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ORIGINAL PAPER

Stink bug *nezara viridula* sustains late MAPKs phosphorylation status and induces expression of genes related with cell wall rearrangement in developing soybean seeds

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Abstract
The southern green stink bug (*Nezara viridula*) is a serious invasive pest in United States and South America that decreases the quantity and quality of soybean seeds. Plants respond to insect attack recognizing cell injury and oral secretions, triggering mitogen-activated protein kinases (MAPK) pathway and inducing defenses against herbivores. Our field studies were conducted to evaluate late MAPKs involvement in defense modulation and the transcriptional response of soybean genes implicated in cell wall modification after stink bugs fed on developing seeds. We observed an induction in MPK3 and MPK6 transcription at 24 and 72 h after insects attacked soybean pods, while MPK6 was the only gene up-regulated after mechanically damaging the seeds. Exposure to JA and SA stimuli increased MPK3 and MPK6 levels. While SA triggered MPK4 activation, MPK3 and MPK6 were phosphorylated after both JA and SA treatments. Stink bugs feeding and SA treatment specifically increased the expression of expansine (EXP), xyloglucan endo-transferase (EXT), pectate lyase (PL), and polygalacturonase (PG) genes, all involved in the relaxation and restructuration of the cell wall. Moreover, examination of safranin-stained seed sections revealed that stink bug damage resulted in thickening of cell walls even on distal undamaged areas of cotyledons. Our study shows that stink bug damage elicits activation of MAPK signal in soybean seeds and induced SA that may induce genes related with cell wall restructuration, and could increase resistance to new insect attack by hardening cell walls.

Keywords Herbivory · MAPK signaling · *Glycine max* L. · *Nezara viridula* L. · Plant–insect interaction · Jasmonic acid · Salicylic acid · Cell wall

Introduction

Southern green stink bug (*Nezara viridula*; Hemiptera) is one the most important pest in soybean (*Glycine max* L.) crops in South and North America. This polyphagous insect feeds on different families of plants, but with preference for legumes, specially developing pods and seeds (Panizzi 1997; Baur et al. 2000). Through the years, several studies have been conducted to analyze the impact of stink bug's feeding affecting yield, quality, and germinative potential of soybean seeds (Todd and Turnipseed 1974; Corrêa-Ferreira and De Azevedo 2002).

Damage caused by stink bugs is initiated through mechanical injury by probing with their stylets (Corrêa-Ferreira and De Azevedo 2002; Depieri and Panizzi 2011), and followed by a burst of oxidative stress due to the injection of saliva in the tissues (Depieri and Panizzi 2011; Peiffer and Felton 2014). We have recently found that developing seeds of soybean perceived damage from *Nezara viridula* by inducing the expression an activation of mitogen-activated protein kinases (MAPK) 3, 4, and 6 (Giacometti et al. 2016). In addition, stink bug herbivory induced an early peak of jasmonic acid (JA) and salicylic acid (SA) accumulation, and ethylene (ET) emission in developing seeds after 3 h, which induced chemical defenses and seeds were less preferred

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by stinkbugs (Giacometti et al. 2016). Recently, it has been suggested that JA may regulate defenses against stink bugs in soybean. Exogenous application of JA induced flavonoid concentrations in soybean pods and reduced growth of the stink bug, *Euschistus heros* (da Graça et al. 2016). However, insect feeding on developing seeds not only should confront chemical defenses, such as isoflavonoids naturally induced in soybean pods by solar UV-B radiation (Zavala et al. 2015), but also structural defenses that make seed cell walls harder to be penetrated and may decrease the mechanical injury by stylets of stink bugs.

Plant cell walls are complex and dynamic structures with several functions, being a physical barrier against pathogens and herbivores (Kaloshian and Walling 2005; Hamann 2012). Primary cell walls consist of non-oriented cellulose microfibrils, which are mainly interlocked by a matrix of hemicellulose and pectin polysaccharides and structural proteins (Houston et al. 2016). Many enzymes are able to modify matrix polysaccharides, and the genes coding for these enzymes are activated through signaling pathways that include various protein kinases (Vorwerk et al. 2004). Since MAPK cascades can perceive stylet of stink bugs and are key factors in internalizing and transducing environmental signals, we propose that *N. viridula* damage triggers specific MAPKs and induce the expression of genes related with enzymes that produce conformational changes of cell wall in damaged seeds.

To our knowledge, no information is available yet about the genes downstream MAPKs involved in structural reorganization of cell wall of developing seeds after stink bugs attack. To evaluate the impact of stink bugs damage on cell wall of developing seeds of field-grown soybean, we analysed MAPKs expression and activation on damaged seeds and its effect on genes that take a role in cell wall modification. Here, we report the late signaling outcome produced by *N. viridula*. Our work provides evidence that seeds' defenses against piercing sucking insects like *Nezara* involves not only an early, but also a late activation of MAPKs as key regulators of JA- and SA-regulated genes involved in the restructuration of the cell wall, and may harden the seeds and hinder new attacks.

Materials and methods

Plant growth, insects, and treatments

Soybean seeds from the commercial cultivar Williams 82 (PI 518,671) 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 five days under controlled conditions (25 ± 3 °C, 60 ± 9% relative humidity, and photoperiod of 15:9 L:D). 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 before they were placed on pods, to enhance their feeding activity. When field-grown plants reached the reproductive stage of R6 (full seed; Fehr et al. 1971), 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 puncture on pods and seeds, (3) pods sprayed with 1.5 mM salicylic acid (SA; SIGMA), or (4) pods sprayed with 100 µM methyl jasmonate (MeJA; SIGMA) as described by Cerrudo et al. (2012) and Shang et al. (2011). To determine gene expression and MAPK activity, pods were collected 24 and 72 h after stink bugs or mechanical damage were performed. For the stink bug damage treatments, samples were collected after the visual confirmation of stylet damage by stink bug. All samples were flash frozen in liquid nitrogen and stored at 80 °C until use. The experiments were performed twice, the first one during summer of 2014–2015 and the second one in 2015–2016, 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 conform a total of six replicates from six independent plants ($n = 6$).

Fixation, sectioning, and histochemical staining

For light microscopy, cotyledons were fixed in FAA (formalin 33%, acetic acid 100%, and ethanol 70% in a ratio of 5:5:90 [vol/vol/vol]) and then embedded in paraffin according to Johansen (1940). Cross sections (10 µm) were prepared with a microtome and stained with safranin (1:25,000 in 50% ethanol) for 30 min and then with Fast Green (Sigma) (1:25,000 in 3:1 xylene: ethanol) for 5 min during the dehydration process. The sections were cleared in xylene, mounted on a slide before observation with a Carl Zeiss light microscope.

Gene expression determination

To analyze soybean seeds, transcripts traditional reverse transcriptase-PCR was used. Briefly, total RNA was isolated from developing seeds using TRIZOL reagent (Invitrogen) according to the manufacturer's procedure. RNA isolated from plants was treated with DNaseI (Ambion). RNA quality and quantity were assessed spectrophotometrically and also on gels before the cDNA was synthesized using the Super-Script First-Strand Synthesis System kit for RT-PCR (Invitrogen) and then used as template. Primer sequences used in this study are detailed in Table 1. 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 to 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 analyzed during the exponential phase of amplification. We performed reactions without reverse transcriptase to control the presence of contaminating DNA. The soybean elongation factor (*ELF1b*), which is a part of the ribosomal protein translation complex, was considered to be a constitutively expressed mRNA and used as an internal control (Tucker et al. 2007; Jian et al. 2008). 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 (Abramoff et al. 2004).

Table 1 Primers used in this study

Name	Sense	Sequence 5′–3′
RT1-MPKK1	Forward	AGCAGGTGCTGAAGGGTCTA
RT2-MPKK1	Reverse	TTCCTGGCTTCCATTGATTC
RT1-MPK3	Forward	CGTTTGCTCGCTGTTGAATA
RT2-MPK3	Reverse	TGGAGCGAATGATATGGTGA
RT1-MPK4	Forward	ATAGGTTCGCCAGATGATGC
RT2-MPK4	Reverse	GGTGCAAACAGGTTCCTCAT
RT1-MPK6	Forward	GAACTCGGAGACGAATGAGC
RT2-MPK6	Reverse	GTACTTCAACCCACGGAGGA
RT1-ELF1b	Forward	AAGGGAGGCTGCTAAAAAGC
RT2-ELF1b	Reverse	CAACTGTCAAGCGTTCCTCA
RT1-EXP	Forward	GCTTCCTGTGGTCAGTGCTA
RT2-EXP	Reverse	CACTGAACCTAACCCCTCCA
RT1-EXT	Forward	TTGGCCGAAACTACGTGCCC
RT2-EXT	Reverse	TTCTTTCGTGGGATCAAACC
RT1-PG	Forward	ATCAGCTGTGGCAGTTTCAG
RT2-PG	Reverse	TTCAACTCCCCATATGCTTC
RT1-PL	Forward	TCGCAAGAGACATGGTGATC
RT2-PL	Reverse	CACCATAGCATTCCCACCTT
RT1-XET	Forward	TGTGGTGGTGGCTACTTTTG
RT2-XET	Reverse	CACAAGCTTGAGCTGCATGT
RT1-XFT	Forward	GTACCGCAAAGCTTCTCCTC
RT2-XFT	Reverse	TTCGATCAGTGAGGATGGCA
RT1-CAD	Forward	TTGGGAACCATGGACTATAT
RT2-CAD	Reverse	AGGAAGCTCAAGGGGTTTGT
RT1-CEL	Forward	TGACCACTATTGCTGGATGC
RT2-CEL	Reverse	ATTTCGAATAGGCCTTGTTG

MAPK determination

Assays to analyze MAPK protein levels and kinase activity were conducted as described previously (Giacometti et al. 2016). Briefly, seeds were grounded in liquid nitrogen with a mortar and pestle, and were suspended in the extraction buffer: 20 mM HEPES-NaOH, pH 7.5, 10 mM KCl, 10 mM $MgCl_2$, 10% v/v glycerol, 1% v/v Triton X-100, 40 mM β-glycerophosphate, 10 mM NaF, 1 mM Na_3VO_4, 5 mM DTT, and one tablet of 'Complete mini' protease mix with EDTA per 10 ml (Roche). 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. (1951) using bovine serum albumin as standard. Total MAPK activity was measured using a non-radioactive method based on the detection of the phosphorylation of the substrate myelin basic protein (MBP-Millipore) using Western blot technique. The phosphorylated substrate was analyzed 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 MBP, 100 µM ATP, 10 mM $MgCl_2$, 1 mM DTT, 20 µM PKC inhibitor peptide (Catalog # 12–121, Upstate), and 2 µM PKA inhibitor peptide (PKI) (Catalog # 12–151, Upstate). 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 MAPK levels and MAPK activity, proteins from soluble extracts were resolved by 10–12% SDS–PAGE and were analyzed by Western Blot. Triplicate gels were run for all of the sample preps. Gels were transferred to PVDF (polyvinylidene fluoride) membranes, blocked the membranes in TBS with 5% non-fat dried milk, and incubated at 4 °C overnight with anti-*A. thaliana* MPK3, AtMPK4, or AtMPK6 antibodies (1:1000, cat# M8318, A7104 and A6979 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 MAPK (Erk1/2) (Thr202/Tyr204) rabbit monoclonal antibody (cat# 4370) from Cell Signaling Technology 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, cat# 05-429, Upstate). 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 MAPKs expression level analysis, equal loading and protein transfer were monitored by gel staining with Coomassie Brilliant Blue and Ponceau S staining of the membranes.

To determine MAPK specific activation, protein extracts (approximately 1 mg) from collected seeds were incubated with protease inhibitor and EDTA (Roche) overnight at 4 °C, together with 200 µl of A-Sepharose protein beads conjugated with anti-AtMPK3, AtMPK4, or AtMPK6 antibodies (SIGMA). After several washing steps with 20 mM Tris–HCl, pH 7.5, 5 mM EGTA, 100 mM NaCl, and 1 mM Triton X-100, immunoprecipitates were analyzed by immunoblot using Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) monoclonal antibody (cat# 4370) from Cell Signaling Technology.

Statistical analysis

Data were analyzed with Stat View, version 5.0 (SAS, Cary, NC). The relative expression values and kinase activities were analyzed by ANOVA followed by Tukey's test comparisons in all experiments using several separations of means (p 0.05, p 0.01, p 0.0001).

Accession numbers

The orthologs of *Arabidopsis thaliana* here tested were identified in the soybean genome by reciprocal BLASTn between the National Center for Biotechnology Information and Phytozome databases. Sequence 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*, Glyma17g260400 to *EXP*, Glyma16g045000 to *EXT*, Glyma19g006200 to *PG*, Glyma17g126500 to *PL*, Glyma15g169100 to *XET*, Glyma07g022300 to *XFT*, Glyma10g262400 to *CAD*, Glyma09g018500 to *CEL*, and Glyma03g26490.1 to *ELF1b*.

Results

Nezara induced a late MPK3 and MPK6 transcriptional response in seeds

Stink bug damage induced early gene expression of MAPKs in developing seeds of soybean crop (Giacometti et al. 2016). To determine whether *GmMPKs* 3, 4, and 6 are also induced after sustained amount of time, pods of field-grown soybean were exposed to insect feeding or mechanical damage and seeds were harvested and analysed after 24 and 72 h (Fig. 1a).

Whereas 24 h after stink bugs damage the expression of *MPKK1, MPK3* (2.7-fold), and *MPK6* (2.5-fold) was increased, *MPK3* expression remained high up to 72 h after damage (Fig. 1a). *MPK4* expression was not significantly affected by stink bug damage. Also, 24 h after mechanical damage, only *MPK6* expression was up-regulated (2.1-fold, Fig. 1a). Total MAPK activity in response to wounding and insect feeding was analyzed (Fig. 1b, c). Stink bug feeding induced significantly higher phosphotransferase activity levels in developing seeds than mechanical damage after 24 and 72 h (Fig. 1c).

SA and JA stimuli induced MAPK transcription in a similar fashion to stink bug feeding

A previous study showed that developing seeds perceive stink bug injury activating MAPKs, which up-regulates plant defenses modulated by SA, JA, and ET (Giacometti et al. 2016). To determine whether *GmMPKs* expression is regulated at late time points by either JA or SA in developing soybean seeds, we exogenously applied both phytohormones. Application of JA induced late transcription of *MPK3* and *MPK6* 24 and 72 h after treatment ($p < 0.0001$; Fig. 2a). In addition, JA induced *MPKK1* expression 72 h after treatment (1.4-fold; $p < 0.001$; Fig. 2a). Although 24 h after SA application, transcription of *MPKK1*, and *MPK3* and *MPK6* were increased (twofold), *MPK4* did not respond to any of these treatments (Fig. 2a).

Both SA and JA application increased total MAPK activity in developing seeds 24 h after treatments ($p < 0.0001$; Fig. 2b, c).

MAPK-specific activity is modulated by SA and JA hormones

To identify the phosphorylation status of MAPKs, we assessed immunoprecipitation assays and the immunological

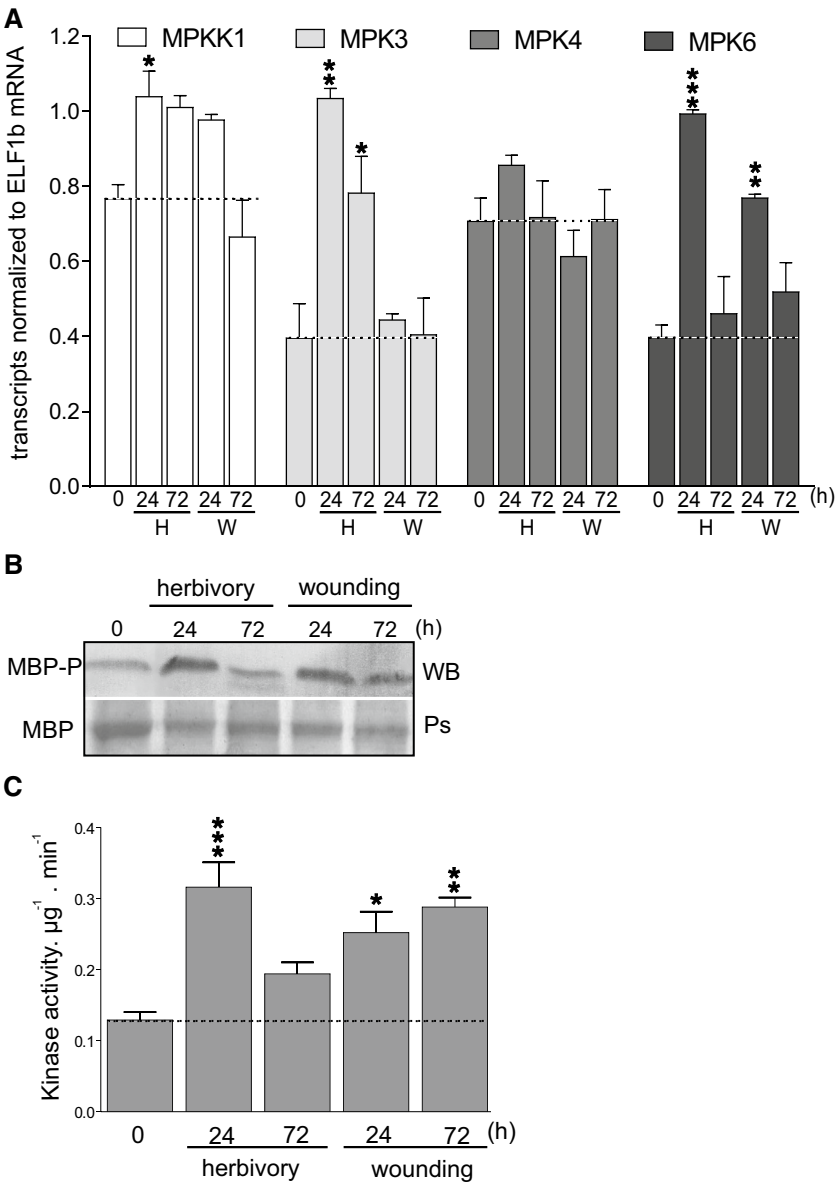


Fig. 1 MAPKs response to *N. viridula* attack. **a** Analysis of *MPKK1, MPK3, MPK4*, and *MPK6* transcription. Densitometric quantification was normalized to *ELF1b* mRNA, **b** in vitro phosphorylation of MBP substrate in the presence of ATP by soluble extracts (100 µg), and **c** quantified phosphorylation levels from undamaged, stink bug (H) or mechanical damaged (W) developing seeds collected 10 and 20 min after treatments. To measure MAPK activity, the reactions were run on 12% SDS–PAGE gels and transferred to PVDF (Polyvinylidene fluoride) membranes for Western blotting using anti-phospho-MBP antibody (Upstate). MBP phosphorylation levels were quantified using ImageJ software. After normalization to the total amount of MBP loaded and transferred determined by Ponceau S staining of the membranes, relative levels of MBP phosphorylation were represented using the software Prism 4.0. Values are the mean ± SE from 2 independent experiments with 3 replicates per year ($n = 6$). The asterisks represent significant differences between treatments versus the control group, determined by one-way ANOVA: $^{**}p < 0.01$, $^{***}p < 0.001$

recognition of activated forms of MAPKs. Only MPK3 and MPK6 were phosphorylated 24 h after JA application, while spraying the pods with SA triggered activation of MPK3, MPK4, and MPK6 in soybean seeds (Fig. 3a, b). No fluctuations in MAPK individual proteins expression were found after treatments (Fig. 3a).

Expression of genes encoding cell wall proteins in response to stink bug attack

To analyze the transcriptional response of genes encoding proteins involved in the biosynthesis, modification, and/or degradation of the cell wall of developing seeds, we applied to soybean pods JA and SA hormones and compared the transcriptional response with stink bug and mechanical damage (Fig. 4). Stink bug damage increased the expression of expansine (*EXP*), xyloglucan endo-transferase (*EXT*), polygalacturonase (*PG*), and pectate lyase (*PL*) genes after 72 h ($p < 0.001$ and $p < 0.05$, respectively; Fig. 4). With the exception of PG transcript, mechanical damage induced the same genes as herbivory ($p < 0.001$, $p < 0.05$; Fig. 4). While SA applied to pods induced high expression levels of *PG, PL*, xyloglucan fucosyltransferase (*XFT*), and xyloglucan endo-transglycosylase (*XET*) transcripts, JA application only up-regulated *EXP* and *XFT* expression ($p < 0.001$; Fig. 4).

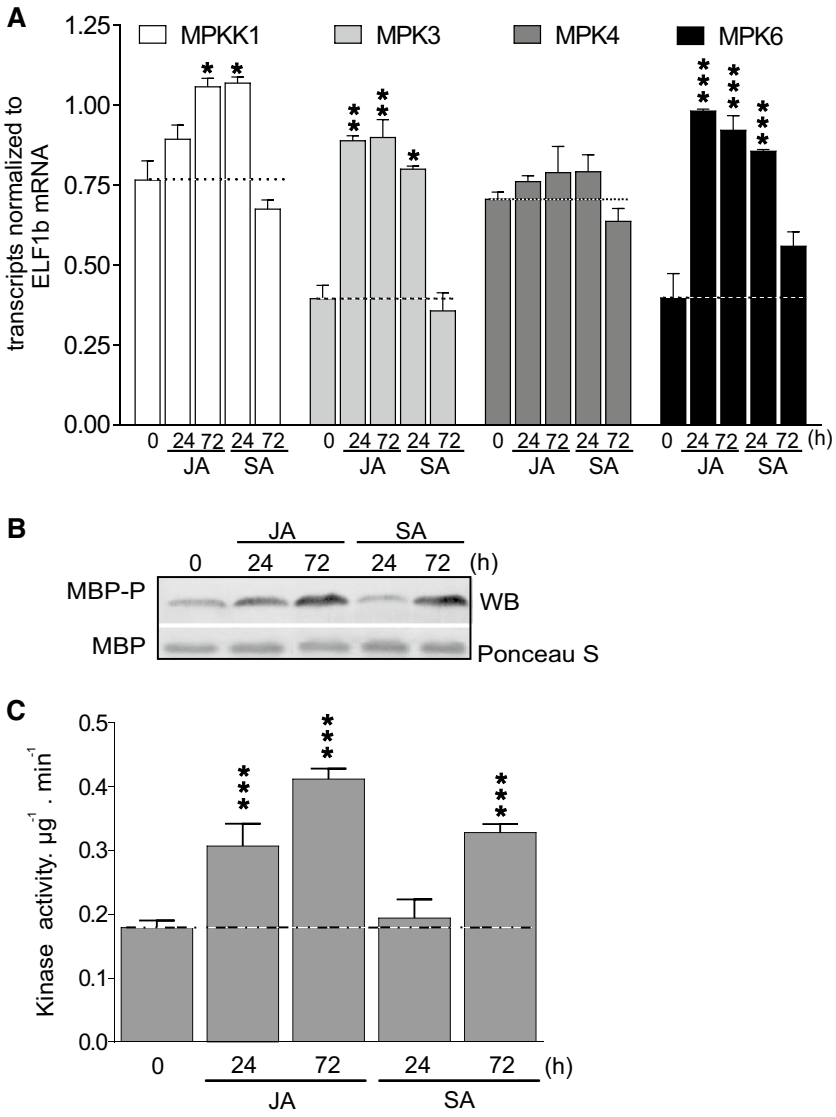


Fig. 2 MAPKs response to JA and SA treatments. **a** Analysis of *MPKK1, MPK3, MPK4*, and *MPK6* transcription. Densitometric quantification was normalized to *ELF1b* mRNA. **b** in vitro phosphorylation of MBP substrate by seeds soluble extracts (100 µg) was assessed in the presence of ATP, and **c** phosphorylation levels from methyl jamonate (JA), and salicylic acid (SA) sprayed seeds collected at 24 and 72 h after treatments were quantified. To measure total MAPK activity, the reactions were run on 12% SDS–PAGE gels and transferred to PVDF (Polyvinylidene fluoride) membranes for Western blotting using anti-phospho-MBP antibody (Upstate). MBP phosphorylation levels were quantified using ImageJ software. After normalization to the total amount of MBP loaded and transferred determined by Ponceau S staining of the membranes, relative levels of MBP phosphorylation were represented using the software Prism 4.0. Values are the mean ± SE from 2 independent experiments with 3 replicates per year ($n = 6$). The asterisks represent significant differences between treatments versus the control group, determined by one-way ANOVA: $^{**}p < 0.001$, $^{***}p < 0.0001$

Effect of stink bug feeding on developing seeds histology

We studied the effects of herbivory on cotyledon anatomy by observing safranin-fast green-stained distal sections from damaged zone under a light microscope (Fig. 5). Although 120 h after both stink bug and mechanical damage of developing seeds showed red staining on the undamaged tissue, the seeds damaged by the stink bugs showed bigger and brighter stained red area than those damaged mechanically (Fig. 5a–c). In addition, a larger number of palisade mesophyll cells of the cotyledons exposed to herbivory were stained red (Fig. 5c) versus those seeds damaged mechanically (Fig. 5b).

Since lignin is known to produce UV-stimulated autofluorescence (Hammond and Lewis 1987), we examined the safranin-stained sections for the presence of autofluorescence (Fig. 5d–f). We found that brighter fluorescence was produced by cells staining red (Fig. 5e, f). These results indicate an increase in the wall thickness possibly due to lignin deposition among other cellular components.

Discussion

Developing soybean seeds recognition of *Nezara viridula* damage leads to rapid transcription and activation of MAPK signaling pathway, inducing JA/ET- and SA-regulated defenses that in turn decreased feeding activity of stink bugs (Giacometti et al. 2016). However, MAPKs induced by stink bug damage may also activate defense pathways that could modify structural defenses and make seed cell walls harder. Our results from field-grown soybean experiments demonstrated that stink bug damage and exogenous

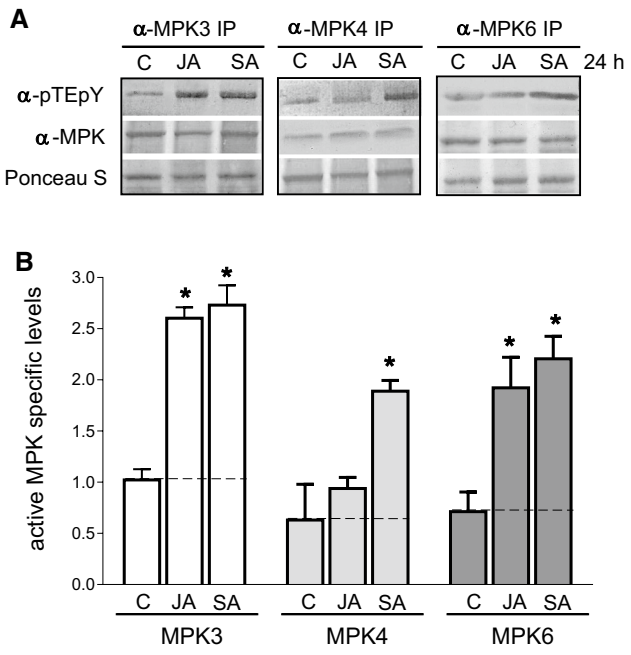


Fig. 3 Soybean-specific MAPK activation in response to phytohormones stimuli. **a** Pull-down assay of specific MAPKs and immunological recognition and activity quantification of MPK3, MPK4, and MPK6 from control (C), methyl jamonate (JA), and salicylic acid (SA) sprayed seeds collected 24 h after treatments. **b** MAPK phosphorylation levels were quantified using ImageJ software. After normalization to the total amount of MAPK immunoprecipitated and loaded determined by Ponceau S staining of the membranes, relative levels of MAPK specific phosphorylation were represented using the software Prism 4.0. Values are the mean ± SE from 2 independent experiments with 3 replicates per year ($n = 6$). The asterisks represent significant differences between treatments versus the control group, determined by one-way ANOVA: $^{**}p < 0.01$; $^{***}p < 0.001$

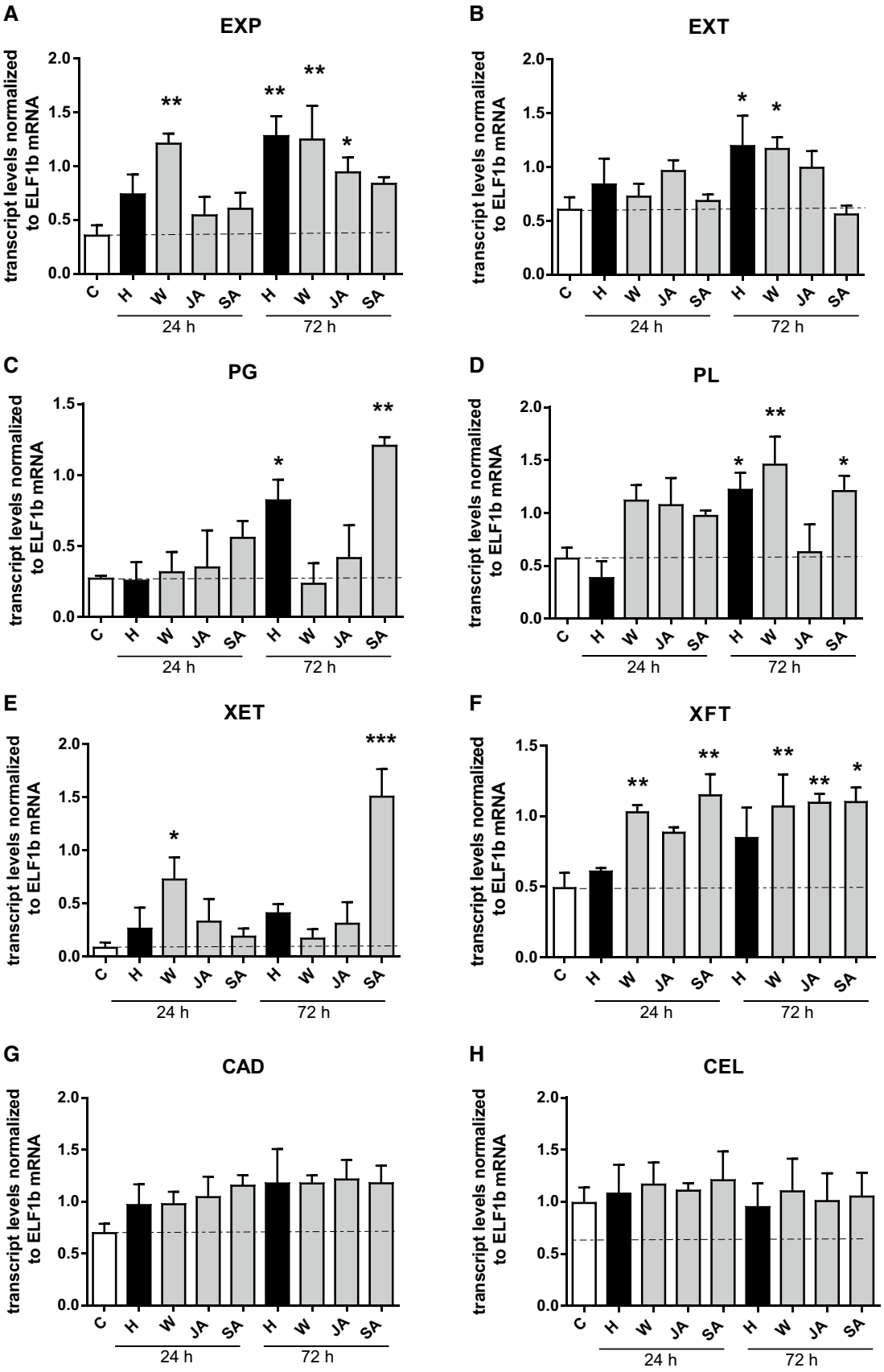
application of SA and JA induced expression and late phosphorylation of MAPK3 and MAPK6, while MAPK4 activity was only induced 24 h after the application of SA (Figs. 1, 2, 3). In addition, stink bug and mechanical damage, as well as SA treatment induced expression of genes related with cell wall reorganization, such as *expansine (EXP), xyloglucan endo-transferase (EXT), pectate lyase (PL), and polygalacturonase (PG)* (Fig. 4). Moreover, both mechanical- and stink bug-damaged seeds resulted in changes on cell walls that were revealed by brighter red staining of the undamaged areas of cotyledons (Fig. 5). Our results suggest that stink bug damage induced the restructuration of cell walls of developing seeds in field-grown soybean. To our knowledge, no study has shown before the consequences of stink bug attack on seed cell walls.

Enzymes present in stink bug's saliva may relax cell walls to facilitate stylet movement. In our field experiments, cells of developing seeds responded to stink bug damage in a similar fashion to SA treatment, by increasing the expression of an array of genes coding for enzymes ready to reconfigure the wall structure, like expansine (*EXP*), xyloglucan endotransferase (*EXT*), pectate lyase (*PL*), and polygalacturonase (*PG*) (Fig. 4). While expansins allow polymer chain movement and stress relaxation and thereby may accommodate cell wall (Cosgrove 2000), xyloglucan endo-transferases mediate interchange of cross-links in the cell wall matrix and functions as a key enzyme responsible for regulation of the cell wall reconstruction (Nishitani and Tominaga 1992). Likewise, polygalacturonases are commonly found in the separation layers of cell walls, and are involved in depolymerization by cleaving the 1,4-linkages of α-d-galacturonic acid in the homogalacturonan polymer (Markovič and Janeček 2001; Mohnen 2008). Other enzymes involved in the relaxation and restructuration of the cell wall are the pectate lyases, which are capable of cleaving α-1,4-glycosidic linkages in demethylated pectin by β-elimination (Palusa et al. 2007).

While expansins (EXP) allow polymer chain movement and stress relaxation and thus accommodating cell wall, xyloglucan fucosyltransferase (XFT), among the other hemicellulose biosynthetic enzymes, is involved in synthesis of various xylan and xyloglucan molecules (Zhong and Ye 2003; Del Bem and Vincentz 2010; Vuttipongchaikij et al. 2012; Zabotina et al. 2012; Voiniciuc et al. 2015). Since *EXP* and *XFT* genes were up-regulated by both JA and SA treatments in our experiments, we cannot rule out the involvement of JA in the defense response mediation. Our results suggest that stink bug attack induced the expression of cell wall genes leading to chemical rearrangements that might be mainly regulated by SA.

Based on our results, it is likely that stink bug damage also induces direct defenses through cell wall modification that may interfere with herbivore feeding preferences. Changes produced in cell walls of damaged seeds after 120 h of stink bug attack were reflected in distal zones from puncture sites where red staining was observed (Fig. 5). The undamaged tissue of attacked seeds showed alteration of the epidermis and the endosperm, and also the wall thickness was increased possibly due to lignin deposition. Lignin depositions can be detected by UV-stimulated autofluorescence of safranin-stained sections of seeds (Hammond and Lewis 1987). Dehydrating and strengthening cell walls by lignin accumulation may reduce the seed susceptibility towards a new attack. Our results suggest that stink bug damage and saliva may elicit a plant self-protection response, and this response may be regulated by activation MAPKs that induce SA-mediated defenses.

Rapid and transient activation of MAPKs is a well-established process in response to some abiotic and biotic stresses (Rodriguez et al. 2010), though its late activation and prolonged involvement in plant defense signaling against insects have not been studied. Our soybean field experiments showed that developing seeds respond to herbivore feeding


A
EXP
B
EXT
C
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CEL
transcript levels normalized to ELF1b mRNA
C
H
W
JA
SA
24 h
72 h

◂**Fig. 4** Transcriptional profile of seeds cell wall-modifying enzymes under herbivory. Densitometric quantification of RT-PCR analysis of **a** expansine (*EXP*), **b** xyloglucan endo-transferase (*EXT*), **c** polygalacturonase (*PG*), **d** pectate lyase (*PL*), **e** xyloglucan endo-transglycosylase (*XET*), **f** xyloglucan fucosyltransferase (*XFT*), **g** cinnamyl alcohol dehydrogenase (*CAD*), and **h** cellulase (*CEL*). Developing seeds were harvested 24 and 72 h after the following treatments: undamaged (control), stink bug (H), mechanical damaged (W) methyl jamonate (JA), and salicylic acid (SA). Images of the bands were analyzed using ImageJ software and values were normalized to *ELF1b* mRNA. Values are the mean ± SE from 2 independent experiments with 3 replicates per year ($n = 6$). The asterisks represent significant differences between treatments versus the control group determined by one-way ANOVA: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

not only by maintaining MAPK activity high, but also inducing transcription of *MPKK1, MPK3*, and *MPK6* at 24 and even 72 h after insect attack (Fig. 1). In a previous study, we found that stink bug damage induced early expression of these kinases, resulting in an early peak of JA and SA up to 72 h after damage (Giacometti et al. 2016). The late expression of *MPK3* and *MPK6* found in our study suggests the importance of these kinases in the amplification and keeping up-regulated defensive responses against stink bugs. *MPK4* was also expressed at later times after stink bug damage (Fig. 1), probably involved in controlling SA levels (Giacometti et al. 2016).

Main defensive responses of plants against herbivores are regulated by MAPKs that perceive the damage and induce the phytohormones JA/ET and SA that modify expression of genes related with plant resistance (Farmer et al. 2003; Kang et al. 2006; Wu et al. 2007). Exogenous application of JA increased flavonoid concentrations in soybean pods and not only reduced growth of the stink bug *Euschistus heros*, but also decreased feeding activity of *Nezara viridula* (da Graça et al. 2016; Giacometti et al. 2016). These studies suggest that JA could be an important modulator of chemical defenses against stink bugs in soybean. However, stink bug attack strongly induced SA accumulation in developing soybean seeds, and exogenous application of SA to developing seeds also decreased stink bug feeding activity (Giacometti et al. 2016). In our study, exogenous application of SA induced a concomitant expression and activation of MAPK3 and MAPK6, as well as the expression of genes related with cell wall restructuration like *PG, PL, XFT*, and *XET* (Figs. 2, 3, 4). Our previous study showed that MAPKs perceived stink bug damage and increased SA synthesis (Giacometti et al. 2016), and in this study, we showed that SA and stink bug damage trigger the restructuration of cell walls in developing soybean seeds, which may decrease the feeding activity of stink bugs.

Whereas most herbivorous insects cause extensive damage to plant tissues when feeding, many piercing sucking

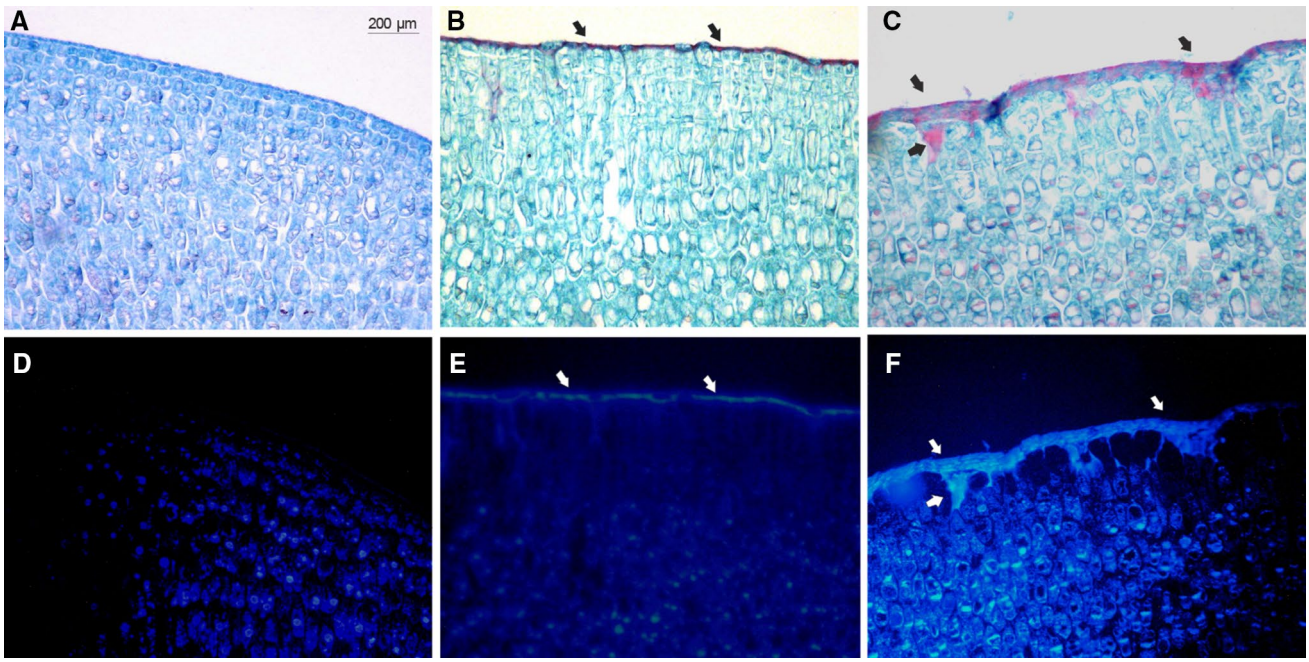

Fig. 5 Microscopic analysis of seeds after stink bug attack. Developing soybean seeds after mechanical damage or damaged by *Nezara viridula* were used for sectioning. Light photomicrographs of cross sections of control seeds (**a**), distal section from damage zone in mechanical wounded seed (**b**), or damaged by the stink bug (**c**) after 120 h. Distribution of autofluorescence in cells from control (**d**), mechanical wounded (**e**), and damaged by stink bug (**f**) after 120 h. Sections stained with safranin showing lignified cells are pointed with black arrows; white arrows indicate cells with autofluorescence and possible lignification

insects feed from the contents of tissues by inserting the stylet in the cells, thus limiting the damage and minimizing induction of a wounding response. Phloem-feeding aphids, whiteflies, and stink bugs induced expression of genes associated with SA and hardly induced JA-related defenses in attacked plants (Walling 2000; Moran and Thompson 2001; de Vos et al. 2007; Giacometti et al. 2016). Since up-regulation of SA inhibits the induction of JA, and JA-regulated compounds are effective defenses against these insects, up-regulation of SA in damaged tissue by piercing sucking insects may be a way to manipulate plant defenses (Mewis et al. 2005; da Graça et al. 2016; Pieterse et al. 2012; Giacometti et al. 2016; Züst and Agrawal 2016). However, our study suggests that increasing SA in tissue damaged by piercing sucking insects could induce changes in the cell walls of attacked seeds. Hardening cell walls of damaged tissue can be a good defense against piercing sucking insects and may reduce susceptibility towards new attack. We propose that *Nezara viridula* attack not only elicits a quick activation of MAPK signal in soybean seeds, but also maintains a phosphorylation status of these kinases after days that prompt the plant trigger downstream responses to defend non-wounded regions of the developing seed by hardening cell walls.

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Author Contributions RG and JZ conceived and designed research. RG and NI conducted experiments. JZ and EP contributed new reagents or analytical tools. RG and NI analyzed data. RG and JZ wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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