MEKK1 Is Required for MPK4 Activation and Regulates Tissue-specific and Temperature-dependent Cell Death in Arabidopsis*

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Innate immunity signaling pathways in both animals and plants are regulated by mitogen-activated protein kinase (MAPK) cascades. An Arabidopsis MAPK cascade (MEKK1, MKK4/MKK5, and MPK3/MPK6) has been proposed to function downstream of the flagellin receptor FLS2 based on biochemical assays using transient overexpression of candidate components. To genetically test this model, we characterized two mekk1 mutants. We show here that MEKK1 is not required for flagellin-triggered activation of MPK3 and MPK6. Instead, MEKK1 is essential for activation of MPK4, a MAPK that negatively regulates systemic acquired resistance. We also showed that MEKK1 negatively regulates temperature-sensitive and tissue-specific cell death and H2O2 accumulation that are partly dependent on both RAR1, a key component in resistance protein function, and SID2, an isochorismate synthase required for salicylic acid production upon pathogen infection.

The recognition of pathogen-derived molecules, so called pathogen-associated molecular patterns (PAMPs), triggers innate immunity responses in both animals and plants (1). In animals, PAMP recognition is often mediated by receptors such as Toll and Toll-like receptors containing leucine-rich repeats (2). In plants, PAMPs are also recognized by leucine-rich repeat-containing receptor kinases. For example, Arabidopsis FLS2 is a receptor kinase required for innate immune responses triggered by a bacterial flagellin (3). Similar to animal pathogens, many plant pathogens introduce effector proteins that are able to suppress PAMP-triggered immune responses (4). Plants, on the other hand, recognize such effectors, directly or indirectly by resistance (R) proteins and trigger rapid defense responses, including localized cell death (4).

The signal transduction following pathogen recognition by R proteins and receptor kinases in plants are thought to be mediated by MAPK cascades, since a number of MAPK components are known to be activated in these processes (5, 6). However, the specificity or requirement of individual MAPK signaling components in disease resistance is poorly defined, since many of these components also are activated by various environmental cues (7). Recently, a transient reverse genetic approach based on virus-induced gene silencing provided genetic evidence for requirement of some MAPK cascade components in disease resistance. For example, tobacco MEK2, a MAPK kinase (MAPKK), and its substrate MAPKs, SIPK and WIPK, were shown to be essential for full N-mediated resistance against Tobacco mosaic virus (8). The likely upstream activator of MEK2 is MAPKKα, which is essential for Pto-mediated resistance in tomato and Nicotiana benthamiana (9). Consistently, silencing MEK2, SIPK, or WIPK resulted in reduction of localized cell death associated with Pto resistance (9). In addition, another MAPK cascade, NPK1-MEK1-NTF6, is also essential for N-mediated resistance and Pto-dependent cell death (9–11). These data suggest that multiple MAPK cascades operate in order to establish disease resistance.

Using a transient gain of function approach in Arabidopsis protoplast, a complete MAPK cascade consisting of MEKK1, MKK4/MKK5 (MEK2 orthologs), and MPK3/MPK6 (WIPK/SIPK orthologs, respectively) was proposed as a downstream signaling process of the flagellin receptor FLS2 (5). In these experiments, transiently expressing a MEKK1 derivative lacking the N-terminal regulatory domain (MEKK1-NΔ) resulted in activation of MKK5. Similarly, a constitutive active form of either MKK4 (MKK4α) or MKK5 (MKK5α) activated MPK3 and MPK6. Fg22 activates MPK3 and MPK6 in a FLS2-dependent manner. Furthermore, transient expression of MEKK1-NΔ, MKK4α, or MKK5α led to enhanced resistance against Pseudomonas syringae pv. tomato (Pst), supporting the model for the complete MAPK cascade in defense responses (5). However, whether full-length MEKK1 directly interacts with and activates MKK4 and MKK5 remains to be shown. Instead, MEKK1 was shown to directly interact with MKK1 and MKK2, but not with MKK4, in yeast two-hybrid assays (12, 13). Interestingly, the N-terminal regulatory domain of MEKK1 also interacts with MPK4, but not MPK3, indicating that MEKK1 may act as a scaffold to establish specific phosphorylation relays for the
MEKK1-MKK1/MKK2-MPK4 cascade (12, 13). Consistent with these observations, flg22 treatment activates MKK1 to phosphorylate and activate MPK4 (14, 15).

Because of these potential discrepancies in the specificity of signaling pathways during defense response, we tested the existing hypotheses by isolating two mutants of MEKK1 to examine the genetic requirement of this protein for activation of the various MAPKs downstream of FLS2. We show here that MEKK1 is required for activation of MPK4, but not MPK3 and MPK6, in response to flg22. Similar to the mpk4 mutants, the mekk1 plants exhibit constitutive expression of certain defense genes, such as PR1, PR2, and PR5. These data suggest that MEKK1 and MPK4 are the components of a MAPK cascade regulating plant defense responses. Furthermore, loss of MEKK1 results in temperature-dependent \( \text{H}_2\text{O}_2 \) accumulation and cell death in a tissue-specific manner. Interestingly, the \( \text{H}_2\text{O}_2 \) accumulation and cell death is partly dependent upon the GABI-Kat program (16, 17). T-DNA insertion mutants (Col background) were generated at SALK and in the Arabidopsis (Arabidopsis thaliana) group of H. Hirt (University of Vienna, Vienna, Austria) (Fig. 1A). The T-DNA insertion site in mekk1-1 was found in the first exon (nucleotide 82 from the start codon), and mekk1-2 was found in the first intron (nucleotide 1203) of

### EXPERIMENTAL PROCEDURES

#### Plant Materials and Growth Conditions—The Arabidopsis

mekk1-1 (SALK_052557) and mekk1-2 (Gabi-Kat 813G10) mutants (Col background) were generated at SALK and in the context of the GABI-Kat program (16, 17). T-DNA insertion sites were determined by direct sequencing of PCR products using T-DNA left border and gene-specific primers (see below). The mekk1 mutants used in this study were backcrossed at least twice. Arabidopsis mutants rar1-21 (18), edm1(sgt1b) (19), and sid2-2 (20) were kindly provided by J. Dangl, M. Tor, and M. Wildermuth, respectively. For the sterile condition, seedlings were grown on Murashige and Skoog (MS) agar media supplemented with vitamins and 1% sucrose at 22 °C in the long day condition (16 h light/8 h dark). Methods for staining tissue with X-gluc (2 mM; Sigma), trypan blue (1.25 mg/ml; Sigma), aniline blue (0.01% Sigma), and 3,3’-diaminobenzidine (DAB; 1 mg/ml; Sigma) were described previously (21–24).

#### Genotyping—The following gene-specific primers were used for genotyping: MEKK1 (mekk1-1 F1, 5’-TAGGCAAGAATCCCTAGCTGTTG-3’; mekk1-1 R1, 5’-CAAACAACACACACCGACCTACAAC-3’; mekk1-2 F1, 5’-GGAGAACAAACCACATGTGTG-3’; mekk1-2 R1, 5’-CCTGGATTGAGCAAAGCTAGCAAG-3’); SGT1b (edmid1-F1, 5’-GGTTCCTCTCCCTACCCATTGG-3’; edmid1-R1, 5’-TAGGGCTGGTATGACTGGCTGGT-3’); and mekk1-1 R1, 5’-CGTTGATGGCAAATGGTACGCAATG-3’). Total RNA Preparation and RT-PCR Analysis—Tissue samples were frozen in liquid nitrogen, and RNA was extracted using Tri Reagent (Sigma) according to the manufacturer’s instructions. DNase I-treated RNA was extracted with phenol/chloroform, precipitated with ethanol, and dissolved in H\( _2 \)O. RT reactions using Superscript III (Invitrogen) were performed with 1 \( \mu \)g of RNA, 0.5 \( \mu \)g of oligo(dT)\( _{12–18} \) primers according to the manufacturer’s instructions. ExTaq polymerase (Takara Bio, Japan) was used for RT-PCR according to the manufacturer’s instructions. The following primers were used for RT-PCR: PR1 (PR1-F1, 5’-GTCCTTTATGCTGATTGGAAG-3’; PR1-R1, 5’-CATTAGTATGGCTGGTCCTGCATAC-3’); PR2 (PR2-F1, 5’-GTCCTTTCCTCTTACCACACACGC-3’; PR2-R1, 5’-CGTGATGGCAAATGGTACGCAATG-3’); SAG12 (SAG12-F1, 5’-GGTTAAAACATACTGCAATT-3’; SAG12-R1, 5’-GCCAGACCACAGAAGAAG-3’); PDF1.2-R1, 5’-CATGGTAACTGGCTCCCTC-3’; PDF1.2-F1, 5’-CTTGTGACAA-3’; and Actin2-R1, 5’-TTCGAGTTTTAGGCATCTAC-3’; Actin2-F1, 5’-TTCGAGTTTTAGGCATCTAC-3’).

#### Protein Extraction, Immunocomplex Kinase Assay, and Immunoblot Analysis—Tissue samples of seedlings were frozen in liquid nitrogen, and proteins were extracted with a buffer containing 100 mM HEPES-KOH, pH 7.4, 5 mM EDTA, 5 mM EGTA, 10 mM Na\( _2 \)VO\( _4 \), 10 mM NaF, 50 mM \( \beta \)-glycerophosphate, 5 mM dithiothreitol, protease inhibitor mixture (Roche Applied Science), and phosphatase inhibitor mixture (Roche Applied Science) as described (27). Equal amounts of proteins were separated by the NuPAGE Novex Bis-Tris gel (Invitrogen) and transferred onto polyvinylidene difluoride membrane (Bio-Rad). Immunoblot analysis and immunoprecipitation were performed as described previously (27, 28). After immunoprecipitates were washed with 1 ml of reaction buffer without ATP (25 mM Tris-HCl, pH 7.5, 2 mM EGTA, 12 mM MgCl\( _2 \), 1 mM dithiothreitol, 0.1 mM Na\( _2 \)VO\( _4 \)) (29), kinase assays were performed in 20 ml of the same buffer containing 25 mM ATP, 1 \( \mu \)Ci of [\( \gamma \)-\( ^{32} \)P]ATP, and myelin basic protein as a substrate at 30 °C for 30 min. The reaction was stopped by the addition of sample buffer. After electrophoresis on a 4–12% gradient gel, the phosphorylated myelin basic protein was visualized by autoradiography.

#### Antibodies—A rabbit polyclonal antibody for MPK3 was raised using a peptide from the N terminus (MTNGGGQYTD-PAVEC) (Eurogentec, Seraing, Belgium). MPK4-specific antibody (Ab4NT1) was raised from rabbit against synthetic peptides corresponding to the N-terminal peptide (FGSSGIDQSSSKGVA) of MPK4 (Sawady Technology Inc., Tokyo, Japan). Conjugation to carrier protein, affinity purification, and MPK6-specific antibody (Ab6NT1) were previously described (27).

#### RESULTS

The mekk1 Mutants Exhibit Dwarfism and Lethality—To investigate functions of MEKK1 (At4g08500) in defense responses, we obtained two T-DNA insertion lines of MEKK1 in the Col background, which we designated as mekk1-1 (SALK_052557) and mekk1-2 (Gabi-Kat 813G10) in agreement with the group of H. Hirt (University of Vienna, Vienna, Austria) (Fig. 1A). The T-DNA insertion site in mekk1-1 was found in the first exon (nucleotide 82 from the start codon), and mekk1-2 was found in the first intron (nucleotide 1203) of
MEKK1. Both mekk1-1 and mekk1-2 mutants were indistinguishable from wild type (WT) Col-0 plants 5 days after germination on plates (Fig. 1B). However, as soon as the first true leaves expanded, we noticed aberrant morphologies in mekk1 mutants (Fig. 1B, bottom). The true leaves were extremely small and curled to the abaxial side. The petiole elongation was also greatly inhibited. Both mekk1-1 and mekk1-2 exhibited dwarfism and eventually died after approximately 5 weeks on soil. The mutant plants also showed early senescence in cotyledons (Fig. 1C). We maintained mekk1-1 and mekk1-2 lines as heterozygous plants, which were morphologically indistinguishable from WT. The segregation of WT and dwarf/lethal phenotypes in the F2 generation was a 90:35 (3:1, \( \chi^2 = 0.53; p = 0.47 \)) and 320:118 ratio (3:1, \( \chi^2 = 0.83; p = 0.36 \)) for mekk1-1 and mekk1-2, respectively. These data indicate that the phenotypes were caused by the loss of MEKK1 function.

MEKK1 Is Dispensable for MPK3 and MPK6 Activation—The complete MAPK cascade proposed by Asai et al. (5) predicted that MEKK1 has a positive regulatory role in the FLS2 signaling pathway, leading to the phosphorylation of MPK3 and MPK6 via activation of MKK4 and MKK5. To test this model genetically, we treated WT and mekk1 young seedlings with flg22 peptide (100 nM) and performed an immunocomplex kinase assay for MPK3, MPK4, and MPK6 activities upon flg22 elicitation. Seedlings (0.2 g) of WT and mekk1-1 grown on MS agar plates at 22 °C for 2 weeks were transferred on water in plastic plates and then incubated for 24 h at 22 °C to eliminate mechanical shock. Then seedlings were treated with 100 nM flg22, and samples were frozen in liquid nitrogen at the indicated time points. MAPK activity was examined by an immunocomplex kinase assay as described under “Experimental Procedures.” B, immunoblot analysis of MPK3, MPK4, and MPK6 proteins in WT and mekk1-1. Samples were taken 0 and 10 min after flg22 elicitation and used for immunoblot analysis. Equal protein loading was confirmed by staining membrane with Ponceau S. C, RT-PCR analysis of MPK3 and MPK4 expression in mekk1-1. Equal RNA loading was confirmed by actin expression.

MPK4 Activation by flg22 Requires MEKK1—MPK4 was shown to be activated upon flg22 treatment (14, 15). To investigate if MEKK1 is involved in MPK4 activation by flg22, we performed a similar immunocomplex kinase assay for MPK4. Flg22 activated MPK4 in WT but weakly, if at all, in mekk1 plants (Fig. 2A). Since protein kinase activities of MPK3, MPK4, and MPK6 were changed in mekk1, we compared protein levels of these MAPKs in WT and mekk1 by immunoblot analysis. We found that MPK3 and MPK4 accumulated to higher levels in mekk1, whereas MPK6 levels were unchanged in the mutant (Fig. 2B). To examine if increased protein levels of MPK3 and MPK4 in mekk1 are due to increased accumulation of transcripts, we performed RT-PCR analysis. We observed up-regulation of MPK3 and MPK4 transcripts in mekk1 (Fig. 2C). Thus, it is possible that increased activity of MPK3 observed in mekk1 is due to the increased protein levels in the mutant. These data also show that loss of MPK4 activity in mekk1 in response to flg22 is not due to reduced MPK4 protein levels. These data indicate that MEKK1 is required for activation of MPK4 triggered by flg22.

Loss of MEKK1 Resulted in Tissue-specific Cell Death—The dwarfism and late lethality phenotypes with early senescence are common to cell death mutants that express constitutive defense responses (31). Thus, we postulated that the phenotypes of mekk1 mutants may be due to microscopic cell death.
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To test this hypothesis, we stained 2-week-old mekk1 mutant plants grown at 22 °C with trypan blue, a common dye for visualizing dead cells (32). We observed dark-stained cells in the vasculature of leaves in both mekk1-1 and mekk1-2, whereas WT plants showed very faint, if any, stained cells around the same region (Fig. 3A, top panels). No mesophyll cells between the vasculature were stained at this stage of development. At higher magnification, elongated cells in the vasculature were stained much darker in the mekk1 mutants (Fig. 3A, bottom panels). Four weeks after germination, we also observed stained mesophyll cells close to vascular tissue (Fig. 3B). Based on these data, we concluded that MEKK1 negatively regulates tissue-specific cell death.

Tissue-specific H2O2 Accumulation in mekk1 Mutants—Cell death caused by either depletion of negative regulators of programmed cell death or activation of R proteins upon pathogen infection often associates with accumulation of H2O2 (33, 34). To study H2O2 production in mekk1 mutants, we stained 2-week-old leaves with DAB, which polymerizes locally in an H2O2 dose-dependent manner (24). In WT plants, only xylem and trichomes were weakly stained (Fig. 3 and data not shown). In contrast, many cells in the vasculature were stained with DAB in both mekk1-1 and mekk1-2 mutants (Fig. 4, top). This result clearly correlated with the trypan blue staining pattern of mekk1 mutants. In addition, we found that many guard cells were also stained in mekk1 mutants (Fig. 4, bottom). In this case, however, the DAB polymerization was found only the side facing the stomatal pore in the guard cell (Fig. 4, bottom). The DAB staining in the guard cells did not correlate with the trypan blue staining pattern, since we did not observe trypan blue-stained guard cells. These results strongly suggest that H2O2 accumulation is associated with cell death in the vasculature but not in the guard cells.

Constitutive Defense Responses in mekk1 Mutants—Mutants that express constitutive defense responses and spontaneous cell death phenotype often accumulate callose, which can be visualized by aniline blue (23). We observed callose deposition in the mesophyll cells in the leaf of mekk1 mutants when stained with aniline blue, whereas only weak staining was visible in the vasculature of WT plants (Fig. 5A). In contrast to trypan blue and DAB staining, callose deposition was mainly observed in the mesophyll cells and only weakly in the vasculature in mekk1.

Next, we tested if mekk1 mutants exhibited expression of defense-related genes in the absence of a pathogen or elicitor. We analyzed accumulation of PR1, PR2, PR5, SAG12, and PDF1.2 transcripts, markers for salicylic acid (SA), senescence, and ethylene and jasmonate pathways (35). We found that expression of PR1, PR2, PR5, and SAG12 genes was constitutively up-regulated in mekk1 mutants, whereas that of PDF1.2 was slightly down-regulated (Fig. 5B). We were not able to measure disease resistance in these mutants because of their dwarfism and lethality. Taken together, these data suggest that loss of MEKK1 function leads to constitutive activation of the defense-associated responses.
We examined cell death and H$_2$O$_2$ accumulation in containers still exhibited dwarfism and lethality at 22 °C. Next, pressing the phenotypes, since mekk1 plants grown at 28 °C for 19 days were indistinguishable from the WT (Fig. 7A). Four-week-old leaves were stained with trypan blue (D) or DAB (E). Bars, 5 mm. F, immunocomplex kinase assay of MPK3, MPK4, and MPK6. Seedlings of WT, mekk1-1, and mekk1-2 grown on MS agar plates at 28 °C for 3 weeks were treated with either 0.1% Me$_2$SO (−) or 100 nM flg22 (+) for 10 min as described under “Experimental Procedures.”

**FIGURE 7.** High temperature suppresses tissue-specific cell death, H$_2$O$_2$ accumulation, and hyperactivation of MPK3 and MPK6 in mekk1. A, WT (Col-0) and mekk1-1 were germinated and grown on MS agar plates at 22 °C for 10 days. Seedlings were then transferred onto the same media in a closed container and incubated until 4 weeks old at either 22 or 28 °C. Bars, 1 cm. B, WT (Col-0) and mekk1-1 were germinated and grown on MS agar plates at 28 °C for 19 days. Bars, 1 cm. C, WT (Col-0) and mekk1-1 were germinated and grown on MS agar plates for 2 weeks at 22 °C or 26 °C. Bars, 5 mm. D, and E, WT (Col-0) and mekk1-1 were treated as in Fig. 7A at the indicated temperature. Four-week-old leaves were treated with trypan blue (D) or DAB (E). Bars, 0.5 mm. F, immunocomplex kinase assay of MPK3, MPK4, and MPK6. Seedlings of WT, mekk1-1, and mekk1-2 grown on MS agar plates at 28 °C for 3 weeks were treated with either 0.1% Me$_2$SO (−) or 100 nM flg22 (+) for 10 min as in Fig. 2A at 28 °C. Protein extraction and immunocomplex MAPK assay were performed as described under “Experimental Procedures.”

**FIGURE 6.** Tissue-specific expression of MEKK1. A 3-kb fragment of MEKK1 5’ upstream was transcriptionally fused to a GUS reporter gene. The MEKK1 promoter::GUS transgenic seedlings were germinated and grown on MS media. Two-week-old seedlings were stained with X-gluc. One representative of 17 independent lines is shown. A, cotyledon. Bar, 0.5 mm. B, first true leaf. Bar, 0.5 mm. C, transverse section of the first leaf. Bar, 50 μm. D, guard cells on cotyledon. Bar, 100 μm.

**Tissue-specific Expression of MEKK1—**Since cell death and H$_2$O$_2$ accumulation in mekk1 mutants were observed in the vasculature, we investigated if the MEKK1 expression profile correlates with this pattern. A 3-kb DNA fragment containing the MEKK1 promoter was transcriptionally fused to the GUS reporter gene and introduced into Arabidopsis. We found that the GUS reporter was expressed predominantly in vascular tissues, especially evident in cotyledons and true leaves (Fig. 6, A and B). Higher expression of GUS in mesophyll cells was also visible in emerging true leaves (data not shown) but weaker in expanded leaves (Fig. 6B). A transverse section of an expanded true leaf clearly indicated preferential expression of MEKK1 in the vasculature (Fig. 6C). We also found strong GUS expression in guard cells (Fig. 6D). Seventeen independent lines showed similar expression patterns. Thus, MEKK1 expression is tissue-specific and correlates well with the cell death phenotype and H$_2$O$_2$ accumulation in leaves.

**Suppression of mekk1 Phenotypes at High Temperature—**Activation of R protein signaling and constitutive defense responses in the cell death mutants are often suppressed by high temperature and/or high humidity (23, 36, 37). To test whether these conditions affect the mekk1 phenotypes, we performed growth recovery assays by a temperature shift. Plants were grown on plates at 22 °C for 10 days and then transferred to closed containers in growth chambers at 22 or 28 °C until 4 weeks old. Although mekk1 plants kept at 22 °C were extremely small with impaired leaf expansion and stem elongation, growing the plants at 28 °C greatly suppressed the severe dwarfism (Fig. 7A). Similarly, mekk1 plants grown from seeds on plates at 28 °C for 19 days were indistinguishable from the WT (Fig. 7B). By contrast, mekk1 plants grown at 26 °C were only slightly larger than those at 22 °C (Fig. 7C). High humidity did not suppress the phenotypes, since mekk1 plants grown in the closed containers still exhibited dwarfism and lethality at 22 °C. Next, we examined cell death and H$_2$O$_2$ accumulation in mekk1 plants kept at 28 °C after the temperature shift. Compared with plants at 22 °C, the number of trypan blue- and DAB-stained cells was greatly reduced (Fig. 7, D and E). Thus, the growth defect phenotypes observed in mekk1 were correlated with the level of cell death and H$_2$O$_2$ accumulation. These data also indicate that the temperature-dependent process is upstream of cell death and H$_2$O$_2$ accumulation.

To investigate if the dwarfism and lethality can be unlinked to MAPK activities in mekk1 mutants, we carried out immunocomplex MAPK assays using WT and mekk1 plants grown at 28 °C for 3 weeks (Fig. 7F). Flg22 treatments activated MPK3, MPK4, and MPK6 in WT plants, indicating that the signaling pathways were intact at this temperature. In contrast, MPK4 was only slightly activated, if at all, in mekk1 mutants. Thus, reduction of MPK4 activation by flg22 in mekk1 mutants is not due to dwarfism and lethality. Furthermore MPK3 and MPK6 activities were unchanged in mekk1 mutants, supporting our conclusion that MEKK1 is dispensable for MPK3 and MPK6 activation by flg22. Interestingly, unlike at 22 °C, MPK3 and MPK6 were not hyperactivated at this temperature. Thus, hyperactivation of MPK3 and MPK6 was tightly linked to dwarfism and lethality in mekk1 plants. And the hyperactivation is likely to be due to stresses caused by the dwarfism.

**Partial Suppression of mekk1 Phenotypes in rar1 or sid2—**The temperature-dependent cell death phenotypes in mekk1 plants indicated that R-protein-dependent signaling pathways
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A. Col edm1 rar1

B. mekk1 mekk1/edm1 mekk1/rar1 mekk1/sid2

C. mekk1 mekk1/edm1 mekk1/rar1 mekk1/sid2

D. mekk1 mekk1/edm1 mekk1/rar1 mekk1/sid2

FIGURE 8. Suppression mekk1 phenotypes by rar1 or sid2 at 26 °C. A, WT (Col-0) and the mutant plants, edm1, rar1-21, sid2-2, mekk1-1, mekk1-1/edm1, mekk1-1/rar1-21, and mekk1-2/sid2-2 were germinated and grown on MS agar plates at 26 °C. Photographs of 2-week-old seedlings were taken. Bar, 5 mm. The same magnification was used in all pictures. True leaves taken from seedlings grown as described in the legend to Fig. 7A were stained by trypan blue (B), DAB (C), or aniline blue (D). Bars, 100 μm.

may be activated in the mutant. To test this hypothesis, we crossed mekk1 into rar1 or edm1 (sgt1b deletion) mutants that exhibit loss of R-protein-dependent disease resistance against particular isolates of Pst and Hyaloperonospora parasitica (18, 19). There was little or no suppression of the dwarfism in mekk1/edm1 or mekk1/rar1 double mutants compared with mekk1 when grown at 22 °C (data not shown). Interestingly, however, the dwarf phenotype was greatly recovered in the mekk1/rar1 at 26 °C, whereas mekk1/edm1 plants were only slightly bigger than mekk1 (Fig. 8A). To examine if cell death and H2O2 accumulation patterns were also altered in mekk1/rar1 double mutants, we stained leaf tissues grown at 26 °C with trypan blue and DAB. We noted that trypan blue- or DAB-stained cells in the mesophyll area in mekk1 plants grown at 26 °C. The number of trypan blue- or DAB-stained cells in both vasculature and mesophyll area was greatly reduced in mekk1/rar1 but only slightly in mekk1/edm1 (Fig. 8, B and C). The callose deposition observed in mesophyll cells of mekk1 mutants grown at 22 °C was also suppressed by rar1 at 26 °C (Fig. 8D). Thus, the suppression effect by rar1 was not limited to the vasculature.

R protein-triggered resistance, cell death, H2O2 accumulation, and callose deposition are often dependent on SA (33, 38). To test if the cell death, H2O2 accumulation, and callose deposition in mekk1 is also controlled by SA-dependent pathways, mekk1 was introduced into sid2 mutant plants, which are not able to accumulate SA upon pathogen infection (20, 39). We found that the mekk1 growth defect, cell death, and H2O2 accumulation, callose deposition phenotypes were not suppressed at 22 °C in sid2 (data not shown). However, similar to rar1, sid2 partially suppresses these mekk1 phenotypes at 26 °C, although the suppression effect was not as strong as in rar1 (Fig. 8, A–D). These data indicate that the growth defects associated with cell death and H2O2 accumulation and callose deposition are partially mediated by RAR1 and SA.

DISCUSSION

Arabidopsis MEKK1 was proposed to play a positive role in plant immunity signaling, because overexpression of HAtagged MEKK1-A N (kinase domain alone) specifically activates positive regulators of defense responses, including MKK5, MPK6, and WRKY29, in a protoplast transient assay system (5). However, our genetic analysis of mekk1 mutants presented here shows that in contrast to the current paradigm, MEKK1 is dispensable for flg22-triggered activation of positive regulators of basal defense, MPK3 and MPK6. There are several possibilities to explain this apparent discrepancy. First, there may be other MAPKKKs functionally redundant to MEKK1 in terms of MPK3 and MPK6 activation. Second, the interpretation of overexpression phenotypes using MAPKKKs without the N-terminal regulatory domain is potentially complicated, because this domain may be involved in the specificity of interactions with downstream MAPK components (40). For example, animal MEKK1 without its N-terminal regulatory domain can phosphorylate MEK1 but is unable to signal further downstream (41). Similarly, the N-terminal domain of Arabidopsis MEKK1 contains the MPK4 binding site (13) and, therefore, may act as a scaffold to specify the phosphorylation targets. Third, ectopic overexpression of MAPK cascade components may also result in activation of similar but nonphysiological downstream signaling components. In any case, MPK3 and MPK6 can be activated by flg22 without MEKK1, indicating that there must be other MAPKKK(s) involved in activation of MPK3 and MPK6.

We showed that MEKK1 is essential for flg22-triggered activation of MPK4. Since the N terminus of MEKK1 interacts directly with MPK4, but not MPK3 or MPK6 in yeast (13), the simplest model is that MEKK1 and MPK4 are components of a single MAPK signaling module. Since MPK4 negatively regu-

5 T. Mizoguchi, K. Ichimura, and K. Shinozaki, unpublished results.
lates expression of particular defense genes (42), MEKK1, an upstream component of MPK4, may also be considered as a negative regulator of such defense gene expression. Indeed, similar to mpk4, PR1, PR2, and PR5 are constitutively expressed in mekk1. Strong expression in vascular tissues and guard cells but weak expression in mesophyll cells is shared also by both MEKK1 and MPK4, consistent with the idea that MEKK1 functions through MPK4 (42). The possible MAPKK for mediating the flg22 signal from MEKK1 to MPK4 is MKK1. MKK1 interacts with both MEKK1 and MPK4 when expressed in yeast and is activated by flg22 or H2O2 in protoplasts (12, 14). Furthermore, MKK1 is able to phosphorylate MPK4 but not MPK3 or MPK6 upon flg22 and H2O2 treatment (14). However, phenotypes of mekk1 are not identical to those of mpk4. For instance, the mekk1 plants are lethal in normal growth conditions at 22 °C, whereas mpk4 are severely dwarfed but not lethal (42). MEKK1 also may function through other MAPKs that belong to the same MAPK subgroup B as MPK4 (43). Similarly, another unidentified MAPKKK may weakly activate the MPK4 pathway, since minor activation of MPK4 in mekk1 was detected (Figs. 2A and 7F).

Our data and data of others showed that PAMPs, such as flg22, trigger activation of both positive (MPK3 and MPK6) and negative (MPK4) regulators for expression of particular defense genes, such as PR1, PR2, and PR5 at the same time (15). Activation of both types of regulators is transient, peaking at 10 min after treatment. This indicates that defense gene induction is controlled by a finely tuned balance of positive and negative regulators at the early time point. Loss of MEKK1 not only abolishes activation of the negative regulator MPK4 but also leads to hyperactivation of positive regulators, suggesting that MEKK1 may play an important role in positive and negative regulation of parallel MAPK cascades. MPK3 mRNA levels are up-regulated in mekk1. Because MPK3 mRNA is induced by stress conditions (44), it is possible that enhanced MPK3 expression is due to pleiotropic effects of cell death induced in mekk1. However, MPK6 protein levels remain unchanged, yet MPK6 is hyperactivated upon flg22 treatment in mekk1, suggesting that hyperactivation of positive regulators should also occur post-transcriptionally.

We show here that MEKK1 is highly expressed in the vasculature and acts as a novel negative regulator in preventing cell death and H2O2 accumulation. It appears that the mekk1-triggered cell death and H2O2 accumulation are developmentally controlled, since the phenotypes appear only after true leaves emerge in our growth conditions. The nature of the signal that triggers tissue-specific cell death and H2O2 accumulation remains unknown (see also below). One possible candidate for such a signal is H2O2, a known cell death inducer in plants (45). H2O2 is a relatively stable and mobile signaling molecule that can transmit through the vasculature (46). Similar to OMTK1, a MEKK1 ortholog in alfalfa, MEKK1 is stabilized and activated upon H2O2 treatment (47).6 In the absence of MEKK1, particular cell types, such as cells in the vasculature, may become less sensitive to H2O2 and result in H2O2 accumulation and cell death. We also noted weak expression of MEKK1 in mesophyll cells where callose deposition but not strong cell death and H2O2 accumulation were detected in mekk1 plants. Thus, expression levels of MEKK1 and the defense phenotypes are highly correlated (see also below).

The dwarf and lethal phenotypes in mekk1 are suppressed by high temperature and partially in rar1 and sid2, indicating that MEKK1 is not essential for plant development or growth per se. Instead, this suppression effect may imply a potential link between MEKK1- and R-protein-dependent defense signaling pathways, many of which are temperature-sensitive and require RAR1 and/or SID2 (23, 36, 37, 39, 48). RAR1 interacts with HSP90 and SGT1 and positively contributes to stabilization of R proteins (18, 26, 36, 49, 50). Furthermore, the RAR1-dependent stability of barley R proteins MLA1 and MLA6 is temperature-sensitive (36). Similarly, cell death controlled by tobacco N protein that requires RAR1 is also suppressed at a high temperature (37). Thus, it is plausible that MEKK1 negatively regulates cell death pathways activated by certain R protein(s) that are unstable at high temperature (28 °C) and unable to accumulate without RAR1 at 26 °C. If this is the case, these R protein-dependent pathways can be regulated either directly or indirectly by MEKK1. The former possibility is explained by the “guard hypothesis,” which predicts that R proteins “guard” proteins important for basal defense and that removal or change of the “guardee” activates them (51). In this scenario, MEKK1 is guarded by a particular R protein, which is activated by removal of MEKK1. Based on the expression analysis, such an R protein-MEKK1 complex would be abundant in the vasculature but less so in mesophyll cells. The steady state levels of such a complex in mesophyll cells may not be sufficient to induce cell death (strong response) but enough for callose deposition (weak response). This could explain why the cell death phenotype was much stronger in the vasculature but not in mesophyll cells in mekk1 mutants. This idea is consistent with the threshold model previously proposed for R protein complexes in defense induction (36, 49). To support this hypothesis, callose deposition in mekk1 mesophyll cells was also suppressed in the absence of RAR1, which is required for R protein stability (Fig. 8D).

A large number of MAPKKK, MAPKK, and MAPK genes are found in the Arabidopsis genome, but few of them have been genetically analyzed. Several complete MAPK cascades were proposed but were rarely confirmed by the genetic analyses. Information on the temporal and spatial expression pattern of those MAPK cascade components is also largely missing. Further systematic genetic and expression analyses as well as in planta protein interaction studies of the individual MAPK components are crucial to dissect complex signaling pathways involved in defense responses.

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6 H. Nakagami and H. Hirt, personal communication.
