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Mitogen-activated Protein Kinases Play an Essential Role in Oxidative Burst-independent Expression of Pathogenesis-related Genes in Parsley*

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Plants are continuously exposed to attack by potential phytopathogens. Disease prevention requires pathogen recognition and the induction of a multifaceted defense response. We are studying the non-host disease resistance response of parsley to the oomycete, *Phytophthora sojae* using a cell culture-based system. Receptor-mediated recognition of *P. sojae* may be achieved through a thirteen amino acid peptide sequence (Pep-13) present within an abundant cell wall transglutaminase. Following recognition of this elicitor molecule, parsley cells mount a defense response, which includes the generation of reactive oxygen species (ROS) and transcriptional activation of genes encoding pathogenesis-related (PR) proteins or enzymes involved in the synthesis of antimicrobial phytoalexins. Treatment of parsley cells with the NADPH oxidase inhibitor, diphenylene iodonium (DPI), blocked both Pep-13-induced phytoalexin production and the accumulation of transcripts encoding enzymes involved in their synthesis. In contrast, DPI treatment had no effect upon Pep-13-induced *PR* gene expression, suggesting the existence of an oxidative burst-independent mechanism for the transcriptional activation of *PR* genes. The use of specific antibodies enabled the identification of three parsley mitogen-activated protein kinases (MAPKs) that are activated within the signal transduction pathway(s) triggered following recognition of Pep-13. Other environmental challenges failed to activate these kinases in parsley cells, suggesting that their activation plays a key role in defense signal transduction. Moreover, by making use of a protoplast co-transfection system overexpressing wild-type and loss-of-function MAPK mutants, we show an essential role for post-translational phosphorylation and activation of MAPKs for oxidative burst-independent *PR* promoter activation.

In most circumstances plants are able to defend themselves against pathogen attack. This is primarily facilitated through recognition mechanisms, which plants use to sense the presence of the pathogen (1–3), and through triggering intrinsic defense mechanisms that either kill the pathogen or limit its spread to the site of immediate infection (4, 5). Parsley (*Petroselinum crispum*) exhibits a non-host resistance response to attack by the oomycetes, *Phytophthora infestans* and *Phytophthora sojae* (6, 7). Defense reactions are triggered through the recognition of an abundant cell wall transglutaminase present and conserved in all but one tested member of *Phytophthora* (8). This protein was previously characterized as a 42-kDa glycoprotein purified from *P. sojae* that was able to trigger phytoalexin accumulation when added to cultured parsley cells (9, 10). Within this protein resides a conserved peptide sequence of 13 amino acids (Pep-13) that is necessary and sufficient for its elicitor activity (11). The ability of Pep-13 to trigger defense responses in parsley requires its interaction with a 100-kDa receptor protein present in the plasma membrane of parsley cells (12, 13), since all mutations made within the Pep-13 sequence that prevented binding to the receptor also inhibited the elicitation of defense reactions (11, 14–17). The defense response itself is multifaceted and involves the generation of reactive oxygen species (ROS)[1] (15), the synthesis of antimicrobial furanocoumarin phytoalexins (10), and the expression of defense-related genes including pathogenesis-related (*PR*) genes (18). Pep-13-induced defense gene activation is temporally regulated (18). Transcripts of immediate early genes, including the *WRKY1*, *-3*, *-4*, and *–5* transcription factor genes, accumulate rapidly after elicitation apparently without the requirement of *de novo* protein synthesis (19). With a slight delay, transient activation of another group of early genes is observed, among these are the *PR1* and *PR2* genes (18, 20, 21). Many *PR*-type defense-related genes appear to be regulated by WRKY transcription factors (22, 23), which have been analyzed in particular for the parsley *PR1* promoter (21, 24). Transcripts encoding enzymes implicated in phenylpropanoid metabolism and the synthesis of the furanocoumarin phytoalexins, including phenylalanine ammonia-lyase (PAL), 4-coumarate:CoA ligase (4CL), and *S*-adenosyl-L-methionine:bergaptol *O*-methyltransferase (BMT) accumulate even later (20). Treatment of

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y12875, AY173415, AY173414, and AY17343.

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[1] The abbreviations used are: ROS, reactive oxygen species; PR, pathogenesis-related; MAPK, mitogen-activated protein kinase; GST, glutathione *S*-transferase; GUS, β-glucuronidase; LUC, luciferase; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MBP, myelin basic protein; DPI, diphenylene iodonium; Me_2SO, dimethyl sulfoxide; BMT, *S*-adenosyl-L-methionine:bergaptol *O*-methyltransferase; ERM kinase, elicitor-responsive MAPK; 2,4-D,2,4-dichlorophenoxy acetic acid.

parsley cells with diphenylene iodonium chloride (DPI) blocks both the induction of the oxidative burst and phytoalexin biosynthesis by elicited parsley cells (15). Moreover, it has been shown that the generation of O_2^- via the oxidative burst is necessary and sufficient to drive phytoalexin biosynthesis by the cells (15). Calcium influx through Pep-13-responsive ion channels of the plasma membrane (15, 17) followed by elevation of cytosolic calcium levels (14) were found to be located upstream of the oxidative burst and the activation of a mitogen-activated protein kinase (MAPK) (14, 16). The DPI insensitivity of this Pep-13-induced MAPK activation positions this kinase between calcium influx and oxidative burst or indicates bifurcation of the signaling pathway into DPI-sensitive and -insensitive branches (16).

Pharmacological and ^{32}P-labeling studies have long since indicated the importance of protein phosphorylation and protein kinase activities in bringing about pathogen defense responses both in parsley and other systems (25, 26). Among the many implicated protein kinases, the activation of MAPKs has been shown to be a consistent and common response of plant cells following infection and exposure to microbial elicitors (27, 28). Based upon analysis of the fully sequenced *Arabidopsis thaliana* genome, plants appear to contain more putative MAPKs than any other known organism, including humans (29). *Arabidopsis* possesses at least 20 MAPK-encoding genes that fall into a minimum of four subgroups (30, 31). In all systems whereby MAPK activity has been studied with respect to elicitor responses, activation of members of the AtMPK6 subgroup has been described (1, 27). This includes the responses of tobacco SIPK to general elicitors, such as Harpin and elicitins, TMV infection, and race-specific elicitation (32–36); alfalfa SIMK to chitin, ergosterol, and β-glucans (37); *A. thaliana* AtMPK6 to bacterial elicitors including the flg22 peptide from flagellin (38) and Harpin (39). It was recently demonstrated for *A. thaliana* that MAPKs can also act as negative regulators of defense responses, as shown for AtMPK4 mutants (40); however, this would appear to be contradictory to the activation of this kinase described in response to Harpin (39). Members of a second closely related class of MAPKs, initially characterized in tobacco as being activated following wounding (WIPK) (41, 42), and having homology to AtMPK3, have also been implicated in pathogen defense signaling (34, 43, 44). Our previous studies demonstrated the activation of such a homologue, described as ERM kinase, following treatment of parsley cells with the Pep-13 elicitor (16).

Evidence indicating the importance of MAPK activation for the elicitation of defense reactions has recently emerged from gain-of-function experiments whereby MAPKs themselves, or constitutively active forms of their upstream activators, MAPK kinases (MAPKKs), were transiently overexpressed in tobacco and *Arabidopsis* leaves (45–47). This resulted in a hypersensitive response-type phenotype in leaves in addition to activation of genes implicated in the biosynthesis of defense-related antimicrobial compounds. These observations have recently been supported by the identification of a complete MAPK cascade from *A. thaliana* that is triggered through recognition of flg22 (48). This resulted not only in the accumulation of transcripts of a group of defense-related genes, but also in increased resistance to attack by both fungal and bacterial pathogens (48). In addition to these functions in defense, AtMPK6 homologues have been shown to be activated in response to various abiotic stresses including osmotic stresses, ozone exposure, oxidative stress, cold stress, drought, and treatment with salicylic acid (32, 49–58). It has therefore been suggested that members of this class of MAPKs may function as points of cross-talk between various stress signaling pathways in plants (3, 27, 30).

In this article we demonstrate the existence of parallel pathways that operate to induce the transcriptional activation of particular sets of defense-related genes in parsley. One pathway is triggered downstream of the oxidative burst and controls genes implicated in phytoalexin biosynthesis. The second pathway is independent of the oxidative burst, but is dependent on MAPK activity. The MAPKs involved are activated in parsley cells through receptor-mediated recognition of the Pep-13 elicitor and other elicitors of defense reactions, but appear largely insensitive to abiotic stresses, suggesting that their activation is primarily associated with pathogen defense. Furthermore, by utilizing a protoplast transient transfection system employing loss-of-function MAPK mutants, we demonstrate a requirement of MAPK activity for the elicitor-mediated oxidative burst-independent activation of *PR* genes, which represent classical markers for pathogen defense responses in plants.

EXPERIMENTAL PROCEDURES

Elicitor Preparations—The Pep-13 elicitor was chemically synthesized as previously described (13). *Pseudomonas syringae* HrpZ was expressed and purified as a recombinant protein from *Escherichia coli* (59). Synthetic *N*-acetyl chitoheptaose was provided by Naota Shibuya (University of Tsukuba, Tsukuba, Japan).

Cell Culture Handling, Treatment, and Protoplast Isolation—Cultured parsley cells were maintained in modified Gamborg's B5-Medium containing 1 mg/liter of 2,4-D as previously described (60). Protoplasts were isolated 5 days following transfer of the culture to fresh medium according to previously described methods (61). Cells were treated by addition of the stimulus to cells previously washed and allowed to equilibrate for 30 min in fresh medium. All treatments were performed by direct application from appropriate stock solutions, or in the case of hypo-osmotic treatment, following dilution in four volumes of medium lacking the osmoticum (sucrose-free). Following appropriate time points cells were collected by vacuum filtration, quickly frozen in liquid N_2, and stored at −80 °C until use.

RNA Isolation and Reverse Transcription (RT)-PCR Analysis—Total RNA was extracted from parsley cells at different times after elicitor treatment by using the TRIzol reagent (Invitrogen) according to the manufacturer's guidelines. For RT-PCR analysis cDNA was synthesized from 2 μg of total RNA by using reverse transcriptase and oligo(dT) or 18Sr primers. The cDNA was amplified by PCR with the following gene-specific primers: PR2f (5′-AGGGCTTTCTTCTTGACAT-3′), PR2r (5′-CTTCGATTGACTTTATTATTCTTA-3′), BMTf (5′-CAAAGCTGGCCCTGGTAACTATT-3′), BMTr (5′-GGCGTCTCCTTTTGGCACAC-3′), WRKY1f (5′-AATCATAACCATCCAAAGC-3′), WRKY1r (5′-CATATTTCAAACAAGGTACACT-3′), PAL2f (5′-TG AAATTGCGATGGCTAG-3′) PAL2r (5′-TTTAAGTAGCAAGAGCCTT-3′), 18Sf (5′-GATGGTAGGATAGTGGCCTA-3′), and 18Sr (5′-TGGTTCAGACTAGGACGATA-3′). PCR was performed in a 50-μl reaction volume containing 1× *Taq*PCR buffer (Promega, Madison, WI), 0.25 mM dNTPs, 0.5 units of *Taq*, and 0.5 μM concentrations of each primer. The PCR cycle consisted of 2 min at 94 °C, 18 cycles of 30 s at 94 °C, 45 s at 50 °C, 80 s at 72 °C, and one final extension step of 7 min at 72 °C. The products were analyzed by agarose gel electrophoresis.

Acquisition and Analysis of MAPK cDNAs—A λ-ZAP™ II (Stratagene, Heidelberg, Germany) phage cDNA library was prepared from mRNA of elicited and un-elicited cultured parsley cells according to the manufacturer's guidelines and screened with radioactively labeled probes corresponding to the open reading frames of the MAPK-encoding genes *MMK1* (62, 63) and *MMK4* from alfalfa (55). Each probe was used to screen 6×10^5 plaques and resulted in the acquisition of 8 cDNA clones encoding 4 different full-length open reading frames. Sequence analysis of the cDNAs and their encoded proteins were performed using the DNASIS 2.1 software (Hitachi, Tokyo, Japan).

Site-directed Mutagenesis—Single point mutations were introduced into MAPK sequences present within vector pGEM-T (Promega, Mannheim, Germany) by PCR-based site-directed mutagenesis using the GeneEditor system (Promega, Mannheim, Germany) and the following 5′-phosphorylated oligonucleotides: PcMPK6Y214F, 5′-GATTTTATGACAGAATTTGTTGTTACAAGATGG; PcMPK6D348N, 5′-CTGCACGACATCAGTAACGAGCCTGTATGTG; PcMPK4Y200F, 5′-GATTTTATGACAGAATTTGTTGTTACTCGCTGG. The manufacturer's guidelines were followed, and the resulting mutants were verified by sequencing.

Generation and Bacterial Expression of Glutathione S-Transferase Fusion Proteins—MAPK-encoding open reading frames were cloned as *Bam*H1/*Xho*I PCR fragments into vector pGEX 2T-2 (Amersham Biosciences) for expression in *E. coli* (strain BL-21) as fusion proteins containing an N-terminal GST moiety. Recombinant proteins were subsequently purified using glutathione-Sepharose 4B according to the manufacturer's guidelines.

Protoplast Transfection and Co-transfection—For MAPK activity measurements wild-type or mutated open reading frames were cloned as *Nco*I/*Bam*H1 fragments into vector pRT100 (64) behind an introduced c-Myc-encoding sequence and the 35S-cauliflower mosaic virus promoter. 30 μg of each construct were then used to transfect 2×10^6 protoplasts (~200 μl). Protoplasts and DNA were mixed before the addition of 600 μl of 25% (w/v) polyethylene glycol (PEG) 6000, pH 9.0, containing 100 mM $Ca(NO_3)_2$ and 45 mM mannitol. Following a 20-min incubation, the protoplasts were collected by centrifugation after the addition of 7 ml of 0.275 M $Ca(NO_3)_2$, pH 6.0, then resuspended in 4 ml of B5-sucrose solution (0.28 M sucrose, 1 mg/ml 2,4-D, 3.2 mg/ml B5 medium (solid)) and divided into two Petri dishes. Following 24 h of incubation, the dishes were treated with either 100 nM Pep-13 or water for 15 min. The protoplasts were then collected by centrifugation following the addition of 25 ml of 0.24 M $CaCl_2$ and quickly frozen in liquid N_2. Co-transfection experiments were performed as already described with the following modifications: 20 μg of MAPK constructs were transfected in combination with 5 μg of PR2-promoter-GUS (β-glucuronidase) construct (24) and 5 μg of the normalization plasmid, pRTLUC (65). Following an 8-h incubation in B5-sucrose medium, the protoplasts were treated either with water or 100 nM Pep-13 and incubated for a further 14 h. Protoplasts were then collected and stored as described.

Protein Extraction—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 $MgCl_2$, 1 mM dithiothreitol, 1 mM NaF, 0.5 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1% (v/v) Tween 20) followed by centrifugation ($23{,}000 \times g$) for 10 min at 4 °C. Protoplasts were extracted in the same buffer by vortexing for 30 s. For studies involving luciferase (LUC) and GUS measurements protoplasts were extracted in K_2HPO_4/KH_2PO_4, pH 7.5, containing 1 mM dithiothreitol.

GUS and 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 $MgSO_4$, 1 mM $(MgCO_3)_4/Mg(OH)_2{\cdot}5H_2O$, 0.1 mM EDTA, 30 mM dithiothreitol, 300 μM coenzyme A, 500 μM ATP, 500 μM luciferin) and measured for 5 s in a luminometer (Luminoscan Ascent plate reader, Labsystems, Frankfurt, Germany). For GUS activities, 10 μl of protoplast extract was mixed with 40 μl of substrate (50 mM Na_2HPO_4/NaH_2PO_4, pH 7, 10 mM mercaptoethanol, 2 mM 4-methylumbelliferyl β-D-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 M $Na(CO_3)_2$ fluorescence was measured at 360 nm excitation/440 nm emission using the Cytofluor II apparatus (Biosearch, Bedford, MA).

In-gel Protein Kinase Assays—Cell extracts containing 20 μg of protein per lane were separated on 10% PAGE gels containing 0.1 mg/ml myelin basic protein (MBP) (Sigma). All subsequent denaturation, renaturation, kinase activity, and washing steps were performed as previously described (66). Protein kinase activity was visualized by phosphorimaging (Molecular Dynamics, Krefeld, Germany).

Antibody Production—Peptides were synthesized corresponding to amino acid sequences 2–15 in PcMPK6 (DGSTQPSDTVMSDAC); 1–11 in PcMPK3b (MANPGDGQYDC); and 360–374 in PcMPK4 (CEQHALTEEQMRE). The peptides were then coupled to keyhole limpet hemocyanin and used to raise antiserum following immunization of rabbits (Eurogentec, Seraing, Belgium).

Western Blotting—SDS-PAGE gels were semidry-blotted onto nitrocellulose membrane (Porablot-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% (w/v) skimmed milk powder or 5% (w/v) bovine serum albumin. Primary antibody solutions were prepared in blocking solution at the following dilutions: 1:10,000 anti-PcMPK6, 3, or 4; 1:500 monoclonal anti-c-Myc (Sigma); 1:15,000 anti-ACTIVE™ MAPK (Promega, Mannheim, 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 terazolium/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 MAPK-specific or c-Myc antibodies coupled to protein A- or protein G-Sepharose (Amersham Biosciences). Subsequent washing and *in vitro* MBP phosphorylation reactions were as described previously (16). Reactions were stopped by the addition of SDS sample buffer and boiling. The proteins were then separated by SDS-PAGE, and MBP phosphorylation was determined by phosphorimaging.

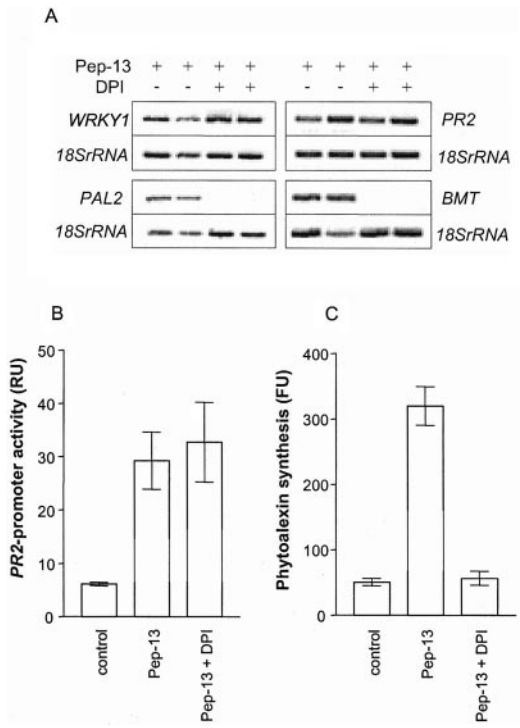

FIG. 1. **Differential activation of parsley defense-related genes through oxidative burst-dependent and -independent pathways in response to the Pep-13 elicitor.** *A*, RT-PCR analysis of transcript accumulation demonstrates the existence of parallel independent pathways leading to defense gene expression. Parsley cells were pretreated for 30 min with either 10 μM DPI (+) or an equivalent volume of Me_2SO (−) prior to addition of 100 nM Pep-13 (+). Cells were then harvested at the following time points: *WRKY1*, 1 h post-elicitation; *PR2*, 4 h; *PAL2*, 8 h; and *BMT*, 24 h. RNA was isolated and used for RT-PCR analysis in order to determine defense gene transcript levels. The transcript level of 18 S rRNA was also determined for each time point for normalization purposes, and each treatment is shown in duplicate. *B*, promoter activity studies confirm oxidative burst-independent transcriptional activation of the *PR2* gene. Parsley protoplasts were transfected with a *PR2* promoter fused to the gene encoding GUS in addition to an 35S-promoter-driven LUC construct (*35S-LUC*). Protoplasts were then treated with 10 μM DPI or a corresponding volume of Me_2SO 30 min prior to addition of 100 nM Pep-13. Following a further 14-h incubation, the protoplasts were harvested, extracts were generated, and GUS and LUC activity determinations were performed. The data are expressed as GUS/LUC activities for each treatment ($n = 4$). *C*, fluorescence of the culture medium was also measured prior to protoplast harvesting to confirm the inhibitory effect of 10 μM DPI upon phytoalexin production.

RESULTS

Differential Activation of Defense Genes through Oxidative Burst-dependent and -independent Pathways—We sought to identify genes whose transcriptional activation occurred independently of the oxidative burst signaling pathway, by performing RT-PCR analysis of defense transcript accumulation in Pep-13- and DPI-treated parsley cells. Prior to RNA isolation, treated cells were tested to ensure that 10 μM DPI had effectively blocked Pep-13-induced phytoalexin production, measured 24 h after elicitation (not shown). Transcripts were examined belonging to each of the "immediate early," "early," and "late" responses in addition to an 18 S rRNA control, and typical results were seen as illustrated by the duplicate treatments shown in Fig. 1*A*. The *WRKY1* transcription factor and *PR2* genes are characteristic immediate early and early elici-

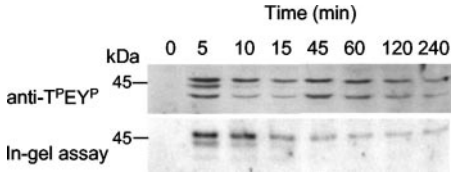

FIG. 2. **The Pep-13 elicitor activates at least three independent MAPKs in cultured parsley cells.** Cells treated with 100 nM Pep-13 were harvested after various time periods and cell extracts were prepared. Proteins (20 μg/lane) were then separated by SDS-PAGE, blotted, and probed with antibodies cross-reacting with activated MAPKs (*anti-T^{P}EY^P*, *upper panel*), or separated on SDS-PAGE gels containing 0.1 mg/ml MBP to test in-gel kinase activities (*lower panel*). Both techniques revealed the activation of at least three MAPKs (M_r ~46, 44, and 42) in Pep-13-treated cells.

tor-responsive genes, respectively (24, 67). As illustrated in Fig. 1*A*, the transcriptional activation of each gene, measured at 1 and 4 h post-elicitation, respectively, was unaffected by DPI treatment. This was in contrast to genes characteristic of the late response, including *PAL2* and *BMT* genes (20), whose activation was inhibited by DPI treatment at all time points tested (Fig. 1 displays the 8-h *PAL2* and 24-h *BMT*). In addition to the genes shown in Fig. 1*A*, other genes were examined that were either sensitive, such as the *S*-adenosyl-L-methionine: caffeoyl-CoA *O*-methyltransferase gene, or insensitive to DPI, such as the *PR1-3* gene. In principle, those genes encoding enzymes of phenylpropanoid metabolism were most strongly affected (data not shown). The only unexpected variation to this theme was 4CL whose transcript accumulation reproducibly showed no inhibition by DPI under the conditions tested. However, as illustrated by Fig. 1*A*, which clearly and consistently showed inhibition by DPI of *PAL* gene expression, transcript accumulation of the *PR2* gene was not affected, suggesting that this gene is regulated by an oxidative burst-independent pathway. To further test this hypothesis we performed additional experiments aimed at studying *PR2* promoter activity in Pep-13- and DPI-treated transfected protoplasts. Parsley protoplasts were co-transfected with a plasmid containing a *PR2* promoter-*GUS* construct (24, 67) in addition to a 35S-promoter-*LUC* construct for normalization. Twenty-four hours after elicitation, the protoplasts were first tested for phytoalexin synthesis prior to their harvesting and the determination of GUS and LUC activities in extracts. Fig. 1*C* shows that 10 μM DPI effectively blocked phytoalexin synthesis by the transfected protoplasts, which is in agreement with the responses seen in cells. However, this treatment had no effect upon the elicitor responsiveness of the *PR2* promoter (Fig. 1*B*), whose activation was indistinguishable to that seen in solvent-treated cells in response to Pep-13. These data support the hypothesis that there exist parallel signaling pathways leading to defense gene expression in parsley cells, one being mediated through the oxidative burst, while the other appears independent of this response and results in the activation of *PR2* and *WRKY1* genes.

Treatment of Cultured Parsley Cells with the Pep-13 Elicitor Induces the Activation of At Least Three MAPKs—We reported previously that in parsley cells a MAPK is activated in a receptor-mediated manner following treatment with the Pep-13 elicitor peptide (16). This activation was shown to be DPI-insensitive, suggesting that these activities are located upstream or independent of the oxidative burst and may be involved in the oxidative burst-insensitive pathway leading to PR gene expression. By using a modified MBP in-gel assay we found that in fact three MBP kinases were rapidly activated in response to Pep-13 treatment (Fig. 2, *lower panel*). The largest kinase had an apparent molecular weight of 46 kDa and showed a sustained activation lasting for up to 240 min, while

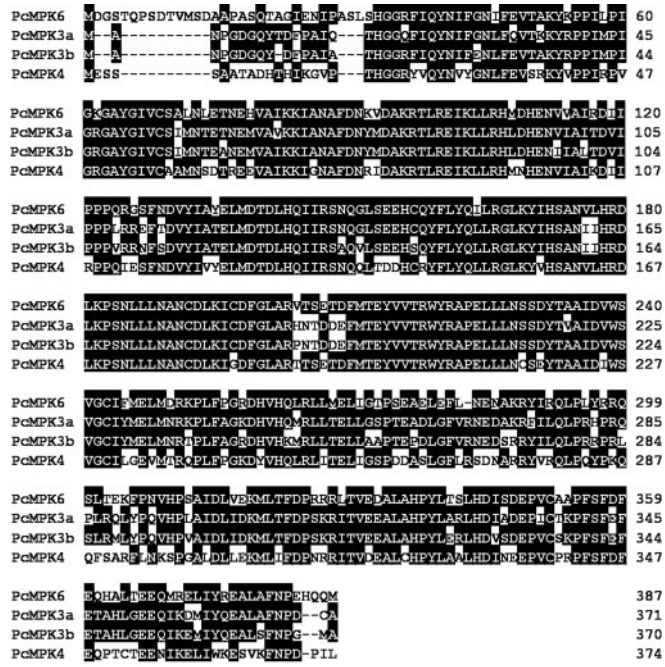

FIG. 3. **Sequence alignments of the encoded proteins of four parsley MAPK cDNA clones.** Four MAPK encoding cDNA clones were isolated from a library generated from a mixture of Pep-13-treated and untreated parsley cells. Based upon the homology to *A. thaliana* MAPKs the parsley MAPK are referred to as PcMPK6, 3a, 3b, and 4. Alignments between the encoded amino acid sequences are shown, and fully conserved residues are indicated in *black boxes*.

two other proteins (44 and 42 kDa in size) showed a more transient activation profile. As the MBP in-gel kinase assay is a sensitive detection method for activated MAPKs, and as the size of the detected kinases are in agreement with those of this class of protein (31, 68), we hypothesized that all the elicitor-responsive MBP kinases are indeed MAPKs. To verify this we used an antiserum that recognizes the dually phosphorylated T^{P}EY^P motif, that is present in the activation loop of most MAPKs from mammals and yeast (68), and also from plants (31). The phosphorylation of this motif is mediated by dual specificity upstream MAPKKs, and leads to the activation of the kinase activity of the MAPKs (30, 68). In Western blot experiments with protein extracts from elicitor-treated cells, this anti-T^{P}EY^P antiserum detected three bands of sizes identical to those seen in the in-gel kinase assay (Fig. 2, *upper panel*). In contrast to this, no signals were detected in protein extracts from non-treated cells, confirming that the elicitor-responsive MBP kinases are MAPKs. The activation characteristics of the 46- and 44-kDa kinases matched the pattern seen in the in-gel assays. In contrast to this, the 42-kDa MAPK gave a relatively stronger signal in the Western blot experiments and was detectable up to 240 min after initiation of elicitor treatment.

Cloning of Parsley MAPK cDNA Clones—In order to identify the MAPKs detected in the Western blotting and in-gel kinase assays, and to address the question of their function in elicitor signal transduction, we initiated efforts to clone a variety of different MAPK-encoding cDNAs. Screening of a parsley cDNA library generated from a mixture of elicited and un-elicited cells with a DNA probe derived from the alfalfa SIMK/MMK1 cDNA (52, 55) was performed. This resulted in the identification of five independent cDNAs, out of which four contained complete open reading frames. Comparison of the deduced amino acid sequences of the encoded kinases (Fig. 3) indicated that they fall into three characteristic subgroups. One cDNA encoded a 46-kDa MAPK with strongest homology to a subclass

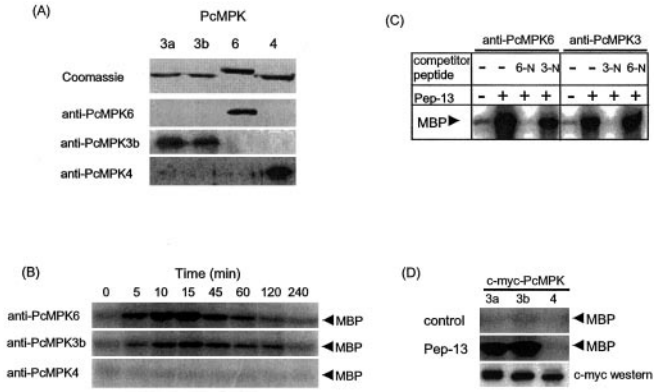

FIG. 4. **Use of MAPK-specific antisera and immunoprecipitation/protein kinase assays identify PcMPK6, 3a, and 3b as Pep-13-responsive.** *A*, specificity of peptide antibodies raised against peptide sequences contained within PcMPK6, 3b, and 4. Antisera cross-reactivity was tested by Western blotting against each of the recombinant MAPKs (100 ng/lane). *B*, immune complex-protein kinase assays. Cultured parsley cells were elicited with 100 nM Pep-13, and cell extracts containing 200 μg of protein were immunoprecipitated with the indicated antiserum coupled to protein A-Sepharose. The immune complexes were then tested for kinase activity by measuring incorporation of ^{32}P into MBP visualized following separation by SDS-PAGE. *C*, specificity of antisera in immune kinase assays. MAPKs were immunoprecipitated from extracts (200 μg of protein) of Pep-13-elicited parsley cells with PcMPK6 and PcMPK3b sera in the presence or absence of competitor peptides (20 μg/ml) corresponding to the N termini of PcMPK6 (6-N) and PcMPK3a and 3b (3-N). Kinase activity of the immune complexes was again determined using MBP as substrate. *D*, Pep-13 activates both PcMPK3a and PcMPK3b. PcMPK3a and 3b possessing an N-terminal c-Myc epitope tag were transiently expressed in parsley protoplasts through the activity of the 35S-promoter. Protoplasts were elicited with Pep-13 for 15 min 24 h after transfection. Proteins (100 μg) were extracted and immunoprecipitated with an antibody to the c-Myc tag. The kinase activity of the immune complexes was then determined using MBP as substrate.

of enzymes containing AtMPK6 from *A. thaliana*, and thus we refer to this sequence as PcMPK6. Additionally, two parsley cDNAs showed high sequence homology to one another (89% identity) and encode proteins of indistinguishable molecular weight (~44 kDa). These proteins exhibit closest homology to a subgroup of plant MAPKs containing *Arabidopsis* AtMPK3 and we thus refer to them as PcMPK3a (formerly described as ERMK, Ref. 16) and PcMPK3b. The final cDNA encodes a MAPK of 44 kDa with closest homology to AtMPK4 and is therefore named PcMPK4.

Use of Specific Antisera Reveal Pep-13-induced Activation of PcMPK6 and 3a/b—We next wished to determine, whether any of the parsley MAPK cDNAs we had cloned encoded one of the elicitor-responsive enzymes seen in the in-gel assay and Western blotting experiments. For this purpose antibodies discriminating between the different MAPK subgroups were produced by immunizing rabbits with synthetic peptides corresponding to the extreme N-terminal amino acid sequences of PcMPK6 and 3b and the extreme C terminus of PcMPK4, respectively. The specificity of the obtained antisera was tested in Western blot experiments with recombinant MAPKs produced as GST fusion proteins in *E. coli* (Fig. 4*A*). The antiserum generated against the peptide sequence of PcMPK6 only detected MPK6, while anti-PcMPK4 peptide antiserum only cross-reacted with MPK4. As predicted from the amino acid sequence conservation, the antiserum generated against the N-terminal peptide of PcMPK3b detected both PcMPK3b and 3a recombinant kinases with equal affinity but did not recognize either of the other tested MAPKs (Fig. 4*A*).

The antisera were then used in coupled immunoprecipitation/*in vitro* MBP kinase assays. Immunoprecipitations performed with anti-PcMPK6 and 3b sera precipitated MBP kinase activity from extracts of Pep-13-treated cells (Fig. 4*B*). These activities increased rapidly, within 5 min of elicitor treatment, and persisted at high levels for up to 240 min thereafter. To further test the specificity of the antisera in the immunoprecipitation experiments we performed competition studies using the peptides to which the antisera were generated. Fig. 4*C* demonstrates that the addition of a large excess of the peptide corresponding to the N terminus of PcMPK6 (6-N) prevented the immunoprecipitation of the activated PcMPK6 from elicited extracts. In contrast, addition of the peptide corresponding to the N terminus of the PcMPK3 proteins did not affect the immunoprecipitation of PcMPK6 by this antibody. Fig. 4*C* also shows the same pattern for the immunoprecipitation of the activated PcMPK3(s), whose immunoprecipitation was only blocked by addition of the N-terminal peptide of PcMPK3b (3-N). In contrast, the antiserum specific for PcMPK4 failed to immunoprecipitate an activated protein kinase from Pep-13-treated cells. These observations demonstrate that PcMPK6 and PcMPK3a and/or 3b are activated following Pep-13 treatment while PcMPK4 is not.

Transient Protoplast Transformation Confirms Pep-13-induced Activation of both PcMPK 3a and 3b—As described above and shown in Fig. 4*A*, the antiserum that immunoprecipitates activated PcMPK3 is unable to discriminate between the PcMPK3a and 3b homologues. Peptides that diverge in the highly homologous MPK3a and MPK3b proteins were found to be unsuitable for antibody production. We therefore decided to test, whether both these kinases were activated during the elicitor response by employing a protoplast transient expression system. N-terminal c-Myc-tagged PcMPK3a, 3b or 4 were overexpressed through the activity of the 35S-promoter in parsley protoplasts. The protoplasts were then treated with Pep-13, and immunoprecipitations were performed on cell extracts using c-Myc antibodies. The kinase activities of the immunoprecipitated epitope-tagged MAPKs were then determined by MBP phosphorylation. Equal expression of the constructs was verified by Western blotting with the c-Myc antiserum. As shown in Fig. 4*D*, both c-Myc-PcMPK3a and c-Myc-PcMPK3b were activated following Pep-13 treatment, suggesting that both kinases are activated in the parsley elicitor response, and make up together one of the activated MAPKs seen in the initial in-gel and Western blot experiments. In contrast to this, and in agreement with the immunoprecipitation experiments performed with the kinase-specific antibodies, c-Myc-PcMPK4 was not activated following treatment with Pep-13 (Fig. 4*B*).

Responses of PcMPK6 and PcMPK3 to Biotic and Abiotic Stress Stimuli—Studies performed in other plant systems have demonstrated that MAPK activation occurs as a common feature of many plant stress responses (30). In order to determine whether any of the parsley MAPKs plays a more general role in plant stress adaptation, we tested whether a selection of commonly studied stress treatments would induce activation of PcMPK6 and PcMPK3a/b. A range of treatments was applied to parsley cell cultures based upon conditions shown to activate MAPK signaling in cell cultures or protoplasts of alfalfa, tobacco, and *Arabidopsis* (32, 49–58). Immunoprecipitation/MBP phosphorylation assays were then performed and kinase activities were expressed against that seen in response to treatment with 100 nM Pep-13, which reproducibly gave the strongest kinase activation. The results of these investigations are presented in Fig. 5. No significant activation of either the PcMPK6 or PcMPK3a/b kinases were observed following treatments of parsley cells with 1 μM *N*-acetyl chitoheptaose (chitin), 250 μM salicylic acid, 250 mM NaCl, 500 nM sorbitol, or 4 volumes of hypotonic buffer (hypo-osmotic). These treatments did also not stimulate phytoalexin synthesis in parsley cells (not shown).

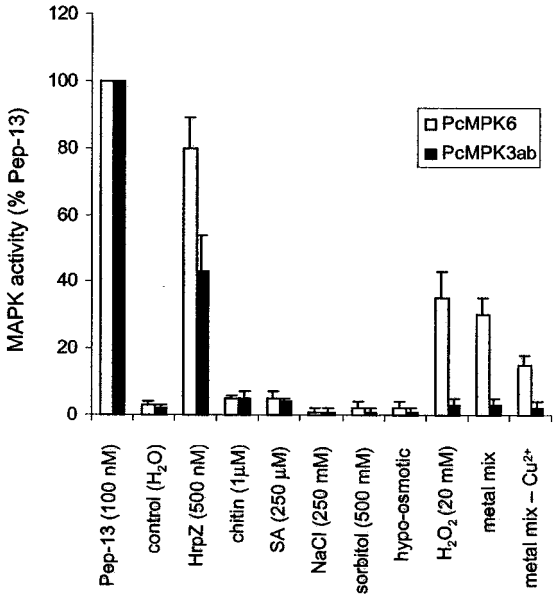

FIG. 5. **The responses of PcMPK6 and 3a/b to a range of biotic and abiotic stresses.** The responses of the PcMPK6 and PcMPK3a/b in parsley cells treated with various abiotic and biotic stress stimuli were determined by immunoprecipitation/protein kinase assays using MBP as substrate. All treatments were applied for 15 min. The metal mix contained 100 μM $CdCl_2$, 250 μM $ZnCl_2$, and 250 μM $CuCl_2$. Proteins (100 μg) were extracted and immunoprecipitated with either PcMPK6- or PcMPK3a/b-specific antisera. Kinase activities in response to each treatment were determined in triplicate by phosphorimage analysis and plotted against the maximum measurable response seen following treatment of cells with 100 nM Pep-13. The kinase activity of PcMPK6 is represented by the *white bars* and for PcMPK3a and 3b by the *black bars*.

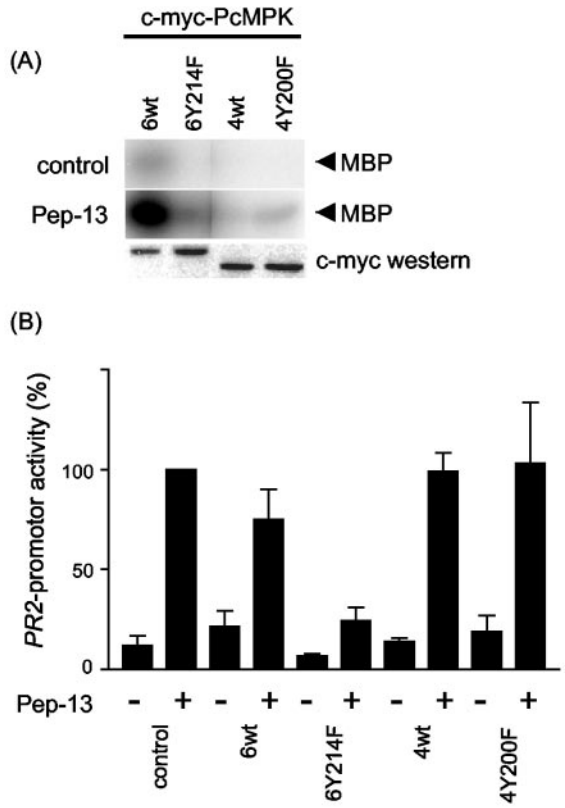

FIG. 6. **PcMPK6 activation through phosphorylation of Tyr-214 is required for Pep-13-induced *PR2* promoter activity.** *A*, activity of MAPK mutants in transfected protoplasts treated with 100 nM Pep-13. PcMPK6 and PcMPK4 wild-type and mutant constructs containing single point mutations in the activation loop motif, TEY (6Y214F, 4Y200F), were generated and transfected into protoplasts as c-Myc-tagged versions. Following 24 h expression under the control of the 35S-promoter the protoplasts were treated with either water (control) or 100 nM Pep-13 for 15 min. Proteins (100 μg) were extracted and MAPKs immunoprecipitated with an anti-c-Myc antibody. Kinase activities present in the immune complexes were then determined by MBP phosphorylation (*upper* and *middle panels*). Correct and equal expression of all constructs was tested by Western blotting of 10 μg of protein with c-Myc antibody (*lower panel*). *B*, transient expression of a dominant negative form of PcMPK6 (6Y214F) blocks the elicitor responsiveness of the parsley *PR2* promoter. Parsley protoplasts were co-transfected with a *PR2* promoter construct fused to *GUS*, together with the MAPK constructs shown in *A* or empty vector (*control*), and a 35S-promoter-*LUC* construct for normalization. Eight hours after transfection the protoplasts were treated with either water (−) or 100 nM Pep-13 (+). Following another 14-h incubation, the protoplasts were harvested, and total extracts were prepared and assayed for GUS and LUC activities. The influence of each co-transfected MAPK construct on the *PR2* promoter activity was determined in triplicate and plotted against the effect of co-transfection with the empty vector (*control*).

Treatment of cells with 500 nM recombinant HrpZ from *P. syringae* pv. *phaseolicola* activated both PcMPK6 (~80% of Pep-13 response) and the PcMPK3 kinases (~45% of Pep-13 response). HrpZ also acted as an elicitor of parsley cells and induced phytoalexin synthesis with an EC_{50} in the nanomolar range.[2] The concentration of 500 nM HrpZ used here gave maximal responses with respect to phytoalexin synthesis by parsley cells (not shown). Only two treatments were able to separate the activation of the different elicitor-responsive MAPKs. Treatment with 20 mM H_2O_2 induced the activation of PcMPK6 (~35% of Pep-13 response), but did not activate the PcMPK3 kinases. PcMPK6 was found to be activated by H_2O_2 concentrations of between 2 and 20 mM in a dose-dependent manner, whereas concentrations up to 1 mM had no effect (not shown). PcMPK6 was also activated in the absence of PcMPK3a/b activity following addition of a combination of heavy metals. This suggests that under some circumstances the elicitor-responsive MAPKs can be activated independently of one another, possibly during oxidative or heavy metal stress signaling. All activity measurements were performed in triplicate 15 min following the application of the treatment. All treatments were also analyzed after 30 min (data not shown) and yielded identical results to those shown for 15 min (Fig. 5). Neither H_2O_2 nor heavy metals stimulated phytoalexin accumulation 24 h after elicitation (not shown).

PcMPK6 Activation through Phosphorylation of Tyrosine 214 Is Required for PR Gene Promoter Activity Following Pep-13 Treatment of Parsley Protoplasts—Previous work had suggested that activated MAPKs might play roles in the control of elicitor-responsive gene expression in parsley (16). In order to directly test this, we performed transient expression experiments using dominant negative MAPK mutants to address, through a loss-of-function approach, the involvement of MAPKs in the activation of Pep-13-induced defense gene activation. Single point mutations predicted to influence kinase activity were introduced into the elicitor-responsive PcMPK6 and the un-responsive PcMPK4. The conserved tyrosine residue present within the TEY activation loop motif was mutated to phenylalanine in both PcMPK6 (6Y214F) and PcMPK4 (4Y200F). These mutations were predicted to render the protein kinases incapable of activation by upstream MAPKK-type activities (69). Both constructs contained an N-terminal c-Myc tag that enabled the determination of expression levels via Western blotting in addition to kinase activities through immunoprecipitation/MBP kinase assays on protoplast extracts. As shown in Fig. 6*A*, wild-type c-Myc-PcMPK6 was activated following treatment of transfected protoplasts with Pep-13.

[2] J. Lee and T. Nürnberger, personal communication.

This supported the previous data that demonstrated activation of this MAPK in parsley cells. However, we were unable to detect any activation of the PcMPK6 Y214F mutant in Pep-13-treated transfected protoplasts. Given that the expression levels were equal to those of the wild-type construct (Fig. 6*A*, lower *panel*), it appears that the Y214F mutation renders PcMPK6 incapable of activation through upstream MAPKK activities. For this reason the PcMPK6Y214F construct provided us with an ideal dominant negative form of the MAPK for further analysis of its influence on defense gene expression. As expected, and also shown in Fig. 6*A*, the PcMPK4 wild-type and 4Y200F kinases were again seen to be un-responsive to Pep-13 elicitor treatment.

The parsley *PR2* gene promoter has been studied in much detail (67, 70), and we have already demonstrated that its activation, like MAPK activation, occurs independently of the Pep-13-triggered oxidative burst response (Fig. 1, *A* and *B*). Therefore, we selected this promoter for use in co-transfection assays, to determine whether overexpression of an inactive MAPK impairs *PR2* promoter activation. Plasmids of the *PR2-GUS* construct (24) were co-transfected along with the constructs shown in Fig. 6*A* and an 35S-promoter-*LUC* construct for normalization purposes. Following 8 h of incubation, the protoplasts were treated with 100 nM Pep-13 and then left for a further 14 h. Protoplasts were then harvested, and GUS and LUC activity determinations were performed upon protein extracts. A typical data set for these co-transfection experiments is shown in Fig. 6*B*. Transfection with the PcMPK6 wild-type construct led to little or no reduction (~20%) in Pep-13-induced promoter activity compared with the co-transfections performed with the empty vector controls. However, co-transfection of the dominant negative form (6Y214F) of PcMPK6 led to a dramatic reduction in Pep-13-induced *PR2* promoter activity (~80% inhibition). In addition to this, the basal (non-elicited) levels of activity were also reduced, suggesting that the PcMPK6Y214F construct has a strong negative effect on both, activity and Pep-13 responsiveness of this promoter. Importantly, Fig. 6*B* also shows that co-transfection with either wild-type or Y200F forms of PcMPK4 had no effect on the Pep-13 responsiveness of the promoter. This agrees well with the fact that PcMPK4 is not activated in response to the Pep-13 elicitor and is therefore unlikely to trigger downstream events of the defense response.

DISCUSSION

Receptor-mediated perception of plant pathogens results in the activation of intracellular signaling pathways that function in triggering downstream defense reactions (3, 4). Defense reactions themselves are characterized by large-scale transcriptional activation of genes, whose products are believed to be actively involved in resisting pathogen attack (20, 71). Our studies have demonstrated that particular signaling pathways are responsible for the transcriptional activation of distinct subsets of defense genes. It is clear that both oxidative burst-dependent and -independent pathways play roles in this response. Previous studies, and those presented here, have demonstrated that the generation of O_2^-, most likely through the activity of an NADPH oxidase homologue(s), is necessary and sufficient to drive the synthesis of antimicrobial phytoalexins in parsley cells (15). The use in these studies of the NADPH oxidase inhibitor, DPI, to block Pep-13-induced phytoalexin biosynthesis, correlated with its ability to inhibit the transcript accumulation of genes encoding enzymes involved in this process. The transcriptional activation of these genes belongs to the late reactions of elicited parsley cells (18, 20). In contrast, transcript accumulation of genes involved in the immediate early and early reactions (21, 23) was unaffected by this treatment suggesting that a separate, albeit parallel, oxidative burst-independent pathway controls the transcriptional activation of such genes.

Changes in protein phosphorylation have long been known to occur as a consequence of treatment of plant cells with microbial elicitors (25, 26). Among the many protein kinases believed to be involved in these events, members of the MAPK family are becoming increasingly recognized as playing important roles in defense signaling (27, 28). In the present study we have shown that in parsley cells at least four different MAPKs are activated in a receptor-dependent manner by the *Phytophthora*-derived elicitor peptide, Pep-13. Three of these MAPKs could be identified by molecular cloning, immunoprecipitation, and transient transformation assays, and they were found to be homologous to MAPKs implicated in defense signaling in other plant species (3, 27, 31, 48). The initial in-gel and Western blotting experiments also suggest that at least one elicitor-responsive MAPK remains to be identified. Based upon the activation profile seen for each of the kinases with these methods, and compared with the activities determined through specific immunoprecipitation/kinase assays, this remaining kinase would appear to be activated more transiently than the PcMPK6 and PcMPK3 kinases. Given the lack of any cross-reacting antisera we have as yet been unable to identify this additional activity.

The MAPKs we have identified as Pep-13-responsive have homology to those seen to be implicated in elicitor signaling in other systems, *i.e.* homologues of the AtMPK6 and AtMPK3 MAPKs from *Arabidopsis* (27). In addition, we isolated a parsley homologue of AtMPK4, a MAPK shown to be a negative regulator of disease-resistance responses in *Arabidopsis* (40). This MAPK was not responsive to elicitors (Pep-13 or HrpZ) in parsley cells, and we cannot say whether it is functionally homologous to the *Arabidopsis* MAP kinase 4, which was previously described as being activated in response to Harpin treatments (39). We also isolated two parsley MAPKs belonging to the AtMPK3 class and have shown that both become activated following Pep-13 treatment. Whether these two kinases share a common function remains to be determined. One might suppose that they could have, despite their high degree of sequence identity, slight differences with respect to substrate specificities and interaction with activators and deactivators, or even that their expression profile *in planta* might differ. In *Arabidopsis* quite a number of such highly homologous MAPK pairs have been identified (29, 31), and it will be interesting in the future to learn to what extent their functions are redundant.

The other Pep-13-responsive MAPK was shown to be PcMPK6, a homologue of the AtMPK6, SIPK, and SIMK MAPKs from *Arabidopsis*, tobacco, and alfalfa, respectively, each of which has been shown to be activated following elicitation (32, 35, 39). As reflected in their nomenclature, many of these kinases have also been shown to become activated following abiotic stress treatments including salicylic acid (54), salt, or hyper-osmotic (52), hypo-osmotic, and oxidative stresses (51, 58). It was therefore surprising that no significant increases in PcMPK6 or PcMPK3 activities were observed when cultured parsley cells were placed under conditions described to activate MAPKs in other systems. The exceptions, from the conditions tested, were H_2O_2 and heavy metal treatments that activated PcMPK6 alone. This may reflect a role for this class of MAPKs in responses to oxidative stress, which has been suggested, with respect to treatment with millimolar concentrations of H_2O_2, by the activation of AtMPK6 in *Arabidopsis* (51, 58). It has also been shown that treatment of plants with micromolar concentrations of heavy metals, including copper, results in the

transcript accumulation of many oxidative stress-protective and -responsive genes (72). AtMPK6 class MAPKs may therefore operate as components of signal cascades initiated by these environmental stimuli. In this case the specificity of the outcome may be determined by the relative duration of the kinase activation (as in our hands, the oxidative stress PcMPK6 activation was more transient than that seen in response to elicitors, not shown) or in the contribution made by parallel signaling pathways. Perhaps significantly, none of the abiotic treatments described resulted in the activation of PcMPK3a or 3b. Even with respect to PcMPK6, the highest and most persistent levels of activity strictly correlated with treatments that induce phytoalexin synthesis in parsley cells, *i.e.* elicitors. *P. syringae* HrpZ and the NPP1 protein from *P. parasitica* effectively and strongly activated both PcMPK6 and PcMPK3, although to levels not quite that seen following Pep-13 treatment (not shown) (73).[3] However, these activities also remained significantly higher than the activity of PcMPK6 during the oxidative stress responses. This alone suggests that these MAPKs play important roles in plant defense signaling. The use of different elicitors also highlights the way in which different perception mechanisms can and do converge upon these kinases, as has also been reported in other systems (37). The identification of the sequential upstream components of these MAPK cascades, and the determination of the initial convergence points will be of significant interest in the future.

What functions do MAPKs have in plant defense responses? Recent gain-of-function experiments in tobacco and *Arabidopsis* leaves overexpressing constitutively active MAPKK or wild-type SIPK resulted in the formation of hypersensitive response-like necrotic lesions (45–47). In addition, accumulation of transcripts associated with defense responses was observed. This clearly shows that SIPK/AtMPK6 homologues or their upstream MAPKK activities when overexpressed can trigger defense-related reactions. The mechanism by which this is achieved remains, however, unclear and corresponding loss-of-function approaches were not presented. The recent complete functional identification of a MAPK cascade from *Arabidopsis* that is sufficient to provide increased resistance to pathogen attack has now confirmed the importance of MAPK signaling for plant defense (48). We chose to investigate the importance of MAPK activity for the induction of downstream defense responses that occurred independently of the oxidative burst, using a loss-of-function approach. Our studies have shown that *PR* gene expression (this study) and MAPK activation (16) in parsley cells occurred upstream or independently of the oxidative burst. It was therefore of interest to see whether one response was linked to the other. Overexpression of PcMPK6 in parsley protoplasts followed by Pep-13 treatment resulted in activation of the kinase in a manner indistinguishable to that observed in cells. However a Y-F activation loop mutant could not be activated in this system, confirming this tyrosine phosphorylation reaction as essential for kinase activation during the elicitor response that likely results from activation of an upstream MAPKK activity. Moreover, in co-transfection experiments, this Y-F mutant gave a strong inhibition of the elicitor responsiveness of the *PR2* promoter activity. As *PR* gene expression is regarded as a classical marker for plant defense, we can conclude that PcMPK6 plays an essential role in the induction of these defense reactions. It is unlikely that the kinase is solely responsible for this activity, since, as we demonstrated here with respect to oxidative stress, it can be activated by treatments that do not trigger typical defense reactions. We therefore believe PcMPK6 activation to be a necessary, but not sufficient component for *PR2* gene expression during defense. Interestingly, co-transfection experiments using a Y-F activation loop mutant of PcMPK3b also showed a degree of inhibition of the *PR2* promoter, although not to the levels shown for the PcMPK6 mutant construct (not shown). This may perhaps represent some redundancy in MAPK signaling pathways during defense, where in almost all cases studied to date, activity of the MPK3 class kinases is seen in addition to the MPK6 class. However, hypotheses of this type need to be addressed with the use of specific knockouts, which is seen as difficult in plants, or the identification of specific substrates. It is most likely that the overexpression of the PcMPK6Y214F construct blocks the correct activation of the endogenous wild-type activity and results in a reduced level of phosphorylation of a protein(s) that regulates *PR2* promoter activity. WRKY-type transcription factors were first identified in parsley as proteins that bind to response elements in these promoters (24), which are not present in promoters of Pep-13-responsive parsley genes encoding phytoalexin biosynthetic enzymes activated *via* the oxidative burst (74). WRKY transcription factors have since been implicated in disease resistance responses of *Arabidopsis*, occurring downstream of MAPK signaling (48). Future studies should address the link between MAPK and WRKY activities and will require the identification of MAPK substrates, which at present remain unknown. The identification of these unknown proteins represents a major future challenge for research in plant MAPK signaling and function in mediating plant defense.

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