Running title: MtZPT2-2 negatively regulates salt tolerance

Zinc finger transcription factor MtZPT2-2 negatively regulates salt tolerance in *Medicago truncatula*

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ABSTRACT

Zinc finger proteins (ZFPs) are transcription factors involved in multiple cellular

functions. We identified a C2H2 type ZFP (MtZPT2-2) in *Medicago truncatula* and demonstrated that it localizes to the nucleus and inhibits transcription of two genes encoding high-affinity potassium transporters (*MtHKT1;1* and *MtHKT1;2*). *MtZPT2-2* transcripts were detected in the stem, leaf, flower, seeds and roots, with the highest level in the xylem and phloem of roots and stems. *MtZPT2-2* transcription in leaves was reduced after salt stress. Compared to the wild type (WT), transgenic lines overexpressing *MtZPT2-2* had decreased salt tolerance, while *MtZPT2-2*-knockout mutants showed increased salt tolerance. *MtHKT1;1* and *MtHKT1;2* transcripts and Na^+ accumulation in shoots and roots, as well as in the xylem of all genotypes of plants, were increased after salt treatment, with higher levels of *MtHKT1;1* and *MtHKT1;2* transcripts and Na^+ accumulation in *MtZPT2_2*_knockout mutants and lower levels in *MtZPT2-2*-overexpressing lines compared with the WT. K^+ levels showed no significant difference among plant genotypes under salt stress. Moreover, MtZPT2-2 was demonstrated to bind with the promoter of *MtHKT1;1* and *MtHKT1;2* to inhibit their expression. Antioxidant enzyme activities and the gene transcript levels were accordingly upregulated in response to salt, with higher levels in *MtZPT2-2*-knockout mutants and lower levels in *MtZPT2-2*-overexpressing lines compared with WT. The results suggest that *MtZPT2-2* regulates salt tolerance negatively through down-regulating *MtHKT1;1* and *MtHKT1;2* expression directly to reduce Na^+ unloading from the xylem and regulates antioxidant defense indirectly.

INTRODUCTION

Soil salinization is a worldwide problem that largely reduces crop production. About 20% of the cultivated land area is affected by salinity (Munns, 2005). Plants accumulate excessive Na^+ under salinity condition, which leads to osmotic stress and inhibits K^+ uptake (Almeida et al., 2017). Long-term salt stress leads to reduced photosynthesis and oxidative damage to plants and, ultimately, death as a result of Na^+ toxicity (Zhu et al., 2017).

Maintaining ion homeostasis through K^+ and Na^+ transporters/channels is the major mechanism for plants to avoid Na^+ toxicity under salinity conditions (Almeida et al., 2017). The SALT OVERLY SENSITIVE (SOS) pathway plays a key role in maintaining ion balance in *Arabidopsis thaliana* (Zhu, 2002). SOS1 is a plasma membrane-localized Na^+/H^+ antiporter, mediating both Na^+ excretion from cytosol to apoplast and long-distance Na^+ transport between roots and leaves (Zhu, 2002). NHX is a tonoplast-localized Na^+/H^+ antiporter and functions to transport Na^+ into vacuoles to avoid its accumulation in the cytoplasm (Deinlein et al., 2014). In addition, redistribution of Na^+ is also important for plant adaptation to salinity. When plants are exposed to salt stress, the loading of Na^+ into the xylem or unloading Na^+ from the xylem is reduced to avoid excess Na^+ accumulation caused by toxicity in shoots (Shabala et al., 2013).

High-affinity potassium transporters (HKTs) are involved in the transport and redistribution of Na^+ in plants during salt stress (Kronzucker and Britto, 2011). HKT1;1 mediates Na^+ loading in the shoot phloem and unloading in the root xylem, resulting in decreased Na^+ accumulation in the shoot and increased salt tolerance (van

Zelm et al., 2020). Salt-responsive *Oryza sativa HKT1;1* is mainly expressed in the phloem of leaves and protects young leaves by accumulating Na^+ in old leaves under salt conditions (Wang et al., 2012). *OsHKT1;4* is mainly expressed in the vascular bundle tissues of shoots and confers the unloading of Na^+ from leaves (Suzuki et al., 2016). *OsHKT1;5* is expressed in the xylem in roots and leaf sheath and confers unloading of xylem Na^+ for reduced Na^+ accumulation in shoots and leaves (Ren et al., 2005). GmHKT1;1 improves salt tolerance by regulating Na^+ and K^+ homeostasis in soybean (*Glycine max*) (Chen et al., 2014). HvHKT1;5 mediates Na^+ absorption in the root epidermis and Na^+ transport from roots to shoots through the xylem in the stele and negatively regulates salt tolerance in barley (*Hordeum vulgare*) (Huang et al., 2020). However, understanding of transcriptional regulation of *HKTs* expression in response to salt stress is still limited, although several transcription factors binding with the promoter of *HKTs* have been reported (Kumar et al., 2017). ABSCISIC ACID-INSENSITIVE 4 (ABI4) and CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 6 (CAMTA6) can directly bind to the ABA-responsive ABEs motif (GCGGCTTT) and AREB motif (ACGTGT) in the promoter of *HKT1;1*, respectively, to regulate salt tolerance in *Arabidopsis* (Shkolnik et al., 2013; Shkolnik et al., 2019). *OsHKT1;1* transcript is regulated by OsMYBc, a MYB coiled-coil transcription factor, which binds with the AAANATNC (C/T) sequence in the *OsHKT1;1* promoter region in rice (*Oryza sativa*) (Xiao et al., 2022). However, it remains unknown whether members of the zinc finger protein (ZFPs) family directly regulate the expression of *HKTs*.

ZFPs are a class of proteins containing zinc finger domains that have multiple functions, including transcription regulation, RNA binding, apoptosis regulation, and protein-protein interactions. C2H2 type of ZFPs is the most common and regulates plant growth and development, as well as stress responses (Han et al., 2020). ZINC FINGER OF *ARABIDOPSIS THALIANA* 6 (ZAT6) enhances salt, drought and pathogen resistance by directly activating the expression of stress-responsive *CBFs* and pathogen-related genes (Shi et al., 2014) and regulates the expression of *GSH1*, which encodes γ-glutamyl-Cys synthetase involved in phytochelatins (PC) synthesis, to improve cadmium tolerance (Chen et al., 2016). ZAT10 plays dual roles in regulating salt tolerance; both gain- and loss-of-function enhance salt tolerance (Mittler et al., 2006). ZAT11 participates in oxidative stress-induced programmed cell death and negatively regulates nickel resistance (Liu et al., 2014). ZAT18 plays a positive role in regulating drought resistance (Yin et al., 2017). PeZAT10/STZ1 (SALT TOLERANCE ZINC FINGER1) confers salt tolerance by scavenging ROS through directly regulating the expression of *PeZAT12* and *PeAPX2* (*ASCORBATE PEROXIDASE2*) in populus (*Populus euphratica*) (He et al., 2020). PhZFP1 (C2H2-TYPE ZINC FINGER PROTEIN 1) regulates galactinol accumulation and cold tolerance in *Petunia hybrida* by activating *GALACTINOL SYNTHASE 1-1* (*PhGolS1-1*) expression (Zhang et al., 2022).

Medicago truncatula is a diploid model legume. The biological function of ZPTs in *M. truncatula* remains unknown. In an early investigation, transcripts of two zinc finger protein-encoding genes, *MtZPT2-1* and *MtZPT2-2*, in roots were induced by

111 salinity in two genotypes, Jemalong A17 and R108. The hairy roots in R108
112 overexpressing *MtZPT2-1* or *MtZPT2-2* from Jemalong A17 showed increased growth
113 compared with its WT under salinity conditions, but the hairy roots in Jemalong A17
114 showed no difference from its WT (de Lorenzo et al., 2007). Jemalong A17 has higher
115 salt and drought tolerance than R108 (Luo et al., 2016), while R108 has high
116 transformation efficiency, allowing it to be widely used for genetic studies. In our
117 previous investigation on the response of *M. truncatula* (R108) to salt stress, a greatly
118 reduced *MtZPT2-2* transcript in leaves by salinity was observed (R.S. Huang,
119 unpublished data). The objectives of this study were to investigate the role of
120 MtZPT2-2 from R108 in regulating salt tolerance using overexpressing lines and
121 gene-edited mutants and the underlying mechanisms. The downstream target genes
122 and physiological changes were also documented.

123

124 **RESULTS**

125 **Molecular characterization of *MtZPT2-2***

126 A 753 bp of open reading frame (ORF) of *MtZPT2-2* was cloned from R108
127 (MTR_1g106730). It encodes a peptide of 250 amino acids with two zinc-finger
128 C2H2 domains and ethylene-responsive element binding-factor-associated
129 amphiphilic repression (EAR) domain (GCC box), showing 95% identity with
130 MtZPT2-2 from A17 in amino acid sequence (Figure S1). Thirty-nine *MtZFPs* and
131 thirty *MsZFPs* were obtained through a genome-wide search for *ZFP* genes in the
132 genome database of *M. truncatula* (A17) and *M. sativa* (Xinjiang Daye) by using

133 *Arabidopsis ZFP* genes as query sequences. Phylogenetic analysis showed that
134 MtZPT2-2 is mostly close to AtZAT10 and MsZAT15 (Figure S2).

135 The subcellular localization of MtZPT2-2 was analyzed. Compared to GFP,
136 whose fluorescence was shown in the cytoplasm and nucleus, the fluorescence signal
137 of MtZPT2-2 fused with GFP was shown only in the nucleus and overlapped with that
138 of OsMADS, a nucleus-localized protein marker (Figure 1A), indicating that
139 MtZPT2-2 was localized in the nucleus. The auto-transactivation assay showed that
140 neither MtZPT2-2 infusion with the GAL4 DNA-binding domain nor the negative
141 control (BD) activated the reporter gene (Figure 1B). A dual luciferase reporter
142 experiment was further performed to examine whether MtZPT2-2 has transcription-
143 repressing activity. Compared to the control (35S::GAL4 DB empty vector), relative
144 LUC/REN expression was reduced in the leaf transformed with 35S::GAL4 DB-
145 MtZPT2-2 (Figure 1C to E). The results indicated that MtZPT2-2 functions as a
146 transcriptional repressor.

147 The tissue-specific expression analysis showed that *MtZPT2-2* transcript level
148 was higher in roots than in stems, leaves, flowers, and seeds (Figure 2A). We further
149 generate transgenic *Arabidopsis* expressing *GUS* reporter gene driven by the promoter
150 of *MtZPT2-2* ($P_{MtZPT2-2}$::*GUS*) for examining the spatial expression. GUS activity was
151 observed in the epicotyls, roots, and veins of the seedling and mature leaves (Figure
152 2B). Semithin sections showed that GUS expression was mainly confined to the stele
153 in the mature roots and the phloem and xylem in the stems (Figure 2B). In addition,
154 cellular localization of *MtZPT2-2* in stems and roots of *M. truncatula* was observed

using *in situ* PCR. The *MtZPT2-2* transcript signal was predominantly detected in both phloem and xylem in the stem and roots and was stronger in roots than in the stem (Figure 2C). The results indicated that *MtZPT2-2* was substantially expressed in the conducting tissues.

MtZPT2-2 mediated salt response negatively

The response of *MtZPT2-2* to salinity was examined. *MtZPT2-2* transcript was increased in roots after 2 h of treatment with 125 mM NaCl but decreased after 24 h (Figure S3A). In contrast, the *MtZPT2-2* transcript in leaves was greatly reduced after 2 h of treatment with 125 mM NaCl and maintained at low levels within 24 h (Figure S3B). The results indicated that MtZPT2-2 might have different functions in shoots and roots in response to salinity.

Transgenic *M. truncatula* plants overexpressing *MtZPT2-2* and loss-of-function mutants using CRISPR/Cas9-based editing were generated. Two transgenic lines (OE7, OE12) showing increased *MtZPT2-2* transcript levels and two *MtZPT2-2*-knockout mutants (C1, C2) were selected for further investigations (Figure S4A, S4B). Lower transcript levels were observed in C1 and C2 compared to WT (Figure S4C). In order to verify whether off-target effects occurred in the mutants (lines C1 and C2), six potentially targeted genes were selected for sequencing based on analysis using the online software (http://cbi.hzau.edu.cn/cgi-bin/CRISPR). The results indicated that off-target effects did not occur since the sequences in the mutants were not altered compared with those in WT (Figure S4D).

Seed germination and root length of seedlings in response to salinity were

177 measured. Seed germination rate showed no significant difference among all
178 genotypes of plants on 1/2 strength MS medium and reached almost 100% after 4 d of
179 germination. It was decreased on 1/2 strength MS medium supplemented with NaCl,
180 with higher levels in the knockout mutants and lower levels in *MtZPT2-2*-
181 overexpressing lines compared with WT (Figure 3A, 3B). Root length was decreased
182 in all genotypes after salt treatment. Compared to WT, *MtZPT2-2*-overexpressing
183 lines had shorter root length, whereas knockout mutant lines had longer root length
184 after treatments with 100 mM and 125 mM NaCl (Figure 3C, 3D).

185 The physiological responses and survival rate of four-week-old plants growing in
186 soil were further evaluated after irrigating 200 mM NaCl solution or water as control.
187 The maximum photochemical efficiency of photosystem II (F_v/F_m) is usually used to
188 evaluate abiotic stress tolerance (Dai et al., 2022). F_v/F_m showed no difference among
189 all genotypes of plants under control conditions. It was decreased after 14 d of salt
190 treatment, with lower levels in *MtZPT2-2*-overexpressing lines but higher levels in the
191 knockout mutants compared with WT (Figure 4A, 4B). Compared to a 40% survival
192 rate in WT after salt treatment, a 13% to 28% survival rate was observed in *MtZPT2-*
193 *2*-overexpressing lines and 63% in *MtZPT2-2*-knockout mutants (Figure 4C, 4D). The
194 above results indicated that MtZPT2-2 regulated salt tolerance negatively.

195 **MtZPT2-2 expression altered Na^+/K^+ homeostasis under salinity conditions**

196 Maintenance of Na^+/K^+ homeostasis plays a key role in plant survival under
197 salinity conditions. Na^+ and K^+ concentrations in roots and shoots showed no
198 significant difference among the genotypes under control conditions, while Na^+

concentration was increased and K^+ concentration was decreased in roots and shoots of all plants after salt treatment (Figure 5). Higher levels of Na^+ in *MtZPT2-2*-overexpressing lines but lower levels in *MtZPT2-2*-knockout mutants were observed as compared with WT after salt treatment (Figure 5A, 5B). K^+ levels showed no significant difference in either shoots or roots among all genotypes after salt treatment (Figure 5C, 5D). The results indicated that *MtZPT2-2* expression affected Na^+ homeostasis but not K^+. Salt treatment resulted in an increased Na^+/K^+ ratio in both shoots and roots in all genotypes, with higher levels in *MtZPT2-2*-overexpressing lines and lower levels in *MtZPT2-2*-knockout mutants compared with WT (Figure 5E, 5F).

Analysis of transcripts of the genes involved in ion homeostasis

Although lots of ion transporters are involved in maintenance of ion homeostasis, *MtHKT1;1* (Zhang et al., 2019), *MtHKT1;2* (Zhang et al., 2019), *MtSRLK* (*Salt-induced receptor-kinase*) (de Lorenzo et al., 2009) and *MtSOS1* (Zhang et al., 2022) were reported to participate in maintaining or regulating Na^+/K^+ homeostasis in *M. truncatula* under salinity. *MtHKT;1*, *MtHKT1;2*, *MtSRLK* and *MtSOS1* were selected for analysis of the expression in response to salinity in the present study. *MtHKT1;1* transcript level showed no difference among the genotypes under control condition, while higher levels of *MtHKT1;2* transcript in *MtZPT2-2*-knockout mutants and lower levels in *MtZPT2-2*-overexpressed lines were observed compared with WT. *MtHKT1;1* and *MtHKT1;2* transcripts were induced after salt treatment, but lower levels in *MtZPT2-2*-overexpressing lines and higher levels in *MtZPT2-2*-knockout

mutants were detected as compared with WT (Figure 6A, 6B). *MtSRLK* and *MtSOS1* transcripts were induced in all plants after salt treatment, but they showed no significant difference among the genotypes under either control or salinity conditions (Figure S5). The results indicated that MtZPT2-2 negatively regulated *MtHKT1;1* and *MtHKT1;2* expression.

MtZPT2-2 expression affected Na^+ concentration in xylem sap but not in phloem under salt stress

Given that *MtZPT2-2* was substantially expressed in the xylem and phloem, Na^+ concentration in the xylem and phloem in shoots was analyzed. Na^+ concentration in the xylem saps showed no significant difference among the genotypes under control conditions (Figure 6C). It was greatly increased in all plants after salt treatment; a higher level was observed in *MtZPT2-2*-overexpressing lines and a lower level in *MtZPT2-2*-knockout mutants compared with WT (Figure 6C). Na^+ concentration was not altered in the phloem of all plants under either control or salinity conditions (Figure 6D). The results indicated that MtZPT2-2 disrupted Na^+ translocation in the xylem but not in the phloem.

MtZPT2-2 expression affected ROS homeostasis under salt stress

Reactive oxygen species accumulate and lead to oxidative damage to plants when plants are exposed to salt stress. Trace amounts of $O^{2\cdot-}$ and H_2O_2 were observed in the root tips of all plants under control conditions. Salt stress resulted in the accumulation of $O^{2\cdot-}$ and H_2O_2; lower levels were observed in *MtZPT2-2*-knockout mutants and higher levels in overexpressed lines compared with the WT (Figure S6A,

B). The antioxidant defense system functions to scavenge ROS for the maintenance of ROS homeostasis in plant cells. SOD and CAT activities showed no significant difference among plant genotypes under control conditions. They were increased in all plants after salt treatment, with lower levels in *MtZPT2-2*-overexpressing lines and higher levels in *MtZPT2-2*-knockout mutants than the WT (Figure 7A, 7B). APX activity was higher in *MtZPT2-2*-knockout mutants than in WT and at a lower level in overexpressed lines under both control and salinity conditions (Figure 7C). In addition, salt stress resulted in induced transcripts of *CAT1, cAPX1*, *cAPX2*, *cpAPX1*, *cpAPX2*, *Cu, Zn-SOD1*, *Cu, Zn-SOD2* and *Cu, Zn-SOD3* in all plants, except *Cu, Zn-SOD1* and *Cu, Zn-SOD2*, and lower levels were maintained in *MtZPT2-2*-overexpressing lines and higher levels in *MtZPT2-2*-knockout mutants compared with WT (Figure 7D to 7K). *Cu, Zn-SOD1* transcripts showed no significant difference between genotypes, except that line OE7 had a lower level than other lines under control conditions and line OE12 had a lower level than knockout mutants under salinity (Figure 7I). *Cu, Zn-SOD2* transcript showed no difference among plant genotypes under both control and salinity conditions (Figure 7J). The results indicated that MtZPT2-2 regulated the expression of antioxidant genes and enzyme activities negatively.

MtZPT2-2 interacted with the promoter of *MtHKT1;1* and *MtHKT1;2* but not with that of antioxidant genes

To further understand whether *MtHKT1;1*, *MtHKT1;2*, *MtCAT1*, *MtcAPX1*, *MtcAPX2*, *MtcpAPX1* and *MtcpAPX2* genes were directly regulated by MtZPT2-2, the

plasmids of the promoter of *MtHKT1;1*, *MtHKT1;2*, *MtCAT1*, *MtcAPX1*, *MtcAPX2*, *MtcpAPX1* or *MtcpAPX2*-driven *LUC* (luciferase) were used as reporters, while the plasmid 35S::*MtZPT2-2* was used as effector (Figure S7A) to co-transform *Nicotiana benthamiana* leaves. Fluorescence was detected in the leaves in a dual-luciferase reporter (DLR) assay. The results showed that the *LUC* expression driven by the promoters of *MtHKT1;1* and *MtHKT1;2* was repressed by MtZPT2-2, while the expression driven by the other promoters was not affected by MtZPT2-2 (Figure S7B).

Interaction of MtZPT2-2 with the promoters of *MtHKT1;1* and *MtHKT1;2* was further examined. Transgenic *M. truncatula* plants expressing *MtZPT2-2-Flag* were used for ChIP assay. MtZPT2-2 protein could be detected in the transgenic plants using Western blot analysis (Figure 8A). Several A (G/C) T-$X_{3\sim4}$-A (G/C) T motifs (P1 to P8) were found in the promoters of *MtHKT1;1* and *MtHKT1;2*, while the motifs are bound with AZFs and ZAT10 in *Arabidopsis* (Xie et al., 2019). ChIP-qPCR analysis showed that P1, P2 and P4 regions in the promoter of *MtHKT1;1* and P5, P6 and P7 regions in the promoter of *MtHKT1;2* were enriched in the transgenic plants (Figure 8B, C). Yeast-one-hybridization (Y1H) assay confirmed the interaction of MtZPT2-2 with the promoter of *MtHKT1;1* and *MtHKT1;2* (Figure 8D). The results indicated that MtZPT2-2 could bind to at least three sites in the promoter of *MtHKT1;1* and *MtHKT1;2*, respectively, to repress *MtHKT1;1* and *MtHKT1;2* expression.

DISCUSSION

MtZPT2-2 was a transcription repressor in the regulation of salt tolerance

Zinc finger proteins (ZFPs) are a large family of proteins, divided into nine subfamilies based on the order and number of cysteine and histidine residues (Ciftci-Yilmaz and Mittler, 2008). MtZPT2-2 contains two zinc-finger C2H2 domains and an EAR motif, while C2H2 domains usually exist in the C2H2 type ZFP subfamily (Ohta et al., 2001), indicating that MtZPT2-2 is a member of the C2H2 ZFP family. The EAR motif confers transcription repression via recruitment and action of chromatin modifiers to regulate responses to diverse stresses in *Arabidopsis* negatively (Kagale et al., 2011). Consistently, MtZPT2-2 showed transcriptional inhibitory activity in *Nicotiana benthamiana*, indicating that MtZPT2-2 was a transcription repressor.

ZFPs are involved in salt stress adaptation. *AZF1*, *AZF2* and *ZAT10* transcripts are rapidly up-regulated in *Arabidopsis* under salt stress (Sakamoto et al., 2004). Most ZATs regulate salt tolerance positively in *Arabidopsis* (Shi et al., 2014). *PeSTZ1* confers salt tolerance by scavenging ROS through regulating *PeZAT12* and *PeAPX2* expression in *Populus euphratica* (He et al., 2020). In contrast to an early observation that *MtZPT2-2* transcripts were induced by 12 and 25 folds, respectively, in roots of both salt-sensitive (R108) and salt-tolerant (Jemalong A17) genotypes after 4 d of salt treatment (de Lorenzo et al., 2007), *MtZPT2-2* transcript was greatly and rapidly reduced in shoots of R108 after 2 to 24 h of salt treatment in the present study. The results revealed that MtZPT2-2 might have different functions in shoots and roots in response to salinity. Overexpression of *MtZPT2-2* led to reduced salt tolerance based on assays of germination rate, seedling growth, F_v/F_m and survival rate, while

MtZPT2-2-knockout mutants showed increased salt tolerance. The results indicated that MtZPT2-2 negatively regulates salt tolerance in *M. truncatula*. By contrast, the hairy roots overexpressing *MtZPT2-2* from Jemalong A17 in the sensitive genotype R108 but not in Jemalong A17 showed increased root growth under salt stress compared with WT (de Lorenzo et al., 2007). Unfortunately, the authors did not use transgenic plants to evaluate salt tolerance.

MtZPT2-2 directly repressed *MtHKT1;1* and *MtHKT1;2* expression to reduce Na^+ accumulation in xylem

Salt stress resulted in increased Na^+ but decreased K^+ concentrations in both roots and shoots in *M. truncatula* plants. Higher levels of Na^+ were observed in *MtZPT2-2*-overexpressing lines but lower levels in *MtZPT2-2*-knockout mutants as compared with WT, while K^+ level showed no significant difference in either shoots or roots among the genotypes after salt treatment. The results suggested that *MtZPT2-2* expression altered Na^+ accumulation under salt stress. The unloading (recycling) of Na^+ from the xylem is an important pathway to minimize Na^+ accumulation in shoots and leaves (Ismail and Horie, 2017). This process indeed occurs in several plant species, such as *Arabidopsis*, rice, and wheat, which is mediated by a number of HKTs (Horie et al., 2009). In consistence, *MtHKT1;1* and *MtHKT1;2* transcripts were induced after salt treatment, but lower levels were maintained in *MtZPT2-2*-overexpressing lines and higher levels in *MtZPT2-2*-knockout mutants as compared with WT, suggesting that the altered Na^+ distribution was associated with *MtHKT1;1* and *MtHKT1;2* transcripts. Similarly, *MtHKT1;1* and *MtHKT1;2* transcripts are

negatively regulated by MtCML40, which plays an important role in unloading Na^+ from the xylem (Zhang et al., 2019). MtSRLK is a salt-induced receptor-kinase; *srlk* mutant showed longer roots, lower Na^+ content in roots and leaves and lower transcription levels of *MtZPT2-1* and *MtZPT2-2* under salt stress (de Lorenzo et al., 2009). MtSOS1 functions to extrude Na^+ through the plasma membrane (Zhang et al., 2022). *MtSRLK* and *MtSOS1* transcripts showed no significant difference among the genotypes, although they were induced under salt stress. The results indicated that *MtSRLK* and *MtSOS1* expression was not involved in the altered salt tolerance regulated by *MtZPT2-2*.

The unloading of Na^+ from xylem vessels to xylem parenchyma cells is controlled by AtHKT1;1, which is localized at the plasma membrane of xylem parenchyma cells in shoots in *Arabidopsis* (Sunarpi et al., 2005). *OsHKT1;5* is expressed in the xylem in roots and leaf sheath and confers unloading of xylem Na^+ for reduced Na^+ accumulation in shoots (Ren et al., 2005). *HvHKT1;5* is expressed in xylem parenchyma and pericycle cells adjacent to xylem vessels for involvement in Na^+ translocation from roots to shoots and negatively regulates salt tolerance in barley (*Hordeum vulgare*) (Huang et al., 2020). Consistent with these results, *MtZPT2-2* was highly expressed in the xylem and phloem of roots and stems based on GUS staining and *in situ* hybridization assays. *MtZPT2-2* expression affected Na^+ concentration in xylem sap but not in phloem under salt stress. The binding of MtZPT2-2 with three regions in the promoters of *MtHKT1;1* and *MtHKT1;2* was documented based on Y1H assay, DLR assay and ChIP-qPCR, indicating that MtZPT2-2 regulated

MtHKT1;1 and *MtHKT1;2* expression directly. Several regulators of *HKT1* expression have been reported. ABI4 and CAMTA6 bind to the promoter of *HKT1* (Shkolnik-Inbar et al., 2013; Shkolnik et al., 2019) in *Arabidopsis*. OsbZIP72 and OsMYBc are direct regulators of *OsHKT1;1* in rice (Wang et al., 2021; Xiao et al., 2022). Our results suggested that MtZPT2-2-repressed *MtHKT1;1* and *MtHKT1;2* expression was associated with reducedNa^+ accumulation in the xylem (Figure 9).

MtZPT2-2 affected the antioxidant enzyme defense system under salt stress

Reactive oxygen species accumulate in plants after salt treatment, causing membrane lipid peroxidation and oxidative damage to plants. The antioxidant defense system functions to scavenge the accumulated ROS to maintain ROS homeostasis (Miller et al., 2010). ROS was accumulated in all genotypes of plants after salt stress, with accordingly increased activities of SOD, CAT and APX. Lower activities of SOD, CAT and APX and more accumulated ROS were observed in *MtZPT2-2*-overexpressing lines, and higher activities and less accumulated ROS were in the mutants. The results suggest that the altered salt tolerance was associated with the antioxidant defense system. Moreover, transcripts of the antioxidant enzyme encoding genes were accordingly altered with enzyme activities in response to salt stress. Transcripts of *CAT1*, *cAPX1*, *cAPX2*, *cpAPX1*, *cpAPX2*, *Cu, Zn-SOD1*, *Cu, Zn-SOD2* and *Cu, Zn-SOD3* were induced in all plants, indicating that they were responsive to salt stress. Lower transcript levels of *CAT1*, *cAPX1*, *cAPX2*, *cpAPX1*, *cpAPX2*, and *Cu, Zn-SOD3* were maintained in *MtZPT2-2*-overexpressing lines, and higher levels in *MtZPT2-2*-knockout mutants, indicating that they were associated with *MtZPT2-2*

expression. The transcript levels of *Cu, Zn-SOD1* and *Cu, Zn-SOD2* were not consistent with the differential SOD activity among genotypes, indicating that the alterations were not associated with *MtZPT2-2* expression. However, MtZPT2-2 showed no interaction with the promoters of *CAT1*, *cAPX1*, *cAPX2*, *cpAPX1* and *cpAPX2*, indicating that MtZPT2-2 regulated the antioxidant genes indirectly (Figure 9). Similarly, *ZAT10* regulates *APX1* and *APX2* expression in *Arabidopsis* (Sakamoto et al., 2004); PeZAT10 confers salt tolerance by regulating the expression of *PeAPX2* in *Populus euphratica* (He et al., 2020).

CONCLUSIONS

A transcription repressor MtZPT2-2 and its negative regulation on salt tolerance in *M. truncatula* were documented. *MtZPT2-2* transcript was greatly reduced in shoots after salt stress, and its down-regulation resulted in increased salt tolerance, which was associated with its direct regulation on *MtHKT1;1* and *MtHKT1;2* expression for increasing xylem Na^+ unloading in shoots and indirect regulation on antioxidant defense system for maintenance of ROS homeostasis under salt stress (Figure 9). The results implied that the homolog of *MtZPT2-2* in crops should be a potential target for improved salt tolerance using genome editing.

MATERIALS AND METHODS

Plant material and culture conditions

The germinated seeds of *Medicago truncatula* cv. R108 were grown in plastic pots filled with a mixture of peat and perlite (3:1, v/v) in a greenhouse at about 25°C under natural light. Roots, stems and leaves were sampled from six-week-old plants,

while flowers and seeds were sampled during flowering and seed maturing stages for analysis of spatial expression of *MtZPT2-2*. For salt stress treatment, two-week-old seedlings were placed in 1/2 Hoagland solution (pH 6.5) for two weeks of cultivation and transferred into 1/2 Hoagland solution containing 125 mM NaCl before isolating total RNA from leaves. Seedlings of *Nicotiana benthamiana* and *Arabidopsis* (*Arabidopsis thaliana*) were planted in plastic pots filled with a mixture of peat and perlite (3:1, v/v) and grown in a growth chamber at 22°C with 16 h of light for three to four weeks.

Cloning and sequence analysis of *MtZPT2-2*

Total RNA was isolated from leaves of *M. truncatula* cv. R108 using the RNAprep pure Plant Kit (Tiangen Inc., Beijing, China) according to the manufacturer's manual. One μg of total RNA was used for cDNA synthesis using HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). The cDNA was used as template for amplification of ORF of *MtZPT2-2* by PCR using Proofast Super-Fidelity DNA polymerase (ATG Biotech, Nanjing, China), using primers (forward: 5'-CACGGGGGACTCTTGACCATGGCTTTAGAATTACAAACTC-3'; reverse: 5'-CGGGGAAATTCGAGCTGGTCACCCTACACCGTTTCATCATTGTC T-3'). The sequence of MtZPT2-2 protein was analyzed using the software DNAMAN (https://www.lynnon.com/) and SMART (http://smart.embl-heidelberg.de/). The phylogenetic tree was constructed using the software MEGA7 (http://www.megasoftw are.net/). The multiple alignments of MtZFPs proteins were performed using ClustalX with default parameters. Phylogenetic analysis of C1-2i

subclass of C2H2-type *ZFPs* in *M. truncatula*, *M. sativa* and *Arabidopsis* was performed using MEGA X with the maximum-likelihood (ML) method 1000 bootstrap replicates.

Reverse transcription quantitative PCR (RT-qPCR) analysis

Reverse transcription quantitative PCR (RT-qPCR) was performed using the diluted cDNA as templates and TSINGKE TSE202 2×T5 Fast qPCR Mix (TSINGKE, Beijing, China) and performed in Thermal Cycler Dice™ Real Time System Software (Takara, Otsu, Japan) following the manufacturer's instructions. The primers were designed using the online software PrimerQuest Tool (https://sg.idtdna.com/PrimerQuest/Home/Index) and listed in Table S1. A negative control without the cDNA template was always included, and a parallel reaction to amplify *ACTIN* was used to normalize the amount of template. Relative expression was calculated by $2^{-\Delta\Delta Ct}$ based on the data from three biological repeats.

Subcellular localization of MtZPT2-2 protein

The *MtZPT2-2* was fused with *EGFP* at the 5′-end in a pCAMBIA1305 vector driven by the CaMV 35S promoter. The plasmids of pCAMBIA1305-MtZPT2-2-EGFP and pCAMBIA1305-OsMADs-mCherry, a plasma membrane-localized marker, were co-transformed into leaves of four-week-old *Nicotiana benthamiana* using *Agrobacterium* (EHA105)–mediated method. Fluorescence was observed using a confocal laser scanning microscope (Carl Zeiss SAS, Jena, Germany) after 2 d of incubation at 25°C. At least three *Nicotiana benthamiana* leaves were transformed for subcellular localization analysis. The primers are listed in Table S4. The experimental

setup used for the fluorescence microscopy work was as follows: GFP: lasers 488 nm, intensity 0.25%, collection bandwidth 400 to 602 nm, and gains 693V; mCherry: lasers 587 nm, intensity 1.7%, collection bandwidth 602 to 650 nm, and gains 713V.

Generation of transgenic plants

The knockout mutants *mtzpt2-2* (C1 and C2) with R108 background were generated using the method of CRISPR/Cas9-based editing containing a plant expression vector pHSE401 and an intermediate vector pCBC-DT1T2 (Xing et al., 2014). The*MtZPT2-2* coding sequence driven by the CaMV 35S promoter was cloned to pCAMBIA1307 to construct an overexpressing vector (35S::*MtZPT2-2-Flag*), while the 35S promoter in pCAMBIA3301 was replaced by a 1857-bp promoter sequence of *MtZPT2-2* ($P_{MtZPT2-2}$) to construct a spatial expression vector ($P_{MtZPT2-2}$::*GUS*). Mature leaves of *M. truncatula* cv. R108 were transformed by *Agrobacterium* strain EHA105 harboring the *MtZPT2-2-pCBC-DT1T2* or 35S::*MtZPT2-2-Flag*. In addition, the vector $P_{MtZPT2-2}$::*GUS* was transformed into *Arabidopsis* (Col-0) by the method of floral dip. Transgenic plants were obtained based on selection of hygromycin B-resistance (for *MtZPT2-2-pCBC-DT1T2* and 35S::*MtZPT2-2-Flag*) or Basta-resistance (for $P_{MtZPT2-2}$::*GUS*). Homozygous lines were obtained by checking the DNA sequence in the editing sites through PCR using the primers MtZPT2-2-F (5'-ATCACTTCACTTCACTTCCCTA-3') and MtZPT2-2-R (5'-ACCAAGTGCTTGATAAGATGGA-3'). The homozygous *MtZPT2-2*-overexpressing lines and $P_{MtZPT2-2}$::*GUS* lines were selected based on hygromycin B-resistance at T1 and T2 generations. The primers are listed in Table S4.

GUS staining

The seedlings were incubated in GUS staining solution containing 50 mM sodium phosphate buffer (pH 7.2), 2 mM X-Gluc, 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6$, 0.1% (v/v) Triton X-100, and 10 mM EDTA for 6 h at 37°C. For semithin section analysis, root elongation regions of one-week-old seedlings and stems of one-month-old seedlings were incubated in GUS staining solution. The stained samples were dehydrated using an ethanol series and then embedded with the Technovit® 7100 resin (Cat no.14653, Heraeus Kulzer, Wehrheim, Germany). The stained sections (2 μm) were obtained using a Leica microtome (UC7, Leica Biosystems, Wetzlar, Germany) and imaged using a microscope (BX53, Olympus, Tokyo, Japan) with CCD (Charge-coupled Device). Three independent experiments were conducted as replications.

In situ PCR assay

The probe for in situ PCR of *MtZPT2-2* was synthesized by Wuhan servicebio technology (Wuhan, China). The *MtZPT2-2* probe sequence (5'-UUGUUUGAAACGUGGGAAUGGAAUAGUA -3') was labeled with digoxigenin. Fixation/dehydration/paraffin embedding in tissue preparation was performed according to Long's protocol (http://www.its.caltech.edu/~plantlab/protocols/in situ.pdf). In short, the elongation area of the roots and the second stems from four-weeks-old seedlings of *M. truncatula* were immersed in fixative containing 63% (v/v) ethanol, 5% (v/v) acetic acid, and 2% (v/v) formaldehyde for 12 h. After that, the samples were embedded into 5% (w/v) agarose and then sectioned to 50 mm. Samples

were stained using NBT/BCIP chromogenic reagent for 2 h. After staining, the sections were washed and mounted in 40% (v/v) glycerol and then observed under a microscope (model no. DM2500M, Leica, Weztlar, Germany). Three independent experiments were conducted as replications.

Dual-luciferase reporter (DLR) assay

A 2000 bp promoter fragment of *MtHKT1;1*, *MtHKT1;2*, *MtCAT1*, *MtcAPX1*, *MtcAPX2*, *MtcpAPX1* and *MtcpAPX2* was cloned into pGreenII0800-LUC vector to generate a reporter, and the CDSs of *MtZPT2-2* were individually PCR amplified and cloned into the 62-SK vector that contained the 35S promoter as effectors. The effector plasmid and one reporter plasmid were co-transformed into *Nicotiana benthamiana* leaves. Firefly luciferase (LUC) and Renilla luciferase (REN) activities were measured using a Dual Luciferase Reporter Gene Assay Kit (Beyotime, Shanghai, China) in a microplate reader (Infinite 200 Pro; Tecan, Männedorf, Switzerland). The relative LUC activity was normalized to that of REN. At least eight leaves were used for DLR analysis as replications. The primers are listed in Table S4.

Western blot analysis

Total proteins were extracted from leaves of four-week-old seedlings using RIPA lysis buffer (Beyotime, Shanghai, China) containing 1% (v/v) Triton X-100, 1% (w/v) deoxycholate, and 0.1% (w/v) SDS. For Western blot, 7.5 μg of total protein in each sample was separated on 10% SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes (Immobilon-P, Millipore Corporation, Bedford, MA, USA). The membranes were blocked for 1 h in TBST buffer containing 20 mM Tris-HCl (pH

7.5), 100 mM NaCl, and 0.05% (v/v) Tween 20 supplemented with 5% (m/v) no-fat dried milk (Beyotime, Shanghai, China) at room temperature. The membranes were then incubated with the primary antibody (1:1000), followed by cleaning with TBST buffer solution and incubation with horseradish peroxidase-conjugated goat anti-mouse antibody (1:2000). The membranes were visualized by using an enhanced chemiluminescence kit (SB-WB001, Share-bio, Shanghai, China). The antibodies used in western blotting included anti-Flag antibody (A8592, Mouse mAb, Sigma, USA), anti-Actin antibody (CW0264, Mouse mAb, CWBIO, Taizhou, China), and goat anti-mouse lgG-HRP (#91196, Cell Signaling Technology, Boston, USA). Three independent experiments were conducted as replications.

ChIP-qPCR assay

Leaves from four-week-old 35S::*MtZPT2-2-Flag* seedlings growing at 22°C in 1/2 Hoagland solution were cross-linked twice in 1% (v/v) formaldehyde diluted with Buffer I (0.4 M sucrose, 10 mM Tris–HCl pH 8, 1 mM PMSF, 1 mM EDTA and 1% (v/v) formaldehyde) for 15 min each under vacuum, followed by stopping by addition of 0.125 M glycine with vacuum for 5 min. Chromatin was isolated and sheared as described by Saleh et al. (2008). The anti-Flag monoclonal antibodies (A8592, Mouse mAb, Sigma, USA) were used to immunoprecipitate the target proteins (MtZPT2-2-Flag). The immunoprecipitated DNA fragments were dissolved in sterilized water and stored at -80°C before use. qPCR was performed to identify the enriched DNA fragments in the IPs compared to Inputs. Input normalized *MtHKT1;1* and *MtHKT1;2* ChIP fractions were then adjusted for the normalized negative control (IgG), giving

529 the ΔΔCt value (Walley et al., 2008). qPCR reactions were performed three times for
530 each sample, and the expression levels were normalized to the input sample for
531 enrichment detection. The fold enrichment was calculated against the IgG reference
532 by $2^{-\Delta\Delta Ct}$ (Walley et al., 2008). Information about the primers is listed in Table S2.

533 **Yeast one-hybrid (Y1H) assay**

534 The ORF of *MtZPT2-2* was cloned to the vector pGADT7, while the promoter
535 sequence (2000 bp) of *MtHKT1;1* and *MtHKT1;2* was cloned to the vector pHis2,
536 respectively, using Seamless Cloning Master Mix (Sangon Biotech, Shanghai, China).
537 The plasmids (AD-MtZPT2-2 and pHis-*MtHKT1;1* or pHis-*MtHKT1;2*) and the
538 control one (AD-GUS and pHis-*MtHKT1;1* or pHis-*MtHKT1;2*) were co-transformed
539 into yeast strain Y187. The transformants were grown on SD/-Trp-Leu medium at
540 30°C for 3 d, and the plump single clones were simultaneously transferred on SD/-
541 Trp-His-Leu medium with or without 3-amino-1,2,4-triazole (AT) and incubated at
542 30°C for 5 to 7 d. Three independent experiments were conducted as replications. The
543 primers are listed in Table S4.

544 **Evaluation of salt tolerance**

545 The sterilized seeds were placed on 1/2 strength MS medium containing 0, 100,
546 and 125 mM NaCl. Germinated seeds were recorded for calculating germination rates
547 after 4 d of incubation at 22℃. For determination of root length, the germinated seeds
548 on 1/2 strength MS medium were transferred onto new medium containing 0, 100 or
549 125 mM NaCl to allow seedling growth for 10 d vertically in a growth chamber at
550 22℃. Each experiment contained five discs as replications. Six-week-old plants

551 growing in plastic pots in a greenhouse under natural light were irrigated with 200 mL
552 of 200 mM NaCl solution per pot for salt stress treatment. Maximum photochemical
553 efficiency of photosystem II (F_v/F_m) was determined 7 d after treatment, while the
554 surviving plants were counted after 7 d of recovery by rewatering post 14 d of salt
555 treatment to distinguish the surviving and dead plants for calculating the survival rate,
556 as previously described (Dai et al., 2022). Each experiment contained three pots of
557 plants as technical replicates, and three independent experiments were conducted.

558 **Determinations of Na^+ and K^+**

559 Four-week-old *M. truncatula* seedlings were transferred to 1/2 Hoagland solution
560 containing 125 mM NaCl for 7 d of salt treatment. The shoots and roots were
561 harvested and washed with deionized water, followed by drying in an oven. The dried
562 samples (0.05 g) were powdered and decomposed in 2 mL 65% (v/v) nitric acid for 45
563 min at 160 ℃ in a microwave (ETHOS ONE, Milestone, Italy). The extraction was
564 used for the measurement of Na^+ and K^+ using Inductively Coupled Plasma Optical
565 Emission Spectrometry (ICP-OES, Optima 8000; PerkinElmer, USA) as described
566 previously (Dai et al. 2022). For the determination of Na^+ in the xylem, the first drop
567 of exudate was removed with absorbent paper at a distance of 1-2 cm from the roots.
568 A 0.5 mL centrifuge tube containing absorbent cotton was placed upside down at the
569 cut of the four-week-old *M. truncatula* seedling and collected in the incubator at
570 100% humidity for 2 h. The adsorption cotton was placed into the plasmid extraction
571 and purification columns with the adsorption membrane removed, repeated by 50
572 plants, centrifuged at 12000 g for 1 min, and the liquid in the collection tubes was

xylem sap (Li et al., 2009). For the determination of Na^+ in the phloem, four mature leaves were separated from four-week-old *M. truncatula* clover at the base of the petiole. The petiole was re-cut at 15 mM EDTA-K_2 (pH 7.5). Four leaves collected from one plant were placed in a 2 mL centrifuge tube, the leaf stalks were immersed in 1 mL 15 mM EDTA-K_2 (pH 7.5) and collected in an incubator at 100% humidity for 8 h, repeated by 50 plants (Berthomieu et al., 2003; Corbesier et al., 2003). The Na^+ concentration in the xylem sap and phloem exudate were measured using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Optima 8000; PerkinElmer, USA). Each assay was repeated using three independent samples.

Measurement of antioxidant enzyme activity

Fresh roots (0.2 g) were ground in a mortar with a pestle in 3 ml of ice-cold 50 mM phosphate buffer (pH 7.8) for extraction of superoxide dismutase (SOD) and catalase (CAT) or in 3 ml of ice-cold 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM AsA for extraction of ascorbate-peroxidase (APX). Enzyme activities were measured and calculated as described previously (Geng et al., 2021). The measurements were conducted using three independent root samples as replications.

DAB and NBT staining

The root detached from one-month-old plants were immersed in 3, 3-diaminobenzidine (DAB) solution (1 mg mL^{-1}) for 1 h to detect H_2O_2 or in nitroblue tetrazolium (NBT) solution (1 mg mL^{-1}) for 12 h in the dark to detect $O^{2\cdot-}$ (Dai et al., 2022). Three independent experiments were conducted.

Statistical analysis

All data were subjected to analysis of variance according to the model for completely randomized design via SPSS program (SPSS Inc., Chicago, USA). Significant differences were calculated based on Student's t-test or Duncan's new multiple range test (MRT) ($r < 0.05$).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers listed in Supplemental Table S5.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflict of Interest

No conflict of interest is declared.

Author contributions

RH, SJ and MD performed the experiments. RH, HS and HZ analyzed the experimental data. RH and ZG wrote the manuscript. ZG conceived the research and designed the experiments.

Figure Legends

Figure 1 Analysis of subcellular localization and transcriptional activity of MtZPT2-2 protein.

The vectors *MtZPT2-2*::GFP or GFP in combination with mCherry-tagged *OsMADS* were co-transformed into *N. benthamiana* leaves for analysis of subcellular localization (A). Transcriptional activation of full-length MtZPT2-2 in yeast cells was detected, using BD as negative control (B). By using the effector control, effector and reporter vectors (C), a dual luciferase reporter experiment was conducted to observe the fluorescence, bar =1 cm (D). The luciferase intensity was quantified. All data are given as means ± SE (n = 8); the same letter above the column indicates no significant difference at $P < 0.05$ using Student's t-test (E).

Figure 2 Analysis of *MtZPT2-2* tissue-specific expression.

Spatial expression of *MtZPT2-2* in *M. truncatula* was analyzed using qPCR, and total RNA was isolated from roots, stems and leaves in two-month-old seedlings and from

662 flowers and seeds in mature plants (A). Seedlings at cotyledon (I) and rosette stages
663 (II), stem (III), young siliques (IV), root (V) and stem cross section (VI, VII, VIII) in
664 $P_{MtZPT2\text{-}2}$::GUS transgenic *Arabidopsis* were used for GUS staining (B). Stem and root
665 sections were used for *in situ* PCR of *MtZPT2-2* (C). Means of three replicates and
666 standard errors are presented; the same letter above the column indicates no
667 significant difference at $P < 0.05$ using Duncan's test. The letters including C, E, C,
668 N, S, Ph, and Xy labeled in each photo indicates cortex, epider mis, endodermis, stele,
669 phloem, ad xylem, respectively.

670 **Figure 3 MtZPT2-2 regulated salt tolerance of *M. truncatula* in plate assay.**

671 Germination rate was measured at 4 d after seed germination in *MtZPT2-2*
672 overexpressing (OE7, OE12) and knockout lines (C1, C2) in comparison with the
673 wild type (WT) on 1/2 MS medium supplemented with NaCl or without NaCl as
674 control. Bar = 1 cm (A, B). Ten seeds per line were placed on each plate with five
675 replications. The uniform germinated seeds on 1/2 MS medium were transferred to
676 fresh medium containing NaCl or without NaCl as control to allow 12 d of growing
677 (C), followed by measurement of root length. Bar = 1 cm (D). Three seedlings per line
678 in each plate were measured with five replications. All data are given as means ± SE
679 (n = 5); the same letter above the column indicates no significant difference at $P <$
680 0.05 using Duncan's test.

681 **Figure 4 MtZPT2-2 regulated salt tolerance of *M. truncatula* in soil assay.**

682 Forty-five-day-old plants of *MtZPT2-2* overexpressing (OE7, OE12) and knockout
683 lines (C1, C2) in comparison with the wild type (WT) were irrigated with 200 mM

NaCl solution as salinity treatment. The chlorophyll fluorescence images (A) and the value (B) of the maximum photochemical efficiency of photosystem II (F_v/F_m) in leaves were recorded after 14 d of treatment; the images in panel A were digitally extracted for comparison. Bar = 1 cm. Surviving plants were counted for calculating survival rate after 7 d of recovery by re-watering. Bar = 1 cm (C, D). All data are given as means ± SE (n = 3); the same letter above the column indicates no significant difference at $P < 0.05$ using Duncan's test.

Figure 5 Na^+/K^+ homeostasis was affected by MtZPT2-2.

Four-week-old seedlings were treated for 7 d in 1/2 Hoagland solution containing 125 mM NaCl or without NaCl as control. Concentrations of Na^+ in shoots (A) and roots (B) and K^+ in shoots (C) and roots (D) were measured respectively, and Na^+/K^+ ratio in shoots (E) and roots (F) were calculated. All data are given as means ± SE (n = 3); the same letter above the column indicates no significant difference at $P < 0.05$ using Duncan's test.

Figure 6 Analysis of transcript levels of *MtHKT1;1* and *MtHKT1;2* and Na^+ content in xylem and phloem sap in response to salinity.

Transcript levels of *MtHKT1;1* (A), *MtHKT1;2* (B) in roots of four-week-old *M. truncatula* seedlings were analyzed after 6 h of treatment with 125 mM NaCl. Na^+ concentration in xylem sap (C) and phloem sap in stems or petiole of four-week-old *M. truncatula* seedlings measured after 7 d after treatment with 125 mM NaCl. All data are given as means ± SE (n = 3); the same letter above the column indicates no significant difference at $P < 0.05$ using Duncan's test.

Figure 7 Antioxidant enzyme activities and transcript levels of the antioxidant enzyme encoding genes in response to salinity.

SOD (A), CAT (B) and APX activities (C) in roots of *MtZPT2-2* overexpression (OE7, OE12) and knockout lines (C1, C2) in comparison with the WT were analyzed after 7 d of treatment with 125 mM NaCl. Transcript levels of *CAT1* (D), *cAPX1* (E), *cAPX2* (F), *cpAPX1* (G), *cpAPX2* (H), *Cu, Zu-SOD1* (I), *Cu, Zu-SOD2* (J) and *Cu, Zu-SOD3* (K) in roots of four-week-old *M. truncatula* seedlings were analyzed after 6 h of treatment with 125 mM NaCl. All data are given as means ± SE (n = 3); the same letter above the column indicates no significant difference at $P < 0.05$ using Duncan's test.

Figure 8 MtZPT2-2 transcriptionally represses *MtHKT1;1* and *MtHKT1;2* in *M. truncatula* and yeast cells.

(A) Western blot identification of OE7, OE9 and OE12 plant in which a Flag tag was infusion with C terminal of MtZPT2-2 using Flag antibody and Actin antibody. (B) Schematic diagram of the *MtHKT1;1* and *MtHKT1;2* promoter, showing the location of the A (G/C) T-$X_{3\sim4}$-A (G/C) T motifs (P1 to P8) in the promoters of *MtHKT1;1* and *MtHKT1;2* where was detected by qPCR in ChIP experiment. (C) ChIP-qPCR for *MtHKT1;1* and *MtHKT1;2*. (D) MtZPT2-2 binds to the promoter of *MtHKT1;1* and *MtHKT1;2* in the Y1H assay. The combination of AD-GUS + pHis-*MtHKT1;1* or pHis-*MtHKT1;2* served as the control group in this experiment. SD/-T/L: SD/-Trp-Leu. SD/-T/L/H: SD/-Trp-Leu-His. All data are given as means ± SE (n = 3); the double asterisks above the column indicate no significant difference at $P < 0.01$ using

Student's t-test.

Figure 9 A proposed model of MtZPT2-2 regulation of salt tolerance in *M. truncatula*.

MtZPT2-2 represses *MtHKT1;1* and *MtHKT1;2* transcripts by binding to the promoter directly for reduced xylem Na^+ unloading. Downregulated expression of *MtZPT2-2* led to increased expression of *MtHKT1;1* and *MtHKT1;2* for reduced Na^+ in the xylem sap and shoots under salt stress. In addition, downregulated expression of *MtZPT2-2* induced expression of antioxidant enzynme genes for maintenance of ROS homeostasis.

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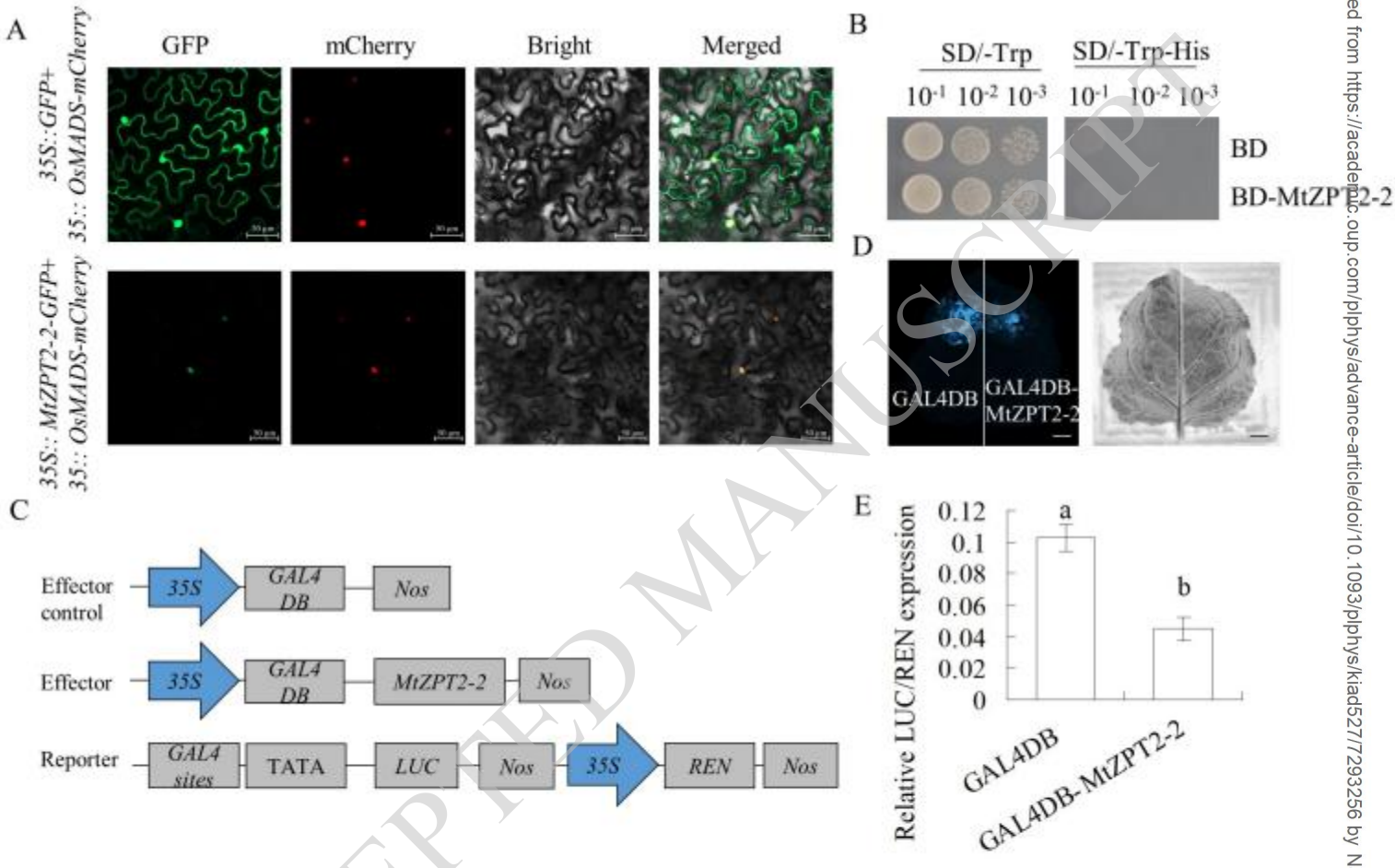

Figure 1 Analysis of subcellular localization and transcriptional activity of MtZPT2-2 protein.
The vectors *MtZPT2-2*::GFP or GFP in combination with mCherry-tagged *OsMADS* were co-transformed into *N. benthamiana* leaves for analysis of subcellular localization (A). Transcriptional activation of full-length MtZPT2-2 in yeast cells was detected, using BD as negative control (B). By using the effector control, effector and reporter vectors (C), a dual luciferase reporter experiment was conducted to observe the fluorescence, bar = 1 cm (D). The luciferase intensity was quantified. All data are given as means ± SE (n = 8); the same letter above the column indicates no significant difference at $P < 0.05$ using Student's t-test (E).

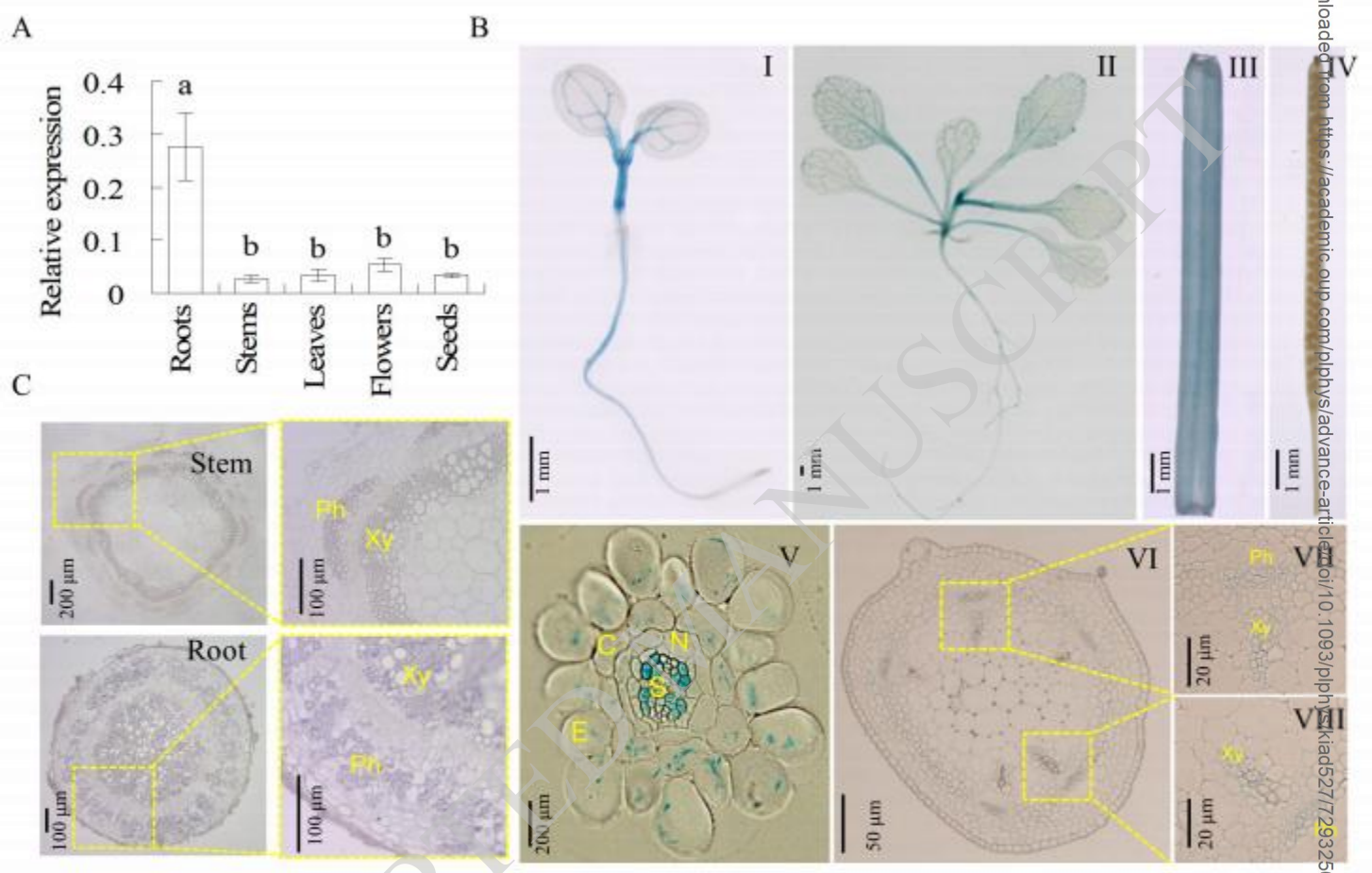

Figure 2 Analysis of *MtZPT2-2* tissue-specific expression.

Spatial expression of *MtZPT2-2* in *M. truncatula* was analyzed using qPCR, and total RNA was isolated from roots, stems and leaves in two-month-old seedlings and from flowers and seeds in mature plants (A). Seedlings at cotyledon (I) and rosette stages (II), stem (III), young siliques (IV), root (V) and stem cross section (VI, VII, VIII) in $P_{MtZPT2\text{-}2}$::GUS transgenic *Arabidopsis* were used for GUS staining (B). Stem and root sections were used for *in situ* PCR of *MtZPT2-2* (C). Means of three replicates and standard errors are presented; the same letter above the column indicates no significant difference at $P < 0.05$ using Duncan's test. The letters including C, E, C, N, S, Ph, and Xy labeled in each photo indicates cortex, epidermis, endodermis, stele, phloem, ad xylem, respectively.

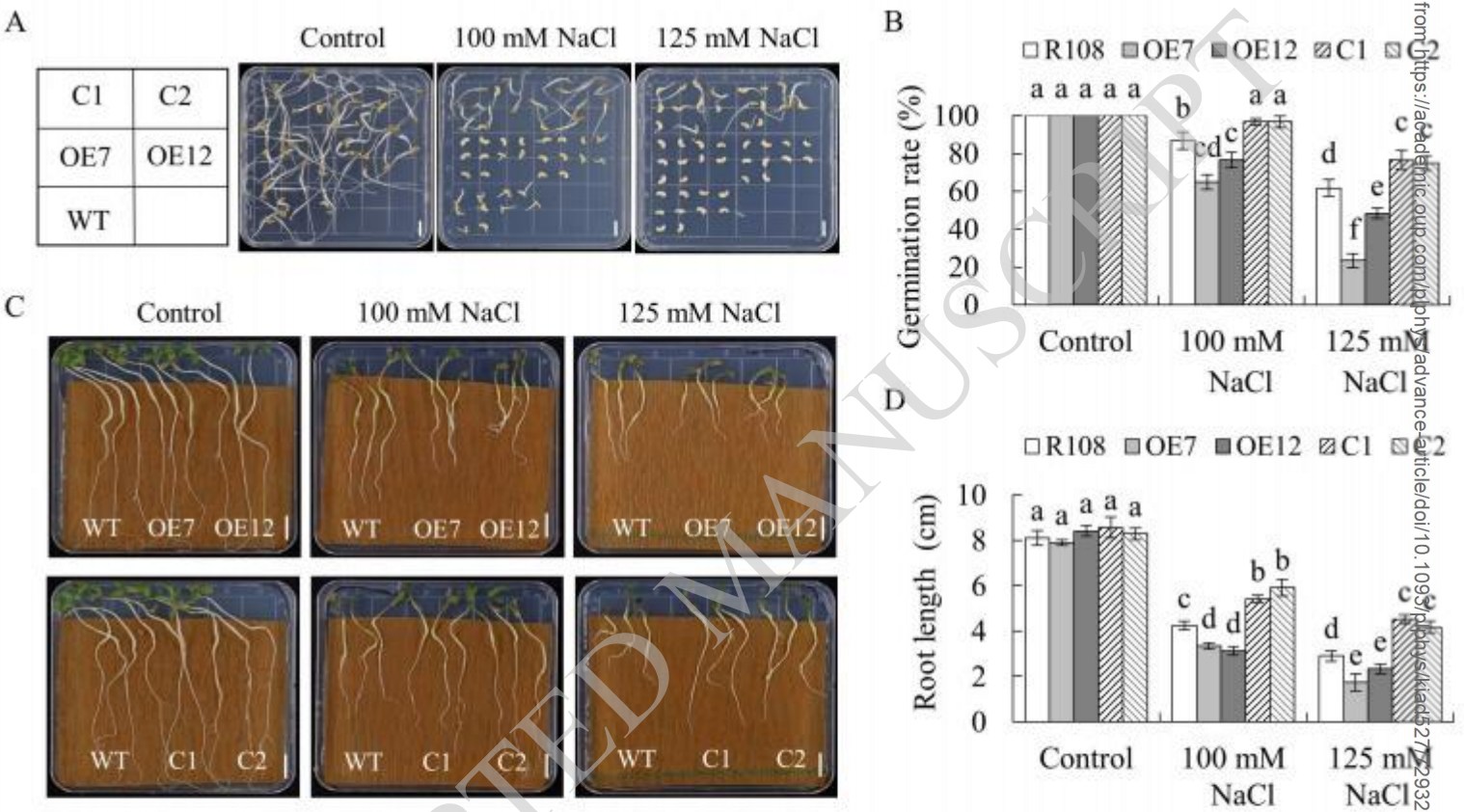

Figure 3 MtZPT2-2 regulated salt tolerance of *M. truncatula* in plate assay.
Germination rate was measured at 4 d after seed germination in *MtZPT2-2* overexpressing (OE7, OE12) and knockout lines (C1, C2) in comparison with the WT on the 1/2 MS medium supplemented with NaCl or without NaCl as control. Bar=1 cm (A, B). Ten seeds per line were placed on each plate with five replications. The uniform germinated seeds on 1/2 MS medium were transferred to fresh medium containing NaCl or without NaCl as control to allow 12 d of growing (C), followed by measurement of root length. Bar = 1 cm (D). Three seedlings per line on each plate were measured with five replications. All data are given as means ± SE (n = 5); the same letter above the column indicates no significant difference at $P < 0.05$ using Duncan's test.

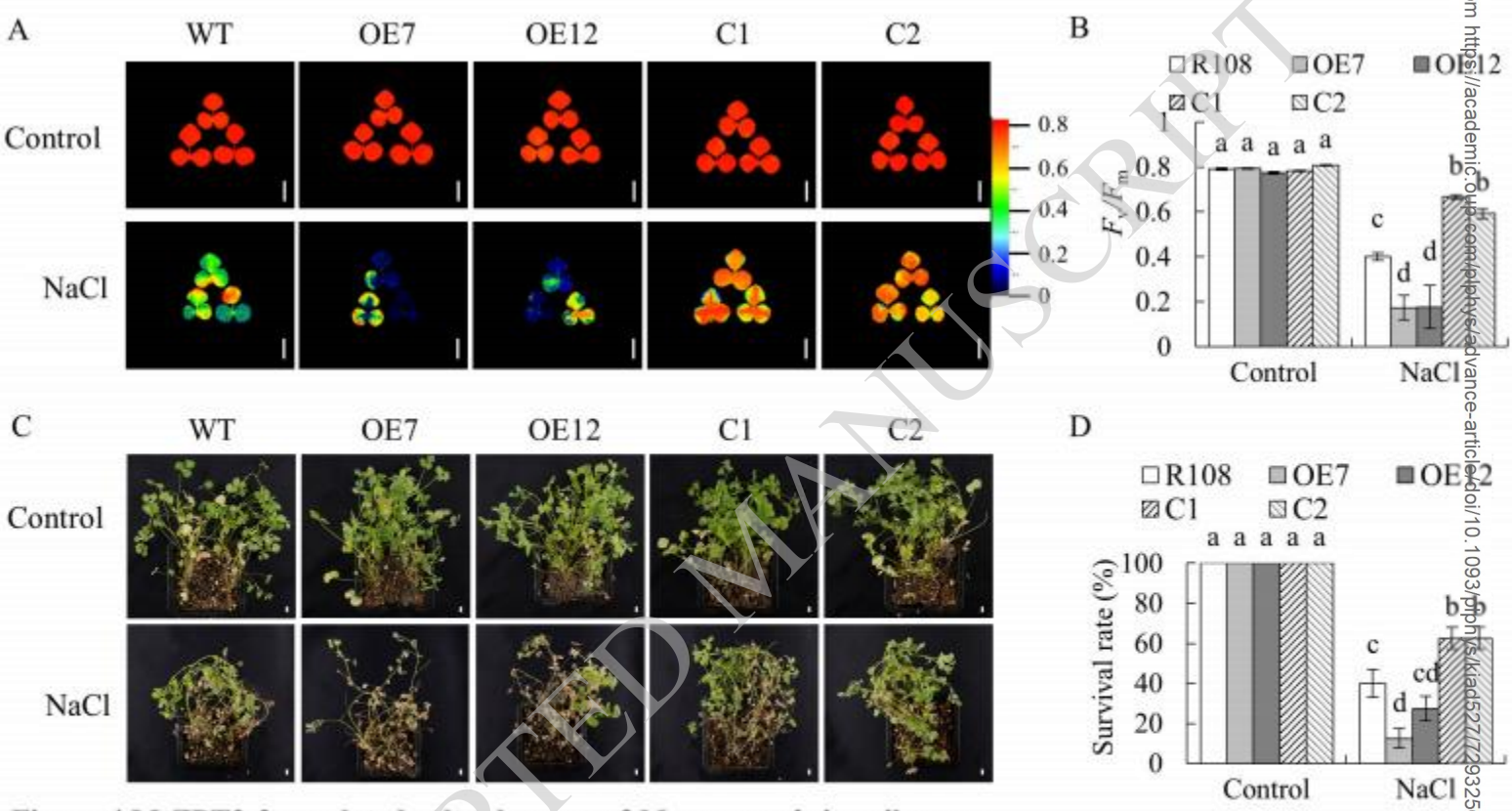

Figure 4 MtZPT2-2 regulated salt tolerance of *M. truncatula* in soil assay.

Forty-five-day-old plants of *MtZPT2-2* overexpressing (OE7, OE12) and knockout lines (C1, C2) in comparison with the WT were irrigated with 200 mM NaCl solution as salinity treatment. The chlorophyll fluorescence images (A) and the value (B) of the maximum photochemical efficiency of photosystem II (F_v/F_m) in leaves were recorded after 14 d of treatment; the images in panel A were digitally extracted for comparison. Bar = 1 cm. Surviving plants were counted for calculating survival rate after 7 d of recovery by re-watering. Bar = 1 cm (C, D). All data are given as means ± SE (n = 3); the same letter above the column indicates no significant difference at $P < 0.05$ using Duncan's test.

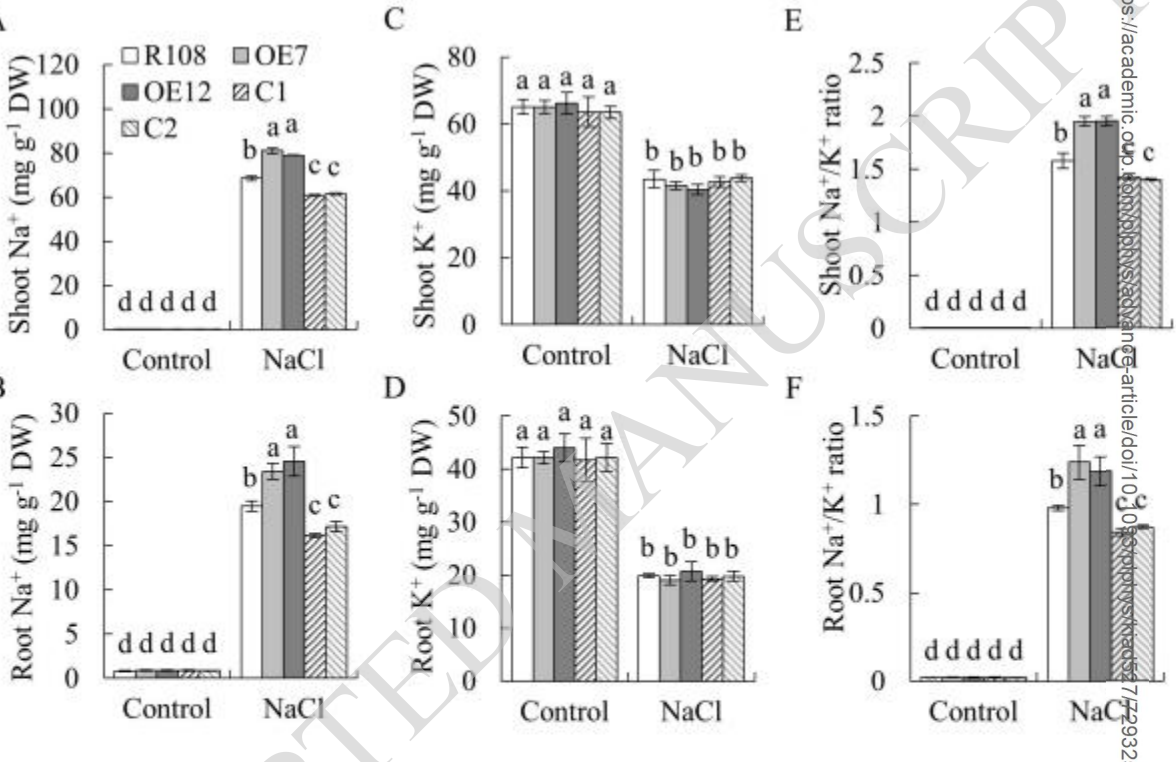

Figure 5 Na^+/K^+ homeostasis was affected by MtZPT2-2.

Four-week-old seedlings were treated for 7 d in 1/2 Hoagland solution containing 125 mM NaCl or without NaCl as control. Concentrations of Na^+ in shoots (A) and roots (B) and K^+ in shoots (C) and roots (D) were measured respectively, and Na^+/K^+ ratio in shoots (E) and roots (F) were calculated. All data are given as means ± SE (n = 3); the same letter above the column indicates no significant difference at $P < 0.05$ using Duncan's test.

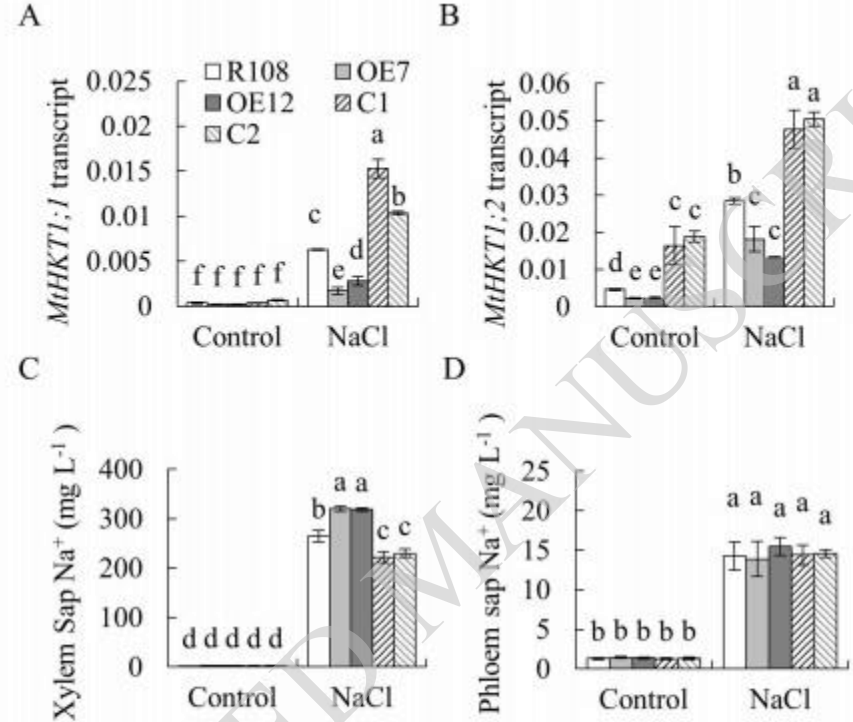

Figure 6 Analysis of transcript levels of *MtHKT1;1* and *MtHKT1;2* and Na^+ content in xylem and phloem sap in response to salinity.

Transcript levels of *MtHKT1;1* (A), *MtHKT1;2* (B) in roots of four-week-old *M. truncatula* seedlings were analyzed after 6 h of treatment with 125 mM NaCl. Na^+ concentration in xylem sap (C) and phloem sap in stems or petiole of four-week-old *M. truncatula* seedlings measured after 7 d after treatment with 125 mM NaCl. All data are given as means ± SE (n = 3); the same letter above the column indicates no significant difference at $P < 0.05$ using Duncan's test.

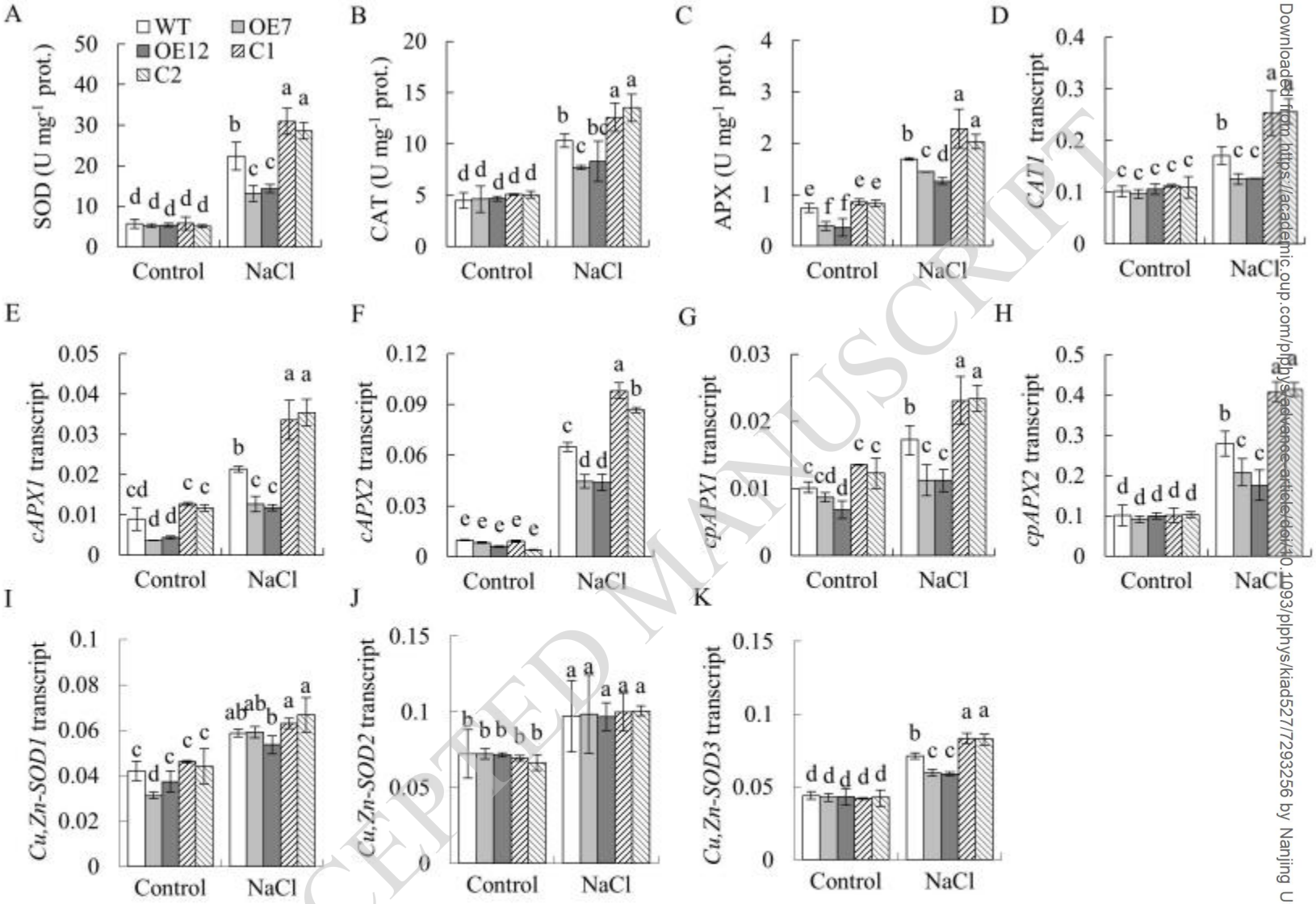

Figure 7 Antioxidant enzyme activities and transcript levels of the antioxidant enzyme encoding genes in response to salinity.

SOD (A), CAT (B) and APX activities (C) in roots of *MtZPT2-2* overexpression (OE7, OE12) and knockout lines (C1, C2) in comparison with the WT were analyzed after 7 d of treatment with 125 mM NaCl. Transcript levels of *CAT1* (D), *cAPX1* (E), *cAPX2* (F), *cpAPX1* (G), *cpAPX2* (H), *Cu, Zu-SOD1* (I), *Cu, Zu-SOD2* (J) and *Cu, Zu-SOD3* (K) in roots of four-week-old *M. truncatula* seedlings were analyzed after 6 h of treatment with 125 mM NaCl. All data are given as means ± SE (n = 3); the same letter above the column indicates no significant difference at $P < 0.05$ using Duncan's test.

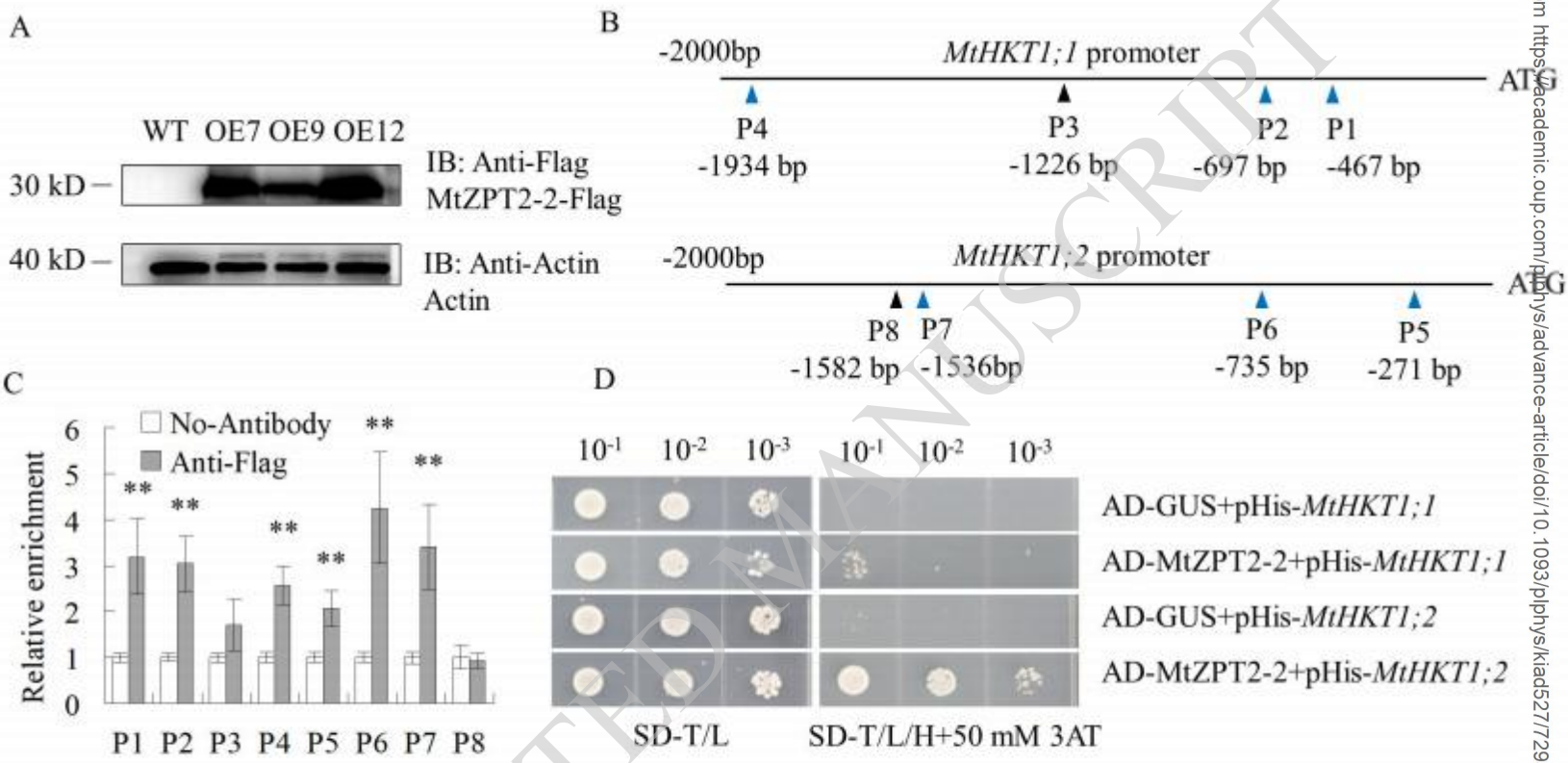

Figure 8 MtZPT2-2 transcriptionally represses *MtHKT1;1* and *MtHKT1;2* in *M. truncatula* and yeast cells.
(A) Western blot identification of OE7, OE9 and OE12 plant in which a Flag tag was infusion with C terminal of MtZPT2-2 using Flag antibody and Actin antibody. (B) Schematic diagram of the *MtHKT1;1* and *MtHKT1;2* promoter, showing the location of the A (G/C) T-X_{3-4}-A (G/C) T motifs (P1 to P8) in the promoters of *MtHKT1;1* and *MtHKT1;2* where was detected by qPCR in ChIP experiment. (C) ChIP-qPCR for *MtHKT1;1* and *MtHKT1;2*. (D) MtZPT2-2 binds to the promoter of *MtHKT1;1* and *MtHKT1;2* in the Y1H assay. The combination of AD-GUS + pHis-*MtHKT1;1* or pHis-*MtHKT1;2* served as the control group in this experiment. SD/-T/L: SD/-Trp-Leu. SD/-T/L/H: SD/-Trp-Leu-His. All data are given as means ± SE (n = 3); the double asterisks above the column indicate no significant difference at $P < 0.01$ using Student's t-test.

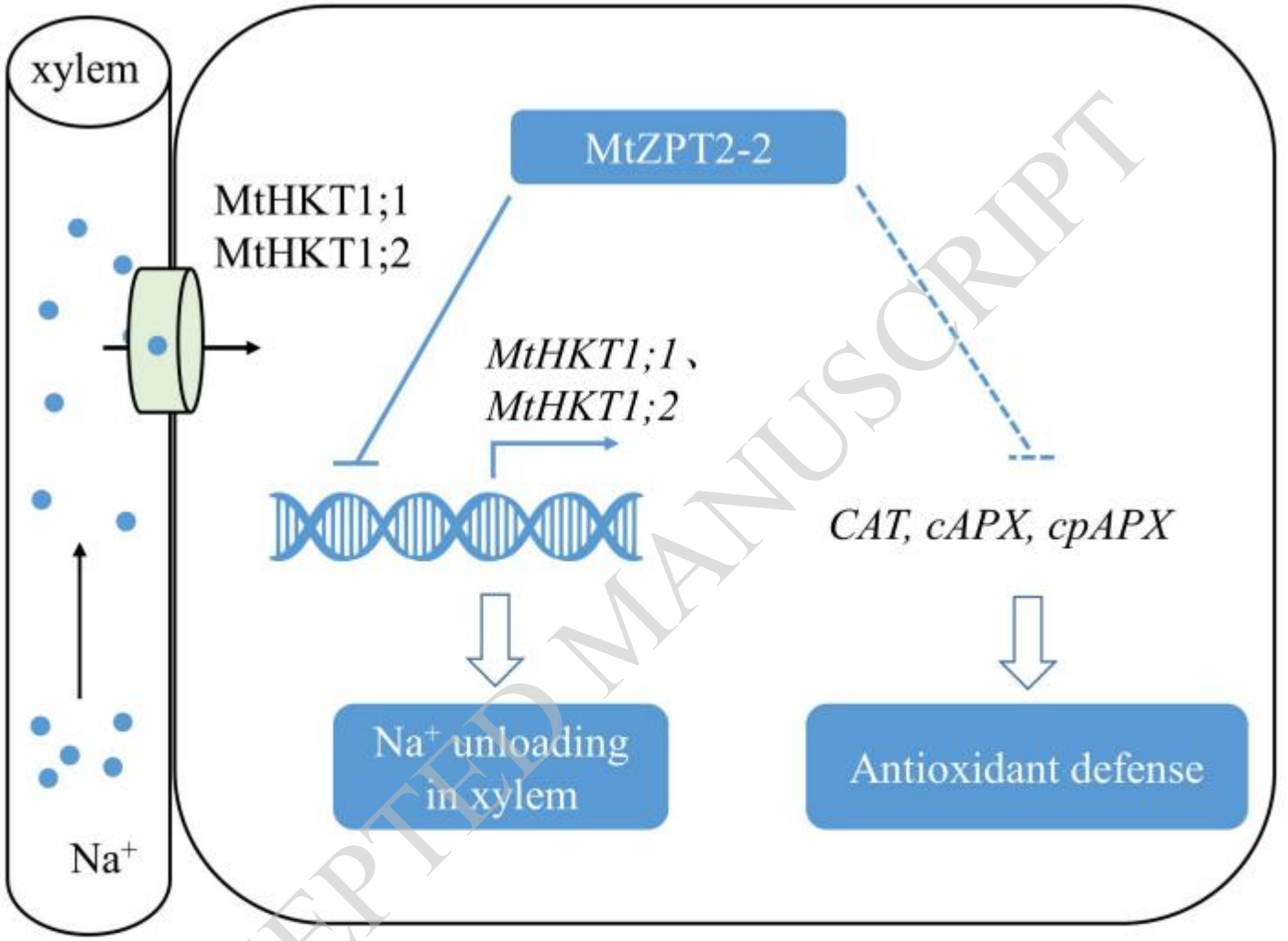

Figure 9 A proposed model of MtZPT2-2 regulation of salt tolerance in *M. truncatula*. MtZPT2-2 represses *MtHKT1;1* and *MtHKT1;2* transcripts by binding to the promoter directly for reduced xylem Na^+ unloading. Downregulated expression of *MtZPT2-2* led to increased expression of *MtHKT1;1* and *MtHKT1;2* for reduced Na^+ in the xylem sap and shoots under salt stress. In addition, downregulated expression of *MtZPT2-2* induced expression of antioxidant enzynme genes for maintenance of ROS homeostasis.

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