization of MePMP3-2, a reporter protein construct was assembled (Figure 3A). The construct was used to transiently express MePMP3-2::GFP fusion proteins in onion epidermal cells. As shown in Figure 3B, in control cells transformed with the pJIT163-GFP plasmid, GFP showed its typical cytoplasmic and nuclear distribution, while in cells expressing the *MePMP3-2*::*GFP* fusion gene, the GFP signal was observed in the plasma membrane, suggesting that native MePMP3-2 may locate to the plasma membrane.

Enhanced salt and drought tolerance of transgenic rice

To further elucidate the biological function of the *MePMP3-2* gene, transgenic rice plants constitutively expressing *MePMP3-2* were generated and analyzed. The expression of *MePMP3-2* was evaluated in T3 generation seedlings of five independent transgenic rice lines by qRT-PCR.

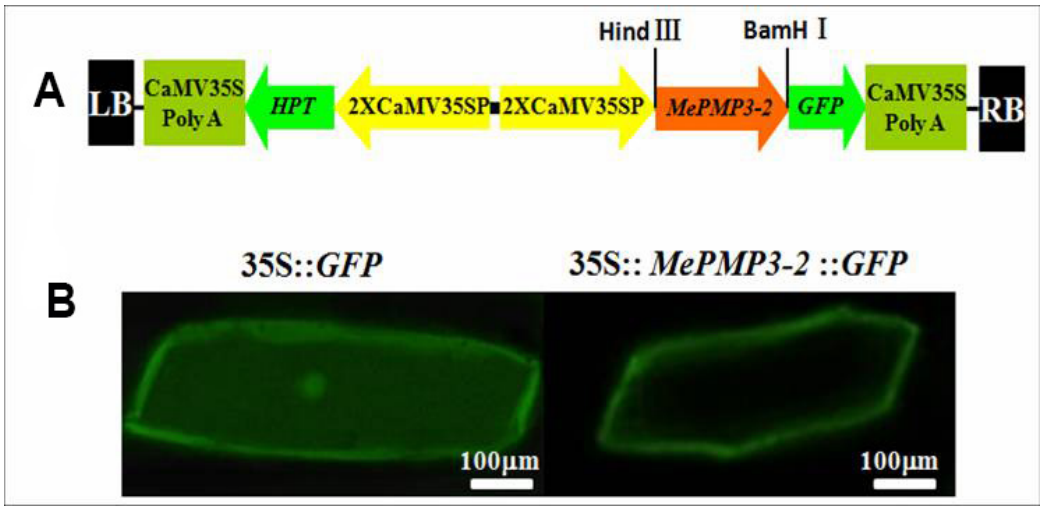

Figure 3. Subcellular localization of the MePMP3-2::GFP fusion protein. **A.** Structure of the fusion gene construct *MePMP3-2::GFP*. LB, T-DNA left border; CaMV35S Poly A, cauliflower mosaic virus 35S terminator; 2X CaMV35SP, double-cauliflower mosaic virus 35S promoter; GFP, green fluorescent protein; RB, T-DNA right border. **B.** Visualization of fluorescence in onion epidermal cells following the transient expression of fusion genes pCaMV35S-*GFP* and *MePMP3-2::GFP*.

Using *UBQ5* as the reference gene, qRT-PCR analysis showed that all five transgenic lines expressed *MePMP3-2* (Figure 4). The two independent transgenic lines with the highest expression of *MePMP3-2*, L-3 and L-5, were chosen for salt and drought stress testing. The transgenic and WT seedlings showed no obvious differences in phenotype under normal growth conditions (Figure 5a and c left). As expected, both salt and drought treatments strongly decreased the survival rate of all plants (Figure 5a and c right). As shown in Figure 5b, under salt treatment, an average of 54.5% transgenic seedlings survived, as compared with 31.1% of control seedlings. Under drought treatment, an average of 50.0% transgenic seedlings, but only 26.7% WT seedlings, survived (Figure 5d). There were no detectable differences in seedling shoot length and root length between the transgenic rice lines and WT when grown on normal ½MS medium (Figure 6a). The shoot and root lengths of the two transgenic rice lines were similar and (considered together) were on average 3.29 ± 0.10 and 3.15 ± 0.06 cm, respectively. The shoot and root lengths of WT were 3.23 ± 0.06 and 3.17 ± 0.06 cm, respectively (Figure 6d and e). The addition of NaCl (140 mM) to the incubation medium significantly reduced the shoot and root lengths for both WT and transgenic rice plants (Figure 6b). However, after salt treatment the shoots and roots of the two transgenic lines were on average 45.9% (2.00 ± 0.12 cm) and 69.9% (1.92 ± 0.10 cm) longer, respectively, than those of the WT (1.37 ± 0.06 and 1.13 ± 0.06 cm, respectively) (Figure 6d and e). Similarly, but less pronounced, was the effect of mannitol (400 mM) on shoot and root lengths, which for the two transgenic lines were, respectively, 47.1% (0.25 ± 0.06 cm) and 18.4% (1.22 ± 0.06 cm) longer on average than those of WT (0.17 ± 0.06 and 1.03 ± 0.06 cm, respectively) (Figure 6c-e).

Altered expression of genes involved in stress-responses

To obtain further insight into the molecular mechanisms accounting for the resistance of transgenic plants to salt (140 mM NaCl) and drought (20% PEG) stresses, we analyzed the

expression of several relevant rice stress-related genes in the *MePMP3-2* transgenic lines and in the WT, including *OsProT* (Igarashi et al., 2000) and *OsP5CS* (Igarashi et al., 1997), which are related to Pro accumulation, and *OsDREB2A* and *OsLEA3* (Xiao et al., 2007), which are markers of ABA-dependent and ABA-independent signaling.

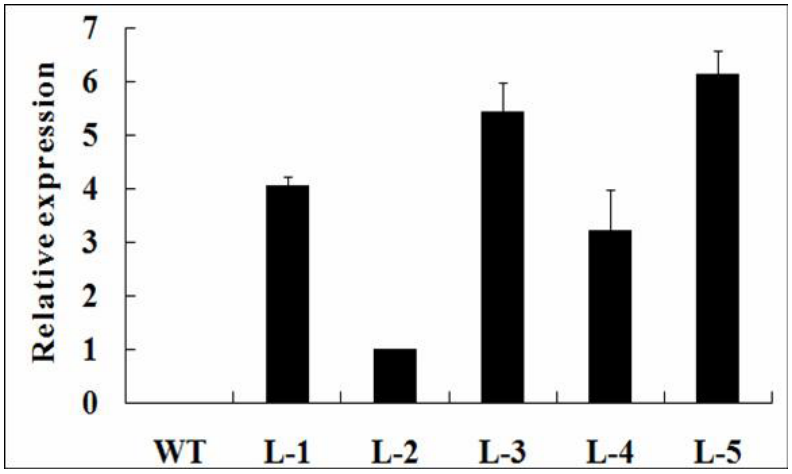

Figure 4. Relative expression levels of *MePMP3-2* in transgenic rice lines and WT plants. Error bars represent ± SD of three independent experiments. WT = wild-type rice; L-1 to L-5 = transgenic lines 1 to 5.

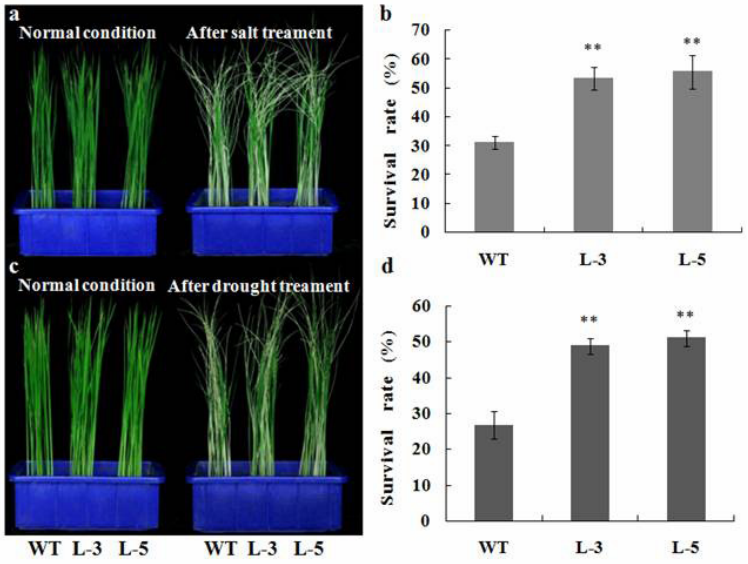

Figure 5. Performance of *MePMP3-2* transgenic and WT seedlings under normal, and salt or drought stress conditions. **a. c.** Left panel, seedlings under normal growth condition for 24 days. **a. c.** Left panel, seedlings after exposure to drought or 140 mM NaCl for 6 days and recovered for 7 days. **b. d.** Survival rates of plants after drought or salt stress and recovered for 7 days. Error bars represent ± SD of three independent experiments. For b and d, **indicates significant differences from the corresponding WT plants in the same treatments at $P < 0.01$. WT = wild-type rice; L-3 and L-5 = transgenic lines 3 and 5.

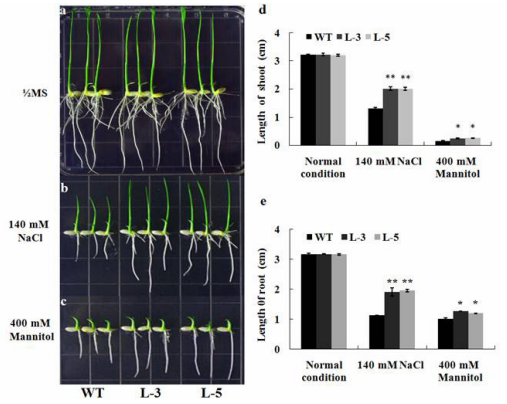

Figure 6. Phenotypes of WT and transgenic seedlings on NaCl or mannitol-supplemented ½MS agar. **a. b. c.** Representative plants grown on ½MS (a), ½ MS with 140 mM NaCl (b) or 400 mM mannitol (c) for 7 days. **d. e.** Effects of NaCl or mannitol treatment on shoot and root lengths of transgenic and WT plants. Error bars represent ± SD for three independent experiments. For d and e, * and **indicate significant differences from the corresponding WT plants in the same treatments at P < 0.05 and P < 0.01, respectively. WT = wild-type rice; L-3 and L-5 = transgenic lines 3 and 5.

There were no significant differences in the levels of *OsProT*, *OsP5CS*, *OsDREB2A*, and *OsLEA3* expression between the *MePMP3-2* transgenic lines and the WT plants under normal growth conditions (Figure 7A-D). However, both transgenic lines showed similar patterns of gene expression, distinct from those seen in the WT, when subjected to stress treatments. Under salt treatment, the levels of *OsProT* and *OsP5CS* expression in *MePMP3-2* transgenic lines were at least 255.6 and 177.9% that of the expression in the WT plants, respectively (Figure 7A and B). However, *OsDREB2A* and *OsLEA3* showed only modest increases in expression of 8.0 and 9.7%, respectively (Figure 7C and D). Similarly, under PEG treatment, the levels of *OsProT*, *OsP5CS*, *OsDREB2A*, and *OsLEA3* expression in *MePMP3-2* transgenic lines were at least 148.8, 350.9, 135.1, and 127.1% that of the expression in WT plants, respectively (Figure 7A-D).

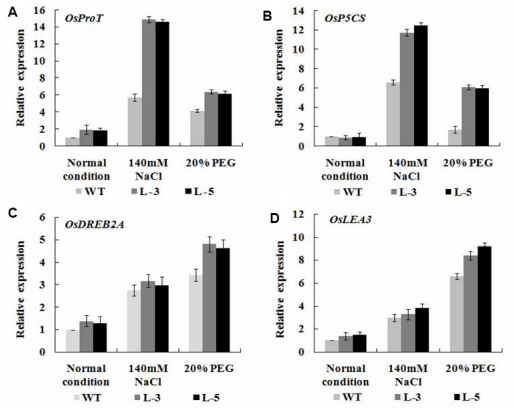

Figure 7. Expression of stress-related genes in *MePMP3-2* transgenic and WT rice. **A. B. C. D.** The expression levels of *OsProT*, *OsP5CS*, *OsDREB2A,* and *OsLEA3* were measured by real-time qRT-PCR under normal condition, or 24 h under salt (140 mM NaCl) or drought (20% PEG) stress for 24 h, respectively. The amplification of *UBQ5* transcripts was used as the internal control. Error bars represent ± SD of three independent experiments. WT = wild-type rice; L-3 and L-5 = transgenic lines 3 and 5.

Altered MDA and Pro contents in transgenic plants after drought or salt stress

To determine whether any correlations existed between drought and salt tolerance and levels of MDA and Pro, their concentrations were measured in seedlings of *MePMP3-2* transgenic lines and in control WT rice grown in nutrient solution alone or in nutrient solution containing either 140 mM NaCl or 20% PEG. Under normal growth conditions, there were no obvious differences in MDA and Pro contents between transgenic lines and WT plants (Figure 8A and B). As shown in Figure 8, compared to the *MePMP3-2* transgenic plants the WT plants accumulated 20.6% more MDA after drought treatment (11.11 ± 0.17 *vs* 9.21 ± 0.18 nmol/g), and more than 42.8% MDA after salt treatment (16.01 ± 0.12 *vs* 11.21 ± 0.55 nmol/g). In contrast, compared to WT plants, the Pro contents of transgenic lines were 26.9% higher after drought treatment (249.23 ± 9.57 *vs* 196.29 ± 5.42 mg/g) and at least 181.7% higher after salt treatment (288.26 ± 10.00 mg/g, Line L-3 *vs* 158.62 ± 6.16 mg/g). Line L-5 plants accumulated somewhat more Pro (346.87 ± 23.32 mg/g) corresponding to an increase of 218.7% compared to that found in WT seedlings.

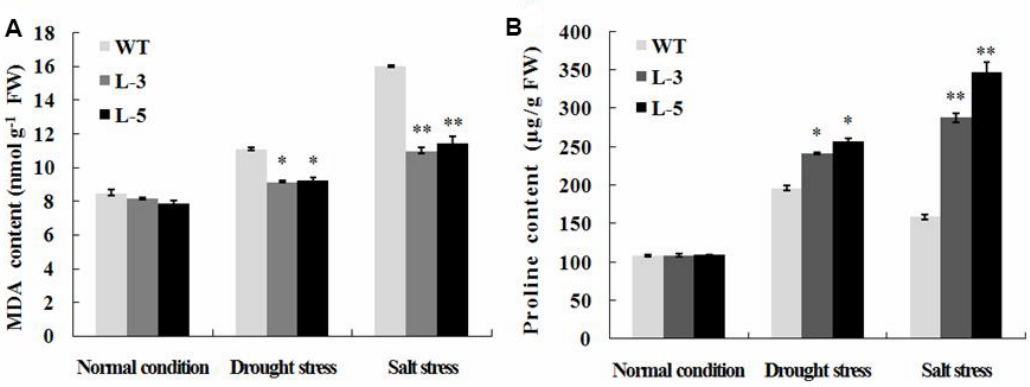

Figure 8. MDA and free Pro levels in *MePMP3-2* transgenic and WT rice under salt and drought conditions. **A.** MDA content. **B.** Free Pro content under normal, 140 mM NaCl or 20% PEG stress conditions, respectively. Error bars represent ± SD for three independent experiments. * and **Indicate significant differences from the corresponding WT plants in the same treatments at $P < 0.05$ and $P < 0.01$, respectively. WT = wild-type rice; L-3 and L-5 = transgenic lines 3 and 5.

DISCUSSION

In this study, a gene homologous to the stress-inducible *OsRCI2-5* (Li et al., 2014), *MePMP3-2*, was isolated for the first time from cassava and its expression was shown to increase in response to salt and drought stresses. Like OsRCI2-5, the predicted MePMP3-2 protein was found to belong to the PMP3 family of small membrane proteins. Bioinformatic analysis showed that, similar to other PMP3s, MePMP3-2 contained two well-conserved centrally located transmembrane domains, which orientate both the N- and C-termini towards the apoplast (Figure1A). *In silico* targeting analysis suggested that MePMP3-2 is likely directed to the secretory pathway and co-localization studies showed that the MePMP3-2::GFP fusion proteins accumulate in the plasma membrane. Taken together, these data strongly suggest that, like yeast Pmp3p (Navarre and Goffeau, 2000), MePMP3-2 is a plasma membrane protein. These observations are in line with previous findings that, except for AtRCI2D::GFP, all plant PMP3::GFP fusion proteins studied are

targeted to the plasma membrane (Medina et al., 2007; Fu et al., 2012). Among these are the GFP fusions of AtRCI2E/F/G, which our phylogenetic analysis showed to be most closely related to MePMP3-2. The association of PMP3s with the plasma membrane corresponds to an expected role of PMP3s in regulating intracellular cation accumulation, as is the case for AtRCI2A (Mitsuya et al., 2006) and AcPMP3-1/2, suggesting that MePMP3-2 may have a similar function.

Plant PMP3s fall into two groups according to size, with the smaller type being 52-58 amino acids (aa). Members of the group of longer PMP3s (70-78 aa) are characterized by an extended hydrophylic C-terminal region. The role of the C-tail is unclear, but while heterologous expression of the shorter AtRCI2A-C/H (52-54 aa) in a yeast *pmp3* deletion mutant can rescue its high sensitivity to Na^+, the longer AtRCID-F (73-77 aa) failed to do so (Medina et al., 2007). However, expression of a deletion derivative of AtRCID that lacks its C-terminal, complemented the salt sensitivity of *pmp3* (Medina et al., 2007). This shows that the C-tails can function as activity modifiers, perhaps by participating in protein-protein interactions. The presence of a longer C-tail does not impede complementation of *pmp3* Na^+ sensitivity by steric hindrance, as was demonstrated with ZmPMP3-2 (72 aa) and ZmPMP3-3 (75 aa) (Fu et al., 2012). *PMP3*s of both sizes appear to co-exist in plant species, as in *Arabidopsis*, rice, and corn. Our study shows a similar situation in cassava, in which the shorter MeLti6a (57 aa) has higher sequence similarity to PMP3s from other plants than to MePMP3-2 (Figure 1B). The existence of multiple PMP3 homologs in plants suggests functional specialization. This has been observed in *Arabidopsis* and corn, in which PMP3 genes are differentially regulated in different organs and in response to environmental challenges (Medina et al., 2007; Fu et al., 2012). It is possible that cassava MePMP3-2 and MeLti6a are differentially regulated in a similar manner.

In cassava, the expression of *MePMP3-2* was up-regulated by NaCl and PEG, and peaked at 3 and 12 h post-treatment (Figure 2A and B). These responses are comparable to those of *ZmPMP3-3*, which is mainly responsive to salt and drought stresses (Fu et al., 2012). Similarly, Li et al. (2014) showed that the expression of rice *OsRCI2-5* could be induced by drought and low temperature stresses. The increased expression of these genes suggests a role for *MePMP3-2* in mediating the responses to triggering stresses.

To elucidate the biological function of *MePMP3-2*, transgenic rice plants constitutively expressing the gene were generated. The *MePMP3-2* transgenic plants showed a higher survival rate (Figure 5b and d), lower MDA content (Figure 8A), and increased Pro content (Figure 8B) relative to WT control plants under stress conditions, and demonstrated higher stress tolerance. It is well known that plants subjected to abiotic stress often accumulate soluble molecules, such as Pro (Liu and Zhu, 1997), betaine, and soluble sugar (Gupta and Kaur, 2005) as osmoprotectants. These compatible osmolites have been reported to play a predominant role in transgenic plants with enhanced tolerance to abiotic stress (Xu et al., 2008). In the present study, the *MePMP3-2* transgenic plants accumulated significantly more Pro under salt stress than the control plants and somewhat more under drought stress. Pro also functions as an antioxidant and reduces oxidative damage under conditions of stress (Székely et al., 2008), and its accumulation in transgenic plants may contribute to their lower relative electrolytic leakage and MDA content under stress conditions. The level of MDA is a measure of membrane lipid peroxidation damage caused by abiotic stresses. *MePMP3-2* transgenic plants had a slightly lower MDA content than WT plants under drought stress, and significantly less MDA under salt stresses. These results are consistent with the relative increases in Pro accumulation observed under drought and stress treatments. A similarly significant lower MDA level under salt treatment was observed by Mitsuya et al. (2006) in *Arabidopsis* plants over-expressing *AtRCI2A*, a homolog of *MePMP3-2*, relative to control plants.

That, for the transgenic plants, higher relative levels of Pro and lower relative levels of MDA were observed after salt treatment than after drought treatment correlates well with the observed higher resistance of the *MePMP3-2*-expressing seedlings to salt than to drought. In summary, the increased survival rate, higher contents of Pro, and lower levels of MDA in the transgenic lines relative to WT rice suggest that *MePMP3-2* may play an important role in the abiotic stress response. It further indicates that MePMP3-2 can participate in pathways of abiotic stress responses in rice, perhaps by mimicking the functions of one or more of the rice PMP3s.

To further elucidate the mechanism underlying the greater accumulation of Pro in the *MePMP3-2*-expressing rice plants under drought and salt stresses, changes in the expression of the P5C synthase (*OsP5CS*) and Pro transporter (*OsProT*) genes were examined in *MePMP3-2*-expressing and WT plants in response to abiotic stress. Over-expression of *OsP5CS* increases Pro production and accumulation, which confers salt tolerance to transgenic lines in a number of crop plants including rice (Karthikeyan et al., 2011), wheat (*Triticum aestivum*) (Vendruscolo et al., 2007), pigeonpea (*Cajanus cajan*) (Surekha et al., 2014), and tobacco (*Nicotiana tabacum*) (Kishor et al., 1995). It has also been reported that up-regulation of *OsP5CS* and *OsProT* in *OsCIPK03*- and *OsCIPK12*-overexpressing rice plants led to greater accumulation of Pro in transgenic rice compared with WT plants under cold and dehydration stresses with a simultaneous increase in stress tolerance. In our study, the expression of *OsP5CS* and *OsProT* in *MePMP3-2* transgenic plants was higher than in WT plants under drought and salt stress conditions (Figure 5a and b). The induced expression of *OsP5CS* and *OsProT* likely led to the increased level of Pro observed in *MePMP3-2* transgenic plants, thus resulting in their enhanced tolerance to drought and salt stresses. *OsDREB2A*, a stress-responsive DRE/CRT transcription factor gene, is involved in ABA-independent dehydration and salt stress responses. Constitutive expression of *LsDREB2A*, *AtDREB2A*, and *ZmDREB2A* in *Arabidopsis* enhanced tolerance to salt and drought stresses (Kudo et al., 2014). LEA proteins are involved in multiple stress responses in plants. *OsLEA3* belongs to group 3 of the LEA gene family, and is induced in an ABA-dependent manner by dehydration and salt (Xiao et al., 2007). Over-expression of *OsLEA3* in rice plants can significantly enhance the dehydration tolerance under field conditions (Xiao et al., 2007). We found that heterologous expression of *MePMP3-2* in only a marginally higher induction of *OsDREB2A* and *OsLEA3* genes under both normal conditions and salt treatment relative than in WT plants (Figure 5c and d). Drought was accompanied by a higher increase in the expression of both *OsDREB2A* and *OsLEA3* in the transgenic plants compared with the WT plants. Therefore, the enhanced tolerance to drought but not salt observed in *MePMP3-2* transgenic rice plants might have been due, in part, to the reinforced expression of *OsDREB2A* and *OsLEA3*. How the constitutive expression of *MePMP3-2* enhanced the expression of *OsP5CS*, *OsProT*, *OsDREB2A,* or *OsLEA3* is not known. This may result from the presence of MePMP3-2 in the membrane affecting membrane components of various signaling pathways.

In summary, this study identified a cassava gene encoding a plasma membrane protein, *MePMP3-2*. Expression of *MePMP3-2* in rice enhanced tolerance to drought and salt stresses, and was accompanied by increased expression of stress-related genes, particularly *OsProT* and *OsP5CS*. Moreover, *MePMP3-2* transgenic plants had lower MDA and increased Pro levels relative to WT plants under stress conditions. Taken together, these data strongly indicate that *MePMP3-2* may play an important role in salt and drought stress tolerance when heterologously expressed in rice.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

Figure S1A. Alignment of predicted amino acid sequences of MePMP3-2 with other PMP3s. The alignment was produced with Clustal Omega (Sievers et al., 2014) and illustrated with GeneDoc (Nicholas and Nicholas, 1997). The amino acid identity levels are by shading in dark gray (>75%) and light gray (>50%). The numbers on the right indicate sequence length. Sequences used included: *Arabidopsis* AtRCI2A-H (NP_187239.1; NP_187240.1; NP_176067.1; NP_179982.1; NP_194794.1; BAH19935.1; NP_974629.1; NP_565897.1); rice OsLti6a/b (NP_001060390.1; NP_001054591.1), OsRCI2-1/3/5/7-9/11 (EAY73580.1; NP_001050200.1; AK070872; NP_001054506.1; NP_001056997.1; NP_001058230.1; NP_001063909.1), corn ZmPMP3-1 to ZmPMP3-8 (NP_001107634.1; ACG26755.1; XP_008658714.1; ACG26760.1; NP_001147403.1; ACG48459.1; NP_001147508.1; NP_001151840.1);

sheepgrass AcPMP3-1/2 (BAD34658.1; BAD34659.1); wheatgrass LeESI3 (P68178.1); alkali grass PutPMP3-1/2 (BAG54793.1; BAG54794.1); cassava MeLti6a (cassava4.1_020620m). **B.** Phylogenetic tree of plant PMP3s inferred using the UPGMA method in MEGA6 (Tamura et al., 2013). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown (Felsenstein, 1985). The evolutionary distances were computed using the JTT matrix-based method (Jones et al., 1992). All positions containing gaps and missing data were eliminated. The sizes (in aa) of the PMP3s are shown after their names. The sequences used are the same as in Figure S1A. The robust cluster containing MePMP3-2 is indicated.

