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ORIGINAL ARTICLE

Possible involvement of MAP kinase pathways in acquired metal-tolerance induced by heat in plants

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Abstract Cross tolerance is a phenomenon that occurs when a plant, in resisting one form of stress, develops a tolerance to another form. Pretreatment with nonlethal heat shock has been known to protect cells from metal stress. In this study, we found that the treatment of rice roots with more than 25 μM of Cu^{2+} caused cell death. However, heat shock pretreatment attenuated Cu^{2+}-induced cell death. The mechanisms of the cross tolerance phenomenon between heat shock and Cu^{2+} stress were investigated by pretreated rice roots with the protein synthesis inhibitor cycloheximide (CHX). CHX effectively block heat shock protection, suggesting that protection of Cu^{2+}-induced cell death by heat shock was dependent on de novo protein synthesis. In addition, heat pretreatment downregulated ROS production and mitogen-activated protein kinase (MAPK) activities, both of which can be greatly elicited by Cu^{2+} stress in rice roots. Moreover, the addition of purified recombinant GST–OsHSP70 fusion proteins inhibited Cu^{2+}-enhanced MAPK activities in an in vitro kinase assay. Furthermore, loss of heat shock protection was observed in Arabidopsis *mkk2* and *mpk6* but not in *mpk3* mutants under Cu^{2+} stress. Taken together, these results suggest that the interaction of OsHSP70 with MAPKs may contribute to the cellular protection in rice roots from excessive Cu^{2+} toxicity.

Keywords Copper ion · Metal stress · Heat shock protein 70 (HSP70) · Mitogen-activated protein kinase (MAPK)

Abbreviations

$CuCl_2$	Copper chloride
CHX	Cycloheximide
CM-H_2DCFDA	5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester
HSP	Heat shock protein
MAPK	Mitogen-activated protein kinase
MAPKK/MEK	MAPK kinase
MAPKKK/MEKK	MAPKK kinase
MBP	Myelin basic protein
ROS	Reactive oxygen species

Introduction

Copper (Cu) is an essential element, because it serves as a cofactor for key enzymes involved in fundamental biological processes. However, when plants absorb excessive amounts, Cu^{2+} can be a toxic element and it retards growth (Jiang et al. 2000). Cu^{2+} toxicity to plants is reportedly involved in damage to cell membranes and in cell death in roots (Wainwright and Woolhouse 1977; Hall 2002; Yeh et al. 2003). In addition, Cu ions can catalyze harmful redox reactions, which result in the oxidation of lipid membranes and generation of reactive oxygen species (ROS) (Hoshino et al. 1999).

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Acquired thermotolerance is a phenomenon that occurs when plants exposed to nonlethal high temperature for short period of time are subsequently able to better resist more severe, even lethal, temperatures. It is well known that in response to heat shock, the expression of a group of proteins called heat shock proteins can be greatly induced. Heat shock protein 70 (HSP70) is one of the major inducible heat shock proteins (Wu 1995). Besides thermotolerance, in mammals, it has been reported that overexpression of HSP70 in several cell lines increases their resistance to stresses like nitric oxide, ROS, UV, and monocyte cytotoxicity (Jaattela and Wissing 1993; Simon et al. 1995; Bellmanna et al. 1996). In plants, it was also reported that a short period of heat stress preceding heavy-metal stress induces a tolerance against metal toxicity in tomato cells and prevents membrane damage (Neumann et al. 1994). This phenomenon has been described as acquired metal-tolerance. On the other hand, heavy metal can induce the expression of heat shock proteins such as small HSP and HSP70 in plants (Neumann et al. 1994; Lewis et al. 2001). This suggests the possibility of crosstalk between the heat and heavy metal, signaling transduction pathways.

Mitogen-activated protein kinase (MAPK) cascades play important roles in plants in response to multiple stresses (Zhang and Liu 2001). Activation of MAPK signaling pathways by heat and heavy metals has been demonstrated previously (Nakagami et al. 2005; Yeh et al. 2007). The MAPK pathways transduce a large variety of external signals, leading to a wide range of cellular responses, including growth, differentiation, defense, and cell death (Herskowitz 1995; Kyriakis and Avruch 1996; Takabatake et al. 2007). The basic assembly of a MAPK cascade is a three kinase module conserved in all eukaryotes. MAPK, the last kinase in the cascade, is activated by dual phosphorylation of the Thr and Tyr residues in a tripeptide motif (Thr–Xaa–Tyr, where Xaa could be Glu, Gly, Pro, or Asp) located in the activation loop (T-loop) between subdomains VII and VIII of the kinase catalytic domain. This phosphorylation is mediated by a MAPK kinase (MAPKK or MEK), which in turn is activated by a MAPKK kinase (MAPKKK or MEKK). Each of the three tiers of kinases in a cell contains multiple members, which contributes to the specificity of the transmitted signal (Zhang and Klessig 2001). In previous studies, our laboratory and others reported that Cu^{2+} induced activation of MAPKs in plants (Jonak et al. 2004; Yeh et al. 2007).

Little is known about how different stresses interact with one another or what are the signaling components that interrelate the responses triggered by different stress types in plants. Recent study has elucidated that the MAPK cascade functions downstream of HSP90 and transduces the cell death signal to mitochondria for N gene-dependent cell death in tobacco against infection of tobacco mosaic virus (Takabatake et al. 2007). To address the relationship between heat shock proteins and MAPK in acquired Cu^{2+}-tolerance, in this study, we applied moderate heat shock condition prior Cu^{2+} stress to rice and/or Arabidopsis *mpk* mutant lines. Our results strongly suggest that the MAPKs and HSP70 may participate in the mechanism of acquired Cu^{2+}-tolerance in rice roots.

Material and methods

Plant materials and treatments

Rice (*Oryza sativa* cv. Japanica TN67; seeds obtained from Agricultural Research Institute, Taichung, Taiwan) were sterilized with 2.5% (v/v) sodium hypochlorite for 15 min, and were germinated in Petri dishes containing distilled water at 37°C in the dark. After 2–3 days of incubation, uniformly germinated seeds were selected and transferred to Petri dishes containing one sheet of Whatman No. 1 filter paper moistened with 10 ml of distilled water or tested solution. Each Petri dish contained 15 germinated seeds. The germinated seeds were grown at 26°C in the dark for 3 days. Rice seedlings were untreated or exposed to heat (42°C, 1 h) in the presence or absence of 100 μM CHX prior to treatment with 0, 25, 50, 100 μM of $CuCl_2$ for 15 min to 3 h, and the measurements of cell death, ROS production, MAPK activity, in vitro gel kinase activity from rice roots were carried out as described in the following sections. Seeds of *Arabidopsis thaliana* cv. Columbia and three MAPK cascade mutant lines (*mpk3*, *mpk6*, and *mkk2*), for which the background ecotype is Columbia, were sterilized with 2.5% (v/v) sodium hypochlorite for 5 min, and were germinated in MS medium. The *mpk3* and *mpk6* were T-DNA insertion mutant lines; their seeds along with seeds of wild type Arabidopsis were obtained from the SALK T-DNA collection (SALK_151594 and SALK_073907, respectively) (Nakagami et al. 2006). The *mkk2* T-DNA null mutant line (Garlic_511_H01.b.1a.Lb3Fa) was obtained from the Syngenta Arabidopsis Insertion Library (SAIL), Torrey Mesa Research Institute (San Diego, CA, USA) (Teige et al. 2004). The Arabidopsis seedlings were grown at 26°C in light for 8 days. The seedlings were untreated or exposed to heat (37°C, 1 h) prior to treatment with 100 μM $CuCl_2$ for 3 h, and the measurements of the cell death and ROS production in roots among wild type and three MAPK cascade mutant lines were carried out as described in the following sections.

Measurement of cell death

Cell death was quantified by the Evans blue (Sigma-Aldrich, St Louis, MO, USA) staining method (Baker and Mock 1994). Rice and Arabidopsis roots after various

treatments were harvested from ten randomly selected seedlings. The roots were stained in 0.25% aqueous Evans blue solution for 15 min at room temperature. The roots were washed twice with ddH_2O, each for 15 min, and left in the distilled water overnight to remove the excessive dye. The apical 5 mm of root tips were excised followed by the extraction of dye in a solution of 50% methanol/1% SDS for 1 h at 50°C. The absorbance at wavelength of 595 nm was measured by a spectrophotometer. In addition, the cell viability was also observed by microscopy analysis after being stained with fluorescein diacetate (FDA) (Stein and Hansen 1999). In brief, roots were stained with 10 μg ml^{-1} FDA in water. After extra dye was removed by washing with deionized water for 1 min, the root-tip was immediately observed by a Leica MPS60 fluorescent microscope equipped with a green filter (excitation 450–490 nm, emission above 515 nm). Exposure times were equal for all samples. Images were captured with a CoolSNAP Cooled CCD Camera (Photometrics, North Reading, MA, USA).

Detection of ROS in rice and Arabidopsis roots

Seedlings of 6-day-old rice and 8-day-old Arabidopsis after various treatments were used to localize the generation of ROS in roots. The roots were labeled with 10 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCF-DA, Molecular Probes, Eugene, OR, USA) for 30 min and then treated with 100 μM $CuCl_2$ for 15 min. A Leica MPS60 fluorescent microscope equipped with a green filter (excitation 450–490 nm; emission 500–530 nm) was used to observe fluorescence images. Exposure times were equal for all samples. Auto-fluorescence was not observed in unstained controls at the same exposure time. Images were captured with a CoolSNAP Cooled CCD Camera (Photometrics).

Preparation of protein extracts

The protein extracts were prepared by grinding the roots in liquid nitrogen, followed by homogenization at 4°C with 2 volumes (w/v) of protein extraction buffer [100 mM Hepes, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM dithiothreitol (DTT), 10 mM Na_3VO_4, 10 mM NaF, 50 mM glycerophosphate, 1 mM phenylmethylsulphonyl fluoride (PMSF), 5 μg ml^{-1} antipain, 5 μg ml^{-1} aprotinin, 5 μg ml^{-1} leupeptin, 10% glycerol]. The slurry was centrifuged at 16,440*g* for 20 min at 4°C. Aliquots of the supernatants were stored at −80°C for later use.

In-gel kinase activity assay

The in-gel kinase assay was performed according to the procedures described previously (Zhang and Klessig 1997) with slight modifications. In brief, rice roots were heat-shocked at 42°C for 1 h before adding 100 μM $CuCl_2$ into medium and incubating for another hour. Protein extracts (10 μg) from roots were electrophoretically separated on 10% SDS-polyacrylamide gels embedded with 0.25 mg ml^{-1} of myelin basic protein (MBP) in the separating gel as a substrate for the kinase. After electrophoresis, SDS was removed by cleansing the gel with washing buffer [25 mM Tris, pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 0.5 mg ml^{-1} bovine serum albumin (BSA), 0.1% (v/v) Triton X-100] thrice, for 30 min, each time at room temperature. The kinases were allowed to renature in 25 mM Tris, pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4, and 5 mM NaF at 4°C overnight with three changes of buffer. The gel for MAP kinase activity assay was then incubated at room temperature in a 90 ml reaction buffer (25 mM Tris, pH 7.5, 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4) for 30 min. Phosphorylation was performed for 1 h at room temperature in 90 ml of the same reaction buffer with 200 nM ATP plus 1.85×10^6 Bq of gamma-^{32}P-ATP. The reaction was halted by transferring the gel into a solution with 5% (w/v) trichloroacetic acid (TCA) and 1% (w/v) sodium pyrophosphate. The unincorporated gamma-^{32}P-ATP was removed by cleansing in the same solution for at least an hour with two changes. The gel was dried and exposed to Kodak BioMax MR Film. Prestained size markers (Bio-Rad, Hercules, CA, USA) were used to calculate the molecular weight of the kinases. All the experiments for the kinase assay were performed at least twice.

Western-blot analysis

The protein extracts (10 μg) were electrophoretically separated on 10% SDS-PAGE. After electrophoresis, the gel was transferred to a polyvinylidene difluoride (PVDF) membrane at 100 V for 70 min at 4°C in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The membrane was incubated in blocking solution containing TBST (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.6) supplemented with 5% (w/v) nonfat dry milk for 1 h at room temperature. It was then washed thrice with TBST buffer. The blots were probed with polyclonal antibodies raised against the conserved subdomain XI of rat ERK1, which recognize ERK1 and ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or against the phosphotyrosine-containing peptide of human p44 MAP kinase (residues 196-209; Cell Signaling Technology, Beverly, MA, USA). The latter antibody recognized only ERK1/2-related polypeptides, which are catalytically activated at Tyr204. The immune complexes were detected by a horseradish peroxidase (HRP)-conjugated secondary antibody (Pierce, Rockford, IL, USA) and revealed by Western Lightning

Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA, USA). Antibodies were used at 1:2,500, 1:1,000, and 1:5,000 dilutions for anti-ERK1, anti-phospho-ERK, and secondary antibodies, respectively.

Expression of GST–OsHSP70 fusion proteins

Sequence of the *Arabidopsis Hsp70* (BAB02269) was used to BLAST the rice cDNA database. A rice *Hsp70* cDNA clone (Accession number NP_001049712) of Arabidopsis *Hsp70* homolog was retrieved, and obtained from the Rice Genome Resource Center (RGRC, Tsukuba, Japan). To overexpress OsHSP70 in *E. coli*, the *OsHsp70* cDNA fragments were amplified by PCR using primers (forward primer, 5′-CGCGGATCCATGGCCGGCAAGAG-3′; reverse primer, 5′-TCCCCCGGGTTAGTCGACTTCCTC GA-3′); and cloned into the *Ba*mHI and *Sma*I sites in pGEX-4T-1 (Amersham, Buckinghamshire, UK). The expression plasmid pGEX-4T1/OsHsp70 was transformed into *E. coli* BL21 (Promega, Madison, WI, USA), and the transformants were grown approximately 16 h at 37°C in 3 ml of Luria-Bertani medium containing 100 μg ml^{-1} ampicilin. To induce the expression of the recombinant protein, 0.5 mM IPTG (isoprorylthio-β-galactoside) was added to the medium, and bacterial culture was allowed to continue growth for 3 h at 30°C. After centrifugation, the bacterial cells were resuspended with phosphate buffer saline solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), and subsequently were lysed by sonication (Sonicator 3000, Misonix, Farmingdale, NY, USA) in 30 s of shock followed with 10 s of suspension for 15 cycles. Bacterial lysates were spanned down at 4°C, and the supernatant was collected. The GST–OsHSP70 fusion protein was purified by GST MicroSpin™ Purification Module (Amersham) according to the manufacturer's instructions.

Immunoprecipiation and in vitro kinase assays

A 200 μl protein extract (1 μg μl^{-1}) from roots was incubated with ERK antibodies (1:500 dilution, v/v) and 30 μl protein-A agarose suspension (Oncogene, San Diego, CA, USA) overnight at 4°C. The agarose bead–protein complexes were pelleted by centrifugation (500*g*, 2 min) and washed thrice in 0.5 ml wash buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, 10 mM β-glycerophosphate, 2 μg ml^{-1} antipain, 2 μg ml^{-1} aprotinin, 2 μg ml^{-1} leupeptin, 0.5% (v/v) Triton X-100, 0.5% (v/v) Nonidet P-40]. After washing, the ERK1-immunoprecipitated protein complex was incubated in 15 μl kinase reaction buffer (50 mM Tris, pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 0.1 mM ATP, and 2.22×10^5 Bq of gamma-^{32}P-ATP) with 5 μg MBP and 1 μg GST–OsHSP70 fusion proteins. The kinase reaction was stopped after 30 min by adding 4 μl SDS loading buffer (5×) and heating for 5 min at 95°C. Reaction products were analyzed by 10% SDS-PAGE. The unincorporated ^{32}P-ATP was removed by cleansing it at least twice in a solution with 5% (w/v) trichloroacetic acid (TCA) and l% (w/v) sodium pyrophosphate for at least 1 h. The gel was dried and exposed to Kodak BioMax MR Film.

Results

The effect of heat shock pretreatment on Cu^{2+} induced cell death in rice roots

Six-day-old rice seedlings were treated with 0, 25, 50, and 100 μM of $CuCl_2$ for 3 h. The cell death of rice roots was examined by using Evans blue staining assay (Kawai and Uchimiya 2000). Evans blue can be excluded from viable cells with intact cell membranes, while those with damaged membranes incorporate the dye. The cell death rate of rice roots surged in response to the increasing concentration of Cu^{2+} in the medium as shown in Fig. 1a. A previous report indicates that a short exposure to heat stress preceding Cd^{2+} stress induces a tolerance in tomato cells to Cd^{2+} toxicity (Neumann et al. 1994). Therefore, we investigated the effect of heat shock pretreatment on Cu^{2+}-induced root cell death. We pretreated 6-day-old rice seedlings with heat shock (42°C, 1 h) in darkness before treating them with $CuCl_2$. As shown in Fig. 1b, heat shock pretreatment significantly reduced Cu^{2+}-induced cell death. An alternative approach by using FDA staining method by which cell viability is correlated with emission of fluorescent light exhibited the same results (Fig. 1c). Therefore, heat shock pretreatment may protect rice root cells from Cu^{2+} toxicity.

To further investigate whether the protection effect of preheated root is dependent on de novo protein synthesis, 6-day-old rice seedlings were exposed to heat shock (42°C, 1 h) before being treated with 50 μM $CuCl_2$ for 3 h in the presence or absence of the protein synthesis inhibitor CHX (100 μM). As shown in Fig. 1d, the protective effect vanished in preheated rice roots in the presence of CHX. This result suggests that preheated rice roots require de novo protein synthesis to increase their tolerance to Cu^{2+} toxicity.

Heat shock pretreatment prevented Cu^{2+}-induced ROS production

Reactive oxygen species (ROS) are either directly or indirectly involved in cell death. We also studied the relationship of ROS production to heat shock-pretreated rice roots and Cu^{2+}-induced cell death by using an ROS-sensitive

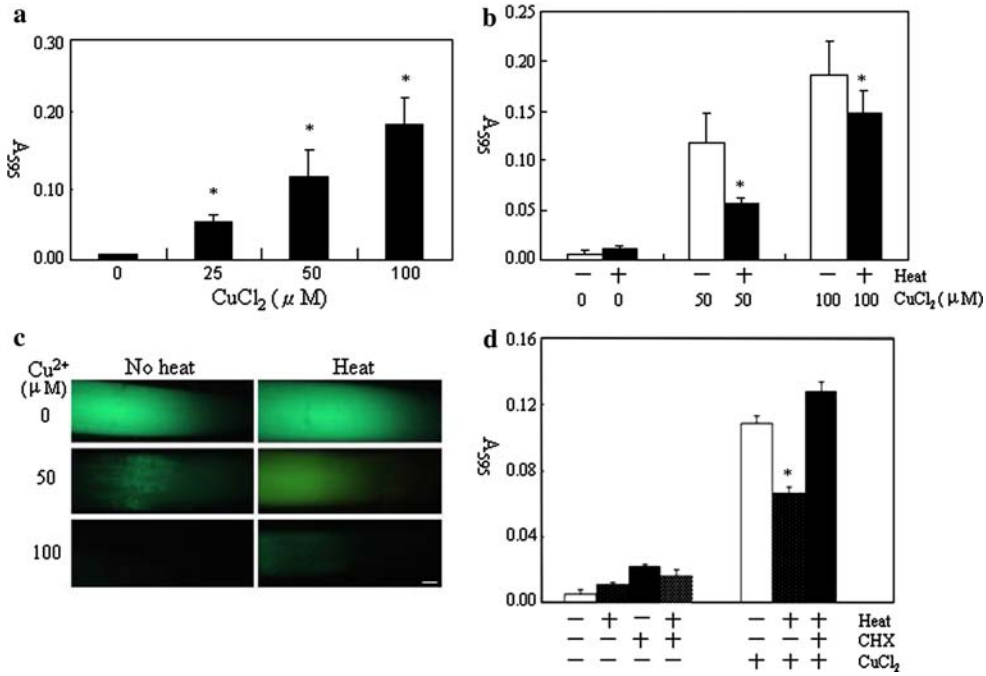

Fig. 1 The cell death effect of rice roots among the interaction of CHX, heat shock, and $CuCl_2$ treatments. The cell death rate of rice roots was measured after following different treatments by either Evans blue (**a**, **b**, **d**) or FDA (**c**) staining methods. **a** Six-day-old rice seedlings were treated with 0, 25, 50, 100 μM of $CuCl_2$ for 3 h. **b** Rice seedlings were pretreated with or without moderate heat shock (42°C, 1 h) before 50 or 100 μM of $CuCl_2$ treatment. **c** Rice seedlings were pretreated with (*right panels*) or without (*left panels*) heat shock (42°C, 1 h) before 0, 50, or 100 μM of $CuCl_2$ treatment. Cellular viability was observed under fluorescent microscope after staining roots with FDA. *Bar* 100 μm. **d** Rice seedlings were pretreated with 100 μM CHX, prior to heat shock and subsequent 50 μM $CuCl_2$ treatments. Values in **a**, **b**, and **d** represent means of three independent experiments. *Bars* SE. The *asterisk* represents a statistically significant difference at $P < 0.05$, according to paired *t* test

dye, CM-H_2DCFDA. This compound is nonfluorescent, but is rapidly oxidized to the highly fluorescent DCF by intracellular ROS (Hoffmann et al. 2005). Rice roots were either pretreated with or without moderate heat shock (42°C) for 1 h before treatment with $CuCl_2$ (100 μM) for 15 min. As shown in Fig. 2, ROS levels in roots rose significantly after Cu^{2+} treatments. However, ROS production fell when the rice roots had been pretreated under moderate heat shock, followed by either the absence or presence of $CuCl_2$ treatment. Taken together, these results suggest that inhibition of Cu^{2+}-induced cell death by heat shock pretreatment probably occurs through the elimination of ROS production in rice root tips.

Heat shock pretreatment inhibits Cu^{2+}-induced MBP kinases and the phosphorylation of ERK-type MAPKs in rice roots

MAPK signaling pathways are involved in multiple cellular stress responses in plants, and MAPK-like kinase activities are induced by heavy metal stress in rice roots (Yeh et al. 2007). To study the effect of MAPK kinase activity in rice roots in response to 0, 50, 100 μM of $CuCl_2$, the crude protein extracts from Cu^{2+}-treated rice roots were separated on 10% SDS-PAGE embedded with MBP as a substrate in an in-gel kinase assay. As shown in Fig. 3a, two MBP kinases, with molecular weights of 40 and 42 kDa, respectively, were activated by Cu^{2+} in a dose-dependent manner.

To further study whether heat shock pretreatment affects the MAPK activation induced by Cu^{2+}, in-gel kinase assay and immunoblot analysis were performed. The in-gel kinase assay indicates that heat pretreatment significantly reduced the activation of both the 40 and 42 kDa MBP kinases activities induced by $CuCl_2$ (Fig. 3b). To further investigate whether MAPK phosphorylation could be affected by heat shock pretreatment, human anti-ERK and anti-phospho-ERK antibodies, which can detect plant MAPKs and phosphorylated MAPKs, respectively, were used for Western-blot analysis. The phosphorylated forms of both 40 and 42 kDa MAPK proteins was significantly reduced upon heat pretreatment as compared with no heat pretreatment, whether or not $CuCl_2$ treatment was subsequently applied (Fig. 3b, middle panels). However, as shown in the lower panels of Fig. 3b, the levels of MAPK proteins in different groups of treatment were approximately the same. This suggests that reduction of phosphorylated MAPK

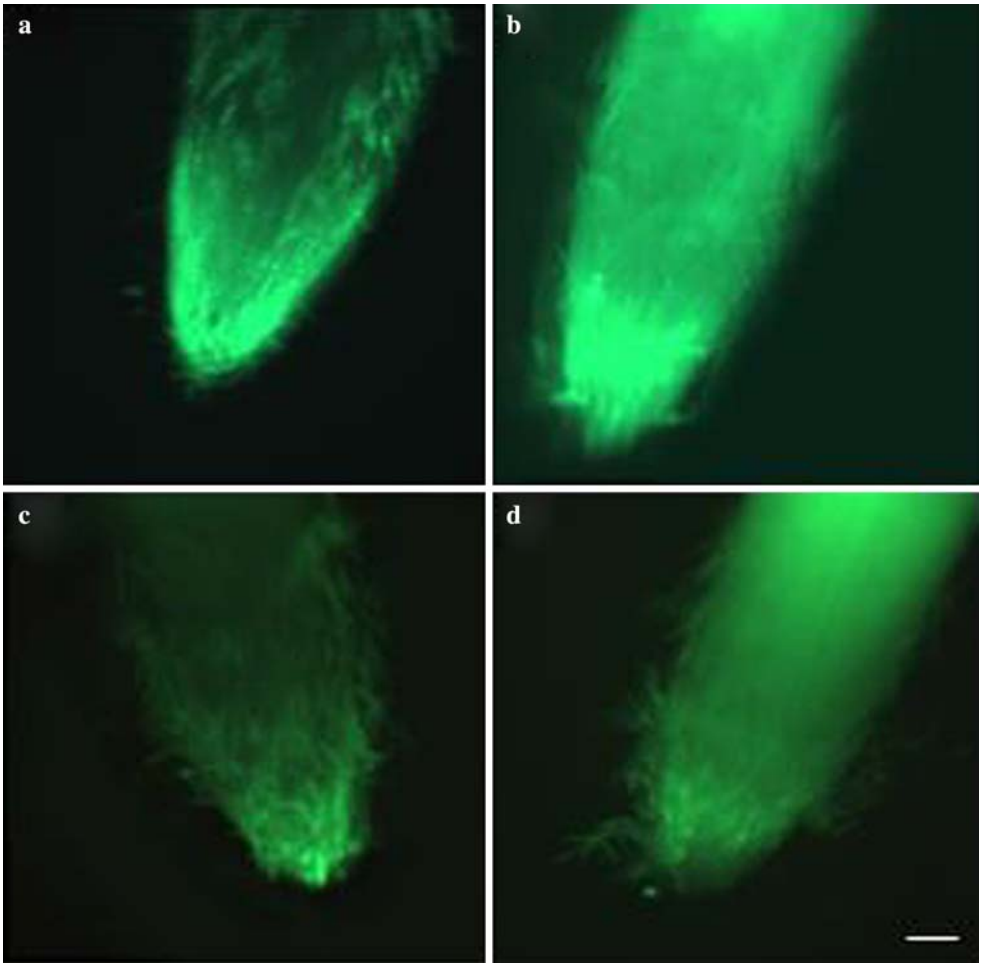

Fig. 2 ROS production in rice roots under Cu^{2+} stress with or without heat shock pretreatment. Rice roots were labeled with 10 μM CM-H_2DCF-DA for 30 min, and the emission level of green fluorescence indicates the amount of ROS production after following treatments: **a** without $CuCl_2$ treatment; **b** with 100 μM $CuCl_2$ treatment at room temperature for 15 min; **c** heat shock pretreatment (42°C, 1 h), but subsequently without $CuCl_2$ treatment; **d** heat shock pretreatment (42°C, 1 h), and subsequently with 100 μM $CuCl_2$ treatment. Ten rice seedlings were assayed for each experiment and similar results were observed. *Bar* 100 μm

upon heat pretreatment is well corelated to the decrease in the activation of 40 and 42 kDa MBP kinase activities by $CuCl_2$.

The time-dependent effect of heat shock pretreatment on the activation of these MAPKs by $CuCl_2$ treatment was also investigated. The phosphorylated forms of 40 and 42 kDa MAPK increased when rice roots were exposed to 100 μM $CuCl_2$ for 1 h (Fig. 3c, d). However, the time-dependent activation of MAPK activities was affected by heat shock pretreatment. Cu^{2+} induced the phosphorylation of 40 and 42 kDa MAP kinases as early as 15 min, and their phosphorylated states were sustained for 60 min. In contrast, the phosphorylated MAPKs began to decrease at 30 min after $CuCl_2$ treatment in preheated roots. This suggested that heat shock pretreatment blocks Cu^{2+}-induced MAPK activations within 30 min.

OsHSP70 downregulates Cu^{2+}-induced MBP kinase activities

Recent study suggests that the interaction between HSP90 and MAPK is required for N gene-dependent hypersensitive cell death in tobacco (Takabatake et al. 2007). To investigate the effect of HSPs on MAPK activities in the acquired Cu^{2+}-tolerance of rice roots, we first focused on HSP70. HSP70 is one of the major heat shock proteins and can be induced by moderate heat treatment in rice roots (Fig. 3c). To determine whether rice HSP70 is involved in the protection against Cu^{2+}-induced cell death, a rice full-length cDNA clone (NP_001049712) of Arabidopsis *Hsp70* homolog was obtained from the Rice Genome Resource Center (RGRC, Tsukuba, Japan). The *OsHsp70* encodes a protein of 650 amino acids, with 92% identity to Arabidopsis HSP70. The recombinant GST–OsHSP70

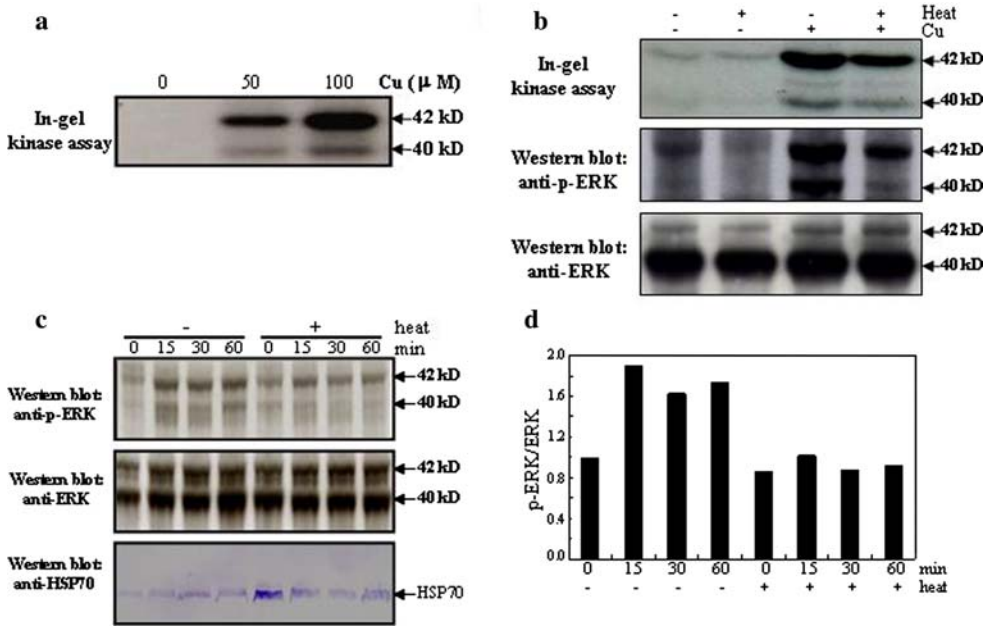

Fig. 3 Inhibition of Cu^{2+}-induced MAPKs activities by heat shock pretreatment. **a** MAPK activation in rice roots is dose-dependent in response to $CuCl_2$ treatment. Proteins were extracted from rice roots, which were exposed to 0, 50, 100 μM of $CuCl_2$ for 1 h. For in-gel kinase assay, root protein extracts (10 μg) were separated by 10% SDS-PAGE embedded with MBP as substrate. MBP phosphorylation was visualized by autoradiography. *Arrows* indicated the kinase-active bands. **b** Heat-shock pretreatment reduces Cu^{2+}-induced MAPKs activities. Rice seedlings were pretreated with or without moderate heat shock (42°C, 1 h) before subsequent treatment with 100 μM of $CuCl_2$. Proteins (10 μg) extracted from rice roots were analyzed by in-gel kinase assay as above. In addition, root protein extracts (10 μg) were separated by 10% SDS-PAGE and detected by immunoblot analysis using anti-phospho-ERK and anti-ERK antibodies, respectively. **c** Time course study of heat shock pretreatment in the inhibition of Cu^{2+}-induced MAPK activation. Rice seedlings were pretreated with or without moderate heat (42°C, 1 h) for different periods of time before subsequent treatment with 100 μM of $CuCl_2$ for 1 h. Protein (10 μg) was extracted from rice roots on different intervals (0, 15, 30, 60 min) after $CuCl_2$ treatment, and were separated by 10% SDS-PAGE and detected by immunoblot analysis using anti-phospho-ERK, anti-ERK, and anti-HSP70 antibodies, respectively. **d** The data obtained from immunoblot experiments (**c**) were analyzed using Photoshop CS3.0 software (Adobe, USA). The basal levels of phosphorylated form of ERK (p-ERK) at time 0 without heat treatment were arbitrarily set to 1; all other values were calculated as multiples of that. The values were corrected for differences in loading based on unphosphorylated ERK (ERK)

fusion protein was overexpressed and purified from *E. coli*. To investigate whether rice HSP70 is directly involved in the reduction of Cu^{2+}-induced MAPK activation, ERK-like kinases were immunoprecipitated from protein extracts of Cu^{2+}-treated rice roots with ERK antibody. The precipitated protein complex was tested for its ability to phosphorylate MBP in vitro in the presence of recombinant GST–OsHSP70 fusion proteins or GST for 30 min at room temperature. As shown in Fig. 4, Cu^{2+} can enhance MAPK activities, but they disappeared in the presence of GST–OsHSP70 fusion protein. In contrast, MAPK activities increased slightly when GST was used as a control. These results indicate that OsHSP70 has an inhibitory effect on Cu^{2+}-induced MAPK activities.

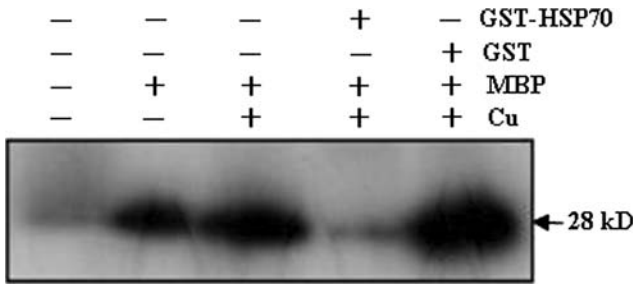

Fig. 4 Recombinant GST–OsHSP70 downregulates Cu^{2+}-enhanced MBP kinase activity. Rice HSP70 fusion protein was overexpressed in *E. coli*. The recombinant GST–HSP70 fusion protein was affinity-purified using glutathione beads. Rice seedlings were untreated or exposed to 100 μM of $CuCl_2$ for 1 h. Proteins (100 μg) extracted from rice roots were immunoprecipitated with anti-ERK antibody. Kinase activity of the immunoprecipitated protein complexes was subsequently assayed by an in vitro kinase assay using MBP as substrate in the presence of proteins (1 μg) from either purified recombinant GST–HSP70 fusion proteins or GST as control. MBP phosphorylation was visualized by autoradiography

Involvement of AtMPK6 and AtMKK2 in the protection of Arabidopsis roots from Cu^{2+} toxicity by heat shock pretreatment

There is little genetic evidence to validate the role of the MAPK signaling pathway in stress response signaling in whole plants. In this study, we used three Arabidopsis MAPK cascade (*mpk3*, *mpk6*, *mkk2*) T-DNA insertion mutant lines (Teige et al. 2004; Nakagami et al. 2006) to

investigate their response to Cu^{2+} stress. The *mpk3* and *mpk6* mutant lines are absent in *MPK3* or *MPK6* transcripts as well as their corresponding proteins resulting from T-DNA disruption (Nakagami et al. 2006). The *mkk2* mutant line carried a single T-DNA insertion in intron 5 of the *MKK2 gene*, leading to mRNA null phenotype (Teige et al. 2004). We found that *mkk2* and *mpk6* null mutant plants were more sensitive to the treatment of 100 μM $CuCl_2$ (Fig. 5a). In contrast, *mpk3* null mutant plants displayed increased tolerance to Cu^{2+} stress when compared to wild-type plants (Fig. 5a). This result suggested that these three Arabidopsis MAPK cascade genes (MKK2, MPK3, MPK6) are involved in basal tolerance of Cu^{2+} stress, and might play an oppositely regulatory role.

To further study whether MAPKs play roles in acquired Cu^{2+}-tolerance, wild type as well as three Arabidopsis MAPK cascade (*mpk3*, *mpk6*, *mkk2*) mutant lines were pretreated under heat shock condition (37°C, 1 h), and subsequently treated with 100 μM $CuCl_2$ for 3 h. Heat shock pretreatment of Arabidopsis seedlings decreased Cu^{2+}-induced cell death (Fig. 5a) and ROS production (Fig. 5b) both in the wild type as well as in the *mpk3* mutant line, but not in *mpk6* and *mkk2* mutant lines. However, both *mpk6* and *mkk2* mutants were more sensitive to Cu^{2+} stress, and exhibited no significant differences with or without heat pretreatment (Fig. 5a, b). This result strongly suggests that AtMKK2 and AtMPK6, but not AtMPK3, participate in heat-induced cellular protection against Cu^{2+} toxicity.

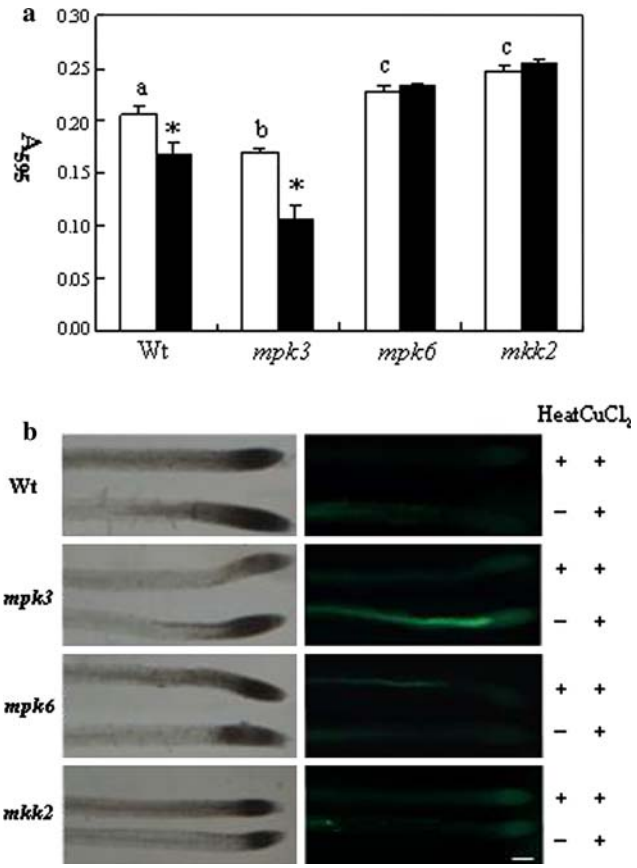

Fig. 5 Effect of Arabidopsis MAPK mutant lines on acquired Cu^{2+}-tolerance. **a** Eight-day-old Arabidopsis seedlings of wild type (Wt) and three MAPK cascade mutant lines (*mpk3*, *mpk6*, and *mkk2*) were pretreated with (*black bar*) or without (*white bar*) moderate heat shock (37°C, 1 h) before 100 μM $CuCl_2$ treatment for 3 h. The cell death was measured by staining with 0.25% Evans blue. Values represent means of three independent experiments. *Bars* SE. The *different letters* and *asterisks* represent a statistically significant difference at $P < 0.05$, according to a paired *t* test. **b** ROS production in three Arabidopsis MAPK cascade mutant lines (*mpk3*, *mpk6*, and *mkk2*) treated with 100 μM $CuCl_2$ with or without heat shock pretreatment (37°C, 1 h), and subsequently labeled with 10 μM CM-H_2DCF-DA for 30 min, the emission level of green fluorescence indicates the amount of ROS production. Ten Arabidopsis seedlings were assayed for each experiment and similar results were observed. *Bar* 100 μm

Discussion

Previously, it has been shown that a short heat treatment preceding heavy-metal (Cd) stress induces a tolerance to metal toxicity in tomato cells (Neumann et al. 1994). To test the effect of transient heat shock on acquired Cu^{2+}-tolerance, we pretreated 6-day-old rice seedlings with moderate heat shock (42°C, 1 h) before excessive $CuCl_2$ treatment. Our results showed that heat shock pretreatment significantly reduced Cu^{2+}-induced cell death in rice roots (Fig. 1b, c). It was also reported that heat shock protein gene expression induced by heat shock was suppressed by treatment with CHX (Mizuno et al. 1997). To investigate whether the protective effect of preheated roots is dependent on a de novo protein synthesis, we incubated rice roots with the protein synthesis inhibitor CHX before heat pretreatment to block the de novo protein synthesis. We found that pretreatment with CHX negates the protective effect of heat shock against Cu^{2+} stress (Fig. 1d). Thus, it seems likely that failure of cells to acquire Cu^{2+}-tolerance is accompanied by CHX blocking de novo protein synthesis of HSPs after heat shock pretreatment. Based on this result, we suggest that heat shock pretreatment protects rice root cells from Cu^{2+} toxicity through inducing the expression of HSPs.

The excessive production of ROS is one of the important factors responsible for plant cell death upon exposure to harmful abiotic and biotic stresses like extreme temperature, UV light, drought, heavy metals, and pathogen infections (Yuasa et al. 2001; Yeh et al. 2007). ROS have been shown to activate MAPK pathways in mammals as well as in plants (Wang et al. 2003; Liu et al. 2007) and are involved in Cu^{2+}-induced MAPK activation in rice root cells (Yeh et al. 2007). Cu^{2+}, like Fe^{2+}, belongs to a group of transition metals, which may induce oxidative stresses via Fenton-type reactions (Wang et al. 2003). To further

test whether heat shock pretreatment affects Cu^{2+}-tolerance through ROS production in rice roots, we treated rice roots with the compound, CM-H_2DCF-DA, a molecular probe of ROS. The results showed that heat shock pretreatment significantly reduced ROS production, which is normally stimulated by Cu^{2+} (Fig. 2). Therefore, it is possible that protection against cell death from Cu^{2+} toxicity by heat pretreatment in rice roots results from a reduction of oxidative stress.

In mammalian cells, it was demonstrated that exposure to heavy metals like As, Cr, Cu, V, and Zn resulted in activation of the ERK, JNK, and P38 MAPK pathways (Samet et al. 1998). It has also been reported that MAPK activation is required for stress-induced apoptosis in human cells (Verheij et al. 1996). In plants, Zhang and Liu (2001) demonstrated that increases of salicylate-induced protein kinase (SIPK) activity alone induce defense gene activation and cell death in tobacco. Previously, it was also found that Cd, Cu, Zn, and Fe could activate MAPKs phosphorylation in rice and alfalfa plants (Yeh et al. 2003, 2007; Jonak et al. 2004; Lin et al. 2005; Tsai and Huang 2006). Moreover, the Cu-induced MAP kinase activation required the involvement of NADPH oxidases, Ca^{2+}-dependent protein kinase (CDPK) and phosphatidylinositol 3-kinase (PI3 kinase). Inactivation of MAPKs was shown to be able to protect cells against environmental stresses (Verheij et al. 1996). In our study, heat shock pretreatment of rice seedlings also led to inhibition of MAPK activation in response to the challenge of excessive $CuCl_2$ stress (Fig. 3b–d). While MAPKs became activated after 15 min of Cu^{2+} treatment in rice root cells, and maintained their activities for 1 h, in preheated cells, MAPK activity subsided at 30 min. This suggested that the protective effect of the heat shock pretreatment is related to a suppression of MAPK activation. Thus, prevention of MAPK activation in preheated rice root cells may be a significant factor in the phenomenon of acquired Cu^{2+}-tolerance.

Induction of a rapid increase in the synthesis of a family of proteins, the so-called heat shock proteins (HSPs), is essential for acquired thermotolerance. HSPs comprise a group of highly conserved proteins that can be induced upon subjecting organisms to high temperature stress. HSPs are molecular chaperones and play critical roles in cellular homeostasis under both normal and adverse growth conditions. HSP70 is one of the major inducible HSPs (Wu 1995). At least two functional roles for HSP70 have been demonstrated in allowing organisms to survive stress. For instance, HSP70 promotes protein folding and assembly from the heat stress-denatured/damaged cellular proteins. Secondly, HSP70 prevents cell death from stress that does not cause detectable protein damage. It is likely that HSP70 can somehow interfere with the apoptotic program initiated by stress factors, thus allowing cells to survive. Mosser et al. (2000) found that HSP70 can affect the apoptotic pathway at the levels of both cytochrome c release and initiator caspase activation. In addition, HSP72 reduces caspase-3-mediated proteolysis of focal adhesion kinase (FAK), an antiapoptotic protein, which is an early target of injury in cells exposed to metabolic inhibitors (Mao et al. 2003). Moreover, increasing levels of HSP70 could prevent apoptosis in a variety of stresses by suppressing JNK activation (Jaattela and Wissing 1993; Rosette and Karin 1996; Gabai et al. 1997; Buzzard et al. 1998). Further studies have suggested two functional domains of HSP70, a chaperone function, and an ATPase function, respectively, which are involved in inhibiting distinctive cellular apoptotic pathways induced by various stress factors. For example, in TNF-induced apoptosis of human fibroblasts, HSP72 specifically interferes with the Bid-dependent apoptotic pathway via inhibition of c-jun N-terminal kinase (JNK), and the chaperone activity of HSP72 is dispensable for suppression of TNF-induced apoptosis (Gabai et al. 2002). In addition, HSP70 blocks heat-induced apoptosis primarily by inhibiting Bax activation and thereby preventing the release of proapoptotic factors from mitochondria, in which both the chaperone and ATPase functions of HSP70 are required for protection from heat shock-induced cell death (Gabai et al. 2002; Stankiewicz et al. 2005; Ruchalski et al. 2006). The ATPase domain of HSP70 is critical for sequestering AIF in the cytosol to prevent nuclear injury and apoptosis in ATP-depleted renal cells (Ruchalski et al. 2003, 2006). Taken together, HSP70 can directly or indirectly interact with the cellular signaling molecules leading to apoptotic pathways to prevent cell death induced by various stress factors. To investigate whether a high level of HSP70 directly affects activation of MAPK in plants, we tested the effect of a purified recombinant OsHSP70 on the activation of MAPK in vitro. *OsHsp70* (NP_001049712) is highly homologous to the cytosolic *Hsp70* (BAB02269) of *Arabidopsis thaliana* in which *AtHsp70* could be highly induced by heat and cold stress (Sung et al. 2001). We thus overexpressed the recombinant GST–OsHSP70 in *E. coli* and examined its protective effects under heavy metal stress. In an in vitro MAPK kinase assay, MAPK was activated in rice roots upon treatment with Cu^{2+} for 1 h (Fig. 4). However, additions of purified recombinant GST–OsHSP70 instead of GST protein repress the activation of MAPKs (Fig. 4). According to the results of our in vitro assay, it is probable that the level of HSP70 in a cell directly regulates the activity of stress-activated kinases in plants.

MAPK cascades play important roles for plants in responses to multiple stresses, including heat and heavy metals (Sangwan et al. 2002; Link et al. 2002; Yeh et al. 2007). There are multiple members of kinases in a cell, which contribute to the specificity of the transmitted signal

(Zhang and Klessig 2001). Arabidopsis MPK6 (AtMPK6) is the ortholog of a tobacco MAPK, termed salicylate-induced protein kinase (SIPK). A number of environmental factors—such as pathogen elicitors, oxidative stress, low temperature, low humidity, hyperosmolarity, and physical stress—lead plants to a rapid and transient activation of AtMPK6 (Ichimura et al. 2000; Nuhse et al. 2000; Yuasa et al. 2001). MKK2 is the specific activator of MPK6 in Arabidopsis, and it is involved in cold and salt stresses response (Teige et al. 2004). In this study, we used three Arabidopsis MAPK cascade (*mpk3*, *mpk6*, and *mkk2*) mutant lines to investigate their roles in acquired Cu^{2+}-tolerance by heat shock pretreatment. As shown in Fig. 5, *mpk3* null mutant displayed increased Cu^{2+}-tolerance compared to wild-type plants. In contrast, *mpk6* and *mkk2* null mutants were more sensitive to Cu^{2+} stress. The results suggest that MPK3, MPK6, and MKK2 participate in basal Cu^{2+}-tolerance. However, MKK2 and MPK6 rather than MPK3 are involved in heat-induced cellular protection against Cu^{2+} toxicity (Fig. 5). The results further support that Cu^{2+}-tolerance is a complex multigenic process, with distinctive gene sets involved in basal and acquired Cu^{2+}-tolerance.

Taken together, we have demonstrated that heat shock pretreatment might enhance Cu^{2+}-tolerance in rice roots through the induction of HSP expression, which in turn, reduces the activation of MAPKs by excessive Cu^{2+} stress. We have also shown that OsHSP70 fusion proteins directly inhibited the activation of Cu^{2+}-induced MAPK in rice roots. The suppression of MAPK activities by OsHSP70 reveals a new phenomenon of acquired Cu^{2+}-tolerance in plants. Therefore, we propose the hypothesis that heat shock pretreatment probably initiates two cellular processes: it rapidly and transiently activates MAPK to a relatively high level but insufficiently turns on the cell death program. On the other hand, it induces accumulation of HSP70 and other HSPs. Subsequently, under excessive Cu^{2+} stress, the accumulated HSP70 suppresses the activation of Cu^{2+}-induced MAPKs and prevent cells from dying. However, further study of the interaction between heat shock proteins and MAPKs is necessary to unveil the mechanism of acquired Cu^{2+}-tolerance.

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