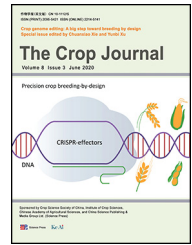


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# Mutagenesis reveals that the rice *OsMPT3* gene is an important osmotic regulatory factor



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## ABSTRACT

Plant mitochondrial phosphate transporters regulate phosphate transport and ATP synthesis. Determining whether they function in abiotic stress response process would shed light on their response to salt stress. We used the CRISPR/Cas9 gene-editing system to mutagenize two mitochondrial phosphate transporters, *OsMPT3;1* and *OsMPT3;2*, to investigate their regulatory roles under salt stress. Two cas9 (CRISPR-associated protein 9)-free homozygous mutants, *mpt33* and *mpt30*, were confirmed to be stable. Both *OsMPT3;1* and *OsMPT3;2* were markedly induced by salt stress, and their mutagenesis strongly inhibited growth and development, especially under salt stress. Mutagenesis sharply reduced the accumulation of ATP, phosphate, calcium, soluble sugar, and proline and increased osmotic potential, malondialdehyde, and  $\text{Na}^+/\text{K}^+$  ratio under salt stress. Both mutants demonstrate normal growth and development in the presence of ATP, revealing high sensitivity to exogenous ATP under salt stress. The mutants showed lowered rates of  $\text{Na}^+$  efflux but also of  $\text{K}^+$  and  $\text{Ca}^{2+}$  influx under salt stress. Mutagenesis of *OsMPT3;2* altered the enrichment profiles of differentially expressed genes involved mainly in synthesis of secondary metabolites, metabolism of glycolysis, pyruvate, tricarboxylic acid cycle, in response to salt stress. The mutant displayed significant accumulation differences in 14 metabolites involved in 17 metabolic pathways, and strongly up-regulated the accumulation of glutamine, a precursor in proline synthesis, under salt stress. These findings suggest that the *OsMPT3* gene modulates phosphate transport and energy supply for ATP synthesis and triggers changes in accumulation of ions and metabolites participating in osmotic

**Abbreviations:** ATP, adenosine triphosphate; CRISPR, clustered regularly interspaced short palindromic repeats; DEG, differentially expressed genes; HPLC, high performance liquid chromatography; MDA, malondialdehyde; MPT, mitochondrial phosphate transporter; MS, Murashige and Skoog medium; NMT, non-invasive micro-test technology; NHXs,  $\text{Na}^+/\text{H}^+$  exchangers; *OsMPT3*, mitochondrial phosphate transporter 3 in *Oryza sativa*; PM, plasma membrane; P5CS, delta-1-pyrroline-5-carboxylate synthase; P5CR, delta-pyrroline-5-carboxylate reductase; ProDH, proline dehydrogenase; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; RT-qPCR, real-time quantitative polymerase chain reaction; SOS1, Salt Overly Sensitive 1; SOS2, Salt Overly Sensitive 2; SOS3, Salt Overly Sensitive 3

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regulation in rice under salt stress, thus increasing rice salt tolerance. This study demonstrates the effective application of CRISPR/Cas9 gene-editing to the investigation of plant functional genes.

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## 1. Introduction

High salinity in soils limits crop growth and development by triggering ion toxicity, osmotic, and oxidative stresses in plants [1]. Plants have evolved a series of physiological mechanisms based on responding to external signals to adapt to environmental changes [2]. Both energy transfer and energy acquisition act as important signaling processes in plant metabolism. The mitochondrion is an organelle that hosts respiration and releases free energy for adenosine triphosphate (ATP) production by oxidative phosphorylation [3]. ATP, the most important energy source in living organisms, directly participates in the regulation of numerous metabolic pathways by controlling the  $\text{Na}^+/\text{K}^+$  balance, redox balance, and signaling pathways in response to salt stress [4,5]. Unlike ATP, as important  $\text{Na}^+/\text{H}^+$  exchangers,  $\text{Na}^+/\text{H}^+$  exchangers (NHXs) transport  $\text{Na}^+$  from the cytoplasm to the vacuole, in a process driven by the establishment of an  $\text{H}^+$  gradient potential regulated by vacuolar  $\text{H}^+$ -pyrophosphatase and  $\text{H}^+$ -ATPase [6,7]. Zhou et al. [8] reported that *STRK1* increases salt tolerance by phosphorylating and activating the catalase domain containing protein (CatC) and thereby regulating  $\text{H}_2\text{O}_2$  homeostasis. Two *Arabidopsis* mutants, *MKK4* and *MKKK20*, have been confirmed to be sensitive to salt stress [9], indicating that mitogen-activated protein kinases (MAPKs) play important regulatory roles in plant signal transduction under osmotic stress. Like MAPK, *SnRK2* is also an ABA-dependent protein family, and participates in signal transduction in response to osmotic stress [10]. Activation of *SnRK2* facilitates the phosphorylation of downstream genes to decompose starch into soluble sugar, thus maintaining the balance of osmotic potentials in plant cells [10].

Mitochondrial phosphate transporters (MPTs) are composed of six transmembrane helices localized to the inner mitochondrial membrane. They are essential for the transport of orthophosphate ( $\text{P}_i$ ) from the cytoplasmic matrix to the mitochondria, thereby providing substrate for the synthesis of ATP in the matrix [11]. Although the first MPT gene was identified in betulaceous tree, most studies of MPT genes have been conducted in *Arabidopsis*. Three typical *AtMPT* genes: *AtMPT1*, *AtMPT2*, and *AtMPT3*, have highly conserved domains and have been characterized in *Arabidopsis* [12,13]. Both *AtMPT2* and *AtMPT3* genes are induced specifically by salt stress, but transgenic lines overexpressing the three *AtMPTs* showed increased sensitivity to salt stress [4]. In addition to this sensitivity, *Arabidopsis* overexpressing the MPT3 gene showed developmental defects including dwarfing, dark green leaf curling, and infertility because the overexpression of the MPT3 gene accelerates electron transfer and causes excessive accumulation of reactive oxygen species (ROS) in plants [14]. Interestingly, transgenic *Arabidopsis* overexpressing either the *AtMPT1* or *AtMPT2* gene showed a normal

phenotype, indicating that the *AtMPT3* gene indeed plays a specific regulatory role in energy transport or accumulation of ROS in the plant [14]. MPT genes are also induced by low phosphorus. Transgenic rice overexpressing *McMPT3* or *SIMPT3;1* accumulated more phosphate in shoots under low phosphorus and showed increased tiller number and grain yield, suggesting that MPT3 modulates plant phosphate transport [15,16].

The CRISPR/Cas9 system offers potential for development of high-yielding crop cultivars by improving stress tolerance via gene editing, and has been applied in *Arabidopsis*, maize, wheat, and rice [17]. Mutagenesis of *OsPPa6* by the CRISPR/Cas9 system markedly lowered alkaline stress tolerance in rice [18]. The MPT-modulated osmotic response mechanism in plants subjected to abiotic stresses is unknown. The modification of rice *OsMPT* genes has not been reported.

In the present study, two rice MPTs, *OsMPT3;1* and *OsMPT3;2*, that are induced by salt stress were simultaneously edited with the CRISPR/Cas9 system, and two mutants with base deletions or insertions, *mpt33* and *mpt30*, were generated. Their physiological responses to osmotic stress were investigated by systematically evaluating the changes in the ATP, phosphate accumulation, compatible metabolites, osmotic potentials, the transcripts of differentially expressed genes, the accumulation and fluxes of the Na, K, and Ca ions in rice. We are expected that this study will provide insight into the regulatory pathway of the mitochondrial phosphate transporters in plants in response to salt stress.

## 2. Materials and methods

### 2.1. Vector construction and plant transformation

The NCBI nucleotide database (<http://www.ncbi.nlm.nih.gov/>) was used to select two MPT genes in *Oryza sativa* L. *japonica* cv. Kitaake, *OsMPT3;1* (Loc4335983) and *OsMPT3;2* (Loc4340459), which share high identity. Two target sequences upstream of the coding regions: 5'-GAAGATTGAGATGTACTCGCCGG-3' for *OsMPT3;1* and 5'-TTCTACGCCGCTGCACGGC CCG-3' for *OsMPT3;2*, were designed with the online guide design tool (<http://crispr.dbcls.jp/>). The underlined CCGs represent PAM (protospacer adjacent motif) sequences. The target sequences were connected with two adapters: 5'-GGCA-3' at the 5' terminus and 5'-AAAC-3' at the 3' terminus. Construction of an expression vector and *Agrobacterium*-mediated transformation followed Wang et al. [18]. Seeds of the  $T_0$  generation were grown in field soil under natural conditions, and leaf genomic DNA was extracted with a DNA extraction kit (EasyPure Genomic DNA Kit, TransGen Biotech, Beijing). Both strands of PCR fragments were sequenced to identify transformants.

## 2.2. Identification of mutants

To determine whether gene editing produced off-target effects, NCBI-Blast-Primer Blast (the method of designing primers) was used to align PCR product sequences and PCR was performed with specific primers (Table S1). Sequencing PCR products showed no off-target effect in genes sharing high identity with the target sequences in both *OsMPT3;1* and *OsMPT3;2*. Two mutants, *mpt33* with a deletion of a single base in *OsMPT3;1* and *mpt30* with an insertion of a single base in *OsMPT3;2*, were selected and used for subsequent experiments.

## 2.3. Plant culture and treatment

Seeds of the wild type (WT) and the  $T_2$  mutants were sterilized and sown on MS solid medium with 120 mmol L<sup>-1</sup> NaCl and grown in a growth chamber for two weeks under a relative humidity of 75%, temperature of 28 °C (day)/22 °C (night), and photoperiod of 16 h light/8 h dark. Both hydroponic and pot-culture experiments were performed. Seeds were placed in water in an incubator at 30 °C in the dark for three days. The germinating seedlings were transferred into Hoagland solution (pH 5.5–5.8) containing 120 mmol L<sup>-1</sup> NaCl and further cultured hydroponically for 10 days in the presence or absence of 20 μmol L<sup>-1</sup> ATP. The culture solutions were renewed every three days with Hoagland solution. Alternatively, germinating seedlings were transferred to pots with peat soil, grown for four weeks in a greenhouse at 26–30 °C under an illumination intensity of 8000–10,000 lx for 12 h with relative humidity of 75%, and then cultured for seven days in Hoagland solution containing 120 mmol L<sup>-1</sup> of NaCl and sprayed three times with 20 μmol L<sup>-1</sup> ATP solution. Leaves of each treatment with three biological replicates were collected for physiological measurements and transcriptome sequencing.

## 2.4. RNA isolation and PCR analyses

Total RNA from rice leaves from hydroponic culture was prepared with an RNA Easy Kit (Takara Biotech, Dalian, China). A total of 1 μg RNA was used for cDNA synthesis by reverse transcription-polymerase chain reaction (RT-PCR) using one-step gDNA removal and a cDNA synthesis SUPERMIX (Vazyme Biotech, Nanjing, China). A total of 2 μL cDNA was added to a 20 μL of reaction solution containing 10 μL of Top Green qPCR Supermix and 0.2 μmol L<sup>-1</sup> of specific primers (Table S2) and real-time quantitative polymerase chain reaction (RT-qPCR) was performed with three independent replicates with a light cycler system (7500ABI, Applied Biosystems, Foster City, USA) under the following conditions: 95 °C for 30 s, 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. The expression fold differences were calculated with the  $2^{-\Delta\Delta Ct}$  formula [19] using  $\beta$ -actin as reference gene (NCBI accession ID: XM\_015774830).

## 2.5. Determination of ATP content in rice leaves

ATP detection followed Wu et al. [20] with modification. Approximately 0.5 g of fresh leaves was extracted from

hydroponically grown plants with 5 mL of ultrapure water at 100 °C for 10 min to obtain ATP extracts of ATP and then cooled to room temperature and filtered through a 0.22 μm filter (Millipore, USA). The filtrate was subjected to high-performance liquid chromatography (UltiMate 3000 BioRS, Thermo Fisher, Germany). An injection volume of 20 μL and column temperature of 30 °C were used to detect the peak absorbance at 254 nm. An Atlantis T3 column (5.0 μm, 4.6 mm × 250.0 mm, Waters, Milford, USA) was used for separation. The mobile phase was phosphate buffer (20 mmol L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>; 20 mmol L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0) at a flow rate of 1.0 mL min<sup>-1</sup>.

## 2.6. Physiological measurements

Physiological measurements were performed with pot-cultured plants. A total of 0.5 g dried leaves was digested with 98% H<sub>2</sub>SO<sub>4</sub> and 30% H<sub>2</sub>O<sub>2</sub> and the digest solution was used for determining the phosphate content by the vanadate-molybdate-yellow colorimetric method using a Continuous Flowing Analyzer (SEAL Analytical AA3, Norderstedt, Germany) [18], and measurements of Na and K ions were performed by atomic absorption (ZEEnit 700P, Jena, Germany). Frozen leaves at -80 °C were compressed in a 25-mL syringe tube and the expressed leaf sap was used to determine osmotic potential with an osmotic pressure dew point meter (Wescor 5520, Logan, UT, USA). A total of 0.5 g fresh leaves was used to isolate the extracts for measuring soluble sugar or proline or malondialdehyde (MDA), respectively [21].

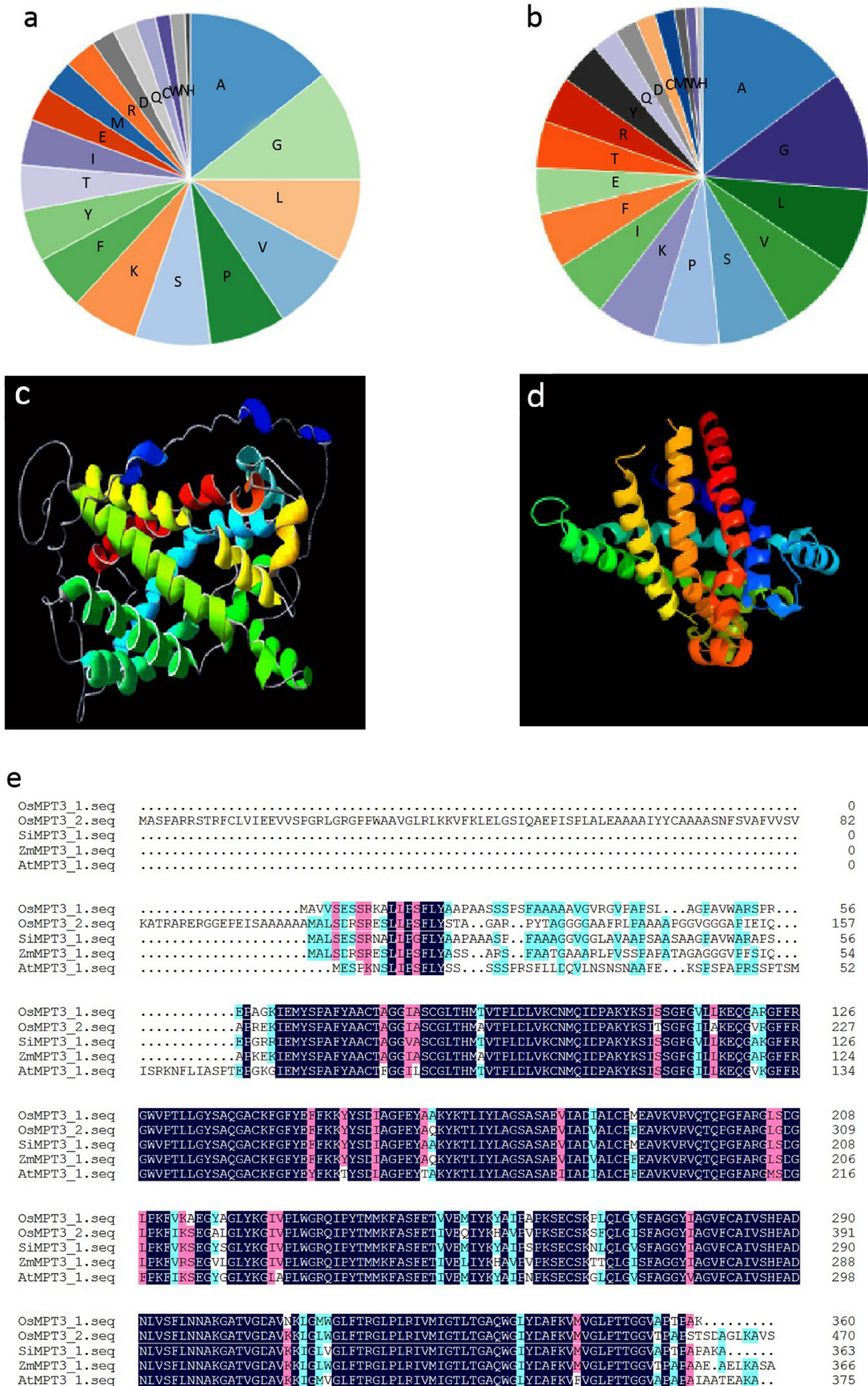
## 2.7. Transcriptome and metabolome sequencing

Leaves from both *mpt30* and the wild type grown in soil culture were separately collected in three biological replicates and used for total RNA isolation and cDNA library construction. Transcriptome sequencing followed He et al. [22]. For metabolome analysis, 50 mg of frozen leaves from pot-culture rice were prepared in six biological replicates, placed in a 1 mL mixture of methanol/acetonitrile/water (2:2:1, v:v:v) containing 20 μL of telmisartan (internal standard), vortexed for 30 s, and fragmented with a grinder (45 Hz) for 4 min and ultrasonicated for 5 min, and then incubated at -20 °C for 1 h. Finally, the isolates were centrifuged at 14,500 ×g at 4 °C for 15 min, and 800 μL of supernatant was transferred to a fresh tube and dried in a freezing vacuum concentrator (LNG-T98, Hualida, Nanjing, China). Extraction solution (acetonitrile:water 1:1, 150 μL) was added to the dry metabolites, the same vortexing, ultrasonication, and centrifugation were performed, and 60 μL of extract was used for metabolite detection by UHPLC/MS (Ultra high-performance liquid chromatography/Mass Spectrometry) (1290, Agilent Technologies).

## 2.8. Flux measurement of Na and K ions in roots

Seeds of both the mutants and the wild type were germinated in water at 30 °C in dark for 3 days and then transferred to Hoagland solution and cultured for 14 days. The two-week-old seedlings were placed for 24 h in Hoagland solution containing 120 mmol L<sup>-1</sup> NaCl and used to determine net fluxes of Na and K ions in the root elongation zone (500 μm proximal to the





root tip). Calibrated electrodes were shifted on the surface of the root tips at 0.3–0.5 Hz for 8 min with non-invasive micro-test technology (NMT) (Xuyue Sci. & Tech. Co. Limited., Beijing, China), and the net Na and K fluxes were determined with Mage Flux software [21].

### 3. Results

#### 3.1. OsMPT3;1 and OsMPT3;2 are homologs

The OsMPT3;1 cDNA encoding 360 amino acids and OsMPT3;2 cDNA encoding 471 amino acids have respective molecular weights of 37.97 and 49.43 kDa. The isoelectric point of OsMPT3;1 is 9.68 and that of OsMPT3;2 is 9.72. Both OsMPT3;1 and OsMPT3;2 proteins are composed mainly of hydrophobic amino acids, including alanine, leucine, valine, and proline (Fig. 1-a, b). Prediction of PHYRE2 (Protein Homology/analogy Recognition Engine V 2.0) shows that the OsMPT3s contain six transmembrane helices and show typical structural characteristics of the mitochondrial phosphate transporter family (Fig. 1-c, d). Multiple alignment shows that OsMPT3;1 shared more than 80% identity with OsMPT3;2 and other MPT3;1 member from the plants *Setaria italica*, *Zea mays*, and *Arabidopsis thaliana* (Fig. 1-e), suggesting that specific structural characteristics of the OsMPT3s might function in transporting Pi from the cytoplasm into the mitochondrial matrix.

#### 3.2. The generated mutants show successful gene editing

PCR amplification showed that both the *mpt33* and *mpt30* mutants were correctly modified at the target sites. As shown in Fig. 2, the *mpt33* mutant shows a deletion of thymine (T) at the 195th position of OsMPT3;1 (Fig. S1-a, b) and the *mpt30* mutant shows an insertion of a T at the 225th position of OsMPT3;2 (Fig. S1-c, d). This insertion or deletion of a single base in the encoding region resulted in early termination of translation in both genes (Fig. S1-e, f). The absence of off-target events was demonstrated as described in Methods.

#### 3.3. Mutants showed lower tolerance to salt stress

Both mutants and WT showed normal growth on MS solid medium under favorable culture (Fig. 2-a). Under salt stress, the growth of both mutants was inhibited, leading to a decrease in biomass compared with the WT, especially for the *mpt30* mutant (Fig. 2-b). No significant phenotypic differences between WT and mutants were observed under favorable culture, but the shoot lengths of the mutants were significantly lower than those of the WT after salt stress (Fig. 2-c, d). The *mpt30* mutant showed a significant decrease in root length compared with the WT under salt stress, but there was no difference between the *mpt33* mutant

and the WT (Fig. 2-d). However, compared with the WT, both mean shoot and root fresh weights of the mutants were significantly decreased under both favorable culture and salt stress, but the differences in biomass between the mutants and WT were obvious under salt stress (Fig. 2-e, f). Thus, the mutants with a loss of function of MPT3 genes showed lower salt tolerance than the WT.

#### 3.4. Mutagenesis of the OsMPT3s genes down-regulated the expression of genes encoding ATPase under salt stress

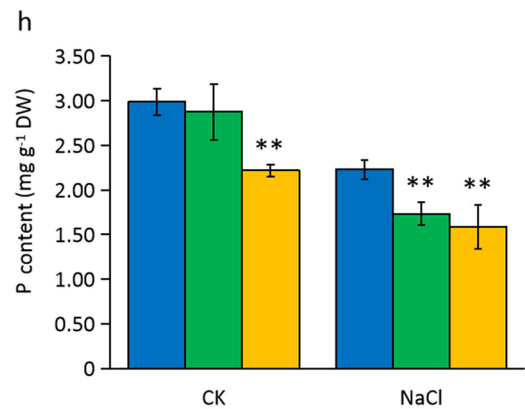
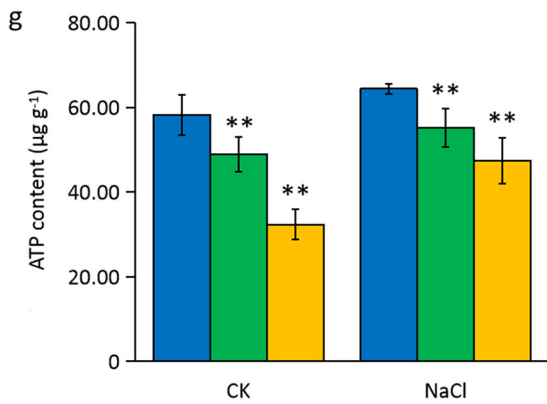
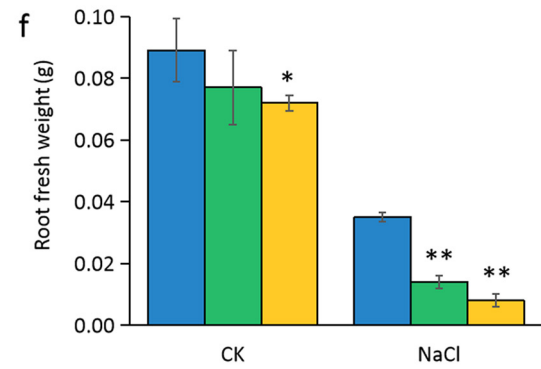
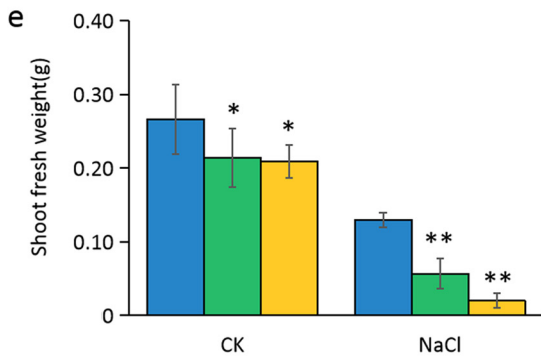
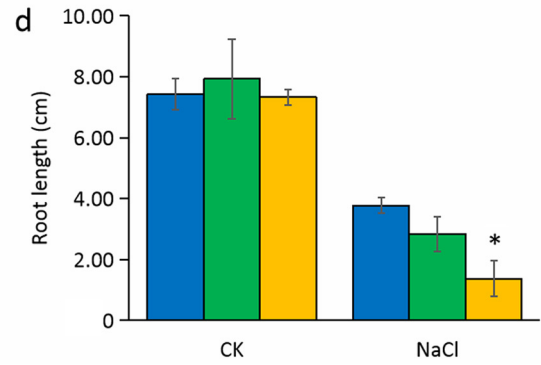
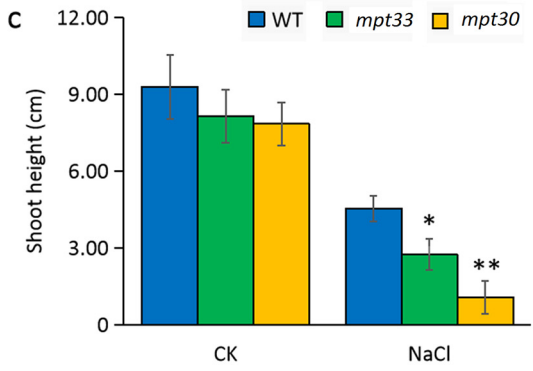
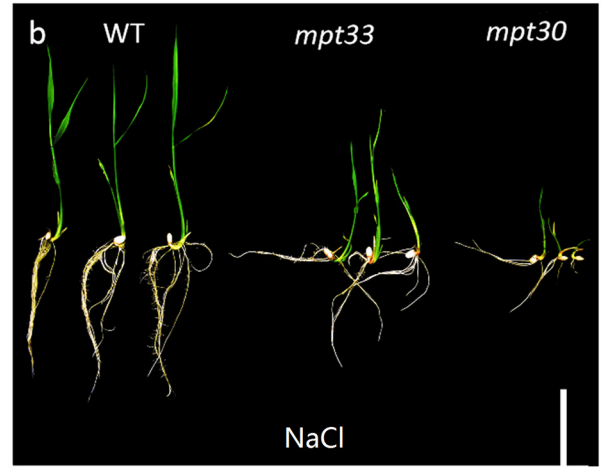
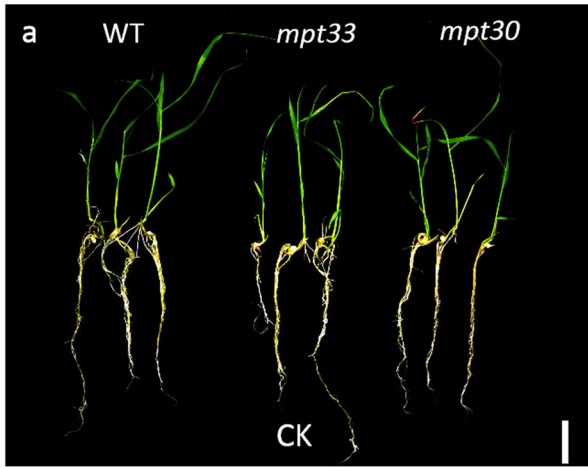
RT-qPCR showed that salt stress significantly increased the transcripts of the OsMPT3;1 and OsMPT3;2 genes in the WT (Fig. S2-a). Besides these two genes, the genes encoding mitochondrial ATP synthase, ATPase1 (Os08g0250200), ATPase2 (Os12g0424180), and ATPase3 (Os12g0207500), are also responsible for catalyzing the reaction of  $\text{ADP} + \text{H}_2\text{O} + \text{Pi} + \text{H}^+ \geq \text{ATP} + \text{H}^+$ . For this reason, we performed RT-qPCR to detect the transcripts of these genes. When rice seedlings were cultured under favorable conditions, the expression level of the gene encoding ATPase1 was higher in the mutants than in the WT (Fig. S2-b), but there were no transcript differences in the genes encoding ATPase2 and ATPase3 compared to WT (Fig. S2-c, d). Salt stress increased the expression levels of the genes encoding ATPase1, ATPase2, and ATPase3, and the expression levels of these genes were significantly lower in the mutants than in the WT.

#### 3.5. The mutants showed altered distributions of ATP, P, K<sup>+</sup>, Na<sup>+</sup>, and Ca<sup>2+</sup> in leaves

The mutants showed decreases of 15.9% of ATP accumulation in *mpt33* and 44.3% in *mpt30* compared with the WT under favorable culture (Fig. 2-g). Salt stress induced synthesis of ATP in the mutants and wild-type, but the ATP contents were still significantly lower in both *mpt33* and *mpt30* than in the WT. In particular, when the mutants were exposed to 120 mmol L<sup>-1</sup> NaCl, the ATP contents in the *mpt33* and *mpt30* decreased by respectively 14.2% and 26.3% compared with the WT (Fig. 2-g). Pi contents in both mutants were decreased in comparison with the WT, but only the *mpt30* mutant showed a clear difference from the WT under favorable culture. Under salt stress, both mutants showed significantly lowered accumulation of Pi in leaves compared with the WT (Fig. 2-h).

Highly accumulation of Na<sup>+</sup> usually hinders the absorption of K<sup>+</sup> and Ca<sup>2+</sup>, thus affecting osmotic potentials in plant cells. Therefore, we measured the contents of K<sup>+</sup>, Na<sup>+</sup>, and Ca<sup>2+</sup> in the mutants. As shown in Table 1, when plants were grown in soil watered with a solution of 0 or 120 mmol L<sup>-1</sup> of NaCl, the mutants showed increased Na<sup>+</sup> contents and lowered K<sup>+</sup> and Ca<sup>2+</sup> contents in shoots in comparison with the WT, indicating that the NaCl treatment significantly altered accumulation profiles of Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup> in the mutants. Before NaCl

**Fig. 1 – Sequence analysis of the OsMPT3;1 and OsMPT3;2 genes. (a) Amino acid sequence of OsMPT3;1. (b) Amino acid sequence of OsMPT3;2. (c) Structural characteristics of the OsMPT3;1 protein. (d) Structural characteristics of the OsMPT3;2 protein. (e) Multiple alignment of OsMPT3;1 (NCBI accession ID: XM\_015778093), OsMPT3;2 (NCBI accession ID: XM\_015785407), SiMPT3;1 (NCBI accession ID: XM\_004975712), ZmMPT3;1 (NCBI accession ID: NM\_001301457) and AtMPT3;1 (NCBI accession ID: NM\_121407).**





**Table 1 – Contents of Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, and Na<sup>+</sup>/K<sup>+</sup> ratio in WT and mutants under salt stress.**

NaCl (mmol L <sup>-1</sup> )	Lines	Na <sup>+</sup> content (mg g <sup>-1</sup> DW)	K <sup>+</sup> content (mg g <sup>-1</sup> DW)	Ca <sup>2+</sup> content (mg g <sup>-1</sup> DW)	Na <sup>+</sup> /K <sup>+</sup> ratio
0	WT	1.64 ± 0.087	29.65 ± 0.145	6.36 ± 0.055	0.06 ± 0.018
	<i>mpt33</i>	1.53 ± 0.114	25.84 ± 0.701**	4.55 ± 0.036**	0.06 ± 0.586
	<i>mpt30</i>	0.94 ± 0.090**	24.97 ± 1.215**	4.29 ± 0.042**	0.04 ± 0.438
120	WT	3.67 ± 0.509	22.45 ± 0.756	4.77 ± 0.040	0.16 ± 0.830
	<i>mpt33</i>	6.77 ± 0.184**	19.09 ± 0.093**	3.65 ± 0.104**	0.35 ± 0.488**
	<i>mpt30</i>	7.31 ± 0.169**	15.87 ± 0.744**	2.86 ± 0.084**	0.46 ± 0.089**

Values represent mean ± standard deviation (n = 3). WT, wild type; *mpt33* and *mpt30* represent two mutant lines. DW, dry weight; \* and \*\* indicate significant difference between the wild type and mutants at the P < 0.05 and P < 0.01, respectively (Student's t-test).

treatment, the *mpt33* mutant and the WT showed no significant difference in Na<sup>+</sup> accumulation, whereas the accumulation of Na<sup>+</sup> was significantly lower in the *mpt30* mutant than in the WT. However, under salt stress, both the *mpt30* and *mpt33* mutants showed significant increases in the contents of Na<sup>+</sup>, by respectively 1.84- and 1.99-fold compared with WT. In contrast, both mutants showed significantly decreased uptake of K<sup>+</sup> compared with WT even under salt stress. Like K<sup>+</sup>, Ca<sup>2+</sup> in the mutants followed identical accumulation profiles. Although no significant differences in Na<sup>+</sup>/K<sup>+</sup> ratio were observed between the mutants and the WT under favorable culture, the mutants showed significantly increased Na<sup>+</sup>/K<sup>+</sup> ratios compared with the WT after salt stress, indicating that the mutations reduced the efflux of Na<sup>+</sup> and the uptake of K<sup>+</sup> and Ca<sup>2+</sup>.

### 3.6. The mutations affected the flux profiles of Na, K and Ca ions in rice roots

When the seedlings were grown in a favorable culture solution, the *mpt30* mutant and the WT showed a similar trend of variation in the fluxes of Na ion in the elongation zone of the root tips, but the *mpt30* mutant showed a lower efflux of Na ion than the WT after 8.5 min (Fig. 3-a). When seedlings were exposed to a 120 mmol L<sup>-1</sup> NaCl solution, both the *mpt30* mutant and the WT showed a gradually increasing trend in the efflux of Na ion, but compared to the WT, the mutant showed a significantly decreased Na ion efflux rate in the elongation zones of the root tips (Fig. 3-b). In contrast to the Na ion, both the mutants and WT showed an influx of K ion in the elongation zones under favorable culture, and the influx flow rates of K ion were significantly higher in the mutant than in the wild type (Fig. 3-c). Under salt stress, the flux of K ion in the *mpt30* mutant was converted from influx to efflux, while the influx rate of K ion in the WT gradually decreased and showed a clear efflux after 9 min (Fig. 3-d). When rice seedlings were grown in favorable solutions, both mutants and WT displayed a gradual decrease of Ca ion efflux with time, but the *mpt30* mutant showed a significantly

increased efflux rates of Ca ion relative to the WT (Fig. 3-e). Under salt stress, the flux profiles of Ca ion showed a similar trend in the mutant and wild type, and the efflux rates of Ca ion in both *mpt30* and WT were markedly increased in comparison with favorable culture (Fig. 3-f).

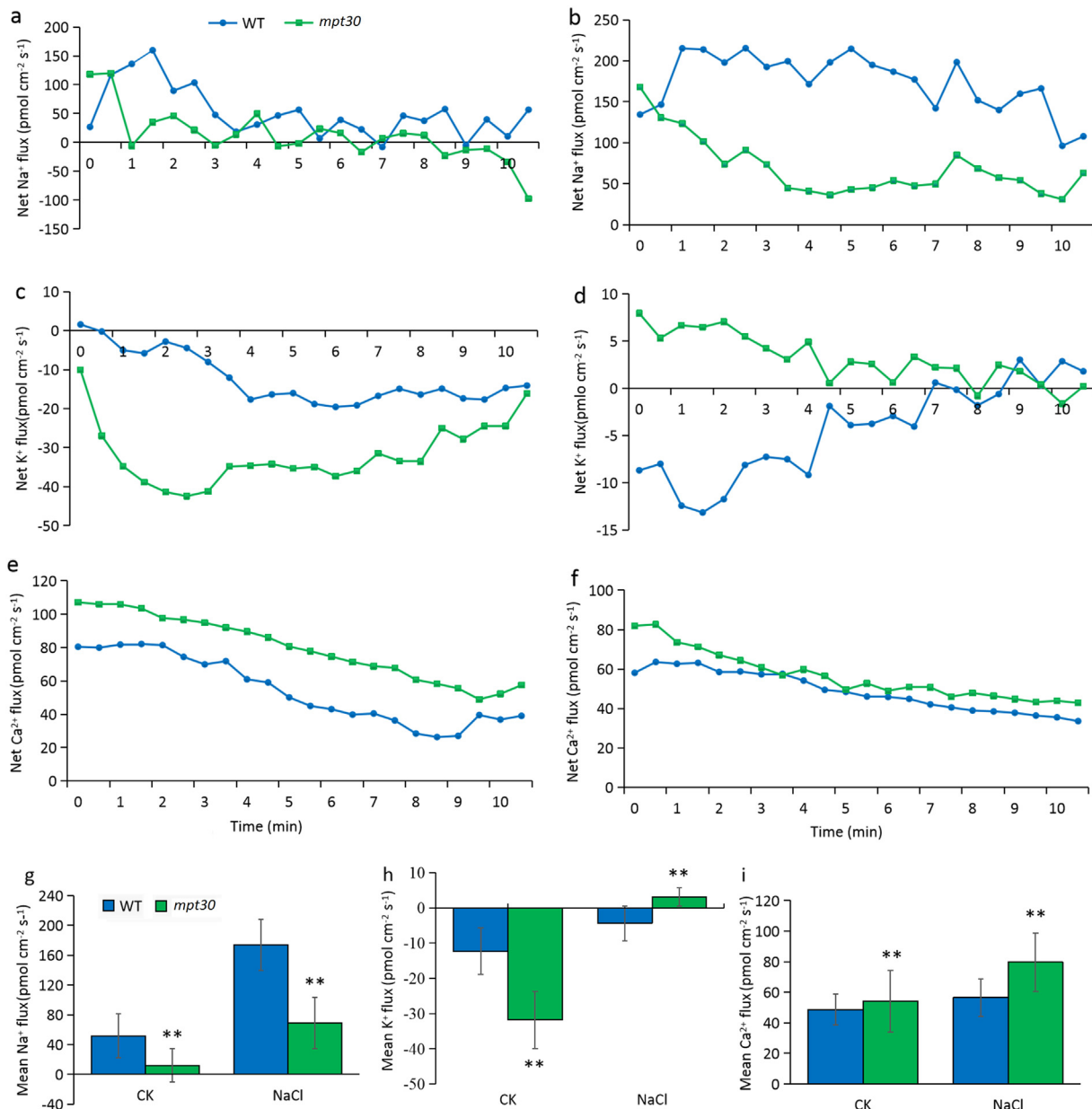
Under either favorable culture or salt stress, the *mpt30* mutant showed significantly decreased net flux rates of Na ion in root tips compared to the WT. The mean efflux of Na ion in the mutant decreased by 76.7% under favorable culture and 60.2% under salt stress (Fig. 3-g). In contrast the Na ion in root tips, the K ion showed influx, and the mean influx rate of K ion was higher in the mutant than in the WT under favorable culture. However, under salt stress, the K ion in the WT showed influx, whereas the *mpt30* mutant showed efflux of K ion (Fig. 3-h). Salt stress increased the efflux of Ca ion in both the mutant and the WT; however, whether rice seedlings were cultured under favorable condition or NaCl stress, the net efflux of Ca ions in the *mpt30* mutant was increased compared with the WT (Fig. 3-i).

### 3.7. The mutants showed high sensitivity to ATP

In hydroponic culture, the mutants and the wild type showed no marked differences in phenotype under favorable conditions, but salt stress reduced the growth of the mutants, a finding similar to that in culture on MS solid medium (Fig. 4-a, b). However, in the presence of exogenous ATP, the *mpt33* mutant showed a restoration in phenotype, while the phenotype of the *mpt30* mutant was partially restored in comparison with the WT (Fig. 4-c), suggesting that plants with mutagenesis of *OsMPT3* are sensitive to exogenous ATP.

The mutant *mpt33* showed no significant differences in osmotic potential or accumulation of soluble sugar, proline, and malondialdehyde compared with the WT under favorable culture, whereas the *mpt30* mutant showed decreased osmotic potential and accumulation of proline in leaves (Fig. 4-d-g). However, both mutants showed significantly increased osmotic potentials and reduced accumulation of soluble sugar and proline in leaves in response to salt stress compared with

**Fig. 2 – Phenotypic characteristics, biomass, ATP content, and phosphate content in rice under salt stress. (a) Phenotypes of wild type (WT) and mutants under favorable culture. (b) Phenotypes of WT and mutants under culture with 120 mmol L<sup>-1</sup> NaCl solution. (c) Shoot lengths of seedlings. (d) Root lengths of seedlings. (e) Shoot fresh weights of seedlings. (f) Root fresh weights of seedlings. (g) ATP content. (h) Total phosphate content. Error bars indicate standard deviation of three biological replicates. DW, dry weight. \* and \*\* indicate significant difference between mutants and WT at P < 0.05 and P < 0.01, respectively (Student's t-test).**

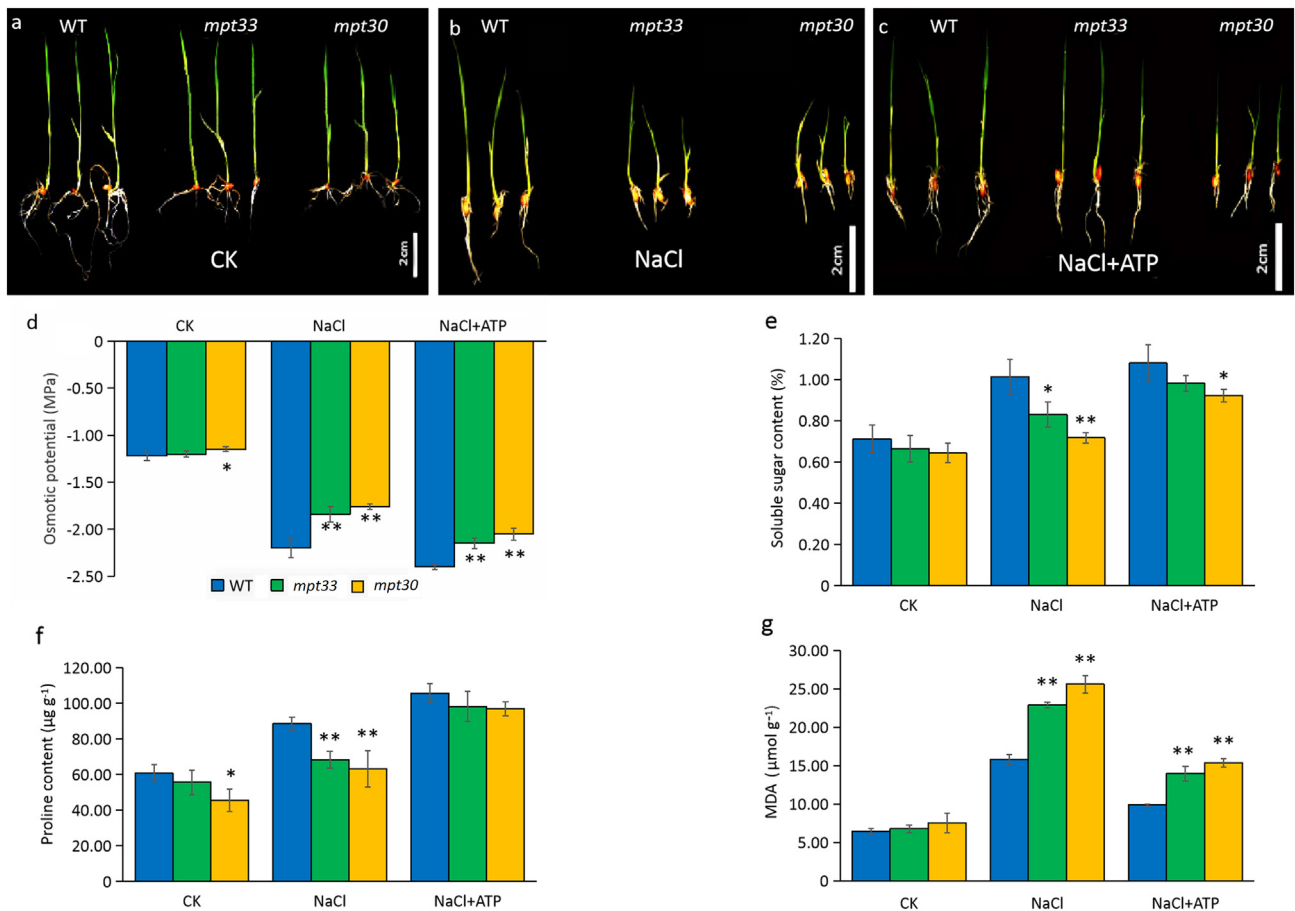


**Fig. 3 – Flux profiles of Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup> in root tip elongation zones of 7-day-old rice seedlings. (a) Net Na<sup>+</sup> fluxes under favorable culture. (b) Net Na<sup>+</sup> fluxes under culture with a 120 mmol L<sup>-1</sup> NaCl solution. (c) Net K<sup>+</sup> fluxes under favorable culture. (d) Net K<sup>+</sup> fluxes under salt stress. (e) Net Ca<sup>2+</sup> fluxes under favorable culture. (f) Net Ca<sup>2+</sup> fluxes under culture with a solution of 120 mmol·L<sup>-1</sup> NaCl. Net effluxes are indicated by positive and net influxes by negative values. (g) Mean Na<sup>+</sup> flux. (g) Mean K<sup>+</sup> flux. (i) Mean Ca<sup>2+</sup> flux. WT, wild type. Error bars indicate standard deviation of three biological replicates. \* and \*\* indicate significant difference between WT and mutants at  $P < 0.05$  and  $P < 0.01$ , respectively (Student's *t*-test).**

the WT (Fig. 4-d-g). When rice seedlings were cultured in NaCl solution, the osmotic potentials of the mutants increased by respectively 16.4% and 20.0% relative to the WT (Fig. 4-d), but the soluble sugar contents in the *mpt33* and *mpt30* mutants were reduced by 18.1% and 29.2% relative to the WT (Fig. 4-e). Proline contents in the *mpt33* and *mpt30* mutants were reduced by 22.7% and 28.6% compared to the WT (Fig. 4-f), while MDA contents in the *mpt33* and *mpt30* mutants increased 1.45- and 1.62-fold, respectively (Fig. 4-g).

As shown in Fig. 4, exogenous ATP lowered the osmotic potentials and accumulation of MDA, but significantly increased the contents of soluble sugar and proline in WT and the mutants under salt stress. In the presence of ATP, the contents of soluble sugar in the WT, *mpt33*, and *mpt30* mutants increased by respectively 6.6%, 18.4%, and 28.6% in comparison with the treatment without addition of ATP (Fig. 4-e). When the mutants were exposed to a solution of 120 mmol L<sup>-1</sup> NaCl in the presence of 20 μmol L<sup>-1</sup> ATP, the





**Fig. 4 – Phenotypic and physiological changes of WT and mutants under favorable culture, salt stress, and ATP treatment. (a) Phenotype of the WT and mutants under favorable culture. (b) Phenotype of the WT and mutants under salt stress in a  $120 \text{ mmol L}^{-1}$  NaCl solution. (c) Phenotype of the WT and mutants under salt stress with ATP treatment. (d) Osmotic potential change. (e) Soluble sugar content. (f) Proline content. (g) MDA content. Error bars indicate the standard deviation of three biological replicates. \* and \*\* indicate significant difference between mutants and WT at  $P < 0.05$  and  $P < 0.01$ , respectively (Student's *t*-test).**

mean contents of proline in the WT, *mpt33* and *mpt30* mutants increased by respectively 17.07, 29.85, and  $33.76 \mu\text{g g}^{-1}$ , (Fig. 4-f), and the MDA contents decreased by respectively 37.50%, 39.09%, and 39.90% under salt stress in the presence of  $20 \mu\text{mol L}^{-1}$  ATP in comparison with the treatment without ATP addition (Fig. 4-g).

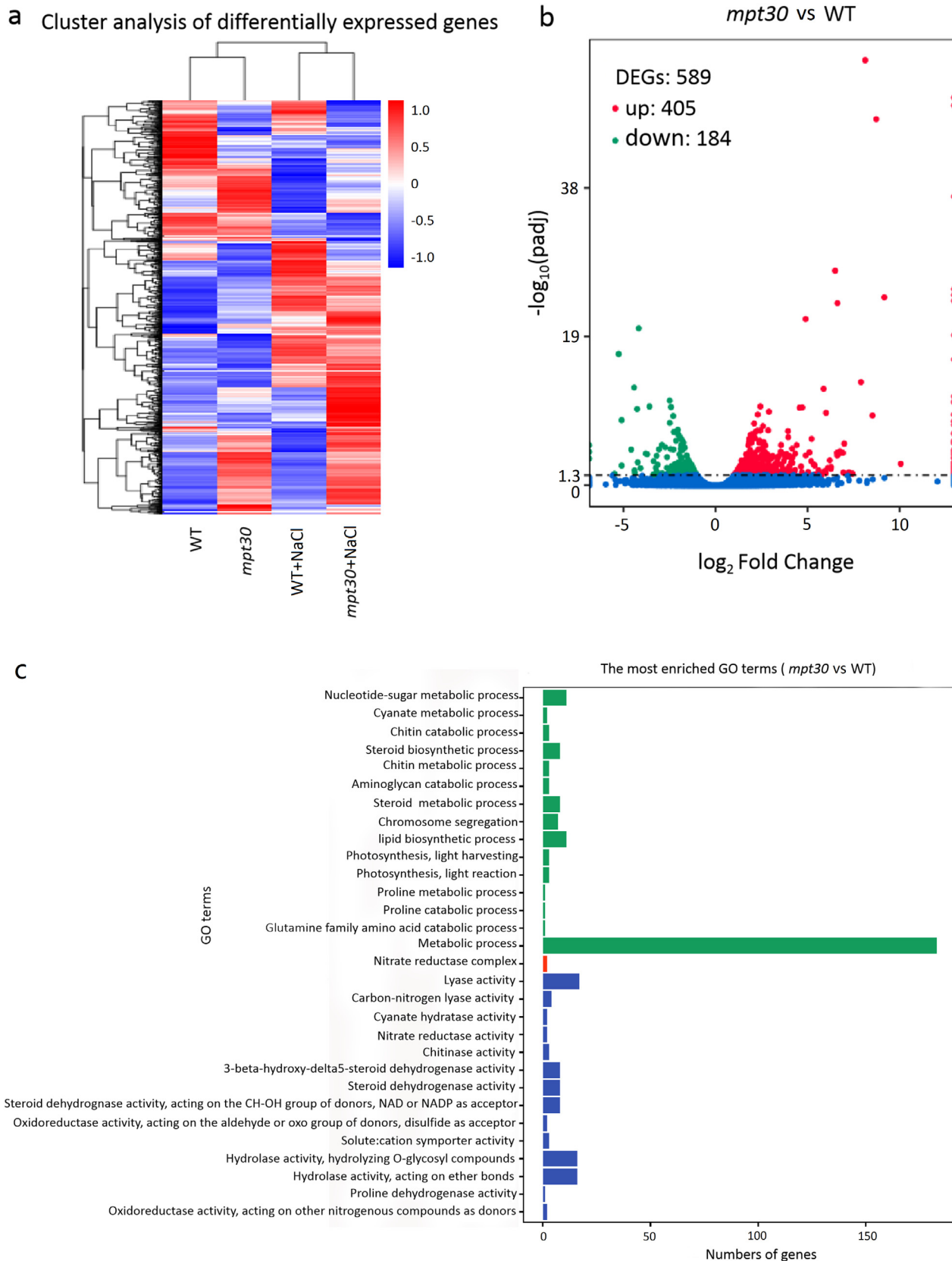
### 3.8. The mutants trigger the alteration of the transcriptional levels

A total of 589 genes were significantly differentially expressed, with 405 of the up-regulated and 184 of the down-regulated genes expressed in the *mpt30* mutant compared with the WT (Fig. 5-b). The GO (Gene Ontology) enrichment profiles of the differentially expressed genes (DEGs) were classified into three categories: biological process, cellular component, and molecular function. The significant GO terms in these three categories and the numbers of DEGs show that the numbers of genes involved in biological regulation of metabolic process were much higher than those in other processes, while the DEGs participating in

molecular functions were grouped mainly in lyase activity and hydrolase activity (Fig. 5-c).

Differentially genes were involved mainly in the synthesis of secondary metabolites, metabolism of starch and carbohydrates, glycolysis, pyruvate metabolism, tricarboxylic acid cycle, and metabolism of arginine and proline. Typically, these processes include glycolysis, pyruvate metabolism, and tricarboxylic acid cycle, which are mainly responsible for providing energy and synthesizing ATP (Fig. 5-d). Arginine and proline are required for increasing plant resistance to abiotic stress, suggesting that the tolerance response of *mpt30* mutant to salt stress may be associated with the inhibition of ATP synthesis and disorders of proline metabolism.

For further profiling the expressions of the differentially expressed genes, 10 DEGs possibly involved in salt stress were presented by transcriptome sequencing (Table 2). They included three down-regulated and seven up-regulated genes. The RT-qPCR results revealed expression profiles identical with those from transcriptome sequencing (Fig. S3). As presented in Table 2, the *Os07g0677200* gene encodes a kind of peroxidase (GO: 0044744) and the *Os07g0152700* gene



**Fig. 5 – Overview and distribution profiles of differentially expressed genes (DEGs) and differentially expressed metabolites in rice in response to salt stress. (a) Cluster analysis of differentially expressed genes. (b) Volcano distributions of DEGs. (c) The most enriched GO terms of DEGs. (d) Differential gene KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment scatter plot. (e) Volcano plot of metabolites carrying a positive charge. (f) The volcano plot of metabolites carrying a negative charge.**

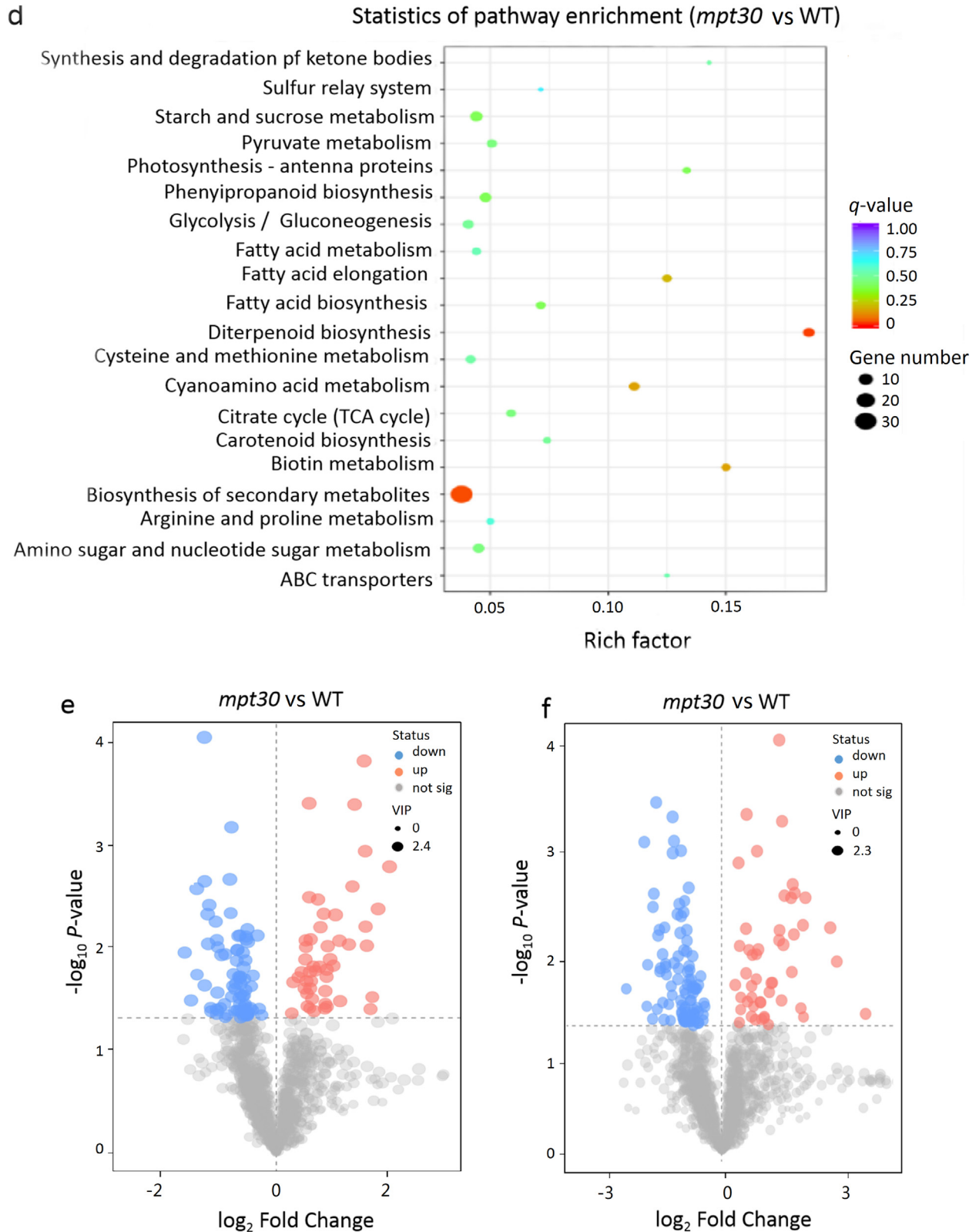


Fig. 5 (continued).

encodes a kind of glutathione transferase (GO: 0006749). As proline dehydrogenase, the *Os10g0550900* gene catalyzes the conversion of proline to delta-1-pyrroline-5-carboxylate (GO: 0010133), while the P5CS2 (*Os01g0848200*) gene encodes a

delta-1-pyrroline-5-carboxylate synthase 2, one of the key enzymes for catalyzing the synthesis of proline (GO: 0006561). The *Os01g0118000* gene functions in catalyzing the conversion of  $\beta$ -D-fructose 1,6-bisphosphate to D-glyceraldehyde 3-

**Table 2 – Expressions of the partial differentially expressed genes of OsMPT3-mediated in rice under salt stress.**

Gene ID	log <sub>2</sub> fold change	P-value	Up/down	Function annotation
Os07g0677200	-2.2571	0.000123	-	Hydrogen peroxide catabolic process
Os07g0152700	-4.5843	7.78E-08	-	Glutathione transferase activity
Os10g0550900	-1.6767	0.000155	-	Proline dehydrogenase
Os01g0848200	2.5816	0.000766	+	Pyrroline-5-carboxylate synthetase 2
Os01g0118000	1.3075	0.000264	+	Fructose-bisphosphate aldolase activity
Os01g0660300	3.0396	0.000002	+	Pyruvate kinase
Os06g0246500	2.7423	0.000002	+	Pyruvate dehydrogenase E1 component subunit alpha-2
Os04g0119400	1.3793	0.000108	+	Pyruvate dehydrogenase E1 component subunit alpha-3
Os08g0431300	2.1257	7.12E-06	+	Dihydrolipoamide acetyltransferase, component of pyruvate dehydrogenase complex
Os02g0625500	1.1453	0.000504	+	Adenosine kinase activity

The  $\beta$ -actin (accession: XM\_015774830) was used as a reference gene; “+” and “-” represent up-regulation and down-regulation, respectively.

phosphate and dihydroxyacetone phosphate, an important step in the glycolysis process (GO: 0006096). Pyruvate kinase (Os01g0660300) could catalyze dihydroxyacetone phosphate into pyruvate and release ATP (GO: 0006096). The Os06g0246500 and the Os04g0119400 genes are two subunits encoding pyruvate dehydrogenase (GO: 0004739), while the Os08g0431300 gene encodes a dihydrolipoic acid transacetylase (GO: 0016746), and these DEGs are responsible for translating the pyruvate into acetyl CoA which finally is accumulated as tricarboxylic acid. The Os02g0625500 gene encoding adenosine kinase (GO: 0004001) could catalyze the transfer of a phosphate group to adenosine when the ATP is catalyzed to produce AMP and ADP.

### 3.9. Mutagenesis of the OsMPT3;2 gene changed the distribution of the differential metabolites

A total of 129 cationic metabolites and 144 anionic differential metabolites were differentially expressed (Fig. 5-e, f; VIP-variable importance in the projection > 1,  $P < 0.05$ ). Analysis of metabolic pathways of differential metabolites by KEGG

(Kyoto Encyclopedia of Genes and Genomes) showed that 17 metabolic pathways were matched and 14 differential metabolites were isolated (Table 3). Glutamine is a precursor substance for protein synthesis and is involved in a variety of metabolic pathways, including pyrimidine metabolism, nitrogen metabolism, proline metabolism and purine metabolism. Under salt stress, amounts of glutamine were significantly increased in the mutant in comparison with the wild type. Like glutamine, adenosine is an important precursor for the synthesis of ATP, and was highly accumulated in the *mpt30* mutant compared with the wild type after salt stress.

## 4. Discussion

### 4.1. Mitochondrial phosphate transporter functions by coordinating ATPase

A mitochondrial phosphate transporter (MPT) is a member of the phosphate transporter family participating in the exchange of phosphate (Pi) between the cytoplasm and

**Table 3 – Expression profiles of differentially metabolites involving in major metabolic pathway in rice in response to salt stress.**

Metabolite	Up/down	Pathway	KEGG
L-Glutamine	+	Pyrimidine metabolism; Nitrogen metabolism; Alanine, aspartate and glutamate metabolism;	C00064
Cytidine	+	Arginine and proline metabolism; Purine metabolism; Aminoacyl-tRNA biosynthesis;	C00475
Serotonin	+	Pyrimidine metabolism	C00780
Taurine	+	Tryptophan metabolism	C00245
Uridine	+	Taurine and hypotaurine metabolism	C00299
Raffinose	+	Pyrimidine metabolism	C00492
Adenosine	+	Galactose metabolism	C00212
Stearic acid	-	Purine metabolism	C01530
L-Arabinose	-	Fatty acid biosynthesis	C00259
7-Methylxanthine	-	Amino sugar and nucleotide sugar metabolism	C16353
alpha-Linolenic acid	-	Caffeine metabolism	C06427
L-Isoleucine	-	alpha-Linolenic acid metabolism	C00407
N-Acetyl-D-glucosamine	-	Valine, leucine and isoleucine biosynthesis; Valine, leucine and isoleucine degradation; Aminoacyl-tRNA biosynthesis	C00140
Histamine	-	Amino sugar and nucleotide sugar metabolism	C00388

“+” and “-” represent respectively up-regulated and down-regulated metabolites.



mitochondria matrix through the Pi/H<sup>+</sup> symport pathway or Pi/OH<sup>-</sup> antiport pathway, and is essential for catalyzing the formation of ATP from ADP and Pi in plants [23]. As a major source of biochemical energy in living cells, ATP acts as a critical signal molecule modulating plant responses to abiotic stress via intercellular communication [24]. In previous studies, two mitochondrial small subunit genes encoding ATP synthase, *RmtATP6* and *AtMtATP6*, conferred salt tolerance in *Oryza sativa* and *Arabidopsis*, respectively [25,26]. More transcripts of the *SpAHA1* gene encoding the plasma membrane H<sup>+</sup>-ATPase also promote salt tolerance by beneficially triggering the physiological changes in rice and transgenic *Arabidopsis* [27]. Similarly, the activities of NHX protein (a Na<sup>+</sup>/H<sup>+</sup> exchanger) and H<sup>+</sup>/ATPase affect the transport of Na<sup>+</sup> in the vacuole in plant [28]. In our study, two mutants with a loss of function of mitochondrial phosphate transporters, the *mpt33* and *mpt30*, showed lower salt resistance than the wild type, indicating that the mutagenesis of the *OsMPT3* genes likely obstructed the transport pathway of Pi from the mitochondrial outer membrane to the inner membrane and reduced ATP synthesis in the mitochondrial matrix, given that both the *mpt33* and *mpt30* mutants showed reduced activities of ATPase and contents of ATP and phosphate under salt stress.

#### 4.2. The *OsMPT3* gene is an osmotic regulator depending on ATP synthesis

Generally, ATP-mediated energy supply modulates the transport of Na<sup>+</sup> and influx of K<sup>+</sup> to alleviate salt damage to the plant [25,29]. Under salt stress, the plasma membrane (PM) H<sup>+</sup>-ATPase activity is activated by J3 (DnaJ homolog3) through inhibiting PKS5 kinase activity, which is required for establishing a proton gradient across the PM and promoting the activity of the Na<sup>+</sup>/H<sup>+</sup> antiporter (SOS1) [30]. SOS3 interacts with SOS2 kinase and activates the phosphorylation of SOS2 and then the SOS3-SOS2 kinase complex mediate Na<sup>+</sup> efflux from the cytoplasm to the apoplast [31]. Two major transporters, NHX1 and NHX2, are essential for mediating K<sup>+</sup> uptake to the vacuole to maintain K<sup>+</sup> homeostasis through vacuolar H<sup>+</sup>-ATPase [6]. In our study, both the *mpt33* and *mpt30* mutants accumulated more Na<sup>+</sup> and lowered K<sup>+</sup>, showing that *OsMPT3* genes play an important regulatory role in maintaining osmotic potentials, which are generally related to the influx or efflux of Na and K ions in the root tips. The efflux profiles of Na and K ions in the roots suggested that the abilities to discharge Na<sup>+</sup> and accumulated K<sup>+</sup> in the mutants were significantly weakened in comparison with the WT, suggesting that the *OsMPT3* genes could regulate the uptake and flux of Na<sup>+</sup> and K<sup>+</sup> to respond to salt stress. Interestingly, the phenotype and physiological metabolism of the mutants were restored in the presence of exogenous ATP, suggesting that the *OsMPT3* gene might mediate rice salt tolerance through a dependent pathway of the synthesis of ATP.

Calcium (Ca<sup>2+</sup>), an important second messenger in eukaryotic cells, is essential for increasing plant salt tolerance [32]. When plants are exposed to high salinity, cytosolic Ca<sup>2+</sup> is rapidly elevated, initiating stress signal transduction pathways for plant protection [33]. Ca<sup>2+</sup> sensors bind Ca<sup>2+</sup> to promote interaction with downstream effectors. Tang et al. [34] reported that as Ca<sup>2+</sup> sensor, both *PbCaM1* and *PbCaM3*

were significantly up-regulated in pear in response to salt stress. The Ca<sup>2+</sup>-binding proteins SOS3 and SCaBP8 were activated by higher cytosolic Ca<sup>2+</sup>, and then activated SOS2, a serine/threonine protein kinase [35–37]. Activated SOS2 activates the plasma membrane (PM) Na<sup>+</sup>/H<sup>+</sup> antiporter SOS1, which is responsible for transporting excess Na<sup>+</sup> into the apoplast [38]. In the present study, both the *mpt30* and *mpt33* mutants showed significantly reduced Ca<sup>2+</sup> contents, higher Ca<sup>2+</sup> efflux rates, and lower ability of retaining calcium than the WT, indicating that intracellular levels of Ca<sup>2+</sup> in cytoplasm were regulated by controlling the influx and efflux of Ca ion. Although the influx of Ca<sup>2+</sup> to cytosol occurs in a passive form via Ca<sup>2+</sup>-channels, its efflux is an active process that is mediated by Ca<sup>2+</sup>/H<sup>+</sup> antiporters and Ca<sup>2+</sup> pumps, and the energy used in this process is provided by ATP hydrolysis and proton motive force [39]. Demidchik et al. [40] reported that increased extracellular ATP elevated the level of Ca<sup>2+</sup> in cytoplasm in *Arabidopsis*, thus up-regulating the transcripts of the *MPK3* gene encoding a kinase involved in salt stress response. Thus, the reduced Ca<sup>2+</sup> uptake and weaker salt tolerance in the mutants may have been triggered by the loss of function of the *OsMPT3* genes and a decrease in ATP.

#### 4.3. *OsMPT3s* play important regulatory role in accumulation of metabolites

When plants are exposed to salt stress, cell permeability generally decreases to maintain the uptake of water, but as an indicator of stress-induced peroxidation of membrane lipids, the malondialdehyde (MDA) is increased, thus lowering the antioxidant capacity in plant cells. Maintaining cell permeability is associated with the levels of soluble sugar and proline [21,22]. In the present study, cell permeability and MDA in the *mpt33* and *mpt30* mutants were significantly increased, but the contents of proline and soluble sugar were lowered, suggesting that the mutagenesis of the *OsMPT3* genes triggered changes in the accumulation profiles of these organic osmolytes. Proline is usually highly accumulated under salt stress, thus alleviating salt damage to the plant [41]. Li et al. [21] and He et al. [22] reported that overexpressing the *ThPP1* gene and *TsPIP1;1* gene increased the contents of proline to increase rice salt tolerance. However, the CRISPR/Cas9-mediated mutagenesis of the *OsPPa6* gene significantly reduced the proline content and alkaline tolerance in rice [18]. As an initial substrate, proline synthesis is performed by the glutamate and ornithine pathways. Proline is first synthesized mainly from glutamate in plants, while delta-1-pyrroline-5-carboxylate synthase (P5CS) and delta-pyrroline-5-carboxylate reductase (P5CR) are the two key enzymes for proline synthesis, and P5CS exerts a strong effect on proline accumulation in the glutamate pathway compared to P5CR [42]. In the presence of ATP, P5CS catalyzes the conversion of L-glutamate to L-glutamyl 5-phosphate, a major step in proline synthesis [43]. Proline dehydrogenase (ProDH) is a main rate-limiting enzyme in proline degradation [44]. In this study, both the *mpt33* and *mpt30* mutants showed significantly reduced accumulation of proline and up-regulation of the proline synthesis gene (*P5CS2-Os01g0848200*) and down-regulation of the proline dehydrogenase gene (*ProDH-Os10g0550900*), indicating that

the mutagenesis of the rice *OsMPT3* gene affects the accumulation of proline. Our metabolome analysis also demonstrates an obvious up-regulation of the precursor of proline synthesis, L-glutamine, in the mutants. Usually, L-glutamine could be converted to L-glutamate by catalyzing a reaction of related enzymes, while P5CS catalyzes L-glutamate to L-glutamyl 5-phosphate through the supply pathway of ATP. In this study, an enhanced decrease in ATP content in the *mpt33* and *mpt30* mutants shows that the transfer process of L-glutamate into L-glutamyl 5-phosphate was impeded, leading to the up-regulation of L-glutamine and decreased accumulation of proline. Thus, mutagenesis of the rice *OsMPT3* gene lowered the accumulation of proline by changing the expression profiles of the *P5CS2* and *ProDH* genes under salt stress, thereby altering osmotic potentials.

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## Author contributions

Shengcai Huang performed the entire experiment and prepared the preliminary manuscript; Shichao Xin and Guoqiang Xie managed the reproduction of rice in the fields; Jiao Han constructed the transformation vector; Zhonglai Liu and Bing Wang participated in physiological measurements; Shuqing Zhang and Qingyu Wu provided guidance for the experimental design and writing; Xianguo Cheng designed the entire experiment and corrected the manuscript.

## Appendix A. Supplementary data

Supplementary data for this article can be found online at <https://doi.org/10.1016/j.cj.2020.02.001>.

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