

# The MAPK Kinase Kinase GmMEKK1 Regulates Cell Death and Defense Responses<sup>1</sup>[OPEN]

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MAPK signaling pathways play critical roles in plant immunity. Here, we silenced multiple genes encoding MAPKs using virus-induced gene silencing mediated by *Bean pod mottle virus* to identify MAPK genes involved in soybean (*Glycine max*) immunity. Surprisingly, a strong hypersensitive response (HR) cell death was observed when soybean *MAPK KINASE KINASE1* (*GmMEKK1*), a homolog of Arabidopsis (*Arabidopsis thaliana*) *MEKK1*, was silenced. The HR was accompanied by the overaccumulation of defense signaling molecules, salicylic acid (SA) and hydrogen peroxide. Genes involved in primary metabolism, translation/transcription, photosynthesis, and growth/development were down-regulated in *GmMEKK1*-silenced plants, while the expression of defense-related genes was activated. Accordingly, *GmMEKK1*-silenced plants were more resistant to downy mildew (*Peronospora manshurica*) and *Soybean mosaic virus* compared with control plants. Silencing *GmMEKK1* reduced the activation of GmMPK6 but enhanced the activation of GmMPK3 in response to flg22 peptide. Unlike Arabidopsis MPK4, GmMPK4 was not activated by either flg22 or SA. Interestingly, transient overexpression of *GmMEKK1* in *Nicotiana benthamiana* also induced HR. Our results indicate that *GmMEKK1* plays both positive and negative roles in immunity and appears to differentially activate downstream MPKs by promoting GmMPK6 activation but suppressing GmMPK3 activation in response to flg22. The involvement of GmMPK4 kinase activity in cell death and in flg22- or SA-triggered defense responses in soybean requires further investigation.

MAPK cascades are evolutionarily conserved signaling hubs that regulate innate immune responses in both plants and animals (Meng and Zhang, 2013). The recognition of nonself by pattern recognition receptors on the cell surface activates a MAPK phosphorylation relay. Initial activation of a MAP kinase kinase kinase (MAP3K/MEKK) leads to the phosphorylation and activation of a downstream MAP kinase kinase (MAP2K/MKK), which subsequently phosphorylates a downstream MAPK. The activated MAPK then phosphorylates its target proteins, such as WRKY transcription factors, and ultimately reprograms gene expression (Pitzschke et al., 2009b; Rasmussen et al., 2012; Meng and Zhang, 2013; Smeets et al., 2013; Pitzschke, 2015).

The genome of Arabidopsis (*Arabidopsis thaliana*) encodes 60 MAP3Ks, 10 MAP2Ks, and 20 MAPKs (Ichimura et al., 2002), indicating that MAPK cascades are not simple linear MAP3K-MAP2K-MAPK modules. Instead, they form tightly regulated signaling networks that coordinate intracellular responses to external and

internal stimuli. Arabidopsis MEKK1, the most extensively studied MAP3K in plants, has been implicated in biotic and abiotic stress responses, cytokinesis, and growth/development (Pitzschke et al., 2009b; Rasmussen et al., 2012; Xu and Chua, 2012; Meng and Zhang, 2013; Xu and Zhang, 2015). Using a truncated version of MEKK1 in protoplasts, a complete MAP kinase signaling module (MEKK1-MKK4/MKK5-MPK3/MPK6-WRKY22/WRKY29) was identified that acts downstream of flagellin receptor, the Flagellin Sensing Locus2 (Asai et al., 2002). Activation of this MAPK cascade confers resistance to both bacterial and fungal pathogens (Asai et al., 2002). However, genetic evidence indicates that, in planta, MEKK1 is dispensable for MPK3/MPK6 activation (Nakagami et al., 2006; Suarez-Rodriguez et al., 2007; Gao et al., 2008; Qiu et al., 2008).

MEKK1 also negatively regulates defense. The *mekk1*, *mkk1/mkk2*, and *mpk4* mutants share similar phenotypes of constitutive defense responses, including dwarfed stature, increased accumulation of salicylic

acid (SA) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), induction of pathogenesis-related (PR) genes, and enhanced pathogen resistance (Petersen et al., 2000; Ichimura et al., 2006; Mészáros et al., 2006; Nakagami et al., 2006; Suarez-Rodriguez et al., 2007; Gao et al., 2008; Qiu et al., 2008; Pitzschke et al., 2009a). Some phenotypes of these mutants can be rescued by the *sid2* mutation or the expression of NahG, both of which cause SA deficiencies, indicating that the activation of defenses is partially SA dependent (Petersen et al., 2000; Suarez-Rodriguez et al., 2007; Gao et al., 2008). Constitutive defense responses and loss of flg22-induced MPK4 activation are seen only in *mkk1/mkk2* double mutants but not in either single mutant, indicating that MKK1 and MKK2 have redundant functions in MPK4 signaling (Gao et al., 2008; Qiu et al., 2008). MKK1/MKK2 interact with both MEKK1 and MPK4 in vivo (Ichimura et al., 1998; Mizoguchi et al., 1998; Gao et al., 2008), and MEKK1 phosphorylates MKK1/MKK2 (Teige et al., 2004; Hadiarto et al., 2006). The activation of MPK4 by flg22 is abolished in *mekk1* and *mkk1/mkk2* mutants (Nakagami et al., 2006; Suarez-Rodriguez et al., 2007; Gao et al., 2008; Qiu et al., 2008). These data establish that MEKK1, MKK1/MKK2, and MPK4 form a linear signaling module to negatively regulate SA-dependent immune responses in Arabidopsis (Suarez-Rodriguez et al., 2007; Gao et al., 2008; Qiu et al., 2008; Pitzschke et al., 2009a).

Interestingly, MPK4 interacts directly with the N-terminal regulatory domain of MEKK1, suggesting that MEKK1 may serve as a scaffold, binding a MAP2K and a MAPK (Ichimura et al., 1998; Nakagami et al., 2006). In support of this idea, the kinase activity of MEKK1 may not be required for flg22-induced MPK4 activation, because a kinase-dead version of MEKK1 (K361M) can largely rescue the activation of MPK4 and the dwarf phenotype of the *mekk1* mutant.

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J.-Z.L. conceived the original research plans and designed the experiments; J.-Z.L., S.A.W., D.R., P.A.N., and J.X. supervised the experiments; J.-Z.L., H.-Y.X., C.Z., Z.-C.L., Z.-R.W., X.-X.J., Y.-F.S., S.-N.T., E.B., Y.M., W.-L.Q., and N.C. performed the experiments; J.X., S.L., and B.W. provided critical technical assistance; J.-Z.L., X.J., M.A.G., and S.A.W. analyzed the data; J.-Z.L., M.A.G., and S.A.W. wrote the article with contributions from all authors.

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These observations suggest that MEKK1 may play a structural role independent of its protein kinase activity (Suarez-Rodriguez et al., 2007). In addition, *avrPto* and *avrPtoB* suppress defense signaling upstream of *AtMEKK1* (He et al., 2006), and the MEKK1-MKK1/MKK2-MPK4 signaling module was proposed to serve as a guarder in effector-triggered immunity (Zhang et al., 2012; Zhou et al., 2014).

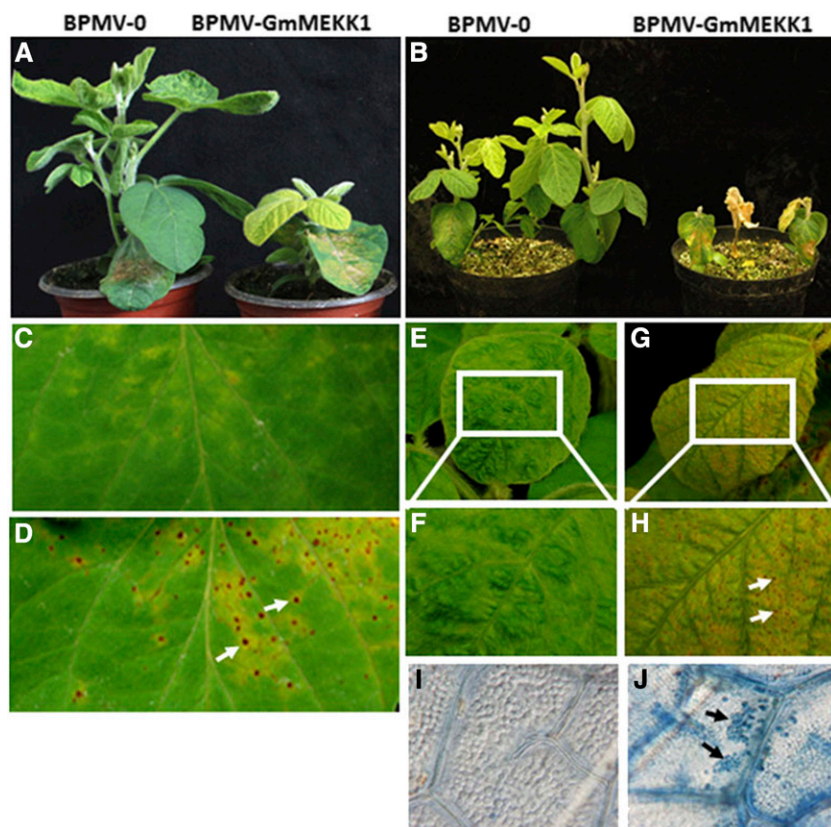
The MEKK1-MKK1/MKK2-MPK4-MKS1-WRKY33-PAD3 module regulates the accumulation of camalexin, a phytoalexin induced in response to bacterial and fungal pathogens in Arabidopsis (Andreasson et al., 2005; Qiu et al., 2008; Ren et al., 2008; Mao et al., 2011). Interestingly, MEKK1 can bypass the MKK1/MKK2-MPK4 module and directly regulate downstream targets (Miao et al., 2007). Phosphorylation of WRKY53 by MEKK1 increases its DNA-binding activity in vitro and increases the transcription of a reporter gene driven by the WRKY53 promoter in vivo. Thus, MEKK1 can bypass MAPK signaling by directly phosphorylating a transcription factor (Miao et al., 2007).

In soybean (*Glycine max*), virus-induced gene silencing (VIGS) mediated by *Bean pod mottle virus* (BPMV) has been used successfully to study MAPK gene function (Liu et al., 2015, 2016). These studies demonstrate that, while soybean *GmMPK4* plays a negative role, *GmMPK6* plays both positive and negative roles in defense responses (Liu et al., 2011, 2014). In this study, we determined to identify the MAP3Ks and MAP2Ks that function upstream of *GmMPK4* or *GmMPK6*. We found that silencing *GmMEKK1*, an Arabidopsis *MEKK1* homolog, resulted in similar but more severe constitutive defense phenotypes than *GmMPK4*-silenced plants. These phenotypes included dwarfed stature, induction of PR genes, increased accumulation of SA and H<sub>2</sub>O<sub>2</sub>, and enhanced pathogen resistance. Reverse transcription quantitative PCR (RT-qPCR) and RNA sequencing (RNA-seq) analyses of *GmMEKK1*-silenced plants confirmed that the constitutively activated defense responses occur at the expense of plant growth and development (Liu et al., 2011). The activated defense in *GmMEKK1*-silenced plants was associated with a gain of resistance to *Soybean mosaic virus* (SMV) and downy mildew (*Peronospora manshurica*). In addition, we provide evidence that *GmMEKK1* plays a positive role in *GmMPK6* activation but a negative role in *GmMPK3* activation, and the correlation between the inactivation of *GmMPK4* and induced cell death is not observed in soybean. Our data suggest that there are similarities but also important differences in MAPK signaling modules of soybean and Arabidopsis.

## RESULTS

### Silencing *GmMEKK1* Results in Both Local and Systemic Hypersensitive Response

Previously, we demonstrated that, while *GmMPK4* negatively regulates defense responses, *GmMPK6* plays



**Figure 1.** Silencing *GmMEKK1* causes both local and systemic hypersensitive response (HR) cell death in soybean. A and B, Control (BPMV-0) and *GmMEKK1*-silenced (BPMV-*GmMEKK1*) plants at 15 d post infection (dpi; A) and 25 dpi (B). C and D, HR response on the bombarded local leaves of BPMV-0 (C) and BPMV-*GmMEKK1* (D); white arrows indicate examples of necrotic lesions. E and F, HR response on systemic leaves of BPMV-0 plants. G and H, HR observed on systemic leaves of BPMV-*GmMEKK1* plants. The images shown in F and H were enlarged from the boxed areas in E and G, respectively. I and J, Trypan Blue-stained systemic leaves of BPMV-0 control (I) and *GmMEKK1*-silenced (J) plants. Black arrows indicate the blued-stained, dead cells.

both positive and negative roles in regulating defense responses (Liu et al., 2011, 2014). To identify the MAP3Ks or MAP2Ks that function upstream of *GmMPK4* or *GmMPK6*, we silenced 20 *GmMP3Ks* and 12 *GmMP2Ks* using BPMV-VIGS. When a soybean MAP3K closely related to Arabidopsis *MEKK1* was silenced [Glyma.04G253500 (Wm82.a2.v1), hereafter *GmMEKK1a*], severe stunting was observed relative to the vector control plants (Fig. 1, A and B). While no HR cell death was observed in control plants (Fig. 1C), inoculated leaves of the *GmMEKK1*-silenced plants displayed a clear HR (Fig. 1D). As silencing progressed, numerous micro-HR lesions appeared on the upper systemic leaves of *GmMEKK1*-silenced plants (Fig. 1, G and H) relative to controls (Fig. 1, E and F). The HR was further confirmed by Trypan Blue staining (Fig. 1, I and J). These results suggest that, like its counterpart in Arabidopsis, *GmMEKK1* plays a negative role in regulating cell death and defense responses (Suarez-Rodriguez et al., 2007; Gao et al., 2008).

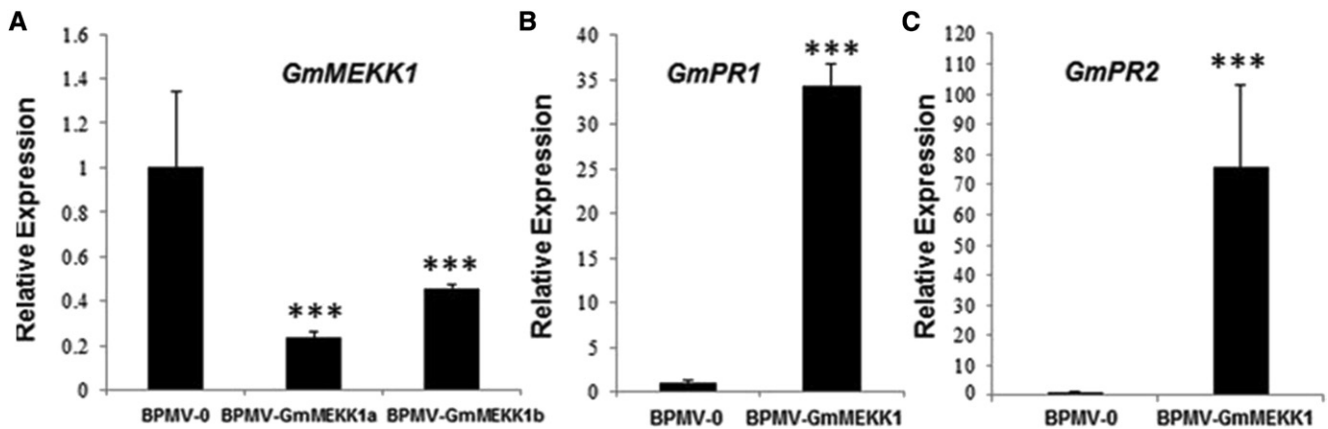
Soybean is a paleopolyploid, and nearly 75% of its genes are present in multiple copies (Schmutz et al., 2010). We have shown previously that *GmMPK4* and *GmMPK6* each have four homologs that can be divided into two paralogous groups. The amino acid identities within the groups are greater than 96%, and for *GmMPK4* we showed that it was necessary to silence all four copies to observe the loss-of-function phenotype (Liu et al., 2011). *GmMEKK1* also is duplicated, with *GmMEKK1a* sharing 94% nucleotide identity with

*GmMEKK1b* [Glyma.06G108900 (Wm82.a2.v1)]. The sequence fragment from *GmMEKK1a* used for silencing shares 95% nucleotide identity with *GmMEKK1b* (Supplemental Fig. S1). Because there is sufficient sequence divergence in the 5' untranslated regions, the expression of *GmMEKK1a* and *GmMEKK1b* could be determined by RT-qPCR using gene-specific primers. These analyses verified that *GmMEKK1a* was silenced, because its transcript level was decreased by 76% in BPMV-*GmMEKK1* plants relative to nonsilenced BPMV-0 controls (Fig. 2A). The transcript level of *GmMEKK1b* also was reduced significantly in the BPMV-*GmMEKK1* plants, indicating that the constitutive HR phenotypes resulted from simultaneously silencing *GmMEKK1a* and *GmMEKK1b*.

HR usually is associated with increased expression of defense marker genes. To test whether the expression of defense-related genes was activated in the *GmMEKK1*-silenced plants, the expression of soybean *GmPR1* (Glyma.13G251600.1) and *GmPR2* (Glyma.03G132700) was examined using RT-qPCR. Consistent with a constitutive defense response phenotype, the levels of both *GmPR1* and *GmPR2* mRNA transcripts were significantly higher in *GmMEKK1*-silenced plants compared with BPMV-0 control plants (Fig. 2, B and C).

#### SA and H<sub>2</sub>O<sub>2</sub> Levels Increase in *GmMEKK1*-Silenced Plants

SA is a key hormone that positively regulates defense responses against biotrophic pathogens, and it



**Figure 2.** The transcript levels of *GmMEKK1s* are reduced and *PR* genes are induced in *GmMEKK1*-silenced plants. A, RT-qPCR analysis of *GmMEKK1a* and *GmMEKK1b* transcript levels in *GmMEKK1*-silenced plants compared with BPMV-0 empty vector control plants. B and C, Transcript levels of *PR1* and *PR2* in *GmMEKK1*-silenced plants compared with the BPMV-0 empty vector control plants. The  $\beta$ -actin gene (Glyma.15G050200) was used as the endogenous reference gene. Error bars represent SD of three replications. Asterisks indicate significant differences from the control (\*\*\*,  $P < 0.001$ , Student's  $t$  test). The experiment was repeated three times with similar results.

overaccumulates in *Arabidopsis mpk4*, *mkk1/mkk2*, and *mekk1* mutants and *GmMPK4*-silenced soybean plants (Petersen et al., 2000; Suarez-Rodriguez et al., 2007; Gao et al., 2008; Qiu et al., 2008; Liu et al., 2011). To test whether SA overaccumulation occurred in *GmMEKK1*-silenced plants, both free SA and bound SA were quantified, and the levels increased 5.3- and 1.95-fold, respectively, in *GmMEKK1*-silenced plants relative to BPMV-0 control plants (Fig. 3, A and B). Increased SA suggested that SA could be involved in the constitutively activated defense responses.

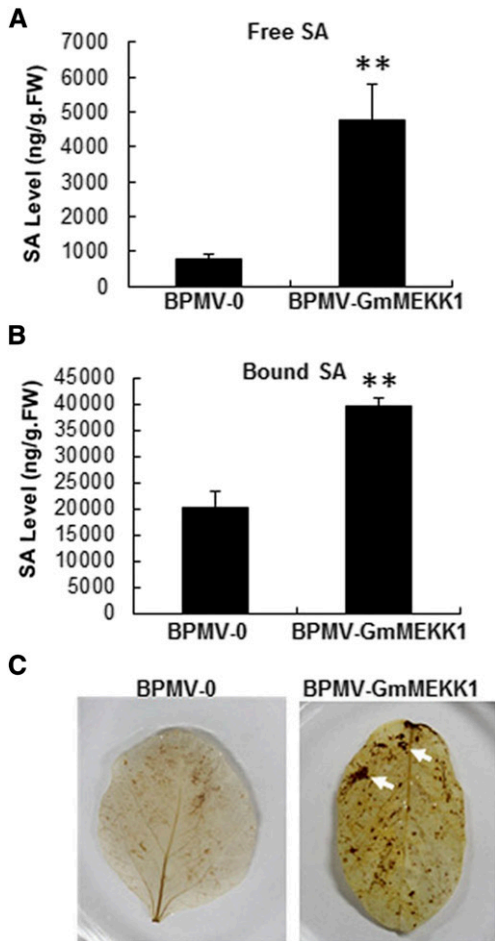
$H_2O_2$  is a potent trigger of cell death (Lamb and Dixon, 1997; Delledonne et al., 1998). To determine whether  $H_2O_2$  overaccumulation occurred in *GmMEKK1*-silenced plants, soybean leaves were stained with 3,3-diaminobenzidine (DAB; Thordal-Christensen et al., 1997; Ren et al., 2002), which forms a brown precipitate when oxidized by  $H_2O_2$ . Brown spots developed in the leaves of *GmMEKK1*-silenced plants that were more intensely colored compared with BPMV-0 control plants (Fig. 3C), indicating that the cell death observed in *GmMEKK1*-silenced plants was associated with elevated  $H_2O_2$  accumulation.

#### Functional Classification of Differentially Expressed Genes between *GmMEKK1*-Silenced and BPMV-0 Vector Control Plants

To identify genes differentially expressed between *GmMEKK1*-silenced and BPMV-0 control samples, RNA-seq analysis was performed (Moran Lauter et al., 2014). We identified 4,007 induced and 4,358 repressed genes (fold change  $\geq 2$  and false discovery rate [FDR]  $\leq 0.001$ ) in *GmMEKK1*-silenced plants relative to BPMV-0 plants (Supplemental Data Sets S1 and S2).

To identify overarching biological processes affected by *GmMEKK1* silencing, we used the SoyBase GO (Gene

Ontology) Term Enrichment Tool ([http://www.soybase.org/goslimgraphic\\_v2/dashboard.php](http://www.soybase.org/goslimgraphic_v2/dashboard.php)) to identify GO biological process terms significantly overrepresented among differentially expressed genes relative to all predicted genes in the soybean genome. In total, 71 and 98 GO terms were significantly overrepresented ( $P < 0.05$ ) among genes induced and repressed, respectively, by *GmMEKK1* silencing (Supplemental Data Sets S3 and S4). To visualize these data, we graphed the 62 significant GO terms with genome counts of 20 or more and in which more than 30% of genes were differentially expressed in our analysis (Fig. 4). Of these GO terms, 10 were associated with genes induced by *GmMEKK1* silencing and included defense- and stress-related responses such as programmed cell death, innate immunity, cell communication, respiratory burst, and phenylpropanoid and camalexin biosynthesis. In contrast, the remaining 52 GO terms representing genes repressed by *GmMEKK1* silencing were associated with photosynthesis, growth, general metabolism, and regulation. Photosynthesis-related GO terms included thylakoid membrane organization, PSII assembly, light reaction, and response to light. Growth responses included multidimensional growth, pattern specification process, and cell differentiation. Metabolism-related GO terms included Cys, starch, chlorophyll, and fatty acid biosynthesis, catabolism of acetyl-CoA, very-long-chain fatty acids, and polysaccharides, and processes related to the pentose-phosphate shunt. Regulation and homeostasis-related GO terms included regulation of translation, protein transport, catalytic activity, and protein phosphorylation, transcription, and mRNA modifications. The down-regulation of genes in growth/development was consistent with the compromised growth and development phenotypes of *GmMEKK1*-silenced plants (Fig. 1, A and B).



**Figure 3.** Elevated accumulation of SA and  $H_2O_2$  in *GmMEKK1*-silenced soybean plants. A and B, Free SA (A) and bound SA (SA-O-glycoside; B) levels were quantified in *GmMEKK1*-silenced and BPMV-0 empty vector control plants at 20 d post BPMV inoculation. Error bars represent SD for three independent samples. Asterisks indicate significant differences from the control (\*\*,  $P < 0.01$ , Student's  $t$  test). FW, Fresh weight. C, Presence of  $H_2O_2$  in soybean leaves visualized by staining with DAB. Oxidized DAB formed a reddish-brown deposit (examples of these deposits are indicated by the white arrows).

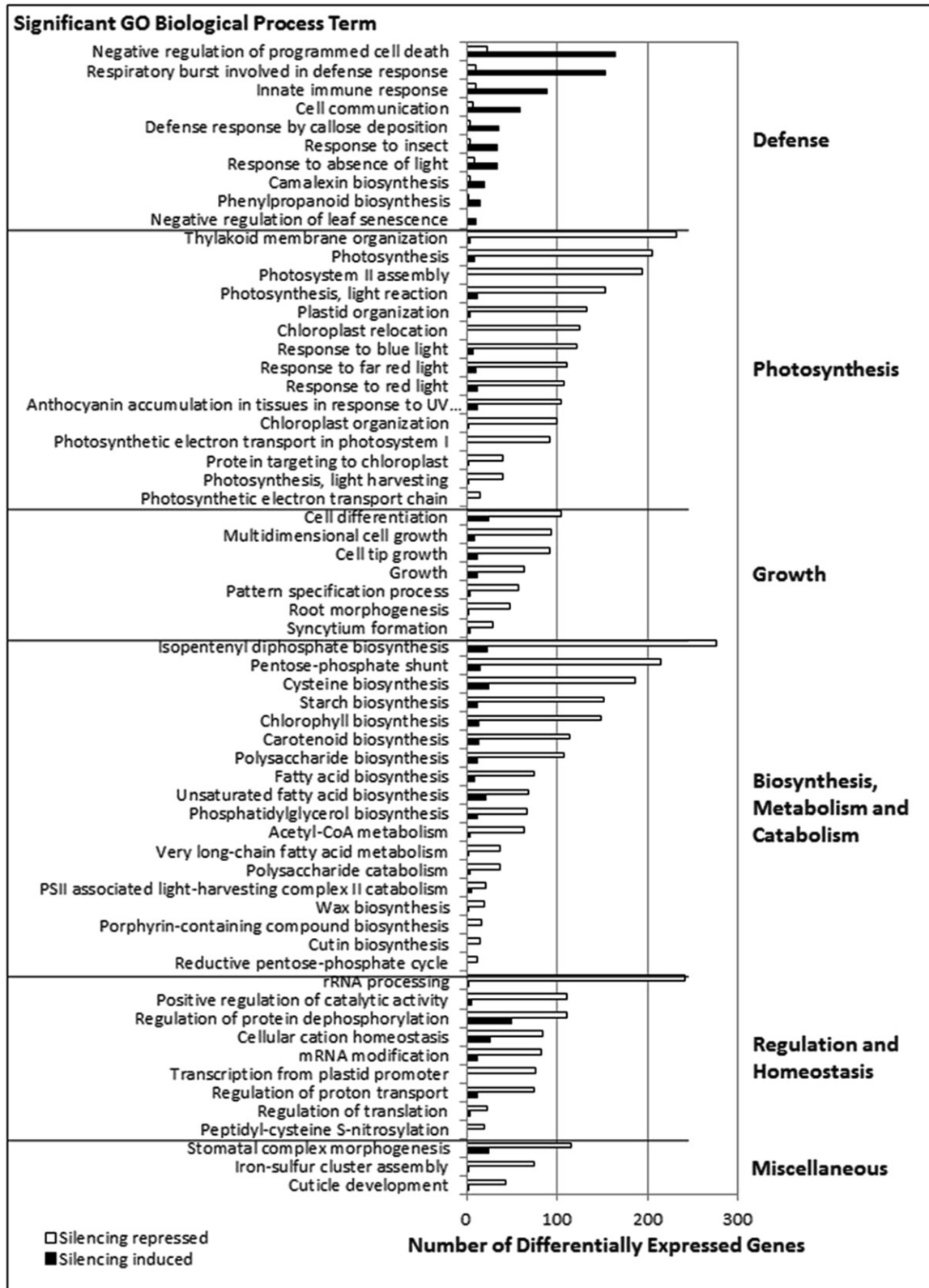
To understand how these complex gene expression networks are regulated downstream of *GmMEKK1*, we took advantage of the SoyDB transcription factor database (Wang et al., 2010) to find transcription factors responding to *GmMEKK1* silencing. Of the 4,946 transcription factors present in the soybean genome (version Wm82.a2.v1), 728 transcription factors, representing 46 families, were differentially expressed in response to *GmMEKK1* silencing (Fig. 5; Supplemental Data Set S5). The 389 repressed transcription factors represented 35 families, while the 339 induced transcription factors represented 37 families. Transcription factor families ARID, C2C2(ZN)YABBY, CSD, HMG, SRS, SSB, WHIRLY, ZF-HD, and ZIM were unique to repressed transcription factors. Transcription factor

families CAMTA, CCAAT, HSF, HTH-FIS, JUMONJI, MADS, NIN-LIKE, PLATZ, SBP, SNF2, TUB, and ZF-A20 were unique to induced transcription factor families. To identify transcription factor families significantly overrepresented among all transcription factors relative to their genome representation, we used Fisher's exact test (Fisher, 1966) and Bonferroni correction (Bonferroni, 1935). Three significantly (corrected  $P < 0.01$ ) overrepresented transcription factor families were identified: WRKY, AUX-IAA-ARF, and ZIM (Fig. 5; Supplemental Data Set S6).

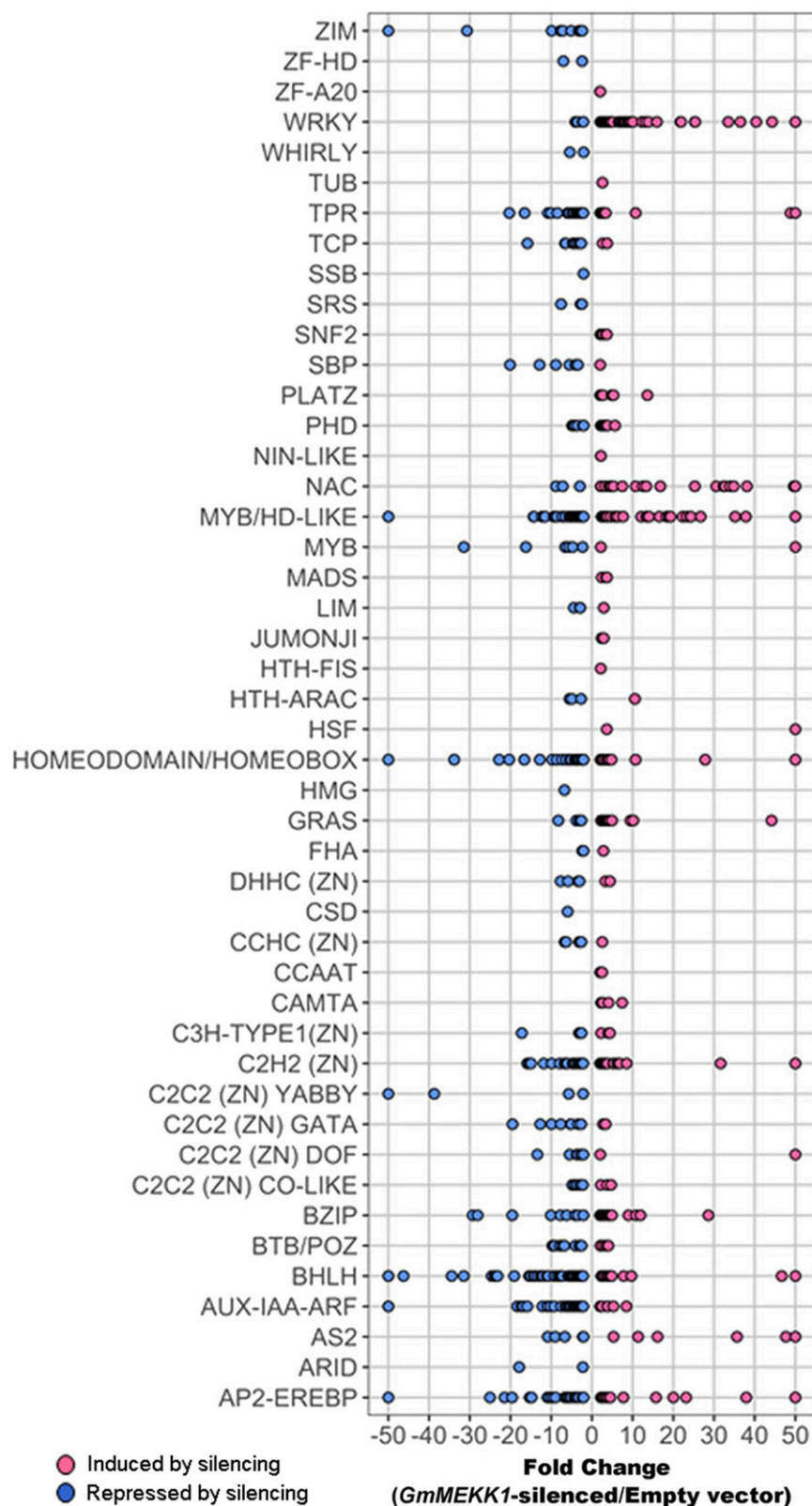
WRKY transcription factors are important regulators of plant defense responses (Cormack et al., 2002; Eulgem and Somssich, 2007; Rushton et al., 2010). We previously showed that, in *GmMPK4*-silenced plants, 45 and three *GmWRKY* genes were induced and repressed, respectively (Liu et al., 2011). Similarly, 64 *GmWRKY* genes were induced and five *GmWRKY* genes were repressed in *GmMEKK1*-silenced plants (Fig. 5; Supplemental Data Sets S5 and S6). Among the 64 induced *GmWRKYs* in *GmMEKK1*-silenced plants, 29 were in common with *GmMPK4*-silenced plants (Liu et al., 2011). Auxin is a major hormone that regulates growth and development. Interestingly, AUX-IAA-ARF transcription factors were repressed similarly in both *GmMPK4* and *GmMEKK1*-silenced plants (Fig. 5; Supplemental Data Sets S5 and S6). Our results suggest that both WRKY and AUX-IAA-ARF transcription factors are coregulated in *GmMPK4*- and *GmMEKK1*-silenced plants. Furthermore, they support the conclusion that both  $H_2O_2$  and SA have inhibitory effects on auxin-responsive gene expression (Nakagami et al., 2006; Wang et al., 2007; Cheng et al., 2011).

### Silencing *GmMEKK1* Enhances Resistance against SMV and Downy Mildew

Enhanced disease resistance often is seen in plants in which defense responses are constitutively activated (Liu et al., 2011, 2014), and it has been reported previously that silencing *NPK1*, which encodes a MAP3K in *Nicotiana benthamiana*, compromises disease resistance (Jin et al., 2002). To test whether disease resistance is enhanced or compromised in *GmMEKK1*-silenced plants, SMV strain N tagged with the GUS marker protein (SMV-N-GUS; Wang et al., 2006) was inoculated via biolistic bombardment onto three individual leaves detached from both vector control plants and *GmMEKK1*-silenced plants. At 3 dpi, the SMV-N-GUS infection was visualized by GUS staining (Fig. 6A). The GUS infection foci were readily seen on the non-silenced empty vector control leaves (Fig. 6A, left). However, the GUS foci on the *GmMEKK1*-silenced plants were much smaller and were visible only with a dissecting microscope (Fig. 6, A, right, and B). These SMV-N-GUS foci were approximately 25% of the diameter of those reported previously on the leaves of *GmMPK4*-silenced plants (Liu et al., 2011), suggesting that *GmMEKK1*-silenced plants are more resistant to SMV than *GmMPK4*-silenced plants.



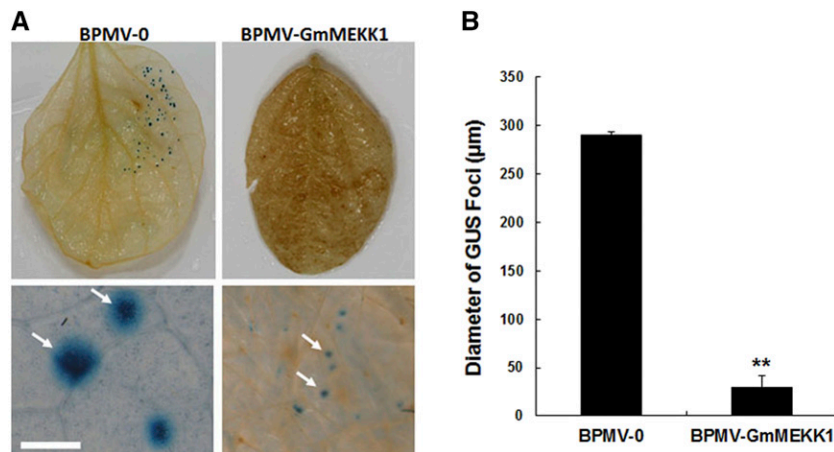
**Figure 4.** GO biological process terms significantly ( $P < 0.05$ ) overrepresented among genes differentially expressed in response to *GmMEKK1* silencing. Significantly overrepresented GO terms were identified using the SoyBase GO Term Enrichment Tool ([https://www.soybase.org/goslimgraphic\\_v2/dashboard.php](https://www.soybase.org/goslimgraphic_v2/dashboard.php)) for genes induced or repressed by *GmMEKK1* silencing relative to vector controls. Due to the large number of significant GO terms identified, GO terms were limited to those terms in



**Figure 5.** Expression of transcription factors in response to *GmMEKK1* silencing. Significantly differentially expressed transcription factors and families were identified using the SoyDB transcription factor database (Wang et al., 2010). A total of 728 transcription factors representing 46 transcription factor families were identified. Fold change is plotted on the x axis, and transcription factor families are plotted on the y axis. For visualization purposes, transcription factors with a fold change greater than 50 were plotted as 50. Similarly, transcription factors with a fold change less than -50 were plotted as -50. For a full list of transcription factors see Supplemental Data Set S5. Transcription factor families ZIM, WRKY and AUX-IAA-ARF are significantly (FDR < 0.001) overrepresented among differentially expressed transcription factors. For additional details, see Supplemental Data Set S6.

**Figure 4.** (Continued.)

which 30% of genes in the genome assigned to an individual GO term were differentially expressed. Only GO terms containing 20 or more genes are included. In addition, any GO terms whose genes completely overlapped were mapped to the largest significantly overrepresented GO term. For all significant GO terms, see Supplemental Data Sets S3 and S4.



**Figure 6.** Silencing *GmMEKK1s* enhances the resistance of soybean plants to SMV. At 15 dpi with BPMV-0 or BPMV-*GmMEKK1*, SMV-N-GUS was biolistically delivered into the detached leaves of silenced and nonsilenced plants. At 3 dpi with SMV-N-GUS, the replication and movement of SMV-N-GUS in the biolistically inoculated leaves was detected by GUS staining. The GUS foci were counted and measured. A, Infection foci of SMV-N-GUS on the leaves of BPMV-0 and BPMV-*GmMEKK1* plants (top), and closeup images from leaves at top taken with a dissecting microscope (arrows at bottom). Bar = 2 mm. B, Comparison of the diameters of SMV-N-GUS foci on the leaves of BPMV-0 and BPMV-*GmMEKK1* plants. Error bars represent sd of the diameters of at least 30 GUS foci measured on each of four independent leaves (at least 120 foci). Asterisks indicate a significant difference from the control (\*\*,  $P < 0.01$ , Student's *t* test).

We have reported that silencing *GmMPK4* and *GmMPK6* also enhances soybean resistance against downy mildew (Liu et al., 2011, 2014). To test whether silencing *GmMEKK1* has the same effect, we inoculated BPMV-0 control plants and *GmMEKK1*-silenced plants (12 dpi) with *P. manshurica*. Symptomatic chlorotic lesions of *P. manshurica* infection were observed on BPMV-0 control leaves at 7 dpi (Fig. 7A, left). However, similar lesions were not observed on *GmMEKK1*-silenced leaves (Fig. 7A, right). To further validate these results, *P. manshurica* mycelia were stained within inoculated leaves using Aniline Blue. Abundant hyphae were readily observed in mesophyll cells of vector control plant leaves with chlorotic lesions (Fig. 7B), while no hyphae were observed within the mesophyll of the *GmMEKK1*-silenced plants (Fig. 7C). As in *GmMPK4*-silenced plants (Liu et al., 2011), sporangia that germinated on the leaves of *GmMEKK1*-silenced plants (Fig. 7D, 1) often produced germ tubes (Fig. 7D, GT) with multiple appressoria (Fig. 7D, 2 and 3), indicating that multiple unsuccessful penetrations were sometimes attempted (Fig. 7D). This behavior was not observed on the leaves of the BPMV-0 control plants, suggesting that the resistance of *GmMEKK1*-silenced plants to *P. manshurica* may have occurred at the penetration stage. Together, these results clearly show that silencing *GmMEKK1* enhanced resistance against SMV and *P. manshurica*.

#### Activation of *GmMPK6* Is Repressed and Activation of *GmMPK3* Is Enhanced in *GmMEKK1*-Silenced Plants in Response to flg22

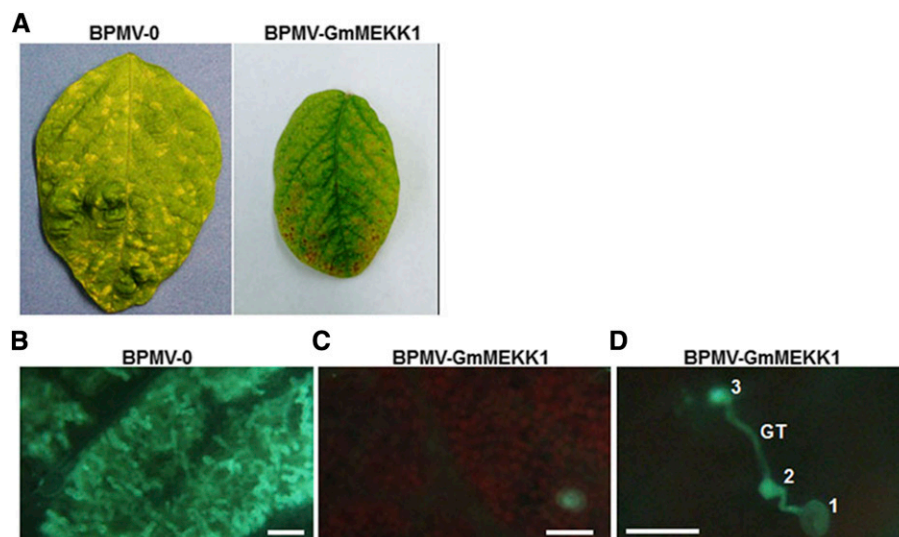
Activation of MPK4 but not MPK3/6 in response to the flg22 peptide is abolished in the Arabidopsis *mekk1*

mutant (Suarez-Rodriguez et al., 2007; Gao et al., 2008). To examine whether *GmMPK4* or *GmMPK3/6* functions downstream of *GmMEKK1*, we tested for *GmMPK4* or *GmMPK3/6* phosphorylation in *GmMEKK1*-silenced plants following flg22 treatment. To detect the phosphorylated forms of these three MPKs, we used the phospho-p44/42 MAPK Erk1/2 antibody that recognizes the phosphorylation of Arabidopsis MPK3/4/6 at the sites corresponding to Thr-202/Tyr-204 of human (*Homo sapiens*) p44 MAPK (Zhao et al., 2014). This phosphorylation site is highly conserved between the human p44 MAPK epitope and Arabidopsis or soybean MPK3/4/6 (Supplemental Fig. S2). *GmMPK3*-, *GmMPK4*-, and *GmMPK6*-silenced plants were used as controls. Compared with BPMV-0 control plants, activation of *GmMPK6* by flg22 was partially dependent on *GmMEKK1*, because less *GmMPK6* phosphorylation was observed in *GmMEKK1*-silenced plants at all time points after flg22 treatment (Fig. 8A). In contrast, *GmMPK3* activation was strongly enhanced in *GmMEKK1*-silenced plants even in the absence of flg22 treatment (Fig. 8A). Interestingly, silencing *GmMPK4* and *GmMPK6* also enhanced the activation of *GmMPK3* by flg22 (Fig. 8, C and D). As expected, activation of *GmMPK3* or *GmMPK6* by flg22 was reduced significantly in the corresponding *GmMPK3*- or *GmMPK6*-silenced control plants (Fig. 8, B and D), respectively.

#### *GmMPK4* Activity Is Not Activated by Either flg22 or SA

To our surprise, *GmMPK4* was not activated in either BPMV-0 control plants or in *GmMEKK1*- or *GmMPK3*-, *GmMPK4*-, and *GmMPK6*-silenced plants (Fig. 8), even





**Figure 7.** Silencing *GmMEKK1* enhances the resistance of soybean to downy mildew. A, Typical chlorotic lesion symptoms of soybean downy mildew detected on the leaves of BPMV-0 empty vector control plants (left) but not on the leaves of *GmMEKK1*-silenced plants (right) 1 week after inoculation with *P. manshurica*. B, *P. manshurica* hyphae observed in the mesophyll of BPMV-0 control plants at 1 week after inoculation. C, Hyphae absent from the mesophyll of *GmMEKK1*-silenced plants 1 week after inoculation with *P. manshurica*. D, Germ tubes (GT) that formed multiple appressoria unable to penetrate the epidermal surface observed on *GmMEKK1*-silenced plants. 1, Sporangium; 2 and 3, appressoria. These experiments were repeated three times (at least three plants each time) with similar results. Bars = 100  $\mu$ m in B and C and 50  $\mu$ m in D.

though MPK3, MPK4, and MPK6 are activated in Arabidopsis (Fig. 8E). This result implied that the antibody does not recognize the phosphorylated GmMPK4. However, the amino acid sequences of the Arabidopsis and soybean MPK3/4/6 peptides, which correspond to the human p44 MAPK epitope used to raise the antibody, are identical (Supplemental Fig. S2). To resolve this issue, an in gel kinase activity assay was performed. Similar to the antibody-based assay, the in gel kinase activity assay also did not detect the activation of GmMPK4 either in the vector control plants or in the plants silenced for *GmMEKK1* or *GmMPK3*, *GmMPK4*, and *GmMPK6* (Fig. 8F). Activation of GmMPK6 was reduced but activation of GmMPK3 was enhanced in *GmMEKK1*-silenced plants, which also was consistent with the antibody-based assay (Fig. 8A). As expected, activation of GmMPK6 and GmMPK3 was reduced in the corresponding *GmMPK6*- and *GmMPK3*-silenced control plants (Fig. 8F).

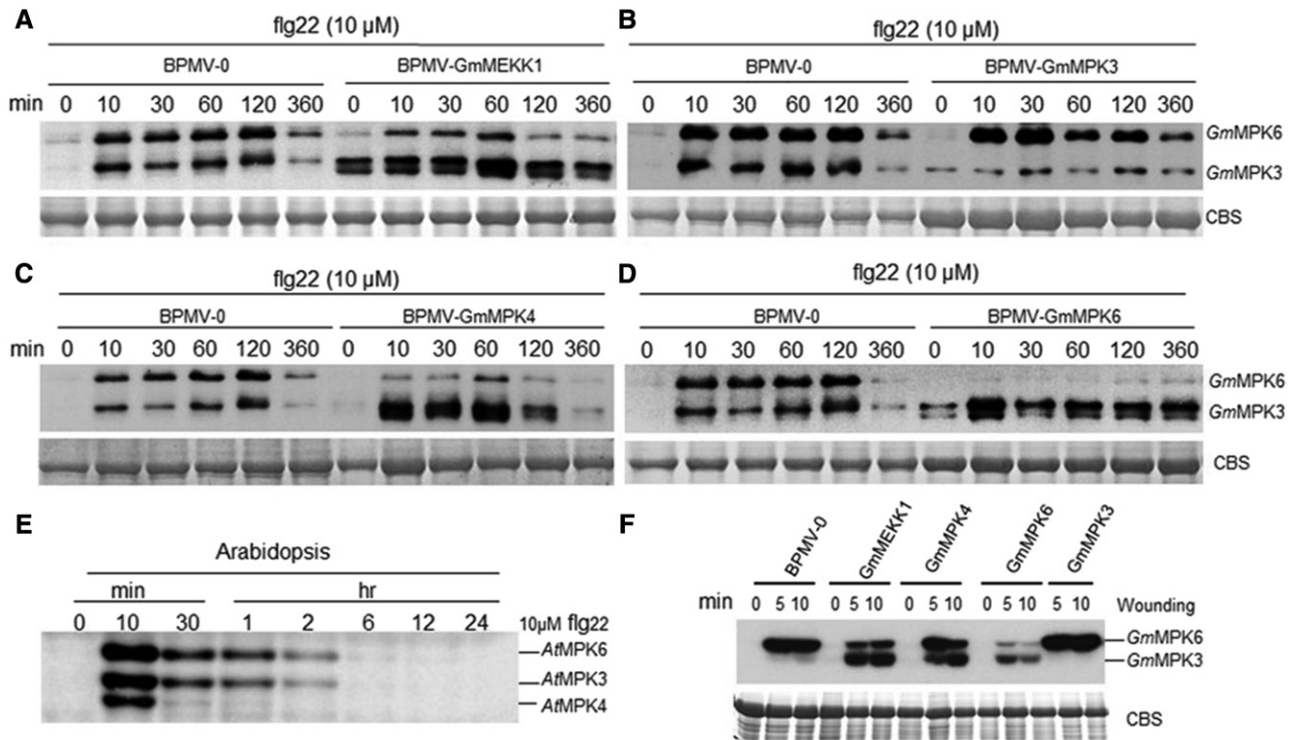
We noticed that activation of GmMPK6 kinase activity was reduced in *GmMPK4*-silenced plants compared with nonsilenced BPMV-0 control plants (Fig. 8C). To investigate the possibility of cross silencing between *GmMPK4* and *GmMPK6*, we performed immunoblot analyses using Arabidopsis MPK3, MPK4, and MPK6 antibodies. The signals detected by these antibodies in soybean were highly related to the amino acid sequence identities between the Arabidopsis epitopes used for raising the antibodies and the corresponding soybean peptides (Supplemental Fig. S3, A and B). The protein levels of *GmMPK3*, *GmMPK4*, and *GmMPK6* were barely detectable in the respective silenced plants

compared with nonsilenced control plants (Supplemental Fig. S3A), indicating that silencing of each gene occurred. Consistent with the kinase activation assay (Fig. 8C), the level of GmMPK6 protein indeed decreased in *GmMPK4*-silenced plants relative to vector control plants (Supplemental Fig. S3A). However, the level of GmMPK4 protein was not reduced in *GmMPK6*-silenced plants (Supplemental Fig. S3A), excluding the possibility of cross silencing between these two genes. Taken together, these results indicated that the silencing mediated by the BPMV-VIGS was specific to the targeted *GmMPK*.

To test the possibility that GmMPK4 activation requires higher concentrations of flg22 and SA and/or has different kinetics from Arabidopsis MPK4, we treated soybean leaf tissues with 20  $\mu$ M flg22 and 1 or 10 mM SA for different periods of time (Fig. 9). Regardless of the concentrations of flg22 and SA or the time points, the activation of GmMPK4 was not observed. Together, these results indicated distinct differences between soybean and Arabidopsis in terms of MPK4 activation, raising the possibility that GmMEKK1 negatively regulates cell death independent of GmMPK4 phosphorylation status.

#### ***GmMEKK1* Interacts with Multiple Downstream GmMKKs**

The MEKK1-MKK1/2-MPK4 module negatively regulates Arabidopsis defense responses (Gao et al., 2008), and MEKK1 interacts directly with MKK1 and MKK2 in yeast two-hybrid (Y2H) and bimolecular fluorescence complementation assays (Ichimura et al., 1998;



**Figure 8.** The activation of *GmMPK3* and *GmMPK6* in response to flg22 is enhanced and reduced, respectively, in *GmMEKK1*-silenced plants. A to D, Leaf discs from the indicated soybean plants were incubated on moist filter paper for 24 h to allow recovery from wounding before treatment with 10  $\mu$ M flg22 or diluted DMSO for the indicated times. The kinase activities were detected by immunoblotting using phospho-p44/42 MAP Erk1/2 antibody. E, Five-day-old whole Arabidopsis seedlings (Columbia-0 ecotype) were treated with 10  $\mu$ M flg22 or diluted DMSO over the indicated time course. The kinase activities were detected by immunoblotting using phospho-p44/42 MAP Erk1/2 antibody. F, Leaf discs from the indicated soybean plants silenced for various *GmMPKs* were wounded for 0, 5, and 10 min. Kinase activities were detected subsequently by in gel kinase assay. Myelin basic protein was embedded in the gel to serve as a substrate for MPKs. CBS, Coomassie Blue staining for a loading control.

Mizoguchi et al., 1998; Gao et al., 2008). MEKK1-MKK4/5 and MKK9 are involved in the activation of MPK3/MPK6 and camalexin production, but their direct interaction has not been reported previously (Ren et al., 2008; Xu et al., 2008). To test whether *GmMEKK1* interacts with *GmMKK1/GmMKK2*, *GmMKK4/GmMKK5*, or *GmMKK9*, Y2H assays were performed. Consistent with Arabidopsis, *GmMEKK1* interacted with *GmMKK1a*, *GmMKK2a*, and *GmMKK2b*, but it also interacted with *GmMKK4* and *GmMKK9* (Fig. 10A), suggesting that *GmMEKK1* might exert its function through multiple downstream *GmMKKs*. However, interaction was not detected between *GmMEKK1* and *GmMKK3* (Fig. 10A).

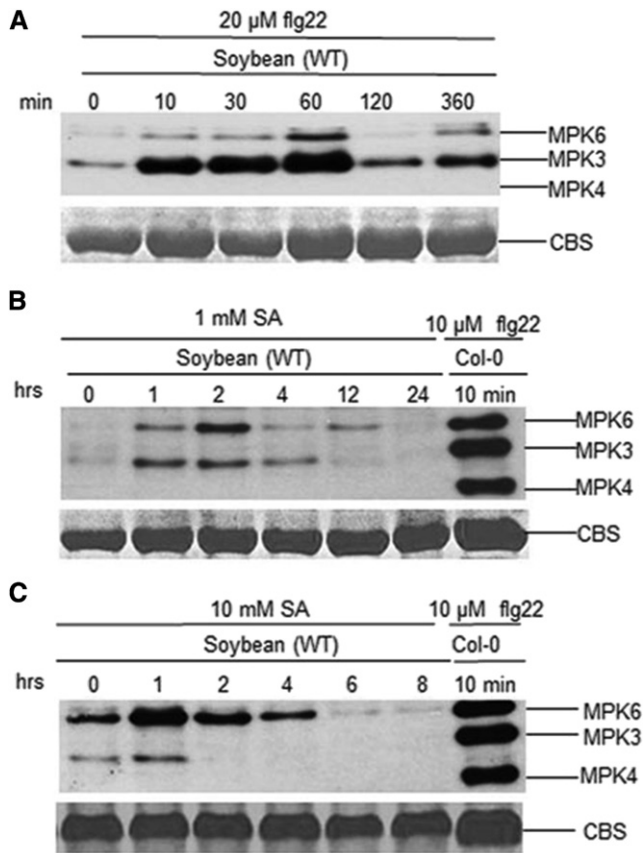
***GmMEKK1* Localizes to the Cytosol and Nucleus**

To investigate the subcellular location of *GmMEKK1*, the full-length *GmMEKK1* was fused to the C terminus of GFP and transiently coexpressed in *N. benthamiana* leaves with a free DsRed-expressing construct via agroinfiltration. 35S::GFP-*GmMEKK1* colocalized with free 35S::DsRed, indicating that *GmMEKK1* was present in

both the cytosol and the nucleus (Fig. 10B). Because the strong constitutive 35S promoter could potentially bias the results, we transiently expressed a *GmMEKK1-GFP* fusion protein driven by the native *GmMEKK1* promoter (~1.2 kb upstream of the start codon). Although the fluorescence intensity was reduced significantly compared with the expression driven by the 35S promoter, *GmMEKK1-GFP* was observed similarly in the cytoplasm and nucleus (Supplemental Fig. S4). The subcellular localization of MEKK1 in other plant species has not been reported previously, even though bimolecular fluorescence complementation assays have shown MEKK1 interacting with MKK1 or MKK2 only at the plasma membrane in Arabidopsis protoplasts (Gao et al., 2008). *GmMEKK1* shares similar subcellular localization with its potential downstream target *GmMPK4* (Liu et al., 2011).

**Transient Overexpression of *GmMEKK1a* in *N. benthamiana* Induces HR**

We have shown previously that *GmMPK6* plays both positive and negative roles in cell death and defense



**Figure 9.** GmMPK4 kinase activation was not detected in the presence of flg22 or SA regardless of concentrations and time points. Leaf discs from wild-type (WT) soybean were incubated on wet filter paper for 24 h to allow recovery from wounding before being treated with 20  $\mu$ M flg22 (A), 1 mM SA (B), or 10 mM SA (C) for the indicated time periods. Kinase activities were detected by immunoblotting using phospho-p44/42 MAP Erk1/2 antibody, which recognizes phosphorylated MPK3, MPK4, and MPK6 across kingdoms. The activation of Arabidopsis MPK3/4/6 by 10  $\mu$ M flg22 was used as a control. CBS, Coomassie Blue staining for a loading control.

pathways (Liu et al., 2014). To test whether *GmMEKK1a* also plays a positive role in inducing HR, we transiently expressed 35S-*GmMEKK1a* using agroinfiltration (Liu et al., 2005). Overexpression of *GmMEKK1a* induced HR cell death in *N. benthamiana* leaves (Supplemental Fig. S5), indicating that it also may promote defense responses. Interestingly, transient overexpression of a kinase-dead version of *GmMEKK1* (K321M, equivalent to K361M of Arabidopsis MEKK1 as described by Suarez-Rodriguez et al. [2007]) did not induce cell death (Supplemental Fig. S5), indicating that the kinase activity of *GmMEKK1* is required to induce cell death. As expected, transient overexpression of 35S::Pto<sup>Y207D</sup> (active Pto) triggered HR-like cell death (Rathjen et al., 1999). This result indicates that *GmMEKK1a* can induce cell death when expressed ectopically in the leaves of *N. benthamiana*, further confirming that the

kinase activity in MAPK signaling pathways must be tightly controlled.

## DISCUSSION

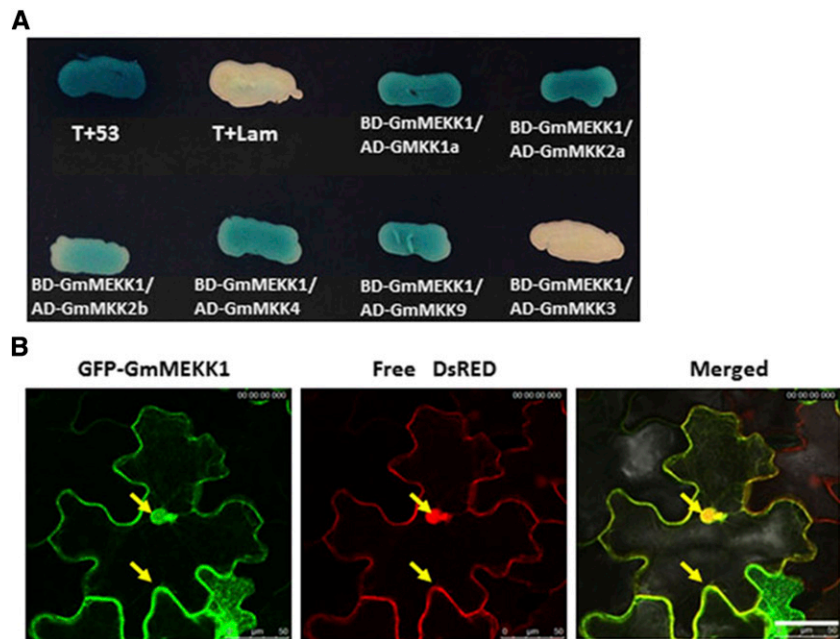
### Does *GmMEKK1* Function Upstream of *GmMPK4*?

Compared with *GmMPK4*-silenced plants, silencing *GmMEKK1* resulted in a more severe constitutively activated defense response, which was manifested by the more severe HR observed on both the inoculated local and systemic leaves (Fig. 1; Liu et al., 2011). For example, *GmMEKK1*-silenced plants typically died by 25 dpi, while *GmMPK4*-silenced plants remained alive (Liu et al., 2011). These constitutive defense phenotypes are consistent with the idea that *GmMEKK1* might be the MAP3K that functions upstream of *GmMPK4*. In Arabidopsis, the *mekk1*, *mkk1/mkk2*, and *mpk4* mutants exhibit a gradient of phenotypic severities, and the activation of MPK4 activity in response to flg22 in *mekk1* and *mkk1/mkk2* mutants is abolished, supporting their proposed functional hierarchy in a signaling cascade (Ichimura et al., 2006; Nakagami et al., 2006; Suarez-Rodriguez et al., 2007; Gao et al., 2008; Qiu et al., 2008).

We have shown previously that silencing *GmMPK4* induced HR and defense responses, and both *GmMEKK1* and *GmMEKK2* interacted with *GmMPK4* in vivo and phosphorylated *GmMPK4* in vitro (Liu et al., 2011). Here, we showed that *GmMEKK1* interacted with *GmMEKK1/2* in Y2H assays (Fig. 10A). In addition, there was a significant overlap in the genes induced in *GmMEKK1*- and *GmMPK4*-silenced plants (Supplemental Data Set S7). It seems that a similar *GmMEKK1*-*GmMEKK1/GmMEKK2*-*GmMPK4* module also might be operating in soybean. However, this signaling module cannot be firmly established because the activation of *GmMPK4* by flg22 or SA was not observed in the BPMV-0 control plants (Figs. 8 and 9). Therefore, we could not determine whether *GmMEKK1* kinase activity is required for *GmMPK4* activation. These results indicate that a distinctive difference exists in MPK4 activation between soybean and Arabidopsis (Supplemental Fig. S6). In addition, unlike Arabidopsis, in which both MPK3 and MPK6 are activated to much higher levels in *mekk1* plants than in wild-type plants in response to flg22 (Ichimura et al., 2006), silencing *GmMEKK1* enhanced the activation of *GmMPK3* but reduced the activation of *GmMPK6* in response to flg22 (Fig. 8, B and D), further supporting that distinctive differences exist between soybean and Arabidopsis in the activation of MAPKs.

As the cell-death phenotype develops in Arabidopsis *mekk1*, *mkk1/2*, and *mpk4* mutant plants under normal growth conditions, the activation of MPK4 is barely detectable even in wild-type Columbia-0 plants (Ichimura et al., 2006; Nakagami et al., 2006; Gao et al., 2008). Therefore, the statement that the inactivation of

**Figure 10.** Identification of GmMEKK1-interacting partners and subcellular localization of GmMEKK1. A, A Y2H assay was performed between GmMEKK1 (Glyma.04G253500) and several GmMCKs, including GmMCK1a (Glyma.15G172600), GmMCK2a (Glyma.17G052400), GmMCK2b (Glyma.13G106900), GmMCK4 (Glyma.08G223400), and GmMCK9 (Glyma.09G172500). T-antigen and p53 were included as a positive interaction control, and Lam and T-antigen were included as a negative interaction control. B, GmMEKK1a localization in both the cytoplasm and nucleus of *N. benthamiana* epidermal cells. *Agrobacterium tumefaciens* carrying 35S::GFP-GmMEKK1a and 35S::DsRed constructs was coinfiltrated into *N. benthamiana* leaves. At 2 dpi, the infiltrated area of the leaves was excised and viewed by confocal microscopy. The left image shows the transient expression of GFP-GmMEKK1a in *N. benthamiana* cells; the middle image shows the transient expression of free DsRed in *N. benthamiana* cells; and the right image shows the merged image. Bar = 100  $\mu$ m.



MPK4's kinase activity is necessary for the activated defense responses observed in *Arabidopsis mekk1*, *mkk1/2*, and *mpk4* mutant plants may not be established unequivocally. Thus, it is possible that a function of MPK4 other than its kinase activity may be required to suppress cell death under normal growth conditions. It will be interesting to test whether a kinase-dead version of MPK4 can rescue the cell-death phenotypes of an *mpk4* mutant, which would parallel the rescue of some *mekk1* phenotypes by a kinase-dead version of MEKK1 (Suarez-Rodriguez et al., 2007). Because GmMPK4 cannot be activated by either flg22 or SA, it may not be involved in the processes triggered by these stimuli. However, we cannot eliminate the possibility that a slight attenuation of MPK4 activation, which is undetectable by the methods we used, is sufficient to trigger the cell death in *Arabidopsis mekk1* and *mkk1/mkk2* mutants and in GmMEKK1-silenced plants.

A recent study showed that constitutively activated MPK3 triggers defense responses in *Arabidopsis*, including dwarfed stature, massive induction of defense-related gene expression, and spontaneous cell death (Genot et al., 2017). Silencing GmMEKK1, GmMPK4, or GmMPK6 increased the activation of GmMPK3 (Fig. 8, A, C, D, and F), which coincides with the spontaneous cell death and constitutive activation of defenses (Liu et al., 2011, 2014). Therefore, the activated defense responses and cell death observed in GmMEKK1-, GmMPK4-, or GmMPK6-silenced plants could be the result of GmMPK3 activation. In contrast to GmMPK3, the activation of GmMPK6 by flg22 was compromised in GmMEKK1-silenced plants (Fig. 8A). Therefore, the activation of GmMPK3 and GmMPK6 is differentially regulated in GmMEKK1-silenced plants. Because GmMEKK1 also interacts with multiple GmMCKs, our results raise the question of whether GmMEKK1

exerts its function through a linear GmMEKK1-GmMCK1/2-GmMPK4 pathway or through a branched network (Supplemental Fig. S6).

An additional band was detected below GmMPK3 in the GmMEKK1- and GmMPK6-silenced plants in our kinase activation assays (see band below GmMPK3 in Fig. 8, A and D), and a band corresponding to *Arabidopsis* MPK4 was not detected in either vector control or any of the silenced soybean plants (Fig. 8). One may argue that the band below GmMPK3 could represent GmMPK4, but the following three lines of evidence may exclude this possibility. First, the band sizes between *Arabidopsis* MPK3 and GmMPK3, MPK4 and GmMPK4, and MPK6 and GmMPK6 were almost identical in our immunoblot analysis using the respective *Arabidopsis* antibodies (Supplemental Fig. S3A). Second, the size of the band below GmMPK3 was significantly larger than *Arabidopsis* MPK4 in our kinase activation assays (Supplemental Fig. S3C). Third, MEKK1 functions upstream of MPK4 in *Arabidopsis*, and the activation of MPK4 is abolished in *mekk1* (Suarez-Rodriguez et al., 2007; Gao et al., 2008). If the function of MEKK1 is conserved between *Arabidopsis* and soybean, the band below GmMPK3 is unlikely to be GmMPK4.

#### Overlapping But Distinct Transcriptome Patterns between GmMEKK1- and GmMPK4-Silenced Plants

Kovtun et al. (2000) showed that the H<sub>2</sub>O<sub>2</sub>-MAPK cascade could repress auxin responses in protoplasts. The *mekk1* and *mpk4* mutants are compromised in the expression of auxin-induced genes (Nakagami et al., 2006; Qiu et al., 2008; Pitzschke et al., 2009a). Genome-wide microarray analysis indicated that even though a large cohort of genes is coregulated in *mpk4*, *mkk1/2*,

and *mekk1* mutants, distinct sets of genes are expressed specifically in each mutant, suggesting both conserved and divergent roles of these kinases in regulating gene expression (Pitzschke et al., 2009a).

Analyses of *GmMPK4*-silenced plants were conducted previously using the soybean Affymetrix GeneChip Soybean Genome Array (Liu et al., 2011). Of the 56,044 genes present in the current genome assembly, 18,619 (33%) were represented on the array. To compare gene expression patterns between *GmMEKK1*- and *GmMPK4*-silenced plants, we converted the *GmMPK4*-silencing data to current gene calls. Of the 2,413 genes induced by *GmMPK4* silencing, 1,243 (52%) also were induced by *GmMEKK1* silencing. Not surprisingly, GO analyses revealed that the commonly induced genes were significantly (corrected  $P < 0.05$ ) overrepresented with GO terms related to defense, immunity, and stress (Supplemental Data Set S7). Similarly, of the 3,471 genes repressed by *GmMPK4* silencing, 1,412 (41%) also were repressed by *GmMEKK1* silencing. These commonly repressed genes were significantly overrepresented with GO terms related to growth, photosynthesis, and metabolism (Supplemental Data Set S7). Since only a subset of genes present in the genome were present on the array used to characterize *GmMPK4*-silenced plants, we cannot absolutely analyze genes unique to *GmMEKK1* silencing, but we can examine genes unique to *GmMPK4* silencing. The 1,170 uniquely induced genes also were significantly overrepresented with GO terms associated with defense and stress responses (Supplemental Data Set S7). The 2,059 uniquely repressed genes were overrepresented with GO terms related to the cell cycle, cell division, methylation, and gene silencing (Supplemental Data Set S7). These results indicate strong overlapping but distinct transcriptome patterns between *GmMEKK1*- and *GmMPK4*-silenced plants. Similar transcriptome patterns also are shared by *mekk1*, *mkk1/mkk2*, and *mpk4* mutants in Arabidopsis (Qiu et al., 2008; Pitzschke et al., 2009a). The distinct expression patterns between *GmMEKK1*- and *GmMPK4*-silenced plants could be explained by the high-level complexity of the MAPK pathway. It is highly possible that *GmMEKK1* can regulate gene expression independent of *GmMPK4* and vice versa.

### Is GmMEKK1 a Double-Edged Sword in Regulating Cell Death?

Transient overexpression of *GmMEKK1* possessing kinase activity was required for HR on *N. benthamiana* leaves (Supplemental Fig. S5). Our numerous efforts to generate transgenic Arabidopsis plants that overexpress wild-type *GmMEKK1* under the control of the constitutive cauliflower mosaic virus 35S promoter or an inducible promoter failed, implying that it also might play a positive role in defense. Similarly, overexpression of *MEKK1* in Arabidopsis is lethal (Suarez-Rodriguez et al., 2007), suggesting that, regardless of plant species, the expression of *MEKK1* must be tightly

regulated to properly control growth/development and defense. Defense responses will be activated if *MEKK1* activity is either too low or too high, which will lead to defense-oriented transcriptomic reprogramming. This seemingly dual role is not unexpected for genes in soybean MAPK pathways based on our previous observation that either silencing or overexpressing *GmMPK6* triggers defense (Liu et al., 2014).

### CONCLUSION

Our results revealed similarities and distinct differences between soybean and Arabidopsis *MEKK1* (Supplemental Fig. S6). Like its Arabidopsis counterpart, *GmMEKK1* is both a positive and negative regulator of soybean cell death and defense responses. Unlike in Arabidopsis, where *MPK4* functions downstream of *MEKK1*, we were unable to demonstrate conclusively that *GmMPK4* functions downstream of *GmMEKK1*. In addition, we found that *GmMEKK1* positively regulates *GmMPK6* but negatively regulates *GmMPK3* activity in response to flg22. These interesting differences in the functional relationships between *GmMEKK1* and *GmMPK3/4/6* will need to be resolved in the future. Furthermore, significantly increased activation of *GmMPK3* in response to flg22 was observed in *GmMEKK1*-, *GmMPK4*-, and *GmMPK6*-silenced plants (Fig. 8, A, C, and D), which is associated with cell death and the constitutively activated defense observed in these plants (Fig. 1; Liu et al., 2011, 2014). Thus, the relevance of the *GmMPK3* activation in cell death and defense will be a focus in future studies. Together, our results indicate that the MAPK signaling pathway in soybean is more complex than expected. It appears that the soybean MAPK components we have studied cooperate in ways not predicted from previous work in Arabidopsis to form an interconnected, multidirectional network rather than unidirectional linear pathways.

### MATERIALS AND METHODS

#### Plant Materials

Seeds of soybean (*Glycine max* 'Williams 82') and *Nicotiana benthamiana* used in this study were harvested from greenhouse-grown plants. Soybean plants were maintained in the greenhouse or growth chamber at 22°C with a photoperiod of 16 h, unless indicated otherwise.

#### BPMV-Mediated VIGS

BPMV strains, BPMV-VIGS constructs, and inoculation of soybean seedlings with DNA-based BPMV constructs via biolistic particle bombardment using a Biolistic PDS-1000/He system (Bio-Rad Laboratories) have been described previously (Zhang et al., 2010, 2013). The orthologs of *MEKK1* were identified by reciprocal best BLASTn searches (cutoff value  $< 0.001$ ) between Arabidopsis (*Arabidopsis thaliana*) *MEKK1* and soybean gene models available in the Phytozome database ([www.phytozome.org](http://www.phytozome.org)). A 360-bp fragment of *GmMEKK1* was amplified by PCR using the following primers: *GmMEKK1a*-F (Glyma.04G253500), 5'-aagGGATCCATGCATTACCTATCTCGGATT-3', and

*GmMEKK1*-R, 5'-ttgGGTACCCCTCGGGAATATCGAGGTTATCG-3'. The underlined sequences are *Bam*HI and *Kpn*I restriction sites, respectively, that were used to clone the PCR fragment into the BPMV-VIGS (IA-D35). The boldface letter indicates an extra nucleotide in reverse primers needed to maintain the reading frame.

## RNA Isolation and RT-qPCR

RNA isolation and RT-qPCR were performed as described elsewhere (Liu et al., 2014). The RT-qPCR tests were performed using an iCycler (Bio-Rad Laboratories) and the Platinum SYBRGreen qPCR SuperMix UDG (Invitrogen). Total RNA samples were treated with DNaseI according to the manufacturer's instructions (Invitrogen). The primers used for qPCR are listed in Supplemental Table S1.

## RNA-Seq Analysis

Sequencing was performed at the National Center for Genome Resources on an Illumina Genome Analyzer II as described by Moran Lauter et al. (2014). In brief, eight multiplex libraries were prepared from four biological replicates of BPMV empty vector control and *GmMEKK1*-silenced plants. Sequencing produced a total of 177,027,059 single-end reads. TopHat (version 2.0.3; Trapnell et al., 2009) was used to align reads to the cv Williams 82 reference genome using default settings (version *G. max* 2.1; Schmutz et al., 2010). SAMtools (Li et al., 2009; Schmutz et al., 2010) was used to remove unreliably mapped reads. The resulting mapping files (bam) were imported into the statistical program R (R Development Core Team, 2006). The Bioconductor package rtracklayer (Lawrence et al., 2009) was used to import the gene feature file corresponding to *G. max* version 2.1. The package GenomicRanges was used to count reads and output a matrix containing gene counts for each sample. Prior to normalization and statistical analysis, counts assigned to *GmMEKK1a* and *GmMEKK1b* were removed, as they could be of viral origin. Genes with counts per million < 1 in at least three of the four biological replicates were eliminated from the analyses. The Bioconductor package edgeR (Robinson et al., 2010) was used for single-factor, pairwise comparisons to calculate normalization factors and determine differential expression. Differentially expressed genes were considered significant if their fold change was greater than 2 with an FDR < 0.001. Significantly differentially expressed genes were annotated using the SoyBase Genome Annotation Report page ([www.soybase.org/genomeannotation](http://www.soybase.org/genomeannotation)), which provided best Arabidopsis homologs, and GO information inferred from Arabidopsis (TAIR version 10; [www.arabidopsis.org](http://www.arabidopsis.org)). Significantly ( $P < 0.05$ ) overrepresented biological process GO terms were identified using the SoyBase GO Term Enrichment Tool ([https://www.soybase.org/goslimgraphic\\_v2/dashboard.php](https://www.soybase.org/goslimgraphic_v2/dashboard.php)), which uses Fisher's exact test (Fisher, 1966) and Bonferroni correction (Bonferroni, 1935). The SoyDB transcription factor database was used to identify transcription factors among differentially expressed genes (Wang et al., 2010). To facilitate comparisons between *GmMEKK1*- and *GmMPK4*-silenced plants, we used the SoyBase Gene Model Correspondence Lookup (<https://www.soybase.org/correspondence/>).

## SA Quantification

SA was quantified using an Agilent 1100 high-performance liquid chromatograph with fluorometric detection (Agilent Technologies) as described previously (Liu et al., 2011). The column was a 4.6-by-75-mm Agilent RR XDB C18 with an isocratic mobile phase comprising 75% (v/v) 20 mM formate (pH 3.8), 20% methanol (v/v), and 5% acetonitrile (v/v) at a flow rate of 0.75 mL min<sup>-1</sup> at 35°C. SA-O-glucoside was measured after converting to free SA by acid hydrolysis. Recovery rates were determined using *o*-anisic acid as an internal standard and were typically greater than 60% (v/v).

## H<sub>2</sub>O<sub>2</sub> Detection by DAB Staining

H<sub>2</sub>O<sub>2</sub> was detected by an endogenous peroxidase-dependent in situ histochemical staining procedure using DAB (Sigma-Aldrich; Ren et al., 2002). Leaves were detached and placed in a solution containing 1 mg mL<sup>-1</sup> DAB (pH 5.5) for 2 h. The leaves were cleared by boiling in 96% ethanol for 10 min and then stored in 96% ethanol (v/v). H<sub>2</sub>O<sub>2</sub> production was visualized as a reddish-brown precipitate in cleared leaves (Ren et al., 2002).

## SMV-N-GUS Inoculation, GUS Staining, and GUS Foci Measurements

SMV-N-GUS inoculation, GUS staining, and GUS foci measurements were described previously (Liu et al., 2011). Briefly, at 15 dpi with BPMV empty vector (BPMV-0) or BPMV-*GmMEKK1* constructs by rub inoculation, the second fully expanded soybean trifoliate leaves counting from the top were detached and biolistically inoculated with SMV-N-GUS (Wang et al., 2006; Zhang et al., 2009). After SMV-N-GUS inoculation, the detached leaves were incubated for 3 d on moist filter paper in petri dishes maintained on a lit growth shelf. GUS staining was then performed as described (Jefferson et al., 1987). The leaves were viewed and photographed using a stereomicroscope (Olympus SZX16). The GUS foci were counted, and their diameters were measured using Image-Pro Plus 6.0 (Media Cybernetics).

## Downy Mildew Infection

The isolate of *Peronospora manshurica* used in these studies was described previously (Liu et al., 2011). Vector control and *GmMEKK1*-silenced soybean plants were inoculated by spraying with a suspension of *P. manshurica* sporangia in deionized water. Plants were kept in the dark at high humidity overnight and then moved to the greenhouse for 7 d. The photographs for the symptoms were taken, and samples for microscopy were collected 1 week after inoculation. Pathogen structures in the infected foci were visualized using a KOH-Aniline Blue staining procedure (Hood and Shew, 1996). Tissue samples were placed in 1 M KOH for 24 h and then heated in 1 M KOH for 30 min at 80°C. Samples were rinsed three times with distilled water and soaked in 0.05% Aniline Blue in 0.7 M K<sub>2</sub>HPO<sub>4</sub> pH 9, for 15 min. Specimens were mounted in the same staining solution and observed with a Leitz Fluovert epifluorescence microscope with UV illumination (exciter filter, BP 340–380; dichroic mirror, RKP 400; barrier filter, LP 430). Autofluorescence was observed in leaf specimens that were fixed in boiling 95% ethanol and cleared for several days in saturated chloral hydrate (Heath, 1984). The cleared specimens were mounted in 50% glycerol and observed with blue illumination (exciter filter, BP 420–490; dichroic mirror, RKP 510; barrier filter, LP 520).

## Subcellular Localization of *GmMEKK1*

The *GmMEKK1* (Glyma.04G253500) open reading frame was amplified by RT-PCR from total RNA extracted from cv Williams 82 soybean plants. The PCR product was initially cloned into the pENTR/D TOPO vector (Invitrogen) and then recombined into the binary destination vector pB7WGF2.0 (Karimi et al., 2002) to generate the GFP-*GmMEKK1* fusion construct. This fusion construct and the free DsRed construct were coinfiltrated into *N. benthamiana* leaves as described (Liu et al., 2005). Images were captured with a confocal laser-scanning microscope (Leica TCS SP5 AOBs).

## Immunoblot Analysis for Detecting Phosphorylated MPKs

Protein was extracted from soybean leaf tissues using native extraction buffer (50 mM Tris-MES, pH 8, 0.5 M Suc, 1 mM MgCl<sub>2</sub>, 10 mM EDTA, 5 mM DTT, and protease inhibitor cocktail S8830 [Sigma-Aldrich]). The extract was centrifuged at 12,000 rpm at 4°C for 30 min. For immunoblotting, proteins were separated by SDS-PAGE (10% acrylamide gel) and transferred to PVDF membranes (Millipore) by semidry electrotransfer (Bio-Rad). The membrane was blocked in 1× TBS buffer containing 5% skim milk powder and incubated further with anti-phospho-p44/p42 MAPK (anti-pTEpY) diluted at 1:2,000 (Cell Signaling Technology) and subsequently with secondary antibody diluted at 1:6,000. Finally, the bands were detected using chemiluminescent horseradish peroxidase (HRP) substrate (Millipore).

## In Gel Kinase Activity Assay

Protein was extracted from soybean leaf tissues as described (Liu and Zhang, 2004), and the concentration was determined using the Bio-Rad protein assay kit with BSA as the standard. In gel kinase assay was performed essentially as described previously using myelin basic protein as the substrate (Zhang and Klessig, 1997).

## Y2H Assay for MEKK1-Interacting Partners

A Y2H screen was performed as described in the BD Matchmaker Library Construction and Screening Kits (Clontech). The complete coding sequences of *GmMEKK1a* (Glyma.04G253500), *GmMCK1a* (Glyma.15G172600), *GmMCK2a* (Glyma.17G052400), *GmMCK2b* (Glyma.13G106900), *GmMCK4* (Glyma.07G0465000 or Glyma.08G223400), and *GmMCK9* (Glyma.09G172500) were fused to either the GAL4 DNA-binding domain (pGBKT7) or activation domain (pGADT7) to generate pGBKT7- or pGADT7-MAP3K or -MAP2K and then cointroduced into yeast (*Saccharomyces cerevisiae*) strain Y187. Detection of the interactions between the MAP2Ks and *GmMEKK1* was performed according to the manufacturer's manual (Clontech). The primers used for making the constructs are listed in Supplemental Table S2.

## Accession Numbers

The full RNA-seq data sets generated in this study were deposited in the National Center for Biotechnology Small Reads Archive under BioProject identifier PRJNA374025 (accession no. SRP099206).

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Nucleotide sequence alignment of the *GmMEKK1a* fragment used for silencing and the corresponding sequence of *GmMEKK1b*.

**Supplemental Figure S2.** Amino acid sequence alignment of the peptide used to raise the phospho-p44/42 MAP Erk1/2 antibody and the corresponding peptide sequences of Arabidopsis and soybean MPK3, MPK4, and MPK6.

**Supplemental Figure S3.** Confirmation of the specificity and effectiveness of *GmMPK* silencing using the BPMV-VIGS system.

**Supplemental Figure S4.** The GFP-*GmMEKK1a* fusion protein is localized in the cytoplasm and the nucleus when its expression is driven by a ~1.2-kb native promoter of *GmMEKK1*.

**Supplemental Figure S5.** Transient overexpression of *GmMEKK1a* in *N. benthamiana* leaves results in HR-like cell death.

**Supplemental Figure S6.** Comparison of MEKK1-regulated cell death pathways in Arabidopsis and soybean.

**Supplemental Table S1.** Primers used for RT-PCR or RT-qPCR.

**Supplemental Table S2.** Primers used for making constructs.

**Supplemental Data Set S1.** Genes induced by *GmMEKK1* silencing relative to vector control plants.

**Supplemental Data Set S2.** Genes repressed by *GmMEKK1* silencing relative to vector control plants.

**Supplemental Data Set S3.** GO terms significantly overrepresented within genes induced by *GmMEKK1* silencing.

**Supplemental Data Set S4.** GO terms significantly overrepresented within genes repressed by *GmMEKK1* silencing.

**Supplemental Data Set S5.** Transcription factors differentially expressed in response to *GmMEKK1* silencing.

**Supplemental Data Set S6.** Significantly overrepresented ( $P < 0.05$ ) transcription factor families in *GmMEKK1*-silenced plants.

**Supplemental Data Set S7.** Comparisons of GO terms significantly overrepresented among genes common to *GmMEKK1*- and *GmMPK4*-silenced plants and genes unique to *GmMPK4*-silenced plants.

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