Tomato MAPKs LeMPK1, LeMPK2, and LeMPK3 function in the systemin-mediated defense response against herbivorous insects

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Systemin is a wound-signaling peptide that mediates defenses of tomato plants against herbivorous insects. Perception of systemin by the membrane-bound receptor SR160 results in activation of MAPKs, synthesis of jasmonic acid (JA), and expression of defense genes. To test the function of MAPKs in the response to systemin, we used virus-induced gene silencing (VIGS) in plants that overexpress the systemin precursor prosystemin (*35S*::*prosys* plants). These transgenic plants accumulate high levels of defense proteins and exhibit increased resistance to herbivorous insects. Cosilencing of the MAPKs *MPK1* and *MPK2* reduced MPK1/2 kinase activity, JA biosynthesis, and expression of JA-dependent defense genes. Application of methyl-JA restored the full defense response. These data show that MPK1 and MPK2 are essential components of the systemin signaling pathway and most likely function upstream of JA biosynthesis. MPK1 and MPK2 are 95% identical at the amino acid level. Specific VIGS of only *MPK1* or *MPK2* resulted in the same reduction of defense gene expression as cosilencing of *MPK1* and *MPK2*, indicating that gene dosage effects may be important for MPK signaling. In addition, VIGS of the closely related *MPK3* also reduced systemin-induced defense responses. The function of MPK1/2 and orthologs in pathogen-induced defenses is well established. Here we show that cosilencing of *MPK1* and *MPK2* compromised prosystemin-mediated resistance to *Manduca sexta* (Lepidoptera) herbivory, demonstrating that MPK1 and MPK2 are also required for successful defenses against herbivorous insects.

jasmonic acid | plant–insect interactions | virus-induced gene silencing

Plants defend themselves against attacks by herbivorous insects via synthesis of toxic secondary metabolites and proteins such as proteinase inhibitors (PIs), polyphenol oxidase, and amino acid-catabolizing enzymes. These wound-response proteins prevent uptake of essential amino acids in insect intestines, thus causing growth and developmental defects (1–3). In tomato plants, the response to insect attacks and mechanical wounding is mediated by systemin, an 18-aa signaling peptide active at femtomolar concentrations (4). Systemin is derived from the precursor protein prosystemin (5). Prosystemin reduction-of-function plants exhibited low levels of defensive proteins and suffered severe defoliation by *Manduca sexta*, whereas larval growth was strongly increased compared with larvae feeding on WT plants (6). Overexpression of prosystemin caused the continuous synthesis and accumulation of defensive proteins. This correlated with increased tolerance to *M. sexta* larvae (1). Systemin is required for a successful systemic wound response (7, 8). However, the plant hormone jasmonic acid (JA), or a JA derivative, is the most likely long-distance wound signal (8–11). Perception of systemin by the membrane-bound receptor kinase SR160 initiates a signaling pathway that involves ion fluxes (3, 12, 13), MAPKs (12, 14, 15), calcium-dependent protein kinases (13, 16), ethylene (17, 18), reactive oxygen species (19, 20), and synthesis and action of JA via the octadecanoid pathway (8, 21–23). The earliest transcriptional response includes activation of JA-biosynthetic genes and prosystemin within 1 hr. These genes are collectively referred to as “early genes.” Transcript levels of effector proteins such as several groups of PIs, polyphenol oxidase, threonine deaminase, arginase, and leucine aminopeptidase start to increase later and reach maximal levels between 6 and 12 hr after systemin application or wounding (3, 7, 20, 24). They are referred to as “late genes.” Basal transcript levels of early genes are present in JA-insensitive *coi1* null-mutant plants but increase in response to exogenous methyl-JA (MeJA) in a CORANATINE INSENSITIVE 1 (COI1)-dependent manner (24). Furthermore, the wound-induced expression of some early genes is partially JA-independent (25). In contrast to the early genes, late gene expression is completely JA-dependent (24).

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It is not known where MAPKs function in the systemin- and wounding-induced signaling pathway. MAPKs are a part of a three-tiered phosphorelay cascade consisting of the MAPKs (MPKs), which are activated by MAPK kinases (MPKK or MKKs), which in turn are activated by MAPKK kinases (MAPKKKs). We had shown earlier that mechanical wounding with a hemostat and systemin application results in the activation of two MAPKs in tomato (*Lycopersicon esculentum*), LeMPK1 and LeMPK2, which belong to the A2 subgroup of plant MAPKs (12, 14, 15). Wounding also activated LeMPK3, which belongs to the A1 subgroup (12, 26). MPK1 and MPK2 are 95% identical at the amino acid level and coordinately activated by all stimuli we tested so far. MPK1/2 activation was not reduced in the *def1* JA-biosynthesis mutant, indicating that these MAPKs function either upstream or in parallel to JA biosynthesis (15). MPK1 and -2 activity is regulated posttranslationally by phosphorylation. Transcript and protein levels were not altered in response to systemin and wounding. In contrast, *LeMPK3* was transcriptionally upregulated in response to wounding, whereas protein levels did not increase (12). Only a few plant MAPK substrates have been identified in *in vivo* studies, such as the 1-aminocyclo-

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Abbreviations: *35S*::*prosys*, *35S*::*prosystemin*; AOC, allene oxide cyclase; AOS, allene oxide synthase; JA, jasmonic acid; LoxD, lipoxygenase D; MeJA, methyl-JA; PI, proteinase inhibitor; sqRT-PCR, semiquantitative RT-PCR; VIGS, virus-induced gene silencing; TRV, tobacco rattle virus; RIDA, radial immunodiffusion assay.

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propane-1-carboxylate synthases 2 and 6, which are phosphorylated and activated by AtMPK6, an LeMPK1/2 ortholog (27).

LeMPK1, -2, and -3 were also activated by the host-specific elicitors AvrPto and AvrPtoB from *Pseudomonas syringae* (28) and infection by *Xanthomonas campestris* (26). Loss-of-function studies had shown they are required for defense against *P. syringae* and *Mi-1*-mediated resistance to aphids (29). In this study, we used virus-induced gene silencing (VIGS) and showed that these LeMPKs also function upstream of JA and are required for expression of a subset of wound-response genes and resistance to herbivorous insects.

Results

Cosilencing of *LeMPK1* and *LeMPK2* Reduces Systemin-Induced Late Gene Expression. To functionally characterize LeMPKs, we used a loss-of-function approach with tobacco rattle virus (TRV)-mediated VIGS that had been optimized for tomato plants (30). Two *Agrobacterium* expression vectors (pTRV1 and pTRV2) carry the bipartite genome of TRV. Following known requirements for efficient gene silencing (31), we designed a cosilencing construct that targets *MPK1* and *-2* (pTRV-*MPK1/2*) but not *MPK3*. For controls, plants were coinfiltrated with pTRV1 and a pTRV2 vector that carries a partial sequence of GFP (pTRV-*GFP*) or an empty pTRV2 vector (pTRV) to exclude the possibility that some of the observed effects are due to general defense responses triggered by TRV or *Agrobacterium*.

To test whether MPK1 and MPK2 are essential components of the systemin signaling pathway, we infiltrated transgenic plants that overexpress prosystemin under the control of the cauliflower mosaic virus 35S promoter (*35S*::*prosys* plants) with the *MPK1/2* cosilencing construct. *35S*::*prosys* plants show a constitutive wound phenotype and slowly accumulate high levels of defensive proteins (32, 33). It is thought that the slow but continuous accumulation of PIs over time is due to the constant release of systemin (3), which leads to slightly higher steady-state JA levels than found in WT plants (23, 34). However, elevated JA levels could be detected only in young plants (23, 34). The transgenic plants had been used to isolate JA-insensitive and -biosynthetic mutants as suppressors of prosystemin-mediated responses, indicating that JA is essential for the prosystemin-mediated wound response (7, 32). We reasoned that mimicking MAPK mutants by using a VIGS approach in *35S*::*prosystemin* (*35S:prosys*) plants will reveal whether MAPKs are essential components of the systemin signaling pathway. However, MAPK activity above background levels found in WT plants could not be detected in untreated *35S*::*prosys* plants in in-gel kinase assays and in immunocomplex kinase assays (Fig. 1 and data not shown). To verify that systemin specifically activates MPKs, we tested systemin-induced MPK activity in systemin-insensitive *spr1* mutant plants (7). Although the oligosaccharide elicitor chitosan and stem excision resulted in increased MPK1, MPK2, and MPK3 activity in the leaves of WT and *spr1* seedlings, systemin activated these MPKs only in WT plants [see supporting information (SI) Fig. 6].

To show that VIGS of *MPK*s results in reduced MPK activity in transgenic plants, MAPK activity was induced by wounding. Compared with control plants, wound-induced MPK1 and MPK2 activity in pTRV-*MPK1/2*-infiltrated plants was reduced by 88% and 40%, respectively (Fig. 1). The combined reduction of MPK1/2 activity determined in in-gel kinase assays was 71 ± 9%. This correlated with reduction of *MPK1* and *MPK2* transcript levels by 77% and 64%, respectively (Fig. 1*B*). *MPK3* transcript levels (Fig. 1*B*) and activity (not shown) were not significantly reduced in *MPK1/2*-cosilenced plants, demonstrating that the pTRV-*MPK1/2* construct specifically targets *MPK1* and *MPK2*. Not all plants exhibited strong silencing, and only plants with a reduction of *MPK1/2* transcript levels by >50% were analyzed in further experiments.

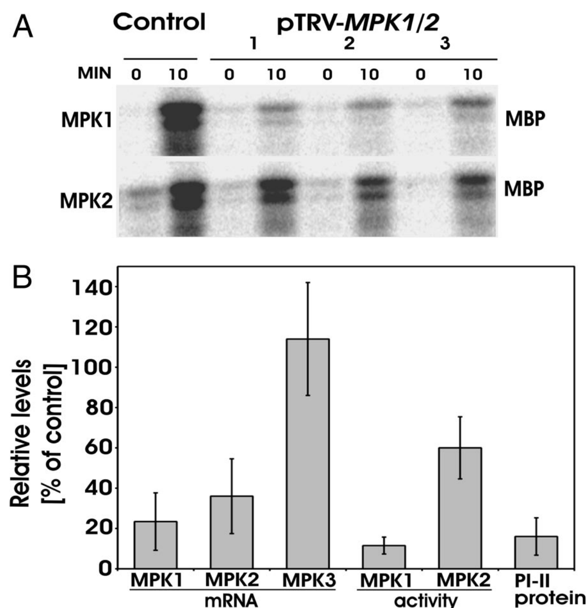

Fig. 1. Cosilencing of *MPK1* and *MPK2* attenuates wound-induced MPK1/2 activity and PI-II synthesis. (*A*) *35S*::*prosys* plants were infiltrated with either pTRV-*MPK1/2* or -*GFP* (Control). Six weeks later, leaves were wounded and analyzed by immunocomplex kinase assays 0 and 10 min after wounding by using specific antibodies against MPK1 and MPK2. Signals represent phosphorylated myelin basic protein, an artificial MAPK substrate. Representative plants (1–3) are shown. (*B*) *35S*::*prosys* plants from independent experiments were analyzed for *MPK1* (n = 9), *MPK2* (n = 9), and *MPK3* (n = 5) mRNA levels by sqRT-PCR, for wound-induced MPK1 (n = 5), and MPK2 (n = 4) activity by immunocomplex kinase assays, and for PI-II protein levels by RIDA (n = 9). The levels in VIGS plants (mean ± SD) were expressed as percentages of the mean levels in control plants which were defined as 100%.

To test whether silencing of *MPK1/2* affects prosystemin-induced defense protein accumulation in untreated plants, we measured protein levels of PI-II, a marker for late gene expression in the tomato wound response. PI-II levels in control plants were high but were reduced by 84% in pTRV-*MPK1/2*-infiltrated plants (Fig. 1*B*). This demonstrates that MPK1 and MPK2 are essential components of the systemin signaling pathway. Because MAPKs are known to function only via their phosphotransfer activity, it is likely they are active in the *35S:prosys* plants. However, this activity cannot be detected as significant in standard kinase assays.

We also tested transcript levels of additional wound response marker genes in pTRV-*MPK1/2*-infiltrated *35S:prosys* plants that showed reduced *MPK1/2* transcript levels. *PI-I* is a late gene and belongs to a different gene family than *PI-II*. *PI-I* and *PI-II* transcript levels were strongly reduced in *MPK1/2*-cosilenced plants. Transcript levels of the early genes *LIPOXYGENASE D* (*LoxD*), *ALLENE OXIDE SYNTHASE2* (*AOS*), and *ALLENE OXIDE CYCLASE* (*AOC*) are up-regulated by systemin (7, 23), but they were not significantly altered in *MPK1/2*-cosilenced plants (Fig. 2). In addition, *LoxD* mRNA increased 1 hr after wounding in *MPK1/2*-cosilenced plants to the same levels as in controls (data not shown). These data show that MPK1 and MPK2 regulate the expression of late JA-dependent genes.

Cosilencing of *LeMPK1* and *LeMPK2* Reduces Systemin-Induced JA Biosynthesis. Because late gene expression is completely JA-dependent (24, 35), we hypothesized that LeMPKs would function upstream of JA synthesis. *35S*::*prosys* plants were infiltrated with pTRV-*MPK1/2*, and 4 weeks later, plants were wounded, and JA levels were determined 1 hr after wounding. Silencing was confirmed by semiquantitative RT-PCR (sqRT-PCR). Un-

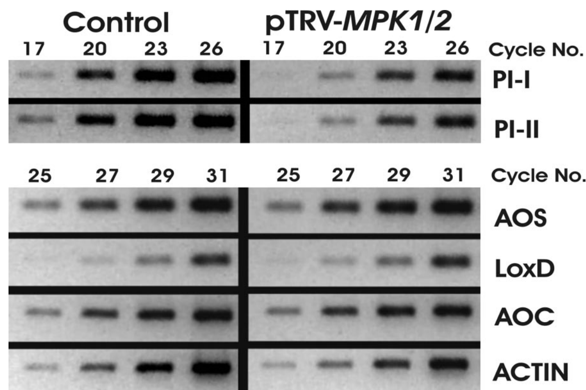

Fig. 2. Cosilencing of *MPK1* and *MPK2* attenuates expression of late systemin-induced wound response genes. *35S*::*prosys* plants were infiltrated with pTRV-*MPK1/2* and -*GFP* (Control). Five weeks later, transcript levels of *Actin* (internal control), the late genes *PI-I* and *PI-II* and the early genes *AOS2*, *LoxD*, and *AOC* were assessed by sqRT-PCR in leaf tissue. Ethidium bromide-stained agarose gels containing RT-PCR products are shown (colors inverted). The experiments are representative of 10 plants from three independent experiments for the late genes and of six plants from three independent experiments for the early genes.

wounded *35S*::*prosys* plants exhibited low background levels of JA. Because JA levels in unwounded control plants were highly variable (108 ± 96 pmol/g), we were unable to detect significant differences between control and cosilenced plants (35 ± 20 pmol/g). However, JA levels increased strongly after wounding in control plants, whereas wound-induced JA levels in cosilenced plants were 60% lower (Fig. 3*A*). Consistent with a role of MAPKs upstream of JA biosynthesis, we found that supplementation of MeJA to *MPK1*/*2*-cosilenced plants restored PI-II synthesis. PI-II levels in control plants were elevated because of the presence of the *Prosystemin* transgene and further increased in response to MeJA. *MPK1*/*2*-cosilenced plants showed low PI-II levels similar to those shown in Fig. 1*B*, but the levels increased to similar levels as in control plants in response to MeJA treatment (Fig. 3*B*). These data indicate that MPK1 and MPK2 function upstream of JA synthesis.

Silencing of *LeMPK1*, *LeMPK2*, and *LeMPK3* Alone Has Similar Effects as Cosilencing of *LeMPK1* and *LeMPK2*. VIGS constructs were generated that specifically target either *MPK1*, *MPK2*, or *MPK3* based on 3′-UTR sequences. The UTRs do not show significant sequence homology to each other. Four weeks after infiltration of *35S*::*prosys* plants with either pTRV-*MPK1* or pTRV-*MPK2*, transcript levels of the targeted MPK, but not of the respective other MPK, were reduced by ≈60% (Fig. 4 *A*–*D*). Reduced *MPK1* and *MPK2* transcript levels both correlated with an 86% and 72% reduction of systemin-induced PI-II synthesis, respectively (Fig. 4 *B* and *D*). *PI-I* and *PI-II* transcript levels were also reduced in both *MPK1*- and *MPK2*-silenced plants (Fig. 4 *A* and *C*). This shows that the presence of both *MPK1* and *MPK2* is required for late gene expression in response to systemin. Transcript levels for early genes were not significantly reduced but were more variable as compared with *MPK1*/*2*-cosilenced plants (data not shown).

In tobacco, the *LeMPK3* ortholog *WIPK* had been shown to regulate wound-induced gene expression and JA synthesis (36, 37). VIGS of *MPK3* reduced transcript levels of *MPK3* by 71% and did not alter transcript levels of *MPK1* and *MPK2* (Fig. 4 *E* and *F*). This correlated with a reduction in systemin-induced PI-II synthesis by 79% (Fig. 4*F*). Transcript levels of the late genes *PI-I* and *PI-II* were also reduced in pTRV-*MPK3*-

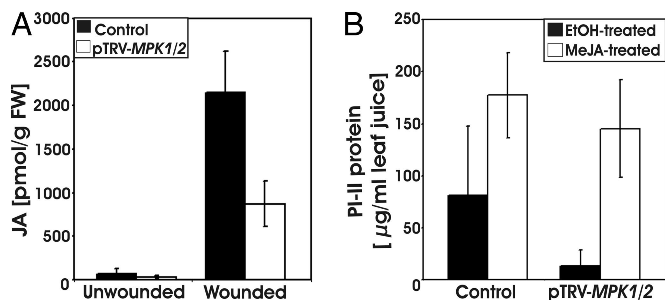

Fig. 3. Cosilencing of *MPK1* and *MPK2* attenuates JA biosynthesis. (*A*) *35S*::*prosys* plants were infiltrated with pTRV-*MPK1/2* or -*GFP* (Control). Four weeks later, leaves were wounded, and JA levels were measured in unwounded and wounded leaves 1 hr later. The bars represent the mean ± SD in 10 plants from three independent experiments. (*B*) *35S*::*prosys* plants were infiltrated with pTRV-*MPK1/2* or -*GFP* (Control). Four weeks later, plants were exposed to MeJA vapor (open bars) or to the solvent ethanol (filled bars) in a closed environment for 12 hr. Twenty-four hours after the start of the experiment, PI-II protein levels in leaves were measured by RIDA. The bars represent mean ± SD ($n \geq 18$; three independent experiments).

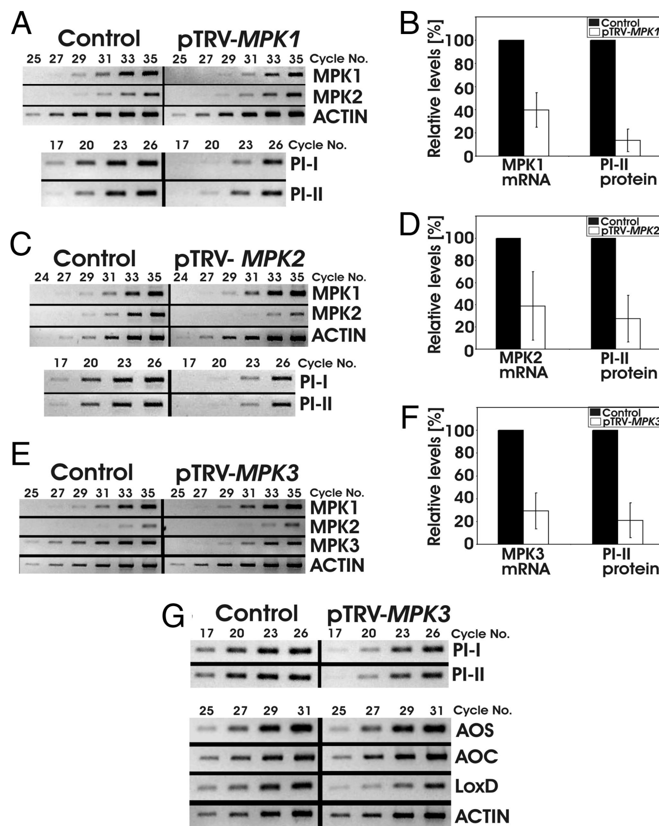

Fig. 4. VIGS of individual MPKs attenuates systemin-induced late gene expression. *35S*::*prosys* plants were infiltrated with pTRV-*MPK1* (*A* and *B*), -*MPK2* (*C* and *D*), -*MPK3* (*E*–*G*), or -*GFP* (Control, *A*–*G*). Four weeks later, *MPK*, *PI-I*, and *PI-II* transcript levels and PI-II protein levels were determined in leaf tissue by sqRT-PCR and RIDAs, respectively. (*A*, *C*, and *E*) *MPK*, *PI*, and *Actin* transcript levels. Ethidium bromide-stained agarose gels of PCR products are shown (colors inverted). (*B*, *D*, and *F*) *MPK* transcript levels and PI-II protein levels. The levels in VIGS plants (mean ± SD) (open bars) were expressed as percentages of the mean levels in control plants (filled bars), which were defined as 100% ($n \geq 5$; ≥ 2 independent experiments; mean PI-II in controls of *B*, *D*, and *F*: 78 ± 15, 61 ± 16, and 75 ± 14 μg/ml leaf juice, respectively). (*G*) Ethidium bromide-stained agarose gels of PCR products corresponding to the early genes *AOS*, *AOC*, and *LoxD*, and to the late genes *PI-I* and *PI-II* in pTRV-*MPK3*- and pTRV-*GFP*-infiltrated control plants ($n = 8$; four independent experiments).

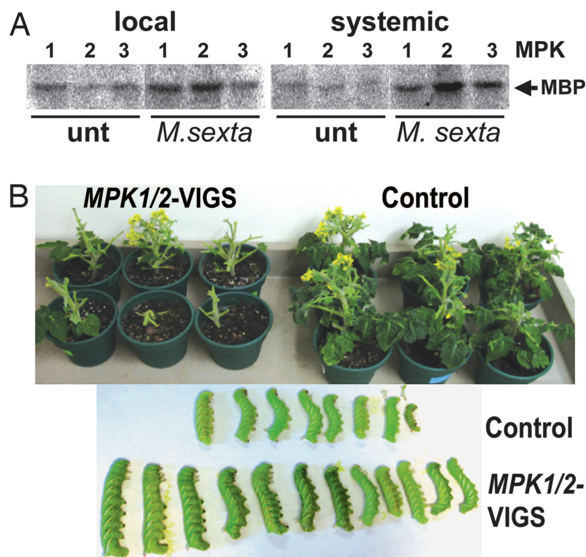

Fig. 5. *M. sexta* herbivory activates MPKs, and cosilencing of *MPK1* and *MPK2* reduces systemin-mediated resistance to *M. sexta* larvae. (*A*) *M. sexta* larvae were allowed to consume one-half of the terminal leaflet of the lower leaf of two-leaf stage WT tomato seedlings. Ten minutes after onset of feeding (*M. sexta*), the wounded (local) and unwounded (systemic) leaf and leaves of unwounded control plants (unt) were assayed for MPK1, MPK2, and MPK3 activity by an immunocomplex kinase assay. Phosphorylated myelin basic protein is shown. The experiment is representative of three similar experiments. (*B*) *35S*::*prosys* plants were infiltrated with pTRV-*MPK1/2* and -*GFP* (Control). Four weeks later, each plant was exposed to one *M. sexta* larva for 11 days. Leaf damage (*Upper*; 12 representative plants are shown) and larval size (*Lower*) were documented photographically. An additional experiment generated similar results (not shown).

infiltrated plants, whereas transcript levels of the early genes *AOS* and *AOC* were not significantly altered (Fig. 4*G*). In addition, silencing of *MPK3* resulted in significant reductions in *LoxD* transcript levels (Fig. 4*G*), unlike silencing of *MPK1* and *MPK2* and cosilencing of *MPK1/2*. Taken together, VIGS of each of the three LeMPKs revealed they all participate in regulating the expression of late wound-response genes.

Cosilencing of *LeMPK1* and *LeMPK2* Reduces Systemin-Mediated Resistance to *M. sexta* Larvae. We have shown earlier that *M. sexta* larvae systemically induce 48-kDa MAPK activity in tomato seedlings (15). Using immunocomplex kinase assays with specific antibodies against MPK1, -2, and -3 (14), we show here that feeding *M. sexta* larvae activated MPK1 and MPK2 in the wounded leaf of two-leaf-stage WT plants. In the unwounded systemic leaf, all three MPKs were activated (Fig. 5*A*). To test whether cosilencing of *MPK1/2* would affect the performance of herbivorous insects, we exposed *35S:prosys* plants to *M. sexta* larvae 4 weeks after infiltration with pTRV-*MPK1/2*. Larvae were placed onto the plants 3–5 days after hatching and allowed to feed for 11 days. Most cosilenced plants were completely defoliated, whereas control plants maintained more than half of their foliage compared with unattacked control plants. The weight of the *M. sexta* larvae on the *MPK1/2*-cosilenced plants was 2.3 ± 0.2-fold higher than the weight of larvae feeding on the control plants (Fig. 5*B*). Silencing of the *MPK1/2*-cosilenced plants was confirmed by sqRT-PCR (data not shown).

Discussion

We investigated the role of tomato MAPKs in the systemin-mediated wound response in tomato plants. Cosilencing of *MPK1* and *MPK2* in WT plants reduced wound-induced 48-kDa MAPK activity and resulted in reduced PI-II synthesis as compared with control plants (data not shown). Wounding not only leads to the release of systemin but also generates additional MAPK-activating signals, such as rapidly propagated mechanical signals (15) or reactive oxygen species and oligosaccharide elicitors released at the site of wounding (3, 20). To specifically investigate the role of MAPKs in the response to systemin, we used transgenic *35S*::*prosys* plants that constitutively produce and accumulate defense proteins without external treatments (32, 33). In these plants, we showed that silencing of *MPK1*, *MPK2*, and *MPK3* attenuated prosystemin-mediated defense protein accumulation, demonstrating that these MPKs are essential components of the systemin-induced signaling pathway. The role that MPK3 plays in the wound response seems to be somewhat different from MPK1 and MPK2. Unlike VIGS of *MPK1/2*, VIGS of *MPK3* lowered the transcript levels of *LoxD* in 35*S*::*prosys* plants, whereas transcripts of the other early genes *AOS* and *AOC* were not affected (Fig. 4*G*). In addition, we found increased MPK3 activity in response to *M. sexta* attack mainly in the systemic leaf, whereas MPK1 and -2 activity increased both in the wounded and the systemic leaf (Fig. 5*A*). Similarly, both different and overlapping roles in the response of *Nicotiana tabacum* and *Nicotiana attenuata* to wounding or *M. sexta* oral secretions were reported for SIPK and WIPK, the tobacco orthologs of tomato MPK1 and MPK3 (38, 39).

JA is an essential signaling component in the systemin and wound-signaling pathways (8). The reduced expression of the strictly JA-dependent late genes in *MPK1*-, *MPK2*-, *MPK1/2*-, and *MPK3*-silenced 35*S*::*prosys* plants (Figs. 1, 2, and 4) indicates these MAPKs could function either upstream or downstream of systemin-induced JA biosynthesis. Restoration of late gene expression by application of MeJA to *MPK1/2*-cosilenced unwounded *35S*::*prosys* plants and reduced wound-induced JA synthesis in *MPK1/2*-cosilenced 35*S*::*prosys* plants demonstrate that these MAPKs function upstream of JA biosynthesis. Consequently, MPK1 and MPK2 (and possibly MPK3) represent a link between the cytosolic part of the systemin signaling pathway and JA synthesis, which is initiated in the chloroplasts. Substrates of the tomato MPKs are not known so far. Because transcript levels of JA-biosynthetic enzymes were not altered in *MPK1/2*-silenced plants, it is conceivable that the MPK substrate(s) regulate the activity of the JA-biosynthetic enzymes directly or indirectly, perhaps via substrate availability.

JA is known to interact with ethylene to activate wound-response genes (18). Recently, it was demonstrated that the LeMPK1/2 ortholog in *Arabidopsis*, AtMPK6, regulates ethylene synthesis through phosphorylation and activation of the cytosolic enzyme 1-aminocyclopropane-1-carboxylic acid synthase (ACS2/6) *in vivo* (27). Ethylene is known to be generated in response to systemin (17, 18), and it is possible that ACS is also the physiological substrate of MPK1/2 in the systemin signaling pathway. Consistent with this scenario is our observation that ethylene synthesis in wounded leaves of *MPK1/2*-silenced *35S*::*prosys* plants is reduced by ≈30% (see SI Fig. 7). This is comparable to the reduction of ethylene synthesis in flagellin-treated *Arabidopsis mpk6* null mutants (27), wounded *SIPK*-silenced tobacco (38), and *N. attenuata* treated with *M. sexta* oral secretions (39). These data do not exclude the possibility that MPK1/2 might recruit additional cytosolic or nuclear substrates.

MPK1 and MPK2 belong to the A2 subgroup of plant MAPKs and are 95% identical at the amino acid level (14). Both are activated by the same upstream MAPKKs, LeMKK2 and LeMKK4 (28). In addition, they are coordinately activated by wounding, systemin, oligosaccharide elicitors, UV-B radiation, and the fungal toxin fusicoccin (12, 14). These data suggested that the two MPKs are functionally redundant. Therefore, we did not expect to find a pronounced effect in plants silenced for only one of the two paralogs. In contrast, silencing of either *MPK1* or

MPK2 resulted in a strong reduction of *PI-I* and *PI-II* transcript accumulation and PI-II protein synthesis (Fig. 4), demonstrating that the presence of one of the two MPKs is not sufficient to induce a strong wound response. The tobacco genome also contains two highly homologous MAPKs of the A2 subgroup, *SIPK* and *Ntf4*. Recently, it was shown that their functions are largely redundant (40). The authors also concluded that some of the published loss-of-function studies that targeted *SIPK* (41, 42) most likely also silenced *Ntf4*, and that systematic loss-of-function studies of each of the two MAPK paralogs are lacking. They further suggested that the presence of two highly homologous MAPKs in certain solanaceous plants may be adaptive, either because the two MAPKs do have some different yet-unknown functions or because of a gene-dosage effect (40). Our results are consistent with the latter scenario. We speculate that the sum of MPK1 and MPK2 molecules per cell has to exceed a critical copy number to effectively signal gene expression, and that VIGS of only one of the two *MPK*s lowered this MPK1/2 copy number below a critical threshold. An investigation of the involvement of *SIPK* in the tobacco response to ozone showed that silencing of *SIPK* by RNAi did not affect *Ntf4* transcript levels. Similar to our results, the *SIPK*-RNAi plants suffered more ozone-induced oxidative damage, indicating that *Ntf4* alone is not sufficient to confer ozone resistance (43). *MPK3*-silencing also attenuated late gene expression. But the presence of *MPK3* in *MPK1/2*-silenced plants did not prevent the reduction of defense protein accumulation in these plants (Fig. 1*B*). This indicates that MPK3 has a different mechanism of action than MPK1 and MPK2, e.g., activation of a different substrate. In tobacco, regulation of *WIPK* gene expression or activity by SIPK had been discussed (43, 44). We did not find evidence for such a scenario in untreated *35S*::*prosys* plants. However, it cannot be excluded that *MPK3* levels are altered in a *MPK1/2*-dependent manner in response to treatments.

LeMPK1/2/3 had been shown to function in host-specific AvrPto-dependent resistance to the bacterial pathogen *P. syringae* (28, 45) and in Mi-1-mediated resistance to aphids (29). Our reduction-of-function study demonstrates that LeMPK1 and LeMPK2 are required for systemin-mediated resistance to *M. sexta* larvae. Antisense expression of prosystemin in transgenic tomato plants resulted in reduced defense protein accumulation and thus loss of resistance to *M. sexta* (6), whereas *35S*::*prosys* plants exhibited high constitutive levels of defense proteins and increased resistance to *M. sexta* (1) and other insects (46). MPK1 and MPK2 play an essential role for systemin-induced defense protein synthesis, and the presence of MPK3 cannot compensate for the loss of MPK1 and MPK2 (Fig. 4). Consistent with these data, VIGS of *MPK1/2* reduced systemin-mediated resistance to *M. sexta* herbivory (Fig. 5*B*). *M. sexta* larvae systemically induced MPK1, MPK2, and MPK3 activity in young tomato seedlings (Fig. 5*A*). Chewing insects generate other MAPK-activating signals in addition to systemin, such as mechanical signals (15) or fatty acid- amino acid conjugates (39). The corresponding signaling pathways all converge on MPK1, MPK2, and MPK3, which can explain why *MPK1/2*-silencing prevented a successful defense response against the attacking insect larvae. Overexpression of *MPK1/2* orthologs or expression of active forms of their upstream MAPKK(K)s did not lead to JA synthesis (47) and had been shown to mimic pathogen-induced responses or confer resistance to microbial pathogens (43, 48–50). But no experiments were aimed at testing the performance of chewing insects on such plants. Because defense responses to pathogens and insects are often mutually exclusive, it is possible this crosstalk prevents MAPK gain-of-function plants from mounting a wound response. However, our reduction-of-function approach using TRV-VIGS revealed that MPK1, MPK2, and MPK3 not only function in pathogen-induced defenses but also are essential signaling components in the wound response that confers resistance to herbivorous insects. It remains to be determined how MAPKs that can be activated by multiple functionally diverse stress signals can signal stress-specific and mutually exclusive defense responses such as wounding- or pathogen-induced responses.

Materials and Methods

Plant Material and Growth Conditions. Tomato plants (*L. esculentum*; alternative nomenclature *Solanum lycopersicon*) of the MicroTom or Castlemart variety were grown in AR66L growth chambers (Percival Scientific, Perry, IA) at 20°C under a 16-hr light [130 ± 20 μE m^{-2}·s^{-1} (E = 1 mol of photons)] and 8-hr dark regime for optimal VIGS conditions. To increase PI protein synthesis, growth temperature was raised to 27°C 5 days before analysis. The transgene in *35S*::*prosys* MicroTom plants had been backcrossed five times from Castlemart (34).

VIGS Constructs. The TRV-based VIGS vectors pTRV1 and pTRV2 (30) were obtained from S. P. Dinesh-Kumar (Yale University, New Haven, CT). For details, see *SI Text*.

Infiltration of pTRV-Containing *Agrobacterium tumefaciens* Cultures into Cotyledons. Eleven- to 12-day-old seedlings (true leaves just emerging) were infiltrated by vacuum infiltration with a mix of pTRV1- and pTRV2-carrying *Agrobacteria* according to Ekengren *et al.* (45). For syringe infiltrations, the same conditions were applied, but the cotyledons of young seedlings were infiltrated with *Agrobacteria* by using a 1-ml syringe.

sqRT-PCR. See *SI Text* for details on sqRT-PCR.

MAPK Activity Assays. Extracts from frozen leaf material were obtained and immunocomplex or in-gel kinase assays with myelin basic protein as an artificial MAPK substrate were carried out as described (14, 15). To measure MPK3 activity, magnesium in the kinase reaction buffer was substituted by manganese (26).

Radial Immunodiffusion Assay (RIDA). PI-II protein levels in expressed leaf juice were measured as described (51). Anti-PI-II goat antiserum was generated by Spring Valley Laboratories (Woodbine, MD).

JA Analysis. The upper (younger) four to five leaves of 4-week-old plants were left untreated or wounded by using a hemostat, and plants were incubated under standard conditions at 27°C. One hour after wounding, one-half of each wounded leaf or unwounded control leaf was excised, weighed, and frozen in liquid nitrogen for JA analysis. Typically, 300–500 mg of leaf material was collected from each plant. Samples were stored at −70°C. Twenty-four hours after wounding, PI-II protein levels in wounded and control plants were analyzed on the remaining portion of leaves by RIDA. The remaining leaf material was also used to determine *MPK1/2* transcript levels by sqRT-PCR 3–4 days after collection of JA samples.

JA analysis using dihydro-JA as an internal standard was performed according to Schmelz *et al.* (52). For details, see *SI Text*.

MeJA Treatment. pTRV-*MPK1/2*-infiltrated plants were exposed to MeJA (Sigma–Aldrich, St. Louis, MO) vapors (7 μl solved in 100 μl of ethanol and applied to cotton wicks) in 2-gallon sealed plastic bags for 12 hr under standard conditions at 27°C. Control plants were exposed to ethanol only. After an additional 12 hr, PI-II protein levels were analyzed by RIDA.

Tissue Sampling for sqRT-PCR, Kinase Assays, and RIDA. Leaf samples for RNA analysis or in kinase assays were collected 4–6 weeks after infiltration. The development and extent of gene silencing

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were assessed by monitoring the photo-bleaching pattern of *PHYTOENE DESATURASE*-silenced plants according to Liu *et al.* (30). From each plant, 20–25 leaf discs from four or five young upper leaves were collected by using a hole puncher. Control (0-min) samples were collected and frozen immediately, whereas another 20–25 leaf discs were punched out in parallel from the same leaves and wounded by using serrated forceps. Wounded leaf discs were floated on MS medium (MS salts, 3% wt/vol sucrose, pH 5.8) for 10–15 min, quickly blotted dry on paper tissue, and frozen in liquid nitrogen for subsequent analysis by kinase assays. One-half of the control (0 min) samples was used for sqRT-PCR. The other half was used for immunocomplex- or in-gel kinase assays. Remaining tissue from these leaves was used for PI-II protein analysis by RIDA.

Herbivory Treatments. *M. sexta* larvae (Carolina Biological Supply, Burlington, NC) were hatched and kept on an artificial diet for 3–5 days, after which they were placed on pTRV-*MPK1/2*- or pTRV-*GFP*-infiltrated plants. The average larval weight at this time was 3.8 ± 0.2 mg for experiment 1 and 5.6 ± 0.9 mg for experiment 2. One larva per plant was placed on the uppermost expanded leaf. Plants were incubated under standard conditions at 27°C. After 11 days, the entire foliage of almost all pTRV-*MPK1/2*-infiltrated plants was consumed and the experiment was stopped. Larval weight was determined, and the damaged plants were documented photographically. Herbivory-induced MPK activity was measured in 13- to 14-day-old *L. esculentum* var. Castlemart seedlings that displayed two unfolded leaves. *M. sexta* larvae (4th or 5th instar) were starved for 3 hr and then allowed to consume half of the terminal leaflet of the lower leaf. To avoid tissue damage by larval feet, larvae were manually placed close to the leaf edges, allowing them to access the leaf only with mandibles. After 3–5 min, the larvae were removed, and the wounded and unwounded leaves were frozen 10 min after onset of insect feeding for analysis by immunocomplex kinase assays.

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