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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.

Keyword words: Copper ion · Metal stress · Heat shock protein70 (HSP70) · Mitogen-activated protein kinase (MAPK)

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).

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.

**Materials and Methods**


**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 100µM 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 (100µM). 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 dye, CM-H$_2$DCFDA. 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$_2$ (100µM) 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 upon heat pretreatment is well correlated to the decrease in the activation of 40 and 42 kDa MBP kinase activities by CuCl₂.

The time-dependent effect of heat shock pretreatment on the activation of these MAPKs by CuCl₂ treatment was also investigated. The phosphorylated forms of 40 and 42 kDa MAPK increased when rice roots were exposed to 100 µM CuCl₂ for 1 h (Fig. 3c, d). However, the time-dependent activation of MAPK activities was affected by heat shock pretreatment. Cu²⁺ 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 CuCl₂ treatment in preheated roots. This suggested that heat shock pretreatment blocks Cu²⁺-induced MAPK activations within 30 min.

**OsHSP70 downregulates Cu²⁺-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 Cu²⁺-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 Cu²⁺-induced cell death, a rice fulllength 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 fusion protein was overexpressed and purified from E. coli. To investigate whether rice HSP70 is directly involved in the reduction of Cu²⁺-induced MAPK activation, ERK-like kinases were immunoprecipitated from protein extracts of Cu²⁺-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, Cu²⁺ 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 Cu²⁺-induced MAPK activities.

**Involvement of AtMPK6 and AtMKK2 in the protection of Arabidopsis roots from Cu²⁺ toxicity by heat shock pretreatment**

There is little genetic evidence to validate the role of the MAPK signaling pathway
in stress response signaling in 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 TDNA 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 100µM CuCl$_2$ (Fig. 5a). In contrast, mpk3 null mutant plants displayed increased tolerance to Cu$^{2+}$ stress when compared to wildtype 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.

**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\(_2\)DCF-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.

**Acknowledgments**

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References

Possible involvement of MAP kinase pathways in acquired metal-tolerance.
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Fig. 1. The cell death effect of rice roots among the interaction of CHX, heat shock, and CaCl₂ 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 CaCl₂ for 3 h. b) Rice seedlings were pretreated with or without moderate heat shock (42°C, 1 h) before 50 or 100 μM of CaCl₂ treatment. c) Rice seedlings were pretreated with (right panel) or without (left panel) heat shock (42°C, 1 h) before 0, 50, or 100 μM of CaCl₂ treatment. Cellular viability was observed under fluorescent microscope after staining roots with FDA. Bars: 100 μm. d) Rice seedlings were pretreated with 100 μM CHX, prior to heat shock and subsequent 50 μM CaCl₂ treatments. Values in a, b, and d represent means of three independent experiments. Bars: SE. The asterisk represents statistically significant difference at *P < 0.05, according to paired t-test.

Fig. 2. ROS production in rice roots under Ca²⁺ stress with or without heat shock pretreatment. Rice roots were labeled with 50 μM CMH₂DCF-DA for 30 min, and the emission level of green fluorescence indicates the amount of ROS production after following treatments: a) without CaCl₂ treatment; b) with 100 μM CaCl₂ treatment at room temperature for 15 min; c) heat shock pretreatment (42°C, 1 h), but subsequently without CaCl₂ treatment; d) heat shock pretreatment (42°C, 1 h), and subsequently with 100 μM CaCl₂ treatment. Ten rice seedlings were assayed for each experiment and similar results were observed. Bars: 100 μm.
Fig. 3. Inhibition of Cu⁺⁺-induced MAPKs activities by heat shock pretreatment. a MAPK activation in rice roots is dose-dependent in response to CuCl₂ treatment. Proteins were extracted from rice roots, which were exposed to 0, 50, 100 μM of CuCl₂ for 1 h. For in-gel kinase assay, root protein extracts (10 μg) were separated by 10% SDS-PAGE, and MBP was used as substrate. MBP phosphorylation was visualized by autoradiography. Arrows indicate the kinase-active bands. b Heat-shock pretreatment inhibits Cu⁺⁺-induced MAPKs activities. Rice seedlings were pretreated with or without moderate heat (42°C, 1 h) before subsequent treatment with 100 μM of CuCl₂. 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.

e Time course study of heat shock pretreatment in the inhibition of Cu⁺⁺-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 CuCl₂ for 1 h. Proteins (10 μg) were extracted from rice roots on different intervals (0, 15, 30, 60 min) after CuCl₂ 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 were analyzed using the software P33.0 (Adobe, USA). The band levels of phosphorylated form of ERK (p-ERK) at each time point were calculated as multiples of the values centered for differences in loading based on unphosphorylated ERK (ERK).

Fig. 4. Recombinant GST–OsHSP70 downregulates Cu⁺⁺-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 CuCl₂ 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 0.1 μM ATP and 1 μg of either purified recombinant GST–HSP70 fusion protein or GST as control. MBP phosphorylation was visualized by autoradiography.
Fig. 5 Effect of Arabidopsis MAPK mutant lines on acquired Cu²⁺-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 CuCl₂ 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 CuCl₂ with or without heat shock pretreatment (37°C, 1 h), and subsequently labeled with 10 μM CM-H₂DCF-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