

Soybean Homologs of MPK4 Negatively Regulate Defense Responses and Positively Regulate Growth and Development^{1[W][OA]}

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Mitogen-activated protein kinase (MAPK) cascades play important roles in disease resistance in model plant species such as *Arabidopsis* (*Arabidopsis thaliana*) and tobacco (*Nicotiana tabacum*). However, the importance of MAPK signaling pathways in the disease resistance of crops is still largely uninvestigated. To better understand the role of MAPK signaling pathways in disease resistance in soybean (*Glycine max*), 13, nine, and 10 genes encoding distinct MAPKs, MAPKKs, and MAPKKKs, respectively, were silenced using virus-induced gene silencing mediated by *Bean pod mottle virus*. Among the plants silenced for various MAPKs, MAPKKs, and MAPKKKs, those in which *GmMAPK4* homologs (*GmMPK4s*) were silenced displayed strong phenotypes including stunted stature and spontaneous cell death on the leaves and stems, the characteristic hallmarks of activated defense responses. Microarray analysis showed that genes involved in defense responses, such as those in salicylic acid (SA) signaling pathways, were significantly up-regulated in *GmMPK4*-silenced plants, whereas genes involved in growth and development, such as those in auxin signaling pathways and in cell cycle and proliferation, were significantly down-regulated. As expected, SA and hydrogen peroxide accumulation was significantly increased in *GmMPK4*-silenced plants. Accordingly, *GmMPK4*-silenced plants were more resistant to downy mildew and *Soybean mosaic virus* compared with vector control plants. Using bimolecular fluorescence complementation analysis and in vitro kinase assays, we determined that *GmMKK1* and *GmMKK2* might function upstream of *GmMPK4*. Taken together, our results indicate that *GmMPK4s* negatively regulate SA accumulation and defense response but positively regulate plant growth and development, and their functions are conserved across plant species.

Activation of mitogen-activated protein kinase (MAPK) cascades is a conserved mechanism for regulating innate immune responses in all eukaryotes (Pitzschke et al., 2009b). A MAPK signaling module consists of three protein kinases sequentially activated through phosphorylation by the upstream component: a MAP kinase kinase kinase (MAPKKK or MEKK), a MAP kinase kinase (MAPKK or MKK), and a MAP kinase (MPK; Mészáros et al., 2006). MPK

cascades act downstream of receptors to transduce extracellular stimuli into adaptive, intracellular responses (Petersen et al., 2000). There are more than 80 putative MAPKKKs, 10 MAPKKs, and at least 20 MPKs in the *Arabidopsis* (*Arabidopsis thaliana*) genome (Ichimura et al., 2002). Among the 20 *Arabidopsis* MPKs, MPK3, MPK4, and MPK6 are implicated in defense responses and have been most extensively studied (Innes, 2001; Pitzschke et al., 2009b). While MPK3 and MPK6 act as positive regulators of defense responses (Asai et al., 2002; Menke et al., 2005; Takahashi et al., 2007; Ren et al., 2008; Mao et al., 2011), MPK4 negatively regulates defense responses (Petersen et al., 2000; Brodersen et al., 2006; Gao et al., 2008). Besides its role in defense, MPK4 also plays a role in cytokinesis (Kosetsu et al., 2010; Beck et al., 2011; Zeng et al., 2011).

The role of *Arabidopsis* MPK4 in disease resistance was uncovered by a transposon-tagged mutant, *mpk4* (Petersen et al., 2000). The *mpk4* plants are severely dwarfed and exhibit constitutive systemic acquired resistance, including elevated salicylic acid (SA) levels, increased resistance to virulent pathogens, and constitutive expression of pathogenesis-related (PR) genes

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(Petersen et al., 2000). Overexpression of an inactive form of MPK4 failed to complement *mpk4* phenotypes, indicating that kinase activity is required for MPK4 function (Petersen et al., 2000). The fact that reducing the endogenous SA levels via the expression of a bacterial salicylate hydroxylase gene (*nahG*) alleviates the majority of the *mpk4* mutant phenotypes indicates that the elevated SA levels account for these phenotypes (Petersen et al., 2000). In rice (*Oryza sativa*), OsMPK6, an ortholog of AtMPK4, functions both as an activator and a repressor in resistance against *Xanthomonas oryzae* pv *oryzae* (Shen et al., 2010). Overexpression of BnMPK4 enhances resistance to *Sclerotinia sclerotiorum* in oilseed rape (*Brassica napus*; Wang et al., 2009). In tobacco (*Nicotiana tabacum*), NtMPK4-silenced plants showed enhanced sensitivity to ozone. Conversely, transgenic tobacco plants overexpressing the constitutively active type SIPKK^{EE} exhibited enhanced resistance to ozone (Gomi et al., 2005).

Interestingly, the induction of jasmonate (JA)-responsive genes was blocked in *mpk4* plants (Petersen et al., 2000), suggesting that while MPK4 negatively regulates SA-mediated defense, it positively regulates the JA pathway. Additional support indicating that MPK4 positively regulates the JA pathway comes from investigation of AP2C1, an Arabidopsis Ser/Thr phosphatase of type 2C, that is a novel stress signal regulator that inactivates MPK4. *ap2c1* mutants produce significantly higher amounts of JA upon wounding and are more resistant to phytophagous mites (Schweighofer et al., 2007).

Genetic studies confirmed that MEKK1 and MKK1/2 act upstream of MPK4, as *mekk1* and *mkk1/mkk2* double mutants display similar constitutive defense responses, including elevated levels of SA and hydrogen peroxide (H₂O₂), spontaneous cell death, constitutive PR gene expression, and pathogen resistance (Petersen et al., 2000; Mészáros et al., 2006; Suarez-Rodriguez et al., 2007; Gao et al., 2008; Qiu et al., 2008b). In addition, both MPK4 and MEKK1 interact with MKK1 and MKK2 in vivo (Gao et al., 2008), and MEKK1 and MKK1/2 are essential for activation of MPK4 (Ichimura et al., 2006; Nakagami et al., 2006; Suarez-Rodriguez et al., 2007; Gao et al., 2008). Lastly, many defense-related genes are similarly deregulated in *mekk1*, *mkk1/2*, and *mpk4* mutants (Qiu et al., 2008b; Pitzschke et al., 2009a). Therefore, a negative regulatory role of the MEKK1-MKK1/2-MPK4 module in defense responses has been firmly established in Arabidopsis.

Yeast two-hybrid screening led to the identification of MKS1 (for MAP kinase 4 substrate 1) as the MPK4 substrate in Arabidopsis, and the MPK4-MKS1 interaction was confirmed biochemically in vitro and in vivo (Andreasson et al., 2005). Interestingly, MKS1 also interacts with the transcription factors WRKY25 and WRKY33 (Andreasson et al., 2005). The interaction of MKS1 with WRKY33 depends on the phosphorylation status of MKS1 by MPK4 (Qiu et al., 2008a). In the absence of pathogens, inactivated MPK4 forms a ternary complex with MKS1 and WRKY33 in the nucleus,

which prevents WRKY33 from functioning as a transcription factor (Qiu et al., 2008a). Upon activation of MPK4 by challenge with *Pseudomonas syringae* or flagellin, MKS1 is phosphorylated, and subsequently, phosphorylated MKS1 and WRKY33 proteins are released from MPK4. The unbound WRKY33 targets the promoter of *PHYTOALEXIN DEFICIENT3* for transcriptional activation (Qiu et al., 2008a). These results reveal elegantly how a plant MAPK can regulate gene expression by releasing transcription factors in the nucleus upon activation.

Information on signaling pathways specifying disease resistance in soybean (*Glycine max*) is relatively limited compared with that in Arabidopsis. NPR1 is a key component in SA-mediated systemic acquired resistance (Durrant and Dong, 2004). The soybean NPR1 orthologs, GmNPR1-1 and GmNPR1-2, can complement an Arabidopsis *npr1* mutant (Sandhu et al., 2009). Key components of SA-mediated defenses, such as *GmEDS1*, *GmNPR1*, and *GmPAD4*, as well as members of the WRKY and MYB transcription factor families were shown by virus-induced gene silencing (VIGS) to be required for *Rpp2* resistance toward *Phakopsora pachyrhizi* (Pandey et al., 2011). In addition, a GmPAL1, an O-methyltransferase (O-MT), and a cytochrome P450 monooxygenase are also required for *Rpp2* resistance against *P. pachyrhizi* (Pandey et al., 2011). RAR1 (for required for Mla12 resistance) and SGT1 (for suppressor of the G2 allele of SKP1) are required for *Rsv1*-mediated extreme resistance to *Soybean mosaic virus* (SMV) and *Rpg-1b*-mediated resistance to *P. syringae* (Fu et al., 2009). It appears that the key components in the signaling pathway of disease resistance are conserved between Arabidopsis and soybean.

MAPKs have been studied in great detail in the model plant Arabidopsis, and there is a need to build on this knowledge to establish their functions in crop plants such as soybean. Here, we show that, like its ortholog in Arabidopsis, GmMPK4 is a central regulator that controls the balance of gene expression between disease resistance and growth and development in soybean, and the constitutively activated defense response observed in *GmMPK4*-silenced plants occurs at the expense of plant growth and development. As expected, the activated defense response in *GmMPK4*-silenced plants is correlated with enhanced resistance to SMV and downy mildew (*Peronospora manschurica*), two unrelated pathogens. In addition, we provide evidence that GmMKK1/2 function upstream of GmMPK4.

RESULTS

Constitutively Activated Defense Responses in *GmMPK4*-Silenced Plants

The successful establishment of VIGS mediated by *Bean pod mottle virus* (BPMV; Zhang et al., 2009, 2010) greatly facilitates the functional investigation of soy-

bean genes involved in defense and other processes, such as MAPKs, that regulate plant disease resistance in model plants (Innes, 2001; Pedley and Martin, 2005; Pitzschke et al., 2009b). Based on BLAST searches, there are at least 56 MAPKs, 80 MAPKKs, and over 100 MAPKKKs in the soybean genome (www.phytozome.org). To begin investigating the functions of MAPK cascades in disease resistance in soybean, 13, nine, and 10 genes encoding distinct MAPKs, MAPKKs, and MAPKKKs were silenced using BPMV-mediated VIGS (Supplemental Table S1). Plants in which *GmMPK4* was silenced had consistent phenotypes characterized by stunted stature (Fig. 1A), rugosity and early senescence (Fig. 1D), necrosis in stems and veins (Fig. 1, B and D, arrows), and spontaneous cell death on the leaves (Fig. 1E). All of these phenotypes were reminiscent of the *Arabidopsis mpk4* loss-of-function mutant (Petersen et al., 2000; Wang et al., 2007). Reverse transcription (RT)-PCR results showed that the transcript level of *GmMPK4* was greatly reduced in BPMV-*MPK4*-treated plants compared with BPMV empty vector control plants (Fig. 1F). As expected, the mRNA transcripts of the *PR2* (*Glyma19g31590*) gene were constitutively induced in *GmMPK4*-silenced plants (Fig. 1F), indicating that these plants exhibited constitutive defense responses.

There are four *GmMPK4* homologs that can be divided into two paralogous groups (Supplemental

Fig. S1). The amino acid identities within the groups are greater than 96%, whereas the identities between the groups are 88.7% (Supplemental Fig. S1). The silencing construct targeted nucleotides 436 to 738 of the *GmMPK4a* (*Glyma16g03670*) open reading frame. This construct can silence both *GmMPK4a* and *GmMPK4b* (*Glyma07g07270*), as the targeted sequence is greater than 97% identical between these two genes. To test whether *GmMPK4c* (*Glyma09g39190*) or *GmMPK4d* (*Glyma18g47140*) was also silenced by this construct, RT-PCR was performed for *GmMPK4c/4d* using a pair of primers that could amplify both. The transcript levels of *GmMPK4c/4d* were reduced in *GmMPK4*-silenced plants (Fig. 1G), demonstrating that all four of the *MPK4* homologs were silenced. As controls, *GmMPK6* was not silenced and *GmMPK3* was induced in *GmMPK4*-silenced plants (Fig. 1G). From here on, the term *GmMPK4*-silenced plants will refer to simultaneous silencing of the four isoforms, as it was not possible to distinguish the contribution of each isoform to the silenced phenotype in this study.

Overaccumulation of SA and H₂O₂ in *GmMPK4*-Silenced Plants

Constitutively activated defense responses are usually associated with increased production of SA (Chen and Klessig, 1991), and the *Arabidopsis mpk4* mutant

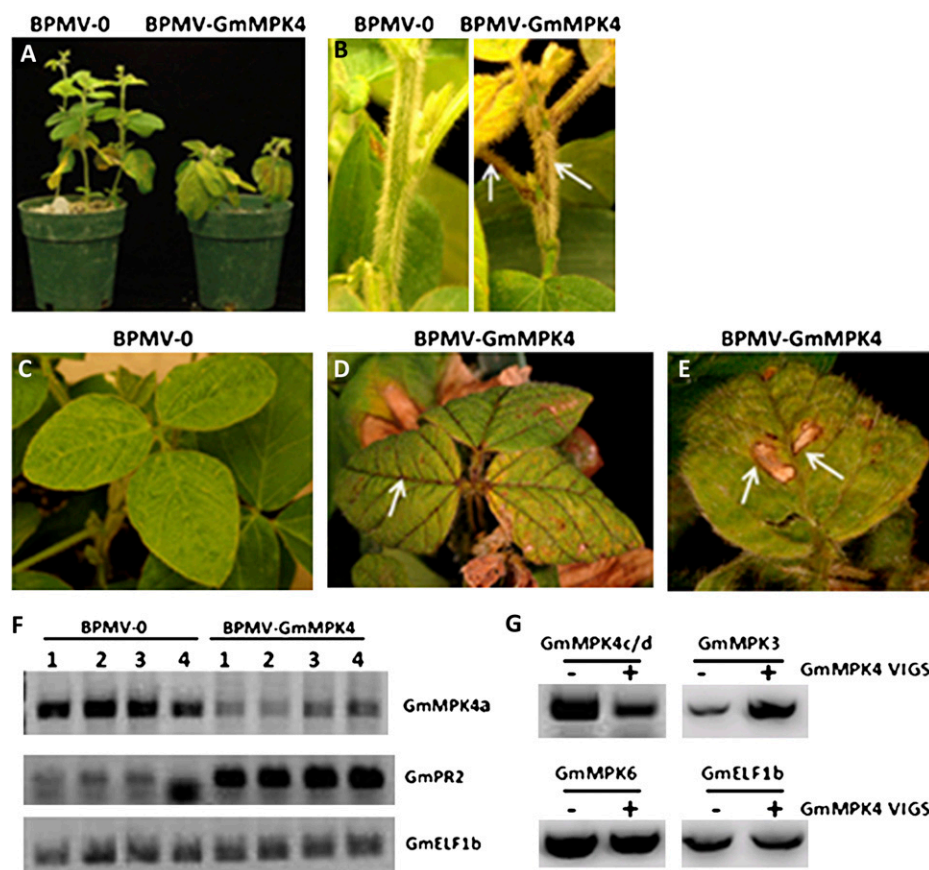


Figure 1. Silencing *GmMPK4* constitutively activates defense responses in soybean plants. A, Stunted stature. B, Purple necrosis on the stem. C, Symptoms of the empty BPMV vector (BPMV-0) on a trifoliate leaf. D, Rough leaves and purple pigmentation in the veins compared with vector control plants. E, Spontaneous cell death on the leaves of *GmMPK4*-silenced plants at 20 dpi. F, RT-PCR showing that the transcript levels of *GmMPK4* and *GmPR2* were reduced and induced, respectively, on the leaves of *GmMPK4*-silenced plants. *GmELF1b* served as a control. The results shown are from four individual *GmMPK4*-silenced plants (BPMV-*GmMPK4*) and BPMV vector control plants (BPMV-0). G, RT-PCR showing that *GmMPK4c/4d* was silenced, whereas *GmMPK3* and *GmMPK6* were not silenced, in *GmMPK4*-silenced plants.

plants accumulate 9- and 25-fold higher SA and salicylic acid glycoside (SAG), respectively, than the wild type (Petersen et al., 2000). To test whether SA accumulation was altered in *GmMPK4*-silenced plants, the levels of both SA and SAG were determined in the *GmMPK4*-silenced and vector control plants. As expected, SA and SAG levels increased 8.6- and 7.2-fold, respectively, in *GmMPK4*-silenced plants compared with vector control plants (Fig. 2A), indicating that the constitutively activated defense response is probably SA dependent.

H₂O₂ is a cell death executioner (Lamb and Dixon, 1997; Delledonne et al., 1998), and its levels are expected to be increased in plants with spontaneous cell death and constitutive defense responses, such as the *GmMPK4*-silenced plants (Fig. 1). To determine whether H₂O₂ accumulation was elevated, 3,3'-diaminobenzidine (DAB) staining (Thordal-Christensen et al., 1997; Ren et al., 2002) was performed on both *GmMPK4*-silenced plants and vector control plants. The brown color indicative of oxidized DAB was more intense on the leaves of *GmMPK4*-silenced plants than on vector control plants (Fig. 2B), indicating that the cell death observed in *GmMPK4*-silenced plants is associated with constitutive H₂O₂ accumulation.

Microarray Analysis

To investigate the effects of silencing *GmMPK4* on the soybean transcriptome, we analyzed gene expression in *GmMPK4*-silenced plants versus vector control plants using the GeneChip Soybean Genome Array (Affymetrix), which contains approximately 37,500 probe sets representing 35,611 soybean transcripts. Two treatments (*GmMPK4*-silenced versus BPMV vector control) and four biological replicates were used in this analysis, with each replicate comprising a pool of three plants. The top fully expanded trifoliolate leaves, petioles, and stem segments about 1 to 2 cm in length

were harvested at 20 d post BPMV inoculation (dpi), when the silencing phenotype was evident (Fig. 1). Total RNA was extracted for the synthesis of labeled copy RNA that was hybridized to the microarrays. The full microarray data sets generated from the eight GeneChips used in this study were deposited in the Gene Expression Omnibus (accession no. GSE29653; Edgar et al., 2002) and the Plant Expression database (accession no. GM25; Wise et al., 2007). The data were normalized using the robust multiarray average method, and probe sets identifying differentially expressed transcripts were determined by generating adjusted *P* values to control for the false discovery rate (FDR). Using the following cutoffs, *P* < 0.001, FDR < 1% (*q* < 0.01), and absolute value of fold change > 2, 4,205 and 5,267 probe sets were induced and repressed, respectively, in *GmMPK4*-silenced plants, demonstrating that a massive transcriptome reprogramming occurred. Such altered mRNA transcript profiles were not unexpected, because similar dramatic transcriptome changes have been previously shown in rice plants overexpressing OsMCK4^{DD} (Kishi-Kaboshi et al., 2010). Because of the extensive changes in gene expression, we analyzed the expression of 48 rRNA soybean probe sets and found that only one rRNA gene was identified as differentially expressed between vector control and *GmMPK4*-silenced plants (soybean_rRNA_1692_RC_at; *P* = 0.00085, *q* = 0.003; Supplemental Table S2). These data confirm that the large number of changes in gene expression were not due to sampling or technical errors and demonstrate that our microarray analysis of *GmMPK4*-silenced plants is biologically meaningful.

Functional Classification of Differentially Expressed Genes in *GmMPK4*-Silenced Plants

The annotations of the differentially regulated genes in *GmMPK4*-silenced plants relative to vector control plants were retrieved from SoyBase (<http://soybase.org/AffyChip/>), and the induced and suppressed gene sets were classified according to the Gene Ontology (GO) biological processes component. GO functional classes that were significantly overrepresented (*P* < 0.05) in the gene lists were obtained by using Fisher's exact test (Fisher, 1966; Draghici et al., 2003) with a Bonferroni correction to adjust for repetitive sampling. The majority of genes in the GO functional classes of biotic and abiotic defense responses were induced, whereas the majority of genes in GO functional classes of growth and development, cell cycle, and cell proliferation were repressed in *GmMPK4*-silenced plants relative to vector control plants (Table I; Fig. 3). The GO functional classes of biotic defense responses include SA-mediated systemic acquired resistance (GO:0009862 and GO:0009627), compatible and incompatible defense response to bacterium (GO:0042742 and GO:0009816), defense response to fungus (GO:0050832 and GO:0009817), and response to other organism (GO:0051707; Supplemental Table S3). GO functional classes corresponding to stress responses include water

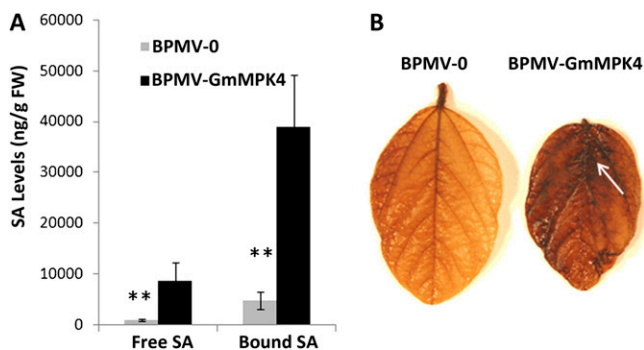


Figure 2. Overaccumulation of both SA and H₂O₂ in *GmMPK4*-silenced soybean plants. A, Both free SA and bound SA (SAG) levels were measured in *GmMPK4*-silenced and vector control plants at 20 dpi. Error bars represent SD for five independent samples; ** *P* < 0.01, Student's *t* test. FW, Fresh weight. B, The presence of H₂O₂ in the soybean leaves was visualized by staining with DAB. Oxidized DAB forms a reddish-brown deposit.

Table 1. Overrepresented GO biological process categories identified from microarray analysis of GmMPK4-silenced plants

GO Identifier	GO Description	Chip Count	Data Count	Fisher Test Two-Tailed <i>P</i>	Bonferroni Correction	No. of Genes		
						Fold Change > 2	Fold Change < 2	Up/Down
Biotic defense related								
GO:0009817	Incompatible interaction to fungus	38	24	6.9E-08	1.1E-04	23	1	23.00
GO:0031347	Regulation of defense response	37	24	3.2E-08	5.0E-05	22	2	11.00
GO:0009862	SA-mediated systemic acquired resistance	33	20	2.2E-06	3.5E-03	17	3	5.67
GO:0050832	Defense response to fungus	124	60	1.0E-10	1.6E-07	49	11	4.45
GO:0009607	Response to biotic stimulus	70	37	2.2E-08	3.3E-05	28	9	3.11
GO:0009627	Systemic acquired resistance	47	28	3.6E-08	5.5E-05	21	7	3.00
GO:0006952	Defense response	512	216	0.0E+00	0.0E+00	153	63	2.43
GO:0009816	Incompatible interaction to bacterium	112	56	1.0E-10	1.6E-07	37	19	1.95
GO:0042742	Defense response to bacterium	275	99	1.9E-07	2.9E-04	65	34	1.91
GO:0051707	Response to other organism	112	66	0.0E+00	0.0E+00	42	24	1.75
Abiotic stress related								
GO:0009414	Response to water deprivation	306	107	3.8E-07	5.9E-04	70	37	1.89
GO:0006979	Response to oxidative stress	402	149	0.0E+00	0.0E+00	96	53	1.81
GO:0009611	Response to wounding	284	125	0.0E+00	0.0E+00	79	46	1.72
GO:0009651	Response to salt stress	769	223	1.2E-05	1.9E-02	120	103	1.17
Metabolism and catabolism related								
GO:0051555	Flavonol biosynthetic process	50	29	4.7E-08	7.3E-05	20	9	2.22
GO:0009813	Flavonoid biosynthetic process	102	50	2.6E-09	4.1E-06	34	16	2.13
GO:0009809	Lignin biosynthetic process	109	53	1.8E-09	2.8E-06	35	18	1.94
GO:0009715	Chalcone biosynthetic process	17	12	2.8E-05	4.4E-02	11	1	11.00
GO:0010120	Camalexin biosynthetic process	27	18	9.6E-07	1.5E-03	18	0	–
GO:0009407	Toxin catabolic process	57	32	2.8E-08	4.4E-05	27	5	5.40
GO:0009805	Coumarin biosynthetic process	22	16	6.3E-07	9.9E-04	13	3	4.33
GO:0042362	Fat-soluble vitamin biosynthesis	12	10	1.2E-05	1.9E-02	8	2	4.00
GO:0006073	Glucan metabolic process	51	30	1.8E-08	2.7E-05	12	18	0.67
GO:0006629	Lipid metabolic process	237	116	0.0E+00	0.0E+00	41	75	0.55
GO:0005975	Carbohydrate metabolic process	403	178	0.0E+00	0.0E+00	45	133	0.34
Cell fate and proliferation								
GO:0035264	Multicellular organism growth	7	7	2.7E-05	4.3E-02	7	0	–
GO:0007275	Multicellular organismal development	162	84	0.0E+00	0.0E+00	75	9	8.33
GO:0045165	Cell fate commitment	9	9	1.3E-06	2.1E-03	3	6	0.50
GO:0010584	Pollen exine formation	19	13	2.2E-05	3.4E-02	3	10	0.30
GO:0010158	Abaxial cell fate specification	24	18	5.6E-08	8.7E-05	0	18	0.00

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Table I. (Continued from previous page.)

GO Identifier	GO Description	Chip Count	Data Count	Fisher Test Two-Tailed <i>P</i>	Bonferroni Correction	No. of Genes		
						Fold Change > 2	Fold Change < 2	Up/Down
GO:0042127	Regulation of cell proliferation	14	12	8.2E-07	1.3E-03	0	12	0.00
GO:0051726	Regulation of cell cycle	91	40	3.6E-06	5.5E-03	1	39	0.03
Hormone related								
GO:0009690	Cytokinin metabolic process	12	10	1.2E-05	1.9E-02	9	1	9.00
GO:0009751	Response to SA stimulus	212	79	7.5E-07	1.2E-03	65	14	4.64
GO:0009753	Response to JA stimulus	300	109	2.7E-08	4.2E-05	68	41	1.66
GO:0009695	JA biosynthesis	116	50	5.0E-07	7.7E-04	24	26	0.92
GO:0009733	Response to auxin stimulus	456	146	1.4E-06	2.1E-03	51	95	0.54
Others								
GO:0009915	Phloem loading	18	13	8.6E-06	1.3E-02	11	2	5.50
GO:0007047	Cell wall organization	12	10	1.2E-05	1.9E-02	8	2	4.00
GO:0006468	Protein amino acid phosphorylation	1,246	431	0.0E+00	0.0E+00	258	173	1.49
GO:0006355	Regulation of transcription	1,154	338	2.8E-08	4.4E-05	198	140	1.41
GO:0007165	Signal transduction	251	87	6.1E-06	9.6E-03	44	43	1.02
GO:0006508	Proteolysis	547	165	1.7E-05	2.6E-02	63	102	0.62
GO:0006869	Lipid transport	117	49	1.9E-06	2.9E-03	11	38	0.29
GO:0009828	Plant-type cell wall loosening	39	23	7.6E-07	1.2E-03	7	16	0.44
GO:0007169	Transmembrane receptor protein Tyr kinase signaling pathway	163	85	0.0E+00	0.0E+00	19	66	0.29
GO:0007017	Microtubule-based process	45	27	5.1E-08	7.9E-05	5	22	0.23
GO:0007018	Microtubule-based movement	66	34	2.4E-07	3.8E-04	3	31	0.10
GO:0006334	Nucleosome assembly	89	50	0.0E+00	0.0E+00	3	47	0.06

deprivation (GO:0009414), oxidative stress (GO:0006979), wounding (GO:0009611), and salt stress (GO:0009651). In addition to defense pathways, most of the genes in hormone pathways associated with defense responses, such as SA (GO:0009751) and JA (GO:0009753), as well as genes in secondary metabolism pathways, including chalcone (GO:0009715), flavonoid (GO:0009813), flavonol (GO:0051555), lignin (GO:0009809), and camalexin (GO:0010120) biosynthetic processes, were also significantly induced in *GmMPK4*-silenced plants (Table I; Supplemental Fig. S2).

WRKY transcription factors positively and negatively regulate defense responses (Eulgem et al., 2000; Cormack et al., 2002; Rushton et al., 2010). Significantly, in our gene list, 71 probe sets corresponding to genes encoding WRKY transcription factors were induced at least 2-fold, while only four WRKY genes were repressed by 2-fold (Fig. 3; Supplemental Table S4). Interestingly, the soybean ortholog of Arabidopsis WRKY33 (Glyma11g29720), which is negatively

regulated by MPK4 in Arabidopsis (Andreasson et al., 2005), was the most highly induced WRKY in *GmMPK4*-silenced plants (greater than 16-fold). MapMan analysis (Thimm et al., 2004) indicated that most differentially expressed PR genes and other defense-related genes were significantly induced in *GmMPK4*-silenced plants (Fig. 3; Supplemental Table S3). In addition to WRKYs, the expression of genes encoding transcription factors such as MYBs and bHLHs was also significantly altered in *GmMPK4*-silenced plants (data not shown). Taken together, these results clearly showed that the defense responses were constitutively activated in *GmMPK4*-silenced plants.

The phytohormone auxin has been linked to disease susceptibility, and both H₂O₂ and SA have inhibitory effects on auxin-responsive gene expression (Nakagami et al., 2006; Chen et al., 2007; Wang et al., 2007). As expected, a large number of genes (95 out of 456; GO:0009733) responsive to auxin were repressed in *GmMPK4*-silenced plants (Table I). Through manual

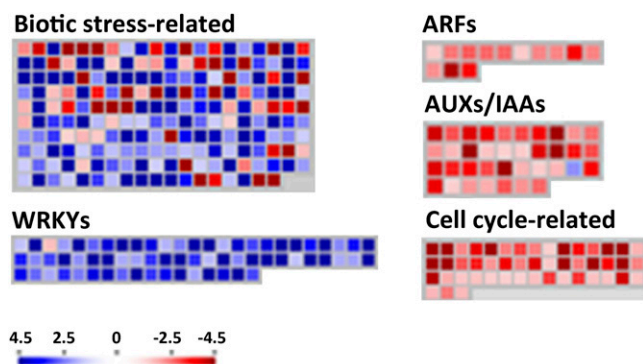


Figure 3. Examples of up- and down-regulated genes or gene families in *GmMPK4*-silenced plants versus vector control plants. Fold changes (\log_2) of gene expression values in *GmMPK4*-silenced plants versus vector control plants ($q < 0.01$) are shown. Blue indicates higher expression in *GmMPK4*-silenced plants, whereas red indicates higher expression in vector control plants. The diagrams were generated using the pathway analysis program MapMan (<https://gabi.rzpd.de/projects/MapMan/>; Thimm et al., 2004). Each square represents the fold change of one gene. Numbers +4.5 to -4.5 on the color scale represent relative \log_2 fold change. The minimum \log_2 fold change is 1 or -1.

searching, we identified an additional 37 differentially expressed genes either related to auxin signaling or auxin transport that were repressed at least 2-fold in *GmMPK4*-silenced plants, increasing the total number of repressed auxin-related genes to 132 (Supplemental Table S5). These genes include members of families encoding ARF and AUX/IAA (Fig. 3), auxin receptors (*TIR1* and *ABF1*), auxin transporter, and the auxin-inducible genes (Supplemental Table S5). Interestingly, some genes that are down-regulated in response to auxin were up-regulated in *GmMPK4*-silenced plants (data not shown). Together, these data indicated that auxin responses were impaired in *GmMPK4*-silenced plants. The down-regulation of auxin-induced genes as well as the genes in growth/development and cell cycle/proliferation (Fig. 3) was well correlated with the compromised growth and development phenotypes displayed by *GmMPK4*-silenced plants. These gene expression profiles indicate that GmMPK4s are central regulators that control the balance of gene expression between defense responses and growth/development. Apparently, the constitutively activated defense response in *GmMPK4*-silenced plants occurs at the expense of plant growth and development (Fig. 1).

To investigate whether the overproduction of SA was associated with the increased accumulation of transcripts of SA biosynthetic genes, we examined the mRNA transcript levels of genes responsible for SA biosynthesis in our microarray analysis. We found that the transcript level of *Isochorismate synthase1* (*ICS1*), a key gene in SA biosynthesis (Wildermuth et al., 2001), was not induced but repressed 8-fold, whereas eight out of 10 phenylalanine ammonia-lyase (*PAL*) genes were significantly induced in *GmMPK4*-silenced plants (data not shown), suggesting that the increased SA

level in *GmMPK4*-silenced plants was PAL dependent but *ICS1* independent, or that SA production was *ICS1* dependent but the expression of *ICS1* was feedback inhibited by its product, SA.

Silencing of *GmMPK4* Enhances Resistance against SMV and Downy Mildew

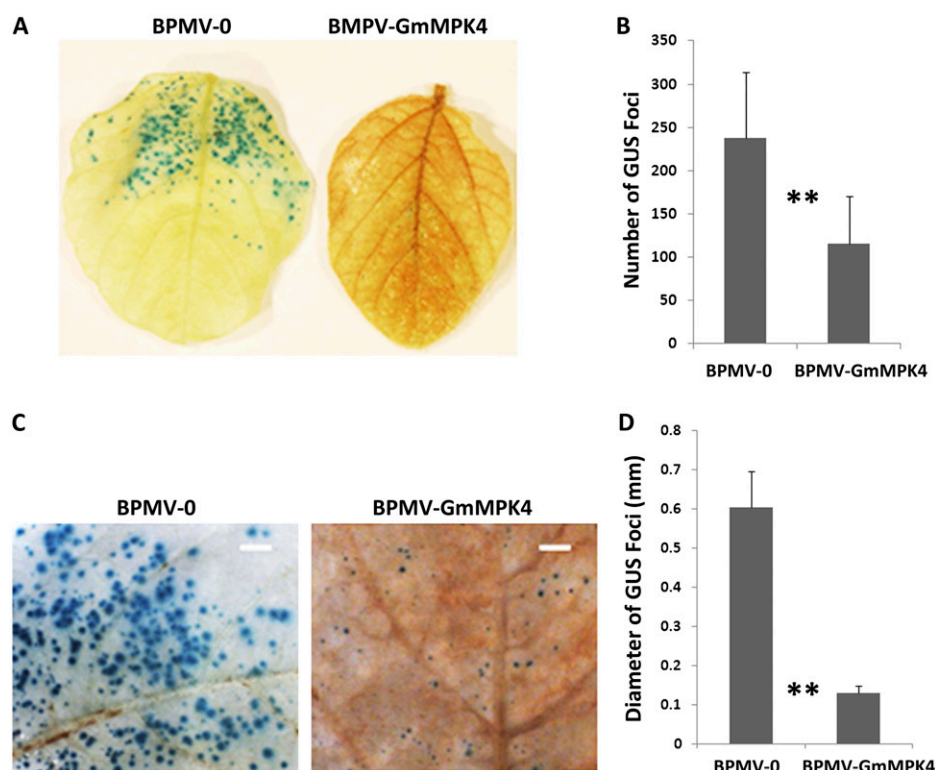
Constitutively activated defense responses usually correlate with enhanced disease resistance (Wang et al., 2007). To test this, *GmMPK4*-silenced and empty vector control plants were inoculated with an infectious clone of SMV that expresses the GUS enzyme (SMV-N-GUS; Wang et al., 2006; Fig. 4). Four individual leaves from four independent vector control plants or *GmMPK4*-silenced plants were used in this assay. At 3 dpi, the SMV-N-GUS infection was visualized by GUS staining (Fig. 4, A and C). The numbers of GUS foci were counted and the diameters of GUS foci were measured (Fig. 4, B and D). The numbers and sizes of GUS foci were significantly decreased (Student's *t* test, $P < 0.01$) by 2- and 4.65-fold, respectively, on *GmMPK4*-silenced leaves compared with vector control leaves, demonstrating that silencing *GmMPK4s* enhances resistance against SMV infection.

We next tested the effect of *GmMPK4* silencing on resistance to downy mildew infection. Chlorotic lesions caused by *P. manshurica* infection were observed on leaves of vector control plants at 7 dpi (Fig. 5A), whereas no lesions were observed on the leaves of *GmMPK4*-silenced plants (Fig. 5, B and D). To further confirm these results, *P. manshurica* mycelium was observed within infected leaves using a KOH-aniline blue staining procedure and fluorescence microscopy. As shown in Figure 5C, abundant hyphae were observed in mesophyll tissue of vector control plants showing chlorotic lesions, while no hyphae were observed within the mesophyll of the *GmMPK4*-silenced plants (Fig. 5D). Sporangia that germinated on the leaves of *GmMPK4*-silenced plants often produced germ tubes with multiple appressoria and no successful penetrations (Fig. 5E). This was not observed on the leaves of the vector control plants. The observation that *GmMPK4*-silenced plants exhibited enhanced resistance to SMV and downy mildew, two unrelated obligate pathogens, is consistent with the results observed for *Arabidopsis mpk4* mutants (Petersen et al., 2000) and *MPK4* mutant or RNA interference lines in rice (Shen et al., 2010).

GmMPK4a Subcellular Localization

To investigate the subcellular location of GmMPK4s, the full-length cDNA of *GmMPK4a* was fused to the C terminus of GFP and codelivered into onion (*Allium cepa*) epidermal cells with free DsRed via biolistic bombardment. GFP-GmMPK4a colocalized with free DsRed, indicating that GmMPK4a was present in both the cytosol and the nucleus (Fig. 6). This subcellular localization of GmMPK4a is consistent with the sub-

Figure 4. Silencing *GmMPK4s* enhances the resistance of soybean plants against SMV. At 18 dpi with BPMV constructs, SMV-N-GUS was biolistically delivered into detached leaves of either BPMV-0 or BPMV-*GmMPK4* plants. The number and diameter of GUS foci were determined at 3 dpi with SMV-N-GUS. A, Comparison of infection foci of SMV-N-GUS on the leaves of BPMV-0 and BPMV-*GmMPK4* plants. B, Comparison of the number of SMV-N-GUS foci on the leaves of BPMV-0 and BPMV-*GmMPK4* plants. C, Closeup images of GUS foci shown in A with a dissecting microscope. Bars = 2 mm. D, Comparison of the size of SMV-N-GUS foci on the leaves of BPMV-0 and BPMV-*GmMPK4* plants. The data shown in B and D are mean values of four individual leaves from four different plants. Error bars in B and D represent SD for four independent leaves. At least 30 GUS foci from each of four independent leaves were measured in D. ** $P < 0.01$, Student's *t* test.



cellular location observed for MPK4 in *Arabidopsis* (Andreasson et al., 2005). Interestingly, the rice MPK4 homolog is localized exclusively in the nucleus (Shen et al., 2010). As a control, a soybean WRKY transcription factor (Glyma04g39620) was exclusively localized in the nucleus (data not shown).

Determination of Upstream GmMKKs for GmMPK4a Using Bimolecular Fluorescence Complementation

In *Arabidopsis*, a negative regulatory role of the MEKK1-MKK1/2-MPK4 module in defense responses has been established (Nakagami et al., 2006; Suarez-Rodriguez et al., 2007; Gao et al., 2008; Qiu et al., 2008b). MKK1 and MKK2 are two closely related MKKs that interact with MPK4 and MEKK1 both in yeast and in vivo (Gao et al., 2008; Qiu et al., 2008b). To test whether a similar signaling module is also present in soybean, bimolecular fluorescence complementation (Walter et al., 2004) was performed to identify the GmMKKs that function upstream of MPK4a. In the soybean genome, there are two *AtMKK1* orthologs, *GmMKK1a* (Glyma15g18860) and *GmMKK1b* (Glyma09g07660), and two *AtMKK2* orthologs, *GmMKK2a* (Glyma17g06020) and *GmMKK2b* (Glyma13g16650). Both N-terminal yellow fluorescent protein (nYFP) and C-terminal (cYFP) fusions were constructed for all these kinases. The reciprocal combinations of nYFP and cYFP fusion plasmids of either GmMKK1/2 or GmMPK4a were cotransformed into onion epidermal cells. Regardless of C- or N-terminal YFP fusion combination, YFP

signals were detected in both the cytosol and the nucleus when GmMKK1 or GmMKK2 was coexpressed with GmMPK4a (Fig. 7, top and second panels). As a positive control, a strong YFP signal was observed in the nucleus when nYFP-AtbZIP and cYFP-AtbZIP were coexpressed (Fig. 7, third panel). As expected, no YFP signal was detectable when the cYFP-GmMPK4 fusion was coexpressed with the nYFP-GmMKK4 fusion (Glyma07g00520), regardless of combinations of C- or N-terminal fusions (Fig. 7, bottom panel).

Both GmMKK1 and GmMKK2 Phosphorylate GmMPK4a in Vitro

Because GmMKK1/2 interacted with GmMPK4, we tested whether GmMPK4a is phosphorylated by GmMKK1/2. Both MBP-GmMKK1/2 and MBP-GmMPK4a fusion proteins were expressed in *Escherichia coli* and purified for in vitro kinase assays. MBP-GmMPK4a had residual autophosphorylation activity (Fig. 8, lane 1). Even though no autophosphorylation activity was detectable for MBP-MKK1 or MBP-MKK2 (Fig. 8, lanes 2 and 3), both MBP-MKK1 and MBP-MKK2 could transphosphorylate MBP-GmMPK4a in vitro (Fig. 8, lanes 4 and 5), suggesting that GmMKK1/2 might function upstream of GmMPK4a. It appeared that MBP-GmMKK1 had higher transphosphorylation activity than MBP-GmMKK2 (Fig. 8, lanes 4 and 5). As a control, GmMPK4a was not phosphorylated by GmMKK4 (data not shown). Interestingly, several at-

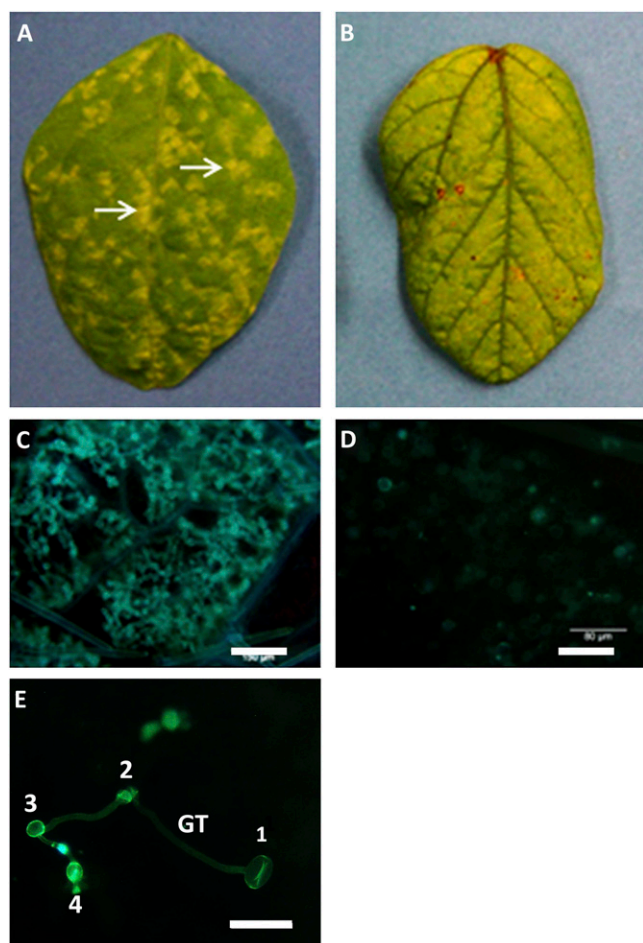


Figure 5. Silencing *GmMPK4s* enhances the resistance of soybean plants against downy mildew. A and B, Chlorotic lesions typical of soybean downy mildew were detected on the leaves of vector control plants (A) but not on the leaves of *GmMPK4*-silenced plants 1 week after inoculation with *P. manschurica* (B). C and D, *P. manschurica* hyphae were observed in the mesophyll of vector control plants (C) but not in the mesophyll of *GmMPK4*-silenced plants 1 week after inoculation with *P. manschurica* (D). E, Germ tubes (GT) with multiple appressoria that were not able to penetrate the epidermal surface were often observed on *GmMPK4*-silenced plants. 1, Sporangium; 2 to 4, appressoria. Bars = 150 μ m in C and D and 80 μ m in E.

tempts to silence *GmMKK1/2* alone or in combination by VIGS did not result in the expected constitutively activated defense response, as observed in *GmMPK4*-silenced plants (data not shown).

DISCUSSION

GmMPK4 Negatively Controls SA and H₂O₂ Levels

Induction of SA and a burst of reactive oxygen species (ROS) are common features of cell death and defense responses that are under both positive and negative regulation (Apel and Hirt, 2004; Jones and Dangl, 2006). The MEKK1-MKK1/2-MPK4 pathway has been estab-

lished as a negative regulator of SA and ROS signaling in Arabidopsis (Pitzschke et al., 2009a). Increased SA and ROS accumulation not only trigger cell death and defense, but they are also the consequence of the activation of MAPK signaling pathways (Yoshioka et al., 2003; Rentel and Knight, 2004; Nakagami et al., 2006; Pitzschke et al., 2009b). Here, we used BPMV-VIGS of *GmMPK4s* to test their function in soybean. Silencing *GmMPK4s* resulted in cell death on the leaves and stems, and it led to elevated levels of SA and H₂O₂ and constitutively activated defense responses (Figs. 1 and 2). These phenotypes are consistent with the spontaneous cell death, seedling lethality, and increased SA and H₂O₂ that were observed for Arabidopsis *mpk4*, *mkk1/2*, and *mekk1* mutants (Nakagami et al., 2006; Suarez-Rodriguez et al., 2007; Gao et al., 2008; Qiu et al., 2008b). Therefore, we conclude that, in soybean, the function of *GmMPK4s* as negative regulators of SA, ROS, and defense responses is conserved with the Arabidopsis homolog.

SA can be derived from chorismate or Phe. ICS1 is the key enzyme for SA biosynthesis derived from chorismate in response to pathogen attack, and the expression of Arabidopsis *ICS1* is induced locally and systemically upon bacterial and fungal infections (Wildermuth et al., 2001). Unexpectedly, we found that the transcripts of *ICS1* (Glyma01g25690) were repressed by 8-fold in *GmMPK4*-silenced plants. This suggests that the overaccumulation of SA in *GmMPK4*-silenced plants occurs in an *ICS1*-independent manner, possibly from precursors derived from the PAL pathway (Ribnicky et al., 1998; Huang et al., 2010), which would be consistent with the induction of genes encoding *PALs*. Alternatively, SA production is *GmICS1* dependent, but *GmICS1* expression is under negative feedback regulation by its own product, SA. The latter possibility is supported by the fact that treatment of Arabidopsis plants with SA suppressed the induction of *ICS1* expression triggered by ozone (Ogawa et al., 2007).

Increasing evidence indicates that there is antagonism between the SA and JA pathways (Durrant and Dong, 2004). In the Arabidopsis *mpk4* mutant, the SA pathway is activated while the JA pathway is repressed (Petersen et al., 2000). To our surprise, among 109 differentially regulated genes responsive to JA stimulus, 68 of them

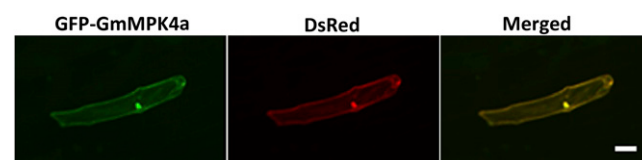
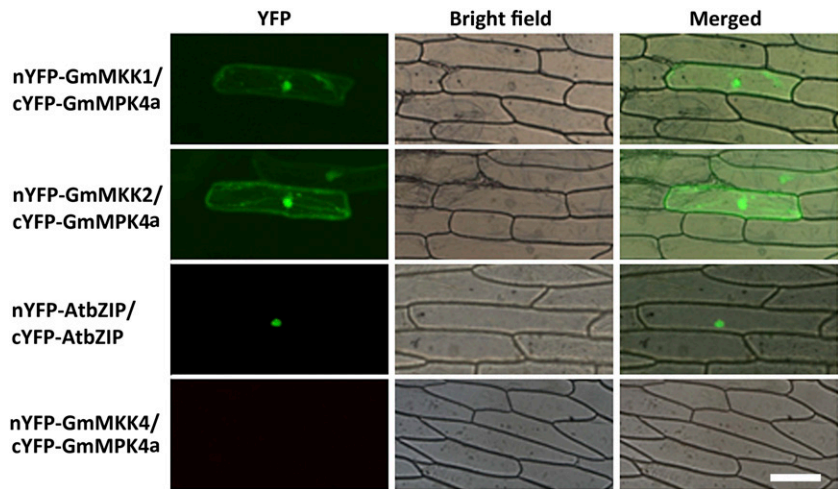


Figure 6. *GmMPK4a* is localized to both cytoplasm and nucleus. The GFP-*GmMPK4a* and free DsRed constructs driven by the 35S promoter were cotransformed into onion epidermal cells. The left panel shows the transient expression of GFP-*GmMPK4a* in onion cells; the middle panel shows the transient expression of free RFP in onion cells; and the right panel shows the merged image. Bar = 100 μ m.

Figure 7. Both GmMKK1 and GmMKK2 interact with GmMPK4a. YFP epifluorescence (left panels), bright-field (middle panels), and merged (right panels) images of onion epidermal cells cobombarded with constructs expressing different fusion proteins as indicated are shown. Coexpression of nYFP-AtbZIP and cYFP-AtbZIP was used as a positive control, and cobombardment of nYFP-GmMKK4 and cYFP-GmMPK4a was used as negative control. Bar = 100 μ m.



were up-regulated in *GmMPK4*-silenced plants relative to vector control plants (Table I). This observation indicates that JA signaling might be activated in *GmMPK4*-silenced plants, opposite to the repression observed in the Arabidopsis *mpk4* mutant. Significantly, a hallmark JA-responsive gene (Glyma13g35320) encoding defensin had the greatest fold change (315-fold) in *GmMPK4*-silenced plants (Supplemental Table S3). These data raise the possibility that the role of MPK4s in regulating the JA signaling pathway is different in soybean and Arabidopsis.

Repression of Growth and Development Is a Common Theme of Constitutively Activated Defense Responses

The robust and consistent phenotype of *GmMPK4*-silenced plants allowed us to couple VIGS with a variety of additional assays. Transcriptome analysis showed that genes positively associated with growth and development were suppressed, whereas defense-related genes were induced, in *GmMPK4*-silenced plants (Table I; Supplemental Tables S3 and S5). This gene expression profile is well correlated with the phenotypes of *GmMPK4*-silenced plants that included impaired growth and development (Fig. 1) and enhanced resistance to viral and oomycete pathogens (Figs. 3 and 4). The specificity of these observations is further substantiated when the expression of gene family members associated with plant defenses or growth and development is compared. For example, *MYB84* (Glyma08g04670.1) is a transcription factor required for resistance to Asian soybean rust (Pandey et al., 2011), and it was induced 16-fold in *GmMPK4*-silenced plants. However, *GmMYB091* (Glyma03g19030.1), an ortholog of Arabidopsis AS1/AtMYB091 that has been shown to play a role in leaf symmetry (Theodoris et al., 2003), was suppressed 3.5-fold. These results support the conclusion that constitutively activated defenses are negatively correlated with plant growth and development.

Constitutively activated defense responses and stunted growth patterns, two intricately intertwined

processes, are common features of Arabidopsis mutants such as *mpk4*, *mkk1/2*, *mekk1*, *lsd1*, *snc1*, *bap1*, and *cpr6* that have elevated SA levels (Dietrich et al., 1997; Yang and Hua, 2004; Wang et al., 2007; Yang et al., 2007; Pitzschke et al., 2009a). The *mpk4* mutant phenotype is SA dependent and NPR1 independent, because overexpression of *nahG* partially suppresses *mpk4* dwarfism and fully suppresses its constitutively activated defense responses, whereas *mpk4/npr1-1* double mutants fully retain the *mpk4* dwarf stature and constitutively activated defense response (Petersen et al., 2000). The drastic increase in SA levels of *GmMPK4*-silenced plants (Fig. 2) suggests that their dwarfism and constitutively activated defense response are also SA dependent. However, in some cases, dwarfism and constitutively activated defense responses can be uncoupled in *mpk4 nahG* or *mekk1 sid2* double mutants that have reduced SA levels (Petersen et al., 2000; Nakagami et al., 2006). These results suggest that the dwarfism in MPK4 pathway loss-of-function plants might be only partially SA dependent and that the MPK4 pathway has additional roles in regulating growth and development beyond negatively regulating SA levels.

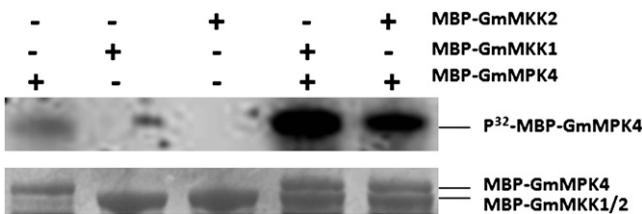


Figure 8. GmMKK1 and GmMKK2 phosphorylate GmMPK4a in vitro. The top panel is an autoradiograph of the phosphorylation assay, and the bottom panel is the same SDS gel stained with Coomassie Brilliant Blue. Lanes are as follows: MBP-GmMPK4a alone (lane 1), MBP-GmMKK1 alone (lane 2), MBP-GmMKK2 alone (lane 3), MBP-GmMPK4a + MBP-GmMKK1 (lane 4), and MBP-GmMPK4a + MBP-GmMKK2 (lane 5).

A mechanistic link between constitutively activated defense responses and dwarfism is revealed by studies focused on pathogen-auxin interactions. Arabidopsis plants with constitutively activated defense responses often display morphological phenotypes that are reminiscent of auxin-deficient or auxin-insensitive mutants, suggesting a role for auxin in these phenotypes (Wang et al., 2007). This inhibitory effect on the auxin signaling pathway is SA and ROS dependent (Kovtun et al., 2000; Nakagami et al., 2006; Wang et al., 2007). SA treatment causes global repression of auxin-related genes (Wang et al., 2007), and ROS-overaccumulating mutants, including *mekk1* and *mpk4*, have reduced expression of several auxin-inducible marker genes (Nakagami et al., 2006). Consistent with this conclusion, our transcriptome analysis showed that 132 genes associated with auxin-related pathways were repressed at least 2-fold in *GmMPK4*-silenced plants, which contain significantly higher amounts of both SA and H₂O₂ (Fig. 2; Supplemental Table S5). The fact that the number of auxin-related genes that are repressed in *GmMPK4*-silenced plants is significantly higher than in SA-treated Arabidopsis plants (132 versus 22; this study versus Wang et al., 2007) suggests that H₂O₂, SA-H₂O₂ interaction, or other functions of GmMPK4s might account for the repression of the additional auxin pathway genes.

WRKY Transcription Factors Are Central Regulators of Defense Transcriptional Networks in Soybean

Our microarray analysis showed that a genome-wide transcriptome reprogramming occurred in *GmMPK4*-silenced plants (Table I; Supplemental Tables S3–S5). The classes of transcription factors with the most dramatically altered mRNA expression included WRKYs (Supplemental Table S2), MYBs, and bHLHs. Plant immune responses are closely associated with the concerted modulation of a large number of different WRKYs (Eulgem and Somssich, 2007). In Arabidopsis, about 50 *AtWRKY* genes are differentially expressed upon triggering SA-dependent defense responses (Dong et al., 2003), and 46 soybean WRKY probe sets were differentially regulated at the mRNA level in response to Asian soybean rust infection (van de Mortel et al., 2007). Similarly, in *GmMPK4*-silenced plants, 71 WRKY probe sets were induced and only four WRKY probe sets were suppressed by at least 2-fold (Supplemental Table S4), suggesting that GmWRKYs are heavily involved in soybean defense responses. The W box, a WRKY-binding motif, is enriched in the promoter regions of WRKY-regulated genes (Dong et al., 2003). Interestingly, the W box is also enriched in the promoter regions of some *AtWRKY* genes, suggesting that their expression is autoregulated by other *AtWRKY* proteins (Eulgem and Somssich, 2007). The expression of *AtNPR1* is regulated by WRKYs, and its promoter region is enriched with three W boxes (Yu et al., 2001). As is the case in Arabidopsis, we found that various numbers of W boxes are present in the promoter

regions of some *GmWRKY* genes (Supplemental Table S3), *GmNPR1a/1b*, as well as many defense-related genes (data not shown). In addition, we found that the expression of other families of transcription factors may also be under the control of WRKYs. For example, *GmMYB84* (Glyma05g35050.1 or Glyma08g04670), which is required for *Rpp2*-mediated resistance against Asian soybean rust (Pandey et al., 2011), was induced 16-fold in *GmMPK4*-silenced plants, and three W boxes were identified in its promoter region (Glyma05g35050; Pandey et al., 2011).

WRKY33 in Arabidopsis is an indirect target of MPK4 mediated by MKS1 (Qiu et al., 2008a). MPK4 functions to sequester WRKY33 in the nucleus and prevent it from activating downstream genes (Qiu et al., 2008a). Interestingly, *GmWRKY33* was induced more than 16-fold in *GmMPK4*-silenced plants (Supplemental Table S4), indicating that GmMPK4s negatively control GmWRKY33 function not only at the posttranslational level but also at the transcriptional level. The differential expression of a large number of WRKY transcription factors coupled with the altered expression of genes with W boxes in their promoters suggests that many WRKYs function in signaling networks that are negatively regulated by MPK4 in both Arabidopsis and soybean.

Arabidopsis WRKY28 and WRKY46 are transcriptional activators of *ICS1* and *PBS3* (for *avrPphB* susceptible 3), respectively (van Verk et al., 2011). In our microarray analysis of *GmMPK4*-silenced plants, *GmWRKY46* was induced by 2-fold and the expression of *GmWRKY28* was unchanged (Supplemental Table S4). Accordingly, *GmPBS3* (Glyma3g30590) was induced (2-fold) in *GmMPK4*-silenced plants, suggesting that the function of WRKY46 may be conserved between Arabidopsis and soybean. The fact that *GmWRKY28* was not induced and *GmICS1* was repressed in *GmMPK4*-silenced plants indicated that, unlike in Arabidopsis, SA production in *GmMPK4*-silenced plants might be independent of GmWRKY28 and GmICS1.

Potential Redundancies among MKK Homologs in Soybean

Because GmMKK1 and GmMKK2 could interact with and phosphorylate GmMPK4 (Figs. 7 and 8), we expected to see the *GmMPK4*-silenced phenotype in plants infected with BPMV constructs targeting *GmMKK1* and *GmMKK2* for silencing. In Arabidopsis, the *mpk4* mutant phenotype is seen only in the *mkk1 mkk2* double mutant but not in *mkk1* or *mkk2* single mutants (Gao et al., 2008; Qiu et al., 2008b). However, silencing *GmMKK1* and *GmMKK2* individually or together resulted in phenotypes similar to the empty vector control (data not shown), suggesting that other *GmMKK* genes are functionally redundant with *GmMKK1* and *GmMKK2*. This statement is supported by the fact that there is an additional MKK1/2-like gene, *Glyma02g32980*, in the soybean genome. Alter-

natively, it is possible that BPMV VIGS did not adequately silence both *GmMCK1* and *GmMCK2* to the level needed to observe the *GmMPK4*-silenced phenotype. It will be interesting to further investigate whether simultaneous silencing of multiple *GmMCK1*, *GmMCK2*, and *GmMCK1/2*-like genes will result in cell death and constitutive defense responses.

Enhanced Resistance against SMV and Downy Mildew in *GmMPK4*-Silenced Plants

The constitutively activated defense responses in *GmMPK4*-silenced plants (Figs. 1 and 3; Table I) correlated with enhanced resistance against viral and oomycete pathogens (Figs. 4 and 5), both obligate biotrophs. However, distinct attributes of the constitutive defense response are likely to underlie the mechanisms of resistance to each pathogen. Downy mildew hyphae were not observed in the mesophyll of *GmMPK4*-silenced plants (Fig. 5, B and D), but germ tubes with multiple appressoria were observed on the surface (Fig. 5E), strongly suggesting that downy mildew could not penetrate through the epidermal cells of *GmMPK4*-silenced plants. This could be due to the changes in cell wall structure and/or components in *GmMPK4*-silenced plants. Lignin is produced by plants to fortify cell walls. Conversion of Phe to trans-cinnamic acid by PAL and the transfer of a methyl group from caffeic acid to ferulate by *O*-MT are the two key steps in lignin biosynthesis. When either *GmPAL1* (Glyma02g47940) or *GmO-MT* (Glyma07g05480) is silenced, *Rpp2*-mediated resistance against Asian soybean rust was compromised in *Rpp2* plants (Pandey et al., 2011). Interestingly, we observed 10.2- and 13.7-fold inductions of *GmPAL1* (Glyma02g47940) and *GmO-MT* (Glyma07g05480), respectively, in *GmMPK4*-silenced plants in comparison with vector control plants. In addition, many genes mapping to the phenylpropanoid and lignin pathways were up-regulated in *GmMPK4*-silenced plants (Supplemental Fig. S2). These observations indicate that increased lignin biosynthesis might contribute, at least partly, to the enhanced resistance against downy mildew observed in *GmMPK4*-silenced plants (Fig. 5). Additionally, other antimicrobial compounds (phytoalexins) produced from precursors of the phenylpropanoid pathway may also contribute to enhanced downy mildew resistance (Table I).

The effect of knocking out or knocking down *GmMPK4s* on virus infection has not been investigated previously in any plant species, to our knowledge. We showed that silencing *GmMPK4s* led to reduced sizes and numbers of SMV-N-GUS infection foci (Fig. 4). The compromised SMV-N-GUS infection could be due to reduced replication, reduced movement, or both. Viruses require cytoskeletal components, such as microtubules and microfilaments, for intracellular and intercellular movement (Liu et al., 2005; Harries et al., 2010; Niehl and Heinlein, 2011). For instance, silencing of a soybean actin gene (Glyma08g15480) resulted in reduced SMV-N-GUS foci (Zhang et al., 2009). Inter-

estingly, our microarray data suggested that genes in microtubule-based process (GO:0007017) and microtubule-based movement (GO:0007018) were significantly down-regulated in *GmMPK4*-silenced plants (Table I). In addition, the majority of actin genes, including Glyma08g15480, were also down-regulated in *GmMPK4*-silenced plants (data not shown). Together, these data suggest that the reduced size of SMV-N-GUS foci on *GmMPK4*-silenced plants could be due, in part, to compromised intracellular and/or intercellular movement resulting from the down-regulation of genes encoding microtubules and microfilaments. The alternative possibility is that viral replication/movement are impaired at the same time in *GmMPK4*-silenced plants, due to the induction of inhibitory factors and/or the down-regulation of factors necessary for efficient replication and movement.

CONCLUSION

Our goal is to develop an understanding of the defense signaling networks in the row crop plant soybean, which will rely on developing novel information about soybean defenses as well as building upon and transferring the knowledge gained from model systems like *Arabidopsis*. This work is enabled by the soybean genome sequence and functional genomics resources such as BPMV VIGS that can be used to develop hypotheses and test gene functions. In model plants such as *Arabidopsis*, MPK4 has been established as an important node in the regulation of defense responses as well as growth and development. Our results indicate that soybean *GmMPK4s* are negative regulators of defense responses and positive regulators of growth and development, suggesting that MPK4 functions are evolutionarily conserved across plant species.

MATERIALS AND METHODS

Plant Materials

Seeds of soybean (*Glycine max* 'Williams 82') used in this study were harvested from greenhouse-grown plants previously indexed for the absence of BPMV and SMV (Zhang et al., 2009, 2010). Soybean plants were maintained in a greenhouse or growth chamber at 22°C with a photoperiod of 16 h.

BPMV-Mediated VIGS

BPMV strains, BPMV VIGS constructs, and inoculation of soybean seedlings with DNA-based BPMV constructs via biolistic particle bombardments using a Biolistic PDS-1000/He system (Bio-Rad Laboratories) have been described previously (Zhang et al., 2009). The orthologs of *Arabidopsis* (*Arabidopsis thaliana*) MPKs or MKKs in the soybean genome were identified by reciprocal BLASTN between the National Center for Biotechnology Information and Phytozome databases. The primers used for the *GmMPK4* silencing construct are *GmMPK4a-F* (Glyma16g03670) (5'-AAGGGATCCCTGTATCAATTGTTACGAGGGCT-3') and *GmMPK4a-R* (5'-TTGGGTACCCCTCTGTGATAAGTCTCAGCTGATGA-3'). The primers used for silencing the 3' untranslated region of *GmMPK4a* are *GmMPK4a-3'UTR-F* (5'-AAGGGATCCCTATATACATTTTATGACCACTACTTGGC-3') and *GmMPK4a-3'UTR-R* (5'-AAGGGATCCCACTATGGACCTTGAATCTAAAAAGA-3'). The primers

used for amplifying both *GmMPK4c/4d* are *GmMPK4c-F* (5'-ATGGCTCTT-GAGTCAGCTCCT-3') and *GmMPK4c-R* (5'-TCAATAAATAGGTGGATCAG-GATTG-3'). The primers used for MKK1/MKK2 fusion constructs were *GmMKK2-F* (5'-AAGGGATCCAAAAGGATATATGGAGTTGGGACT-3'), *GmMKK2-fu-R* (5'-CAGCCTTAAACGTTCACATTCTAAGGTTGCAAGAG-GAGATCCT-3'), *GmMKK1-fu-F* (5'-AGGATCTCCTCTTGCAACCTTAGAA-AGTGAACGTTTAAGGCTG-3'), and *GmMKK1-R* (5'-TGGGTACCCCT-TGTGCTGATTGATTAATTTTAGCTC-3'). The *GmMKK2* and *GmMKK1* fragments were amplified, respectively, using *GmMKK2-F/GmMKK2-fu-R* and *GmMKK1-fu-F/GmMKK1-R*. The *GmMKK2-GmMKK1* fusion was then amplified using *GmMKK2-F/GmMKK1-R* as primer pair and the amplified *GmMKK1* and *GmMKK2* fragments as templates. The underlined sequences are *Bam*HI and *Kpn*I restriction sites, respectively, in front of forward and reverse primers. The boldface letter indicates an extra nucleotide in reverse primers needed to maintain the reading frame.

RNA Isolation and RT-PCR

RNA isolation and RT-PCR were performed as described elsewhere (Liu et al., 2005). For microarray analysis, the top fully expanded trifoliolates, petioles, and stems of about 1 to 2 cm in length were harvested at 20 dpi. The RNA samples extracted were treated with DNaseI according to the manufacturer's instructions (Invitrogen). Primers used in this study are *GmMPK4-F* (5'-ATGTCGTCTT-GAGTCAGCTG-3'), *GmMPK4-R* (5'-TCAGTAGACTGGTGAACAGGATT-3'), *GmELF1b-F* (5'-ACCGAAGAGGGCATCAAATCCC-3'), *GmELF1b-R* (5'-CTCA-CTGTCAAGCGTTCCTC-3'), *GmPR2-F* (5'-TGAAATAAGGGCCACGAGT-CCAAATG-3'), and *GmPR2-R* (5'-ATGGTACATGCAGACTTCAAGATGCA-GAT-3').

SA Quantification

SA was quantified using an Agilent 1100 HPLC apparatus with fluorometric detection (Agilent Technologies). The column was a 4.6- × 75-mm Agilent RR XDB C18 used with an isocratic mobile phase composed of 75% 20 mM formate, pH 3.8, 20% methanol, and 5% acetonitrile at a flow rate of 0.75 mL min⁻¹ at 35°C. SAG 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%.

H₂O₂ Detection by DAB Staining

H₂O₂ 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⁻¹ DAB (pH 5.5) for 2 h. The leaves were cleared by boiling in ethanol (96%) for 10 min and then stored in 96% ethanol. H₂O₂ production was visualized as a reddish-brown precipitate in cleared leaves (Karimi et al., 2002).

Microarray Labeling, Hybridization, and Scanning

Microarray labeling, hybridization, and scanning were performed at the Iowa State University GeneChip Facility (www.biotech.iastate.edu/facilities/genechip/Genechip.htm). Briefly, RNA samples were adjusted to a concentration of 0.1 µg µL⁻¹, and RNA concentration and quality were determined by RNA Nano LabChip on a 2100 Bioanalyzer (Agilent Technologies). The synthesis of labeled target copy RNA used 5 µg of total RNA and was performed using the GeneChip One-Cycle Target Labeling and Control Reagents kit (Affymetrix) according to the manufacturer's instructions. Fragmented copy RNA (10 µg) was hybridized to GeneChip Soybean Genome Arrays (Affymetrix) according to the manufacturer's instructions. The quality of fragmented copy RNA was verified on an Agilent 2100 Bioanalyzer with an RNA Nano LabChip. Washes were performed using the EukGE-WS2v5_450 washing protocol, and microarrays were scanned with a GCS3000 7G scanner (Affymetrix).

Microarray Data Analysis

Data were normalized using the robust multiarray average method (Irizarry et al., 2003) as implemented in the Bioconductor R package *affy* (Gautier et al., 2004). A linear model analysis of the normalized data was conducted for each gene using the Bioconductor R package *limma* (Smyth,

2004, 2005). Because the data were collected using a completely randomized design, each linear model included only a single factor, treatment, with two levels (VIGS vector control and MPK4 silenced). A *t* test for differential expression was conducted as part of each linear model analysis. The *P* values from these tests were adjusted using the method of Benjamini and Hochberg (1995) to enable the identification of differentially expressed genes while maintaining approximate control of the FDR.

Probe Annotation

In order to annotate the differentially expressed probes, we took advantage of the SoyBase Affymetrix GeneChip Soybean Genome Array annotation Web page (www.soybase.org/AffyChip). This resource allows users to download annotation information for any probes of interest. Annotation data include matching cDNAs from the whole soybean genome assembly (Schmutz et al., 2010), best BLAST (Altschul et al., 1997) matches from the uniref100 protein database (Apweiler et al., 2004), and best Arabidopsis matches and corresponding GO information (Ashburner et al., 2000) from The Arabidopsis Information Resource (www.arabidopsis.org).

Identification of Overrepresented GO Categories

Custom Perl scripts were used to count and compare individual GO Biological Process categories from our differentially expressed probe list to all probe sets available on the entire soybean genome array. Fisher's exact test (Fisher, 1966) was used to identify probe sets overrepresented in our data set when compared with the array. To correct for repeat sampling, a Bonferroni correction was applied. Only GO categories significant after Bonferroni correction (*P* < 0.05) are reported. The display and gene classifications are based on MapMan (Thimm et al., 2004; <http://gabi.rzpd.de/projects/MapMan/>).

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

At 18 dpi with *BPMV* vector only (*BPMV-0*) or *BPMV-GmMPK4* constructs, second fully expanded soybean trifoliolate leaves counting from the top were detached and biolistically inoculated with SMV-N-GUS (Wang et al., 2006; Zhang et al., 2009). Following SMV-N-GUS inoculation, the detached leaves were put into petri dishes with moist filter papers and kept on a lighted growth shelf for 3 d before GUS staining. GUS staining was performed as described (Jefferson, 1989). Photographs of the leaves with GUS foci were taken using a stereo microscope (Olympus SZH10). The numbers of GUS foci were counted, and the diameters of GUS foci were measured using Soft Image System analysis (IA Package; Olympus).

Downy Mildew Infection

The isolate of *Peronospora manschurica* used in these studies was obtained from naturally infected plants in Ames, Iowa, in 2008. The isolate is maintained by periodic transfer on soybean plants (cv Williams 82) in the greenhouse. Vector control and *GmMPK4*-silenced soybean plants were inoculated by spraying with a suspension of *P. manschurica* sporangia in deionized water (10⁴ sporangia mL⁻¹). Plants were held in the dark at high humidity overnight and then moved to the greenhouse for 7 d. Symptoms were observed and samples for microscopy were collected 1 week after inoculation. Pathogen structures on and in plant tissues were visualized using a KOH-aniline blue staining procedure (Hood and Shew, 1996). Tissues were excised from plants 1 week after inoculation. 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 in three changes of distilled water and soaked in 0.05% aniline blue in 0.7 M K₂HPO₄, 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 GmMPK4 and Bimolecular Fluorescence Complementation Analysis

The full-length cDNA of *GmMPK4* (Glyma16g03670) was amplified by RT-PCR from total RNA extracted from Williams 82 soybean plants. The PCR product was initially cloned into pENTR/D TOPO vector (Invitrogen) and then recombined into the binary destination vector pB7WGF2.0 (Karimi et al., 2002) to generate the GFP-GmMPK4 fusion construct. This fusion construct and the free DsRed construct were cobombarded into onion (*Allium cepa*) epidermal cells (Biolistic PDS-1000/He system; Bio-Rad Laboratories) as described (Zhang et al., 2009). Images were captured with an inverted AxioPhot microscope (Zeiss) equipped with a digital camera (Diagnostic Instruments).

The full-length cDNAs of *GmMKK1* (Glyma15g18860), *GmMKK2a* (Glyma17g06020), *GmMKK2b* (Glyma13g16650), *GmMKK4* (Glyma07g00520), and *GmMPK4* (Glyma16g03670) were cloned into pENTR/D vector (Invitrogen) as described above and then recombined into destination vectors pE-SPYCE-GW and pE-SPYNE-GW, which contain the C-terminal and N-terminal regions of YFP, respectively (Walter et al., 2004), via attLattR reaction (Invitrogen). All constructs were confirmed by sequencing. The different combinations of constructs expressing SPYCE and SPYNE fusion proteins were cobombarded into onion epidermal cells, and images were captured as described above. The primers used for full-length cDNA amplification are *GmMPK4-F* (5'-CACCATGCTCTGCTGTGA-GTCAGCTG-3'), *GmMPK4-R* (5'-CAGTAGACTGGTGGAAACAGGATT-3'), *GmMKK1-F* (5'-CACCATGAAGAAAGCAGGGAGCATAAG-3'), *GmMKK1-R* (5'-CTATATGGTTGCAAGTGACATCCTG-3'), *GmMKK2a-F* (5'-CACCAT-GAAGAAAGGAAAGTGGGTCTT-3'), *GmMKK2a-R* (5'-TTATAAGGTTG-CAAGAGGAGATCC-3'), *GmMKK2b-F* (5'-CACCATGAAGAAAGGAAAC-TTGGGTCTT-3'), *GmMKK2b-R* (5'-TTATAAGGTTGCAAGAGGAGATCC-3'), *GmMKK4-F* (5'-CACCATGAGGCCGATGCAACTG-3'), and *GmMKK4-R* (5'-CTA-GGAAGGAAGAGGCCTTGG-3').

Protein Purification and in Vitro Kinase Assay

Full-length cDNAs encoding GmMPK4a, GmMKK1, and GmMKK2 were cloned into pMAL-c2 (New England Biolabs) to generate MBP-GmMPK4a, MBP-GmMKK1, and MBP-GmMKK2 fusion constructs. The constructs were verified by sequencing. The fusion proteins were expressed and purified by amylose-affinity chromatography (New England Biolabs) and quantified by Bio-Rad protein assay reagent.

The in vitro phosphorylation assays were performed as described (Lee et al., 2008). Briefly, each glutathione *S*-transferase (GST)-MPK (0.5 μ g) was incubated in 25 μ L of kinase reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM β -glycerolphosphate, 2 mM dithiothreitol, 10 mM MgCl₂, 0.1 mM Na₃VO₄, 0.1 mM ATP, and 3 μ Ci of [γ -³²P]ATP) either with or without GST-MKK1 or GST-MKK2 (0.2 μ g) at 30°C for 45 min. The reaction was terminated by the addition of concentrated SDS-PAGE sample buffer followed by boiling for 8 min. Reaction products were analyzed using SDS-PAGE, autoradiography, and Coomassie Brilliant Blue staining.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Dendrogram of GmMPK4 homologs and AtMPK4.

Supplemental Figure S2. Expression of genes in secondary metabolism pathways in *GmMPK4*-silenced plants versus vector control plants.

Supplemental Table S1. Soybean MAPK, MAPKK, and MAPKKK genes and primers for VIGS cloning.

Supplemental Table S2. FDR (*q* value) and log₂ fold change (FC) of rRNA probe sets.

Supplemental Table S3. FDR (*q* value) and log₂ fold change (FC) of defense-related probe sets.

Supplemental Table S4. WRKY probe sets differentially expressed in *GmMPK4*-silenced plants.

Supplemental Table S5. Differentially expressed probe sets corresponding to auxin-regulated genes in *GmMPK4*-silenced plants.

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