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Relation	

Constitutive overexpression of rice metallothionein-like gene *OsMT-3a* enhances growth and tolerance of Arabidopsis plants to a combination of various abiotic stresses

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Abstract

Metallothioneins (MT) are primarily involved in metal chelation. Recent studies have shown that MT proteins are also involved in the responses of plants to various environmental stresses. The rice metallothionein-like gene *OsMT-3a* is upregulated by salinity and various abiotic stressors. A DNA construct containing the complete *OsMT-3a* coding sequence cloned downstream to the CaMV35S promoter was transformed into Arabidopsis and homozygous single-copy transgenic lines were produced. Compared to wild-type plants, transgenic plants showed substantially increased salinity tolerance (NaCl), drought tolerance (PEG), and heavy metal tolerance ($CdCl_2$) as individual stresses, as well as different combinations of these stresses. Relevantly, under unstressed control conditions, vegetative growth of transgenic plants was also improved. The shoot Na^+ concentration and hydrogen peroxide in transgenic plants were lower than those in wild-type plants. *OsMT-3a*-overexpressing Arabidopsis lines accumulated higher levels of Cd^{2+} in both shoots and roots following $CdCl_2$ treatment. In the transgenic MT-3a lines, increased activity of two major antioxidant enzymes, catalase and ascorbate peroxidase, was observed. Thus, rice *OsMT-3a* is a valuable target gene for plant genetic improvement against multiple abiotic stresses.

Keywords: Gene expression, Metallothioneins, OsMT-3a, Salinity stress, Stress combination

1. Introduction

Soil salinity, drought, and heavy metal stresses are among the major natural factors that adversely affect plant growth and development (Assaha et al. 2016; Mittler 2006; Wangsawang et al. 2018). It is known that biotic and abiotic stressors increase the production of reactive oxygen species (ROS) in plant cells at certain stress levels (Abdelaziz et al. 2018; Assaha et al. 2017b; Yassin et al. 2019). High levels of ROS are extremely toxic, inducing secondary stress termed oxidative stress, characterized by the oxidation of biomolecules, including lipids, proteins, and nucleic acids, that result in lipid peroxidation, membrane injury and deactivation of the enzymes (Zhang et al. 2007). Plants have developed various defensive mechanisms to mitigate the impact of oxidative stress (Mittler 2002). These include ROS-detoxifying enzymes, such as superoxide dismutase, ascorbate peroxidase, catalase, and glutathione reductase, as well as antioxidants of low molecular mass, such as ascorbate, glutathione, carotenoids, and metallothioneins (MTs) (Jin et al. 2010). Metallothioneins act as antioxidants by reducing ROS-induced cellular injury, irrespective of their metal sequestration feature (Chiaverini and De Ley 2010). Through the oxidation of the thio group (-SH) of cysteine residues, the cysteine groups in MTs are directly involved in the elimination of ROS, and therefore shield against cellular injury and indirectly scale back the assembly of cellular ROS (Hassinen et al. 2011).

A number of plant studies addressing tolerance to various abiotic stresses identified a group of plant MTs that are correlated with environmental stress resistance (Klaassen et al. 1999). Plant MTs are low molecular weight (7-10 kDa) family of Cys-rich metal-chelating proteins comprised of four types, MT1, MT2, MT3, and MT4, based on the Cys distribution pattern (Cobbett and Goldsbrough 2002). Although many studies have delineated the expression of plant *MT* genes in different tissues and in response to various biotic and abiotic stressors, the roles of plant MTs remain poorly understood. The characteristic arrangement of the preserved cysteine residues within the four types of plant MTs and their tissue-specific expression implies different functions for the four types of MT (Freisinger 2011; Leszczyszyn et al. 2013). However, MTs are commonly suggested to be involved in a number of processes, including homeostasis and tolerance of metal ions (Cobbett and Goldsbrough 2002; Zimeri et al. 2005), oxidative stress mitigation

(Akashi et al. 2004; Wong et al. 2004), root development and seed germination (Yuan et al. 2008), pathogen defense signaling (Wong et al. 2004), and the senescence program (Guo et al. 2003). Therefore, to some extent, MTs could enable plants to adapt to different environmental stresses.

Most investigations aimed at elucidating the mechanisms of plant responses to abiotic stresses are performed primarily in response to single stress factors. However, under field conditions, plants are exposed to multiple abiotic stresses (Colmenero-Flores 2014), whose combined effect may be adversely more important to plants than single stress factors. Hence, stress combination is considered a critical threat faced by plants. Therefore, further studies are required to determine the responses of plants to a mixture of stresses to boost crop production with higher tolerance under field conditions (Kim and Kang 2018; Kumar et al. 2012; Pandey et al. 2015; Turchi et al. 2012).

In our previous study (Mekawy et al. 2018b), we identified a single gene, a homolog of the *Oryza sativa* MT type 3 (OsMT3), from a cDNA library screen under conditions of high salinity. The function of this rice MT-like type 3 (*OsMT-3a*) gene was then characterized in *Escherichia coli* cells and its expression was analyzed in two rice cultivars that contrasted with salinity tolerance. In the study, overexpression of the gene complemented the salt sensitivity in the KNabc *E. coli* mutant cells, and additionally, improved *E. coli* metal tolerance, particularly tolerance to Cd^{2+}. Furthermore, *E. coli* overexpressing the *OsMT-3a* gene, and the tolerant rice variety with enhanced expression of the *OsMT-3a* gene maintained lower ROS levels than their controls. Thus, it was suggested that *OsMT-3a* plays a pivotal role in salinity tolerance through ROS detoxification. However, the biological function of MTs in response to the combination of various abiotic stresses has not been elucidated. The aim of the present study was to evaluate the function of the rice *OsMT-3a* gene in plant systems under single and multiple stresses to gain more insight into the role of plant MTs in stress tolerance. Thus, we cloned the open reading frame of *OsMT-3a* from rice in the present study and investigated its role in transgenic *Arabidopsis thaliana* (L.) Heynh. (Arabidopsis) plants under the stress induced by Cd^{2+}, Na^{+}, and PEG. The effects of individual and combined stress of salinity, drought, and $CdCl_2$ were investigated in wild-type and transgenic Arabidopsis lines. Arabidopsis was selected for

the study based on the advantages of the species for genetic transformation, including shorter life cycles compared to other model plants (Hays 2002).

2. Materials and methods

2.1 Gene transformation in Arabidopsis

The *OsMT-3a* gene has a 189 bp full-length coding sequence and encodes a 62 amino acid protein (the *OsMT-3a* nucleotide sequence was registered under the accession number LC331297 in the DDBJ / EMBL / GenBank database) (Mekawy et al. 2018b). The Gateway system was used to construct the binary vectors for Arabidopsis transformation. The gene *OsMT-3a* was cloned into the pDONR221 vector as an entry vector. pGWB2 was used as the destination vector (Nakagawa et al. 2007). The *Agrobacterium tumefaciens* C58 strain was transformed with the pGWB2-*OsMT-3a* vector construct and recombinant *A. tumefaciens* colonies were selected on a medium containing kanamycin, hygromycin, rifampicin, and gentamycin. Arabidopsis (ecotype Columbia) plants were transformed with *A. tumefaciens* harboring the pGWB2-*OsMT-3a* vector construct, via the floral dip method (Clough and Bent 1998). Arabidopsis T0 seeds were germinated on 0.5× MS (Murashige-Skoog) medium under kanamycin and hygromycin selection. Phenotypic analyses were performed on T3 homozygous lines.

2.2 Stress tolerance of transgenic Arabidopsis

Wild-type seeds and the transgenic T3 (homozygous) Arabidopsis seeds (L1, L2, and L3) were surface-sterilized with 70% ethanol for 1 min, followed by 1% NaClO solution for 3 min, and then rinsed three times in sterile water and kept in the dark for 3 days at 4 °C before being moved to a growth room (25 °C, 16 h light/8 h dark cycle). For the germination test under different stress combinations, WT and transgenic plant seeds were plated on 0.5× MS medium supplemented with filter-sterilized PEG6000 (2%, 5%, and 10%), NaCl (50, 100, and 200 mM), $CdCl_2$ (50, 100, and 200 μM), 50 mM NaCl + 50 μM $CdCl_2$, 100 mM NaCl + 100 μM $CdCl_2$, 50 μM $CdCl_2$ + 2% PEG, 100 μM $CdCl_2$ + 5% PEG, 50 mM NaCl + 2% PEG, or 100 mM NaCl + 5% PEG. Germinated seeds on MS medium were used as a control. When the radicles

were 1 mm long, seeds were assumed to have germinated. The percentage of germination was determined as the number of seeds germinated from the total number of seeds tested. Germination tests were repeated three times. Photographs were taken on the 12th d after the stress treatments.

Seeds of WT and transgenic plants were germinated on 0.5× MS agar medium for stress testing at the early seedling stage. Seven-day-old WT and transgenic seedlings were transplanted onto MS medium (as a control) and MS medium supplemented with either 50 μM $CdCl_2$, 50 mM NaCl, 2% PEG, 50 mM NaCl + 50 μM $CdCl_2$, 50 μM $CdCl_2$ + 2% PEG, or 50 mM NaCl + 2% PEG. To visually compare root growth, the plates were placed vertically on the shelves. Photographs were taken after the stress treatments between the 7th day and 14th day.

2.3 Na^+, K^+, and Cd^{2+} uptake in Arabidopsis plants

WT and transgenic Arabidopsis lines (L1, L2, and L3), 14-days-old, were treated without (control) or with one of the following solutions: 100 μM $CdCl_2$, 100 mM NaCl, 5% PEG, 100 mM NaCl+100 μM $CdCl_2$, 100 μM $CdCl_2$ + 5% PEG, or 100 mM NaCl+5% PEG for 7 d. In deionized water, roots and shoots were washed. The samples were dried for 3 days at 70 °C to assess dry weight (eight plants used as one replicate, $n = 3$). At temperatures between 80 and 200 °C, the dried plant materials were digested with concentrated ultrapure grade HNO_3 and $HClO_4$ (2:1 v/v), diluted in 0.1 N HNO_3, and then measured for ion (Na^+ and K^+) content using a flame photometer (ANA 135, Tokyo Photoelectric, Tokyo, Japan). The Cd^{2+} concentration was determined using inductively coupled plasma-atomic emission spectrometry (ICP-AES, iCAP6300 Duo, Thermo Fisher Scientific). Briefly, the samples digested using HNO_3 and $HClO_4$ were vaporized, atomized, and ionized using argon plasma, and emission of electromagnetic radiation at 228.80 nm was detected in an axial viewing. Standard solutions were purchased from Fujifilm-Wako Pure Chemical, Co.

2.4 Quantification of H_2O_2 in WT and transgenic Arabidopsis plants

To measure the H_2O_2 concentration, the shoots of three plants were ground in liquid nitrogen as one replicate and homogenized in 4 mL of cold acetone. The homogenate was centrifuged at 8,000 × *g* for 15 min at 4 °C. Then, 100 µL of each sample was added to 1 mL of the reaction buffer and allowed to stand for 1 h at room temperature. The H_2O_2 levels were quantified spectrophotometrically at 560 nm and calculated by comparison with the standards (Suharsono et al. 2002).

2.5 CAT and APX antioxidant enzyme activities

The concentration of proteins in the enzyme extract was determined using a protein assay kit and bovine serum albumin, as directed by the manufacturer. For catalase and ascorbate peroxidase activities, fresh samples were used to extract enzymes according to the Takagi and Yamada method (Takagi and Yamada 2013). For the assay method of both CAT and APX activities, 1 mL of the assay mixture was used as described previously (Mekawy et al. 2018a). In the CAT assay, H_2O_2 decreases were tracked at 240 nm and the activity is represented as mmol H_2O_2 consumed per min. For the APX assay, ascorbate oxidation was estimated at 290 nm and the concentration was calculated using 2.8 mM^{-1} cm^{-1} as the extinction coefficient. One unit of APX was defined as 1 µmol of oxidized ascorbate per min.

2.6 Statistical analyses

The data obtained were statistically analyzed using one-way variance analysis in version 21 of the SPSS statistics program, and the means were segregated by Duncan's post hoc test using the multi-range test at $p \leq 0.05$. The mean values ± SE of at least three replicates are represented by all data in this analysis.

3. Results

3.1 Characterization and overexpression confirmation of *OsMT-3a* gene

The *OsMT-3a* gene was cloned in our previous study (Mekawy et al. 2018b), which contained a short 189 bp ORF and encoded a 62 amino acid protein. Sequence alignment analysis indicated that OsMT-3a shared high homology with many MT3-like genes from several other plant species (Mekawy et al. 2018b). The

gene expression levels of *OsMT-3a* in the different T3 transgenic Arabidopsis lines were analyzed using RT-PCR. Three homozygous *OsMT-3a* transgenic lines, L1, L2, and L3, were selected for further analysis. The analysis with semi-quantitative RT-PCR confirmed the significant constitutive expression of *OsMT-3a* transcripts in the three transgenic lines (Fig. 1).

3.2 Overexpression of *OsMT-3a* gene improved the germination rate of transgenic Arabidopsis seeds under single and multiple stresses

In the above three chosen transgenic Arabidopsis and wild plants, the effects of either PEG, NaCl, $CdCl_2$, or the combination of these treatments on seed germination were examined (Fig. 2). Under normal growth conditions, the germination rate assay showed no variation in seed germination between transgenic and WT plants (Fig. 2a). However, under single and multiple stresses, the transgenic seed germination rates were significantly higher than the WT (Fig. 2a–d). At 50 μM $CdCl_2$ treatment, the transgenic lines L1, L2, and L3 displayed higher seed germination rates by 86%, 80%, and 90%, respectively, compared to those of WT plants (60%). Under 100 μM $CdCl_2$ conditions, seed germination rates were 60%, 90%, 93%, and 23% in the transgenic lines L1, L2, L3, and WT plants. Under 200 μM $CdCl_2$ conditions, it was 33%, 73%, and 50% in the lines L1, L2, and L3, respectively, while no seed germination was observed in WT seeds (0%). Under 50, 100, and 200 mM NaCl conditions, all transgenic lines showed higher germination rates at 80%–96% in line L1, 73%–96% in line L2, 33%–46% in line L3, and 16%–73% in WT plants. Under 2%, 5%, and 10% PEG conditions, germination rates were 63%–100%, 50–100%, 73%–96%, and 40%–63% in the transgenic lines L1, L2, L3, and WT plants, respectively. These findings suggest that MT-3a functions are critically important in the maintenance of seed germination under stress conditions. Seed germination rates were also examined under the combined stress conditions (Fig. 2d). Overall, the combined stress decreased the germination rate of WT plants. Under 50 mM NaCl + 50 μM $CdCl_2$ and 100 mM NaCl + 100 μM $CdCl_2$, germination rates were 70%, 90%, 76%–90%, and 30%–33% in the transgenic lines L1, L2, L3, and WT plants, respectively. Under 2% PEG + 50 μM $CdCl_2$ and 5% PEG + 100 μM $CdCl_2$ treatments, germination rates were 70%–80%, 90–93%, 90%, and 26%–70% in the transgenic lines L1, L2, L3, and WT plants,

respectively. Under 2% PEG + 50 mM NaCl and 5% PEG + 100 mM NaCl treatments, germination rates were 33%–53%, 33–70%, 60%–70%, and 20%–33% in the transgenic lines L1, L2, L3, and WT plants, respectively. These results showed that *OsMT-3a* overexpression in Arabidopsis promoted multiple stress tolerance during the germination stage.

3.3 Overexpression of *OsMT-3a* gene enhanced vigor and vegetative growth of the transgenic Arabidopsis plants at the early seedling stage

The effects of either PEG, NaCl, $CdCl_2$, or the combination of these treatments on the growth of transgenic Arabidopsis and WT plants were examined during the early seedling stage (Fig. 3). Under control, non-stressed conditions, transgenic Arabidopsis lines showed significant phenotypic differences from WT plants. Two independent transgenic lines (L1 and L3) had longer roots, larger-sized leaves, and greater biomass than their respective WT plants. However, the growth of the transgenic and WT plants was inhibited when the medium contained 50 μM $CdCl_2$, 50 mM NaCl, 2% PEG, 50 mM NaCl + 50 μM $CdCl_2$, 50 μM $CdCl_2$ + 2% PEG, or 50 mM NaCl + 2% PEG, even though the transgenic Arabidopsis lines grew better than their WT counterparts. Vegetative growth of transgenic plants was more vigorous compared to that of WT plants (Fig. 3). Under control conditions, shoot and root dry weights of the L1 and L3 transgenic lines were significantly higher than those of the WT plants (Fig. 4). After stress treatment, 100 μM $CdCl_2$, 100 mM NaCl, 5% PEG, 100 mM NaCl + 100 μM $CdCl_2$, 100 μM $CdCl_2$ + 5% PEG, or 100 mM NaCl + 5% PEG, the dry weights (DWs) of transgenic and WT lines were drastically affected. However, transgenic lines DWs were less affected than the shoots and roots DW of WT plants, specifically under $CdCl_2$, PEG, and $CdCl_2$+PEG stresses (Fig. 4a, b). These results show that *OsMT-3a* overexpression could improve growth under normal conditions and confer tolerance to Arabidopsis lines under both single $CdCl_2$ and combined $CdCl_2$ and osmotic stress conditions.

3.4 *OsMT-3a* overexpression affected ion accumulation in the transgenic Arabidopsis plants under NaCl and $CdCl_2$ stresses

To investigate whether overexpression of the *OsMT-3a* gene affects ion uptake and accumulation, the Na^+ and Cd^{2+} concentrations in the transgenic Arabidopsis and WT plants were measured. In the control medium, Na^+ (Fig. 5) and Cd^{2+} (Fig. 6) concentrations did not differ significantly between the transgenic and WT seedlings. However, the concentration of Na^+ in *OsMT-3a*-transgenic and WT seedlings increased sharply, with significantly lower levels in shoots and roots of the transgenic Arabidopsis lines when plants were grown on medium containing either 100 mM NaCl, 100 mM NaCl+100 μM $CdCl_2$, or 100 mM NaCl+5% PEG. Although K^+ concentrations dropped in both shoots and roots of the transgenic plants by most of the stress treatments compared to WT plants, no significant differences were observed in the Na^+/K^+ ratios between the transgenic and WT plants (Fig. 5). When exposed to 100 μM $CdCl_2$, 100 mM NaCl+100 μM $CdCl_2$, or 100 μM $CdCl_2$+5% PEG, a sharp increase in Cd^{2+} concentrations was observed in the transgenic and WT plants. Cd^{2+} concentrations were significantly higher in both roots and shoots of only one of the *OsMT-3a*-transgenic lines (L1) under $CdCl_2$ stress treatment (Fig. 6).

3.5 Overexpression of *OsMT-3a* gene increased ROS scavenging ability of the transgenic Arabidopsis plants

Accumulation of H_2O_2 was observed in transgenic lines as well as in WT plants under stress conditions. Compared to WT plants, transgenic lines maintained significantly lower concentrations of H_2O_2 under either NaCl, NaCl+$CdCl_2$, or PEG+NaCl stresses. However, an increase in H_2O_2 levels was observed in only one of the transgenic lines (L2) subjected to NaCl stress (Fig. 7). Fig. 8a, b shows the activity of the antioxidant enzymes, APX and CAT, in the shoots of WT and *OsMT-3a*-transgenic lines. The activity of APX displayed a sharp and significant induction (2–6 fold) in the transgenic lines using most of the different stress treatments compared to that in WT plants, but not under $CdCl_2$+PEG stress treatment. On the other

hand, CAT activity was significantly higher in the transgenic lines under both $CdCl_2$ and PEG stress treatments than in WT plants, while CAT activity was reduced by the other stress treatments. (Fig. 8b).

4. Discussion

Plants have established a range of cellular mechanisms that can be implicated in heavy metal detoxification, and therefore, metal stress tolerance. High-affinity ligands are theoretically a very critical pathway for chelating metals. These include amino acids, organic acids, and two groups of cysteine-rich peptides, phytochelatins (PCs), and metallothioneins (MTs). In the metal-thiolate cluster, metallothioneins bind to metal ions and thus lead to metal detoxification by buffering the cytosolic metal concentration. Metallothioneins have significant effects on plant growth when plants are subjected to various abiotic stresses (Jin et al. 2017; Lee et al. 2004; Patankar et al. 2019).

In the present study, transgenic Arabidopsis plants had significantly higher seed germination levels and more robust seedling growth than non-transgenic (WT) plants under high concentrations of $CdCl_2$, NaCl, PEG, applied singly, or in combination. These results suggest that *OsMT-3a* is involved in heavy metal, salt, and osmotic stress adaptation of transgenic Arabidopsis plants. It has been suggested to have variable specificities and affinities for different heavy metals (Foley et al. 1997). Previous *in vitro* experiments showed that both isoforms, OsMTI-1b and OsMTII-1a, have different Cd^{2+} binding capabilities (Nezhad et al. 2013). Furthermore, a previous study showed that Arabidopsis MT3 improved Cd^{2+} tolerance when expressed in *Vicia faba* guard cells (Lee et al., 2004). In addition, when *Avicennia marina* type 2 MT (*AmMT2*) was expressed in *E. coli* cells, it improved heavy metal resistance to Zn^{2+}, Cu^{2+}, Pb^{2+}, and Cd^{2+} by binding to these metals, with the highest affinity for Cd^{2+} (Huang and Wang 2010). Similarly, our previous results (Mekawy et al. 2018b) showed that the *OsMT-3a* gene conferred increased Cd^{2+} tolerance and Cd^{2+} accumulation in *E. coli* cells and rice plants. In the present study, Cd^{2+} accumulation was relatively higher in transgenic Arabidopsis plants (more tolerant to Cd^{2+}) than in the WT plants. Moreover, Arabidopsis MT1 knock-down lines were found to be hypersensitive to Cd^{2+} and contained lower Cd^{2+} concentrations than WT plants in another report (Zimeri et al. 2005). The OsMT-3a protein binds Cd^{2+} in

the cytoplasm, thereby preventing Cd^{2+} from freely interfering with cytoplasmic components or accessing organelles. This mode of action may result in reduced Cd^{2+} damage to transgenic plants, but damage to WT plants, which may justify the differences in DWs (Fig. 4a, b). The DWs of shoots and roots of both *OsMT-3a*-overexpressed plants and WTs were reduced by $CdCl_2$ alone or by the combination of $CdCl_2$+NaCl or $CdCl_2$+PEG (Fig. 4a, b). Nevertheless, this decrease in growth in WT plants was much more serious than in controls. Although the shoots and roots of transgenic Arabidopsis plants accumulated higher concentrations of Cd^{2+} (Fig. 6), they showed a higher dry mass than WT plants, suggesting that OsMT-3a has a possible role in the chelation and detoxification of Cd^{2+} in transgenic lines, thus conferring metal tolerance. However, the effect of MTs on Cd^{2+} tolerance and Cd^{2+} accumulation requires further study to elucidate their function. Na^+ concentration in *OsMT-3a*-overexpressed plants was significantly lower than that in WT plants under single NaCl stress and in combination with NaCl+$CdCl_2$ or NaCl+PEG stress. To prevent excessive Na^+ accumulation during salinity exposure, plants may reduce Na^+ in the cells through the activation of Na^+ transporter genes, which store Na^+ in vacuoles, or export Na^+ to the external medium (soil or the apoplast) via plasma membrane Na^+/H^+ antiporters (Assaha et al. 2015; Assaha et al. 2017a; Chuamnakthong et al. 2019; Elsawy et al. 2018; Mekawy et al. 2015; Mekawy et al. 2018a; Shi et al. 2002; Ueda et al. 2013). Therefore, the lower Na^+ concentration in *OsMT-3a*-transgenic lines and the enhanced tolerance to salt stress may be the result of the interaction of the *OsMT-3a* gene with Na^+ transporter genes, but this needs further investigation. Overexpression of *OsMT-3a* in transgenic lines might have induced the transport of Na^+ from the plant. One more probability is the Na^+-binding ability of the OsMT-3a protein to chelate and detoxify the cellular Na^+ damaging effects, as suggested by Zhang et al. (2014) and Mekawy et al. (2018b), although this property should be validated further. Lower concentrations of Na^+ in the *OsMT-3a*-transgenic plants likely reduced plant damage and improved resistance to Na^+ stress. This result further reveals a possibly new pathway involving the participation of MTs in Na^+ homeostasis under salt stress conditions.

When plants are subjected to drought, heavy metals, and salts, increased production of ROS may occur, leading to changes in the balance between production and scavenging of ROS (Miller et al. 2010;

Mittler 2002). Compared to WT plants, *OsMT-3a*-transgenic Arabidopsis plants produced relatively lower H_2O_2 concentrations. In previous studies, several species of transgenic seedlings had less H_2O_2 than in control plants under specific stresses, such as the genes *BcMT1* and *BcMT2* from *Brassica campestris* (Lv et al. 2013), *Elsholtzia haichowensis EhMT1* (Xia et al. 2012), and *Gossypium hirsutum GhMt3a* (Xue et al. 2009). Thus, the *OsMT-3a* gene mediates H_2O_2 scavenging upon exposure to various abiotic stresses and results in much lower levels of H_2O_2 in the transgenic plants. Therefore, OsMT-3a can serve as an antioxidant to mitigate the toxicity of ROS under single and multiple stresses.

In the ROS scavenging process, H_2O_2 accumulates by the action of SOD, which decomposes the more hazardous superoxide anions into H_2O_2. Elevated levels of cellular H_2O_2 are toxic to plant cells; therefore, it is necessary to remove them instantly. Thus, synchronization between CAT and APX (which has a high affinity for H_2O_2) is essential to tolerate stress (Gill and Tuteja 2010). H_2O_2 serves as a substrate for both CAT and APX, so raising the activity of these two enzymes is correlated with a reduction in the level of H_2O_2. Examining the activities of antioxidant enzymes showed that APX was significantly elevated in the shoots of the transgenic plants, and this could demonstrate the lower concentration of H_2O_2 detected under single and multiple stresses. Similar observations were reported for the tolerant varieties of flax, rice, and barley with lower H_2O_2 levels and induced activities of antioxidant enzymes under salinity stress (Abdelaziz et al. 2018; Elsawy et al. 2018; Mekawy et al. 2019). In addition, the steep increase in APX activity (2–6 fold) in transgenic lines compared to that in WT plants under various stress treatments shows that *OsMT-3a*-over-expressing Arabidopsis has attained a more effective antioxidant mechanism with increased enzyme activity to properly deal with oxidative stress. Our results confirm the previous report, which shows that overexpression of the *OsMTI-1a* gene in rice substantially increased peroxidase, catalase, and ascorbate peroxidase activity by H_2O_2 application (Yang et al. 2009). However, further research is needed to explore the correlation between this gene overexpression and antioxidant enzyme activity.

5. Conclusion

In conclusion, the rice *OsMT-3a* gene was overexpressed in Arabidopsis plants (*in planta* analysis) to show its contribution to various abiotic stresses. Enhanced seed germination, seedling growth, and increased tolerance to various stress combinations in transgenic plants were observed. Furthermore, H_2O_2 and Na^+ content in the transgenic lines were lower than those in the controls. These results suggest that a role of OsMT-3a in impacting plant response to $CdCl_2$, osmotic, or salt stresses may be direct, through the binding of ions and activation of other genes, or indirect through the improvement of the ROS scavenging ability, and shoot Na^+ exclusion. Thus, enhanced tolerance of transgenic plants to multiple stresses, as shown here, indicates that OsMT-3a is of paramount importance in genetic engineering of plant stress tolerance for field application and hence enhanced crop production.

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Contribution

The authors have made the following declarations about their contributions:

Conceived and designed the experiments: AMMM, AU.

Performed the experiments: AMMM.

Analyzed the data: AMMM.

Wrote the paper: AMMM, DVMA, AU.

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Figure legend

Fig. 1 RT-PCR of the transgenic Arabidopsis plants. L1, L2, and L3 correspond to the independent transgenic lines, WT, wild type plant.

Fig. 2 Seed germination of transgenic plants under single and combination of different abiotic stresses. (**a**) Seed germination on medium supplemented with 0 (Control); 50, 100, 200 μM $CdCl_2$; 50, 100, 200 mM NaCl; 2, 5 or 10% PEG single stresses, (**b**) Seed germination on medium supplemented with 50 mM NaCl+50 μM $CdCl_2$, 100 mM NaCl+100 μM $CdCl_2$, 50 μM $CdCl_2$+2% PEG, 100 μM $CdCl_2$+5% PEG, 50 mM NaCl+2% PEG, or 100 mM NaCl+5% PEG combined stresses, using the Arabidopsis wild type (WT) and transgenic plants (L1, L2, and L3). Photos were taken on the 12^{th} day after the stress treatments. (**c**) Germination rate of transgenic plants under single stresses, and (**d**) Germination rate of transgenic plants under multiple combined stresses. Data represents the mean of 3 replicates ± SE (n = 3). The same letters indicate no significant differences ($P \leq 0.05$).

Fig. 3 Relative stress tolerance of WT and the *OsMT3*-a-overexpressing transgenic Arabidopsis plants (L1, L2, and L3) at the early seedling stage. 7-d-old At*OsMT-3a* and WT seedlings were transplanted onto MS medium (as a control) and MS medium supplemented with either 50 μM $CdCl_2$, 50 mM NaCl, 2% PEG, 50 mM NaCl+50 μM $CdCl_2$, 50 μM $CdCl_2$+2% PEG, or 50 mM NaCl+2% PEG. The plates were positioned vertically on shelves in order to compare root growth visually. Photos were taken between the 7th and 14th day after the stress treatments.

Fig. 4 Dry weights of (**a**) shoots and (**b**) roots of WT and the transgenic Arabidopsis plants (L1, L2, and L3) at the seedling stage. 14-d-old At*OsMT-3a* and WT seedlings were grown onto MS medium (as a control) and MS medium supplemented with either 100 μM $CdCl_2$, 100 mM NaCl, 5% PEG, 100 mM NaCl+100 μM $CdCl_2$, 100 μM $CdCl_2$+5% PEG, or 100 mM NaCl+5% PEG for 7 days. Data represents the mean of 3 replicates ± SE (n = 3). The same letters indicate no significant differences ($P \leq 0.05$).

Fig. 5 Na^+ and K^+ concentrations and Na^+/K^+ ratios in the shoots (**a-c**) and roots (**d-f**) of WT and the transgenic Arabidopsis plants (L1, L2, and L3) at the seedling stage. 14-d-old At*OsMT-3a* and WT seedlings were grown onto MS medium (as a control) and MS medium supplemented with either 100 μM $CdCl_2$, 100 mM NaCl, 5% PEG, 100 mM NaCl+100 μM $CdCl_2$, 100 μM $CdCl_2$+5% PEG, or 100 mM NaCl+5% PEG for 7 days. Data represents the mean of 3 replicates ± SE (n = 3). The same letters indicate no significant differences ($P \leq 0.05$).

Fig. 6 Cd^{2+} concentration in the (**a**) shoots and (**b**) roots of WT and the transgenic Arabidopsis plants (L1, L2, and L3) at the seedling stage. 14-d-old At*OsMT-3a* and WT seedlings were grown onto MS medium (as a control) and MS medium supplemented with either 100 μM $CdCl_2$, 100 mM NaCl, 5% PEG, 100 mM NaCl+100 μM $CdCl_2$, 100 μM $CdCl_2$+5% PEG, or 100 mM NaCl+5% PEG for 7 days. Data represents the mean of 3 replicates ± SE (n = 3). The same letters indicate no significant differences ($P \leq 0.05$).

Fig. 7 H_2O_2 concentration in the shoots of WT and the transgenic Arabidopsis plants (L1, L2, and L3) at the seedling stage. 14-d-old At*OsMT-3a* and WT seedlings were transplanted onto MS medium (as a control) and MS medium supplemented with either 100 μM $CdCl_2$, 100 mM NaCl, 5% PEG, 100 mM NaCl+100 μM $CdCl_2$, 100 μM $CdCl_2$+5% PEG, or 100 mM NaCl+5% PEG for 7 days. Data represents the mean of 3 replicates ± SE (n = 3). The same letters indicate no significant differences ($P \leq 0.05$).

Fig. 8 Antioxidant enzyme activity of ascorbate peroxidase (APX) (**a**) and catalase (CAT) (**b**) in the shoots of WT and the transgenic Arabidopsis plants (L1, L2, and L3) at the seedling stage. 14-d-old At*OsMT-3a* and WT seedlings were transplanted onto MS medium (as a control) and MS medium supplemented with either 100 μM $CdCl_2$, 100 mM NaCl, 5% PEG, 100 mM NaCl+100 μM $CdCl_2$, 100 μM $CdCl_2$+5% PEG, or 100 mM NaCl+5% PEG for 7 days. Data represents the mean of 3 replicates ± SE (n = 3). The same letters indicate no significant differences ($P \leq 0.05$).

493 **Fig.1**

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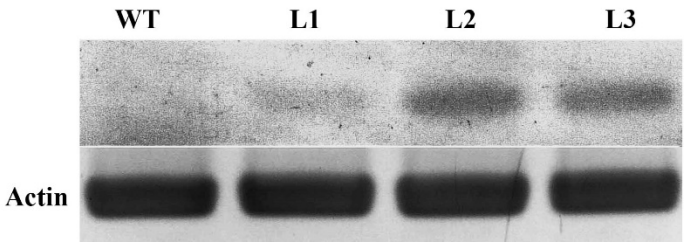

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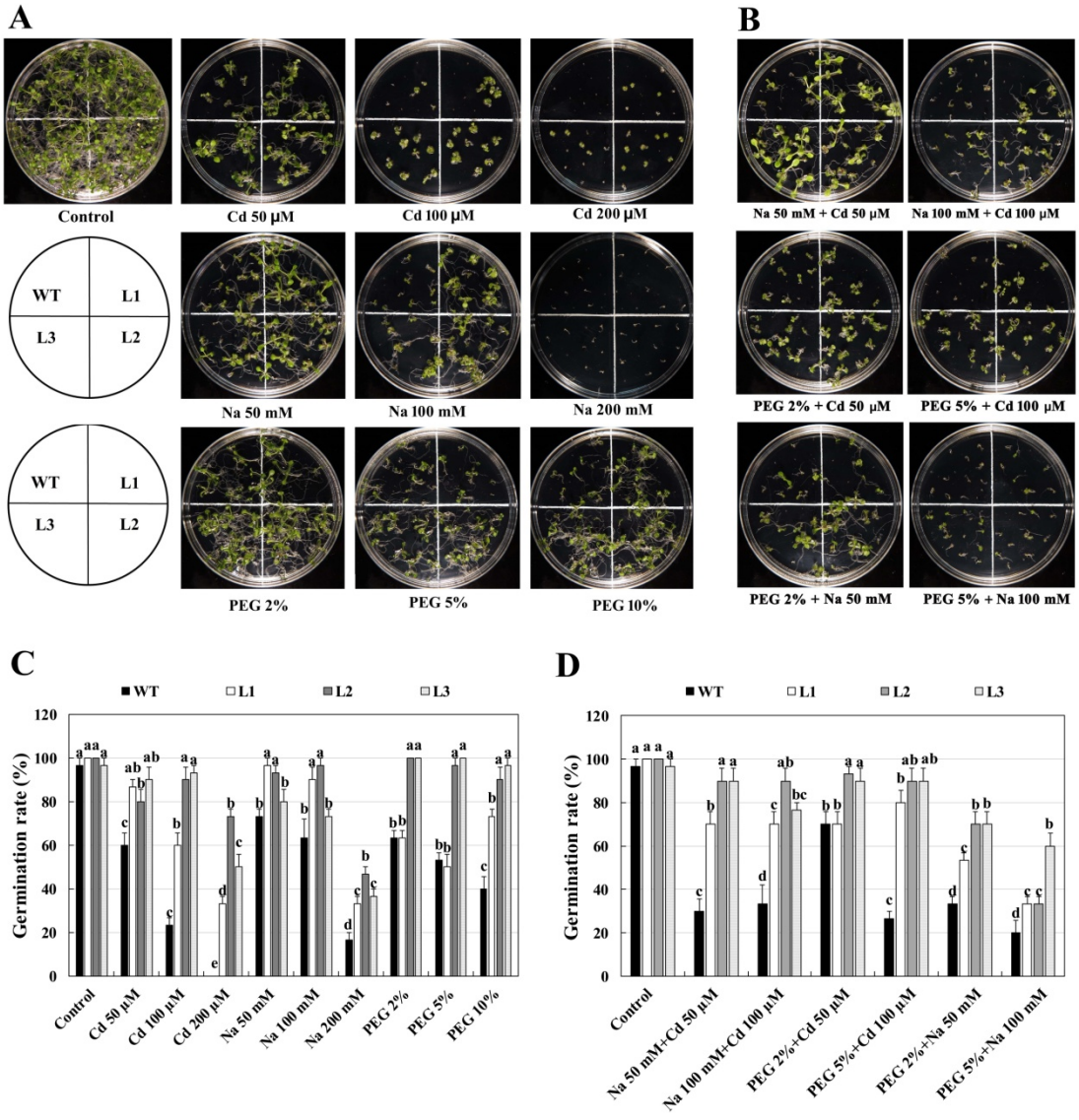
Fig.2



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511 **Fig.3**

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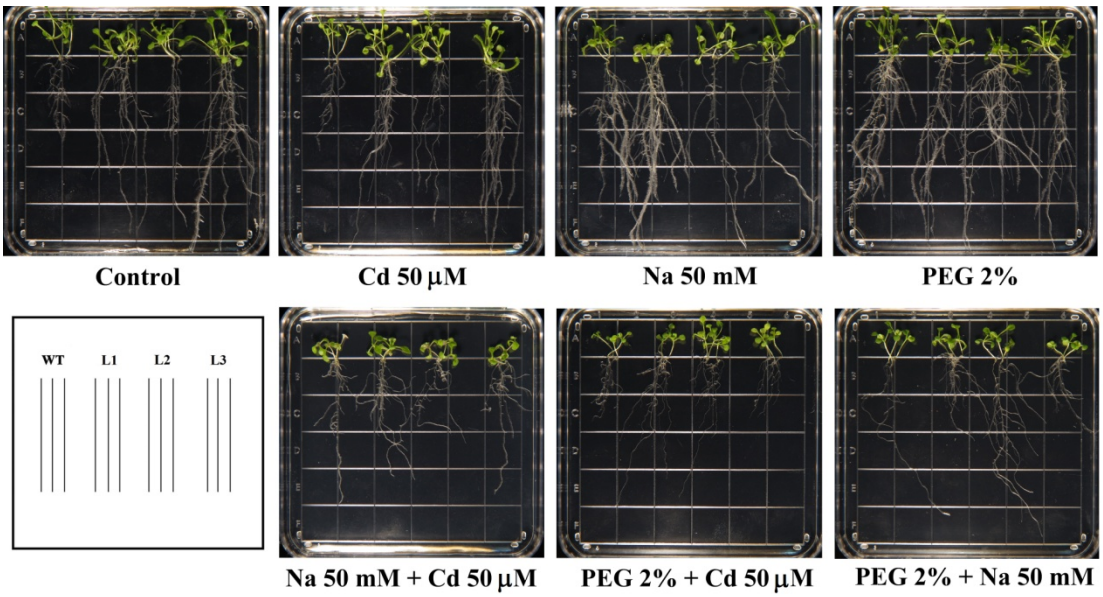

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520 day after the stress treatments.

521 **Fig.4**

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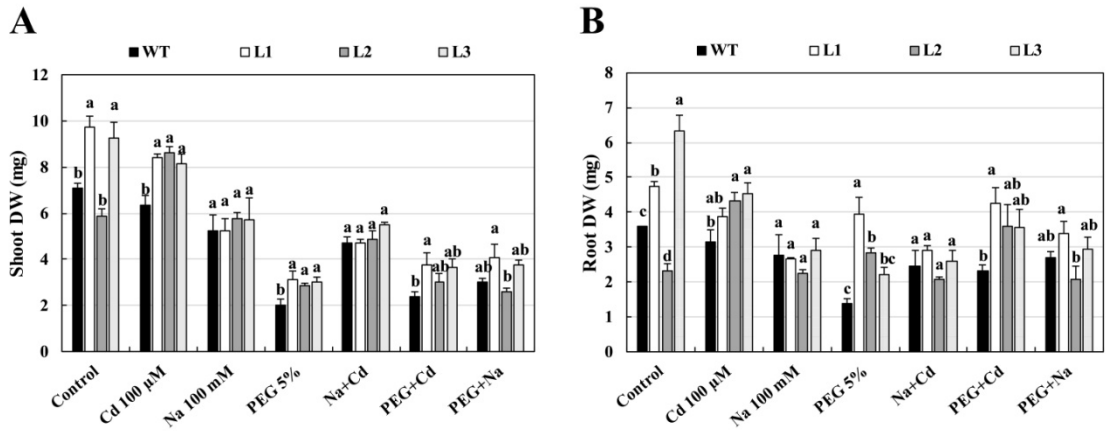

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525 **Fig. 4.** Dry weights of (A) shoots and (B) roots of WT and the transgenic *Arabidopsis* plants (L1, L2, and
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530 **Fig.5**

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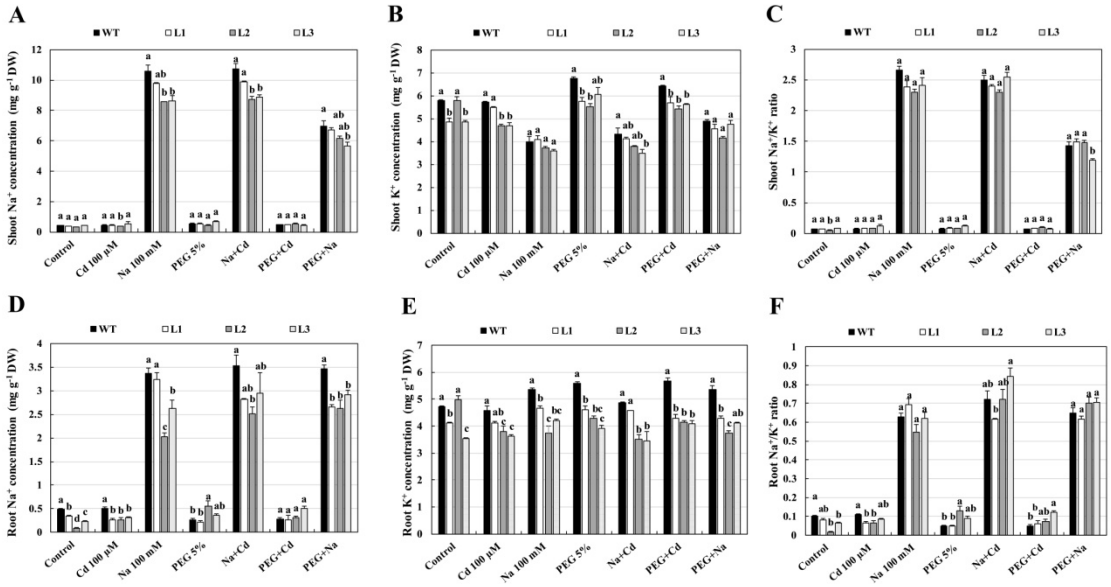

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534 **Fig. 5.** Na^+ and K^+ concentrations and Na^+/K^+ ratios in the shoots (A-C) and roots (D-F) of WT and the
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Fig.6

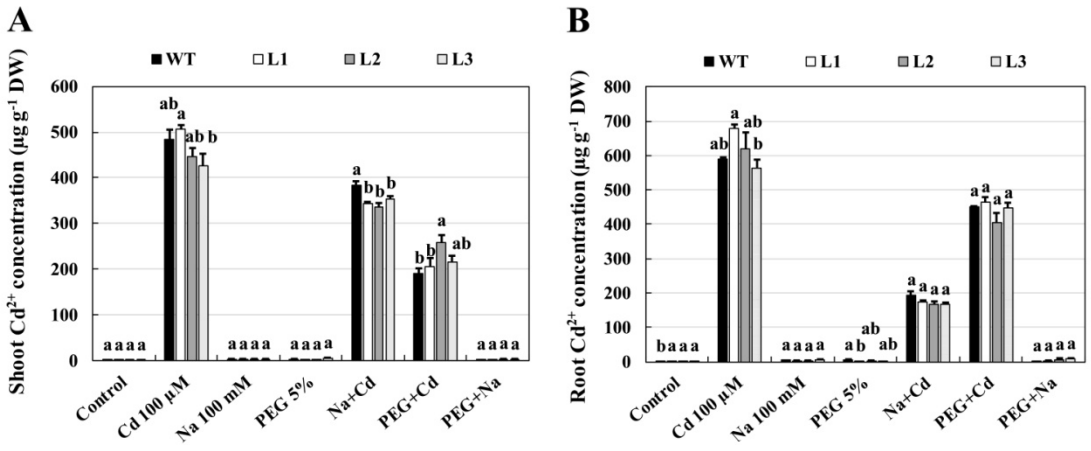

Fig. 6. Cd^{2+} concentration in the (A) shoots and (B) roots of WT and the transgenic *Arabidopsis* plants (L1, L2, and L3) at the seedling stage. 14-d-old At*OsMT-3a* and WT seedlings were grown onto MS medium (as a control) and MS medium supplemented with either 100 μM $CdCl_2$, 100 mM NaCl, 5% PEG, 100 mM NaCl+100 μM $CdCl_2$, 100 μM $CdCl_2$+5% PEG, or 100 mM NaCl+5% PEG for 7 days. Data represents the mean of 3 replicates ± SE (n = 3). The same letters indicate no significant differences ($P \leq 0.05$).

549 **Fig.7**

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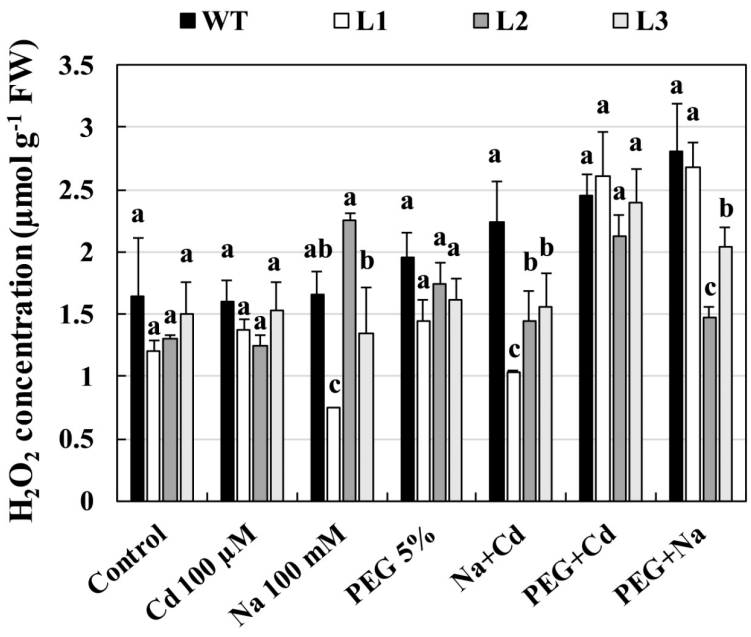

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553 **Fig. 7.** H_2O_2 concentration in the shoots of WT and the transgenic *Arabidopsis* plants (L1, L2, and L3) at
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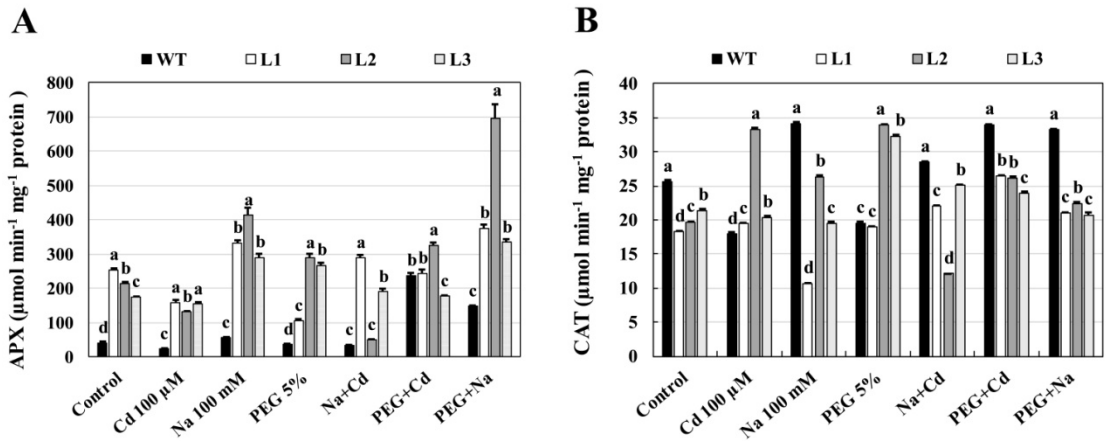
Fig.8



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