Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2014) 15(2), 145–160 DOI: 10.1111/mpp.12075

Overexpression of *GmCaM4* in soybean enhances resistance to pathogens and tolerance to salt stress

SURYADEVARA S. RAO[1], MOHAMED H. EL-HABBAK[1], WENDY M. HAVENS[1], AJAY SINGH[1], DANMAN ZHENG[2], LAURA VAUGHN[2], JAMES S. HAUDENSHIELD[3], GLEN L. HARTMAN[3,4], SCHUYLER S. KORBAN[2] AND SAID A. GHABRIAL[1,*]

[1]*Department of Plant Pathology, University of Kentucky, Lexington, KY 40546-0091, USA*
[2]*Department of Natural Resources and Environmental Sciences, University of Illinois, Urbana, IL 61821, USA*
[3]*USDA Agricultural Research Service, Urbana, IL 61801, USA*
[4]*Department of Crop Sciences, University of Illinois, Urbana, IL 61801, USA*

SUMMARY

Plant diseases inflict heavy losses on soybean yield, necessitating an understanding of the molecular mechanisms underlying biotic/abiotic stress responses. Ca^{2+} is an important universal messenger, and protein sensors, prominently calmodulins (CaMs), recognize cellular changes in Ca^{2+} in response to diverse signals. Because the development of stable transgenic soybeans is laborious and time consuming, we used the *Bean pod mottle virus* (BPMV)-based vector for rapid and efficient protein expression and gene silencing. The present study focuses on the functional roles of the gene encoding the soybean CaM isoform GmCaM4. Overexpression of GmCaM4 in soybean resulted in enhanced resistance to three plant pathogens and increased tolerance to high salt conditions. To gain an understanding of the underlying mechanisms, we examined the potential defence pathways involved. Our studies revealed activation/increased expression levels of pathogenesis-related (*PR*) genes in *GmCaM4*-overexpressing plants and the accumulation of jasmonic acid (JA). Silencing of *GmCaM4*, however, markedly repressed the expression of *PR* genes. We confirmed the *in vivo* interaction between GmCaM4 and the CaM binding transcription factor Myb2, which regulates the expression of salt-responsive genes, using the yeast two-hybrid (Y2H) system and bimolecular fluorescence complementation assays. GmCaM4 and *Glycine max* CaM binding receptor-like kinase (GmCBRLK) did not interact in the Y2H assays, but the interaction between GmCaM2 and GmCBRLK was confirmed. Thus, a GmCaM2–GmCBRLK-mediated salt tolerance mechanism, similar to that reported in *Glycine soja*, may also be functional in soybean. Confocal microscopy showed subcellular localization of the green fluorescent protein (GFP)-GmCaM4 fusion protein in the nucleus and cytoplasm.

*Correspondence: E-mail: saghab00@email.uky.edu

INTRODUCTION

Soybean [*Glycine max* (L.) Merr] is the major oilseed crop in the world, with an annual value of nearly 14 billion dollars in the USA, which is the largest producer and exporter of soybean (Hartman *et al*., 2011). Soybean is a main source of oil and high-quality protein for both humans and animals worldwide. Plant diseases inflict heavy losses on soybean yield with a significant negative impact on the US economy, with fungal diseases, collectively, causing the most losses. Implicit in the high economic value of this crop is the importance of a thorough understanding of the molecular mechanisms underlying biotic/abiotic stress responses.

Ca^{2+} is an important universal messenger for the conveyance of signals and the regulation of numerous aspects of plant growth and development and plant responses to stresses. A change in cytoplasmic Ca^{2+} concentration is implicated in the management of diverse physiological processes and plant defence and stress responses (Hepler, 2005; Reddy, 2001; White and Broadley, 2003). The cell signalling process via Ca^{2+} has three stages: the production of a Ca^{2+} signature, sensing of the signature and transduction of the signal to downstream effectors (Reddy and Reddy, 2004). Protein sensors recognize cellular changes in Ca^{2+} in response to diverse signals. Calmodulins (CaMs) are the prominent Ca^{2+} sensors which, on binding Ca^{2+}, interact and alter the activity of other proteins involved in a large number of plant processes. Therefore, protein–protein interactions are key players in Ca^{2+}/CaM-mediated processes in signalling pathways.

Pathogen infection results in the induction and/or suppression of different plant CaM isoforms (Garcia-Brugger *et al*., 2006; Heo *et al*., 1999; Kim *et al*., 2002; Takabatake *et al*., 2007; Yamakawa *et al*., 2001). Multiple isoforms of CaM occur in higher plants (Luan *et al*., 2002; Reddy, 2001). Of the five known soybean *CaM* genes (*GmCaM1–5* or *SCam1–5*), two divergent CaM isoforms (GmCaM4 and GmCaM5) are highly induced by either fungal elicitors or pathogen attack, whereas the three other genes encoding conserved CaMs are not inducible (Lee *et al*., 1995). Transgenic tobacco plants overexpressing *GmCaM4* or *GmCaM5* show spontaneous necrotic lesions and constitutive expression of systemic acquired resistance (SAR)-associated genes independent of

salicylic acid (SA) production (Heo *et al*., 1999). Moreover, these transgenic plants exhibit enhanced resistance to a wide spectrum of pathogens, including *Phytophthora parasitica* var. *nicotianae*, *Pseudomonas syringae* pv. *tabaci* and *Tobacco mosaic virus*, suggesting that specific CaM isoforms are part of an SA-independent signalling cascade leading to disease resistance (Heo *et al*., 1999; Kim *et al*., 2009). Furthermore, CaM is recognized as a major sensory molecule involved in the decoding of Ca^{2+} signatures associated with salt stress (Bouché *et al*., 2005; Yang *et al*., 2010). GmCaM4 has been demonstrated to mediate salt-induced Ca^{2+} signalling by direct interaction with an R2R3-type Myb2 transcription factor which regulates salt- and dehydration-responsive genes (Yoo *et al*., 2005).

The production of stable transgenic soybeans is still considered to be laborious and time consuming (Govindarajulu *et al*., 2008). Given the economic importance of soybean, faster and more efficient methods are required for the analysis of the molecular mechanisms governing plant development and the biotic/abiotic stress response (Burch-Smith *et al*., 2004). One such important tool includes the *Bean pod mottle virus* (BPMV)-based vector, which can be used for both heterologous gene expression and silencing of endogenous gene expression in soybean (Zhang and Ghabrial, 2006). The BPMV-based vector is derived from RNA2, which codes for a putative cell-to-cell movement protein (MP) and two coat proteins (CPs; large, L-CP; small, S-CP). BPMV RNA2 was modified so that target sequences could be inserted between regions encoding the MP and L-CP. For the recombinant RNA2 polyprotein to be processed correctly, additional proteinase cleavage sites flanking the sites of target sequence insertion were generated. The BPMV vector has been used to study soybean defence responses to diverse plant pathogens (Fu *et al*., 2009; Kachroo *et al*., 2008; Selote and Kachroo, 2010; Singh *et al*., 2011).

Previous studies using the BPMV vector to silence soybean endogenous genes, e.g. genes encoding phytoene desaturase (*PDS*) and omega-3 fatty acid desaturase (*FAD3*), have shown silencing phenotypes throughout the soybean plants in which virus replication takes place (Singh *et al*., 2011; Zhang and Ghabrial, 2006). To date, the BPMV vector has not been used to overexpress endogenous genes in soybean. Overexpression of heterologous genes (e.g. the green fluorescent protein gene, *GFP*), however, has shown efficient GFP fluorescence throughout the plant and in all soybean tissues in which the viral genome is translated (Zhang and Ghabrial, 2006). Although stable *GmCaM4* overexpression has been achieved using transgenic technology in heterologous systems, *GmCaM4* overexpression has not been studied in soybean itself. The overexpression of other endogenous genes has been successfully accomplished in transgenic soybeans (e.g. Gm*EREBP*; Mazarei *et al*., 2007). As *GmCaM4* overexpression confers pathogen resistance in tobacco and tolerance to salinity stress in *Arabidopsis thaliana* (Heo *et al*., 1999; Park *et al*., 2004), we studied the responses of *GmCaM4*-overexpressing (*GmCaM4*-OE) or silenced (*GmCaM4*-SI) soybean plants to infection with different soybean pathogens and to salinity stress.

RESULTS

Virus accumulation in *GmCaM4*-SI and *GmCaM4*-OE plants

Two BPMV-based constructs were produced: overexpression and silencing constructs. For overexpression of the *GmCaM4* gene from the BPMV RNA2 vector, a cDNA fragment (450 bp) containing the complete coding sequence of *GmCaM4*, excluding the termination codon, was inserted into the *Bam*HI/*Msc*I sites of the BPMV vector (Fig. S1a, see Supporting Information). The recombinant RNA2-*GmCaM4* mRNA was detected by Northern blot analysis of total RNA from infected plants when the blot was probed with a *GmCaM4*-specific probe (see below). The RNA2-*GmCaM4* mRNA was translated into chimeric RNA2-GmCaM4 polyprotein, which was processed by a virally encoded protease (Lomonossoff and Ghabrial, 2001) into the mature viral proteins: RNA2 replication cofactor (CR)/MP, L-CP and S-CP (Fig. S1a) and GmCaM4. To construct the *GmCaM4* silencing construct, we amplified a 198-bp fragment corresponding to a portion of the 5'-untranslated region (UTR) of the *GmCaM4* gene. The reason for choosing the 5'-UTR sequence is the high percentage of sequence identity (88%) between the coding sequences of *GmCaM4* and *GmCaM5* (Fig. S1b). The percentage sequence identity scores, based on full-length or coding sequences, between the five known soybean *GmCaM* genes [*GmCaM1*–*GmCaM5*; (Lee *et al*., 1995)] are shown in Fig. S1b. The sequence of the 5'-UTR 198-nucleotide silencing fragment is unique to *GmCaM4* and is not shared by the other *GmCaM* 5'-UTRs.

Western blot analysis and enzyme-linked immunosorbent analysis (ELISA) testing, using an antiserum against BPMV CPs, showed that approximately two-fold higher levels of viral CP could be detected in plants inoculated with either the overexpression transcript or the silencing transcript than the empty vector control. The intensity of L-CP and S-CP bands in the Western blot suggests slightly higher levels of viral CPs in *GmCaM4*-SI relative to *GmCaM4*-OE plants and the empty vector treatments (Fig. S2, see Supporting Information).

The results of the Western blot also suggested that correct processing of the chimeric GmCaM4-RNA2 polyprotein had occurred at the cleavage site between GmCaM4 and L-CP, as the L-CP generated from the RNA2-GmCaM4 polyprotein was indistinguishable in size from that derived from empty vector polyprotein.

Phenotypes of *GmCaM4*-OE/*GmCaM4*-SI soybean plants

GmCaM4-OE, *GmCaM4*-SI and empty vector-infected soybean plants were reduced in size compared with mock control plants

(Fig. 1a). The *GmCaM4*-OE plants at the V4 growth stage (fully expanded fourth trifoliolate leaf growth stage) were clearly smaller than those of other treatments. Leaves from the empty vector treatment showed moderate mottling symptoms of BPMV. However, leaves from *GmCaM4*-OE and *GmCaM4*-SI plants showed relatively more severe phenotypic changes including some blistering, which was more apparent in *GmCaM4*-SI plants (Fig. 1b). The severity of symptoms may be related to the BPMV titre, which was higher in *GmCaM4*-OE and *GmCaM4*-SI plants than in the empty vector control plants, as assessed by ELISA and confirmed by Western blot analysis (Fig. S2). Interestingly, necrotic spots were noted on the older leaves of soybean plants that overexpressed the *GmCaM4* gene. However, no such necrotic spots were noted on the leaves of vector control or *GmCaM4*-SI plants (Fig. 1c). These experiments were carried out at least five times with reproducible results. Similar necrotic spots were also reported on transgenic tobacco overexpressing *GmCaM4* or *GmCaM5* (Heo *et al*., 1999).

Variation in *GmCaM4* transcript levels among treatments

A semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) approach was used to evaluate the abundance of the endogenous *GmCaM4* mRNA and the chimeric BPMV RNA2-*GmCaM4* transcript. Using primers designed to amplify the full-length coding region of *GmCaM4*, the endogenous *GmCaM4* mRNA was detected in mock control plants only after 20 cycles of RT-PCR. No band was detectable after 15 cycles, suggesting its low abundance (Fig. 2a). These results are consistent with those of Heo *et al*. (1999), who reported very low levels of endogenous *GmCaM4* mRNA at normal conditions in soybean cells. The level of *GmCaM4* mRNA is clearly higher after 25 RT-PCR cycles in plants infected with the empty vector than in control mock plants, suggesting that *GmCaM4* is induced as a result of virus infection. However, *GmCaM4* mRNA levels were obviously lower in *GmCaM4*-SI plants than in mock or vector plants; this became evident after 25 cycles of RT-PCR (Fig. 2a). Quantification by densitometry suggested ~50% knock down of the *GmCam4* gene as revealed following 30–35 RT-PCR cycles (Fig. S3, see Supporting Information). In *GmCaM4*-OE plants, high levels of the chimeric BPMV-RNA2-*GmCaM4* transcript were easily detected after 15 cycles of RT-PCR (Fig. 2a).

Northern blot analysis was carried out to assess *GmCaM4* expression levels using *GmCaM4*- and BPMV RNA2-specific probes. Consistent with the RT-PCR data, which predicted very low levels of endogenous *GmCaM4* mRNA, no RNA band corresponding to the endogenous *GmCaM4* mRNA was detected in total RNA or poly(A) RNA preparations from mock, empty vector or *GmCaM4*-SI plants. However, the chimeric RNA2-*GmCaM4* transcript band was visible only in *GmCaM4*-OE samples (Fig. 2b). The hybridization signal was so high in the *GmCaM4*-OE treatment that we removed the blots after exposure for less than 30 min. No endogenous RNA was observed even after 10 days of exposure (Fig. 2b, arrow indicates predicted position of *GmCaM4* mRNA). We also carried out Northern hybridization analysis to detect the vector RNA2. No transcript was observed in mock plants, whereas strong hybridization signals were observed with the remaining three treatments: the empty vector, *GmCaM4*-OE and *GmCaM4*-SI plants (Fig. 2b). Furthermore, Northern hybridization analysis demonstrated the stability of the inserts, as bands of the predicted size of recombinant RNA containing the coding sequence of *GmCaM4* (450 bp; lane *GmCaM4*-OE) or the sequence of the silencing fragment (198 bp; lane *GmCaM4*-SI) were resolved (Fig. 2b, bottom panel).

Western blot confirmation of GmCaM4 protein overexpression in soybean

Using an antiserum raised against bacterially expressed GmCaM4, very faint or faint bands corresponding to the endogenous GmCaM4 (molecular mass of 17 kDa) were detected in mock, empty vector and *GmCaM4*-SI plants (Fig. 3). In addition to the endogenous GmCaM4 band, *GmCaM4*-OE plants contained a more intense band (~20 kDa) of the size predicted for the recombinant GmCaM4 protein (GmCaM4 protein plus 19 virus-derived amino acids; Fig. 3). The chimeric GmCaM4 protein was stable and functional as it accumulated in the *GmCaM4*-OE plants, which showed a distinct phenotype and enhanced resistance to pathogens and salt stress.

Resistance of *GmCaM4*-OE soybean plants to pathogens

Soybean plants inoculated with the different transcripts were tested for their responses to three pathogens, including the oomycete *Phytophthora sojae* (the root and stem rot pathogen of soybean) and two necrotrophic fungal pathogens, *Alternaria tenuissima* and *Phomopsis longicolla*, causal agents of Alternaria leaf spot and pod and stem blight of soybean, respectively. Stem inoculations with *Py. sojae* race 3 were performed using susceptible cv. Harosoy 63 plants. For all recombinant viral transcript treatments, a brown lesion developed initially at the inoculation site. *GmCaM4*-OE plants were able to restrict lesion formation to a few millimetres around the incision, but, in *GmCaM4*-SI plants, stem lesions developed more slowly than in the controls and the leaves remained green over the duration of the experiment. Mock- and empty vector-inoculated plants of comparable age to *GmCaM4*-SI plants were killed as the stem lesions expanded extensively as a result of pathogen infection (Fig. 4a). Interestingly, Heo *et al*. (1999) reported that transgenic tobacco plants constitutively expressing *GmCaM4* or *GmCaM5*

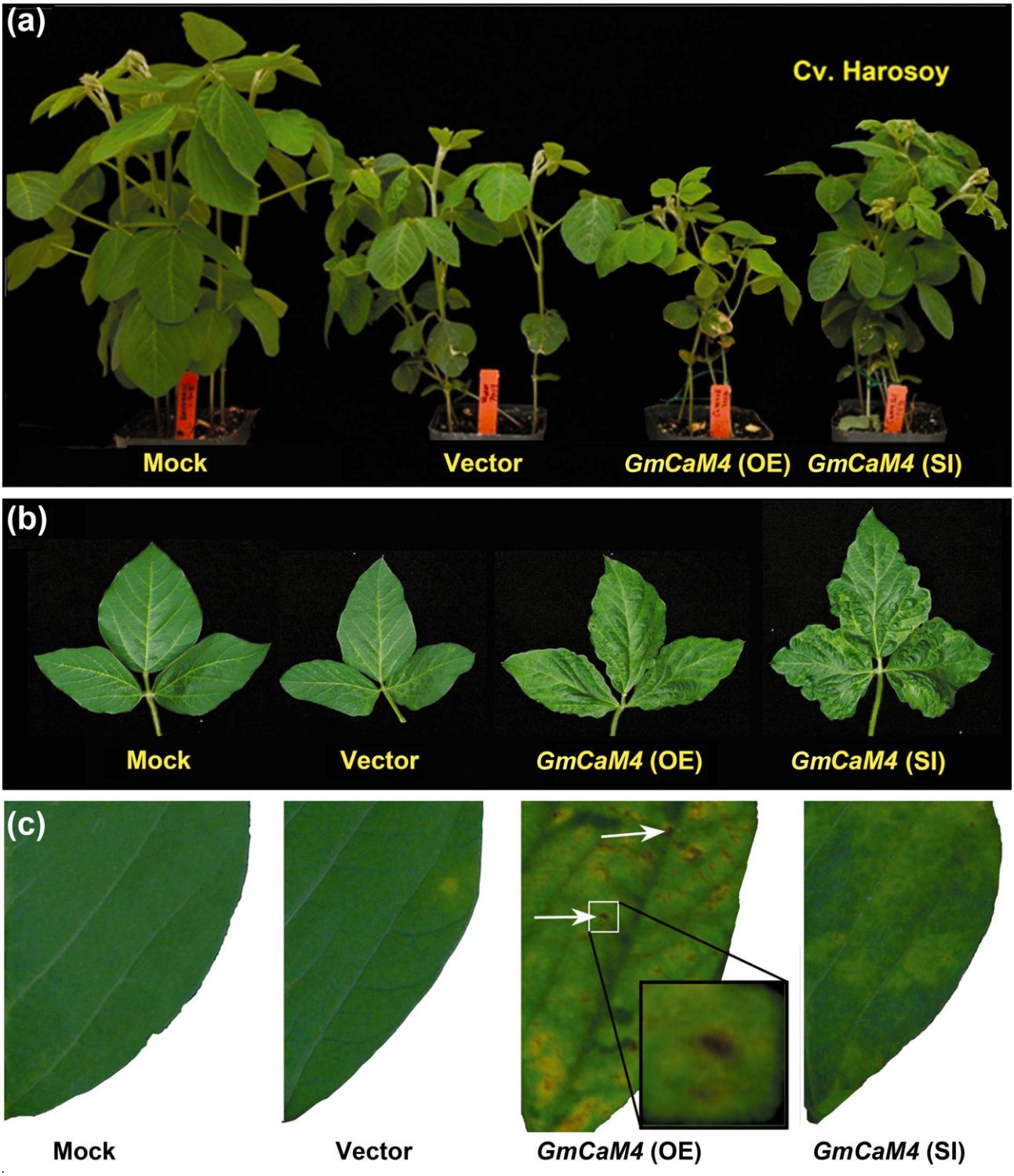

Fig. 1 Phenotypes of *GmCaM4*-overexpressing (*GmCaM4*-OE), *GmCaM4*-silenced (*GmCaM4*-SI) and empty vector soybean plants compared with mock. (a) Representative plants (3 weeks post-inoculation) showing the relatively smaller size of the *GmCaM4*-OE plants compared with the other treatments. (b) Individual trifoliolate leaves from the different treatments with more severe symptoms exhibited by the *GmCaM4*-SI plants. (c) Necrotic spots (arrows) are noted only on the *GmCaM4*-OE plants.

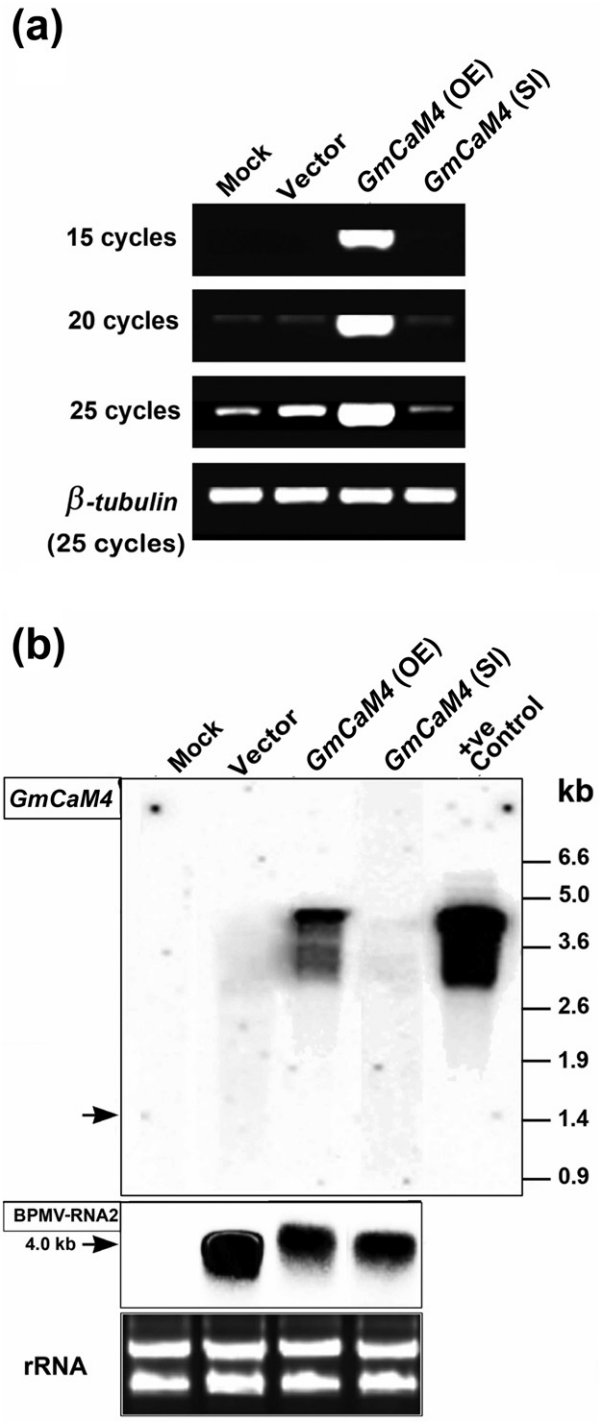

Fig. 2 Reverse transcription-polymerase chain reaction (RT-PCR) and Northern blot analysis of transcripts in soybean plants inoculated with empty or recombinant *Bean pod mottle virus* (BPMV)-*GmCaM4* vector. (a) Semi-quantitative RT-PCR to assess the transcript levels in representative plants using a pair of primers designed to amplify the entire coding sequence of *GmCaM4* (450 bp). (b) Northern hybridization analysis using *GmCaM4*-specific probe (top panel) and BPMV-RNA2-specific probe (bottom panel). RNA (10 μg) was loaded per lane. Arrow indicates predicted position of *GmCaM4* endogenous mRNA. Transcript derived from the *GmCaM4*-overexpressing (*GmCaM4*-OE) construct was used as a positive control. Note that the *GmCaM4*-specific probe comprises only the CaM4 coding sequence, but lacks the 5'-untranslated region (5'-UTR) sequence. Therefore, it did not detect the insert in the *GmCaM4*-silenced (*GmCaM4*-SI) transcript which contains a 198-bp fragment derived from the 5'-UTR.

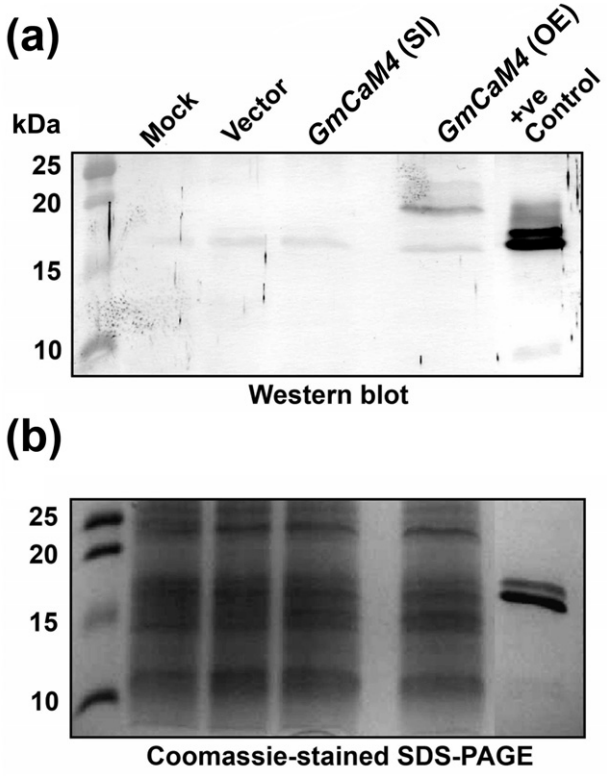

Fig. 3 (a) Western blot analysis of protein extracts from different soybean samples using an antiserum to bacterially expressed GmCaM4. Very faint or faint bands corresponding to the endogenous GmCaM4 (molecular mass of 17 kDa) were detected in mock, empty vector and *GmCaM4*-silenced (*GmCaM4*-SI) plants. In addition to the endogenous GmCaM4 band, a more intense band (~20 kDa) of the size predicted for the recombinant GmCaM4 protein (GmCaM4 protein plus 19 virus-derived amino acids) was detected in the *GmCaM4*-overexpressing (*GmCaM4*-OE) plants. (b) A Coomassie-stained gel showing equal loading of the protein samples. The *Escherichia coli*-expressed nickel column-purified GmCaM4 was used as a positive control. SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis.

showed a resistance response to *Phytophthora parasitica* var. *nicotianae*.

Detached leaves were used to test the responses of soybean plants to infection with *A. tenuissima* and *P. longicolla* using the soybean cultivars Harosoy and Essex, respectively. Disease assessment was performed at 7 days post-inoculation (dpi) for

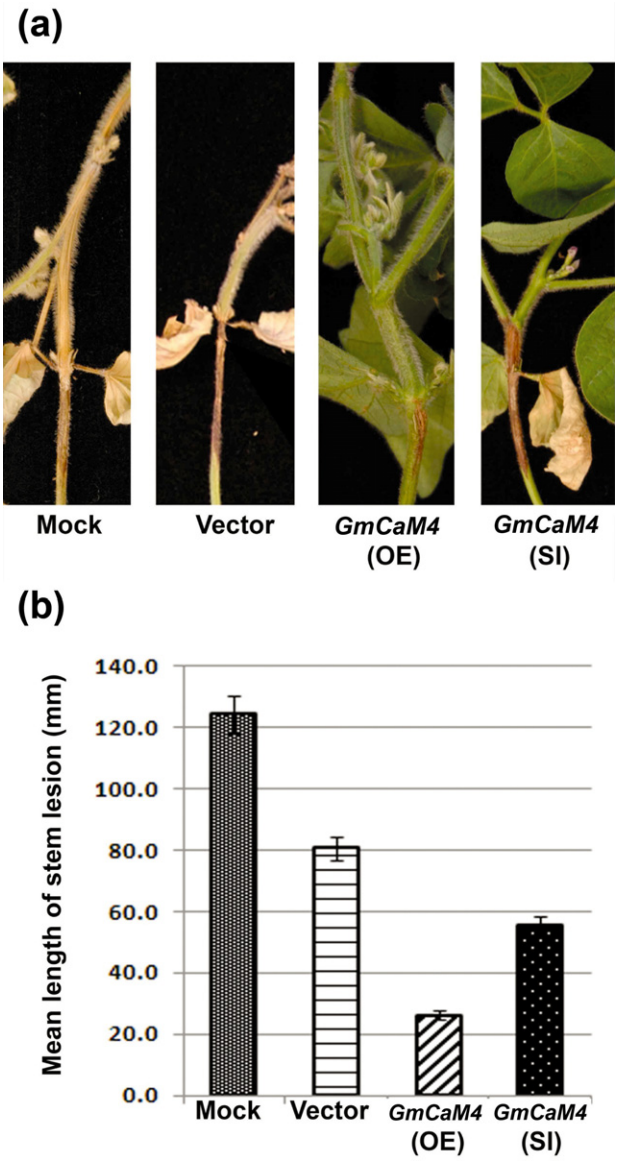

Fig. 4 Responses of soybean plants previously infected with empty and recombinant *Bean pod mottle virus* (BPMV) vectors to inoculation with *Phytophthora sojae*. (a) Close-up examination of stem lesions following inoculation with *Py. sojae* race 3 (R3). (b) Histogram showing differences in the lengths of stem lesions among the different treatments: mock, vector, *GmCaM4*-overexpressing (*GmCaM4*-OE) and *GmCaM4*-silenced (*GmCaM4*-SI) plants. Error bars indicate standard error (n = 12 plants).

A. tenuissima and 10 dpi for *P. longicolla* by comparing the size of the light-brown macerated area around the inoculation site. *GmCaM4*-OE plants developed a remarkable resistance response observed as a hypersensitive reaction (HR) at the inoculation site in the case of *A. tenuissima* or as substantial reduction in lesion development for *P. longicolla* (Fig. 5a,b, respectively). Empty vector-infected plants and *GmCaM4*-SI plants were as susceptible as the mock-inoculated plants.

Expression levels of pathogenesis-related (*PR*) gene transcripts in *GmCaM4*-OE/*GmCaM4*-SI plants

In the absence of pathogens, transgenic tobacco plants overexpressing *GmCaM4* showed higher levels of *PR* gene transcripts than normally found during pathogen infection or in response to SA or jasmonic acid (JA) (Heo *et al*., 1999). This led us to evaluate the transcript abundance of four different *PR* genes differentially regulated by either SA or JA (van Loon *et al*., 2006). The results showed activation of *PR4* and increased expression levels of *PR1a*, *PR2* and *PR3* in *GmCaM4*-OE plants relative to mock-inoculated and empty vector control plants. Interestingly, the expression level of these *PR* genes was markedly repressed in *GmCaM4*-SI plants (Fig. 6).

Accumulation of JA in *GmCaM4*-OE plants

Results of enhanced resistance to plant pathogens and increased transcript accumulation of *PR* genes in *GmCaM4*-OE plants, as well as the spontaneous appearance of necrotic lesions on older leaves, strongly suggest a corresponding increase in the signal molecules SA and JA. Heo *et al*. (1999) presented several lines of evidence indicating that GmCaM4 activates plant disease resistance responses via an SA-independent pathway(s). We therefore examined JA levels in *GmCaM4*-OE in comparison with empty vector and *GmCaM4*-SI plants. The results indicated that JA accumulation in *GmCaM4*-OE plants was three- to four-fold higher than in empty vector or *GmCaM4*-SI plants (Fig. 7).

Salinity tolerance of *GmCaM4*-OE soybean plants

GmCaM4 overexpression has been shown previously to enhance salt tolerance in transgenic Arabidopsis (Yoo *et al*., 2005). This provided the impetus to study salt tolerance in *GmCaM4*-OE and *GmCaM4*-SI soybean plants together with mock-inoculated and vector control plants (using cultivar Williams 82) at NaCl concentrations of 100, 150 and 200 mM. When treated with 100 mM NaCl, all plants showed leaf scorch within 10 days of salt treatment. In the case of *GmCaM4*-SI plants, the scorched leaves turned necrotic at the tip and the necrosis slowly spread to other parts of the leaf. However, *GmCaM4*-OE plant leaves did not develop any necrotic symptoms, and all plants treated with 100 mM NaCl survived (nine of nine per treatment) and produced pods and seeds. When the NaCl concentration was increased to 200 mM, all plants (nine plants per treatment) were killed within 3 weeks. Mock-inoculated and *GmCaM4*-OE plants survived longer than the empty vector control and *GmCaM4*-SI plants, with the *GmCaM4*-SI plants dying earlier than the vector control plants. When treated with 150 mM NaCl, only the *GmCaM4*-OE plants (five of nine plants) survived (Fig. 8).

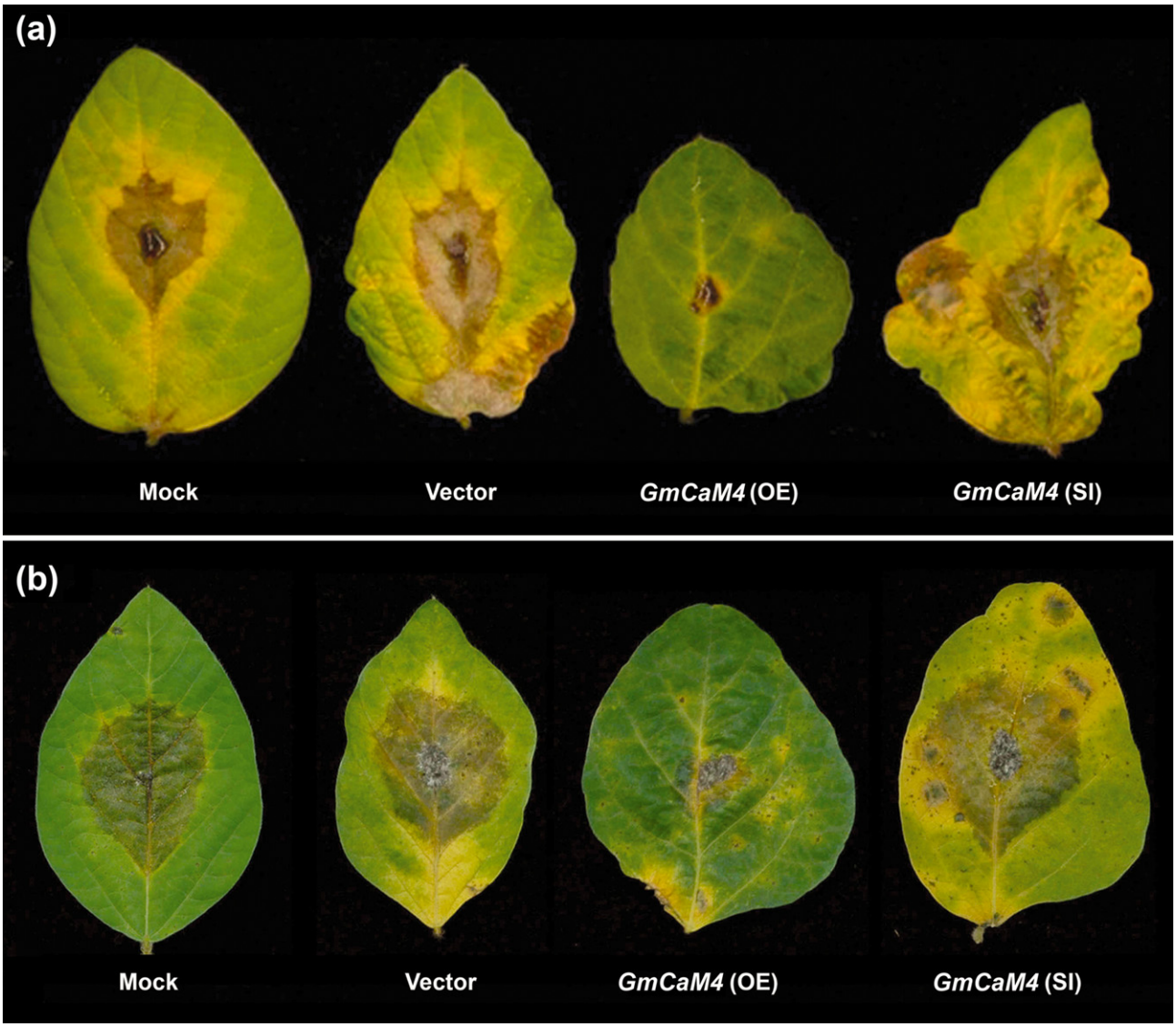

Fig. 5 Changes in plant susceptibility to pathogens as a result of *GmCaM4* overexpression. In detached leaf assays, three leaflets representing three different plants for each treatment or controls were inoculated with a drop of spore suspension of *Alternaria tenuissima* (a) or a drop of mycelial homogenate of *Phomopsis longicolla* (b). Each experiment was carried out three times with reproducible results. OE, overexpressing; SI, silenced.

In vivo interaction of GmCaM4 protein with transcription factor Myb2 using yeast two-hybrid (Y2H) assays

To understand the role played by other soybean proteins in the *CaM4*-mediated resistance to high salt, a Y2H approach was adopted. For this purpose, the entire coding regions of *GmCaM4*, a soybean homologue of Arabidopsis *Myb2* transcription factor (Myb2-like) and a soybean homologue of *Glycine soja* CaM binding receptor-like kinase (CBRLK-like) were used. Furthermore, the predicted CaM binding region in the *Myb2*-like gene was removed and the resulting mutant transcription factor gene (ΔMyb2) was expressed in yeast cells. Pair-wise interactions between CaM4 and the other selected proteins were tested in both activation (AD) and binding (BD) domain combinations by plating on selective medium lacking adenine, histidine, leucine and tryptophan with 100 mM 3-amino-1,2,4-triazole (3-AT) added to the medium. Co-transformations of the AD or BD recombinant transformants with AD or BD plasmids and, also, only AD and BD transformants were included as negative controls. As yeast cells expressing these combinations of vectors that include an empty vector normally do not survive on selective medium, they served as negative controls. However, we observed that the CaM4 BD recombinant clone consistently showed transcriptional activation, and thus it could survive the selection. In cases of self-activating proteins in the BD orientation [e.g. GmCaM4 and GmCaM2 (this

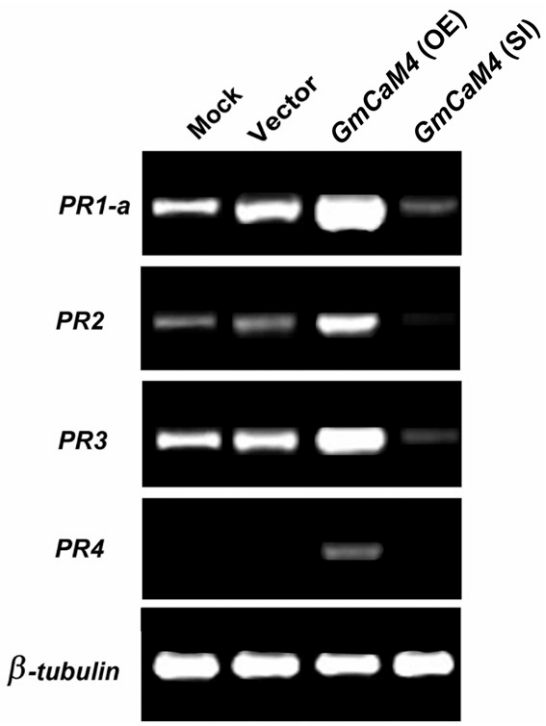

Fig. 6 Changes in the expression of pathogenesis-related (*PR*) genes in soybean plants overexpressing (OE) or silenced (SI) for *GmCaM4*. Specific primer pairs were used to amplify fragments of *PR1a*, *PR2*, *PR3* and *PR4*. *β-Tubulin* was amplified from the same cDNAs as an internal control. Polymerase chain reaction (PCR) was carried out for 40 cycles.

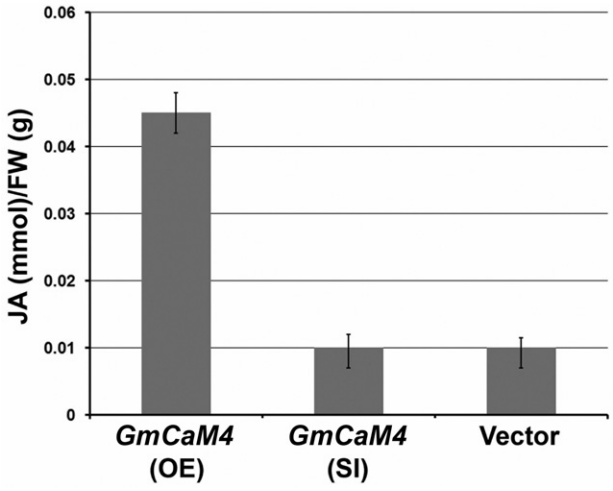

Fig. 7 Levels of jasmonic acid (JA) accumulation in soybean plants overexpressing (OE) GmCaM4. JA accumulation in *GmCaM4*-OE plants was three- to four-fold higher than in empty vector or *GmCaM4*-silenced (*GmCaM4*-SI) plants.

study) or GsCaM (Yang *et al*., 2010)], interpretation of Y2H assays relies on the interactions of combinations involving only the AD orientation (e.g. CaM4 AD × vector BD compared with the combination CaM4 AD × Myb2 BD; positions 2 and 8 in Fig. 9a).

The Y2H results showed interaction between CaM4 and the full-length Myb2-like transcription factor from soybean in both AD and BD combinations tested (Fig. 9a). Comparatively fewer yeast cells were observed to grow on the selection medium when the CaM interacting site was removed from the Myb2 transcription factor (ΔMyb2), suggesting that this transcription factor from soybean may have an additional CaM4 binding site (Fig. 9a). A putative CaM binding site (LHSRWGNRWSKIAQY) was identified near the N-terminal region of the truncated Myb2 (ΔMyb2) using a CaM binding site search program (http://calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html).

No yeast growth was observed when dual transformants of CaM4 and the CBRLK homologue from soybean were placed on the selective medium (Fig. 9a). These results suggest that CaM4-mediated salt tolerance involves the Myb2 transcription factor, but not CBRLK. Because *G. soja* CaM (GenBank accession number DT083281), which is a homologue of GmCaM2 (GenBank accession number AAA03580), interacts with *G. soja* CBRLK (Yang *et al*., 2010), we used Y2H to verify that GmCBRLK interacts with GmCaM2. The results shown in Fig. 9b confirmed the interaction between GmCaM2 and GmCBRLK.

Subcellular localization of GmCaM4 protein

Confocal microscopy was used to study the localization of the GFP-GmCaM4 fusion protein in leaves of *Nicotiana benthamiana* that had been infiltrated previously with an *Agrobacterium* strain transformed with the *GFP-GmCaM4* construct. Results showed that GmCaM4 was localized to both the nucleus and cytoplasm (Fig. 10a; GFP). This result was not surprising because GmCaM4 is thought to interact with transcription factors (e.g. SCa1; soybean Ca^{2+}-ATPase 1). Using the Y2H system, we demonstrated an interaction between GmCaM4 and the soybean Myb2 transcription factor protein (Fig. 9a). We further confirmed the nuclear localization of GmCaM4 by infiltrating *N. benthamiana* leaves expressing red fluorescent protein (RFP) fused to histone-2B (H-2B; Martin *et al*., 2009) with an *Agrobacterium* strain transformed with a *GFP-GmCaM4* construct. Confocal microscopy of the infiltrated *N. benthamiana* leaves confirmed that GmCaM4 was localized to the nucleus. This was further verified by merging images of both GFP- and RFP-expressing cells (Fig. 10a). Western blot analysis of total protein from *N. benthamiana* leaves previously infiltrated with the *GFP-GmCaM4* fusion construct verified that the source of fluorescence observed by confocal microscopy is the GFP-GmCaM4 fusion protein (Fig. 10b). Using the protein localization predictor WoLF PSORT and CELLO programs (Horton *et al*., 2007; Yu *et al*., 2006), GmCaM4 was predicted to localize in the chloroplasts in addition to the cytoplasm and nucleus. Localization of GmCaM4 in chloroplasts might not be surprising as earlier immunocytochemical localization studies have revealed that CaM is generally associated with plastids and elements of the cytoskeletal system in pea seedlings (Dauwalder *et al*., 1986).

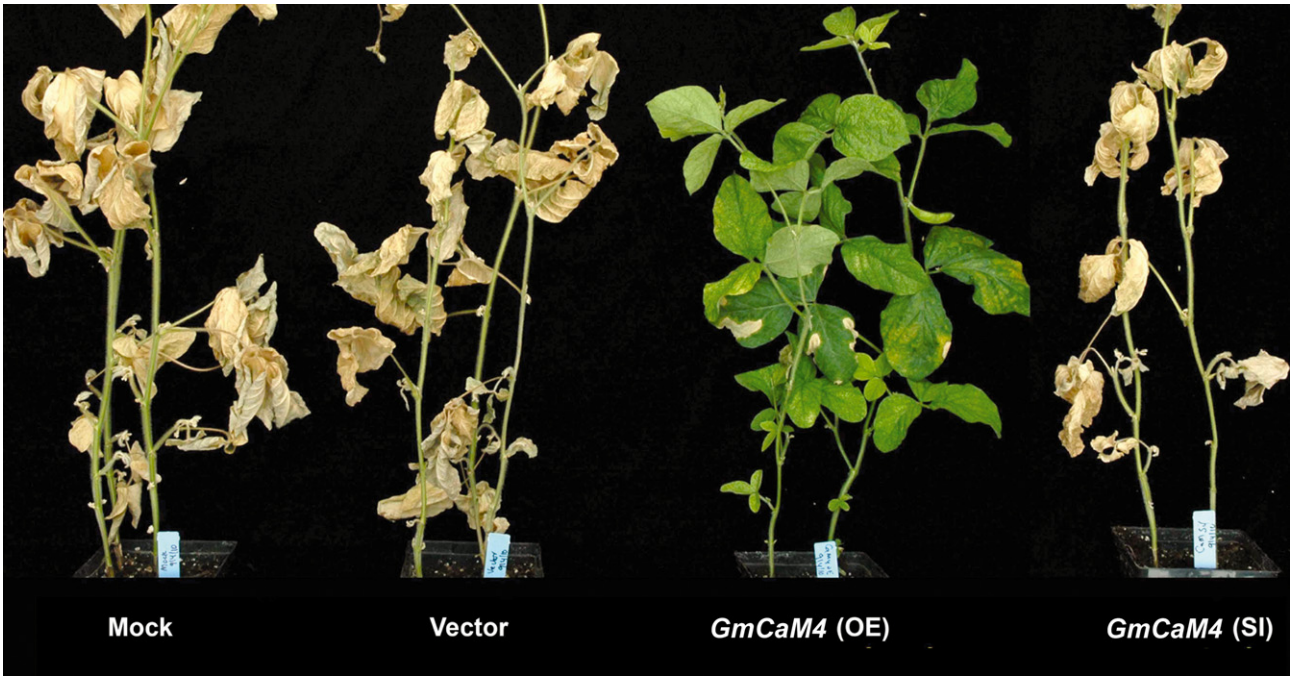

Fig. 8 Effect of *GmCaM4* overexpression (OE) or silencing (SI) on the tolerance of soybean (cv. Williams 82) plants to salt stress. Plants were supplied with 150 mM NaCl solution every other day for 2 weeks.

Interaction between GmCaM4 and GmMyb2 *in planta* using bimolecular fluorescence complementation (BiFC) assay

YFP fluorescence was observed in *N. benthamiana* leaves that expressed both pSITE-BiFC-GmCaM4 and pSITE-BiFC-Myb2, indicating the interaction between these two proteins *in planta* (Fig. 11a). The leaves that were infiltrated with either of the clones alone showed no fluorescence. Negative controls included N-terminal or C-terminal yellow fluorescent protein (YFP) fragments (nYFP or cYFP) fused to glutathione transferase (GST) and GmCaM4 (Fig. 11b,c). No YFP fluorescence was observed when *N. benthamiana* leaves were infiltrated with nYFP-GmCaM4 and nYFP-GST constructs (Fig. 11b) or with GmCaM4-cYFP and GST-cYFP constructs (Fig. 11c). The BiFC interaction further confirmed the Y2H results obtained with GmCaM4 and Myb2 proteins. Similar to GFP-GmCaM4 cellular localization (Fig. 10a), YFP is also localized in the cytoplasm and nucleus (Fig. 11a), indicating that Myb2–GmCaM4 interaction did not alter the GmCaM4 localization in plants. Although we did not use a nuclear marker in our BiFC studies, our GFP-GmCaM4 experiments with H-2B-RFP confirmed the nuclear localization of GmCaM4.

DISCUSSION

We demonstrated that the overexpression of GmCaM4 protein using a BPMV vector enhanced soybean resistance to the oomycete *Py. sojae* and the necrotrophic fungal plant pathogens *A. tenuissima* and *P. longicolla* (Figs 4 and 5). Moreover, the *GmCaM4*-OE plants showed spontaneous necrotic lesions and activation/increased expression of *PR* genes (Fig. 6). Similar results have been reported previously from transgenic tobacco overexpressing GmCaM4 or GmCaM5 proteins (Heo *et al*., 1999). The conclusions of these authors, however, relied entirely on gain-of-function experiments. No loss-of-function strategy was employed, and thus the physiological roles of GmCaM4 and GmCaM5 could not be established. Furthermore, because there are multiple CaM isoforms in soybean, redundancy might make the analysis of simple loss-of-function mutants difficult. Virus-induced gene silencing (VIGS) is a powerful reverse-genetic strategy that allows gene functional analysis in species not readily amenable to stable genetic transformation, e.g. soybean. It is also suitable for targeting multiple isoforms of a single gene family. We have demonstrated recently the efficacy of the BPMV vector in silencing several endogenous soybean genes, and in overexpressing heterologous proteins (Fu *et al*., 2009; Singh *et al*., 2011; Zhang and Ghabrial, 2006). A unique silencing fragment based on the 5'-UTR sequence of *GmCaM4* RNA was used and found to silence only *GmCaM4*, but not *GmCaM5* or the highly conserved *GmCaM*s (*GmCaM1–3*; data not shown). Interestingly, the expression of *PR* genes was repressed in the *GmCaM4*-SI plants, with expression levels of PR1a, PR2 and PR3 lower than in mock and empty vector plants.

It is worth noting that virus infection alone (empty vector treatment) did not alter host responses to biotic or abiotic stresses, even though the empty vector-infected plants at 3 weeks post-infection (Fig. 1) were reduced in size compared with mock-inoculated plants (Figs 4, 5 and 8; compare mock and empty vector treatments).

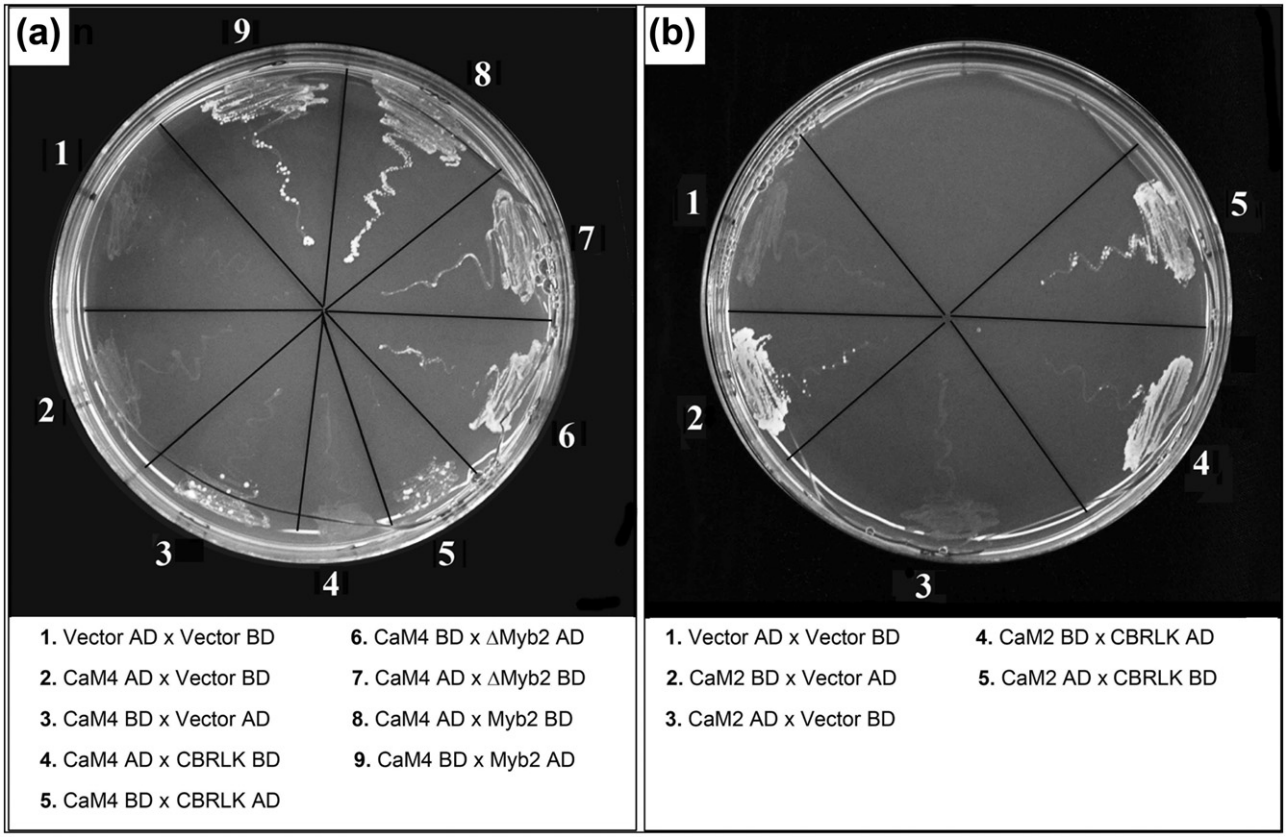

Fig. 9 (a) Yeast two-hybrid interaction studies involving GmCaM4 and soybean homologues of Arabidopsis Myb2 transcription factor and *Glycine soja* calmodulin binding receptor-like kinase (CBRLK). Interaction between GmCaM4 and full-length Myb2 transcription factor was observed, as shown in interactions 8 and 9. Interaction was still seen between GmCaM4 and truncated Myb2 transcription factor (ΔMyb2) which lacks an established calmodulin (CaM) binding site (interactions 6 and 7). Although less yeast growth can be seen when the GmCaM4 binding domain was used in combination with either empty vector AD or GmCBRLK AD (interactions 3 and 5), GmCaM4 self-interaction is clearly evident. No interactions were noted when GmCaM4 AD was tested against either empty vector BD or GmCBRLK BD (interactions 1 and 4). (b) Yeast two-hybrid interaction studies involving GmCaM2 and the soybean homologue of GmCBRLK. Yeast growth can be seen when the GmCaM2 binding domain was used in combination with empty vector AD, indicating self-interaction (interaction 2). No interactions were noted when empty vector AD was tested against empty vector BD or when GmCaM2 AD was tested against empty vector BD (interactions 1 and 3, respectively). GmCaM2 interaction with GmCBRLK, however, can be observed in both AD and BD combinations (interactions 4 and 5).

Although the overexpression of *GmCaM4* in transgenic tobacco or soybean inoculated with BPMV-*GmCaM4* recombinant RNA2 vector was found to induce/enhance constitutive *PR* gene expression and increase disease resistance, little is known about the pathway via which GmCaM4 activates the defence signal and up-regulates the *PR* genes. Heo *et al*. (1999) presented several lines of evidence indicating that activation of SAR and *PR* genes is SA independent. However, we have determined that CaM4-overexpressing soybean plants accumulated higher levels of the phytohormone JA. Consistent with this is the activation of *PR4* and the up-regulation of the expression of the JA-mediated defence-related *PR3* gene (Thomma *et al*., 1998). Whether JA accumulation is antagonistic to SA-mediated defence (Kachroo and Kachroo, 2007) in the *GmCaM4*-OE soybean plants is not known, but would be consistent with the finding of Heo *et al*. (1999). Both PR3 and PR4 possess endochitinase (antifungal) activities, with the basic chitinase PR3 and the hevein-like protein PR4 dependent on a pathway involving at least JA as a signal molecule (Thomma *et al*., 1998).

It is of interest that the silencing of *GmCaM4* resulted in substantial repression of *PR* gene expression. It may be relevant in this regard to note that *GmCaM4*-SI plants did not show as severe symptoms to infection with *Py. sojae* as mock and empty vector control plants. This may be explained by the potentially lower glucanase and endochitinase activities in these plants, as the expression levels of the pertinent *PR* genes were considerably repressed (Fig. 6). The finding that the *GmCaM4*-SI plants exhibited similarly severe symptoms to controls when infected with two necrotrophic fungal pathogens (Fig. 5) suggests that the host response to the hemibiotroph *Py. sojae* is different. This may be related to the fact that *Py. sojae* specifically inhibits the glucanase activity of soybean by producing inhibitor proteins (Rose *et al*., 2002). Furthermore, the smaller lesions produced by host plants

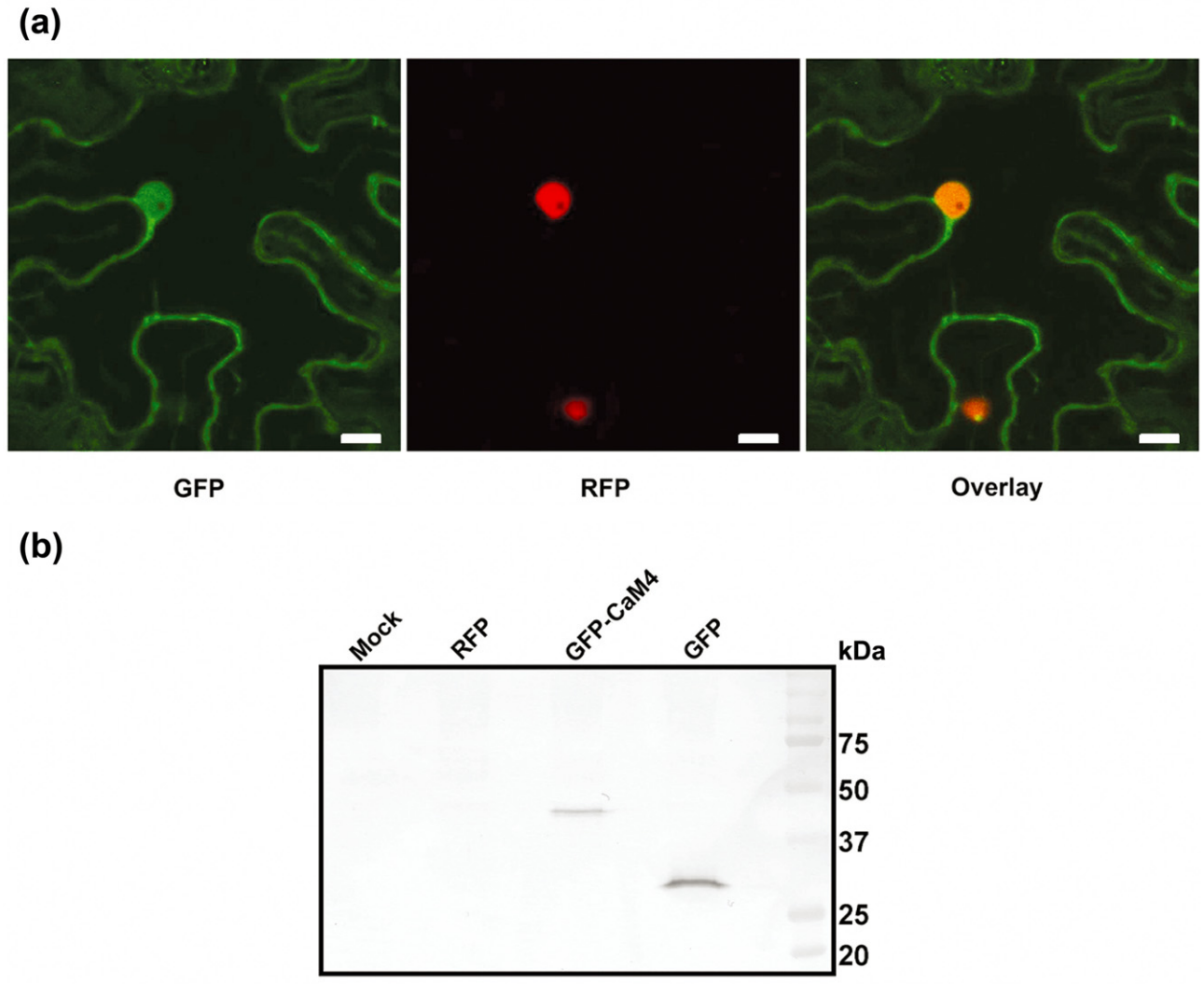

Fig. 10 (a) Cellular distribution of GFP-GmCaM4 fusion protein in *Nicotiana benthamiana* cells. Left (green fluorescent protein, GFP): confocal microscopy demonstrates the localization of GFP-GmCaM4 fusion protein in leaves of *N. benthamiana* previously infiltrated with an *Agrobacterium* strain transformed with the *GFP-GmCaM4* construct; GFP localization is both in the cytoplasm and nucleus. Middle panel (red fluorescent protein, RFP): nuclear localization of GmCaM4 was confirmed by infiltrating *N. benthamiana* leaves expressing RFP fused to histone-2B (H-2B) with an *Agrobacterium* strain transformed with the *GFP-GmCaM4* construct. Right: overlays of images shown in the left and middle panels further verify GFP localization to the nucleus. Two different nuclei from two different cells can be seen. (b) Western blot analysis of GFP and GFP-CaM4 fusion protein. *Nicotiana benthamiana* leaves previously infiltrated with an *Agrobacterium* strain transformed with the *GFP-GmCaM4* construct (see Experimental procedures for details). Protein samples (25 μg) were loaded onto a 12% polyacrylamide gel and subjected to polyacrylamide gel electrophoresis (PAGE) and Western blot analysis. Western blot confirmed the expression of GFP-GmCaM4 fusion protein in the leaves. Lanes are as follows (from left to right): mock, RFP, GFP-CaM4 fusion product (43.5 kDa), GFP (27 kDa) and pre-stained markers.

containing lower levels of PR2 and PR3 in response to *Py. sojae* infection confirm that PR2 and PR3 are important in soybean–pathogen interactions.

CaM is known to physically interact with AtMyb2, a transcription factor that regulates salt-and dehydration-responsive gene expression (Yoo *et al*., 2005). The CaM binding motif of AtMyb2 and of its homologue GmMyb2 are located between arginine-64 and glutamic acid-81, which comprise the R2R3 DNA binding region of Myb2. We have demonstrated *in vivo* interaction between GmCaM4 and Myb2 in yeast and *in planta* using the Y2H system (Fig. 9a) and BiFC (Fig. 11), respectively. *GmCaM4* overexpression in transgenic Arabidopsis has been reported to enhance the transcription of AtMyb2-regulated genes, including that of the proline-synthesizing enzyme P5CS1 (Yoshiba *et al*., 1999), which confers salt tolerance by facilitating the accumulation of higher proline concentrations (Yoo *et al*., 2005). Interestingly, Seo *et al*. (2008) provided evidence supporting the contention that PR3, whose expression is up-regulated in

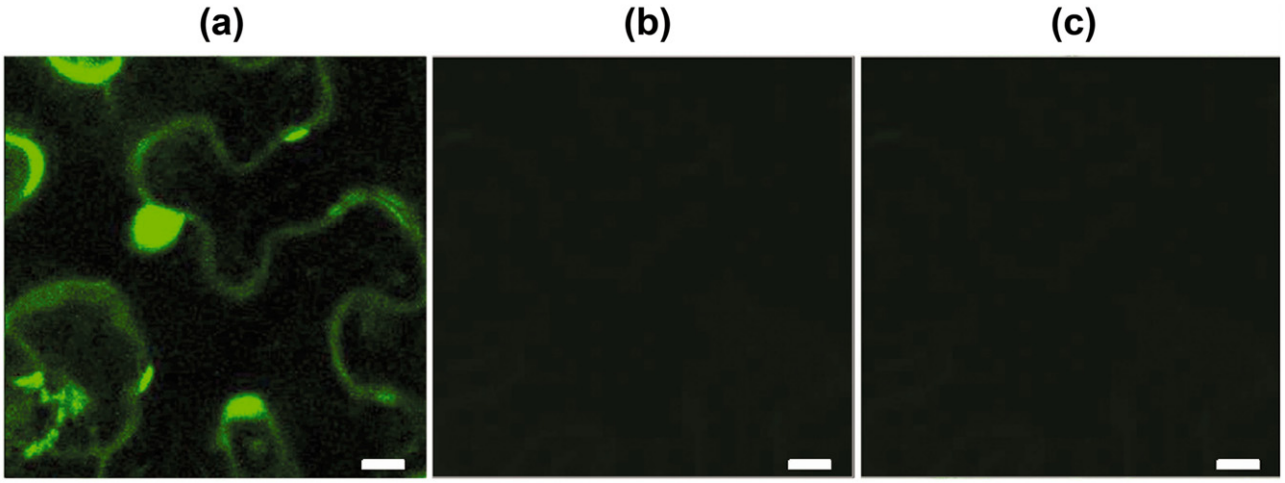

Fig. 11 Confocal micrographs of bimolecular fluorescence complementation (BiFC) showing specific interaction between GmCaM4 and GmMyb2. (a) Yellow fluorescent protein (YFP) fluorescence is observed in *Nicotiana benthamiana* leaves expressing both pSITE-BiFC-GmCaM4 and pSITE-BiFC-GmMyb2. YFP localization is observed in the cytoplasm and nucleus. This indicates that the GmCaM4 localization pattern was not influenced by its interaction with Myb2. Negative controls included N-terminal and C-terminal YFP fragments (nYFP and cYFP) fused to glutathione transferase (GST) and GmCaM4 (b and c). No YFP fluorescence was observed when *N. benthamiana* leaves were infiltrated with nYFP-GmCaM4 and nYFP-GST constructs (b) or with GmCaM4-cYFP and GST-cYFP constructs (c).

GmCaM4-OE plants, plays a role in salt stress regulation of seed germination. PR4, which is induced in *GmCaM4*-OE plants, was also found to contribute to salt regulation (Seo *et al*., 2008). The Y2H experiments also demonstrated a lack of interaction between GmCaM4 and GmCBRLK, but confirmed the interaction between GmCaM2 and GmCBRLK. Thus, a GmCaM2–GmCBRLK-mediated salt tolerance mechanism may be functional in soybean in addition to the GmCaM4–Myb2 mediated one.

Protein–protein interactions are believed to play a major role in the resistance responses of GmCaM4-overexpressing soybean plants to pathogens and high salt. Potentially important candidates in protein–protein interactions involving GmCaM4 are GmNPR1-1 and GmNPR1-2 (nonexpressor of PR1). It is well documented that NPR1 regulates SAR in *Arabidopsis thaliana* (Rochon *et al*., 2006). Complementation analyses in the Arabidopsis *npr1-1* mutant have suggested that the homoeologous *GmNPR1-1* and *GmNPR1-2* genes are orthologous to Arabidopsis *NPR1* (Sandhu *et al*., 2009). Therefore, it is reasonable to propose that the SAR pathway in soybean is most probably regulated by the *GmNPR1* genes. Following binding to GmCaM4, the NPR1 oligomer may become monomeric and moves into the nucleus to activate the transcription of the *PR* genes (Kinkema *et al*., 2000). Of particular interest in this regard is the fact that the GmNPR1 proteins contain two predicted strong CaM binding sites. We demonstrated that GmCaM4 is localized in the nucleus, and it is possible that it is transported to the nucleus via a piggyback mechanism after binding to the nucleus-localized NPR1 proteins (Pfeiffer *et al*., 2012). Constitutive expression of *PR* genes is closely related to the interactions of CaM-activated transcription factors with *cis* elements in *PR* gene promoters. Transcription factors, including some members of the TGA family, are implicated downstream of Ca^{2+}/CaM and NPR1 (Després *et al*., 2000).

In the present study, we used the BPMV-based vector for the overexpression and silencing of the endogenous soybean gene *GmCaM4*. As far as we are aware, plant virus-based vectors have not been used previously for the overexpression of endogenous proteins. The level of endogenous *GmCaM4* mRNA is very low under normal conditions, as reported by Heo *et al*. (1999) and confirmed by our RT-PCR and Northern hybridization analyses (Fig. 2). The recombinant viral mRNA (RNA2-*GmCaM4*), however, accumulates to high levels in *GmCaM4*-OE plants (Fig. 2) and is translated into a chimeric RNA2 polyprotein, which is processed by a viral protease (Sanfaçon *et al*., 2011) to release the chimeric GmCaM4 (Fig. S1). The released chimeric GmCaM4 is stable and functional as it accumulates in *GmCaM4*-OE plants (Fig. 3) and induces necrotic spots in infected tissues and confers resistance to plant pathogens. These characteristics were predicted based on the responses of *GmCaM4*-overexpressing transgenic tobacco plants (Heo *et al*., 1999). Our results thus demonstrate the utility of the BPMV vector as a valuable tool in studies attempting to understand the role of host proteins in response to abiotic and biotic stresses.

EXPERIMENTAL PROCEDURES

Soybean cultivars and plant growth conditions

Soybean cvs Harosoy, Harosoy 63, Williams 82 and Essex were grown in a glasshouse with day and night temperatures of 25 and 20 °C, respectively. Unless otherwise indicated, carborundum-dusted fully unfolded unifoliate leaves above the cotyledonary node (VC growth stage; Pedersen, 2004)

were rub inoculated with the different treatments. Leaves at the V4 growth stage (Pedersen, 2004), which had been inoculated previously on the unifoliate leaves with mock, empty vector, silencing and overexpression vectors, were used in all the experiments described in this study. All experiments were repeated at least three times. All plants used for testing the response to pathogens and salt concentrations were verified for GmCaM4 mRNA content by RT-PCR prior to inoculation with pathogens and salt treatment. To monitor the effectiveness of silencing, a positive control (silencing of the soybean *PDS* gene; Zhang and Ghabrial, 2006) was included in every VIGS experiment.

Construction of viral vectors, *in vitro* transcription and plant inoculation

Two independent BPMV-based vectors were constructed for overexpression and silencing of *GmCaM4*. For overexpression of *GmCaM4* in soybean, a full-length cDNA of the *GmCaM4* coding sequence (excluding the stop codon) was amplified by RT-PCR using soybean cDNA as a template. Following double digestion of the PCR product with *Bam*HI and *Sma*I, the PCR product was cloned into the *Bam*HI-*Msc*I-digested BPMV RNA2 vector (Fig. S1). The BPMV-*GmCaM4*-overexpression vector was designated as BPMV-*GmCaM4*-OE. To construct a BPMV-based vector for *GmCaM4* silencing, a 198-bp silencing fragment was amplified from the 5'-UTR of the *GmCaM4* gene by RT-PCR and cloned into the *Bam*HI-*MSc*I-digested BPMV vector. This region of *GmCaM4* does not share any sequence identity with other soybean *CaM* genes. A stop codon in the 198-bp fragment (TAA at nucleotide positions 160–163) was modified to code for lysine (AAA) using overlap PCR, so that the full-length coding sequence of the viral polyprotein remained translatable. *In vitro* transcription and rub inoculation of carborundum-dusted soybean leaves have been described in detail previously (Diaz-Camino *et al*., 2011; Kachroo and Ghabrial, 2012). Freeze-dried initial transcript-infected tissues were used to prepare inocula for the various treatments (Diaz-Camino *et al*., 2011; Kachroo and Ghabrial, 2012).

RNA extraction, Northern blotting and RT-PCR analysis

Total RNA was extracted from soybean leaf tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Northern blot analysis and the synthesis of random-primed probes were performed as described previously (Kachroo *et al*., 2008). First-strand cDNA synthesis was carried out using SuperScript® II Reverse Transcriptase (Invitrogen). Two to three independent RNA preparations were analysed at least twice by a semi-quantitative RT-PCR procedure involving 15–25 cycles to evaluate the relative differences in transcript levels. Primers were designed for the amplification of either full-length or partial-length coding sequences available in the National Center for Biotechnology Information (NCBI) database, including *GmCaM4* (L01433), *PR1a* (AF136636), *PR2* (M37753), *PR3* (AF202731), *PR4* (BT090788) and β-tubulin (M21297). All primer sequences used in this study are listed in Table S1 (see Supporting Information).

Protein extraction and Western blot analysis

A total of 100 mg of leaf material was ground in 200 μL of 10 mM sodium phosphate buffer (pH 6.9) with 100 μM phenylmethanesulfonylfluoride (PMSF). The protein concentration was determined using the Bio-Rad (Hercules, CA, USA) protein assay. A sample of 20 μg of total protein was electrophoresed on a 12% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and then transferred to Millipore Immobilon-P PVDF membrane (Billerica, MA, USA), as described previously (Li *et al*., 2011). The membrane was blocked with Tris-buffered saline (TBS) containing 5% powdered milk and reacted with chicken anti-GFP antibodies. Membranes were probed with rabbit anti-chicken alkaline phosphatase conjugate, and developed with the substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro-blue tetrazolium chloride (NBT) in AP buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5). The GFP primary and secondary antibodies and the pGD vectors (Goodin *et al*., 2002) were generous gifts from Dr Michael Goodin, Plant Pathology Department, University of Kentucky, Lexington, KY, USA.

Bacterial expression of GmCaM4 protein

The bacterial expression vector pET21a (EMD Millipore) was used to express GmCaM4 protein in *Escherichia coli*. The vector and the PCR-amplified insert were doubly digested with *Bam*HI and *Sal*I restriction enzymes. The reverse primer was designed so that the insert remains in frame with the histidine tag in the vector. After ligation and *E. coli* transformation, the positive clones were screened by colony PCR and confirmed by sequencing. *Escherichia coli* BL21 (DE3) cells were used for GmCaM4 protein expression. After induction by isopropyl-β-D-1-thiogalactopyranoside (IPTG) [both soluble and inclusion body localized proteins were extracted using Bug buster protein extraction reagent (EMD Millipore)], both fractions were purified on a nickel column and fractionated on a 15% PAGE gel. A purified protein band with a molecular mass of ~17 kDa, which corresponds to the known molecular mass of GmCaM4, was observed in the soluble fraction. Approximately 1.0 mg of the purified protein was sent to Cocalico Biologicals, Inc. (Reamstown, PA, USA) for raising GmCaM4 antiserum in a rabbit. CaM from soybean leaves was extracted as described by Ling and Assmann (1992).

Pathogens and disease resistance assays

Soybean plants infected with the BPMV empty vector, or with the recombinant vectors for overexpression or silencing of *GmCaM4*, were tested for their responses to *Py. sojae* race 3, *A. tenuissima* and *P. longicolla*. *Phytophthora sojae* race 3 was provided by Dr Paul Vincelli, Plant Pathology Department, University of Kentucky, Lexington, KY, USA. An isolate of *A. tenuissima* was obtained from local soybean plants and identified by Mohamed El-Habbak (El-Habbak, 2013). The *P. longicolla* isolate previously described in an earlier study (Koning *et al*., 2003) was used. The cultures were maintained at 20 °C on V8 agar for *Py. sojae* or on potato dextrose agar (PDA) for the other two pathogens.

To assay for resistance to *Py. sojae*, soybean cv. Harosoy 63, which is compatible with race 3 of *Py. sojae*, was used. At least four plants were used per treatment and the experiment was carried out three times. At 10 dpi with the different BPMV treatments, rectangular mycelial plugs were removed from 2-week-old cultures of the pathogen and used as inocula. Stem inoculation was carried out by making a 1.5-cm longitudinal incision below the second node on the stem using a sterile scalpel, and then immediately placing a mycelial plug onto the wound. A sterilized water-saturated piece of cheesecloth (3×1.5 cm^2) was wrapped around

the inoculation zone, which was finally sealed by wrapping with parafilm. Inoculated plants were maintained in a controlled environment inside a growth chamber with a 16-h photoperiod at 27 °C/20 °C (day/night) and 90% relative humidity for 10 days. Disease severity was scored by measuring the length of the lesion along the stem.

To assay for resistance to *A. tenuissima*, detached leaves were obtained from soybean plants (cv. Harosoy) at the V4 growth stage. All leaflets were from the fourth trifoliolate position from all infected or control plants in the three trials. In each trial, three leaflets representing three different plants per treatment were tested. A 20-μL drop of spore suspension [(3–4) $\times 10^4$ spores/mL] prepared from a 14-day-old culture of *A. tenuissima* was placed at the centre of each detached leaflet, and the leaflet was then placed in a Petri dish containing a sterile water-soaked filter paper and incubated at 28 °C for 24 h, after which it was transferred to 20 °C for 6 days. The disease was evaluated visually by comparing the lesion size with that of the susceptible mock-inoculated control.

To assay for resistance to *P. longicolla*, the detached leaf assay and disease evaluation were carried out as described for *A. tenuissima*, except that the inoculum was prepared by harvesting mycelia, pycnidia and stroma from a 3-week-old culture and homogenizing the combined material using a sterile mortar and pestle in 3 mL of sterilized water. From the homogenate, a 40-μL drop was placed on each leaflet. The Petri dishes were incubated at 25 °C and the lesions were evaluated at 10 dpi. In another trial, the *P. longicolla* assay was performed using cv. Essex instead of cv. Harosoy.

Salt tolerance evaluation

Eight to 10 individual soybean plants (cv. Williams 82 or Essex) were tested per experiment. High salt treatments were performed as described by Goel *et al*. (2010). Briefly, at 10 dpi with the different BPMV treatments, plants were transplanted into 4-in pots containing a mixture of PRO-MIX (BX; Premium Seed, Louisville, KY, USA) and soil at a 1:1 ratio. Preliminary experiments showed that potting mix alone did not hold water effectively as it dried quickly. The plants were allowed to grow in these pots for 10 more days before regular watering was stopped to let the soil dry (≤5 days). Once it was dry, the plants were subjected to treatment with salt solutions (100, 150 or 200 mM NaCl) on alternate days for 10 days, and then returned to a regular watering and fertilization schedule [watering once a day and fertilization twice a week using 20:10:20 Peat Lite Special (Premium Seed, Louisville, KY, USA) at 250 ppm]. The plants were monitored for salt-related symptoms, e.g. leaf scorch, for 3–4 weeks. The experiments were carried out three times.

Cloning of soybean cDNA homologues of Arabidopsis Myb 2 transcription factor and *G. soja* CBRLK

Nucleotide search of the soybean genome (DFCI *Glycine max* Gene Index) using BLASTN with the Arabidopsis Myb2 transcription factor (AT2G47190) as a query sequence identified a potential homologue in *G. max* for the Myb2 transcription factor (TC355011). The protein coding region of TC355011 was entered into the BLASTP program, which identified a 1433-bp full-length soybean cDNA clone (BT093711). A comparison of the CaM binding regions of the Arabidopsis and soybean Myb2 clones indicated that they share the following identical amino acid sequence: LRWLNYLRPDVRRGNITLE. Oligonucleotide primers were designed to amplify full-length cDNA of the corresponding soybean *GmMyb2* gene. The CaM binding region was identified at the N-terminal part of the gene, 192 nucleotides downstream from the start codon. To PCR amplify *GmMyb2* lacking the CaM binding region, primers were designed so that the first 249 bases at the N-terminal region were omitted. Both full- and partial-length clones that lacked the CaM binding region of GmMyb2 were used to produce Y2H clones. Similarly, the *G. max* CBRLK was identified by BLASTN of the soybean genome database with the *G. soja* GsCBRLK (GQ501043) nucleotide sequence as a query. The corresponding *G. max* cDNA was RT-PCR amplified using soybean total RNA as template. The full-length coding sequence of the *G. max* CBRLK gene was used to clone into Y2H vectors.

Y2H assays

Y2H assays were carried out to study the interactions between GmCaM4 and GmMyb2 transcription factor and GmCaM4 and GmCBRLK. Y2H experiments were performed as described by Hunt *et al*. (2008). The coding regions of *GmCaM4*, *GmMyb2*-like and *GmGBRLK*-like genes were cloned into Y2H vectors after PCR amplification. The PCR products were digested with *Eco*RI and *Sal*I restriction enzymes. The excised fragments were cloned into the Matchmaker™ GAL4 Two-Hybrid System 3 (Clontech Laboratories, Inc., Mountain View, CA, USA), which utilizes two plasmids, pGBKT7 and pGADT7. pGBKT7 was digested with *Eco*RI and *Sal*I to obtain BD clones, whereas pGADT7 was digested with *Eco*RI and *Xho*I to yield AD clones. The AD and BD plasmids were transformed into AH109 yeast competent cells. The AH109 transformants were selected on media lacking leucine and tryptophan (–LW). The –LW-selected colonies were re-streaked onto plates lacking leucine (–L), tryptophan (–W), histidine (–H) and adenine (–A) and supplemented with the histidine analogue 3-AT at 100 mM concentration (SD–L/–W/–H/–A medium). At least six clones were checked for both positive and negative interactions. Positive interactions were those in which all tested colonies grew on the above-mentioned selection medium. Negative controls for these tests included transformations with combinations of plasmids that included unmodified pGADT7 or pGBKT7 and the combination of empty AD and BD vectors.

JA analysis

For JA extraction, 1 g of leaf tissue was ground in cold 100% methanol with dihydro-JA as an internal standard. The methanol extract was passed through a silica-based Sep-Pak 18 column (Waters: Sep-Pak Classic C18 cartridge; Waters Corporation, Milford, MA, USA). The column-purified extract was processed as described by Xia *et al*. (2009) and injected into a gas chromatograph attached to an electron ionization detector (Hewlett Packard GCD Systems, Palo Alto, CA, USA). The JA peaks were identified using mass spectrometry.

Agroinfiltration and confocal microscopy

The full-length coding region of *GmCaM4* was PCR amplified and digested with *Bgl*II/*Apa*I restriction enzymes. The resulting fragment was ligated to the pGD-GFP (pGDG) vector previously digested with the same restriction enzymes; this allowed C-terminal in-frame fusion of GFP with GmCaM4. Positive clones were confirmed by sequencing. *Agrobacterium* strain

LB4404 was transformed with the pGDG-GmCaM4 construct using the freeze–thaw method (An *et al*., 1988). *Nicotiana benthamiana* transformation was carried out as described by Goodin *et al*. (2002). Sections of the infiltrated leaves were observed using a Fluorview FV1000 microscope (Olympus America, Inc., Center Valley, PA, USA) with excitation at 488 nm.

Cloning of *GmCaM4* and *GmMyb2* coding regions into BiFC destination vectors

The full-length *GmCaM4* coding region and CaM binding region of soybean *Myb2* (first 399 bp starting with ATG) were PCR amplified using Gateway (GW; Invitrogen) cloning primers (Table S1). BP recombinase reaction was carried out to recombine the PCR-amplified products to the pDONOR 221 vector to create the entry clones. The *GmCaM4* and soybean *Myb2* inserts from the pDONOR 221 vector were transferred to GW-converted pSITE-BiFC destination vectors (pSITE-3C1 vectors carrying split *YFP* gene) using LR clonase (Martin *et al*., 2009). *Agrobacterium* was transformed with the BiFC plasmids containing *GmCaM4* and *GmMyb2* coding regions. Independent *Agrobacterium* clones expressing pSITE-BiFC-GmCaM4 and pSITE-BiFC-Myb2 grown on separate plates were mixed together before infiltration into the *N. benthamiana* plants. YFP fluorescence emission was captured after exposing the samples to a wavelength of 514 nm.

ACKNOWLEDGEMENTS

This project was funded in part by a grant received from the United Soybean Board (grant #0262 to SSK, SAG and GLH) and by the Kentucky Science and Engineering Foundation (grant # to SAG). We thank A. Crume for maintaining glasshouse plants.

REFERENCES

An, G., Erbert, P.R., Mitra, A. and Ha, S.B. (1988) Binary vectors. In: *Plant Molecular Biology Manual* (Gelvin, S.B. and Schilperoort, R.A., eds), pp. 1–19. Dordrecht, The Netherlands: Kluwer Academic Publishers.

Bouché, N., Yellin, A., Snedden, W.A. and Fromm, H. (2005) Plant-specific calmodulin-binding proteins. *Annu. Rev. Plant Biol.* **56**, 435–466.

Burch-Smith, T.M., Anderson, J.C., Martin, G.B. and Dinesh-Kumar, S.P. (2004) Applications and advantages of virus-induced gene silencing for gene function studies in plants. *Plant J.* **39**, 734–746.

Dauwalder, M., Roux, S.J. and Hardison, L. (1986) Distribution of calmodulin in pea seedlings: immunocytochemical localization in plumules and root apices. *Planta*, **168**, 461–470.

Després, C., DeLong, C., Glaze, S., Liu, E. and Fobert, P.R. (2000) The *Arabidopsis* NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. *The Plant Cell Online*, **12**, 279–290.

Diaz-Camino, C., Annamalai, P., Sanchez, F., Kachroo, A. and Ghabrial, S.A. (2011) An effective virus-based gene silencing method for functional genomics studies in common bean. *Plant Methods*, **7**, 16.

El-Habbak, M.H. (2013) Overexpression/silencing of selected soybean genes alters resistance to pathogens. PhD Dissertation, University of Kentucky, Lexington, KY. Published online: http://uknowledge.uky.edu/plantpath_etds/8.

Fu, D.Q., Ghabrial, S. and Kachroo, A. (2009) *GmRAR1* and *GmSGT1* are required for basal, *R* gene-mediated and systemic acquired resistance in soybean. *Mol. Plant–Microbe Interact.* **22**, 86–95.

Garcia-Brugger, A., Lamotte, O., Vandelle, E., Bourque, S., Lecourieux, D., Poinssot, B., Wendehenne, D. and Pugin, A. (2006) Early signaling events induced by elicitors of plant defenses. *Mol. Plant–Microbe Interact.* **19**, 711–724.

Ghabrial, S.A. and Schultz, F.J. (1983) Serological detection of bean pod mottle virus in bean leaf beetles. *Phytopathology*, **73**, 480–483.

Goel, D., Singh, A.K., Yadav, V., Babbar, S.B. and Bansal, K.C. (2010) Overexpression of osmotin gene confers tolerance to salt and drought stresses in transgenic tomato (*Solanum lycopersicum* L.). *Protoplasma*, **245**, 133–141.

Goodin, M.M., Dietzgen, R.G., Schichnes, D., Ruzin, S. and Jackson, A.O. (2002) pGD vectors: versatile tools for the expression of green and red fluorescent protein fusions in agroinfiltrated plant leaves. *Plant J.* **31**, 375–383.

Govindarajulu, M., Elmore, J.M., Fester, T. and Taylor, C.G. (2008) Evaluation of constitutive viral promoters in transgenic soybean roots and nodules. *Mol. Plant–Microbe Interact.* **21**, 1027–1035.

Hartman, G.L., West, E.D. and Herman, T.K. (2011) Crops that feed the world. 2. Soybean—worldwide production, use, and constraints caused by pathogens and pests. *Food Secur.* **3**, 5–17.

Heo, W.D., Lee, S.H., Kim, M.C., Kim, J.C., Chung, W.S., Chun, H.J., Lee, K.J., Park, C.Y., Park, H.C., Choi, J.Y. and Cho, M.J. (1999) Involvement of specific calmodulin isoforms in salicylic acid-independent activation of plant disease resistance responses. *Proc. Natl. Acad. Sci. USA*, **96**, 766–771.

Hepler, P.K. (2005) Calcium: a central regulator of plant growth and development. *The Plant Cell Online*, **17**, 2142–2155.

Horton, P., Park, K.J., Obayashi, T., Fujita, N., Harada, H., Adams-Collier, C.J. and Nakai, K. (2007) WoLF PSORT: protein localization predictor. *Nucleic Acids Res.* **35**, W585–W587.

Hunt, A., Xu, R., Addepalli, B., Rao, S., Forbes, K., Meeks, L., Xing, D., Mo, M., Zhao, H., Bandyopadhyay, A., Dampanaboina, L., Marion, A., Von Lanken, C. and Li, Q. (2008) *Arabidopsis* mRNA polyadenylation machinery: comprehensive analysis of protein–protein interactions and gene expression profiling. *BMC Genomics*, **9**, 220.

Kachroo, A. and Ghabrial, S. (2012) Virus-induced gene silencing in soybean. *Methods Mol. Biol.* **894**, 287–297.

Kachroo, A. and Kachroo, P. (2007) Salicylic acid-, jasmonic acid- and ethylene-mediated regulation of plant defense signaling. In: *Genetic Engineering: Principles and Methods* (Setlow, J.K., ed.), pp. 55–83. New York: Plenum Press.

Kachroo, A., Fu, D.Q., Havens, W., Navarre, D., Kachroo, P. and Ghabrial, S.A. (2008) An oleic acid-mediated pathway induces constitutive defense signaling and enhanced resistance to multiple pathogens in soybean. *Mol. Plant–Microbe Interact.* **21**, 564–575.

Kim, M.C., Panstruga, R., Elliott, C., Muller, J., Devoto, A., Yoon, H.W., Park, H.C., Cho, M.J. and Schulze-Lefert, P. (2002) Calmodulin interacts with MLO protein to regulate defence against mildew in barley. *Nature*, **416**, 447–450.

Kim, M.C., Chung, W.S., Yun, D.J. and Cho, M.J. (2009) Calcium and calmodulin-mediated regulation of gene expression in plants. *Mol. Plant.* **2**, 13–21.

Kinkema, M., Fan, W. and Dong, X. (2000) Nuclear localization of NPR1 is required for activation of *PR* gene expression. *The Plant Cell Online*, **12**, 2339–2350.

Koning, G., TeKrony, D.M. and Ghabrial, S.A. (2003) Soybean seedcoat mottling: association with soybean mosaic virus and *Phomopsis* spp. seed infection. *Plant Dis.* **87**, 413–417.

Lee, S.H., Kim, J.C., Lee, M.S., Heo, W.D., Seo, H.Y., Yoon, H.W., Hong, J.C., Lee, S.Y., Bahk, J.D., Hwang, I. and Cho, M.J. (1995) Identification of a novel divergent calmodulin isoform from soybean which has differential ability to activate calmodulin-dependent enzymes. *J. Biol. Chem.* **270**, 21 806–21 812.

Li, H., Havens, W.M., Nibert, M.L. and Ghabrial, S.A. (2011) RNA sequence determinants of a coupled termination–reinitiation strategy for downstream open reading frame translation in *Helminthosporium victoriae* virus 190S and other victoriviruses (family Totiviridae). *J. Virol.* **85**, 7343–7352.

Ling, V. and Assmann, S.M. (1992) Cellular distribution of calmodulin and calmodulin-binding proteins in *Vicia faba* L. *Plant Physiol.* **100**, 970–978.

Lomonossoff, G.P. and Ghabrial, S.A. (2001) Comoviruses. In: *Encyclopedia of Plant Pathology* (Maloy, O.C. and Murray, T.D., eds), pp. 239–242. New York: John Wiley & Sons.

van Loon, L.C., Rep, M. and Pieterse, C.M.J. (2006) Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.* **44**, 135–162.

Luan, S., Kudla, J., Rodriguez-Concepcion, M., Yalovsky, S. and Gruissem, W. (2002) Calmodulins and calcineurin B-like proteins: calcium sensors for specific signal response coupling in plants. *Plant Cell*, **14**, S389–S400.

Martin, K., Kopperud, K., Chakrabarty, R., Banerjee, R., Brooks, R. and Goodin, M.M. (2009) Transient expression in *Nicotiana benthamiana* fluorescent marker lines provides enhanced definition of protein localization, movement and interactions *in planta*. *Plant J.* **59**, 150–162.

Mazarei, M., Elling, A.A., Maier, T.R., Puthoff, D.P. and Baum, T.J. (2007) GmEREBP1 is a transcription factor activating defense genes in soybean and *Arabidopsis*. *Mol. Plant–Microbe Interact.* **20**, 107–119.

Park, C.Y., Heo, W.D., Yoo, J.H., Lee, J.H., Kim, M.C., Chun, H.J., Moon, B.C., Kim, I.H., Park, H.C., Choi, M.S., Ok, H.M., Cheong, M.S., Lee, S.M., Kim, H.S., Lee, K.H., Lim, C.O., Chung, W.S. and Cho, M.J. (2004) Pathogenesis-related gene expression by specific calmodulin isoforms is dependent on NIM1, a key regulator of systemic acquired resistance. *Mol. Cells*, **18**, 207–213.

Pedersen, P. (2004) *Soybean Growth and Development*. Ames, IA: Iowa State University Extension.

Pfeiffer, A., Nagel, M.-K., Popp, C., Wüst, F., Bindics, J., Viczián, A., Hiltbrunner, A., Nagy, F., Kunkel, T. and Schäfer, E. (2012) Interaction with plant transcription factors can mediate nuclear import of phytochrome B. *Proc. Natl. Acad. Sci. USA*, **109**, 5892–5897.

Reddy, A.S.N. (2001) Calcium: silver bullet in signaling. *Plant Sci.* **160**, 381–404.

Reddy, V.S. and Reddy, A.S.N. (2004) Proteomics of calcium-signaling components in plants. *Phytochemistry*, **65**, 1745–1776.

Rochon, A., Boyle, P., Wignes, T., Fobert, P.R. and Despres, C. (2006) The coactivator function of *Arabidopsis* NPR1 requires the core of its BTB/POZ domain and the oxidation of C-terminal cysteines. *Plant Cell*, **18**, 3670–3685.

Rose, J.K.C., Ham, K.-S., Darvill, A.G. and Albersheim, P. (2002) Molecular cloning and characterization of glucanase inhibitor proteins: coevolution of a counterdefense mechanism by plant pathogens. *The Plant Cell Online* **14**, 1329–1345.

Sandhu, D., Tasma, I.M., Frasch, R. and Bhattacharyya, M.K. (2009) Systemic acquired resistance in soybean is regulated by two proteins, orthologous to *Arabidopsis* NPR1. *BMC Plant Biol.* **9**, 105.

Sanfaçon, H., Iwanami, T., Karasev, A.V., van der Vlugt, R., Wellink, J., Wetzel, T. and Yoshikawa, N. (2011) Secoviridae. In: *Virus Taxonomy: Classifcation and Nomenclature of Viruses: Ninth Report of the International Committee on Taxonomy of Viruses* (King, A.M.Q., Adams, M.J., Carestens, E.B. and Lefkowitz, E.J., eds), pp. 881–899. San Diego, CA: Elsevier.

Selote, D. and Kachroo, A. (2010) RPG1-B-derived resistance to *AvrB*-expressing *Pseudomonas syringae* requires RIN4-like proteins in soybean. *Plant Physiol.* **153**, 1199–1211.

Seo, P.J., Lee, A.K., Xiang, F.N. and Park, C.M. (2008) Molecular and functional profiling of *Arabidopsis pathogenesis-related* genes: insights into their roles in salt response of seed germination. *Plant Cell Physiol.* **49**, 334–344.

Singh, A.K., Fu, D.Q., El-Habbak, M., Navarre, D., Ghabrial, S. and Kachroo, A. (2011) Silencing genes encoding omega-3 fatty acid desaturase alters seed size and accumulation of bean pod mottle virus in soybean. *Mol. Plant–Microbe Interact.* **24**, 506–515.

Takabatake, R., Karita, E., Seo, S., Mitsuhara, I., Kuchitsu, K. and Ohashi, Y. (2007) Pathogen-induced calmodulin isoforms in basal resistance against bacterial and fungal pathogens in tobacco. *Plant Cell Physiol.* **48**, 414–423.

Thomma, B.P.H., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A. and Broekaert, W.F. (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proc. Natl. Acad. Sci. USA*, **95**, 15 107–15 111.

White, P.J. and Broadley, M.R. (2003) Calcium in plants. *Ann. Bot.* **92**, 487–511.

Xia, Y., Gao, Q.M., Yu, K.S., Lapchyk, L., Navarre, D., Hildebrand, D., Kachroo, A. and Kachroo, P. (2009) An intact cuticle in distal tissues is essential for the induction of systemic acquired resistance in plants. *Cell Host Microbe*, **5**, 151–165.

Yamakawa, H., Mitsuhara, I., Ito, N., Seo, S., Kamada, H. and Ohashi, Y. (2001) Transcriptionally and post-transcriptionally regulated response of 13 calmodulin genes to tobacco mosaic virus-induced cell death and wounding in tobacco plants. *Eur. J. Biochem.* **268**, 3916–3929.

Yang, L.A., Ji, W., Zhu, Y.M., Gao, P., Li, Y., Cai, H., Bai, X. and Guo, D.J. (2010) GsCBRLK, a calcium/calmodulin-binding receptor-like kinase, is a positive regulator of plant tolerance to salt and ABA stress. *J. Exp. Bot.* **61**, 2519–2533.

Yoo, J.H., Park, C.Y., Kim, J.C., Heo, W.D., Cheong, M.S., Park, H.C., Kim, M.C., Moon, B.C., Choi, M.S., Kang, Y.H., Lee, J.H., Kim, H.S., Lee, S.M., Yoon, H.W., Lim, C.O., Yun, D.J., Lee, S.Y., Chung, W.S. and Cho, M.J. (2005) Direct interaction of a divergent CaM isoform and the transcription factor, MYB2, enhances salt tolerance in *Arabidopsis*. *J. Biol. Chem.* **280**, 3697–3706.

Yoshiba, Y., Nanjo, T., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1999) Stress-responsive and developmental regulation of Δ1-pyrroline-5-carboxylate synthetase 1 (P5CS1) gene expression in *Arabidopsis thaliana*. *Biochem. Biophys. Res. Commun.* **261**, 766–772.

Yu, C.-S., Chen, Y.-C., Lu, C.-H. and Hwang, J.-K. (2006) Prediction of protein subcellular localization. *Proteins Struct. Funtc. Bioinformatics*, **64**, 643–651.

Zhang, C.Q. and Ghabrial, S.A. (2006) Development of bean pod mottle virus-based vectors for stable protein expression and sequence-specific virus-induced gene silencing in soybean. *Virology*, **344**, 401–411.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 (a) Genome organization of *Bean pod mottle virus* (BPMV) RNA2 and vector construction strategy. BPMV RNA2 is translated into two overlapping carboxy co-terminal polyproteins. CR, RNA2 replication cofactor; MP, movement protein; L-CP, large coat protein; S-CP, small coat protein. The vector pG7R2V (Zhang and Ghabrial, 2006) contains a green fluorescent protein (GFP) fragment (ΔGFP) inserted between the coding regions of MP and L-CP, and also contains additional restriction sites (*Bam*HI and *Msc*I) for cloning of the gene of interest (GOI). In constructing the vector, the Q/M cleavage site sequence between MP and L-CP (the dipeptide QM plus flanking sequences) was duplicated. A T7 RNA polymerase promoter sequence was engineered upstream of the modified full-length RNA2 cDNA and cloned into plasmid pGEM T easy to generate pG7R2V. The plasmid pG7R2V can be linearized by digestion with *Sal*I prior to transcription. (b) Percentage sequence identity between the five known *GmCaM* genes. Values above the diagonal are based on full-length sequences and values below the diagonal are based on coding region sequences.

Fig. S2 Immunoblot analysis of total protein from soybean plants infected with empty and recombinant *Bean pod mottle virus* (BPMV) vectors. Samples of 20 μg of total protein extracted from mock soybean plants or soybean plants previously inoculated with vector, recombinant *GmCaM4*-overexpressing (*GmCaM4*-OE) and *GmCaM4*-silenced (*GmCaM4*-SI) transcripts were subjected to sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis using an anti-BPMV coat protein (CP) antiserum. The positions of large (L-CP) and small (S-CP) CPs are indicated to the right. An arrowhead points to a minor host protein detectable in the Western blots using the BPMV-CP antiserum. Extracts from the same plants as used for Western blot analysis were also tested by double-antibody sandwich enzyme-linked immunosorbent analysis (ELISA), as described previously (Ghabrial and Schultz, 1983), and the corresponding ELISA values for the different treatments are listed below the Western blot.

Fig. S3 Reverse transcription-polymerase chain reaction (RT-PCR) analysis and quantification by densitometry. (a) Semi-quantitative RT-PCR to assess the *GmCaM4* transcript levels in *GmCaM4*-SI and empty vector soybean plants using a pair of primers designed to amplify the entire coding sequence of *GmCaM4* (450 bp). (b) Quantification of bands generated by RT-PCR was performed using a Bio-Rad Gel-Doc XR+ system and Quantity One software (Bio-Rad Laboratory, Hercules, CA, USA). Briefly, intensity profiles for selected bands are determined and the area under the profile curve to the baseline is integrated, resulting in units of intensity × millimetres.

Table S1 Primers used in this study.