Heavy Metal Stress. Activation of Distinct Mitogen-Activated Protein Kinase Pathways by Copper and Cadmium[1]

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Excessive amounts of heavy metals adversely affect plant growth and development. Whereas some regions naturally contain high levels of heavy metals, anthropogenic release of heavy metals into the environment continuously increases soil contamination. The presence of elevated levels of heavy metal ions triggers a wide range of cellular responses including changes in gene expression and synthesis of metal-detoxifying peptides. To elucidate signal transduction events leading to the cellular response to heavy metal stress we analyzed protein phosphorylation induced by elevated levels of copper and cadmium ions as examples for heavy metals with different physiochemical properties and functions. Exposure of alfalfa (*Medicago sativa*) seedlings to excess copper or cadmium ions activated four distinct mitogen-activated protein kinases (MAPKs): SIMK, MMK2, MMK3, and SAMK. Comparison of the kinetics of MAPK activation revealed that SIMK, MMK2, MMK3, and SAMK are very rapidly activated by copper ions, while cadmium ions induced delayed MAPK activation. In protoplasts, the MAPK kinase SIMKK specifically mediated activation of SIMK and SAMK but not of MMK2 and MMK3. Moreover, SIMKK only conveyed MAPK activation by $CuCl_2$ but not by $CdCl_2$. These results suggest that plants respond to heavy metal stress by induction of several distinct MAPK pathways and that excess amounts of copper and cadmium ions induce different cellular signaling mechanisms in roots.

Heavy metal ions play essential roles in many physiological processes. In trace amounts, several of these ions are required for metabolism, growth, and development. However, problems arise when cells are confronted with an excess of these vital ions or with nonnutritional ions that lead to cellular damage (Avery, 2001; Schützendübel and Polle, 2002; Gaetke and Chow, 2003; Polle and Schützendübel, 2003). Heavy metal toxicity comprises inactivation of biomolecules by either blocking essential functional groups or by displacement of essential metal ions (Goyer, 1997). In addition, autoxidation of redox-active heavy metals and production of reactive oxygen species (ROS) by the Fenton reaction causes cellular injury (Stohs and Bagchi, 1995).

In response to toxic levels of heavy metals, plants synthesize Cys-rich, metal-binding peptides including phytochelatins and metallothioneins. Therefore, heavy metals can be detoxified by chelation and sequestration in the vacuole (Clemens, 2001; Cobbett and Goldsbrough, 2002), and various membrane transport systems play an important role in metal ion homeostasis and tolerance (Hall and Williams, 2003).

Gene expression patterns change when plants encounter excessive amounts of heavy metals. Cadmium- and copper-responsive genes have been shown to code for signal transduction components, such as the Arabidopsis mitogen-activated protein kinase kinase kinase (MAPKKK) MEKK1, transcription factors, stress-induced proteins, proteins participating in protein folding, and sulfur and glutathione metabolism (Xiang and Oliver, 1998; Suzuki et al., 2001; Louie et al., 2003).

Mitogen-activated protein kinase (MAPK) pathways represent an evolutionary conserved signaling mechanism in eukaryotes (Gustin et al., 1998; Xu, 2000; Pearson et al., 2001; Ichimura et al., 2002; Agrawal et al., 2003). Diverse signal transduction pathways use MAPKs to regulate a variety of cellular functions in response to different extracellular stimuli. MAPKs are part of a phosphorylation cascade that is composed of three sequentially activated protein kinases: MAPKKK, MAPK kinase (MAPKK), and MAPK (Schaeffer and Weber, 1999; Widmann et al., 1999). MAPKKKs are Ser/Thr protein kinases that phosphorylate and thereby activate MAPKKs. MAPKKs in turn are dual-specific kinases that phosphorylate MAPKs on a Thr and Tyr residue. The dual phosphorylation of MAPKs renders the enzymes active. MAPKs are Pro-directed Ser/Thr kinases phosphorylating numerous substrates in different cellular compartments. In this way, information is transduced in the form of a phosphorylation cascade from upstream kinases to downstream targets.

Experimental evidence from different plant species indicates that several MAPK pathways are activated in response to environmental challenges, hormones, and during cell division (Tena et al., 2001; Jonak et al.,

2002). For example, in alfalfa (*Medicago sativa*), the MAPKs SIMK and SAMK are involved in the response to pathogen-associated stimuli as well as to abiotic stresses like mechanical stimulation, wounding, drought, and cold (Bögre et al., 1996, 1997; Jonak et al., 1996; Cardinale et al., 2000). Pathogen-derived elicitors also activate MMK2 and MMK3 (Cardinale et al., 2000). Interestingly, MMK3 plays a role not only in pathogen response but also in cytokinesis (Bögre et al., 1999). Moreover, the ethylene precursor amino-cyclopropane-1-carboxylic acid triggers SIMK and MMK3 pathways (Ouaked et al., 2003).

Despite our knowledge regarding ion toxicity and detoxification mechanisms, information about signal transduction induced by heavy metal stress is scarce. In this study, we therefore analyzed protein phosphorylation events in roots of alfalfa in response to excess levels of heavy metals. We report on the identification of several distinct MAPK pathways that are activated in response to copper and cadmium stress.

RESULTS

$CuCl_2$ and $CdCl_2$ Stress Induces Myelin Basic Protein Kinases in Roots

To investigate protein phosphorylation events during the plant response to heavy metal stress we studied heavy metal-induced protein kinase activities in roots of alfalfa. We analyzed activation of protein kinases by $CuCl_2$ as a model for a redox-active micronutrient that is toxic at supraoptimal concentrations and by $CdCl_2$ as a toxic, nonredox, and nonessential metal.

Alfalfa seedlings were challenged for 30 min with increasing concentrations of $CuCl_2$ or $CdCl_2$. Protein extracts from roots of these plants were analyzed for changes in protein kinase activities by in gel-kinase assays using myelin basic protein (MBP) as substrate. As shown in Figure 1, exposure of plants to more than 50 μM $CuCl_2$ activated protein kinases with relative molecular masses of 46 and 44 kD. $CdCl_2$ induced the activity of a 46-kD protein kinase at a concentration of 1 μM, whereas robust activation of protein kinases of 46 and 44 kD were observed at 50 μM $CdCl_2$. These data suggest that heavy metal stress leads to the activation of MBP-phosphorylating protein kinases in roots.

Distinct MAPKs Are Activated by $CuCl_2$ and $CdCl_2$ in a Dose-Dependent Manner

The sizes of the $CuCl_2$- and $CdCl_2$-responsive MBP-protein kinases are reminiscent of MAPKs. To investigate whether the 46 and 44 kD protein kinases correspond to known MAPKs from alfalfa, immuno-kinase assays were performed on plants exposed to $CuCl_2$ or $CdCl_2$ using antibodies that are specific for distinct MAPKs. Root protein extracts were prepared from plants exposed to increasing concentrations of $CuCl_2$ or $CdCl_2$ before immunoprecipitation with the
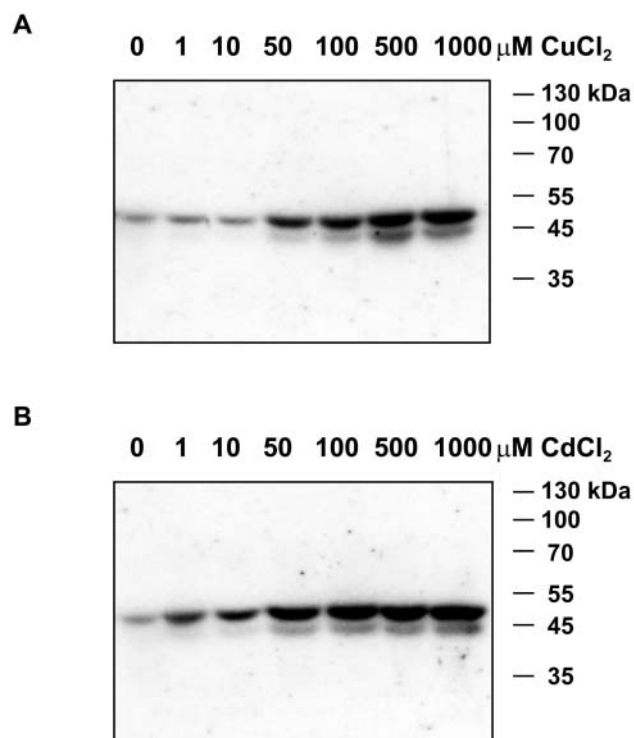

Figure 1. $CuCl_2$ and $CdCl_2$ induce the activation of 44- and 46-kD protein kinases. Alfalfa roots of hydroponically grown plants were exposed for 30 min to 0, 1, 10, 50, 100, 500, and 1,000 μM $CuCl_2$ (A) or $CdCl_2$ (B). In-gel kinase assays were performed with 20 μg of protein extracts using MBP as substrate.

antibodies M23, M11, M14, and M24 that specifically recognize SIMK, MMK2, MMK3, and SAMK, respectively (Munnik et al., 1999; Cardinale et al., 2000). Subsequent kinase assays using MBP as substrate revealed that the activity of SIMK, MMK2, MMK3, and SAMK increased in response to $CuCl_2$ or $CdCl_2$. Concentrations higher than 50 μM $CuCl_2$ induced the activity of SIMK, MMK2, and MMK3, and to a lesser extent SAMK (Fig. 2A). Immunokinase assays of $CdCl_2$-treated roots showed similar dose-dependent MAPK activation (Fig. 2B). These results indicate that plants respond to elevated $CuCl_2$ and $CdCl_2$ concentrations by activation of at least four distinct MAPK pathways.

$CuCl_2$ and $CdCl_2$ Induce MAPK Activation with Different Kinetics

Depending on the plant organ and the extracellular stimulus, the activity of MAPKs can either transiently or constitutively increase. Thus, the kinetics of SIMK, MMK2, MMK3, and SAMK activation was analyzed in response to excess $CuCl_2$ or $CdCl_2$. Application of 100 μM $CuCl_2$ to alfalfa roots rapidly activated SIMK (Fig. 3A, left). SIMK was activated within 5 min and showed maximum activity between 10 and 60 min. Although SIMK activity decreased at later time points, considerable activity was still detected at 180 and
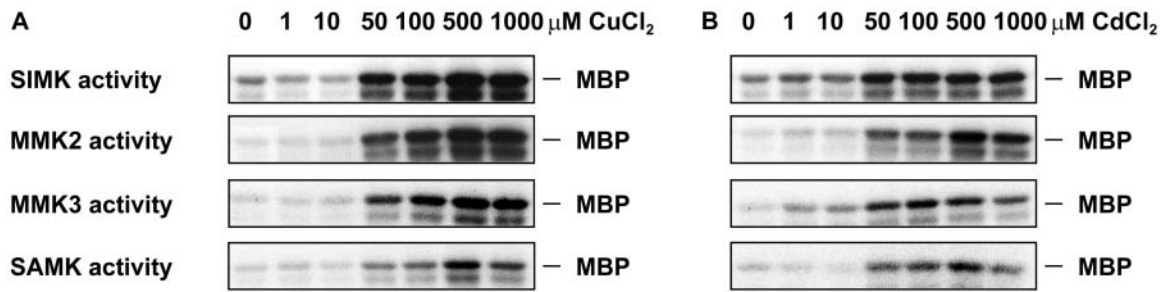
Figure 2. Concentration-dependent activation of MAPKs by CuCl$_2$ and CdCl$_2$. Seedlings were exposed to increasing concentrations of CuCl$_2$ or CdCl$_2$ for 30 min. Protein extracts from roots were immunoprecipitated with M23, M11, M14, or M24 antibodies that are specific for SIMK, MMK2, MMK3, and SAMK, respectively. Subsequently, kinase activities were determined by in vitro kinase assays with γ-[^{32}P]ATP and MBP as substrate. The experiment was performed independently three times showing similar results.

360 min. MMK2, MMK3, and SAMK showed transient activation profiles with a maximum between 10 and 60 min.

When alfalfa seedlings were treated with 100 μM CdCl$_2$, the time course of SIMK activation showed a similar but delayed profile as after treatment with CuCl$_2$ (Fig. 3A, right). While CuCl$_2$ treatment induced maximal SIMK activity within 10 min, maximal activation of SIMK by CdCl$_2$ was observed only at 30 to 60 min after exposure to CdCl$_2$. Similarly, activation of MMK2, MMK3, and SAMK occurred later in response to CdCl$_2$ than to CuCl$_2$. Application of the corresponding amount of water did not activate SIMK, MMK2, MMK3, or SAMK during the experimental period (data not shown).

To determine the MAPK protein levels upon CdCl$_2$ and CuCl$_2$ treatments, aliquots of the same cell extracts that were used for the immunokinase assays (Fig. 3A) were used for immunoblotting. M23, M11, M14, and M24 antibodies detected protein bands of 46, 44, 44, and 44 kD corresponding to the SIMK, MMK2, MMK3, and SAMK proteins, respectively (Fig. 3B). In contrast to the CuCl$_2$- and CdCl$_2$-induced changes in protein kinase activities, the steady-state levels of the proteins
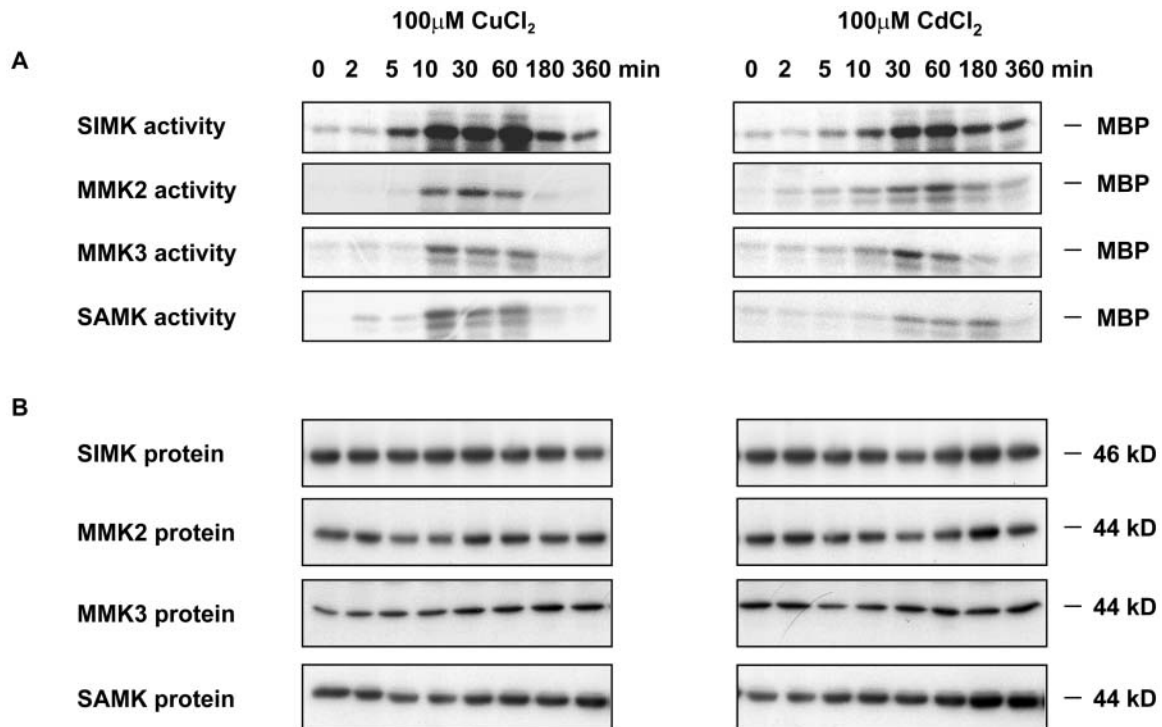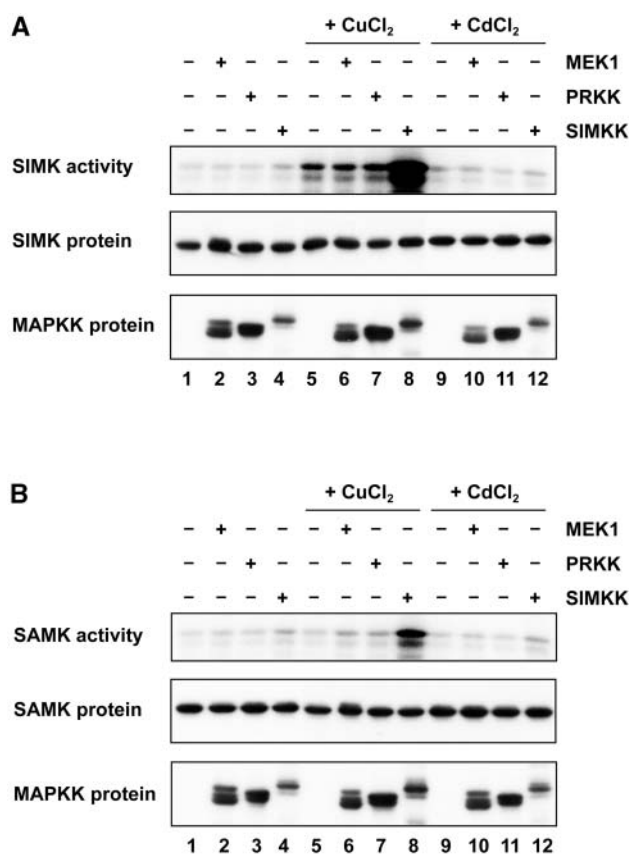

Figure 3. Differential activation of MAPKs by CuCl$_2$ and CdCl$_2$. Seedlings were treated with 100 μM CuCl$_2$ or CdCl$_2$. Roots were harvested at the given time points. A, Immunokinase assays were performed with M23, M11, M14, or M24 antibodies that specifically recognize SIMK, MMK2, MMK3, or SAMK, respectively. B, The same protein extracts were used for protein gel-blot analysis with M23, M11, M14, or M24 antibodies. The experiment was repeated twice revealing similar kinetics in all cases.
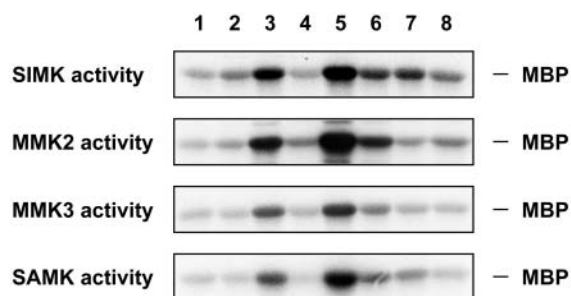
remained constant over the experimental period. These data suggest that the $CuCl_2$- and $CdCl_2$-induced activation of SIMK, MMK2, MMK3, and SAMK occurred by posttranslational mechanisms.

SIMKK Mediates the Activation of SIMK and SAMK by $CuCl_2$ in Vivo

MAPKs are activated by distinct MAPKKs in response to different stimuli. To identify the corresponding upstream component of SIMK, MMK2, MMK3, and SAMK in response to excess copper and cadmium, different MAPKKs were tested for their ability to mediate $CuCl_2$- and $CdCl_2$-induced MAPK activation. For this purpose, hemagglutinin (HA)-tagged versions of SIMK, MMK2, MMK3, and SAMK were transiently expressed in Arabidopsis protoplasts in the

Figure 5. Ion-specific MAPK activation. Seedlings were exposed to (1) water or 100 μM (2) $Al_2(SO_4)_3$, (3) $CdCl_2$, (4) $CoCl_2$, (5) $CuCl_2$, (6) $FeCl_2$, (7) $Pb(NO_3)_2$, or (8) $ZnCl_2$ for 30 min. Root protein extracts were used to immunoprecipitate SIMK, MMK2, MMK3, and SAMK with M23, M11, M14, or M24 antibodies, respectively. Subsequently, kinase activities were determined using MBP as substrate. Independent repetitions of the experiment showed the same activity profile.

Figure 4. SIMKK enhances the activation of SIMK and SAMK by $CuCl_2$. SIMK-HA (A) and SAMK-HA (B) were either expressed alone (1, 5, and 9) or coexpressed with myc-tagged MEK1 (2, 6, and 10), PRKK (3, 7, and 11), or SIMKK (4, 8, and 12) in protoplasts. The protoplasts were treated either for 30 min with 100 μM $CuCl_2$ (lanes 5–8), 100 μM $CdCl_2$ (lanes 9–12), or as control with equal amount of water (lanes 1–4). Subsequently, SIMK and SAMK activities were determined by immunokinase assays using anti-HA antibodies for immunoprecipitation and MBP as substrate. The same protein extracts were analyzed for the presence of the coexpressed proteins by protein gel-blot analysis with anti-HA or anti-Myc antibodies. Three independent experiments yielded comparable results.

absence or presence of the three myc-tagged MAPKKs MEK1, PRKK, or SIMKK.

SIMK, MMK2, MMK3, and SAMK activity was measured from control, $CuCl_2$-, and $CdCl_2$-treated protoplasts by immunokinase assays using an anti-HA antibody. In nontreated cells that expressed the MAPKs alone, extremely low kinase activity was detected (Fig. 4, A and B, lane 1). Incubation of protoplasts with 100 μM $CuCl_2$ induced a moderate activation of SIMK (Fig. 4A, lane 5). Coexpression of SIMK with SIMKK strongly enhanced SIMK activation by $CuCl_2$ (Fig. 4A, lane 8), while MEK1 and PRKK did not have any effect on SIMK activity (Fig. 4A, lanes 6 and 7). Analysis of cells coexpressing SAMK and MEK1, PRKK, or SIMKK revealed that $CuCl_2$-induced activation of SAMK is mediated by SIMKK, but not by MEK1 or PRKK (Fig. 4B, lane 8). In contrast, MMK2 and MMK3 activation by copper could not be stimulated by SIMKK, MEK1, or PRKK (data not shown).

Exposure of protoplasts expressing the MAPKs alone or coexpressing MEK1, PRKK, or SIMKK to 100 μM $CdCl_2$ did not activate SIMK, MMK2, MMK3, or SAMK (Fig. 4, A and B, lanes 9–12; data not shown), indicating that neither MEK1, PRKK, nor SIMKK can convey $CdCl_2$-induced activation of SIMK, MMK2, MMK3, or SAMK.

As shown by protein gel-blot analysis of the same cell extracts that were used for the SIMK and SAMK immunokinase assays (Fig. 4, A and B), equal amounts of SIMK and SAMK were present (Fig. 4, A and B, lower sections), suggesting that SIMK and SAMK were activated by SIMKK in response to $CuCl_2$.

Metal Ion-Specific MAPK Activation

To determine whether MAPK activation is a general response to elevated amounts of metal ions or specific for copper and cadmium, we exposed alfalfa seedlings for 30 min to 100 μM of $Al_2(SO_4)_3$, $CdCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_2$, $Pb(NO_3)_2$, or $ZnCl_2$. Analysis of MAPK activity levels in roots by immunokinase assays showed that
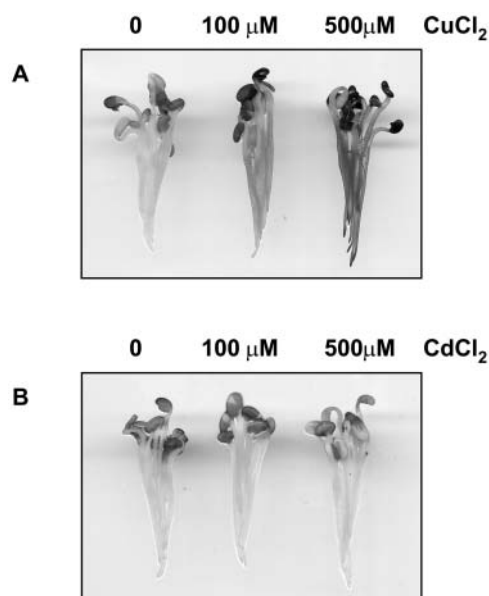
Figure 6. Excess levels of $CuCl_2$ induce hydrogen peroxide accumulation. DAB staining of alfalfa roots exposed to water, 100 μM and 500 μM $CuCl_2$ (A), or 100 μM and 500 μM $CdCl_2$ (B). The experiment was repeated five times showing comparable stainings.

SIMK activity was highly induced by $CdCl_2$ and $CuCl_2$ (Fig. 5, lanes 3 and 5, respectively) and slightly induced by $FeCl_2$ and $Pb(NO_3)_2$ (Fig. 5, lanes 6 and 7, respectively). Little or no increase in SIMK activity was observed after incubation with $Al_2(SO_4)_3$, $CoCl_2$, or $ZnCl_2$ (Fig. 5, lanes 2, 4, and 8, respectively). MMK2, MMK3, and SAMK were also most strongly activated by $CuCl_2$ and $CdCl_2$, but showed some differences in response to treatment with the other metals. Whereas MMK2 was also significantly activated by $FeCl_2$, MMK3 and SAMK poorly responded to this metal (Fig. 5, lane 6). The significance of these observations is presently unclear and requires further studies. Taken together, these data indicate that SIMK, MMK2, MMK3, and SAMK are specifically activated in response to excess levels of particular heavy metal ions.

DISCUSSION

Heavy metals are potentially highly toxic to all organisms including animals and plants. Numerous studies on the physiological responses to excess amounts of heavy metal ions indicate that plants have developed various mechanisms to cope with this environmental threat. Until now, however, the cellular mechanisms of heavy metal stress-induced signaling remained elusive. In this study we have analyzed the role of protein kinases in roots that experience heavy metal stress. We demonstrate that in response to elevated levels of copper and cadmium ions four distinct MAPK pathways are activated. Excess of copper ions rapidly activated SIMK, MMK2, MMK3, and SAMK, while activation of the four MAPKs to

cadmium ions showed similar but delayed profiles. In transient expression assays copper-induced activation of SIMK and SAMK, but not of MMK2 or MMK3, was specifically mediated by SIMKK.

Copper and cadmium are heavy metals with different physiochemical properties and functions. Copper is a vital micronutrient essential for normal plant growth and development. It is a cofactor for many physiological processes including photosynthesis, respiration, superoxide scavenging, ethylene sensing, and lignification. However, excess levels of copper are harmful due to the production of ROS by autoxidation and Fenton reactions.

Cadmium has no known biological function. It is highly reactive and inactivates various enzymatic processes. Consequently, cadmium is generally toxic for all living cells. Although cadmium does not directly interfere with cellular redox reactions it causes oxidative injury. The displacement and thereby the release of redox-active metal ions from various biomolecules as well as the depletion of the antioxidant system by cadmium disturbs the redox balance of the cell.

Since both copper and cadmium can disturb the redox control of the cell, it could be speculated that MAPK activation by excess copper and cadmium is mediated by ROS. Evidence for activation of MAPKs by ROS has been provided by several studies in different plant species (Kovtun et al., 2000; Desikan et al., 2001; Yuasa et al., 2001; Nakagami et al., 2004; Rentel et al., 2004). A possible explanation for the different kinetics of MAPK activation by copper and cadmium ions might therefore stem from the different cellular reaction mechanisms of these two metals. As a redox-active metal, copper ions directly induce the formation of ROS when present at supraoptimal concentrations. In contrast, cadmium ions do not directly
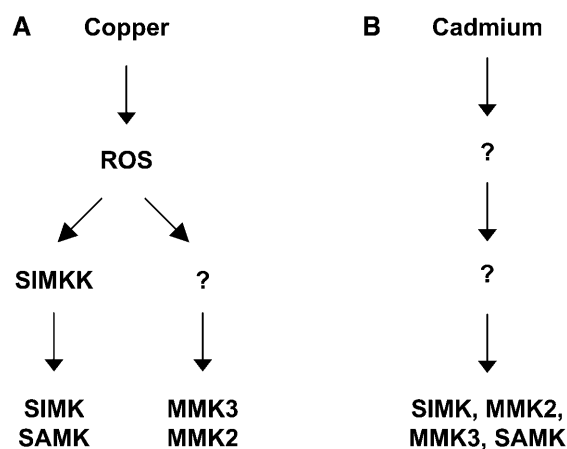
Figure 7. Copper and cadmium-induced MAPK signaling pathways. Excess copper and cadmium ions induce distinct MAPK pathways with different kinetics. A, As a redox-active metal ion, copper leads to the production of ROS that might trigger SIMK and SAMK activation via SIMKK. B, Cadmium activates SIMK, MMK2, MMK3, and SAMK. The upstream components mediating MAPK activation by cadmium remain to be identified.

interfere with cellular oxygen metabolism but cause oxidative injury as a secondary effect. These considerations could suggest that the differential rate of ROS production might account for the delayed induction of SIMK, MMK2, MMK3, and SAMK activity by cadmium. Although this is an attractive hypothesis, the possibility that copper- and/or cadmium-induced activation of some MAPKs is independent of ROS production should not be dismissed and requires further investigation.

Hydrogen peroxide was reported to accumulate in response to excess levels of copper and cadmium (Schützendübel and Polle, 2002). We used 3,3-diaminobenzidine (DAB) stainings to estimate hydrogen peroxide levels in alfalfa roots after treatment with copper or cadmium. $CuCl_2$ induced a dose-dependent hydrogen peroxide production, whereas even prolonged exposure to high concentrations of $CdCl_2$ did not lead to any detectable DAB staining (Fig. 6), indicating that elevated amounts of copper and cadmium ions induce distinct cellular responses in alfalfa. Although very low levels of hydrogen peroxide cannot be visualized by DAB staining, these data make it likely that SIMK, MMK2, MMK3, and SAMK activation by cadmium ions could be mediated by a ROS-independent mechanism.

To investigate the possible involvement of upstream factors in the activation of the four alfalfa MAPKs, three different MAPKKs were coexpressed with the MAPKs in protoplasts before heavy metal stress application. The results revealed that in vivo activation of SIMK and SAMK by excess copper is mediated by SIMKK. It is worth noting that SIMKK also mediates SIMK activation in response to ROS (F. Ouaked and H. Hirt, unpublished data). MEK1 and PRKK could not convey activation of any of the four MAPKs by elevated amounts of copper. Interestingly, copper-induced activation of MMK2 and MMK3 is not mediated by SIMKK, suggesting that multiple signaling components are involved in conveying copper-induced MAPK activations.

In our analysis of the involvement of MAPKKs in cadmium-induced activation of MAPKs, SIMKK did not mediate SIMK and SAMK activation by $CdCl_2$. These results suggest that $CdCl_2$-induced MAPK signaling occurs by a mechanism distinct from that for $CuCl_2$. The different kinetics of MAPK activation by $CuCl_2$ and $CdCl_2$ further support the notion that copper and cadmium ions are transduced by different signaling pathways.

MAPK pathways are involved in many processes, including developmental and hormonal responses (Tena et al., 2001; Jonak et al., 2002). So what is the overall significance of MAPK signaling in response to heavy metal stress? Evidence for a central role of MAPKs in heavy metal stress comes from yeast (Schizosaccharomyces pombe) and animals. In fission yeast, the Sty1/Spc1 MAPK pathway is required for tolerance to many adverse stress conditions, including heavy metals (Degols et al., 1996; Degols and Russell,

1997; Smith et al., 2002). Genetic evidence in Caenorhabditis elegans implicates the MAPKK MEK-1 in heavy metal stress tolerance and nematodes carrying a mek-1 deletion are hypersensitive to copper and cadmium ions (Koga et al., 2000). So far, the evidence for an involvement of a MAPK pathway in heavy metal stress is scarce in plants, but a recent screen for cadmium-responsive genes identified the Arabidopsis MAPKKK MEKK1 to be transcriptionally induced by high concentrations of $CdCl_2$ (Suzuki et al., 2001). Further studies with transgenic and mutant plants will verify the role of distinct MAPK modules during the plant response to copper and cadmium stress.

Taken together, heavy metal stress signaling appears to be highly complex and specific for different metal ions. At least four different MAPK pathways are activated in alfalfa roots in response to excess amounts of copper. Even though SIMK, MMK2, MMK3, and SAMK are also activated by $CdCl_2$, copper- and cadmium-induced stress appear to be mediated by distinct signal transduction pathways (Fig. 7).

Different biotechnological efforts are undertaken to improve plant heavy metal tolerance and to use plants to extract toxic ions from soil. This new, environment-friendly, plant-based technology is known as phytoremediation and focuses on mechanisms of detoxification and homeostasis of heavy metals (Salt et al., 1998; Meagher, 2000). To further improve this technology it is important to understand cellular signaling induced by heavy metal stress. Our findings that distinct MAPK pathways are activated in response to cadmium and copper stress encourage new strategies for improving plant tolerance to heavy metals and phytoremediation.

MATERIALS AND METHODS

Protein Extraction

Roots of hydroponically grown 4-d-old alfalfa plants (Medicago sativa cv Europa) were exposed to indicated concentrations of heavy metals before shock-freezing them in liquid nitrogen. Cell extracts were prepared in extraction buffer (25 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$, 15 mM EGTA, 75 mM NaCl, 1 mM dithiothreitol, 1 mM NaF, 0.5 mM $NaVO_3$, 15 mM p-nitophenyl phosphate, 0.1% Tween 20, 15 mM β-glycerophosphate, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, and 5 μg/mL aprotenin). After centrifugation at 20,000g for 45 min the supernatant was immediately used for further experiments.

In-Gel Protein Kinase Assays

For in-gel protein kinase reactions, cell extracts containing 20 μg of total protein/lane were separated by SDS-PAGE. MBP (0.5 mg/mL) was used as a substrate for the kinase reaction and polymerized in the polyacrylamide gel. Protein denaturation, renaturation, and kinase reactions were performed as described (Usami et al., 1995).

Immunokinase Assays

Immunokinase assays were performed with cell extracts containing 100 μg of total protein as described in Cardinale et al. (2000). The antibodies M23, M11, M14, and M24 were used to specifically immunoprecipitate SIMK, MMK2, MMK3, and SAMK, respectively. The specificity of the antibodies was

previously demonstrated (Munnik et al., 1999; Cardinale et al., 2000). Immunoprecipitations from protoplast protein extracts were performed with anti-HA antibodies.

Immunoblots

For protein gel-blot analysis 15 μg of total protein extracts were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed, either with M23, M11, M14, and M24 antibodies, at a dilution of 1:10,000 (Cardinale et al., 2000) or with HA (Babco/Convance, Denver) and Myc antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:5,000. Alkaline phosphate-conjugated goat anti-rabbit (Santa Cruz Biotechnology) and anti-mouse IgG (Sigma, St. Louis) were used as secondary antibodies, and the reaction was visualized by fluorography using CDP-Star (Amersham Life Science, Buckinghamshire, UK).

Transient Expression Assays

Cloning of HA-tagged SIMK, MMK2, MMK3, and SAMK and myc-tagged SIMKK into the plant expression vector pRT101 was reported in Kiegerl et al. (2000), Cardinale et al. (2002), and Nakagami et al. (2004). The open reading frames of MEK1 and PRKK were fused at their C termini to a double myc epitope and cloned into the vector pRT101. Transient expression experiments were performed with protoplasts from Arabidopsis cells using polyethylene glycol transformation (Ouaked et al., 2003). Twelve to 16 h after transformation with 5 μg DNA of each construct protoplasts were treated with 100 μM $CuCl_2$ or $CdCl_2$ for 30 min and subjected to biochemical analysis.

Hydrogen Peroxide Detection

Hydrogen peroxide accumulation was visualized with DAB according to a procedure adapted from Thordal-Christensen et al. (1997). Briefly, alfalfa seedlings were vacuum infiltrated for 5 min with 1 mg/mL DAB. Subsequently, different concentrations of $CuCl_2$ or $CdCl_2$ were added and DAB staining was assessed visually.

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