Identification of MAPKs and Their Possible MAPK Kinase Activators Involved in the Pto-mediated Defense Response of Tomato*

Kerry F. Pedley‡ and Gregory B. Martin‡§

From the ‡Boyce Thompson Institute for Plant Research, Ithaca, New York 14853-1801 and the §Department of Plant Pathology, Cornell University, Ithaca, New York 14853-4203

The Pto kinase mediates resistance to bacterial speck disease in tomato by activating host defense responses upon recognition of Pseudomonas syringae pv. tomato strains expressing the AvrPto or AvrPtoB proteins. Previous gene-silencing experiments have indicated that mitogen activated protein kinase (MAPK) cascades play a key role downstream of the Pto kinase to activate host defense responses. Here we use biochemical methods to demonstrate that two tomato MAPKs, LeMPK2 and LeMPK3, are activated in leaves in a Pto-specific manner upon expression of AvrPto and AvrPtoB. We show that these same MAPKs are activated upon overexpression of LeMAPKkk, a protein previously demonstrated to be involved in Pto-mediated immunity. We identified two phyleogenetically unrelated MAPK kinases (LeMKK2 and LeMKK4) that when overexpressed in leaves elicit cell death and activate LeMPK2 and LeMPK3. In vitro analysis demonstrated that LeMKK2 and LeMKK4 each phosphorylate the same subset of three MAPKs. Together these data provide biochemical evidence for the involvement of MAPK cascades in Pto-mediated resistance.

Our best understanding of active plant defense responses is derived from studies of the interplay between the products of plant resistance genes (R)1 and pathogen avirulence genes. Many plant R proteins function to detect the presence of disease-causing bacteria and fungi by recognizing specific pathogen "effector" proteins, encoded by avirulence genes, which are produced and delivered into the plant cell during the infection process (1). Detection of effector proteins triggers many host responses including proteolysis, changes in ion fluxes, the production of reactive oxygen and nitrogen species, and the induction of gene expression (2–5). Host defense often culminates in a form of host programmed cell death termed the hypersensitive response, which may delay or restrict the spread of the pathogen beyond the initial point of infection (6).

Although many R genes and their corresponding avirulence genes have been cloned and characterized, less is known about the signal transduction components that link the perception of a pathogen with downstream responses. In some cases, the signaling proteins are pathway specific, functioning in concert with particular R proteins, whereas others appear to be shared by many R gene pathways. Among the defense-related signaling pathways that appear to be conserved are the mitogen-activated protein kinase (MAPK) cascades. All eukaryotes utilize MAPK cascades, which are composed of three sequentially acting protein kinases: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK to transduce intracellular signals (7). In the Arabidopsis thaliana genome there are predicted to be 20 genes for MAPKs, 10 MAPKKs, and more than 60 MAPKKKs (8). To date, functional data are available for relatively few of the corresponding proteins, and their participation in different pathways remains largely unknown.

Perhaps the most extensively characterized plant MAPKs are the tobacco proteins SIPK and WIPK. SIPK, initially identified as a gallic acid-induced protein kinase, is activated rapidly in response to a variety of biotic and abiotic stresses including osmotic and salt stress, wounding, and treatment with non-race-specific elicitors from fungi and bacteria (9–13). SIPK has also been shown to be activated downstream of gene for gene interactions between resistant tobacco plants (NN) challenged with Tobacco mosaic virus and upon Avr9 treatment of C79 transgenic tobacco (14, 15). The kinetics of SIPK activation in response to abiotic stresses is transient, whereas biotic elicitors that induce cell death result in prolonged activation (11, 16). Conditional gain-of-function studies have shown that SIPK overexpression is sufficient to induce both defense gene expression and cell death (17). Together these data indicate that SIPK may be a convergence point for many different stimuli and that the duration of its activation may play a role in the types of downstream processes it mediates.

SIPK activation is often accompanied by the activation of WIPK, which was originally identified as a wound-induced protein kinase (18). In addition to its association with wound- ing, WIPK appears to also play a role in defense signaling. Although many of the same stimuli activate SIPK and WIPK, they differ in several regards. WIPK activation is typically slower than SIPK activation (17). This may be due, in part, to the fact that WIPK is regulated at the transcription level; WIPK mRNA levels accumulate in response to various stresses, most notably wounding (18). Also, unlike SIPK, WIPK overexpression is not sufficient to elicit cell death,
which likely indicates that although these two MAPKs are typically active at the same time, they influence different downstream processes (19).

The dual activation of SIPK and WIPK by the same stimuli is not surprising in light of the discovery that they are both activated by the same upstream MAPKK, NtMEK2 (20). Expression of a constitutively active form of NtMEK2 induces cell death, which is preceded by the activation of SIPK and WIPK and the induction of genes associated with defense responses (20). More recently, NtMEK2 has been shown to activate Nt4, a MAPK that is closely related to SIPK (21). In Arabidopsis, expression of constitutively active forms of two putative orthologs of NtMEK2, AtMKK4 and AtMKK5, induces cell death (22). AtMKK4 and AtMKK5 have also been shown to activate the Arabidopsis SIPK and WIPK orthologs AtMPK6 and AtMPK3, respectively (22).

Based on phylogenetic analysis, genes encoding putative orthologs of SIPK, Nt4, and WIPK have been cloned recently from tomato (23). The tomato proteins LeMPK1 and LeMPK2 are highly similar to SIPK and Nt4, respectively, and LeMPK3 is highly similar to WIPK. At the amino acid level, LeMPK1 and LeMPK2 are share 95% identity with most of the differences occurring at the N terminus outside of their identical conserved kinase domains. Using specific antibodies raised against peptides representing their divergent N termini, Holey et al. (23) showed that both LeMPK1 and LeMPK2 are activated in Lycopersicon peruvianum suspension cells treated with systemin, oligosaccharide elicitors, and ultraviolet-B radiation. These observations suggest that LeMPK1 and LeMPK2 may be redundant or have overlapping functions. In tobacco, separate functions for SIPK and Nt4 can be inferred from the temporal and tissue-specific expression of Nt4 (24). LeMPK3 was also shown to be activated by ultraviolet-B radiation and has been shown to be regulated at the transcript level similar to WIPK (25).

As a model system to investigate active defense responses in plants, we study the interaction between tomato (Lycopersicon esculentum) and the bacterial pathogen Pseudomonas syringae pv. tomato (Pto) (26). As part of the infection process, the bacterium translocates effector proteins directly into the cytoplasm of the host cell via a type III secretion system. In resistant plants, two of these effectors, AvrPto and AvrPtoB, are detected by a Ser/Thr protein kinase encoded by the R gene Pto (27–29). This recognition event activates a variety of defense responses including the hypersensitive response. The Pto-mediated defense response requires another protein, Prf, that contains a nucleotide-binding site and a region of leucine-rich repeats (30). Prf likely functions with Pto during the recognition event, but its role is unknown. Several substrates of Pto have been identified including a Ser/Thr protein kinase, Pti1 (31), and an ERF-like transcription factor, Pti4 (32).

Recently, in a study using virus-induced gene silencing (VIGS), an additional nine genes that contribute to Pto-mediated resistance were identified. Among these, two encode MAPKKs (putative orthologs of NtMEK1 and NtMEK2) and two encode MAPKs (putative orthologs of SIPK and WIPK) (33). In a separate VIGS screen, we recently identified a MAPKK, LeMAPKKKα, that plays a dual role as a regulator of cell death in both Pto-related plant immunity and disease (34). Overexpression of LeMAPKKKα induces cell death and thus enabled us to delineate two downstream MAPK cascades using a combination of overexpression analysis and VIGS (34). Based on these experiments the putative tomato orthologs of NtMEK2 and SIPK were found to constitute a MAPK cascade acting directly downstream of LeMAPKKKα. Together, these gain- and loss-of-function studies established that MAPKs play a role downstream of Pto in the defense response (33, 34).

To further elucidate the role of MAPKs in Pto-mediated resistance, we now provide biochemical evidence that LeMPK2 and LeMPK3 are activated in vivo in response to AvrPto and AvrPtoB and in tomato leaves overexpressing LeMAPKKKα. We identify two phylogenetically unrelated MAPKKs, LeMKK2 and LeMKK4 that when overexpressed elicit cell death and activate LeMPK2 and LeMPK3. Additionally, using LeMPK1, LeMPK2, and LeMPK3 as substrates for phosphorylation assays, we demonstrate that LeMKK2 and LeMKK4 have overlapping substrate specificity.

**EXPERIMENTAL PROCEDURES**

**Cloning of MAPKs and MAPKs—**Two expressed sequence tag clones, cLEF46119 and cTOB17A11, were sequenced and found to contain complete open reading frames of LeMPK2 and LeMPK3, respectively. LeMPK1 was generated by reverse transcriptase-PCR using primers designed from the published sequence (23). The tomato MAPKK genes (LeMKK1, LeMKK2, LeMKK3, and LeMKK4) were obtained as expressed sequence tag clones (cLEKX6523, cLER2B17, cLEH3F10, and cTOP13E16), respectively, that contained complete open reading frames. Kinase inactive mutants (LeMKKKK92R, LeMKKKK92R, LeMKKKK92R, LeMKKKK92R, and LeMKKKK92R) that contain an Arg in place of the catalytic Lys were generated using the QuikChange site-directed mutagenesis kit (Stratagene). All mutations were confirmed by sequencing. The tomato MAPKK genes and their inactive derivatives were cloned into pE8 (35) that was modified to produce proteins containing an N-terminal double hemagglutinin (HA) epitope. To generate maltose binding protein (MBP)-LeMPK fusion proteins, BamHI and Xhol sites were added to LeMKKKK92R, LeMKKKK92R, and LeMKKKK92R via PCR to the 5′ and 3′ ends, respectively, for cloning into the pMAL-c2X vector (New England Biolabs, Beverly, MA). Protein expression and purification with amylose resin were performed according to the instructions of the manufacturer (New England BioLabs).

**Transient Gene Expression in Tomato and Nicotiana benthamiana—**

Agrobacterium tumefaciens GV2260 strains carrying the vector pE8 (35) containing the gene of interest were grown and prepared for infiltration (Aoo1 = 0.03) following a similar procedure as described in He et al. (36). For whole-leaf assays, leaves were sprayed with 5 μl estradiol-17β 48 h post infiltration. For kinase assays and protein expression, 1 cm leaf segments from infiltrated leaves, floated on 20 mM HEPES, pH 7.5, for 2 h, and treated with estradiol (final concentration = 5 μM). At designated time points, leaf disks were frozen in liquid nitrogen and stored at −80 °C.

**Protein Extraction, In-gel Kinase Assay, and Immunoblot Analysis—**Proteins were extracted by grinding leaf tissue in extraction buffer (100 mM HEPES, pH 7.5, 5 mM EDTA, 10 mM dithiothreitol, 10 mM NaVO₄, 10 mM NaF, 50 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1% plant protease inhibitor mixture (Sigma) in 1.5-ml Eppendorf tubes with a microcresette. The mixture was then spun for 5 min in a microcentrifuge to remove cellular debris. The supernant was removed and stored at −80 °C. The concentration of protein extracts was determined by using the Bio-Rad protein assay kit with bovine serum albumin as standard. In-gel kinase assay and immunoblot were performed as previously described (34).

**Immunocomplex Kinase Assay—**The immunocomplex kinase assay was performed as described previously (23) with slight modifications. Briefly, extracts containing 0.2 mg of total protein were incubated for 2 h at 4 °C with anti-LeMPK1, -2, or -3 antisera (1:100 dilution) on a rocking platform shaker in immunoprecipitation buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaVO₄, 1 mM NaF, 10 mM β-glycerophosphate, 1% (w/v) Triton X-100, 0.5% (w/v) Nonidet P-40, 2 mM dithiothreitol, and 1% plant protease inhibitor mixture (Sigma). Approximately 20 μl of packed volume of recombinant protein G-agrose (Roche Applied Science) was added, and the incubation continued for an additional 1.5 h at 4 °C. The complexes were precipitated by a brief centrifugation and washed twice with immunoprecipitation buffer, one time with immunoprecipitation buffer containing 1 mM NaCl, and three times with kinase reaction buffer (without myelin basic protein and ATP). Kinase reactions were performed for 20 min at room temperature at 20 μl of kinase reaction buffer (20 mM HEPES, pH 7.5, 15 mM MgCl₂, 2 mM EGTA, 1 mM dithiothreitol, 0.25 mg ml⁻¹ myelin basic protein, and 25 μM ATP) containing 0.1 μl (γ⁻³²P)ATP. The reaction was stopped by the addition of SDS-PAGE sample buffer. After
Inducible expression of AvrPto causes cell death and increases MAPK activity in RG-PtoR tomato leaf tissue. A. Agrobacterium carrying either the empty vector, pER8, or pER8:avrPto was used to transiently transform leaves of Pto-expressing line RG-PtoR. Two days after Agrobacterium infiltration, transformed leaves were sprayed with estradiol to induce gene expression. Cell death (arrow) in areas expressing AvrPto was observed by 16 h after estradiol application. B, in-gel kinase analysis using myelin basic protein as an artificial substrate to monitor MAPK activity in leaf tissue expressing either AvrPto or AvrPtoB. Total cellular proteins were extracted from leaf tissue after treatment with estradiol at times indicated. Control lanes represent protein extracted from tissue transformed with the empty vector.

**FIG. 1.** Inducible expression of AvrPto causes cell death and increases MAPK activity in RG-PtoR tomato leaf tissue. A. Agrobacterium carrying either the empty vector, pER8, or pER8:avrPto was used to transiently transform leaves of Pto-expressing line RG-PtoR. Two days after Agrobacterium infiltration, transformed leaves were sprayed with estradiol to induce gene expression. Cell death (arrow) in areas expressing AvrPto was observed by 16 h after estradiol application. B, in-gel kinase analysis using myelin basic protein as an artificial substrate to monitor MAPK activity in leaf tissue expressing either AvrPto or AvrPtoB. Total cellular proteins were extracted from leaf tissue after treatment with estradiol at times indicated. Control lanes represent protein extracted from tissue transformed with the empty vector.

In Vitro Kinase Assay—In vitro phosphorylation experiments were performed using HA-epitope tagged LeM KKs and kinase-inactive MBP-LeMPK fusion proteins as substrates. For purification of LeM KKs, 0.1 mg of total protein extracted from N. benthamiana leaves transiently expressing the four LeM KKs were incubated for 1 h at 4 °C in immunoprecipitation buffer containing 100 μl of packed volume anti-HA affinity matrix (Roche Applied Science) on a rocking platform shaker. The beads were collected by a brief centrifugation and were washed one time with immunoprecipitation buffer, one time with immunoprecipitation buffer containing 1 μl of NaCl, and two times with kinase reaction buffer (without myelin basic protein and ATP). The purified LeM KKs were divided equally into four aliquots and combined with ~25 μl of amylase resin, saturated with the substrate MBP-LeMPK recombinant proteins. Kinase reactions were performed for 20 min at room temperature in 20 μl of kinase buffer containing 1.0 μCi of [γ-32P]ATP. The reaction was stopped by the addition of SDS-PAGE sample buffer. After electrophoresis on a 10% (w/v) SDS-polyacrylamide gel, gels were dried, and radiolabeled MBP-LeMPK fusion proteins were visualized by exposure to Kodak Biomax MR film.

Phylogenetic Analysis—The neighbor-joining phylogenetic tree was created by the ClustalW method (37) (MegAlign program, DNAStar, Madison, WI). The human MAPKK protein HsMEK1 (Q02750) was created by the ClustalW method (37) (MegaAlign program, DNAStar, Madison, WI). The human MAPKK protein HsMEK1 (Q02750) was used as an out group to root the tree.

**RESULTS**

Previous VIGS experiments indicated that MAPKs play an important role in the Pto-mediated defense response against avirulent Pst strains. Therefore we investigated whether MAPKs are activated specifically in response to bacterial effector proteins AvrPto and AvrPtoB in Pto-expressing tomato leaves (Rio Grande-PtoR, hereafter RG-PtoR). To synchronize expression of the effector genes in tomato leaves and to study early changes in MAPK activation following detection of these proteins, we used an estradiol-inducible expression system developed for use in plant cells (35). Leaves that were transiently transformed with Agrobacterium containing either AvrPto or AvrPtoB under the control of the inducible promoter were treated with estradiol and monitored for cell death. Only areas that had been transformed with AvrPto or AvrPtoB resulted in cell death following the application of estradiol (Fig. 1A). Expression of AvrPto and AvrPtoB appeared to be tightly regulated because no cell death was detected in transformed tissue that was not treated with estradiol (data not shown).

To determine whether expression of AvrPto or AvrPtoB induced kinase activity in Pto-expressing tomato leaf cells, we used an in-gel kinase assay with myelin basic protein serving as an artificial substrate to detect MAPK activity. To avoid problems associated with wounding that could potentially result from excision of the tissue after treatment with estradiol, 1-cm leaf disks from portions of the leaf that had been transformed with Agrobacterium were removed and floated on buffer for 2 h prior to treatment with estradiol. A time-course analysis demonstrated that expression of either AvrPto or AvrPtoB resulted in an increase of MAPK activity within 1 h after treatment of the leaf disks with estradiol (Fig. 1B). This activity returned to levels comparable with the control tissue by 3 h after induction.

To determine whether the MAPK activity detected could be attributed to Pto- or Prf-mediated host defenses activated in response to AvrPto and AvrPtoB we used two additional tomato lines, RG-prf3 and RG-PtoS. The former is isogenic to RG-PtoR and contains a 1-kb deletion in the Prf gene rendering it non-functional (30), whereas the latter is nearly isogenic to RG-PtoR and carries the Pto haplotype from Lycopersicon esculentum that lacks Pto but contains several closely related genes of the Pto family (26). No increase in MAPK activity could be detected in RG-prf3 tissue in response to expression of AvrPto or AvrPtoB (Fig. 2). In RG-PtoS tissue, a slight increase in MAPK activity was detected in response to AvrPto. This may be attributable to the reported weak recognition of AvrPto by one of the other Pto family members that produces a weak hypersensitive response following infiltration with Agrobacterium expressing AvrPto (38). There was no increase in MAPK activity in RG-PtoS plants following the expression of AvrPtoB. Thus, the MAPK activity we detected is dependent on Pto and Prf and correlates with the phenotypes observed when plants are treated with avirulent Pst.

As discussed in the introduction, Holley et al. (23) developed antibodies for the specific detection of LeMPK1 (similar to NtMk4), LeMPK2 (similar to SIPK), and LeMPK3 (similar to WIPK). We obtained antisera for LeMPK2 and LeMPK3 and used them in a series of immunocomplex kinase assays to determine whether either of these MAPKs are activated in RG-PtoR tissue expressing either AvrPto or AvrPtoB. Both LeMPK2 and LeMPK3 were activated in response to AvrPto.
and AvrPtoB, whereas a much lower background activity was detected in the empty vector control (Fig. 3).

To identify MAPKKs that might act upstream of LeMPK2 and LeMPK3 in the Pto-mediated defense response, we searched the TIGR tomato gene index using the NtMEK2 amino acid sequence and found expressed sequence tags encoding four distinct MAPKKs. An expressed sequence tag encoding a full-length version of each MAPKK was identified and used for further analysis. We used the DNASTAR analysis software to cluster the tomato MAPKKs with related kinases from tobacco, *Arabidopsis*, rice, maize, and alfalfa (Fig. 4A). The tomato MAPKKs clustered into four of the five known MAPKK groups defined by Ichimura *et al.* (8). eLER2B17, which encodes a MAPKK we refer to as LeMKK2, belongs to group C. This group contains tobacco NtMEK2, which has been demonstrated to activate both SIPK and WIPK (20) and causes hypersensitive response-like cell death and activates their downstream MAPKs indicating a role in defense (20, 22). Therefore we predicted that LeMKK2 would function similarly in tomato.

Plant MAPKK proteins can be modified to be constitutively active by replacing the conserved Ser/Thr residues in the activation loop ((S/T)XXX(S/T)) with Asp residues (20, 22). A potentially constitutively-active form of LeMKK2 was developed by replacing the conserved Ser/Thr residues Thr-215 and Ser-221 with Asp; an N-terminal epitope tag was added to monitor protein expression (HA-LeMKK2<sup>215D/221D</sup>, referred to hereafter as LeMKK2<sup>215D/221D</sup>). LeMKK2<sup>215D/221D</sup> caused cell death when expressed from the estradiol-inducible promoter in leaves of both tomato and *N. benthamiana* (Fig. 4B). Unexpectedly, the unmodified wild-type LeMKK2 also resulted in cell death when expressed in tomato leaves, although no cell death was observed when this protein was expressed in *N. benthamiana*. This observation in tomato is in contrast to what was reported in tobacco and *Arabidopsis*, where expression of wild-type NtMEK2 and AtMKK4 and AtMKK5 gave no phenotype (20, 22). However, these previous studies were conducted using the dexamethasone-inducible promoter (39), which is weaker than the estradiol-inducible promoter (35). Expression in leaves of an LeMKK2 protein with a substitution in the ATP-binding site (LeMKK2<sup>KR</sup>) did not cause cell death indicating a requirement of kinase activity for this phenotype (Fig. 4B).

To determine whether any of the other three tomato MAPKK genes would give a similar phenotype, each one was expressed in leaves as wild-type or kinase-inactive versions, and the leaves were monitored for cell death. Expression of the MAPKKs that belong to groups A1 and A2 had no discernible effect, whereas expression of the group D MAPKK, LeMKK4, resulted in cell death in both tomato and *N. benthamiana* leaves (Fig. 4B). This finding was particularly interesting because to date there are no reports regarding the function of group D MAPKKs from any plant species (8). As with LeMKK2, a substitution in the ATP-binding site of LeMKK4 (LeMKK4<sup>KR</sup>) abrogated the cell death phenotype indicating a requirement of kinase activity (Fig. 4B). Expression of each of the MAPKKs and their variants in *N. benthamiana* leaves was verified by Western blot analysis using epitope-tagged versions of the proteins (Fig. 4C).

We used an in-gel kinase assay to investigate whether the cell death that resulted from expression of LeMKK2 and
LeMKK4 was preceded by an increase in MAPK activity. A time course analysis revealed that the expression of both LeMKK2 and LeMKK4 increased an increase in MAPK activity above the control beginning at 3 h after estradiol application and reached maximal levels at 5 h (Fig. 5A). To determine whether there was a correlation between cell death and the increase in MAPK activity, we compared MAPK activity levels resulting from overexpression of all four tomato MAPKKs. Leaves expressing the two MAPKKs that did not induce cell death, LeMKK1 and LeMKK3, showed no increase in MAPK activity (Fig. 5B). In all these experiments, transformed areas of the leaves expressing the MAPKKs were monitored for cell death; cell death in the LeMKK2- and LeMKK4-expressing tissue usually occurred by 16 h after application of estradiol in tomato and by 36 h in N. benthamiana.

Next we addressed whether the MAPKs activated by expression of LeMKK2 and LeMKK4 were the same as those activated by AvrPto and AvrPtoB in RG-PtoR leaves. With antisera specific for LeMKK2 and LeMPK3, we analyzed tomato leaf tissue expressing LeMKK2 and LeMKK4 using the immunocomplex kinase assay. As with our results obtained using protein extracts from RG-PtoR leaves expressing AvrPto and AvrPtoB, both LeMPK2 and LeMPK3 were activated in tissues expressing LeMKK2 and LeMKK4 (Fig. 6).

Based on the previous characterization of NtMEK2 and AtMKK4/AtMKK5 (20, 22) we predicted that LeMKK2 would directly phosphorylate and activate LeMPK2 and LeMPK3. However, we found it interesting that LeMKK4, which belongs to an uncharacterized group of MAPKKs, also activated these two MAPKs. Two possibilities might account for this activation. Either LeMKK4 could directly phosphorylate and activate LeMPK2/LeMPK3, or expression of LeMKK4 could be triggering a cell death pathway that eventually leads to indirect activation of LeMKK2 and its downstream MAPKs. To examine the former possibility, we performed a series of in vitro kinase assays using all four of the LeMKK proteins and LeMPK1, LeMPK2, and LeMPK3 as substrate proteins. For these experiments, HA-tagged LeMKK proteins were expressed and immunoprecipitated from N. benthamiana tissue. For substrates, kinase-inactive versions of LeMPK1, LeMPK2, and LeMPK3 were expressed and purified from E. coli as fusions to MBP. Both LeMKK2 and LeMKK4 phosphorylated LeMPK1 and LeMPK2 and to a lesser degree LeMPK3 in vitro (Fig. 7). LeMKK1 and LeMKK3 were unable to phosphorylate any of the MAPK proteins used in the assay. Based on this analysis, LeMKK2 and LeMKK4 appear to have overlapping substrate specificity.

LeMAPKKKa is predicted (based on epistasis experiments) to function upstream of LeMKK2 (34). Overexpression of LeMAPKKKa results in cell death, which is preceded by an increase in MAPK activity (34). Therefore, we predicted that LeMKK2 and LeMPK3 would be activated in response to LeMAPKKKa. To test this hypothesis we performed an immunocomplex kinase assay on LeMAPKKKa-expressing tissue with the LeMKK2 and LeMPK3 antisera. Expression of either the full-length LeMAPKKKa protein or only its kinase domain (LeMAPKKKaKD) both activate LeMPK2 and LeMPK3 in vivo (Fig. 8).

**DISCUSSION**

We have used biochemical methods to demonstrate the involvement of specific MAPKs in the gene for gene resistance mediated by Pto. Through a combination of overexpression analysis, in vitro kinase assays, and immunological methods we show that two bacterial effector proteins, AvrPto and AvrPtoB, and three putative upstream kinases activate the same subset of MAPKs. Two of these kinases, LeMAPKKKa and LeMKK4/LeMKK5, previously have been implicated in Pto-mediated defense through loss-of-function studies using VIGS. The third kinase, LeMKK4, belongs to a subgroup of plant MAPKK proteins that has not been characterized previously.

Plants have more than 100 genes that encode MAPK-related proteins, yet relatively little is known about their function and contribution to different pathways. To date, only one complete plant MAPK cascade (related to defense signaling) has been elucidated based upon biochemical data. This Arabidopsis cascade involves AtMEKK1, AtMKK4/5, and AtMPK3/AtMPK6 and functions downstream of FLS2-mediated flagellin perception to activate innate immunity. Activation of this pathway...
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LeMPK1<sup>KR</sup>
LeMPK2<sup>KR</sup>
LeMPK3<sup>KR</sup>

LeMPK1<sup>KK</sup>
LeMPK2<sup>KK</sup>
LeMPK3<sup>KK</sup>

FIG. 7. LeMKK2 and LeMKK4 directly phosphorylate LeMPK2 and LeMPK3 in vitro. Tomato MAPKKs expressed in N. benthamiana leaves were purified by immunoprecipitation, washed, and incubated with [γ-32P]ATP and kinase-inactive versions of LeMPK1, LeMPK2, and LeMPK3. Phosphorylated radioactive LeMPK proteins were separated by SDS-PAGE and visualized by autoradiography. All LeMPK proteins were expressed and purified from E. coli as fusions with MBP. Left panels indicate Coomassie blue-stained proteins separated by SDS-PAGE. Right panels show autoradiographs.

FIG. 8. Expression of LeMAPKK<sup>KKα</sup> in tomato activates LeMPK2 and LeMPK3. A, in-gel kinase assay using total cellular proteins extracted from RG-PtoR leaves expressing either LeMAPKK<sup>KK</sup> or LeMAPKK<sup>KKα</sup>-K<sup>R</sup>. B, immunocomplex kinase assay performed on protein extracts used in A. Extracts were prepared and incubated with LeMPK2 or LeMPK3 antisera and protein G-agarose. After washing, immunocomplexes were incubated with [γ-32P]ATP and myelin basic protein. Phosphorylated radioactive myelin basic protein was separated by SDS-PAGE and visualized by autoradiography.

confers resistance to both bacterial and fungal pathogens, suggesting that signaling events initiated upstream by diverse pathogens converge into a conserved pathway (40). In tobacco, resistance to Tobacco mosaic virus involves SIPK, WIPK, and NtMEK2 (41), which are highly homologous to AtMPK6, AtMPK3, and AtMPK4/5, respectively. An upstream MAPKK that activates NtMEK2 in tobacco has yet to be identified.

We recently identified a MAPKKK from tomato, LeMAPKKKα, that mediates host cell death associated with both Pto-mediated immunity and disease. Overexpression of LeMAPKKKα in leaves induces cell death, and this phenotype enabled us to delineate two downstream MAPK cascades using a combination of overexpression analysis and VIGS (34). One of these pathways appeared to involve the putative tomato orthologs of NtMEK2 and NtSIPK (referred to here as LeMPK2 and LeMPK1/LeMPK2, respectively). We have examined LeMPK2 and LeMPK2 in our present study, and our data provide biochemical support for these kinases acting downstream of Pto and LeMAPKKKα.

In addition to triggering host cell death, we found previously that overexpression of LeMAPKKKα activates MAPK activity (34). Here, using specific antisera, we show that this activity is attributable to LeMPK2 and LeMPK3. Although we had not initially proposed that LeMPK3 acts downstream of LeMAPKKKα (34), its placement there is consistent with a previous report showing that VIGS of LeMPK3 compromised resistance to avirulent strains of Pst (33). More recently, LeMPK3 mRNA levels were shown to be induced in resistant plants by avirulent strains of Pst, further supporting its involvement downstream of Pto (25).

Previous work showed that gene silencing of LeMKK2 compromises Pto-mediated resistance and blocks the development of cell death induced by the co-expression of AvrPto and Pto in N. benthamiana and the cell death that results from the overexpression of LeMAPKKKα (34). We have shown here that overexpression of LeMKK2 also activates LeMPK2 and LeMPK3 in vivo. Further analysis of LeMKK2 revealed that it phosphorylates three tomato MAPKs, LeMPK1, LeMPK2, and LeMPK3 in vitro, which strongly supports its role as their upstream kinase. Based on the high degree of sequence identity between LeMPK1 and LeMPK2, it is not surprising that they could both serve as substrates for the same MAPKK. The finding that LeMKK2 also phosphorylates LeMPK3 is consistent with previous reports from work in Arabidopsis and tobacco in which AtMKK4/5 phosphorylates AtMPK3 and AtMPK6 (22) and NtMEK2 phosphorylates NtSIPK and NtWIPK (20), to which LeMKK2 and LeMPK1/LeMPK2 and LeMPK3 are similar. Our data reinforce the supposition that, considering the fact that there are fewer MAPKs than MAPKs in plants (8), any particular MAPKK likely phosphorylates multiple MAPKs.

Our search of the TIGR tomato gene index identified four phylogenetically distinct MAPKKs. One of these, LeMKK4, belongs to a phylogenetic group of MAPKKs for which no function has been reported. Therefore we were surprised that the overexpression of LeMKK4 induced cell death in the leaves of both tomato and N. benthamiana and that it behaved similarly to LeMKK2, activating and phosphorylating the same MAPKs in vivo and in vitro. Although we have yet to be able to assign a biological role to LeMKK4, it may participate in a programmed cell death pathway that leads to the activation of LeMPK1, -2, and -3. Further analysis of LeMKK4, including loss-of-function analysis utilizing VIGS, is in progress.

LeMPK1 and LeMPK2 were previously shown to be activated by systemin, several oligosaccharide elicitors, and ultraviolet-B radiation. In contrast, LeMPK3 was only demonstrated to be activated by ultraviolet-B radiation (23). In our analysis of the Pto pathway, we show that LeMPK2 and LeMPK3 are both activated in response to both AvrPto and AvrPtoB. Because of inconsistent results obtained with the LeMPK1 antisera, we report here only the results of our analysis with the antisera for LeMPK2 and LeMPK3. However, based on the in vitro phosphorylation analysis using LeMPK1 as a substrate for LeMKK2, it is possible that this MAPK is also activated in vivo in response to both AvrPto and AvrPtoB. It is interesting that despite its apparent lack of involvement in response to systemin and oligosaccharide elicitors, LeMPK3 was strongly induced by the bacterial effector proteins tested here. This may indicate that different upstream kinases are required to transduce the signals generated by the perception of these different stimuli.

Based on the previous loss-of-function studies and the biochemical analysis presented here, it is now clear that MAPKs, specifically LeMPK2 and LeMPK3, are required for Pto-mediated resistance. How these MAPKs contribute to the defense response remains to be elucidated. Currently there are no known substrates for plant MAPKs, but potential candidates include specific transcription factors that turn on defense-related gene expression. Several studies implicate a role for WRKY transcription factors working downstream of MAPK.
MAPK Signaling in Pto-mediated Resistance

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Kerry F. Pedley and Gregory B. Martin

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