Tomato MAPKKK is a positive regulator of cell-death signaling networks associated with plant immunity

Shiri Melech-Bonfil and Guido Sessa

Department of Molecular Biology and Ecology of Plants, Tel-Aviv University, 69978 Tel-Aviv, Israel

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*For correspondence (fax 972 3 640 9380; e-mail guidos@post.tau.ac.il).

SUMMARY

Mitogen-activated protein (MAP) kinase cascades are fundamental components of the signaling pathways associated with plant immunity. Despite the large number of MAP kinase kinase kinases (MAPKKK) encoded in the plant genome, only very few of them have an assigned function. Here, we identified MAPKKK gene of tomato (Solanum lycopersicum), SlMAPKKKe, which is required for hypersensitive response cell death and disease resistance against Gram-negative bacterial pathogens. Silencing of SlMAPKKKe compromised tomato resistance to Xanthomonas campestris and Pseudomonas syringae strains, resulting in the appearance of disease symptoms and enhanced bacterial growth. In addition, silencing of NbMAPKKKe in Nicotiana benthamiana plants significantly inhibited the cell death triggered by expression of different R gene-effector gene pairs. Conversely, overexpression of either the full-length SlMAPKKKe gene or its kinase domain in N. benthamiana leaves caused pathogen-independent activation of cell death that required an intact kinase catalytic domain. Moreover, by suppressing the expression of various MAPKK and MAPK genes and overexpressing the SIMAPKKKe kinase domain, we identified a signaling cascade acting downstream of SIMAPKKKe that includes MEK2, WIPK and SIPK. Additional epistasis experiments revealed that SIPKK functions as a negative regulator of SIMAPKKKe-mediated cell death. Our results provide evidence that SIMAPKKKe is a signaling molecule that positively regulates cell death networks associated with plant immunity.

Keywords: tomato, Xanthomonas campestris pv. vesicatoria, Pseudomonas syringae pv. tomato, MAP kinase, plant immunity, virus-induced gene silencing.

INTRODUCTION

A localized cell death, referred to as the hypersensitive response (HR), often occurs in resistant plants challenged by avirulent pathogens (Greenberg and Yao, 2004). The HR is typically triggered upon recognition of microbial virulence proteins, designated as pathogen effectors, by resistance (R) proteins (Martin et al., 2003; Caplan et al., 2008). In addition to the HR, immune responses activated by R proteins include changes in ion fluxes, production of reactive oxygen and nitrogen species, cell wall fortification and accumulation of pathogenesis-related proteins (Hammond-Kosack and Jones, 1996). A similar set of immune responses, but generally excluding the HR, are also activated in plants upon recognition of conserved molecular structures of pathogenic microbes by transmembrane pattern-recognition receptors (PRRs) (Boiler and Felix, 2009).

In recent years it has become clear that mitogen-activated protein (MAP) kinase cascades participate in the activation of plant immune responses mediated by R and PRR proteins (Pedley and Martin, 2005; Pitzschke et al., 2009). The MAP kinase core module includes three protein kinases sequentially activated through phosphorylation by their upstream component: MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK) and MAP kinase (MAPK). These three kinase modules function as molecular switches that turn on the expression of specific sets of genes, resulting in the activation of cellular responses. Components of MAPK cascades are particularly abundant in higher plants: there are 60 putative MAPKKKs, 10 MAPKKs and at least 20 MAPKs in Arabidopsis (Ichimura et al., 2002). Functional information is only available for a
limited number of these proteins (Colcombet and Hirt, 2008).

MAPKKKs represent the most numerous and structurally heterogeneous component of MAPK modules (Ichimura et al., 2002). However, only a few MAPKKKs associated with plant immunity have been identified to date (Pedley and Martin, 2005; Pitzschke et al., 2009). For example, the Nicotiana benthamiana MAPKKK NPK1 and its downstream MAPKK MEK1 and MAPK Nt6 were shown to be required for resistance to the tobacco mosaic virus (TMV) mediated by the R protein N (Jin et al., 2002; Liu et al., 2004). Similarly, Solanum lycopersicum (tomato) MAPKKK is required for the HR mediated by the R protein Pto, and was proposed to activate two MAP kinase modules (del Pozo et al., 2004). Our knowledge of MAPKKKs acting downstream of PRRs is limited to the MAPKKK MEKK1 from Arabidopsis, which is activated upon the sensing of flagellin by the FLS2 kinase (Asai et al., 2002). Finally, the Arabidopsis Raf-like MAPKKK EDR1 was found to negatively regulate plant immunity (Frye et al., 2001).

As model systems for the investigation of signaling pathways involved in plant immunity, we study interactions of tomato plants with the Gram-negative bacteria Pseudomonas syringae pv. tomato (Pst) and Xanthomonas campestris pv. vesicatoria (Xcv), which are the causal agents of bacterial speck and spot diseases, respectively (Jones et al., 1996; Pedley and Martin, 2003). Resistance to Pst in tomato is mediated by the R protein Pto, a Ser/Thr protein kinase, which interacts directly with the Pst effectors AvrPto and AvrPtoB (Scofield et al., 1996; Tang et al., 1996; Kim et al., 2002). The nucleotide binding site/leucine-rich repeats protein Prf is also required for resistance to Pst, and associates with Pto in a high molecular weight complex (Salmeron et al., 1996; Mucyn et al., 2006). Several proteins that participate in signaling pathways downstream of the Pto/Prf complex have been identified, including a Ser/Thr protein kinase, an F-box E3 ligase, transcription factors and, as described above, components of MAP kinase cascades (Zhou et al., 1995; 1997; Ekengren et al., 2003; del Pozo et al., 2004; Mayrose et al., 2006).

Signaling components involved in tomato resistance to Xcv are largely unknown. Our knowledge is limited to candidate proteins encoded by genes that are differentially expressed in resistant plants challenged by avirulent Xcv strains (Bonshtien et al., 2005; Balaji et al., 2007). Here, by a virus-induced gene silencing (VIGS) screen of candidate genes, we identified a tomato MAPKKK, SIMAPKKK, which is required for disease resistance against Xcv and Pst bacteria, and is a positive regulator of cell death. In addition, we provide evidence that NbMAPKK is required for eliciting HR downstream of various R gene/effector gene pairs in N. benthamiana plants. Finally, we dissected an SIMAPKKK-activated MAP kinase cascade, and identified a negative regulator of SIMAPKKK-mediated cell death. These results demonstrate that SIMAPKKK functions as a key regulator of signaling networks activated by R proteins to promote cell death and plant immunity.

RESULTS

**SIMAPKKK** is required for tomato resistance to Xcv expressing avrXv3

Plants of the tomato line Hawaii 7981 are resistant to Xcv bacterial strains expressing the avrXv3 effector gene (Scott et al., 1995). To identify components of signaling pathways involved in tomato disease resistance to Xcv, we used the VIGS technique to silence candidate genes in Hawaii 7981 plants. VIGS experiments were performed using a tobacco rattle virus (TRV)-based vector (Liu et al., 2002), and included genes that are differentially expressed during the tomato resistance response to Xcv, or encoding components of MAP kinase cascades (Balaji et al., 2007) (Table S1). Silenced plants were inoculated with $5 \times 10^6$ colony-forming units (cfu) ml$^{-1}$ of an avirulent Xcv T2 strain expressing avrXv3 [T2(avrXv3)], or were mock-inoculated, and were then screened for breakdown of resistance. As a control, plants were infected with an empty TRV vector and inoculated either with the avirulent Xcv T2(avrXv3) strain or with a virulent Xcv T2 strain. As shown in Figure 1(a), silencing the tomato SIMAPKKK gene using TRV that contains a unique fragment of the gene (nucleotides 3009–3684, hereafter TRV3/MAPKKK; Figure 3a) resulted in the consistent appearance of disease symptoms upon inoculation with Xcv T2(avrXv3). Symptoms in these plants were qualitatively similar to those observed in TRV-only control plants inoculated with virulent Xcv T2, whereas no symptoms were detected in TRV-only control plants inoculated with Xcv T2(avrXv3), or in mock-inoculated SIMAPKKK-silenced plants (Figure 1a). In addition, 4 days after inoculation the Xcv T2(avrXv3), the bacterial population was significantly higher in plants silenced for SIMAPKKK than in control plants, yet lower than growth of Xcv T2 in control plants ($P < 0.05$; Figure 1b). These results indicate that SIMAPKKK is required for tomato disease resistance to Xcv strains expressing avrXv3. Interestingly, the silencing of SIMAPKKK also caused a moderate inhibition of plant growth, suggesting that this gene may also play a role in developmental processes (Figure 2a).

To confirm that the above phenotypes directly result from the silencing of SIMAPKKK, we repeated the experiments using another unique fragment of the gene (nucleotides 898–1308, hereafter TRV5/MAPKKK; Figure 3a). In agreement with our previous findings, silenced plants developed disease symptoms upon inoculation with the avirulent Xcv T2(avrXv3) strain, and supported significantly higher bacterial populations than control plants ($P < 0.05$; Figure 1). Tomato plants silenced with TRV5/MAPKKK did not produce any visible disease symptoms in the absence of...
a pathogen (Figure 1a), but were slightly inhibited in growth (Figure 2a). Importantly, quantitative RT-PCR (qRT-PCR) analysis indicated that endogenous transcript levels of SlMAPKKKe were reduced by approximately 70% in plants silenced with either fragment of the gene (Figure S1a). As a negative control, in the same plants we measured transcript levels of the SlNPK1 gene (homolog of NPK1; Jin et al., 2002), which does not show any sequence similarity to the SlMAPKKKe fragments used for VIGS, but displays the highest nucleotide identity to SlMAPKKKe among tomato homologs of MAPKKK genes involved in plant immunity. As shown in Figure S1(a), SlNPK1 transcript levels were only slightly altered in SlMAPKKKe-silenced plants. Together with the observation that SlMAPKKKe is a single-copy gene in the tomato genome, as predicted by genome sequencing data available in the Sol Genomics Network (SGN) database and confirmed by Southern blot analysis (Figure S2), these results indicate that the break-down of disease resistance to Xcv bacteria expressing avrXv3 was a result of the specific silencing of SlMAPKKKe.

SlMAPKKKe belongs to the A4 subgroup of plant MEKKs

We isolated a full-length cDNA clone of SlMAPKKKe by 5’ RACE PCR using a partial sequence of the gene retrieved in the SGN database. The SlMAPKKKe open reading frame encodes a polypeptide of 1401 amino acids with an N-terminal kinase domain and two armadillo (Arm) repeat domains (Coates, 2003) (Figure 3a). Phylogenetic analysis, which included proteins from different plant species that show high identity to SlMAPKKKe (Table S2), revealed that

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Bootstrap values are shown for each branch as a percentage of 100 replicates. Length measured by the number of amino acid replacements per position. Out-groups in this analysis. The scale bar above the tree represents branch a and MAPKKK related NPK1 from tobacco (Solanum lycopersicum) (Figure 3b). Kinase domain sequences of the immunity-related NPK1 from tomato (Solanum lycopersicum) were used as positive controls (Salmeron et al., 1996). SIMAPKKKc silenced plants were then inoculated with corresponding avirulent bacterial strains: Hawaii 7998 plants were infiltrated with \(5 \times 10^6\) cfu ml\(^{-1}\) of an \(Xcv\) T3 strain expressing \(avrXv\), whereas RG-PtoR plants were dipped in \(5 \times 10^7\) cfu ml\(^{-1}\) of the \(Pst\) strain DC3000. As an additional control, Hawaii 7998 plants infected with empty TRV were inoculated with a virulent \(Xcv\) T3 strain (\(5 \times 10^7\) cfu ml\(^{-1}\)). Plants were monitored for the appearance of disease symptoms and for bacterial growth over 5 days. As shown in Figure 4(a), the silencing of SIMAPKKKc compromised resistance of both lines, as manifested by the appearance of typical disease symptoms that were qualitatively similar to those developed in the respective positive controls (i.e. Hawaii 7998 infected with TRV-only and inoculated with \(Xcv\) T3 strain expressing \(avrXv\)).

SIMAPKKKc is required for tomato resistance to different bacterial pathogens

We next assessed whether SIMAPKKKc plays a role in signaling pathways activated by different R proteins or is specific to the resistance of tomato against \(Xcv\) expressing \(avrXv\). To this aim, we used the tomato lines Hawaii 7998 and Rio Grande PtoR (RG-PtoR). Hawaii 7998 plants carry three non-dominant \(R\) genes and are resistant to \(Xcv\) strains expressing \(avrXv\) (Whalen et al., 1993), whereas RG-PtoR plants carry the \(Pto\) \(R\) gene and are resistant to \(Pst\) strains expressing \(avp\) and \(avp\) (Pedley and Martin, 2003). Plants of the two lines were silenced for SIMAPKKKc with TRV-3’MAPKKKc and, as a positive control, RG-PtoR plants were also silenced for the \(Prf\) gene that is required for \(Pto\)-mediated resistance (Salmeron et al., 1996). SIMAPKKKc silenced plants were then inoculated with corresponding avirulent bacterial strains: Hawaii 7998 plants were infiltrated with \(5 \times 10^6\) cfu ml\(^{-1}\) of an \(Xcv\) T3 strain expressing \(avrXv\), whereas RG-PtoR plants were dipped in \(5 \times 10^7\) cfu ml\(^{-1}\) of the \(Pst\) strain DC3000. As an additional control, Hawaii 7998 plants infected with empty TRV were inoculated with a virulent \(Xcv\) T3 strain (\(5 \times 10^7\) cfu ml\(^{-1}\)). These results suggest that SIMAPKKKc is required for tomato disease resistance against different bacterial pathogens.
**SIMAPKKKc regulates plant cell death**

_NbMAPKKKc_ is required for eliciting cell death triggered by different _R_ gene/effector gene pairs in _N. benthamiana_ plants

To determine whether _MAPKKKc_ is involved in eliciting cell death associated with disease resistance in _N. benthamiana_ plants, we tested the effect of _NbMAPKKKc_ silencing on cell death triggered by the co-expression of the tomato R protein Pto and the _Pst_ effector AvrPto (Tang et al., 1996). _N. benthamiana_ plants are particularly suited for such experiments because of the high uniformity and efficiency of VIGS, and the conservation of signaling pathways acting downstream to Pto-AvrPto recognition in tomato and _N. benthamiana_ (Liu et al., 2002; del Pozo et al., 2004). Plants were silenced with TRV:3’MAPKKKc or TRV:Prf, and 4 weeks later their leaves were infiltrated with _Agrobacterium_ strains for the expression of Pto or AvrPto alone, and for co-expression of Pto and AvrPto. At this time, qRT-PCR analysis revealed that in plants silenced with TRV:3’MAPKKKc, transcript abundance of _NbMAPKKKc_ was reduced by 65%, and there was no effect on expression of the _NbNPK1_ gene used as a negative control (Figure S1c). As shown in Figure 5(a) and Table 1, the silencing of _NbMAPKKKc_ significantly reduced the percentage of leaves developing cell death upon co-expression of Pto and AvrPto, compared with TRV-only infected leaves (30 versus 76%). As expected, no cell death was observed when Pto or AvrPto were expressed alone or in plants silenced for the _Prf_ gene. To confirm that silencing of _NbMAPKKKc_ caused a reduction of cell death, we measured electrolyte leakage in leaves silenced for _NbMAPKKKc_ and _Prf_, or infected with empty TRV. Development of cell death caused by the co-expression of Pto or AvrPto was visible 2 days after _Agrobacterium_ infiltration in control plants, and was associated with increased ion leakage (Figure 5b). In contrast, leaves silenced for _NbMAPKKKc_ or _Prf_, which showed no or weak cell death as a result of Pto and Pto or AvrPto co-expression, had reduced ion leakage compared with control plants. Similarly, no ion leakage was observed in control plants expressing Pto alone (Figure 5b). These results are consistent with the involvement of _NbMAPKKKc_ in signaling pathways mediating cell death triggered by Pto-AvrPto recognition. It is noteworthy that _N. benthamiana_ plants silenced for _NbMAPKKKc_ did not show any spontaneous cell death phenotype, but instead displayed a growth inhibition phenotype, as observed in tomato (Figure 2b).

We next examined whether _NbMAPKKKc_ is a component of cell death signaling pathways activated by additional R protein/effectector protein pairs, such as those involved in the interaction between tomato plants and the fungal pathogen _Cladosporium fulvum_ (Rivas and Thomas, 2005). To this aim, _N. benthamiana_ leaves silenced for _NbMAPKKKc_ were infiltrated with _Agrobacterium_ strains co-expressing Cf4 and Avr4, or Cf9 and Avr9. As shown in Table 1, the silencing of _NbMAPKKKc_ reduced the percentage of leaves developing cell death upon co-expression of Cf4/Avr4 or Cf9/Avr9 compared with control leaves (54 versus 86% and 46 versus 92%, respectively). Together, our results suggest that _NbMAPKKKc_ participates in signaling pathways mediating the elicitation of cell death triggered by different R/effectector protein recognition events.

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**Figure 4.** Silencing of _SIMAPKKKc_ compromises tomato (_Solanum lycopersicum_) resistance to _Xcv_ expressing _avrRxv_ and to _Pst_ DC3000.

(a) Bacterial spot and speck symptoms in _SIMAPKKKc_-silenced Hawaii 7998 and Rio Grande PtoR plants, respectively. Hawaii 7998 plants were infected with TRV-only or TRV:3’MAPKKKc, whereas Rio Grande PtoR plants were infected with TRV:3’MAPKKKc or TRV:Prf. Hawaii 7998 plants were then inoculated with _Xcv_ T3 or _Xcv_ T3 (avrRxv) strains, whereas Rio Grande PtoR plants were inoculated with _Pst_ DC3000. Photographs were taken 5 days after inoculation.

(b) Bacterial populations in leaves of plants treated as described above were determined at 1 h (0 days post-inoculation, dpi) and 4 dpi. Bars represent the average and SE of at least eight independent plants. Letters in bars represent groupings of statistical significance based on ANOVA and comparisons for all pairs using the Tukey-Kramer honestly significant difference test (P < 0.05). Experiments were repeated four times with similar results.

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SIMAPKKK confinement is not involved in cell death associated with tomato disease susceptibility

Once determined that in N. benthamiana plants NbMAPKKK plays a role in cell death associated with plant immunity, we tested whether this gene is also involved in cell death associated with tomato disease susceptibility, as reported previously for MAPKKK (del Pozo et al., 2004). To this aim, we monitored bacterial growth and development of spot and speck disease symptoms in tomato plants silenced for SIMAPKKK and inoculated with virulent Xcv and Pst strains, respectively. For silencing, Hawaii 7981, Hawaii 7998 and RG-PtoS lines were infected with TRV:3’MAPKKKe or TRV only. Four weeks later, Hawaii 7981 and Hawaii 7998 plants were inoculated with virulent Xcv T2 and Xcv T3 strains, respectively. At this time, RG-PtoS plants were inoculated with the Pst strain DC3000. As shown in Figure 6, SIMAPKKK: silenced plants displayed disease symptoms and supported bacterial populations similar to control plants. These results indicate that SIMAPKKK: is not involved in cell death observed during the progression of disease symptoms caused by the compatible interactions between tomato plants and Xcv or Pst bacteria.

Overexpression of SIMAPKKK: causes pathogen-independent cell death

To test whether overexpression of SIMAPKKK: causes cell death, we used Agrobacterium to transiently express the full-length SIMAPKKK: protein or only its kinase domain in active (KD+) or inactive (KD−) forms under the control of an estradiol-inducible system in N. benthamiana leaves. The inactive form of the SIMAPKKK: kinase domain was obtained by substituting the essential lysine in the ATP-binding site (Lys49) with an arginine. As positive controls, we expressed tomato MAPKKKα or its kinase domain alone (KDα), which were previously shown to cause cell death in N. benthamiana leaves (del Pozo et al., 2004). As negative controls, we expressed a kinase-deficient form of KD− (KD−) or infiltrated leaves with Agrobacterium carrying an empty vector. As shown in Figure 7(a), overexpression of SIMAPKKK: or KD resulted in cell death in N. benthamiana leaves within 72 and 48 h after estradiol application, respectively. This was slightly slower than the cell death caused by MAPKKKα and KD that, as previously reported (del Pozo et al., 2004), appeared 48 and 36 h after estradiol application, respectively. However, cell death was not observed following the expression of the inactive SIMAPKKK: kinase domain (KD−), indicating that the kinase activity of SIMAPKKK: is required for the activation of cell death. Similar results were also obtained in tomato plants
for KD\textsubscript{e} and KD\textsubscript{c} (Figure S3). Expression of SIMAPKKK\textsubscript{e}, KD\textsubscript{e} and KD\textsubscript{c} was confirmed by immunoblot analysis (Figure 7b,c). In this analysis, the full-length SIMAPKKK\textsubscript{e} did not run at the predicted molecular weight size (160 kDa), possibly because of post-translational modifications occurring in the protein. These results support the conclusion that SIMAPKKK\textsubscript{e} is a positive regulator of cell death that activates signal transduction pathway(s) by phosphorylation.

**MEK2, WIPK and SIPK act downstream of SIMAPKKK\textsubscript{e}, whereas SIPKK is a negative regulator of SIMAPKKK\textsubscript{e}-mediated cell death**

To identify components of MAP kinase cascade(s) activated by SIMAPKKK\textsubscript{e}, we used epistasis experiments that combine a gain-of-function cell death assay (overexpression of the SIMAPKKK\textsubscript{e} kinase domain) with loss of function based on VIGS of MAPKKs and MAPKs that are known components of immunity-related cascades (Ekengren et al., 2003; Pedley and Martin, 2005). Our assumption was that SIMAPKKK\textsubscript{e}-mediated cell death would be suppressed by silencing key MAPKKs or MAPKs acting downstream of SIMAPKKK\textsubscript{e}. In these experiments, *N. benthamiana* plants were silenced for MEK\textsubscript{1}, MEK\textsubscript{2} or SIPKK (MAPKKs) and for WIPK, SIPK or

![Figure 6. Silencing of SIMAPKKK\textsubscript{e} does not affect cell death associated with disease susceptibility in tomato (*Solanum lycopersicum*) plants. Hawaii 7981 (a), Hawaii 7998 (b) and Rio Grande PtoS (c) plants were silenced with TRV:3’MAPKKK\textsubscript{e} or TRV only. Rio Grande PtoS plants were then inoculated with Pst strain DC3000, whereas Hawaii 7981 and Hawaii 7998 plants were inoculated with the Xcv T2 and Xcv T3 strains, respectively. Photographs (left) were taken 5 days after infection. Bacterial populations (right) in infected leaves were determined at 1 h (0 days post-inoculation, dpi) and 4 dpi. Bars represent the averages and SEs of four independent plants. Letters represent groupings of statistical significance based on ANOVA and comparisons for all pairs using the Tukey–Kramer honestly significant difference test (*P* < 0.05). Experiments were repeated three times with similar results.](image)

![Figure 7. Overexpression of SIMAPKKK\textsubscript{e} in *Nicotiana benthamiana* elicits cell death. (a) Cell death in *N. benthamiana* leaves infiltrated with *Agrobacterium* (OD\textsubscript{600} = 0.06) strains carrying an empty vector (EV) or expressing SIMAPKKK\textsubscript{e}, SIMAPKKK\textsubscript{e} kinase domain in active (KD\textsubscript{e}) and inactive form (KD\textsubscript{c}), MAPKKK\textsubscript{a}, and MAPKKK\textsubscript{a} kinase domain in active (KD\textsubscript{a}) and inactive form (KD\textsubscript{c}). Proteins were tagged with a double hemagglutinin (HA) epitope, and their expression was estradiol-inducible. Photographs were taken 48 h after estradiol application. (b,c) Expression of SIMAPKKK\textsubscript{e}, KD\textsubscript{e} and KD\textsubscript{c} in *N. benthamiana* leaves monitored by immunoblot analysis (left) with anti-HA antibody in leaf extracts sampled 6 h after estradiol application. Ponceau staining of membranes (right) confirmed similar protein loading.](image)
NTF6 (MAPKs). As a negative control, we included plants infected with the TRV empty vector. The efficiency and specificity of silencing was assessed by qRT-PCR analysis that measured the expression of each target gene and its closest homolog in silenced plants relative to control plants. This analysis indicated a decrease of at least 60% in transcript abundance of each gene targeted by VIGS, whereas the abundance of the homologous genes remained at wild-type levels in all instances (Figure S4). In addition, no spontaneous cell death was observed in any of the silenced plants. The kinase domain of SlMAPKKKe (KDKe) and its kinase deficient form (KDKe−) were then transiently expressed in the silenced leaves via Agrobacterium infiltration. As shown in Figure 8(a,b), cell death was typically observed in control plants expressing KDKe 72 h after estradiol application, whereas cell death was not observed in leaf areas expressing KDKe−, or infiltrated with Agrobacterium carrying an empty vector (not shown). However, silencing of MEK2, WIPK and SIPK significantly inhibited cell death caused by the expression of KDKe. Conversely, in leaves silenced for SIPKK, cell death mediated by KDKe developed earlier, and was significantly enhanced compared with control leaves (Figure 8b,c). Finally, the silencing of MEK1 and NTF6 did not affect KDKe− mediated cell death. These results suggest that MEK2, WIPK and SIPK act downstream of SIMAPKKKe in signaling pathways activating immunity-associated cell death. On the other hand, SIPKK appears to function as a negative regulator of SIMAPKKKe-mediated cell death. A role for MEK1 and NTF6 downstream of SIMAPKKKe is unlikely, but it cannot be excluded because of the residual transcript levels of these genes in the silenced plants (Figure S4).

**DISCUSSION**

In this study, we established an important role for SIMAPKKKe in signaling pathways mediating immune responses to Xcv and Pst phytopathogenic bacteria. In addition, by loss- and gain-of-function experiments, we provided evidence that SIMAPKKKe is a key regulator of cell death associated with plant immunity. Epistasis analysis positioned SIMAPKKKe in a cell death signaling pathway, upstream of MEK2 and WIPK/SIPK, and defined SIPKK as a negative regulator of SIMAPKKKe-mediated cell death.

In tomato plants, SIMAPKKKe is required for disease resistance pathways that are activated by different types of genetic interactions (Figure 9). These include both quantitative resistance to Xcv strains expressing avrRxv, which is recognized by three plant R genes (Whalen et al., 1993; Yu et al., 1995), and classical gene-for-gene interactions mediating resistance to Xcv strains expressing avrXv3 (AstuMonte et al., 2000), as well as resistance to Pst strains expressing avrPto and avrPtoB (Pedley and Martin, 2003).
Components of resistance signaling are generally categorized into two groups based on whether they act downstream of a single or multiple structural classes of R proteins (Martin et al., 2003). \textit{SIMAPKKK}\textsubscript{e} can be ascribed to the latter group because silencing of the corresponding gene impaired cell death mediated either by the Ser/Thr kinase Pto (Martin et al., 1993), which acts in concert with the cytoplasmic NBS-LRR R-like protein Prf (Salmeron et al., 1996), or by the transmembrane LRR-containing R protein Cf9 (Jones et al., 1994). Although silencing of \textit{SIMAPKKK}\textsubscript{e} compromised disease resistance, in \textit{SIMAPKKK}\textsubscript{e}-silenced plants the appearance of disease symptoms and bacterial growth remained at lower levels than in susceptible control plants. A similar partial breakdown of resistance was observed when the expression of several genes involved in Pto-mediated resistance to \textit{Xcv} or \textit{AvrXv} effectors by corresponding tomato (\textit{Solanum lycopersicum}) R proteins. The model is based on an analysis of cell death triggered by overexpression of either \textit{SIMAPKKK}\textsubscript{e} (this work) or MAPKKK\textsubscript{e} (del Pozo et al., 2004) in \textit{Nicotiana benthamiana} plants silenced for various MAPKK and MAPK genes. \textit{SIMAPKKK}\textsubscript{e} activates a MAP kinase module, which is negatively regulated by SIPK through its interaction with SIPK or activation of MPK4. MAPKKK\textsubscript{e}, which is regulated by a 14-3-3 protein (Oh et al., 2010), activates two distinct and possibly sequential cascades. Dotted lines indicate possible connections between proteins or cascades.

Overexpression of \textit{SIMAPKKK}\textsubscript{e} results in the activation of pathogen-independent cell death, whereas silencing of \textit{NbMAPKKK}\textsubscript{e} inhibits cell death activated by the co-expression of Pto and AvrPto in \textit{N. benthamiana} plants. This evidence defines \textit{SIMAPKKK}\textsubscript{e} as a positive regulator of R protein-mediated cell death. Epistasis analysis in \textit{N. benthamiana} leaves indicates that MEK2, SIPK and WIPK are required for \textit{SIMAPKKK}\textsubscript{e}-mediated cell death. Based on these data, we propose that after sensing either \textit{Xcv} or \textit{Pst} bacteria, \textit{SIMAPKKK}\textsubscript{e} receives the input signal for the induction of cell death and activates MEK2, SIPK and WIPK as components of a pro-cell death MAP kinase module (Figure 9). A MEK2, SIPK/WIPK MAP cascade activated by an unknown MAPKK was previously implicated in disease resistance to TMV (Jin et al., 2003). This MAPK module was associated with cell death, as constitutively active forms of MEK2 or SIPK were shown to induce pathogen-independent cell death (Yang et al., 2001; Zhang and Liu, 2001). In addition, by overexpression of a constitutively active MEK2 and biochemical analysis, MEK2 was confirmed as the upstream MAPKK of SIPK and WIPK (Yang et al., 2001). Our results strongly support that \textit{SIMAPKKK}\textsubscript{e} can function as an upstream MAPKK to MEK2 in this cascade.
MAPKKKs were previously shown to participate in signaling pathways activated by Pto (del Pozo et al., 2004). Because silencing of either MAPKKKs or NbMAPKKKs compromises Pto-mediated elicitation of cell death in N. benthamiana plants, these two MAPKKKs appear to be both required for disease resistance, rather than act redundantly. In support of this conclusion, MAPKKKs or SIMAPKKKs silencing is sufficient to compromise resistance to Pst strains in N. benthamiana and tomato plants, respectively. However, it should be noted that, in contrast to MAPKKKs, SIMAPKKKs is not involved in cell death associated with disease susceptibility. Based on VIGS and epistasis analysis in N. benthamiana, del Pozo et al. (2004) proposed that two distinct and interconnected MAP kinase cascades act downstream of MAPKKKs (Figure 9). The first cascade consists of MAPKKKs, MEK2 and SIPK, and was proposed to sequentially activate a second MAP kinase module that includes MEK1 and Ntf6. Notably, SIMAPKKKs and MAPKKKs share MEK2 and SIPK as downstream components. Conversely, WIPK is required for cell death elicited only by SIMAPKKKs, and not by MAPKKKs, whereas MEK1 and Ntf6 are specific downstream components of MAPKKKs. Based on these observations, we propose that upon their activation, MAPKKKs and SIMAPKKKs transfer input signals to MEK2 that in turn activates SIPK (Figure 9). Consistent with the inter-relationship between SIPK and WIPK proposed by Liu et al. (2003), activation of SIPK by SIMAPKKKs-MEK2 might induce transcription of the WIPK gene. Once newly synthesized WIPK accumulates, it is then activated by MEK2 and contributes along with SIPK to cell death by phosphorylation of downstream proteins. On the other hand, SIPK activated by MAPKKKs-MEK2 could lead to the activation of an additional MAPKKKs-dependent MAP kinase cascade, including an unknown MAPKKK, MEK1 and Ntf6 that contribute to cell death. A scenario including de novo protein synthesis in pathways activated by SIMAPKKKs, but not by MAPKKKs, is consistent with the later development of cell death observed upon overexpression of SIMAPKKKs, compared with overexpression of MAPKKKs. However, although not required for MAPKKKs-mediated cell death, WIPK could still play a role downstream of MAPKKKs as the WIPK-homolog MPK3 was found to be activated by overexpression of MAPKKKs in tomato plants (Pedley and Martin, 2004). Finally, it remains to be established what the nature of the signals transferred by MAPKKKs and SIMAPKKKs to MEK2 is, and how SIPK output specificity is controlled.

An additional important result of our epistasis analysis is that silencing of the SIPKK gene accelerates cell death activated by overexpression of SIMAPKKKs. This observation strongly suggests that SIPKK acts as a negative regulator of cell death activated by the SIMAPKKKs/MEK2/SIPK-WIPK module. SIPKK could exert its negative effect acting directly on SIPK, as these two proteins were shown to physically interact in a yeast two-hybrid system (Liu et al., 2000). In support of this possibility, SIPK activity was suppressed in tobacco plants by overexpression of SIPKK, either in the wild-type or in a constitutively active form (Gomi et al., 2005). Alternatively, SIPKK may negatively regulate cell death by acting in a MAPK signaling pathway parallel with the SIMAPKKKs-activated module. Consistent with such a mechanism, in tobacco plants SIPKK was shown to activate MPK4, the Arabidopsis homolog of which is a negative regulator of salicylic acid-dependent plant defense responses (Petersen et al., 2000).

Additional research is required to fill the gap between the recognition of AvrPto by the Pto/Prf complex and activation of SIMAPKKKs- and MAPKKKs-initiated MAP kinase modules. A first important step in this direction was the identification and functional characterization of TFT7, a 14-3-3 protein that interacts with MAPKKKs and regulates its signaling ability (Oh et al., 2010). In the future it will be important to determine whether SIMAPKKKs is also regulated by TFT7, what other molecules control its activity, and how the output signals of SIMAPKKKs- and MAPKKKs-dependent cascades eventually lead to the activation of cell death.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains and plant material**

The bacterial strains used are: Xcv T3, Xcv T3 expressing avrRvxv (Bonshtien et al., 2005), Xcv T2 strain 5746 (Xcv T2), and Xcv T2 carrying the avrXv3 gene (Gibly et al., 2004); Pst strain DC3000 (Buell et al., 2003); Agrobacterium tumefaciens strains GV2280 and EHA105.

The tomato cultivars used are: Hawaii 7981 (Scott et al., 1995), Hawaii 7998 (Whalen et al., 1993), RG-PrfR (Pto/Prf and Prf/Prf) and RG-PtoS (pto/pto and Prf/Prf (Pedley and Martin, 2003). Plants were grown using standard glasshouse practices (25 ± 2°C and 16-h light/8-h dark).

**Plant inoculation**

For inoculation with Xcv strains, 5-week-old plants were vacuum infiltrated with bacterial cultures prepared as follows: Xcv strains were grown overnight at 28°C in nutrient yeast glycerol (NYG) liquid medium (Daniels et al., 1984), with the addition of 50 µg ml⁻¹ kanamycin for Xcv T2 (avrXv3) and Xcv T3 (avrRvxv). Bacteria were pelleted, washed twice with 10 mM MgCl₂, and diluted to a concentration of 5 × 10⁶ cfu ml⁻¹ in 10 mM MgCl₂ and 0.005% (v/v) Silwett-L77.

For inoculation with Pst DC3000, 5-week-old plants were dipped for 1 min in a bacterial suspension prepared as follows: cultures were grown overnight at 28°C in King's B (KBM) plates (Martin et al., 1993) in the presence of 100 µg ml⁻¹ rifampicin. On the day of infection, bacteria were scooped from the plates, suspended in 10 mM MgCl₂ and diluted to a concentration of 5 × 10⁶ cfu ml⁻¹ in 10 mM MgCl₂ and 0.025% (v/v) Silwett-L77. Bacterial populations in infected plants were determined as described in the supplementary experimental procedures (Appendix S1).

**Virus-induced gene silencing plasmids and procedures**

Plasmids pTRV1, pTRV2, pTRV2:SiPPrf, pTRV2:NbWIPK, pTRV2: NbSIPK, pTRV2:NtMEK1, pTRV2:NtMEK2 and pTRV2:NbSIPKK have
been previously described (Liu et al., 2002; Ekengren et al., 2003). To generate pTRV derivatives for VIGS of candidate genes in tomato, cDNA fragments of the genes to be silenced were PCR-amplified from tomato cDNA by using the primers listed in Table S1. For VIGS of NbNTF6 (GenBank accession no. AY547494) in *N. benthamiana*, a corresponding cDNA fragment was PCR-amplified from *N. benthamiana* cDNA using the following primers: 5′-GCTCTAGAGTGTTCAAGACAAACCTTTTT-3′ and 5′-GGGGATCCATGACGCTTCCATATGAG-3′. PCR-amplified products were inserted into the multiple cloning site of pTRV2 as *XbaI*- BamHI fragments, and the identity of each construct was confirmed by sequencing. Plasmids were then transformed into *Agrobacterium* strain GV2280, and plants were inoculated as described in Appendix S1.

RNA isolation and quantitative RT-PCR analysis

Total RNA was isolated from leaf samples (50 mg) using the SV Total RNA Isolation Kit (Promega, http://www.promega.com). RNA samples (2 µg) were reverse-transcribed and used for quantitative PCR analysis as described in Appendix S1 and Table S3.

Cloning and mutagenesis of the SIMAPKKKc gene

Based on partial sequences of an SIMAPKKKc expressed sequence tag (EST) that was retrieved from the SGN tomato EST database (SGN-U153382), an SIMAPKKKc full-length cDNA clone was amplified by 5′-RACE with the BD SMARTTM RACE cDNA Amplification kit (BD Biosciences, Clontech, http://www.bdbiosciences.com), inserted at the SalI restriction site of the cloning vector pBlueScript KS+, and sequenced (GenBank accession no. GU192457).

Site-directed mutagenesis of SIMAPKKKc was performed in pBlueScript KS+ containing the SIMAPKKKc coding region fused to a double hemagglutinin (HA) epitope. Mutation of Lys49 to Arg in the SIMAPKKKc kinase catalytic domain (KDc; from amino acid 19–273) was introduced using the Quickchange kit (Stratagene, http://www.stratagene.com) and the following oligonucleotides: 5′-GGAGACTTGGCATTAGACAAGGTTTCTGGAG-3′ and 5′-CTCCAGAGAACTGTCTAATTGCAACAAAGTCTCC-3′.

Alignment and phylogenetic tree analysis

To identify SIMAPKKKc homologs from different plant species, a BlastX search was performed in the National Center for Biotechnology Information (NCBI) database by using the SIMAPKKKc amino acid sequence as a query. Proteins with the highest identity to SIMAPKKKc (Table S2) were selected for analysis. Homologs to SIMAPKKKc were also identified in the maize EST database (http://compbio.dfcii.harvard.edu/cgi/plant.html), and in the *S. pimpenellifolium* genome draft (http://solgenomics.net). A multiple sequence alignment of the retrieved proteins was generated by using *Muscle* v3.0 (Edgar, 2004). A maximum-likelihood phylogenetic tree was created with PhyML (Guindon and Gascuel, 2003) using the LG replacement model.

Protein extraction and immunoblotting

Proteins were extracted by grinding three leaf discs (1 cm in diameter) in extraction buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na3HPO4, 1.5 mM KH2PO4, 1 mM PMSF, 5 µg ml−1 leupeptin and 5 µg ml−1 aprotinin). Extracts were cleared by centrifugation and the supernatant was collected. Protein samples (40 µg) were analyzed by immunoblotting and chemiluminescence visualization (Amersham Biosciences, http://www.gelifesciences.com) using monoclonal HA antibodies (1:1000) (Roche Diagnostics, http://www.roche.com/diagnostics) and horseradish peroxidase-conjugated anti-mouse secondary antibodies (1:5000) (Sigma-Aldrich, http://www.sigmaaldrich.com).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Silencing efficiency and specificity in plants silenced for SIMAPKKKc and NbMAPKKKc.

**Figure S2.** Southern blot analysis of SIMAPKKKc in the tomato (*Solanum lycopersicum*) genome.

**Figure S3.** Overexpression of the SIMAPKKKc kinase domain in tomato (*Solanum lycopersicum*) plants.

**Figure S4.** Silencing efficiency and specificity in *Nicotiana benthamiana* plants silenced for selected MAPKK and MAPK genes.

**Table S1.** Genes targeted by virus-induced gene silencing (VIGS) in *N. benthamiana* (*Solanum lycopersicum*) and Arabidopsis MEKK1 as out-groups. Branch supports were computed using 100 bootstrap replicates.

**Agrobacterium-mediated transient expression**

Constructs for *Agrobacterium*-mediated expression of the gene pairs *PtovavrPto*, *Cf4 AVR4* and *Cf8 AVR8* driven by the CaMV 35S promoter were as previously described (Sessa et al., 2000; Van der Hoorn et al., 2000), and transformed into *Agrobacterium* EHA105 by electroporation. For estradiol-inducible expression, full-length SIMAPKKKc or its kinase catalytic domain in the wild-type (KDc) or mutant (KDc−) form, were cloned with a C-terminal double HA epitope tag into the pE8R binary vector as Xhol-SpeI fragments, and transformed into *Agrobacterium* GV2280. Constructs for the expression of full-length MAPKKKc or its kinase catalytic domain in the wild-type (KDc) or mutant (KDc−) form were as previously described (del Pozo et al., 2004). *Agrobacterium* strains were grown overnight at 28°C in LB medium and used for transient expression, as described in Appendix S1.

Ion leakage measurements

Three discs (1 cm in diameter) were sampled from infiltrated areas for each plant and floated in 50-ml tubes containing 15 ml double-distilled water for 4 h at room temperature (24°C) with gentle shaking. Conductivity was measured with an autoranging EC Temp Meter TH-2400 (El-Hamma Instruments, http://www.elhamma.com).

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issues arising from supporting information (other than missing files) should be addressed to the authors.

REFERENCES


Accession numbers: Newly deposited sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers GU192457 for SIMAPKKCe, and GU205153 for NbMAPKKCe.