Mitogen-activated protein kinase (MAPK) pathways serve as highly conserved central regulators of growth, death, differentiation, proliferation, and stress responses (1–4). A MAPK cascade minimally consists of a MAPKKK-MAPKK-MAPK module that is linked in various ways to upstream receptors and downstream targets. Receptor-mediated activation of a MAPKKK can occur through physical interaction and/or phosphorylation by either the receptor itself, intermediate bridging factors or interlinking MAPKKKs. MAPKKKs are serine/threonine kinases that activate MAPKKs through phosphorylation on two serine/threonine residues in a conserved (S/T)X₁₋₅(S/T) motif (1, 2). In contrast, MAPKKs are dual-specificity kinases that phosphorylate MAPKs on threonine and tyrosine residues in the TXY motif. MAPKs are promiscuous serine/threonine kinases that phosphorylate a variety of substrates including transcription factors, protein kinases, and cytoskeleton-associated proteins (2). Specificity of MAPK cascades functioning within the same cell is generated through the presence of docking domains found in various components of MAPK modules and through a growing number of scaffold proteins (5).

Recently, we have isolated OMTK1 (oxidative stress-activated MAP triple-kinase 1), a novel MAPKKK from alfalfa (Medicago sativa), which among a panel of hormones and stresses tested, was only activated by hydrogen peroxide (H₂O₂) (6). Out of four MAPKs, OMTK1 specifically activated MMK3 resulting in an increased cell death rate. Pull-down analysis between recombinant proteins showed that OMTK1 directly interacts with MMK3 and that OMTK1 and MMK3 are part of a protein complex in vivo. These results indicated that OMTK1 plays a MAPK scaffolding role and functions in activation of H₂O₂-induced cell death in plants. Because of its autotetraploid nature, a genetic analysis of OMTK1 in alfalfa is not possible. Therefore, we investigated the function of MEKK1, which is the most closely related MAPKKK of alfalfa OMTK1, in Arabidopsis. MEKK1 has been implicated in biotic and abiotic stress signaling cascades. MEKK1 transcripts accumulate upon cold, salt and touch stress (7) and a truncated version of MEKK1 activates MAPK pathways induced by the bacterial elicitor flagellin (8) or by cold and salt stress (9). So far, no genetic analysis of the function of MEKK1 was performed. In the present study, knock-out mekk1 mutants were isolated and phenotypically characterized. mekk1 plants showed multiple defects including misregulation of several redox control genes and a lethal phenotype during early leaf development. Biochemical analysis indicated that MEKK1 is regulated by H₂O₂ in a proteasome-dependent manner and regulates ROS-induced MAPK MPK4 activation.

**EXPERIMENTAL PROCEDURES**

Analysis of MEKK1 T-DNA Insertion Lines—The mekk1-1 T-DNA insertion line was isolated from the SALK T-DNA collection (SALK_052557). The T-DNA insertion site was mapped with gene-specific primers LP: 5’-GATTATTCACGAAAC-
ACCGCG-3' and RP: 5'-AGAAATAGCCAAAATCATCAGG-ACC-3' and with a T-DNA specific primer LBA1: 5'-TGGTT-CAGCTAGTGCGCCATCG-3'. Pairs of primers were used to determine if the plants were homozygous or heterozygous for the T-DNA insertion, and the PCR products were sequenced. The mekk1-3 T-DNA insertion line was isolated from the University of Wisconsin pDs-Lox collection (WiscDsLox339H07). The T-DNA insertion site was mapped with gene-specific primers. LP: 5'-GATCATCGGGGATCGTCTTGG-3' and RP: 5'-GAAACTTCCATTTCCATACCCCCC-3' and with a T-DNA specific primer p745-Ws: 5'-AACCTCCGCAATGT-GTTATTAGTTGTC-3'. Pairs of primers were used to determine if the plants were homozygous or heterozygous for the T-DNA insertion, and the PCR products were sequenced. 

**Imaging of Root Hairs—** Sterilized seeds were imbibed at 4 °C for 2–7 days in sterile water to synchronize germination onset and then sown on 1.2% agar 1/2 MS (1% sucrose) in one row of square plates. Size bars were embedded by software analysis of microscopic images with the Cell Profiler analysis software (Olympus DP50 camera and Viewfinder Lite software (Pixera Corporation)). Roots grown synchronously from the second day after sowing. Roots grown synchronously on the surface of agar plates were selected for microscopic analysis. Images of the root hair zone of 7-day-old seedlings were taken with an Olympus BX51 stereo binocular microscope (objective Olympus UplanApo 4× NA 0.16), an Olympus DP50 camera and Viewfinder Lite software (Pixera Corporation). Size bars were embedded by software analysis (Soft Imaging System GmbH).

**H₂O₂ Staining with DAB—** H₂O₂ accumulation in planta was visualized by DAB staining. *Arabidopsis* seedlings were vacuum infiltrated for 2 min with DAB solution (1 mg/ml, pH 3.8) and incubated on a shaker for 1 h. The stain was poured off and chlorophyll removed by incubating overnight in 96% (v/v) ethanol. DAB is polymerized locally in the presence of H₂O₂ giving a visible brown stain.

**Transient Expression Assays—** Transient expression assays were performed with isolated protoplasts from a cultured *Arabidopsis* cell suspension. The isolation, transformation, and cultivation of protoplasts was performed as described (6). The open reading frame of *MEKK1* was fused at its C terminus to a triple HA epitope and cloned into the plant expression vector pRT100. The open reading frame of MPK4 was fused at its C terminus to a triple Myc epitope and cloned into the plant expression vector pRT100. The open reading frame of MPK3 was fused at its C terminus to a triple HA epitope and cloned into the plant expression vector pRT100. The open reading frame of MEKK1 was transiently transformed via polyethylene glycol with 4 μg of plasmid. 12–16 h after transformation, the protoplasts were treated with or without 100 μM proteasome inhibitor MG115, and then treated with or without 2 mM H₂O₂ for 5 min, before extracts were prepared from the protoplasts as described earlier (6) and used for immunokinas assays.

**Protein Extraction—** Cell extracts were prepared in an extraction buffer (25 mM Tris-HCl, pH 7.5, 15 mM MgCl₂, 15 mM EGTA, 75 mM NaCl, 1 mM dithiothreitol, 1 mM NaF, 0.5 mM NaVO₃, 15 mM p-nitrophenylphosphate, 0.1% Tween 20, 15 mM β-glycerophosphate, 0.5 mM phenylmethylsulfonyl fluoride, 5 mg/ml leupeptin, and 5 mg/ml aprotinin). After centrifugation at 20,000 × g for 40 min the supernatant was immediately used for further experiments.

**Immunokines Assays—** Immunokines assays were performed with cell extracts from roots containing 50 μg of total protein as described in Cardinale et al. (10). The supernatant was immunoprecipitated with polyclonal antibodies raised against synthetic peptides encoding the N-terminal 15, C-terminal 16, or C-terminal 7 amino acids of MPK3, MPK4, and MPK6, respectively. Specificities of the antibodies were confirmed by using mpk3, mpk4, and mpk6 mutant plants. Immunoprecipitations from protoplast protein extracts were performed as described previously with anti-Myc or anti-HA monoclonal antibodies (6).

**Immunoblots—** Immunoblots were done according to Nakagami et al. (6), probed either with MPK3, MPK4, and MPK6 antibodies, or with anti-Myc and anti-HA antibodies at a dilution of 1:5,000.

**H₂O₂ and IAA Treatment of Seedlings—** For RT-PCR analysis, sterilized seeds were imbibed at 4 °C for 2–7 days in sterile water to synchronize germination onset and then sown on 1/2 MS, 1% sucrose, 0.7% agar, and incubated in long day conditions (16 h of light/8 h of darkness) at 24 °C. Root growth was monitored starting from the second day after sowing. Seeds grown synchronously on the surface of agar plates were selected for microscopic analysis. Images of the root hair zone of 7-day-old seedlings were taken with an Olympus BX51 stereo binocular microscope (objective Olympus UplanApO 4× NA 0.16), an Olympus DP50 camera and Viewfinder Lite software (Pixera Corporation). Size bars were embedded by software analysis (Soft Imaging System GmbH).

**H₂O₂ Staining with DAB—** H₂O₂ accumulation in planta was visualized by DAB staining. *Arabidopsis* seedlings were vacuum infiltrated for 2 min with DAB solution (1 mg/ml, pH 3.8) and incubated on a shaker for 1 h. The stain was poured off and chlorophyll removed by incubating overnight in 96% (v/v) ethanol. DAB is polymerized locally in the presence of H₂O₂ giving a visible brown stain.

**RT-PCR Analysis—** RNA was isolated from seedlings according to manufacturer’s instruction using TRI reagent (Sigma). Concentration of RNA was determined by measuring OD at 260 nm. First strand cDNA was synthesized with a Revert Aid™ M-MuLV reverse transcriptase (Fermentas). PCRs were performed using following primers. MEKK1, 5'-ATGGGACAGAAATTTCTAGC-TGC-3' and 5'-CTGTCTAGTGTCACAAGGACATA-3'; MPK4, 5'-GGCGGAGGTGTTTCCGAGACCT-3' and 5'-TGAGCCCCAGCAACAACTGTACAG-3'; MPK3, 5'-TTGCC-TTGGCCACACCCGCAAG-3' and 5'-ATCCCGGGGACGTTAGTTGGATTTGATG-3'; MPK6, 5'-ACGTCGACACATGGAGCGTTGTGGTCAGTCAAC-3' and 5'-ATCCCCGGTTGTGATATTTGAGTTAAAGGCTGA-3'; GST1, 5'-AGTTTCCGTCACCAGCTT-C-3' and 5'-AAAGACCTTTGAGACGAG-3'; Zat12, 5'-TATCGGAGATCAGTGCCG-3' and 5'-TGTTCCACCATCTCAGACTCAG-3'; OXI1, 5'-GAATCTCAATTTCTGAGA-3' and 5'-CATTCTGACGGGCAATGTA-3'; APX4, 5'-CTACTAAATCCTGGGAGGCAATG-3' and 5'-TCTGTTGTGCACCTCCTTC-3'; APX5, 5'-AGCTAACCCTGCGGAGGTGG-3' and 5'-GTCCCAAATTGTTAAGGCT-GAGA-3'; tAPX, 5'-CAGAAATGCGGATCTTAGCAACAGG-3' and 5'-AGGC-3', 5'-ATGGCAGACAGCTTCAGCAGTAC-3'; RbohC, 5'-ATGGGACACAGAGCTTCAGCAGTAC-3'; RbohD, 5'-AACGC-3'.
MEKK1 Regulates Accumulation of Reactive Oxygen Species—Because OMTK1 is involved in ROS signaling in alfalfa, we investigated whether MEKK1 might also be part of a ROS-signaling MAPK pathway in Arabidopsis. For this purpose, we determined the accumulation of hydrogen peroxide (H₂O₂) in mekk1 mutants and wild type seedlings using 3,3′-diaminobenzidine (DAB) staining. As seen by the accumulation of brown precipitate, in comparison to wild type plants, mekk1–1 and mekk1–3 mutants accumulated H₂O₂ in cotyledons and true leaves (Fig. 3). As shown in Fig. 3A, young developing leaves accumulate H₂O₂ especially at the tip regions. However, H₂O₂ accumulation occurs at entire region of leaves at later stage (Fig. 3B).

MEKK1 Is Activated by H₂O₂—To test whether MEKK1 could be regulated by H₂O₂, we transiently expressed Myc-tagged MEKK1 under the 35 S Cauliflower Mosaic virus (CaMV) promoter in Arabidopsis protoplasts. Upon plasmid transfection, protoplasts were treated with or without H₂O₂ before MEKK1–Myc was immunoprecipitated by anti-Myc antibody. Subsequently, the kinase activity of MEKK1 was determined using [γ-²³⁵]ATP and myelin basic protein (MBP) as an artificial substrate. As shown in the autoradiogram in Fig. 4, MEKK1 kinase activity was detected at low levels in untreated protoplasts, but became strongly stimulated in response to H₂O₂ treatment. Interestingly, when MEKK1–Myc protein...
amounts were quantified by immunoblot analysis with anti-Myc antibody, MEKK1-Myc levels also showed a strong increase in H$_2$O$_2$-treated samples. These results indicated that MEKK1 is regulated through proteasome-mediated stability. Kinase assays of immunoprecipitated MEKK1-Myc from these extracts also showed increased activity but not to similar levels as seen after H$_2$O$_2$-treatment. In a further experiment, the proteasome inhibitor treatment was combined with the H$_2$O$_2$ treatment. Immunoblotting showed that the combined treatment did not result in higher accumulation of MEKK1-Myc protein amounts than after either treatment alone. Interestingly, the kinase activity of MEKK1-Myc was clearly higher in H$_2$O$_2$+MG115-treated protoplasts than in protoplasts treated with MG115 alone. These data indicate that proteasome-mediated MEKK1 protein stabilization might be a part of H$_2$O$_2$-induced activation of MEKK1, and full activation of MEKK1 by H$_2$O$_2$ might require other, as yet unknown, post-translational modifications.

**mekk1 Mutant Plants Are Impaired in ROS-induced MAPK Activation**—MEKK1 has been shown to be involved in the activation of three MAPKs that play key roles in abiotic stress adaptation and pathogen defense (8, 9). During both pathogen attack and in response to all abiotic stresses, ROS are amply produced and could serve as a common mechanism for activating this pathway. To investigate this possibility in the context of MEKK1, the activation of the endogenous MAPKs MPK3, MPK4 and MPK6 was analyzed in wild type Col-0 and mekk1 mutant plants before and in response to H$_2$O$_2$ treatment. MPK3, MPK4, or MPK6 were immunoprecipitated from root or seedling protein extracts with MAPK-specific antibodies and analyzed by in vitro kinase assays using MBP as a substrate. In root extracts, all three MAP kinases showed low basal kinase activity but were all strongly activated by H$_2$O$_2$ treatment in wild type Col-0 plants (Fig. 5A). Immunoblots of the protein extracts with the respective MAPK antibodies did not reveal significant changes in MAPK protein amounts after H$_2$O$_2$ treatment that would explain the increases in kinase activities of MPK3, MPK4 or MPK6 (Fig. 5A). In mekk1 mutant lines, the basal amounts and activities of all three MAPKs treatment of 10 min resulted in a clear accumulation of MEKK1-Myc protein to similar levels as seen after H$_2$O$_2$-treatment. These results indicated that MEKK1 is regulated through proteasome-mediated stability.
showed similar levels as in wild type plants (Fig. 5A). Whereas MPK6 activation was slightly reduced and MPK3 activation not affected, \( \text{H}_2\text{O}_2 \)-induced activation of MPK4 was completely abrogated (Fig. 5A). Although the protein amounts of all three MAPKs were not affected in roots of \textit{mekk}1 mutants, protein amounts in seedlings differed greatly making the interpretation of the kinase activities complicated (supplementary Fig. S1). From the root data, we conclude that MPK4 is a major downstream target of the \( \text{H}_2\text{O}_2 \)-induced MEKK1-dependent MAPK pathway.

**MEKK1 Activates MPK4**—As the kinase activity in \textit{mekk}1 mutants indicated that MEKK1 is an upstream activator of MPK4, we tested whether MEKK1 can activates MPK4 in response to ROS. For this purpose, HA-tagged MPK4 was co-expressed with Myc-tagged MEKK1 in protoplasts. Upon treating the protoplasts with or without \( \text{H}_2\text{O}_2 \), MPK4-HA was immunoprecipitated before determination of MPK4 kinase activity using MBP as a substrate. As shown in Fig. 5B, MPK4-HA alone was poorly active, and was not notably activated by \( \text{H}_2\text{O}_2 \) treatment. However, co-expression of MEKK1-Myc enhanced the basal activity of MPK4-HA to a certain extent, and caused significant enhancement of MPK4-HA activity upon \( \text{H}_2\text{O}_2 \) treatment. These data strongly support the idea that \( \text{H}_2\text{O}_2 \)-induced MPK4 activation is mediated by MEKK1.

**Phenotypic Similarity between \textit{mekk}1 and \textit{mpk}4 Mutant Plants**—Because we found that MPK4 is a downstream component of the \( \text{H}_2\text{O}_2 \)-induced MEKK1 signaling cascade, we tested whether \textit{mpk}4 would show phenotypic similarities to \textit{mekk}1 mutant plants. So far, a genetic analysis of MPK4 function has only been performed in \textit{mpk}4 mutants in the genetic background of \textit{A. thaliana} accession \textit{Landsberg erecta} (11) which shows major physiological differences to \textit{A. thaliana} accession \textit{Columbia} used in our analysis. To allow a direct comparison between MEKK1 and MPK4 functions, we selected MAPK mutants in the Col-0 background. The \textit{MPK4} T-DNA insertion line was isolated from the SALK T-DNA collection (SALK_056245). RT-PCR of RNA prepared from \textit{mpk}4 plants with MPK4 gene-specific primers indicated that low amounts of MPK4 transcripts are still present (Fig. 6), but no MPK4 protein could be detected in this line by immunoblot analysis with MPK4-specific antibody (data not shown). The MPK3 and MPK6 T-DNA insertion lines were also isolated from the SALK T-DNA collection (SALK_151594 and SALK_073907, respectively). RT-PCR analysis of \textit{mpk}3 and \textit{mpk}6 plants indicated the absence of MPK3 or MPK6 transcripts in the corresponding lines (Fig. 6), and the absence of MPK3 and MPK6 protein in the mutants was also confirmed by immunoblot analysis using MPK3 and MPK6 specific antibodies (data not shown). Like \textit{mekk}1, \textit{mpk}4 but not \textit{mpk}3 and \textit{mpk}6 mutants showed a strongly dwarfed phenotype with wilted cotyledons and curled dwarfed true leaves (supplementary Fig. S2A). When compared with wild type plants or \textit{mpk}3 and \textit{mpk}6 mutants, DAB staining indicated that \textit{mpk}4 mutants accumulate \( \text{H}_2\text{O}_2 \) in their leaves (Fig. 3B and supplementary Fig. S2B). Overall, \textit{mekk}1 and \textit{mpk}4 mutant plants showed similarities with respect to both, phenotype and \( \text{H}_2\text{O}_2 \) accumulation.

**Altered Expression of Genes Related to ROS Signaling and Homeostasis in \textit{mekk}1 and \textit{mpk}4 Mutant Plants**—To further establish a role of MEKK1 and the MAPKs MPK3, MPK4, and MPK6 in \( \text{H}_2\text{O}_2 \) signaling, we analyzed whether expression of genes involved in redox control are affected in mutant plants. For this purpose, we performed RT-PCR analysis of \( \text{H}_2\text{O}_2 \) treated or untreated wild type Col-0, \textit{mekk}1, \textit{mpk}3, \textit{mpk}4, and \textit{mpk}6 plants. As shown in Fig. 6, the expression of \textit{MPK4} was up-regulated in \textit{mekk}1 mutants, conversely \textit{mpk}4 mutants showed enhanced expression of MEKK1. These data suggest that expression of the MEKK1 and MPK4 genes is under negative control of their own pathway.

The expression of several \( \text{H}_2\text{O}_2 \)-inducible genes showed significantly higher induction in \textit{mekk}1 and \textit{mpk}4 mutants than in wild type plants or in \textit{mpk}3 and \textit{mpk}6 mutants. To this group of genes
belong Zat12, a putative transcription factor (12), and OXI1, a protein kinase that is an upstream regulator of MPK3 and MPK6 (13). Basal expression levels of the ROS-inducible marker gene GST1, encoding a glutathione S-transferase (14), was up-regulated in mekk1 and mpk4 mutants. mekk1 and mpk4 mutants also showed increased basal amounts of transcripts of the RbohD gene, encoding a ROS-inducible NADPH oxidase that plays an important role in pathogen defense (15). Upon H2O2 treatment, expression of the aforementioned genes consistently resulted in higher expression levels than observed in wild type plants or in mpk3 and mpk6 mutants. In contrast to these genes, expression of RbohC, encoding an NADPH oxidase (16), was strongly down-regulated in mekk1 and mpk4 mutants both before and after H2O2 treatment. The expression of APX5, and to a lesser extent tAPX, encoding microsomal and thylakoid ascorbate peroxidases (17), was down-regulated in mekk1 and mpk4 mutants. In summary, an overlapping set of genes related to ROS signaling and homeostasis was identified in mekk1 and mpk4 mutants that significantly differed in expression levels from wild type plants or mpk3 and mpk6 mutants.

**mkk1 and mpk4 Mutant Plants Are Compromised in the Expression of Auxin-induced Genes**—A negative regulation of H2O2 signaling on phytohormone responses has been described previously for auxin (18). These authors showed that an H2O2–MAPK cascade could repress auxin-responsive gene expression in protoplasts. Because mekk1 was defective in H2O2–induced MPK4 activation, we performed RT-PCR analysis of RNA extracted from untreated or IAA-treated wild type Col-0 and mekk1 plants. Compared with wild type plants, the basal expression of the auxin-responsive IAA3 and IAA14 genes was reduced in untreated mekk1 mutants (Fig. 7). The auxin-induced accumulation of transcripts of these genes was also markedly lower in mekk1 mutants than in wild type plants. A similar picture was obtained in the RT-PCR analysis of the auxin-inducible IAA5 gene, which showed no basal expression, but

**FIGURE 6. Misregulation of redox-related genes in mekk1 and mpk4 mutant plants.** RNA was extracted from wild type Col-0, mekk1-1, mekk1-3, mpk4, mpk3, and mpk6 seedlings at 10 days after sowing and treatment with or without 10 mM H2O2 for 1 h before RT-PCR analysis. Two different Col-0 samples were prepared from independently treated seedlings.

**FIGURE 7. Reduced expression of auxin-inducible genes in mekk1 and mpk4 mutants.** RNA was extracted from wild type Col-0, mekk1-1, mekk1-3, mpk4, mpk3, and mpk6 seedlings at 10 days after sowing after treatment with or without 20 μM IAA for 1 h before RT-PCR analysis. Two different Col-0 columns represent samples prepared from independently treated seedlings.

strong induction upon auxin treatment. As seen with IAA3 and IAA14, IAA5 transcript levels showed some increase in mekk1 mutants upon auxin treatment, but stayed considerably below those levels of wild type plants.

We also performed RT-PCR analysis of these auxin-inducible marker genes with the mpk3, mpk4 and mpk6 mutants. Basal and auxin-induced levels of IAA3, IAA5, and IAA14 of mpk3 and mpk6 mutants were comparable to those in wild type plants (Fig. 7). In contrast, the transcript profiles for IAA3, IAA5, and IAA14 in mpk4 mutants strongly differed from those of wild type but were highly similar to those seen in the mekk1 mutants (Fig. 7).

These data show that the basal and induced expression levels of auxin-inducible marker genes are compromised in mekk1 and mpk4 mutants but that auxin signaling per se is not affected.

**DISCUSSION**

Our previous analysis of alfalfa OMTK1 indicated that this MAPKKK channels oxidative stress signaling through activation of a downstream MAP kinase (6). Because a genetic analysis of OMTK1 could not be carried out in the autotetraploid alfalfa, we decided to perform a functional analysis of MEKK1 as the most closely related Arabidopsis MAPKKK to OMTK1. In this report, we have isolated and characterized two mekk1 null mutants. Our analysis revealed that mekk1 mutants have multiple defects concerning ROS homeostasis, including the misregulation of a number of genes related to redox control. The enhanced accumulation of hydrogen peroxide in leaves correlates with the appearance of a chlorotic phenotype of the cotyledons early after unfolding the first true leaves. Other evidence indicates that this lethal phenotype is caused by the constitutive defense responses in mekk1 mutant plants.4

In a biochemical analysis, transiently expressed Myc-tagged MEKK1 was detected at low protein and kinase activity levels in untreated Arabidopsis protoplasts. Upon H2O2 treatment, MEKK1 protein amounts and kinase activity strongly increased, indicating that MEKK1 protein amounts and kinase activity are directly regulated by oxidative stress. We also found that the other MAPKKK ANP1, which was shown to activate MPK3 in response to H2O2 treatment in protoplasts (18), is stabilized either by H2O2 or proteasome inhibitor MG115

4 K. Ichimura and K. Shirasu, personal communication.
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treatment (supplementary Fig. S3). However, in contrast to MEKK1, ANP1 cannot be further activated, but is rather inactivated by \( \text{H}_2\text{O}_2 \) after stabilization by MG115. Interestingly, these data suggest that different \( \text{H}_2\text{O}_2 \)-activated MAPKKKs are regulated by different mechanisms.

Previous investigations reported that the Arabidopsis MAPKs MPK3, and MPK6 are activated in response to hydrogen peroxide treatment of cells (18, 19). Moreover, MPK3, MPK4 and MPK6 were activated by MEKK1 in protoplast assays (8, 9). These results prompted us to investigate whether these MAPKs are compromised in mekk1 mutants. In immune complex kinase assays of roots, ROS-induced activation of MPK4 was compromised in mekk1 mutant plants whereas MPK6 was only slightly and MPK3 not at all affected under these conditions (Fig. 5A). Furthermore, we found that MEKK1 activates MPK4 in response to \( \text{H}_2\text{O}_2 \) treatment in protoplasts. These data reveal MEKK1 as a positive regulator of MPK4, and demonstrate that MPK4 is a downstream target of MEKK1. It should be noted that in aerial parts of mekk1 plants, quite different MAPK activation profiles were observed (supplementary Fig. S1). Although \( \text{H}_2\text{O}_2 \)-induced activation of MPK4 was also completely abrogated when we analyzed whole seedlings, MPK3 and MPK6 activity levels increased strongly. These results could superficially be interpreted to mean that MEKK1 is a negative regulator of MPK3 and MPK6. However, this effect could at least to some extent be caused by the increased MAPK protein levels in the aerial parts, especially when considering levels of other prominent proteins such as the Rubisco subunits, which showed a strong reduction in mekk1 seedlings under these conditions.

To allow a direct comparison between MEKK1, MPK3, MPK4, and MPK6 functions, mpk3, mpk4, and mpk6 mutants were selected in the same genetic background as the mekk1 mutants. In contrast to mpk3 and mpk6 mutants, mekk1 and mpk4 accumulated hydrogen peroxide when compared with wild type plants (Fig. 3B and supplementary Fig. S2B). When compared with wild type or mpk3 and mpk6 mutants, expression of a set of genes related to ROS homeostasis was found to be equally affected in mekk1 and mpk4 mutants (Fig. 6). These results clearly indicate that MPK4 and MEKK1 are located on the same signaling pathway. However, phenotypic differences between the mekk1 and mpk4 mutants (supplementary Fig. S2A) suggest that additional factors such as MPK6 or other group B MAPKs, which are related to MPK4 might contribute to the full repertoire of the MEKK1 signaling pathway.

Our data indicate that MPK4 is a downstream target of MEKK1 in Arabidopsis whereas in Medicago sativa, the MAPK MKM3 can be targeted by OMTK1 (6). At first sight, it might seem surprising that the related MAPKKKs MEKK1 and OMTK1 might not target the most closely related homologous MAPKs in these two organisms. However, it should be noted that MPK4 and MKM3 both belong to the group B MAPKs and that a number of closely related MAPKKKs exist in both Arabidopsis and Medicago (20). The observation that MEKK1 interacts with MPK4 in yeast (21), and our finding that MEKK1 directly interacts with group B MAPKs, including MPK4, MPK5, and MPK13 in vitro (supplementary Fig. S4), strongly indicates that MEKK1 acts as a scaffold protein to target MPK4 activation in the same manner as MMK3 activation by OMTK1. Our data also do not exclude the possibility that other group B MAPKs are also targeted by MEKK1 and OMTK1, respectively.

In an alternative approach to the isolation of loss-of-function mekk1 mutants, we also tried to produce gain-of-function MEKK1 mutants by overexpressing MEKK1 under the 35 S CaMV or a dexamethasone-inducible version of the 35 S CaMV promoter in wild type plants. Of the few transgenic plants that we obtained (data not shown), none of the lines expressed the transgenes, suggesting that overexpression of MEKK1 is lethal and that plants have to tightly control expression levels of this gene.

Kovtun et al. (18) showed that the \( \text{H}_2\text{O}_2 \)-MAPK cascade could repress auxin responses in protoplasts. Because mekk1 and mpk4 mutant plants accumulate high levels of ROS, we investigated whether mekk1 and mpk4 mutants are compromised in auxin signaling. In line with the role of ROS as a negative regulator of auxin responses, RT-PCR of wild type plants and mekk1 and mpk4 mutants indicated that mekk1 and mpk4 mutants are not compromised in auxin-induction of several marker genes, but reduce expression levels of these genes before and after auxin treatment.

In summary, our data reveal a role of MEKK1 in mediating ROS-induced MAPK MPK4 signaling in A. thaliana and define MEKK1 as a pathway that regulates a variety of processes including ROS homeostasis and hormone responses.

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