Dynamic Changes in the Localization of MAPK Cascade Components Controlling Pathogenesis-related (PR) Gene Expression during Innate Immunity in Parsley*

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The activation of mitogen-activated protein kinase (MAPK) cascades is an important mechanism for stress adaptation through the control of gene expression in mammals, yeast, and plants. MAPK activation has emerged as a common mechanism by which plants trigger pathogen defense responses following innate immune recognition of potential microbial pathogens. We are studying the non-host plant defense response of parsley to attempted infection by Phytophthora species using an experimental system of cultured parsley cells and the Phytophthora-derived Pep-13 peptide elicitor. Following receptor-mediated recognition of this peptide, parsley cells trigger a multifaceted innate immune response, involving the activation of three MAPKs that have been shown to function in the oxidative burst-independent activation of defense gene expression. Using this same experimental model we now report the identification of a MAPK kinase (MAPKK) that functions upstream in this pathway. This kinase, referred to as PcMKK5 based on sequence similarity to Arabidopsis thaliana AtM KK5, is activated in parsley cells following Pep-13 treatment and functions as an in vivo activator of all three MAPKs previously shown to be involved in this response. Gain- and loss-of-function mutant versions of PcMKK5, when used in protoplast co-transfection assays, demonstrated that kinase activity of PcMKK5 is required for PR gene promoter activation following Pep-13 treatment. Furthermore, using specific antibodies and immunofluorescent labeling, we demonstrate that activation of MAPKs in parsley cells correlates with an increase in their nuclear localization, which is not detectable for activated PcMKK5. These results suggest that activation of gene expression through MAPK cascades during innate immune responses in plants involves dynamic changes in the localization of the proteins involved, which may reflect the distribution of key protein substrates for the activated MAPKs.

Received for publication, January 31, 2004, and in revised form, February 27, 2004
Published, JBC Papers in Press, March 4, 2004, DOI 10.1074/jbc.M401099200

Innate immunity describes common systems by which eukaryotes sense and respond to the majority of potential pathogens to prevent infection (1, 2). These well studied systems of mammals and Drosophila employ receptor-mediated perception of pathogen-associated molecular patterns to trigger common signal transduction pathways leading to the transcriptional activation of genes that act against the potential pathogens. It has become increasingly clear that non-host disease resistance in plants exhibits strong similarities to the innate immune protection systems of mammals and Drosophila (3).

Our previous work studying the non-host resistance response of parsley during interactions with Phytophthora species has supported this analogy. Cultured parsley cells respond to a diversity of pathogen-associated molecular pattern-like structures, referred to as elicitors, with the transcriptional activation of pathogen defense genes and the synthesis of antimicrobial phytoalexins (4). Foremost in our studies has been the characterization of responses induced by the Pep-13 elicitor, which represents a surface-exposed peptide part of, and essential for the function of, a secreted transglutaminase present in all tested species of Phytophthora (5–7). The interaction of Pep-13 with its 100-kDa plasma membrane receptor (5, 6) is necessary and sufficient to trigger signal transduction and gene expression events implicated in the innate immune response of parsley. The temporal activation of elicitor-responsive defense genes has been the focus of considerable study in parsley, and a range of activated genes have been classified, according to their sensitivity to protein synthesis inhibitors, as either “immediate early,” “early,” or “late” transcriptional events (8). Members of the “immediate early” and “early” transcripts include WRKY transcription factors and pathogenesis-related (PR)1 genes, respectively (9–12). The signaling pathways leading to these transcriptional changes are multifaceted. Pep-13 treatment of parsley cells has been shown to stimulate rapid influx of Ca2+ leading to a sustained increase in the cytosolic Ca2+ ion concentration [Ca2+]i (13). An oxidative burst response involving the activity of a diphenylene iodonium-sensitive NADPH oxidase activity is located downstream of this. The generation of O2·− via this response has been shown to be necessary and sufficient to trigger gene expression changes resulting in the production of furanocoumarin phytoalexins after Pep-13 treatment (14).

1 The abbreviations used are: PR, pathogenesis-related; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAP kinase kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; GST, glutathione S-transferase; DTT, dithiothreitol; LUC, luciferase; GUS, β-glucuronidase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MBP, myelin basic protein; DAPI, 4′,6-diamidino-2-phenylindole.

* This work was supported by the European Commission (HPRN-CT-2000-00093, CRISP) and the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF533301 and AF533302.
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MAPK Regulation of Gene Expression during Plant Defense

Other Pep-13-induced changes in gene expression that are triggered independently of the oxidative burst require protein phosphorylation and protein kinase activities (15, 16). MAPK activation is a common element of plant responses to elicitors (17). Moreover, MAPK signaling has been implicated in a wide diversity of plant stress situations involving both abiotic and biotic challenges (18–21). Pep-13 treatment of parsley cells stimulates the rapid activation of at least three MAPKs referred to as PcMPK6, PcMPK3a, and PcMPK3b (16). These MAPKs were seen to be activated most strongly, specifically by stimuli that induce phytoalexin synthesis in parsley cells, namely elicitors. Subsequent loss-of-function experiments demonstrated that post-translational activation of these MAPKs plays an essential role in an oxidative burst-independent signaling pathway leading to the expression of “early” PR genes (16).

In all studied organisms, MAPKs form one component of a “three-kinase” module involving an upstream activator, MAP kinase (MAPKK), and a MAPKK kinase (MAPKKK) (17, 21). MAPKKK activities that function upstream of MAPK activation have been described for osmotic stress signaling in alfalfa (22), during tobacco N-gene mediated race/cultivar-specific recognition of tobacco mosaic virus (23), following Phytophthora-derived elicitor perception in tobacco (24), as a consequence of a range of abiotic stresses in Arabidopsis (25, 26) and following perception of the flagellin-derived elicitor, Flg-22, by Arabidopsis (27). In the latter case, while identifying a complete MAPK signaling cascade triggered following perception of bacterial flagellin, a direct requirement for the activity of the MAPKK in triggering downstream defense responses was demonstrated (27). Full sequence analysis of the Arabidopsis genome has resulted in the identification of 10 genes encoding MAPKKs (28). Based upon sequence similarities within the family and with MAPKK-encoding genes from other plant species, a phylogenetic nomenclature has been suggested for the classification of MAPKKs into sub-groupings (29). Whether or not specific members of these sub-groups share similar functions remains to be established.

Many stress applications when applied to plants result in the activation of the same subset of MAPKs. A major question is, therefore, how (or even if) response specificity can be maintained. One explanation that has arisen from studies in the alfalfa system is that an individual MAPK may have different activation characteristics (duration etc.) when it is acted upon by differing upstream MAPKKs (22). Another important means by which specificity is maintained during MAPK signaling, as exemplified by studies in yeast, is the use of strict control of the localization of pathway components. Studies in mammals and yeast have shown that stimulus-induced activation of MAPKs correlate with dynamic changes in their localization, whereby the proteins often translocate to, and accumulate in, the nucleus of the cell. This is often required due to the nuclear localization of key MAPK substrates, including transcription factors, involved in the control of gene expression (30–34).

We have previously shown that Pep-13 treatment of parsley cells stimulates a rapid nuclear localization of a MAPK originally referred to as ERMK (15), which was subsequently renamed as PcMPK3a (16). We now present a more detailed analysis of the dynamic regulation of MAPK cascade proteins that function in the Pep-13-mediated activation of “early” defense gene expression. The identification of the upstream MAPKK, referred to as PcMKK5 because of its high sequence similarity (84%) to AtMKK5, that is activated in elicited parsley cells and is responsible for the activation of PcMPK6, PcMPK3a, and PcMPK3b is presented, and its role in controlling the transcriptional activation of the parsley PR2 gene is demonstrated. Further detailed immunolocalization studies are also presented that show rapid nuclear translocation and accumulation of PcMPK3a/b and PcMPK6 but a constitutive cytosolic localization of PcMKK5 following Pep-13 treatment of parsley cells. These results provide an insight into the regulation of defense gene expression through the activation and dynamic changes in localization of MAPK cascade components operating during innate immunity in plants.

EXPERIMENTAL PROCEDURES

Elicitor Preparations—The Pep-13 elicitor was chemically synthesized as previously described (5). Pseudomonas syringae pv. phaseolicola HrpZ was expressed and purified as a recombinant protein from Escherichia coli (35).

Cell Culture Handling, Treatment, and Protoplast Isolation—Cultured parsley cells were maintained in modified Gamborg’s B5 medium containing 1 mg/liter 2,4-dichlorophenoxyacetic acid as previously described (5). Protoplasts were isolated 5 days following transfer of the culture to fresh medium according to previously described methods (16). Cells were treated by direct addition of the elicitor to cells previously washed and allowed to equilibrate for 30 min in fresh medium. Following appropriate time points, cells were collected by vacuum filtration, quickly frozen in liquid N2 and stored at −80 °C until use.

Acquisition and Analysis of PcMKK-encoding cDNA—Degenerate primers, matching highly conserved peptide sequences in plant MAPKK, were designed and used to amplify DNA fragments by reverse transcription-PCR. The primers GTKRCARCAAYARTGAGC and RTCYCTRTGDATDATATG amplified a 300-bp fragment of group A MAPKs (29); similarly the primers CCTGAAGGTGATCTACGGCAAYCAGAGA and TGGCTGGGACATGCAGATNGRCACAT were employed to amplify a 600-bp fragment of group C MAPKs. A Lambda Zap-Express™ (Stratagene, Heidelberg, Germany) cDNA library of parsley was separately screened with these two DNA fragments to yield the respective cDNA clones. The clones from each class that contain the complete open reading frames were used for further studies. These were named PcMKK2 and PcMKK5, respectively, according to the genes with highest sequence similarities in Arabidopsis thaliana (29).

Site-directed Mutagenesis—Clones encoding the desired mutations (see results section) were produced with the aid of the QuikChange™ mutagenesis kit (Stratagene, Heidelberg, Germany). All clones were sequenced to verify the mutations.

Generation and Baculovirus Expression of His-tagged or GST Fusion Recombinant Proteins—MAPK-encoding open reading frames were cloned as BamHI/Xhol PCR fragments into vector pGEX 2T-2 (Amer sham Biosciences, Freiburg, Germany) for expression in Escherichia coli (strain BL21) as fusion proteins containing a N-terminal glutathione S-transferase (GST) moiety. Recombinant proteins were subsequently purified using glutathione-Sepharose 4B according to the manufacturer’s guidelines. For bacterial expression of the recombinant MKKs, the corresponding DNA fragments were cloned as EcoRI (for PcMKK2 or EcoRI/EcoRV (for PcMKK5) into pUC40 (36) and transformed into BL21(DE3)pLysS cells. To express only the N-terminal regions of the MKKs, appropriate double-stranded oligonucleotides containing a stop codon were ligated into the SacI/BamHI (PcMKK2) or HindIII/BamHI (PcMKK5) restriction sites internal to the open reading frame. The resulting clones encode His-tagged 69- or 70-amino acid N-terminal sequences of PcMKK2 or PcMKK5, respectively.

Protoplast Transfection and Co-transfection—For kinase activity measurements wild-type or mutagenized (-d or -kr) PcMKK2 and PcMKK5 open reading frames were cloned into vector pRT100-c-myc containing the 35S-CaMV promoter (16). Details of thecloning steps for these clones are available on request. Five micrograms of each PcMKK-effecter construct was then used to transfect 2 × 105 protoplasts (~200 μl) in combination with 5 μg of the same empty vector or plasmid containing either PcMPK6, PcMPK3a, or PcMPK3b as previously described (16). Following 24-h incubation in B5-sucrose solution (0.25 μM sucrose, 1 mg/ml 2,4-dichlorophenoxyacetic acid, 3.2 mg/ml B5 medium) the protoplasts were collected by centrifugation following the addition of 25 ml of 0.24 mM CaCl2 and quickly frozen in liquid N2, Co-transfection experiments were performed as already described with the following modifications: For loss-of-function experiments 20 μg of PcMKK-kr constructs was transfected in combination with 5 μg of Pβ2 promoter-GUS construct and 5 μg of the normalization plasmid, pRTLUC (16). Following an 8-h incubation in B5-sucrose medium, the protoplasts were treated either with water or 100 μM Pep-13 and incubated for a further 14 h. For gain-of-function experiments, 5 μg of
PcMKK-dd constructs were co-transfected with PR2-GUS and pRTLUC and incubated for 24 h. Following the incubations the protoplasts were collected and stored as described above.

**Protein Extraction**—For assays of kinase activity, proteins were extracted by grinding frozen cells in extraction buffer (25 mM Tris/HCl, pH 7.8, 75 mM NaCl, 15 mM EGTA, 15 mM glycerophosphate, 15 mM 4-nitrophenylphosphate, 10 mM MgCl2, 1 mM DTT, 1 mM NaF, 0.5 mM Na3VO4, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 0.1% (v/v) Tween 20) followed by centrifugation (20,000 × g) for 10 min at 4 °C. Protoplasts were extracted in the same buffer by vortexing for 30 s. For studies involving luciferase (LUC) and β-glucuronidase (GUS) measurements, protoplasts were extracted in K-HPO4/KH2PO4, pH 7.5, containing 1 mM DTT.

**Activation of LUC Determinations**—For LUC activities, 10 μl of protoplast extracts were mixed with 90 μl of LUC substrate (20 mM Tricine, pH 7.8, 2.5 mM MgSO4, 1 mM MgCl2/OH-, pH 7, 10 mM mercaptoethanol, 2 mM 4-methylumbelliferyl β-g-glucopyranoside, 0.1 mM EDTA, 0.1% (v/v) Triton X-100) and incubated at 37 °C for 1 h. Following addition of 200 μl of 0.4 mM Na2HPO4, fluorescence was measured at 360 nm excitation/440 nm emission using the Cytofluor H instrument (Biosearch, Bedford, MA).

**Antibody Production**—Recombinant polypeptides corresponding to amino acids 1–69 of PcMKK2 and 1–70 of PcMKK5 were produced as His-tagged versions in E. coli. The purified His-tagged polypeptides were then used to raise antiserum following immunization of rabbits (Eurogentec, Seraing, Belgium).

**Western Blotting**—SDS-PAGE gels were semi-dry blotted onto nitrocellulose membrane (Parablot-NCL, Machery-Nagel, Düren, Germany). Membranes were blocked at 4 °C overnight in either TBS (20 mM Tris/HCl, 150 mM NaCl, 0.1% (v/v) Tween 20 (TBST)) containing 5% BSA or 5% skimmed milk powder or 5% (w/v) BSA. Primary antibody solutions were prepared in blocking solution at the following dilutions: 1:5000 for anti-PcMKK2, 1:6000 for anti-PcMKK5, and 1:500 for monoclonal anti-myelo (Sigma–Aldrich, Taufkirchen, Germany). Secondary antibodies coupled to either horseradish peroxidase or alkaline phosphatase were also prepared in blocking solution. All washes were performed in TBST. Blots were developed using either enhanced chemiluminescence (Amersham Biosciences) or nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate precipitate formation.

**Immunoprecipitation Protein Kinase Assays**—Cell or protoplast extracts containing 100 μg of protein were immunoprecipitated for 1 h at 4 °C with either anti-PcMPK6, anti-PcMKK2, anti-PcMKK5, or c-Myc antibodies, coupled to protein-A or protein-G-Sepharose (Amersham Biosciences). Subsequent washing and in vitro phosphorylation reactions were as described previously (16). PcMKK activity measurements were performed by the phosphorylation of 2 μg of “kinase-re- combinant” PcMPK6-kr, 3b-kr, or 4-kr as substrates. PcMPK activities were determined using the myelin basic protein (MBP) as substrate. Reactions were stopped by the addition of SDS sample buffer and boiling. The proteins were then separated by SDS-PAGE, and MAPK or MBP phosphorylation was determined by phosphorimaging.

**Immunolocalization of PcMPKs and PcMKKs**—Assays were performed using tobacco (accession numbers: A. thaliana, Zm, Zea mays; and Os, Oryza sativa). The proposed MAPK family was indicated by the letters A–D with the subsequent number representing subgroups.

**RESULTS**

**Cloning of Two MAPKK-encoding cDNAs from Parsley Cells**—To identify MAPKK activities that function upstream of the activation of the MAPKs, PcPK6, 3a, and 3b, in the Pep-13 signal transduction cascade, we initiated a screen to clone MAPKK-encoding cDNAs from a parsley cDNA library. Studies in other plant systems have implicated specific members of the MAPKK sub-families, in particular the sub-group C (37, 38) and possibly the sub-group A (22), as being involved in triggering plant defense reactions. To clone putative homologues of these MAPKKs from parsley, we first performed sequence alignments of representative members of each phylogenetic sub-class. Degenerate primers, based on conserved regions of these MAPKKs, were used to amplify the corresponding gene fragments via reverse transcription-PCR. These PCR fragments were then used as probes for cDNA library screening to acquire full-length sequences (see “Experimental Procedures” for details). These screens led to the acquisition of two cDNAs encoding MAPKKs from parsley. The first screen identified a cDNA clone with highest sequence similarity to NtSIPKK from tobacco (39) and LeMK2 from tomato (40), which are classified into the MAPKK sub-group A1 (Fig. 1) (29). To follow the proposed nomenclature for MAPK cascade components (29), we named this PcMKK2 on the basis of its sequence similarity to AtMKK2. The second MAPKK-encoding cDNA, referred to as PcMKK5, showed highest sequence similarity to NtMK2 from tobacco (37) and AtMKK5/4 from Arabidopsis, which all belonged to sub-group C (Fig. 1) (29).

**Generation of Specific Antiserum and Use in Coupled Immunoprecipitation Kinase Assays**—The first screen identified a cDNA clone with highest sequence similarity to NtSIPKK from tobacco (39) and LeMK2 from tomato (40), which are classified into the MAPKK sub-group A1 (Fig. 1) (29). To follow the proposed nomenclature for MAPK cascade components (29), we named this PcMKK2 on the basis of its sequence similarity to AtMKK2. The second MAPKK-encoding cDNA, referred to as PcMKK5, showed highest sequence similarity to NtMK2 from tobacco (37) and AtMKK5/4 from Arabidopsis, which all belonged to sub-group C (Fig. 1) (29).
and the requirement for generating MKK-specific antisera. The resulting antisera were then tested for cross-reactivity against each of the full-length His-tagged recombinant proteins. Fig. 2A (left-hand panels) demonstrates that the antibody raised against the polypeptide corresponding to PcMKK2 cross-reacted with the full-length recombinant PcMKK2 protein but showed no cross-reactivity toward the PcMKK5 protein. Similarly, the antibody raised using the polypeptide sequence from PcMKK5 showed cross-reactivity only toward the PcMKK5 protein. Addition of an excess of the polypeptides used for immunizations resulted in complete competition of this activity, further supporting the specificity of the sera (Fig. 2A, right-hand panels). Taken together, these approaches provided us with specific antisera that could be further employed in immunoprecipitation-protein kinase assays to monitor the activity of the MKK proteins in Pep-13-treated parsley cells.

Preliminary data using recombinant MKKs or proteins immunoprecipitated from cell extracts showed that the MKKs only weakly phosphorylated MBP (not shown). Because our objective was to identify a Pep-13-activated MAPKK that would use a Pep-13-activated MAPK as substrate, we generated, by site-directed mutagenesis, recombinant proteins corresponding to a “kinase-dead” form of PcMPK6 (-kr) and PcMPK3b-kr (-kr) for use as substrates in immunoprecipitation-protein kinase assays. In addition, we generated the corresponding mutant form of PcMPK4 (-kr), which is a MAPK that is not activated in Pep-13-treated cells, for use as a negative control. These mutant MAPKs had a conserved ATP-binding lysine to arginine substitution that prevented both autophosphorylation and kinase activity of the recombinant proteins (not shown). However, the activation loop TEY motif that acts as the site of phosphorylation by MAPKK activities was intact and thus provided us with an ideal substrate with which to monitor PcMKK activities.

Extracts from control-treated (−) parsley cells or from cells treated for 15 min with Pep-13 (P) were immunoprecipitated with either pre-immune serum (Pre-2 or Pre-5 in Fig. 2B) or antisera for PcMKK2 (anti-PcMKK2) or PcMKK5 (anti-PcMKK5), and the immunoprecipitates were analyzed for kinase activity toward the recombinant PcMPK6-kr, PcMPK3b-kr, or PcMPK4-kr proteins purified as GST fusions (Fig. 2B). The left-hand panel of Fig. 2B demonstrates that no Pep-13-mediated activation of PcMKK2 was detectable using any of the recombinant MAPKs as substrates. However, a weak activity of immunoprecipitated PcMKK2 toward the phosphorylation of PcMPK4 was visible. The level of this activity was identical in both control and Pep-13-treated extracts, and therefore suggested that PcMKK2 not to be activated during Pep-13 signaling. This result is in accordance with in vitro experiments performed with recombinant AtMEK1 (41), renamed AtMKK1 (29), suggesting that in such an in vitro assay, basally active PcMKK2 is able to use PcMPK4 as a phosphorylation substrate. In contrast, Pep-13 treatment led to a clear activation of PcMKK5 as determined by its increased phosphorylation of both PcMPK6-kr and PcMPK3b-kr (right-hand panels of Fig. 2B). No activity was detected toward the PcMPK4-kr substrate. These results demonstrate that PcMKK5 is activated in Pep-13-treated cells and is able to phosphorylate both PcMPK6 and PcMPK3b in in vitro kinase assays.

For a more complete temporal analysis of Pep-13-mediated MAPKK activation, antibodies specific for PcMKK2, PcMKK5, and PcMPK6 were used to precipitate the corresponding proteins over a time course of Pep-13 or HrpZ treatment of parsley cells. The Pseudomonas syringae HrpZ protein is a bacterial elicitor of plant defense reactions (53) and has previously been shown to activate the parsley orthologues of MPK6 and MPK3 (16), via a mechanism that is independent of the Pep-13 receptor. The immunoprecipitates from control or elicited cells were subjected to in vitro kinase phosphorylation assays using either PcMPK6-kr (for PcMKK5) or MBP (for PcMPK6) as substrate. Fig. 2C demonstrates that 5 min of Pep-13 treatment resulted in the activation of PcMKK5 in parsley cells as determined by the phosphorylation of the PcMPK6-kr substrate. This activation was rapid and persisted for several hours post-Pep-13 treatment (Fig. 2C). Moreover, the temporal activation of PcMKK5 matched exactly that observed for the immunoprecipi-
tated PcMPK6 from the same material supporting the notion that PcMKK5 may be responsible for PcMPK6 activation following Pep-13 treatment. A tight temporal correlation between these activities was also seen following treatment of parsley cells with HrpZ, which overall gave a slightly delayed activation of both proteins in comparison with Pep-13 (Fig. 2C). This delay probably reflects the slower mobility of the 35-kDa HrpZ elicitor through the plant cell wall compared with the smaller oligopeptide, Pep-13. In contrast to the situation for PcMKK5, we were unable to detect Pep-13-mediated activation of PcMKK2 using either PcMPK6-kr or myelin basic protein as substrate at any time point (data not shown, but see Fig. 2B for the analysis of the 15-min treatment). These data indicate that PcMKK5, but not PcMKK2, is activated in parsley cells following Pep-13 and HrpZ treatments and that this activation correlates temporally with that of PcMPK6.

Protoplast Transfection Assays Using Constitutively Active MAPKK Mutants Identify PcMKK5 as an In Vivo Activator of the MAPKs, PcMPK6, PcMPK3a, and PcMPK3b—The studies described above suggested that PcMKK5 may function as an activator of MAPK signaling in parsley cells treated with the Pep-13 elicitor. We therefore wanted to test directly whether the protein was involved in this response in vivo. Pep-13 treatment of parsley cells induced the activation of at least three MAPKs described as PcMPK6, PcMPK3a, and PcMPK3b (16). A fourth parsley MAPK, PcMPK4, was shown not to be activated in response to Pep-13 treatment. Because the available antiserum for studying the activity of the PcMPK3 kinases cannot discriminate between the 3a and 3b isoforms, it was necessary to perform individual protoplast transfection experiments to monitor the activation of the two kinases independently (16). To test the ability of PcMKK2 and PcMKK5 to activate the MAPKs in vivo, mutant MKK DNA constructs were generated by double aspartic acid exchange site-directed mutagenesis of threonine 220 and threonine 226 of PcMKK2 and threonine 211 and serine 217 of PcMKK5. These residues are present in the activation loop of the MAPKKs and normally act as phosphorylation sites for upstream activating MAPKK kinases (37). The resulting mutants (PcMKK2-dd and PcMKK5-dd) were expressed as recombinant proteins and displayed the expected constitutive activities (not shown).

Protoplast co-transfection assays were then performed using plasmids containing 35S-CaMV promoter-c-myc-tagged PcMKK2-dd or PcMKK5-dd in combination with either empty vector or 35S-c-myc PcMPK6-, 3a-, 3b-, or 4-containing plasmids. Eight hours after transfection, extracts were generated and immunoprecipitated using a c-Myc antibody. Kinase activities of the immunoprecipitates were then determined by MBP phosphorylation. Because MBP is not used efficiently as a substrate by MAPKKs, the kinase activities observed here can mainly be attributed to the activated MAPKs. Fig. 3 (upper panel) demonstrates that constitutively active PcMKK5-dd phosphorylated and activated the MAPKs, PcMPK6, 3a, and 3b, in transfected protoplasts. No activation of PcMPK4 was detected, which agrees well with the lack of activation of this kinase in Pep-13-treated cells (16). Constitutively active PcMKK2-dd was unable to activate any of the MAPKs in these assays (Fig. 3, lower panel). These data suggest that PcMKK5 functions as an in vivo activator of the Pep-13-responsive MAPKs, PcMPK6, 3a, and 3b, whereas PcMKK2 functions in pathways that are independent of the activation of these proteins.

PcMKK5 Activity Is Required for Pep-13-induced Transcriptional Activation of PR Genes—Previous studies have demonstrated the activation of PcMPK6, 3a, and 3b to be a necessary component of the oxidative-burst-independent signaling pathways leading to transcriptional activation of pathogenesis-related genes in Pep-13-treated parsley cells (16). These genes represent members of the “early” transcriptional events in parsley in response to elicitation. To confirm that PcMKK5 functions in the PcMPK6-, 3a-, and 3b-mediated pathway leading to PR gene expression, we tested the ability of constitutively active PcMKK5-dd to activate the parsley PR promoter in the absence of Pep-13 treatment using protoplast co-transfection experiments. Plasmid containing a PR2 promoter-GUS fusion was co-transfected with 35S promoter-driven effector plasmids containing either the c-Myc-PcMKK5-wt or -dd constructs, or the corresponding constructs of PcMKK2, along with a 35S promoter-luciferase (35S-LUC) normalization plasmid. After 24 h protoplast extracts were generated and relative GUS activities were determined as an indication of PR2 promoter activity. Fig. 4A demonstrates that co-transfection of a plasmid containing PcMKK5-dd was sufficient to activate the PR2 promoter in the absence of elicitation. This effect was specific to the constitutive active form, because neither PcMKK5-wt nor any of the tested PcMKK2 constructs were able to induce this promoter activity (Fig. 4A). These gain-of-function experiments suggest that constitutive active PcMKK5 functions upstream of the activation of PcMPK6, 3a, and 3b and is sufficient for inducing the expression of “early” defense-related transcripts following Pep-13 elicitation of parsley cells.

To further support this hypothesis we also performed the corresponding loss-of-function experiments with the goal of investigating Pep-13-induced PR2 promoter activity in the presence of dominant negative forms of PcMKK5. For these experiments PcMKK5 and PcMKK2 were further subjected to site-directed mutagenesis whereby the conserved ATP-binding lysine (Lys-99 for PcMKK2/Lys-95 for PcMKK5) was mutated to arginine to generate a kinase-dead mutant. These mutant constructs (PcMKK2-kr and PcMKK5-kr), in addition to wild-type versions (PcMKK2-wt/PcMKK5-wt), were co-transfected as previously described along with the PR2-GUS and 35S-LUC constructs. After 8 h the protoplasts were treated with Pep-13 and then incubated for a further 14 h. Protoplast extracts were then generated, and relative PR2-GUS activities were determined. Fig. 4B demonstrates that co-transfection of PcMKK5-kr invoked a strong and specific inhibitory effect upon the Pep-13-mediated activation of the PR2 promoter. This represented an approximate 80% inhibition of the promoter activity seen in co-transfections performed with either the empty vector control, or with either PcMKK2-wt or PcMKK2-kr,
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**DISCUSSION**

Plants are continuously exposed to potential microbial pathogens and must therefore have the ability to quickly recognize the potential invader and to trigger defense mechanisms. Non-host resistance exhibited by a particular plant species against a whole species of potential pathogens has been likened to the innate immunity seen in animals (3). Our previous studies using parsley cells and their receptor-mediated recognition of, and response to, the *Phytophthora*-derived Pep-13 elicitor have broadened this analogy (5–7, 15, 16). An important component of the innate immune signaling pathways of plants involves the activation of MAPKs and is a common response following recognition of pathogen-derived signals (17, 21). However, as we have previously demonstrated using the parsley/Pep-13 model system, MAPK activation is only one component of the overall signaling response, and the challenge, therefore, is to determine the contribution of MAPK signaling pathways in triggering defense reactions.

**Fig. 4.** PcMKK5 is required for Pep-13-mediated PR gene activation. A, expression of the constitutive-active MAPKK form, PcMKK5-dd, is sufficient to drive the activation of the parsley PR2-promoter activity in protoplasts. B, loss-of-function PcMKK5 (PcMKK5-kr) can inhibit the Pep-13 induction of PR2-promoter activity.

which had no influence on the Pep-13 responsiveness of the promoter. Fig. 4B demonstrates co-transfection of wild-type PcMKK5 to also have an inhibitory effect upon promoter activation. This was a consistent effect and was similar to that seen in previous work using MAPK constructs, where often the presence of overexpressed wild type PcMPK6 was seen to interfere with the responsiveness of the PR2 promoter (16). This may therefore reflect a disruption of the normal signaling following overexpression of one of the key proteins involved. However, significantly in both cases, the respective loss-of-function constructs further accentuate the observed inhibition of Pep-13-mediated PR2 promoter activity. Taken together, these results demonstrate that PcMKK5 functions in the Pep-13-induced signaling pathway leading to expression of “early” defense response genes in parsley cells and that this is most probably mediated through its activation of PcMPK6, 3a, and 3b. The lack of effect of PcMKK2 supports the above observations that suggested this protein to function in alternative signaling pathways.

**Pep-13 Treatment of Parsley Cells Induces the Translocation and Accumulation of PcMPK6 and 3ab in the Nucleus, Whereas PcMKK5 Remains Cytosolic**—Previous work, and that described here, has identified elements of Pep-13-inducible MAPK cascades that function in the control of “early” defense gene expression. It was previously shown that a parsley MAPK rapidly translocates to the nucleus following Pep-13 treatment (15). The timing of this nuclear translocation of ERMK (now renamed PcMPK3a) correlated closely with its activation as determined by immunoprecipitation–kinase assays. The localization of MAPK pathway components is key to understanding how the specificity of signaling is maintained and signaling is further propagated. We employed our specific antisera for immunofluorescence analysis of the localization of the different MAPks and MAPKKs in both control cells and those that had been treated with the Pep-13 elicitor. Treated parsley cell cultures were fixed and then embedded in paraffin. Six-micrometer sections were then taken and immunostained with antibodies specific for PcMPK3b (Fig. 5, A and B), PcMPK6 (C and D), PcMPK4 (E and F), PcMKK2 (G and H), and PcMKK5 (I and J). The panels A–I of Fig. 5 display immunofluorescent localization of each protein, whereas the corresponding right panels, marked A′–J′, display the DAPI stain indicating the position of the nuclei.

Fig. 5 demonstrates that, in control-treated cells (A, C, E, G, and I), all the studied proteins predominantly exhibit a cytosolic localization with only occasional staining of the nucleus. After 5 min of Pep-13 treatment an increase in the nuclear staining of PcMPK6 and PcMPK3b can already be seen (not shown). Following 15 min of treatment the staining intensity and frequency of nuclear staining further increased (Fig. 5, B and D) with an approximate 60% of all visible nuclei showing positive staining (Fig. 5K). This suggests that, during the activation time course of the PcMPK6 and 3 kinases, translocation activity is increased and the proteins begin to accumulate in the nucleus. In contrast to this, immunostaining of PcMPK4 showed constitutive cytosolic localization during treatment (Fig. 5, E and F). Because this MAPK is not activated during Pep-13 signaling, this may suggest that activation of the protein is necessary for nuclear translocation. Significantly, despite its activation by Pep-13, no or little nuclear localization of PcMKK5 was detectable (Fig. 5, I and J). In fact, following 15 min of Pep-13 treatment, immunostaining was often seen in the perinuclear region (compare J and J′ of Fig. 5). This suggests that, in contrast to the situation for its substrate MAPks, PcMKK5 does not accumulate in the nucleus following Pep-13 treatment. Similarly, and as expected based on the lack of activation of the protein, no change in the localization of PcMKK2 was detected following Pep-13 treatment (Fig. 5, G and H).
We have previously reported the rapid activation of 3 MAPKs in Pep-13-treated parsley cells and have shown these activities to be necessary for triggering "early" defense gene activation, via a signaling pathway that is independent of the oxidative burst response (16). Roles for MAPK activation in triggering defense gene expression have also been demonstrated in *A. thaliana* following FLS2-mediated perception of bacterial flagellin (27) and in tobacco in response to *Phytophthora*-derived elicitors (37). In both these experimental systems at least one upstream activator of the MAPKs involved has been identified (27, 37). According to the recently established MAPK pathway nomenclature (29) these upstream MAPKKs belong to the sub-class C based upon primary amino acid sequence similarity. The PcMKK5 identified in this work is also a member of this sub-class. In addition to the C class members another MAPKK, belonging to the A1 sub-class, was implicated.
in elicitor signaling from alfalfa. This MAPKK, referred to as MsPRKK, was shown to increase the activation levels of the alfalfa MAPKs, MsSIMK and MsSAMK, when heterologously co-expressed in parsley protoplasts subsequently treated with the Pep-13 elicitor (22). The PcMKK2 presented here is also a member of the A1 sub-group but, as described throughout this work, is not activated during Pep-13 signaling and does not appear to trigger the signaling pathways downstream of MAPKK activation that lead to the activation of “early” defense genes. This suggests that MAPKK members that are classified into the same sub-groups based upon sequence similarity may not necessarily have identical functions. Alternatively, the result involving MsPRKK may be a consequence of inappropriate overexpression of multiple kinases in the transfected protoplasts.

The essential starting criteria for a MAPKK as being implicated in a particular signaling chain is the direct demonstration of a change in the protein activity following addition of a stimulus. We have shown herein that PcMKK5 is activated rapidly in parsley cells following treatment with the Pep-13 elicitor and following treatment with HrpZ from Pseudomonas syringae. Because these elicitors are perceived by parsley cells via different receptors, this one again points to a convergence of the signaling pathways in the plant innate immune response (3, 4); it would appear in this case that the convergence point lies somewhere upstream of the activation of PcMKK5. Moreover, the temporal activation profile of PcMKK5 is identical to the activation of the downstream MAPK, PcMPK6. This suggests that sustained MAPK activity is required for sustained MAPK activity. Studies performed in alfalfa have indicated that different phosphatase activities are involved in the deactivation of MAPKs and MAPKKs (42). This suggests that for deactivation of the Pep-13-stimulated MAPK signaling pathway, MAPKK inactivation via dephosphorylation through protein phosphatases will probably precede MAPK deactivation by other or the same uncharacterized phosphatase activities.

PcMKK5 was also demonstrated here to be an in vivo activator of the three MAPKs shown to be activated in parsley cells following Pep-13 treatment. In this respect, PcMKK5 is not only similar at the amino acid sequence level to NtMEK2 of tobacco and AtMKK4/AtMKK5 of Arabidopsis but also functionally analogous to these proteins, which have been shown to be in vivo activators of SIPK and WIPK of tobacco, and AtMPK6 and AtMPK3 of Arabidopsis, respectively (27, 37, 43). Although direct activation of AtMKK4 and AtMKK5 following Flg22 treatment of Arabidopsis cells was not shown, the existence of two close and functionally related MAPKK homologues in the Arabidopsis genome might indicate that functional redundancy at the level of MAPKK signaling may occur in plants. For this reason, PcMKK5 may not be the sole elicitor-activated MAPKK in parsley cells. Nevertheless, the blocking of PR2 promoter-driven reporter gene expression by the “loss-of-function” PcMKK5-kr construct indicates that any other MKK5 homologues that may be present are likely redundant in function. Previous work has indicated that at least one Pep-13-activated MAPK remains to be identified (16), and we cannot say whether activation of PcMKK5 (or redundant homologues) is/are responsible for the activation of this as yet uncharacterized MAPK.

Further support for PcMKK5 as being an authentic upstream activator of the Pep-13-activated MAPKs came from both gain- and loss-of-function experiments that demonstrated a role in the activation of “early” defense genes, typified by the parsley PR2 gene (16). Because this gene was previously shown by loss-of-function studies to be controlled via the activation of PcMPK6, this provides solid evidence for a functional role for PcMKK5 in this signaling chain. In plants, with the exception of work performed studying MAPK signaling following Flg22 treatment of Arabidopsis (27), most studies have linked changes in MAPKK activity with downstream defense responses using only gain-of-function approaches. These studies have implicated activated MAPKKs in triggering gene expression, 

H₂O₂ production, and hypersensitive-like cell death (43, 44). However, the corresponding loss-of-function information was not shown that would unequivocally demonstrate a function for these activated MAPKKs in these processes. Our ability to demonstrate, through both loss- and gain-of-function approaches, a role for activation of PcMKK5 in controlling Pep-13-induced defense gene expression provides convincing evidence for an authentic function for this protein in mediating this response.

As discussed earlier, despite many experiments being performed using transient overexpression of PcMKK2 and PcMKK5, and respective mutant constructs, there is still a high level of specificity with respect to protein activation and function. One way in which such high levels of specificity are believed to be maintained is through interactions with other proteins and strict regulation of protein localization and the localization of protein substrates. MAPK and MAPKK localization has been studied extensively in both the mammalian and yeast systems under various conditions that are often seen to induce changes in the localization of the proteins, which correlate with changes in their activity states (33, 45, 46). With respect to studies in plants, variations in cellular localizations of MAPKs have been reported during the cell cycle of tobacco (47), in alfalfa cells (48), and embryogenesis of microspores and other plant differentiation processes of several plant species (49, 50). A dynamic re-localization of SIMK from the nucleus to the growing tips was also reported during root hair formation in alfalfa (51). To date there exists only one demonstration of rapid changes in localization of MAPKs during plant stress responses (15). However, in this previous work, it was not certain if the Pep13-stimulated nuclear translocation of MAPKs involved PcMPK6 or PcMPK3a/b or both. The studies presented here have now provided a more complete picture of the dynamic relationship of MAPKs and MAPKKs that function in this response. The use of specific antibodies demonstrated unequivocally that the MAPKs, PcMPK6, 3a, and 3b, show a rapid nuclear translocation that correlates with their activation following Pep-13 treatment. The proteins appear to accumulate in the nucleus over time, to such an extent that after 15-min Pep-13 treatment over 60% of the visible nuclei showed strong positive immunofluorescence.

In contrast, MAPKs that are not activated, as shown here for PcMPK4, remain cytosolic. PcMKK2, which is not activated in Pep-13-treated cells similarly shows no nuclear accumulation. The upstream activator of the elicitor-activated MAPKs, PcMKK5, despite itself being activated also shows no nuclear accumulation and remains distributed throughout the cytosol or in perinuclear regions. Based upon these observations and upon studies reported in yeast and mammals (31–33, 52), we may hypothesize that in resting cells PcMKK5 functions as an anchor in keeping PcMPK6, 3a, and 3b in the cytosol. Following Pep-13 treatment PcMKK5 becomes activated, leading to phosphorylation and activation of the MAPKs. The MAPKs then translocate to, and accumulate in, the nucleus where they may phosphorylate specific substrate proteins implicated in inducing defense gene expression. How MAPK translocation is mediated in plants is currently unknown, because they do not appear to have classic nuclear localization signals and should be the subject of future studies. However, based upon our available data we might envisage one of two scenarios: 1) the
activated MAPKs dissociate from the anchoring PcMKK5 following elicitation (Fig. 5F) by jasmonate-mediated mechanism. Thus, the higher frequency of pe-振兴ated MAPKs dissociate from the anchoring PcMKK5-con-