Soybean Homologs of MPK4 Negatively Regulate Defense Responses and Positively Regulate Growth and Development

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Soybean Homologs of MPK4 Negatively Regulate Defense Responses and Positively Regulate Growth and Development

Abstract
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.

Disciplines
Agricultural Science | Agriculture | Plant Pathology

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

Jian-Zhong Liu, Heidi D. Horstman, Edward Braun, Michelle A. Graham, Chunquan Zhang, Duroy Navarre, Wen-Li Qiu, Yeunsook Lee, Dan Nettleton, John H. Hill, and Steven A. Whitham* 

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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 (GmMAPK4s) 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 GmMK1 and GmMK2 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 SIPKKE 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 (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 Plakhapsora 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 Rso1-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 manshurica), 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 trifoliolate 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.

H$_2$O$_2$ 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 H$_2$O$_2$ 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 H$_2$O$_2$ 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 OsMKK4DD (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
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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).

### Table I. (Continued from previous page.)

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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. mansonchurica 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. mansonchurica 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-
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 (c)YFP fusions were constructed for all these kinases. The reciprocal combinations of nYFP and cYFP fusion plasmids of either GmMKK1/2 or GmMPK4a were cobombarded 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-GmMPK4 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-
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 H$_2$O$_2$ 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 established 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 H$_2$O$_2$ and constitutively activated defense responses (Figs. 1 and 2). These phenotypes are consistent with the spontaneous cell death, seedling lethality, and increased SA and H$_2$O$_2$ 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...
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* and *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 7](https://www.plantphysiol.org/figures/Figure_7.png)

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 co bombarded with constructs expressing different fusion proteins as indicated are shown. Coexpression of nYFP-AtbZIP and cYFP-AtbZIP was used as a positive control, and co bombarment of nYFP-GmMKK4 and cYFP-GmMPK4a was used as negative control. Bar = 100 μm.

![Figure 8](https://www.plantphysiol.org/figures/Figure_8.png)

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 in 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 WRY28 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.
natively, it is possible that BPMV VIGS did not adequately silence both GmMKK1 and GmMKK2 to the level needed to observe the GmMPK4-silenced phenotype. It will be interesting to further investigate whether simultaneous silencing of multiple GmMKK1, GmMKK2, and GmMKK1/2-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 viral movement 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). Interestingly, 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 biostatic particle bombardments using a Biostatic 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 GmMPK4-R (Glyma16g03670) (5'-AAGGGATCCCT-GTATCATCAATTGTTACGAGGGCT-3') and GmMPK4s-R (5'-TGGGTACCCC-TGCTGATAAGTCTCAGCIGA-3'). The primers used for silencing the 3′ untranslated region of GmMPK4s are GmMPK4-3′ -UTR-F (5′-AAGGGAT- CCACTCAGGACC-GCCG-3′) and GmMPK4-3′-UTR-R (5′-AAGGGATCCAACTGAGCCTTGAATCTAAAAAAGA-3'). The primers...
used for amplifying both GmMPK4c/A and GmMPK4c-F (5'-ATGGCTCTTGAGTCAAGCTCCTTC3') and GmMPK4c-R (5'-TCAATAAATAGGTTGATGACGATGTTGACTC3'). The primers used for MKK1/MKK2 fusion constructs were GmMKK2-F (5'-AAAGGATCCAAAAAGGATATGAGTTGGAGGACT3'), GmMKK2-fu-R (5'-CAGGATCCACTTCTTCAAGGGTGCAAGAGGAGAGATCTC3'), GmMKK1-R (5'-ATGCTCTCTCTGTCGATGACTACATGATTG-3'), and GmMKK1-R (5'-TGGTGATGACTGTAATTTTGA3'). The GmMKK2 and GmMKK1 fragments were amplified, respectively, using GmMKK2-F/GmMKK2-fu-R and GmMKK1-R/GmMKK1-R as primer pair and the amplified GmMKK1 and GmMKK2 fragments as templates. The underlined sequences are BamHI and PstI restriction sites, respectively, in front of forward and reverse primers. The boldface letter indicates an extra nucleotide in reverse strands 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 GmMKK4-F (5'-ATGGCTCTCTTGAGTCAAGCTCCTTC3'), GmMKK4-R (5'-TCAATAAATAGGTTGATGACGATGTTGACTC3'), GmPR2-F (5'-ACCGAAGGACGCATTCC3'), GmPR2-R (5'-CCTCAACTGTCACAGTTCC3'), GmPR2-F (5'-TCAATAAATAGGTTGATGACGATGTTGACTC3'), GmPR2-R (5'-ATGGCTCAATGCAAGATTCCAAATG-3'), and GmPR2-F (5'-ATGGCTCAATGCAAGATTCCAAATG-3').

RNA Isolation and RT-PCR

RNA isolation and RT-PCR were performed as described elsewhere (Li et al., 2005). For microarray analysis, the top fully expanded trifoliolate leaves, petioles, and stems of about 1 to 2 cm in length were harvested at 20 dpi. The RNA samples extracted (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 analyses were used in 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 manshurica 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. manshurica sporangia in deionized water (10^5 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 K3HPO4, 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 pB7WG2D (Karimi et al., 2002) to generate the GFP-GmMPK4 fusion construct. This fusion construct and the free dsRed construct were coinjected 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 Axioskop microscope (Zeiss) equipped with a digital camera (Diagnostic Instruments).

The full-length cDNAs of GmMKK1 (Glyma15g18860), GmMKK2a (Glyma17g06020), GmMKK2b (Glyma13g16600), GmMKK4 (Glyma07g05820), 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 and pE-SPYNE-GW, which contain the C-terminal and N-terminal regions of YFP, length cDNA amplification are cobombarded into onion epidermal cells (Biolistic PDS-1000/He system; Bio-Rad Laboratories) as described (Zhang et al., 2009). Images were captured with an inverted Axioskop microscope (Zeiss) equipped with a digital camera (Diagnostic Instruments).

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