Research Article

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Early perception of stink bug damage in developing seeds of field-grown soybean induces chemical defences and reduces bug attack

Romina Giacometti,[a,b] Jesica Barneto,[a,b] Lucia G Barriga,[c] Pedro M Sardoy,[b,d] Karina Balestrasse,[a,b] Andrea M Andrade,[e] Eduardo A Pagano,[a] Sergio G Alemano[e] and Jorge A Zavala[a,b*]

Abstract

BACKGROUND: Southern green stink bugs (*Nezara viridula* L.) invade field-grown soybean crops, where they feed on developing seeds and inject phytotoxic saliva, which causes yield reduction. Although leaf responses to herbivory are well studied, no information is available about the regulation of defences in seeds.

RESULTS: This study demonstrated that mitogen-activated protein kinases MPK3, MPK4 and MPK6 are expressed and activated in developing seeds of field-grown soybean and regulate a defensive response after stink bug damage. Although 10–20 min after stink bug feeding on seeds induced the expression of *MPK3*, *MPK6* and *MPK4*, only MPK6 was phosphorylated after damage. Herbivory induced an early peak of jasmonic acid (JA) accumulation and ethylene (ET) emission after 3 h in developing seeds, whereas salicylic acid (SA) was also induced early, and at increasing levels up to 72 h after damage. Damaged seeds upregulated defensive genes typically modulated by JA/ET or SA, which in turn reduced the activity of digestive enzymes in the gut of stink bugs. Induced seeds were less preferred by stink bugs.

CONCLUSION: This study shows that stink bug damage induces seed defences, which is perceived early by MPKs that may activate defence metabolic pathways in developing seeds of field-grown soybean.

Supporting information may be found in the online version of this article.

Keywords: herbivory; jasmonic acid; MAPK signalling; plant–insect interactions; salicylic acid; *Glycine max* L; *Nezara viridula* L

1 INTRODUCTION

Field soybean (*Glycine max*) crops, the most important grown seed legume in the United States and in South America, are invaded by southern green stink bug (*Nezara viridula* L.).[1] Stink bugs preferentially feed on young developing seeds and inject noxious saliva, which causes further tissue damage and discoloration, or may even cause abortion or deformation, producing yield reduction.[2,3]

Plants have evolved sophisticated signalling networks that allow them to perceive and to cope with leaf herbivory. Herbivore attack alters the levels of the defensive phytohormones jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) in plants (Wu and Baldwin, 2010), resulting in a reconfiguration of their transcriptomes and proteomes.[4,5] Leaves damaged by chewing herbivores accumulate chemical defences, such as proteinase inhibitors (PIs), polyphenol oxidase, isoflavonoids or alkaloids.[6,7] In addition, piercing-sucking insects, such as aphids, induce defences in leaf tissues to avoid or deter attack.[8]

Mitogen-activated protein kinases (MPKs) are part of the signalling networks that convert the extracellular stress stimuli into intracellular defence responses.[9,10] Three consecutive elements (MPKKK, MPKK or MEK and MPK) comprise the signal cascade in

* Correspondence to: JA Zavala, Cátedra de Bioquímica/Instituto de Investigaciones en Biociencias Agrícolas y Ambientales, Facultad de Agronomía, Universidad de Buenos Aires, Avda. San Marín 4453, C1417DSE Buenos Aires, Argentina. E-mail: zavala@agro.uba.ar

a Cátedra de Bioquímica/Instituto de Investigaciones en Biociencias Agrícolas y Ambientales, Facultad de Agronomía, Universidad de Buenos Aires, Buenos Aires, Argentina

b CONICET – Consejo Nacional de Investigaciones Científicas, Buenos Aires, Argentina

c Cátedra de Biomoléculas, Facultad de Agronomía, Universidad de Buenos Aires, Buenos Aires, Argentina

d Cátedra de Zoología, Facultad de Agronomía, Universidad de Buenos Aires, Buenos Aires, Argentina

e Laboratorio de Fisiología Vegetal, Universidad de Rio Cuarto, Río Cuarto, Argentina

which MPK is finally activated by the dual phosphorylation of the receptor motif Ser/Thr-Pro. The phosphorylation of the substrate MPK can occur in both the cytoplasm and nucleus, and then targets transcription factors related to defences in plant tissues.[11,12] The *Arabidopsis thaliana* orthologue salicylic-acid-induced protein kinase (SIPK), named MPK6, together with the wound-induced protein kinase (WIPK) or its *A. thaliana* orthologue MPK3, balances the accumulation of JA and SA in response to mechanical or herbivore-induced wounding produced by Lepidoptera.[13] Whereas SA levels are regulated by SIPK and WIPK in *Nicotiana attenuata* through LECTIN RECEPTOR KINASE1,[14] only SIPK regulated herbivory-induced ET.[7] Conversely, AtMPK4 is a negative regulator of SA-mediated defences and a positive modulator of the antagonist JA.[15]

While the defensive roles of MPKs have been well characterised in leaves of *Arabidopsis*, tobacco (*Nicotiana*) and tomato (*Solanum lycopersicum*), information on MPK in soybean is scarce. A soybean MPK1 homologue reported to be activated in response to salt stress[16] and a 49 kDa MPK induced by elevated phosphatidic acid have been characterised.[17] A further two MPKs identified in soybean leaves (MPK3 and MPK6) activated by *Phytophthora sojae* were described as interactive partners with MKK1.[18] Transient silencing *GmMPK4* expression with virus-induced gene silencing (VIGs), which seems to be functioning downstream of MPKK1, greatly increased SA levels in soybean leaves and thus provided elevated resistance to downy mildew (*Peronospora manshurica*) and soybean mosaic virus.[19] A more recent study demonstrated that transient *GmMPK6*-silenced plants were also more resistant to downy mildew and accumulated elevated levels of the conjugated form of SA, suggesting that MPK6 functions in defence responses of soybean leaves.[20] Although we presume that seeds might regulate responses to insect attack similarly to leaves, so far no experiments have been carried out to evaluate the role of MPKs in developing seeds as a response to piercing-sucking insects feeding on soybean pods. Previous studies showed that *N. viridula* attack induced levels of isoflavonoids in soybean seeds,[21] and elevated high levels of daidzin and genistin in pods reduced insect damage and preference.[22] Surprisingly, no information is available about the regulation of defences in seeds, the main components of fitness in natural species and the final product of many field crops.

To examine the role of seed's MPKs in modulating chemical defences against herbivores, pods of field-grown soybean were either attacked by stink bugs placed on pods and enclosed with a fine mesh bag or induced by application of phytohormones related to defences (JA and SA). Specifically, we examined whether MPK signalling responses are present in developing seeds, whether *GmMPK3*, *GmMPK4* and *GmMPK6*, as well as *GmMPKK1*, are differentially expressed after either mechanical (punctured with a needle) or stink bug damage and whether MPK activity and expression are related to the upregulation of defence genes induced by either JA/ET or SA as a result of herbivory. Our results demonstrate that MPKs are expressed in developing seeds of field-grown soybean and activated by stink bug feeding or mechanical damage. Expression of *GmMPK4* was only detected a few minutes after stink bug damage. Our results show that stink bug damage to developing soybean seeds triggers JA/ET and SA and their mediated defences through early MPK gene transcription and phosphorylation, thus activating specific MPKs in a time-dependent manner. Defensive responses of developing seeds reduced the digestive enzyme activity of stink bugs and modified their feeding behaviour.

2 EXPERIMENTAL METHODS

2.1 Plant growth and treatments

Soybean seeds from the commercial cultivar Williams 82 (PI 518671) were grown at the experimental fields of the University of Buenos Aires, Argentina. Regular agronomic practices and planting dates were carried out. In order to test the effects of herbivory on developing seeds of field-grown soybean, adults of *Nezara viridula* L. (Heteroptera: Pentatomidae) were collected from several soybean agricultural fields of rural locations near Buenos Aires and kept for at least 5 days under controlled conditions (25 ± 3 °C, 60 ± 9% relative humidity and 15:9 L:D photoperiod). Stink bugs were allowed to feed on artificial diet, consisting of rehydrated soybean and sunflower seeds, and water-imbibed cotton (supplemented with 0.5% w/v ascorbic acid). Eggs were collected, and a laboratory population was established. Young unmated adults (5–15 days old) from this population were used in the experiments. Insects were starved for 24 h to enhance their feeding motivation. When field-grown plants reached the reproductive stage R6 (full seed),[23] pods of similar sizes and from the same node position within the plant were selected for treatments. Plants were protected against insect attack before treatments were applied.

Four treatments and one untreated (control) were performed: (1) stink bug damage – insects were placed on pods and enclosed with a fine mesh bag; (2) mechanical damage – to mimic stink bug damage, we used a needle to produce punctures on pods and seeds; (3) pods sprayed with 1.5 mM of salicylic acid (SA) (Sigma, St Louis, MO); (4) pods sprayed with 100 μM of methyl jasmonate (MeJA) (Sigma), as described by Cerrudo *et al.*[24] and Shang *et al.*[25] To determine JA, ET and SA levels and gene expression, treated and untreated pods were collected 3, 24 and 72 h after treatments were initiated. To determine MPK expression and activity, pods were collected 10 and 20 min and 24 h after stink bug or mechanical damage. For the stink bug damage treatments, samples were collected after visual confirmation of stylet damage by stink bugs. All samples were flash frozen in liquid nitrogen and stored at −80 °C until use. The experiments were performed twice, firstly during the summer of 2011–2012 and secondly in 2012–2013, in four plots of 18 rows, 0.35 m apart and 2.5 m long. One pod per plant at the time of harvest and treatment was collected to form a replicate, and three replicates per year were collected to total six replicates from six independent plants ($n = 6$). Seeds were separated from pods for chemical analysis.

2.2 Preference choice experiments and proteolytic activity determination

Manipulative choice experiments were carried out in the field using adult insects from the natural population. Two neighbour plants were covered with a voile bag and connected to a wooden platform (30 cm). Stink bugs were starved for 24 h and then released in the centre of the platform, where they were allowed to choose between soybean branches (bearing pods) that had previously (72 h) been either (i) untreated (control) or (ii) treated with MeJA or (iii) treated with SA. All four possible paring combinations of treated and untreated plants were tested. Plants for the choice experiments were at the R5 growth stage (beans beginning to develop). The tests lasted 3 h, and the response variable

was the number of insects found sucking on the pods, which was used to evaluate feeding preferences in paired comparisons between treatments. There were three independent replicates for each experiment of paring combination, and ten new stink bugs were used in each experiment and replicate.

One stink bug per plant was placed on a pod at the R5 stage and enclosed with a fine mesh bag on 16 plants. Eight stink bugs after 24 or 72 h were removed at each time point for analysis of gut cysteine protease activity. Insects were dissected under cold saline solution (215 mM of NaCl) under a stereoscopic microscope, and two midguts from each time point were combined to form one replicate ($n = 4$) and stored at −20 °C. Digestive cysteine protease activity extracts were obtained as described in Zavala *et al.*,[26] with slight modification. Tri-K citrate buffer (30 mM, pH 6.0) was added to the guts in a 3:1 (μL:mg) ratio, and tissues were homogenised using micropestles. After 30 min on ice, samples were centrifuged at 12 000 × *g* for 15 min at 4 °C, and the supernatant was used for activity determination. Cysteine protease activity was estimated with 2 μL of the gut extract in a final volume of 100 μL and with a specific chromogenic substrate (0.38 mM of *p*-Glu-Phe-Leu-pNA) in 0.1 M of sodium phosphate, 0.3 M of KCl, 0.1 mM of EDTA and 3 mM of DTT (pH 6.0). A calibration curve was constructed using increasing concentrations of pNA to convert from OD units to nmol pNA (R2 = 0.999). Absorbance at 405 nm from wells on the microtitre plate containing gut extracts and reaction mixture was measured at 20 s intervals for 30 min at 37 °C. Initial rates of hydrolysis were estimated from the slopes of the resulting absorbance versus time graphs. Protease activity was calculated as nmol pNA released mg^{-1} fresh gut tissue min^{-1}.

2.3 Gene expression determination

To analyse soybean seed transcripts, traditional reverse transcriptase-PCR was used. Briefly, total RNA was isolated from developing seeds using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's procedure. RNA isolated from plants was treated with DNaseI (Ambion, Austin, TX, USA). RNA quality and quantity were assessed spectrophotometrically and also on gels before the cDNA was synthesised using the SuperScript First-Strand Synthesis System kit for RT-PCR (Invitrogen) and then used as template. The primer sequences used in this study are detailed in supporting information Table S1. Samples were denatured at 94 °C for 2 min, followed by 15–35 cycles, depending on the transcript linearity (94 °C for 45 s, 50–58 °C for 45 s and 72 °C for 30 s). The levels of amplified products were determined at several cycle intervals to ensure that samples were analysed during the exponential phase of amplification. We performed reactions without reverse transcriptase to control the presence of contaminating DNA. The soybean elongation factor (*GmELF1b*), which is a part of the ribosomal protein translation complex, was considered to be a constitutively expressed mRNA and was used as an internal control.[27,28] A series of dilutions for each cDNA sample was prepared and run with the RT1-ELF1b and RT2-ELF1b primer pair to determine the efficiency of amplification of a 260 bp product in each of the cDNA synthesis samples as internal mRNA loading control. In order to determine the accuracy of amplified cDNA, sequences were cloned and sequenced. Transcripts were quantified using ImageJ.[29] Although soybean is an ancient tretaploid and many genes have multiple copies, to analyse MPK expression, we used a general primer set to amplify each MPK family (MPK3, MPK4 and MPK6) owing to the high identity among nucleotide sequences (supporting information Fig. S4).

2.4 MPK determination

To analyse MPK protein levels and kinase activity, seeds were ground in liquid nitrogen with a mortar and pestle and were suspended in the extraction buffer: 20 mM of HEPES-NaOH, pH 7.5, 10 mM of KCl, 10 mM of $MgCl_2$, 10% v/v glycerol, 1% v/v Triton X-100, 40 mM of β-glycerophosphate, 10 mM of NaF, 1 mM of Na_3VO_4, 5 mM of DTT and one tablet of cOmplete mini protease mix with EDTA per 10 mL (Roche, Mannheim, Germany). All manipulations were thereafter performed at 4 °C. The resulting suspension was spun down in a microcentrifuge at maximum speed for 30 min, and the supernatant was centrifuged for 45 min at 100 000 × *g*. This second supernatant was used immediately for enzymatic assays. Protein concentration was determined by the method of Lowry *et al.*[30] using bovine serum albumin as standard. Total MPK activity was measured using a non-radioactive method based on the detection of the phosphorylation of the substrate myelin basic protein (MBP) (Millipore, Darmstadt, Germany) using the western blot technique. The phosphorylated substrate was analysed by immunoblot analysis probing with a phospho-specific monoclonal MBP antibody. The reactions were carried out under conditions of linearity with respect to the amount of extract and the incubation time. The phosphorylation reactions were performed in a final volume of 50 μL mixture containing 5 μg of MBP, 100 μM of ATP, 10 mM of $MgCl_2$, 1 mM of DTT, 20 μM of PKC inhibitor peptide (catalogue number 12–121; Upstate Biotechnology, Lake Placid, NY) and 2 μM of PKA inhibitor peptide (PKI) (catalogue number 12–151; Upstate Biotechnology). The protein extract was added to the mixture to initiate the phosphorylation reaction, and the incubation was performed at 30 °C for 30 min.

In order to detect and quantify specific MPK levels and MPK activity, proteins from soluble extracts were resolved by 10–12% SDS-PAGE and were analysed by western blot. As C-termini of MAPKs in soybean have not diverged significantly from their *A. thaliana* orthologues, commercial *A. thaliana* antibodies were used in this study. Previous publications have shown that these react in a specific manner with soybean MAPKs.[19,20] Triplicate gels were run for all of the sample preps. Gels were transferred to polyvinylidene fluoride (PVDF) membranes, followed by blocking of the membranes in TBS with 5% non-fat dried milk and incubation at 4 °C overnight with anti-*A. thaliana* MPK3, AtMPK4 or AtMPK6 antibodies (1:1000) (Sigma). Immunological detection was performed using anti-rabbit IgG conjugated to alkaline phosphatase (1:5000) (Sigma) in TBS with 5% non-fat dried milk at room temperature for 2 h with gentle shaking. For active MAPK detection, the primary antibody used was Phospho-p44/42 MPK (Erk1/2) (Thr202/Tyr204) rabbit monoclonal antibody (catatlogue number 4370) from Cell Signaling Technology (Danvers, MA, USA) diluted (1:2000) in TBST–5% BSA (Sigma). The blots were then incubated with the secondary antibody, anti-rabbit IgG conjugated to alkaline phosphatase (1:5000) (Sigma) in TBST–1% (w/v) BSA at room temperature for 2 h with shaking. To detect phosphorylated MBP substrate, membranes were probed with anti-phospho-specific MBP-clone P12 monoclonal antibody (1:500) (catalogue number 05–429; Upstate Biotechnology). The blots were then incubated with the secondary antibody, anti-mouse IgG conjugated to alkaline phosphatase (1:5000) (Sigma) in TBST–1% (w/v) BSA at room temperature for 2 h with shaking. In all cases, the blots were washed and then developed using a standard NBT/BCIP substrate mixture. For MPK expression level analysis, equal loading and protein transfer were monitored by gel staining with Coomassie Brilliant Blue and Ponceau S staining of the membranes.

For the purpose of assessing specific MPK activation, sampled seeds were used for protein extracts (approximately 1 mg) in the presence of the cOmplete mini protease inhibitor cocktail with EDTA (Roche) and incubated overnight at 4 °C with 200 µL of Protein A-Sepharose beads conjugated with anti-AtMPK3, AtMPK4 or AtMPK6 antibodies (Sigma). After several washing steps with 20 mM of Tris–HCl, pH 7.5, 5 mM of EGTA, 100 mM of NaCl and 1 mM of Triton X-100, immunoprecipitates were analysed by immunoblot using Phospho-p44/42 MPK (Erk1/2) (Thr202/Tyr204) monoclonal antibody (catalogue number 4370) from Cell Signaling Technology.

2.5 Phytohormone determination

For JA/ET and free SA determinations, 0.2 g dry weight (DW; g plant^{-1}) of seeds was used. Plant material was homogenised in an Ultraturrax T25 basic instrument (IKA; Staufen, Germany) with 5 mL of deionised water. D6-JA and D6-SA were used as internal standards, and 50 ng of each was added to samples. Samples were centrifuged at 1540 × *g* for 15 min. The supernatant was adjusted to pH 2.8 with 15% (v/v) acetic acid and extracted twice with diethyl ether. The organic fraction was evaporated under vacuum. The dried extracts were dissolved in 1 mL of methanol and filtered on a vacuum manifold at a flow rate of <1 mL min^{-1}. The eluate was evaporated at 35 °C under vacuum in a SpeedVac SC110 (Savant Instruments, New York, NY). The assay employed four biological replicates ($n = 4$).

JA and SA were separated from tissues by reversed-phase high-performance liquid chromatography (HPLC). An Alliance 2695 separation module (Waters, Milford, MA) equipped with a Restek C18 column (100 mm × 2.1 mm, 3 µm) was used to maintain the performance of the analytical column. Fractions were separated using a gradient of increasing methanol concentration, a constant glacial acetic acid concentration (0.2% in water) and an initial flow rate of 0.2 mL min^{-1}. The gradient was increased linearly from 40% methanol/60% water–acetic acid at 25 min to 80% methanol/20% water–acetic acid. After 1 min, the initial conditions were restored, and the system was allowed to equilibrate for 7 min. The identification and quantification of hormones were performed on a quadrupole tandem mass spectrometer (Quattro Ultima; Micromass, Manchester, UK) fitted with an electrospray ion (ESI) source. The spectrometry software used was MassLynx v.4.1 (Waters).

Ethylene emission was determined by introducing each soybean pod into a 110 mL glass container that was tightly sealed with a silicon septum. A quantity of 1 mL of the head-space gas was extracted after 3 and 24 h. Ethylene was quantified on a model 4890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) fitted with a flame ionisation detector and a stainless steel Porapak N column (3.2 mm × 2 m; 80/100 mesh). The injector, oven and detector temperatures were 110, 90 and 250 °C respectively. Carrier gas N_2 was used at a flow rate of 0.37 mL s^{-1}. Five independent replicates were evaluated ($n = 5$).

2.6 Statistical analysis

Data were analysed with StatView, v.5.0 (SAS, Cary, NC). The relative expression values and kinase activities were analysed by analysis of variance (ANOVA) followed by least significant difference (LSD) test comparisons in all experiments, using several separations of means ($P = 0.05$; $P = 0.01$; $P = 0.001$). For ethylene measurements, statistical significance was determined by one-way ANOVA. The model assumptions of homogeneity of variance and normality were tested using Levene's test and the Shapiro–Wilk's test respectively. When these assumptions were not satisfied, data were transformed into ln for further analysis. Treatment means were compared with the control using Dunnett's test ($P < 0.05$). For JA and SA quantification, analyses were performed separately, and, when necessary, varFunc = varIdent function was used to stratify the variances within the levels of a factor (40). The activity of cysteine proteinases in the gut of stink bugs was analysed by *t*-test and preference choice experiments by the chi-square test.

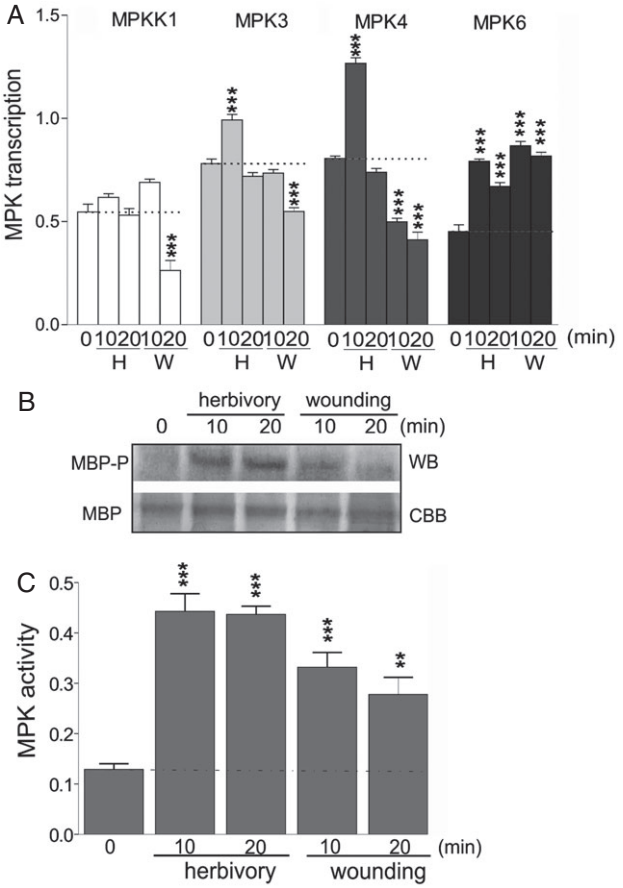

Figure 1. MPK early response to *N. viridula* and wounding treatments. (A) RT-PCR analysis of *GmMPKK1*, *GmMPK3*, *GmMPK4* and *GmMPK6* genes. Densitometric quantification of bands normalised to *ELF1b* mRNA. (B) *In vitro* phosphorylation of MBP substrate in the presence of ATP by soluble extracts (100 µg). (C) Quantified phosphorylation levels from undamaged, stink-bug-damaged (H) or mechanically damaged (W) developing seeds collected 10 and 20 min after treatments. To measure MPK activity, the reactions were run on 12% SDS-PAGE gels and transferred to PVDF membranes for western blotting using anti-phospho-MBP antibody (Upstate Biotechnology). MBP phosphorylation levels were quantified using ImageJ software. After normalisation to the total amount of MBP loaded, determined by Coomassie staining of the gels, the relative levels of MBP phosphorylation were represented using Prism 4.0 software. Values are the mean ± SE from two independent experiments with three replicates per year ($n = 6$). The asterisks represent significant differences between treatments versus the control group, determined by one-way ANOVA: **, $P < 0.01$; ***, $P < 0.001$.

2.7 Accession numbers

The orthologues of *Arabidopsis thaliana* MPKK, MPKs and genes tested here were identified in the soybean genome by reciprocal BLAST N between the National Centre for Biotechnology Information and Phytozome databases. Sequence

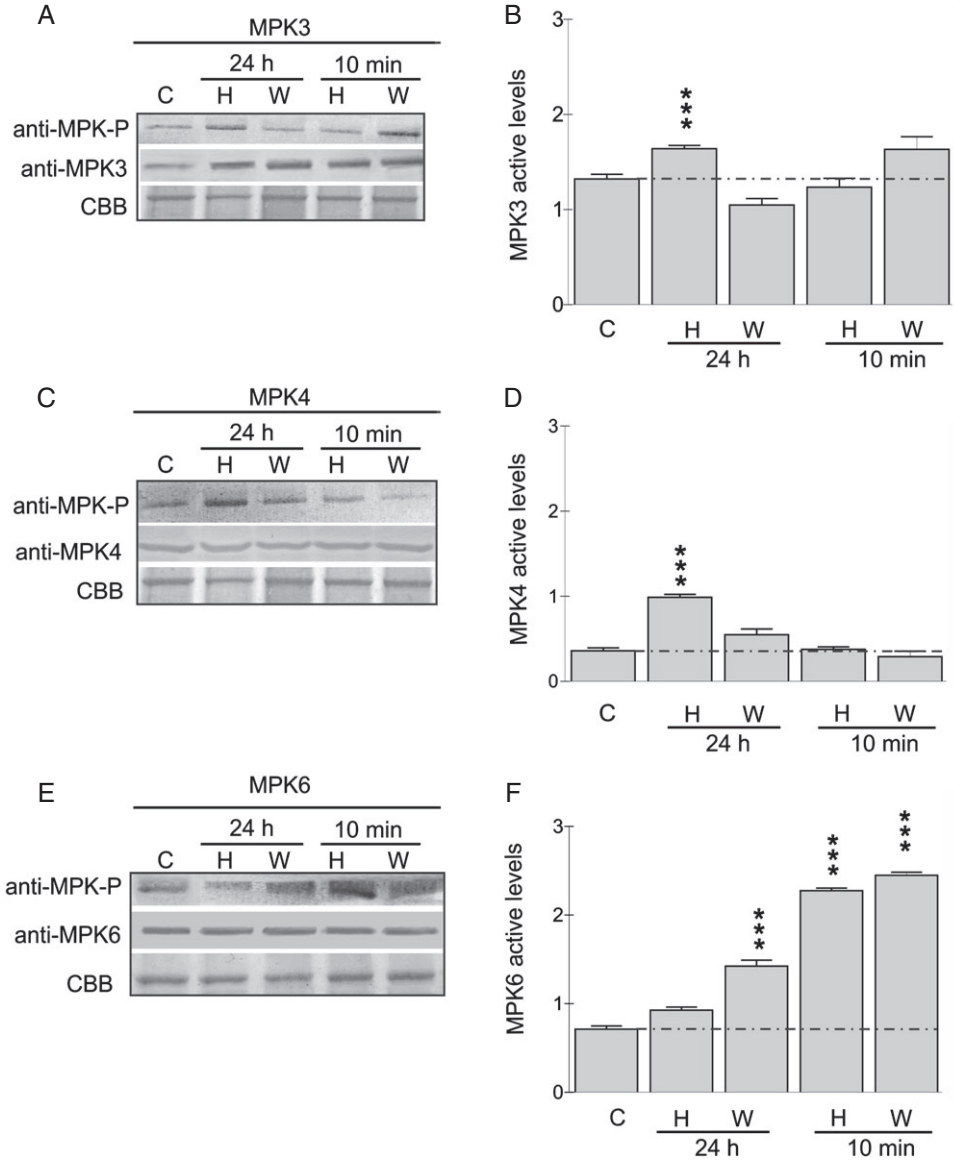

Figure 2. Soybean-specific MPK activation in response to herbivory and phytohormone stimuli. Pull-down assay of specific MPKs and immunological recognition and activity quantification of (A, B) MPK3, (C, D) MPK4 and (E, F) MPK6 from undamaged (C), stink-bug-damaged (H) or mechanically damaged (W) developing seeds collected 10 min and 24 h after treatments. MPK phosphorylation levels were quantified using ImageJ software. After normalisation to the total amount of MPK immunoprecipitated and loaded, determined by Coomassie (CBB) staining of the gels, the relative levels of MPK-specific phosphorylation were represented using Prism 4.0 software. Values are the mean ± SE from two independent experiments with three replicates per year ($n = 6$). The asterisks represent significant differences between treatments versus the control group, determined by one-way ANOVA: **, $P < 0.01$; ***, $P < 0.001$.

data used in this article can be found in databases under the following accession numbers: Glyma15g18860 corresponding to *MPKK1*, Glyma12g07770 to *MPK3*, Glyma16g03670 to *MPK4*, Glyma02g15690 to *MPK6*, Glyma03g26490.1 to *ELF1b*, Glyma03g181600.1 to *PAL2*, Glyma01g43880.1 to *CHS7*, Glyma13g24200.1 to *IFS2*, Glyma13g32560 to *PR1*, U51855 to *CystPI R1*, U51854 to *CystPI N2* and AF314823 to *KTI I*.

3 RESULTS

3.1 Stink bug damage induced early MPK transcriptional responses and total MPK activity

As MPKs are rapidly transcribed after wounding[7,31] and accumulation of MPKs in their inactive form increases the amount of substrate necessary to reach high activity levels, MPK activity and transcription were analysed in developing seeds of field-grown soybean harvested 10 and 20 min after stink bug attack or mechanical damage (Fig. 1). Stink bug feeding induced high expression levels of *MPK3* and *MPK6* after 10 min, and *MPK6* expression remained high up to 20 min after insect attack (Fig. 1A). Interestingly, while *MPK4* expression was highly induced by stink bug damage after 10 min in developing seeds, it was downregulated after 10 and 20 min of mechanical damage (Fig. 1A). In addition, 20 min after mechanical damage, *MPKK1* and *MPK3* expression were downregulated, and only *MPK6* expression turned out to be upregulated (Fig. 1A).

To investigate protein phosphorylation in response to damage and insect feeding, total MPK activity in seeds was measured (Figs

1B and C). After 10 and 20 min, stink bugs induced significantly higher phosphotransferase activity levels in developing seeds (fourfold) than mechanical damage (threefold) (Fig. 1C).

3.2 Specific MPK activation in response to herbivory and wounding

Activation of MPKs must occur in a signal-specific manner to communicate precise signals and induce effective defence responses against herbivores.[10] To discriminate the phosphorylation state of the MPKs analysed, immunoprecipitation assays were performed, and the immunological recognition of activated MPKs in response to treatments was assessed (Fig. 2). At the early time point of 10 min after stink bug and mechanical damage, only the activation state of MPK6 was induced in developing seeds of field-grown soybean (Figs 2E and F). Interestingly, at the late time point of 24 h, stink bug damage increased the activation of MPK3 and MPK4 (Figs 2A to D), while mechanical damage only induced MPK6 phosphorylation in developing seeds (Figs 2E and F). No fluctuations in MAPK individual protein expression were found after treatments, except for a slight increase in MPK6 translation at 20 min after stink bug feeding (see Figs 2A, C and E and supporting information Fig. S1).

A regression analysis showed no relation between expression of MPKs and kinase specific activities in developing seeds (supporting information Fig. S2), suggesting that the increased specific MPK activity levels after damage are not the result of higher amounts of protein, but rather of post-translational modifications by upstream MPKKs.

3.3 SA and JA/ET in soybean seeds

Leaf perception of herbivore damage activates MPKs and upregulates defence responses by modulating the production of JA/ET and SA.[7] To determine whether stink bug feeding on developing seeds of field-grown soybean increases the production of JA, SA and ET, damaged seeds and seeds treated with MeJA or SA were analysed (Fig. 3). While stink bug damage triggered only an early peak of JA accumulation (fourfold) after 3 h in developing seeds, free SA levels increased fourfold after 3 h and continued to rise up to 72 h (fivefold) after insect damage (Figs 3A and B). MeJA application on developing seeds induced JA accumulation 3 and 24 h after treatment (Fig. 3B). Although ET emission was induced 3 h after stink bug damage and MeJA and SA application on developing seeds, mechanical damage induced the highest ET emission levels (tenfold) 3 and 24 h after elicitation (Fig. 3C).

3.4 Gene expression in response to stink bug damage

Activated MPKs regulate gene expression by the phosphorylation of DNA-binding transcription factors or by directly activating certain enzymes, which in turn upregulate defences against herbivory.[11] Although the two critical genes involved in flavonoid synthesis in developing seeds, phenylammonia-lyase (*PAL*) and chalcone synthase (*CHS*) were upregulated in all treatments, only stink bug and mechanical damage increased isoflavone synthase (*IFS*) expression (Fig. 4 and supporting information Fig. S3), an enzyme responsible for the synthesis of the isoflavonoids daidzein and genistein. While both inducible cysteine protease inhibitor (PI) genes *N2* and *R1*[26] were induced by all treatments, trypsin PI (Kunitz TPI: *KTI I*) expression in developing seeds was only upregulated by mechanical damage and MeJA (Fig. 4 and supporting information Fig. S3).

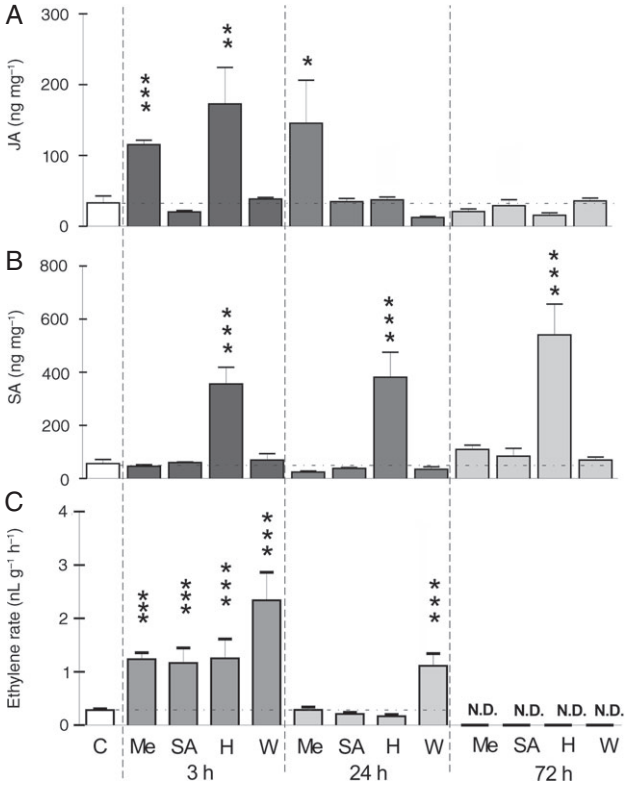

Figure 3. Soybean seed phytohormone accumulation in response to herbivory. (A) Jasmonic acid (JA), (B) salicylic acid (SA) and (C) ethylene levels from undamaged (C), methyl-jamonate-treated (Me), salicylic-acid-treated (SA), stink-bug-damaged (H) or mechanically damaged (W) developing seeds collected 3, 24 and 72 h after treatments. Values are the mean ± SE from pods of five independent plants ($n = 5$). Asterisks represent significantly different levels between control and treatments (*, $P < 0.1$; **, $P < 0.05$; ***, $P < 0.01$). N.D. indicates no detectable values.

As expected, SA-inducible gene *PR1* was upregulated in developing seeds 72 h after SA application, while MeJA downregulated *PR1* expression (Fig. 4 and supporting information Fig. S3). Interestingly, 24 h after stink bug damage or SA application, *PR1* was downregulated, suggesting that either undamaged field-grown plants may present already high *PR1* expression levels or both treatments downregulate *PR1* expression (Fig. 4 and supporting information Fig. S3). The results suggest that JA/ET and SA defences against herbivores are regulated after stink bug damage in developing seeds of field-grown soybean in a time-dependent manner.

3.5 Stink bugs avoid feeding on induced developing seeds

Herbivory-induced proteinase inhibitors that reduce the activity of digestive proteases and the performance of insects have been found to be induced after feeding on leaves.[6,26] Stink bugs that fed on developing seeds of field-grown soybean for 72 h had lower cysteine protease activity than those that fed either for 24 h on developing seeds or on artificial diet (Fig. 5A). To test the hypothesis that developing seeds with induced defences, regulated by either JA or SA, are less preferred by stink bugs than uninduced ones, we performed binary choice bioassays. Stink bugs preferred to feed on uninduced (control) developing seeds of field-grown soybean rather than on those that were defence-induced by application of either MeJA or SA 72 h before the experiments were initiated (Fig. 5B). Interestingly, more stink

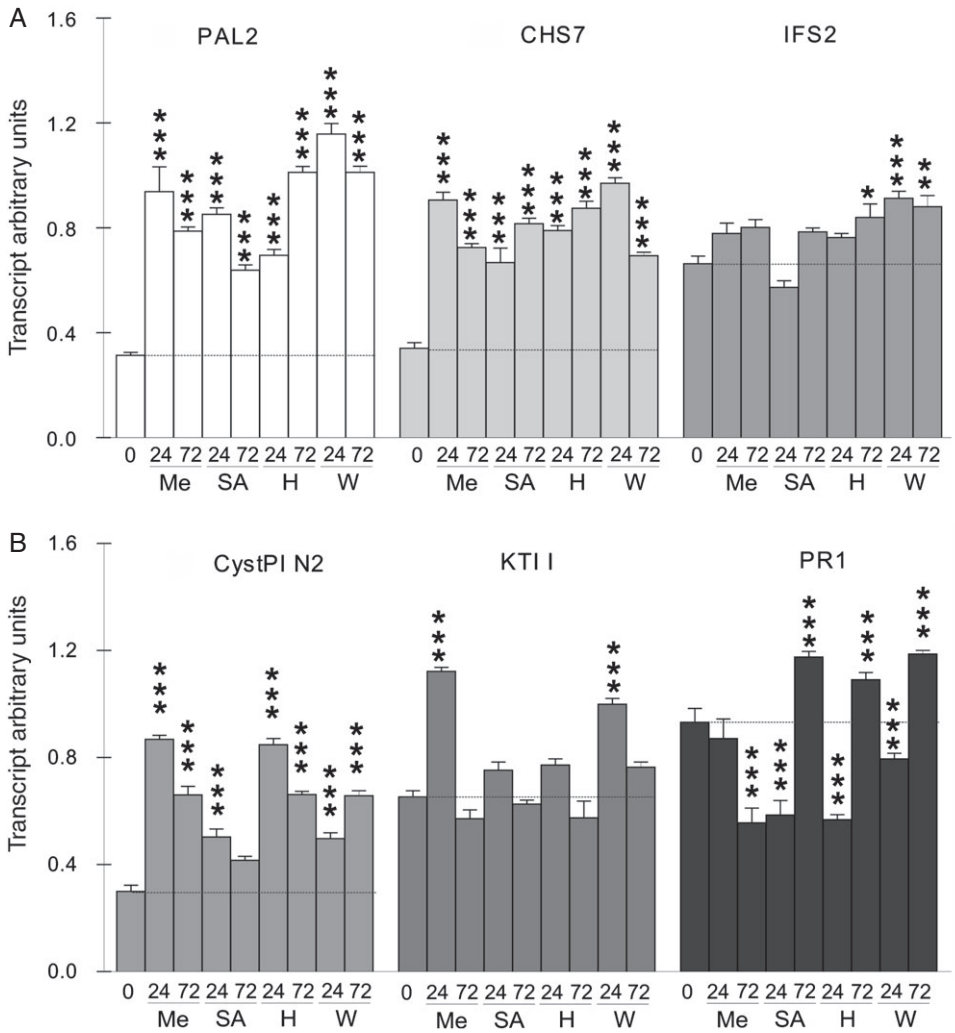

Figure 4. Transcriptional profile of enzymes involved in secondary metabolite accumulation involved in plant defences. Densitometric quantification of RT-PCR analysis of transcripts of (A) phenyl ammonia-lyase (*PAL*), chalcone synthase (*CHS7*) and isoflavone synthase (*IFS2*) and (B) cysteine protease inhibitor (*CystPI N2*), Kunitz trypsin protease inhibitor (*KTI I*) and pathogenesis-related protein 1 (*PR1*) from undamaged (0), methyl-jamonate-treated (Me), salicylic-acid-treated (SA), stink-bug-damaged (H) or mechanically damaged (W) developing seeds collected 24 and 72 h after treatments. Images of the bands were analysed using ImageJ software and values were normalised to *ELF1b* mRNA. Values are the mean ± SE from two independent experiments with three replicates per year ($n = 6$). The asterisks represent significant differences between treatments versus the control group, determined by one-way ANOVA: **, $P < 0.01$; ***, $P < 0.001$.

bugs preferred to feed on developing seeds induced by MeJA than on those induced by SA (Fig. 5B).

4 DISCUSSION

Leaf recognition of pathogen and herbivore damage leads to rapid transcription and activation of MAPK signalling pathways that induce JA/ET- and SA-regulated defences in many plant species.[7,15,19,20,32] This study demonstrated that *MPK3*, *MPK4* and *MPK6*, as well as an MPKK (*MPKK1*), are expressed in seeds of field-grown soybean, and they are differentially transcribed and activated after herbivore perception (Figs 1 and 2). Although 10 and 20 min after stink bug damage the developing seeds induced expression of *GmMPK3*, *GmMPK6* and *GmMPK4*, only MPK6 was phosphorylated after damage (Figs 1 and 2). MPK6 regulates herbivore-induced JA/ET and SA in leaves of *N. attenuata* and *Arabidopsis*.[7,33] Our field experiments demonstrated that stink bug feeding damage induced an early peak of JA accumulation and ET emission after 3 h in developing seeds, whereas SA was also induced early, and at increasing levels up to 72 h after stink bug damage (Fig. 3).

In general, responses of plants to sucking insects are considered to be JA-independent signalling mediated by SA.[34,35] Additionally, expression levels of the JA-inducible *Pin2* and the SA-inducible *PR1* genes were not affected by saliva of marmorated stink bug (*Halyomorpha halys*) in tomato.[2] In this study, stink bug herbivory concomitantly modified the expression and activation of the MPKs in developing soybean seeds and the expression of genes typically modulated by SA or JA/ET (Fig. 3). Moreover, MeJA and SA treatments induced seed defences, which reduced the activity of digestive enzymes in the gut of stink bugs, and induced seeds were less preferred by stink bugs (Fig. 5). Our results suggest that stink bug damage is regulated by seed defences, and damage is perceived early by MPKs and may activate defence metabolic pathways in developing seeds, the main components of crop yield.

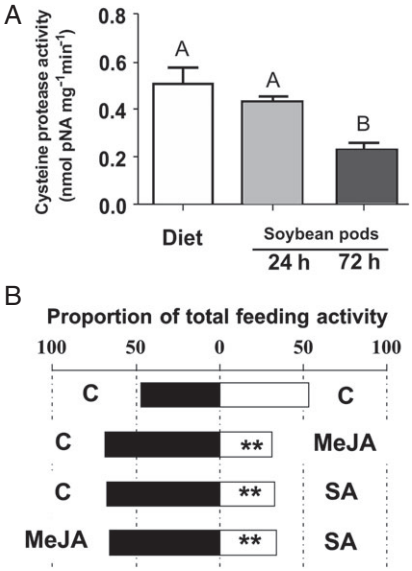

Figure 5. Induced defences of developing seeds of field-grown soybean reduced activity of digestive enzymes of stink bugs and affected feeding behaviour. (A) Cysteine proteinase activities in the gut of stink bugs that fed on artificial diet and developing seeds for 24 or 72 h, expressed as nmol pNA released mg^{-1} gut tissue min^{-1}. Values are the mean ± SE from four independent replicates ($n = 4$). Different letters indicate statistical differences (ANOVA–LSD test; $\alpha = 0.05$). (B) Choice experiments testing the preference of stink bugs for developing seeds untreated (C) or treated with either methyl jasmonate (MeJA) or salicylic acid (SA). Stink bugs were allowed to feed on artificial diet before the starvation period of 24 h. Values are the average of three independent replicates ($n = 3$), with ten adult stink bugs each time. Asterisks denote significant differences in the chi-square test: *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

Developing seeds responded to herbivore feeding mainly by upregulation of SA, which is the major hormone-regulating defence response against piercing-sucking insects.[34,35] Stink bug damage induced early (3 h) SA levels that increased up to 72 h after herbivory (Fig. 3B), and SA application, compared with MeJA, was a stronger determinant of feeding preference (Fig. 5B), highlighting the importance of this hormone in defence regulation in developing seeds of soybean. Interestingly, 24 h after stink bug damage or SA application, the SA-regulated gene *PR1* ws downregulated, suggesting that this gene was regulated by other pathways besides SA. Another non-exclusive possibility could rely on the fact that *PR1* expression levels were already up in the undamaged field-grown plants used as control (Fig. 4). Whereas both JA and SA regulate the synthesis of phenolic compounds by regulating phenyl ammonia-lyase (*PAL*), the expression of isoflavone synthase (*IFS*) and chalcone synthase (*CHS*) regulates specific isoflavonoid accumulation in developing soybean seeds.[36] Soybean seeds damaged by stink bugs increased the accumulation of daidzin and genistin under laboratory conditions,[21] and these isoflavonoids reduced soybean acceptance to *N. viridula* under field conditions.[22]

The early role of JA in defence responses of developing soybean seeds to stink bug damage was evidenced 24 h after the application of MeJA, which induced typical JA-regulated genes and downregulated SA-responsive genes (*PR1*) (Figs 3A and 4). Seed damaged by stink bugs after 24 h induced and downregulated gene responses similarly to MeJA treatment, with some exceptions, such as the JA-regulated *KTPI* (Fig. 4). Whereas TPIs are an effective defence against herbivores with trypsin as digestive proteases, such as lepidopteran larvae,[6] TPIs are not the target for cysteine proteases, the digestive enzyme in stink bugs.[37] However, both JA-regulated CystPI genes *N2* and *R1* were induced by stink bug damage and the rest of the treatments (Fig. 4 and supporting information Fig. S3), and thus CystPIs may inhibit the digestive cysteine proteases of stink bugs (Fig. 5A). Conversely, *IFS2* expression was only upregulated by mechanical and stink bug damage in developing seeds, suggesting a possible role of the early ET burst (Figs 3C and 4). Nevertheless, the regulation of SA- and JA/ET-related defences as response to stink bug damage can be explained by MAPKs fine-tuning.

Although both MPK3 and MKP6 redundantly regulate SA and JA in different plant species, only MPK6 regulates ET emission.[7,32] Silencing MPK6 but not MPK3 impaired herbivory-induced ET levels in *N. attenuata* and *Arabidopsis* by reducing the expression of CDPKs.[7,33] In field-grown soybean, after a few minutes of stink bug damage to developing seeds, MPK6 phosphorylation was increased as well as JA/ET and SA being induced (Figs 2 and 3). Transient or stable overexpression of *GmMPK6* in *N. benthamiana* and in *Arabidopsis* resulted in hypersensitive-response-like (HR-like) cell death.[20] Stink bug damage on developing seeds of field soybean normally produces HR-like cell death surrounding the puncture,[38] suggesting that high activity levels of MPK6 may also induce HR-like cell death in damaged seeds. Although both mechanical and stink bug damage increased MPK6 activity, only stink bug feeding amplified a full defensive response and induced both SA and JA/ET signalling and the early transcription of the three MAPKs studied (Figs 1 to 3). It is considered that saliva of stink bugs can suppress plant defences,[2,39,40] and might also carry yeasts that can grow within attacked seeds;[41] however, the components of saliva responsible for mediating adverse effects on crop production are still unknown.[42] The results of this study suggest that saliva of *N. viridula* is somehow recognised by developing soybean seeds inducing tailored defence responses against stink bugs, resulting in a decreased preference for induced seeds (Fig. 5).

While MPK3 and MPK6 are involved in the amplification of defensive responses, MPK4 plays a role in repressing SA biosynthesis. MPK4 in *Arabidopsis* blocks the SA signalling pathway by inhibiting EDS1 and PAD4 proteins and releasing the JA/ET pathway.[15,43] *GmMPK4*-silenced soybean accumulated SA, and SA-regulated genes were upregulated in leaves.[19] Whereas stink bug damage induced early transcription of *GmMPK4* in developing seeds, mechanical damage downregulated the expression of *GmMPK4*, which may explain the increase in ET emission 3 h after damage and the decrease in expression of the SA-regulated *PR1* gene (Figs 3 to 5). High ET levels can eliminate negative SA–JA interaction[44] and may allow the early accumulation of both SA and JA in developing seeds after stink bug damage (Fig. 3). Therefore, the lack of later (24 and 72 h) ET emission initiated negative SA–JA interaction, reducing JA accumulation and increasing SA accumulation and expression of the SA-regulated *PR1* gene in developing seeds after stink bug damage (Figs 3 and 4). Whereas early phosphorylation of MPKs induces rapid responses to insect damage, accumulation of MPK transcripts amplifies later responses by increasing the quantity of substrate to be phosphorylated. As stink bug damage induced increasing levels of free SA (Fig. 3), early transcription and later phosphorylation of MPK4 (24 h) may function as negative feedback to stop biosynthesis of SA once chemical defences are already upregulated as part of a hypersensitive response (Fig. 2).

The accumulation of signalling proteins in their inactive form and their prompt activation under stress situations can contribute to the accumulation of short-term stress imprints.[45] In *Arabidopsis*,

priming by the SA analogue BTH induced the accumulation of inactive MPK3 and MPK6,[46] which amplifies response levels to pathogen infection, thus enhancing defensive gene expression and induction of antifungal metabolites. Although the damage produced on developing seeds up- or downregulated the expression of three MPKs, only the activity and protein levels of MPK6 were increased (Figs 1 and 2 and supporting information Fig. S1). Furthermore, a regression analysis showed no relation between the expression of MPKs and kinase-specific activities (Fig. S2), suggesting that different levels of specific MPK activities were not the result of variation in expression levels, but rather of post-translational modifications (i.e. phosphorylation) by upstream MPKKs.

5 CONCLUSION

Our field experiments demonstrated that stink bug damage is perceived by MKPs of developing soybean seeds. Although insect damage induced an early peak of JA accumulation and ET emission in seeds, developing seeds responded to herbivore feeding mainly by upregulation of SA, which induced plant defences and reduced stink bug preference. As the defensive roles of MPK6, MPK4 and MPK3 have been well characterised in leaves of many species and no information is available about the regulation of defences in seeds, other MPKs could also be phosphorylated and be involved in signalling perception in developing seeds. This study suggests a possible role for MPK activation in fine-tuning the defensive response against herbivory in developing seeds, the selection unit in natural species.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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