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RESEARCH ARTICLE

Early wound-responsive cues regulate the expression of WRKY family genes in chickpea differently under wounded and unwounded conditions

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Abstract Insect wounding activates a large number of signals that function coordinately to modulate gene expression and elicit defense responses. How each signal influences gene expression in absence of wounding is also important since it can shed light on changes occurring during the shift to wound response. Using simulated *Helicoverpa armigera* herbivory on chickpea, we had identified at least 14 WRKY genes that showed 5–50 fold increase in expression within 5–20 min of wounding. Our studies show that contrary to their collective effects upon wounding, individual chemical cues show distinct and often opposite effects in absence of wounding. In particular, jasmonic acid, a key early defense hormone, reduced transcripts of most WRKY genes by > 50% upon treatment of unwounded chickpea leaves as did salicylic acid. Neomycin (a JA biosynthesis inhibitor) delayed and also reduced early wound expression. H_2O_2 transiently activated several genes within 5–20 min by 5–8 fold while ethylene activated only a few WRKY genes by 2–5 fold. The summation of the individual effects of these chemical cues does not explain the strong increase in transcript levels upon wounding. Detailed studies of a 931 nt region of the *CaWRKY41* promoter, show strong wound-responsive GUS expression in *Arabidopsis* even in presence of neomycin. Surprisingly its expression was lost in the *coi1, ein2* and *myc2myc3myc4* mutant backgrounds suggesting the requirement of intact ethylene and JA signaling pathways (dependent on MYCs) for wound-responsive expression. The studies highlight the complexity of gene regulation by different chemical cues in the presence and absence of wounding.

Keywords Jasmonic acid · Ethylene · Hydrogen peroxide · Neomycin · *Helicoverpa armigera*

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Introduction

During growth, a plant is constantly exposed to various microbes and organisms that depend on it for survival. While most biotic interactions with the plant may be benign or beneficial, some predatory interactions can be detrimental to its growth or even survival. Insect herbivory by chewing and sucking pests is one of the most common predatory interactions that a plant has to protect itself against. In response to mechanical wounding and insect damage, a large number of changes occur at the site of wounding and its surrounding tissue. These include activation of early and late wound responses that address the immediate damage caused and subsequent infections. The early signaling steps include depolarization of the plasma transmembrane potential (Vm), rise in cytosolic Ca^{++}, production of reactive oxygen species (ROS), glutamic acid and mitogen-activated protein kinase (MAPK) activity (Erb and Reymond 2019; Miller et al. 2009; Kumari et al., 2019). Glutamate activates GLUTAMATE RECEPTOR-LIKE proteins, which increase intracellular Ca^{++} levels and help in the propagation of long distance signaling through slow wave potential (Toyota et al. 2018; Shao et al., 2020). Early wound responses also bring about a

rapid change in the signaling of different phytohormones such as JA, ethylene, auxin, ABA, SA and GA (Diezel et al. 2009; Erb et al. 2012; Pandey et al. 2017). These, in turn, modulate downstream signaling and activate different pathways which collectively contribute to the diversity of responses. The specific responses to biotic stress factors are often mediated through the action of transcription factors (TFs) of certain families. Important among these are WRKY, MYC and ERF families that regulate responses to various types of organisms. To what extent the individual signal molecules can independently activate their specific targets (such as TFs) on their own, and whether they also simultaneously require other cues to be in an activated state for their full action, is still not clear.

WRKY transcription factors belong to a large plant-specific family of TFs and function either as activators or suppressors of gene expression (Eulgem and Somssich 2007; Pandey and Somssich 2009; Adachi et al. 2015). They have been identified as being important in the development, defense as well as abiotic stress responses. The family is characterized by the presence of a conserved 60 amino acid WRKY domain with the sequence WRKYGQK at its N- terminus followed by one or more zinc finger motifs (Rushton et al. 2010). The WRKY domain binds the W box (TTGACC) in the promoters of target genes to regulate their transcription (Rushton et al. 1996; Ciolkowski et al. 2008).

Many WRKY family members are activated upon pathogen attack and may mediate responses by regulating hormone interactions (Eulgem 2005; Ryu et al. 2006). *NaWRKY3* and *NaWRKY6* from *N. attenuata* mediate herbivory-induced defense responses by modulating JA and JA-Ile/-Leu levels (Skibbe et al. 2008) while *AtWRKY70* mediates R gene resistance by altering the balance between SA and JA-dependent defense pathways (Li et al. 2006; Knoth et al. 2007). Similarly, *AtWRKY33* expression enhances resistance towards the necrotrophic fungi, *Alternaria brassicicola* and *Botrytis cinerea* (Zheng et al. 2006), while *AtWRKY53* and *AtWRKY70* regulate systemic acquired resistance (Wang et al. 2006). Transgenic tobacco lines expressing *Capsicum annum CaWRKY27* show enhanced resistance to *R. solanacearum* due to modulation of SA, JA and ethylene pathways (Dang et al. 2014) while *CaWRKY6* promotes resistance against *R. solanacearum* resistance by activating *CaWRKY40* (Cai et al. 2015). Lines over-expressing *OsWRKY89* show greater resistance to fungal blast and *Sogatella furicifera* by modulating wax deposition on the leaf surface (Wang et al. 2007). *OsWRKY53* acts as a suppressor for herbivore-induced responses (Hu et al. 2015). Other WRKYs such as *SlWRKY70*, *SlWRKY72a* and *72b*, *TaWRKY53* (from wheat), mediate defenses against aphids (Atamian et al. 2012; Bhattarai et al. 2010; Van Eck et al. 2010).

Cicer arietinum L. or chickpea is the primary legume in India (with India as the largest producer) but faces losses in productivity due to prevailing biotic and abiotic stresses (Dhaliwal et al. 2010). The pod borer, *Helicoverpa armigera,* is the major pest of chickpea (Sequeira et al. 2001; Dhaliwal et al. 2010) and alone accounts for losses of up to 30% or more (Dinesh, et al. 2017). Hence, a detailed understanding of the earliest wound responses mounted by chickpea against *Helicoverpa* is necessary so as to develop strategies to deter these as soon as they begin feeding on leaves/pods. In pursuit of these, we had performed simulated herbivory on chickpea leaves using *H. armigera* oral secretions and carried out a comparative analysis of transcriptomes of unwounded leaves with insect saliva-pretreated/mechanically wounded chickpea leaves at 20 min. The analysis revealed differential expression of about 8.4% of the chickpea transcriptome within 20 min of wounding (Pandey et al. 2017). Interestingly, a large number of genes of the WRKY family were identified amongst the earliest wound-inducible genes in the transcriptomic data. In this study, we have tried to dissect the early wound response of these genes to study how various factors contributing to the wound response can individually influence the expression of these genes in absence of wounding. Our studies show that each factor contributes differently to the regulation of each gene and that the wound response is not a summation of individual effects of the major chemical cues.

Materials and methods

Plant material

Chickpea seeds (*Cicer arietinum,* variety Pusa-362) were grown in pots in a growth chamber at 22 ± 2 °C with a 16 h light/8 h dark period at a light intensity of 100 $\mu Em^{-2} s^{-1}$ and 75% relative humidity. For neomycin treatment, chickpea seeds were grown in the field of NBRI from November to March.

Treatments

JA, SA and H_2O_2 treatment Eight-week old chickpea plants were treated either with 100 µM jasmonic acid (JA, dissolved in ethanol), 2 mM salicylic acid (SA, dissolved in ethanol), or 5 mM H_2O_2 by lightly and uniformly spraying on to the surface of leaves of individual plants using a hand sprinkler with constant flow. Samples were collected at intervals of 0, 20, 60, and 120 min after treatment. A set of plants sprayed with water containing the same amount of ethanol (used in the hormone sprays), and kept for

the same time intervals as those of JA/SA treatments, served as a negative control. Samples were frozen in liquid N_2 and stored at -70 °C until further use.

Ethylene treatment For ethylene treatment, chickpea plants were kept in a desiccator and ethylene (10 µl L^{-1}) was injected in the desiccator with a needle. Samples were collected at the time points of 0, 20, 60 and 120 min.

Neomycin treatment Eight-week-old field-grown chickpea plants were pre-treated with 100 µM neomycin solution for 1 h by spraying. Thereafter, the leaves were exposed to oral secretions of *Helicoverpa armigera* (10 µl oral secretions spread over the leaf with a soft brush) and then rapidly wounded with a pair of forceps by repeated pricking about 9–10 times (18–20 punctures) within a span of 10 s. The leaf tissue was collected at time points of 0, 5, 20, 60 and 120 min after simulated herbivory of neomycin-pretreated leaves and qRT-PCR was carried out and compared with wounded, neomycin-untreated leaves.

RNA isolation and real-time PCR analysis

Total RNA was extracted from the frozen chickpea leaves using a plant total RNA isolation kit (Sigma, India) according to the manufacturer's instructions. The cDNAs were synthesized from RNA samples using the REVERTAID MMLV kit (Fermentas). Gene-specific real-time PCR primers are listed in Supplementary Table S1. Real-time PCR was performed in 10 µl for a set of selected WRKY genes using SYBR Green PCR Master Mix (ABI, USA) using the following cycle conditions: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final 5 min extension at 72 °C. *EF1α* and *HSP90* were used as internal controls for the normalization of relative mRNA in different RNA samples. Reactions were carried out on an ABI StepOnePlus real-time PCR system with three biological replicates, each with three technical replicates. Relative gene expression was calculated using the $2^{-\Delta CT}$ method for all comparisons except neomycin treatment where $2^{-\Delta\Delta CT}$ was used (Livak and Schmittgen, 2001).

In silico analysis of promoter sequences

To identify putative *cis* regulatory elements in the promoter region of different wound-inducible WRKYs, a region of 2 kbp upstream from the translational initiation codon was extracted from genomic regions using the desi chickpea genome sequence ICC4958 available in the NCBI database and analyzed using the web-based online program PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), a database of plant *cis*-acting regulatory DNA elements. The accession numbers of the WRKY gene sequences are provided in Supplementary Table S2.

Promoter studies in wild type and mutant Arabidopsis and histochemical GUS assays

The promoter region of one of the WRKY genes, *WRKY41*, was chosen for detailed studies and validation. A region of 931 nt was isolated from genomic DNA of chickpea using gene-specific primers (Table S1) based on the sequence in the chickpea genome database available in NCBI database and cloned first in pTZ57R/T (Fermentas) and then introduced in pBI101.2 at *Sal*I. Primers were designed such that the initiation codon of the gene was in translational fusion with the *GUS* gene. The construct was introduced into the *Agrobacterium* strain GV3101. Promoter studies were carried out in stable transgenic *Arabidopsis thaliana*, ecotype Columbia (Col-0) using the floral dip method as described (Clough and Bent, 1998). Phytohormone treatments for promoter analysis were carried out by treating four week-old transgenic *Arabidopsis* plants expressing the promoter with either JA (100 µM), ethylene (10 µL L^{-1}), SA (2 mM) or mock (water or 0.1% ethanol) for 2 h before color development.

To identify the hormones and factors regulating the function of *CaWRKY41* promoter, the *WRKY41pro* construct was introduced into various *Arabidopsis* mutant backgrounds such as *ein2-1* (defective in ethylene responses), *coi1-1* (defective in JA responses) and *myc2myc3myc4* (defective in multiple MYC functions affecting specific JA responses). All the plants were grown on soilrite in a culture room maintained at 22 ± 2 °C under a 16 h light period (light intensity 100 µE m^{-2} s^{-1}, relative humidity 78 ± 4% at 25 °C).

Histochemical GUS staining was carried out as described (Gattolin et al. 2006). Tissue samples were incubated in 1 mg ml^{-1} X-gluc solution containing 50 mM sodium phosphate buffer pH 7.2, 0.5 mM $K_3Fe(CN)_6$ and 0.5 mM $K_4Fe(CN)_6$ for 16–24 h at 37 °C. After incubation, tissues were destained in 70% ethanol at 37 oC and examined. Light microscopy was performed on a Leica Wild M3Z microscope (Leica Germany).

Statistical analysis

Significant differences between control and treated plants were analyzed using ANOVA: single factor with the help of Analysis tool pack (Data Analysis) in Microsoft Excel 2010 where * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$.

Results

A large number of WRKY genes are activated early in chickpea in response to simulated herbivory by *Helicoverpa armigera*.

A comparative transcriptome sequencing of simulated herbivory on chickpea leaves was previously carried out in our lab using RNA from unwounded and 20 min mechanically wounded chickpea leaves (pre-exposed to *H. armigera* saliva) to assess the early transcriptional responses of chickpea towards wounding (Pandey et al. 2017). One of the most prominent groups to be differentially regulated upon simulated herbivory was the WRKY family, with 32 genes being up-regulated within 20 min of wounding.

In order to validate the transcriptomic data, 14 out of 32 up-regulated WRKY genes showing greater than five-fold change in actual transcript levels ($Log_2FC \geq 2$) were selected for real time PCR validation. These included *WRKY11, WRKY17, WRKY33A, WRKY33B, WRKY33C, WRKY40A, WRKY40B, WRKY40C, WRKY41, WRKY46, WRKY53, WRKY70A, WRKY70B* and *WRKY72*. Of the 14 genes chosen, *WRKY33C, WRKY40A*, *WRKY41* and *WRKY*53 were most strongly up-regulated with 30–45 fold higher expression compared to the unwounded control while the others showed 5–16 fold higher expression within 5–20 min of wounding (Fig. 1). The qRT-PCR expression profiles of the selected *WRKY* genes were consistent with the patterns obtained by RNA-Seq analyses, indicating that changes in expression determined by transcriptome sequencing were true to nature and that the WRKYs responded strongly and rapidly to wound signals.

JA strongly suppresses the expression of most wound-inducible WRKY genes in absence of wounding

Like most plants, chickpea responds to simulated herbivory and mechanical wounding by activating and remodeling several phytohormone pathways (Pandey et al. 2017). Jasmonic acid is the primary defense-related hormone involved in mediating short term and long term responses to herbivory. The JA pathway is also rapidly up-regulated upon simulated herbivory in chickpea (Pandey et al. 2017). To check whether the strong up-regulation observed upon simulated herbivory in chickpea was primarily induced by JA, the expression of the WRKY genes was tested in response to JA treatment under unwounded conditions.

Quite against expectations of what was observed upon wounding, the expression profiles of the majority of the wound-inducible WRKY genes namely *WRKY11, WRKY17, WRKY33A, WRKY40B*, *WRKY40C, WRKY41, WRKY53* and *WRKY70A* showed a rapid reduction in transcript levels upon JA treatment to less than 50% of the control values in 20 min (Fig. 2). Three of these, *WRKY33A*, *WRKY41* and *WRKY53,* were reduced to just 20% within 20 min of JA treatment. *WRKY33B* also showed a JA-dependent reduction in transcript levels but these reduced to below 50% after 1 h. Transcript levels of *WRKY33C* and *WRKY46* did not show much change while *WRKY72* and *WRKY40A* were induced upon JA treatment in 20 min. *WRKY70B* was unusual in showing a slight induction at 20 min after JA treatment, but a strong reduction to less than 20% after 60 min followed again by a rise in transcript levels at 2 h after JA treatment. The results showed that JA strongly inhibited transcript accumulation of most wound-inducible genes in absence of wounding and suggested that it may either not be involved in the early wound response or may only partly contribute to it or that it may have a role reversal depending on the conditions.

Suppression of JA biosynthesis by neomycin treatment only partially suppresses the wound-induced expression of WRKY genes

Although most of the wound up-regulated *WRKYs* in chickpea showed a strong reduction in transcript levels upon treatment with JA in absence of wounding, the possibility existed that these genes may still be activated by JA but only in combination with some accessory factor(s) that are activated upon wounding. To test this, the wound-inducible expression of the WRKY genes, following simulated herbivory, was studied after pretreatment of leaves with neomycin. Neomycin, a poly-cationic aminoglycoside antibiotic, blocks the accumulation of oral secretion-induced Ca^{++} elevation and the conversion of JA to its bioactive form JA-Ile (Vadassery, et al. 2014). Thus, pretreatment with neomycin would prevent formation of active JA-Ile and thereby block the activation of JA-dependent wound-inducible genes but not JA-independent wound-inducible genes.

Interestingly, pre-treatment of chickpea leaves with neomycin, one hour prior to wounding, reduced the transcript levels of nine WRKY genes namely, *WRKYs 11, 17, 33A, 33B, 33C, 40A, 41, 46* and *70A* at 5 min as compared to mechanically wounded chickpea leaves that were pretreated with *H. armigera* oral secretions but not with neomycin (Fig. 3). The reduction ranged from ~ 50–80% at 5 min compared to the neomycin-untreated samples. For *WRKY72*, the reduction was seen at 20 min. In most cases, however, wound-induced transcription in neomycin pretreated leaves increased by 20 min and was close to the high levels observed upon wounding (in absence of

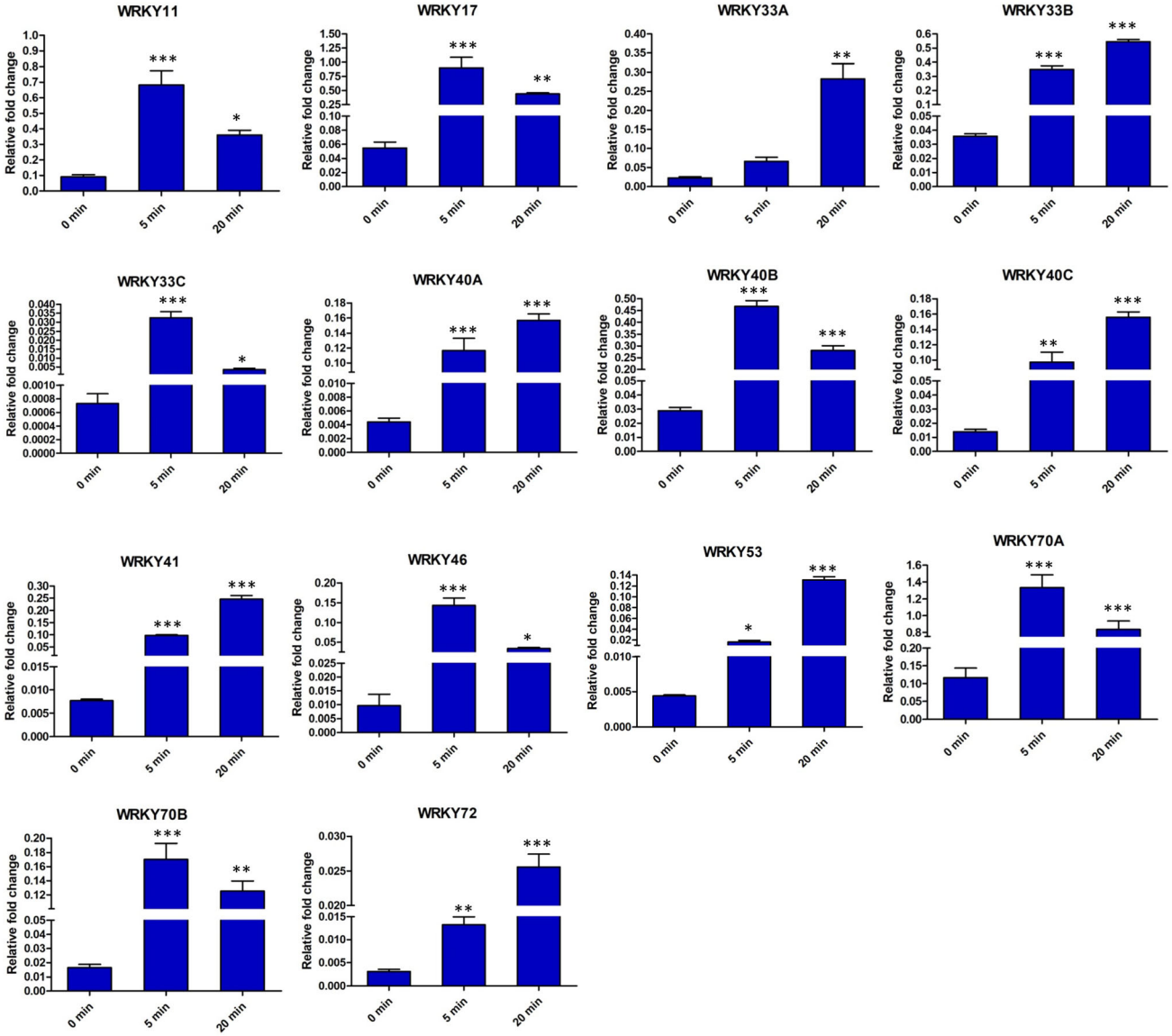

Fig. 1 Real-time PCR validation of expression of the wound-inducible chickpea WRKY genes identified from the transcriptome. Total RNA was isolated from chickpea leaves subjected to simulated herbivory by *H. armigera* saliva and mechanical wounding for 0, 5 and 20 min. The data was normalized against reference genes *EF1α* and *HSP-90*. Expression analysis was performed in technical triplicates on three biological replicates. Error bars show the standard error ± SE of three biological control. * on the bar indicate significant differences at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ with respect to the controls

neomycin treatment) suggesting that the transcript increase upon neomycin pre-treatment was only delayed compared to neomycin-untreated wounding. *WRKY33A* was an exception where pre-treatment with neomycin completely blocked wound-induced increase in transcription suggesting complete dependence on JA. On the other hand, WRKYs *40B, 40C* and *70B* did not undergo any change upon pre-treatment with neomycin and showed the same increase in transcription as seen upon wounding (in absence of neomycin) suggesting that their expression was independent of JA. The results suggested that JA-dependent as well as JA-independent factors contributed to the increase in transcript levels of the WRKYs.

Ethylene induces several WRKY genes while SA suppresses most WRKYs in absence of wounding

Since the ethylene pathway, representing another important defense hormone, was also substantially up-regulated upon simulated herbivory in chickpea within 20 min (Pandey et al. 2017), it was investigated for its contribution to controlling WRKY gene expression. Ethylene, unlike JA, regulated the WRKY genes differently in absence of

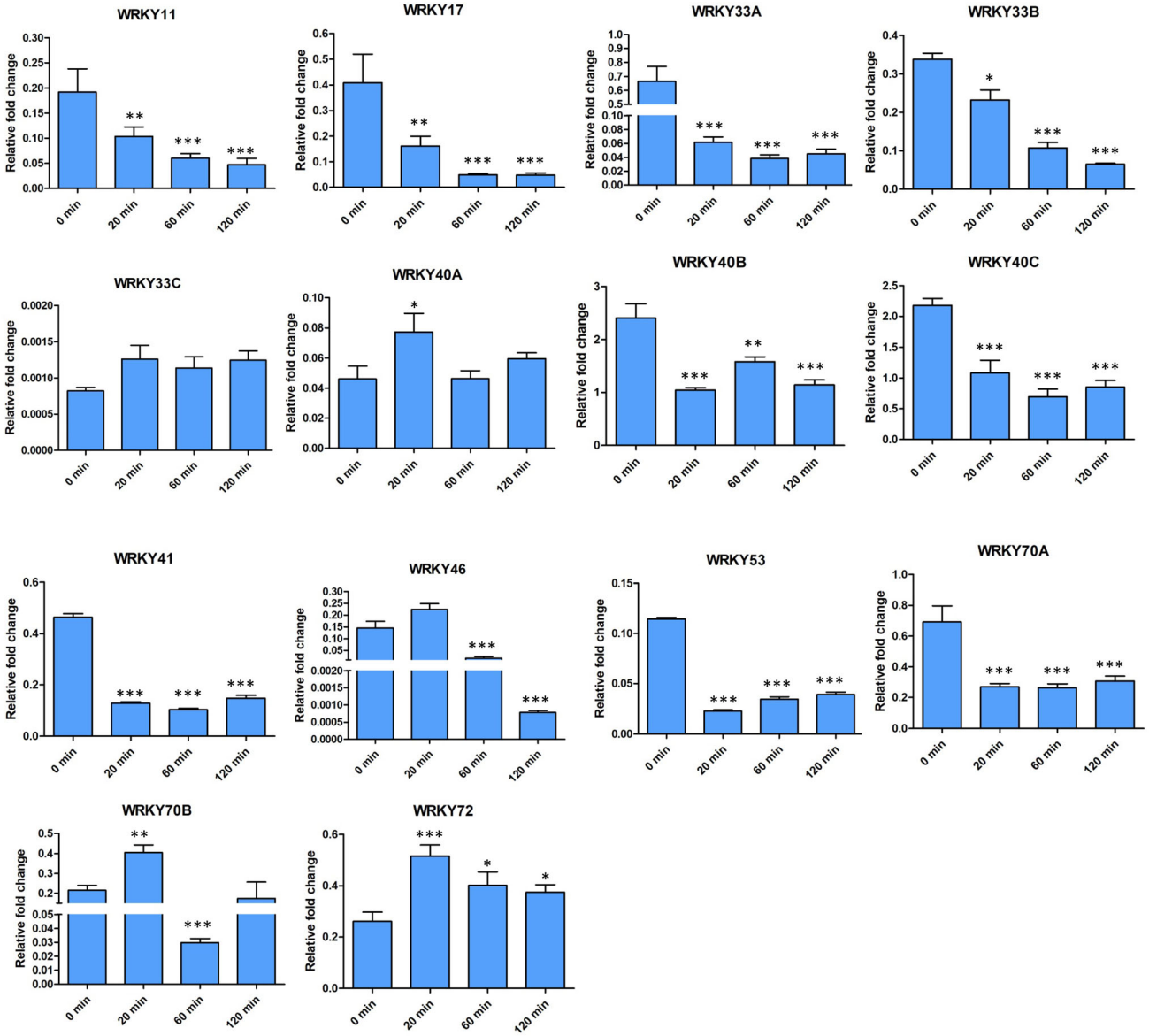

Fig. 2 Real-time expression profiles of WRKY genes in response to jasmonic acid. Leaves of eight-week-old unwounded chickpea plants were treated with 100 µM jasmonic acid. Samples were collected at 0, 20, 60 and 120 min. Reactions were run and analyzed as described in Fig. 1

wounding with several genes being up-regulated. Five of the genes (*WRKY*s *40A, 40B, 70A, 70B* and *72*) were strongly induced by ethylene by 3–10 folds within 20 min of treatment (Fig. 4). For *WRKY70B* and *WRKY72*, the scale of induction in response to ethylene treatment and simulated herbivory was similar, suggesting that wound-induction of these genes may be wholly dependent on ethylene while for the other three (*WRKY40A, WRKY40B* and *WRKY70A*) the scale of induction was much less compared to that observed upon simulated herbivory suggesting other factors being responsible for their induction. Three genes, *WRKY33A*, *WRKY33B* and *WRKY40C* showed only a slight increase (∼ three fold) at 60 min while a fourth, *WRKY46,* increased slightly at 20 min. Four genes, *WRKY11, 17, 33C* and *41,* were not affected by ethylene treatment. *WRKY53* was the only gene that was negatively regulated by ethylene with a transient decrease in transcript levels to less than 50% between 20–60 min.

Salicylic acid signaling, which is activated by biotrophic organisms, antagonizes JA responses in wounding but is known to be activated upon feeding by some insects, thereby enabling their survival (Diezel et al. 2009; Thaler et al. 2012; Rajendran et al. 2014; Schäfer et al. 2011). Treatment with SA, like that of JA, led to a rapid reduction in transcript levels of majority of the WRKY genes namely *WRKY11, WRKY17, WRKY33A, WRKY33B, WRKY40C* and *WRKY53* (Fig. 5) with most being reduced to less than 50% of untreated samples in 20 min while *WRKY33A* was

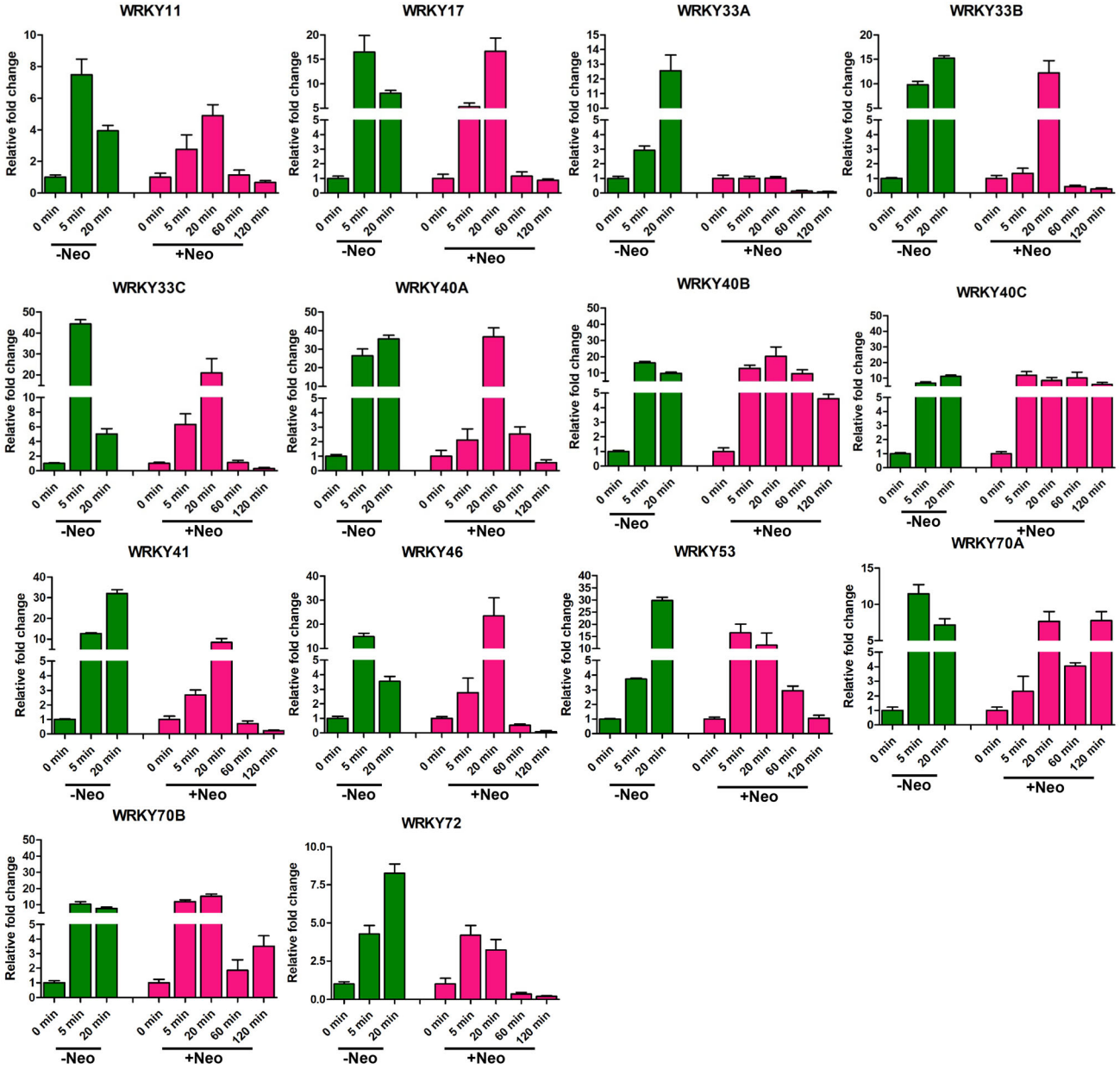

Fig. 3 Comparative real-time PCR analysis of WRKY genes upon simulated herbivory in presence and absence of neomycin. Chickpea plants were pretreated with 100 µM neomycin for an hour prior to simulated herbivory and expression studied at 5, 20, 60 and 120 min after simulated herbivory and compared to 0, 5 and 20 min wound-induced expression of simulated herbivory (neomycin-untreated; Fig. 1) with 0 min being the unwounded, neomycin untreated control in both sets of experiments. Reactions were run and analysed as described in Fig. 1

reduced to less than 10% within 20 min. *WRKY40B* showed a strong but transient reduction in transcript levels to less than 10% within 20 min of SA treatment before reaching background (unwounded) levels. In contrast, some of the *WRKYs,* namely *WRKY33C, 46, 70A* and *70B,* were up-regulated by SA. Of these, the level of up-regulation for *WRKY70A* and *WRKY70B* (4.9 and 4.2 fold, respectively), was very close to their induction in response to simulated herbivory. Three other WRKY genes, namely *WRKY41, WRKY40A* and *WRKY72,* showed only a transient induction at 20 min upon treatment.

H_2O_2 treatment activates the expression of most WRKY genes even in absence of wounding

Reactive oxygen species like hydrogen peroxide and the superoxide radical are common components of the plant defense in response to pathogen and herbivore attacks and

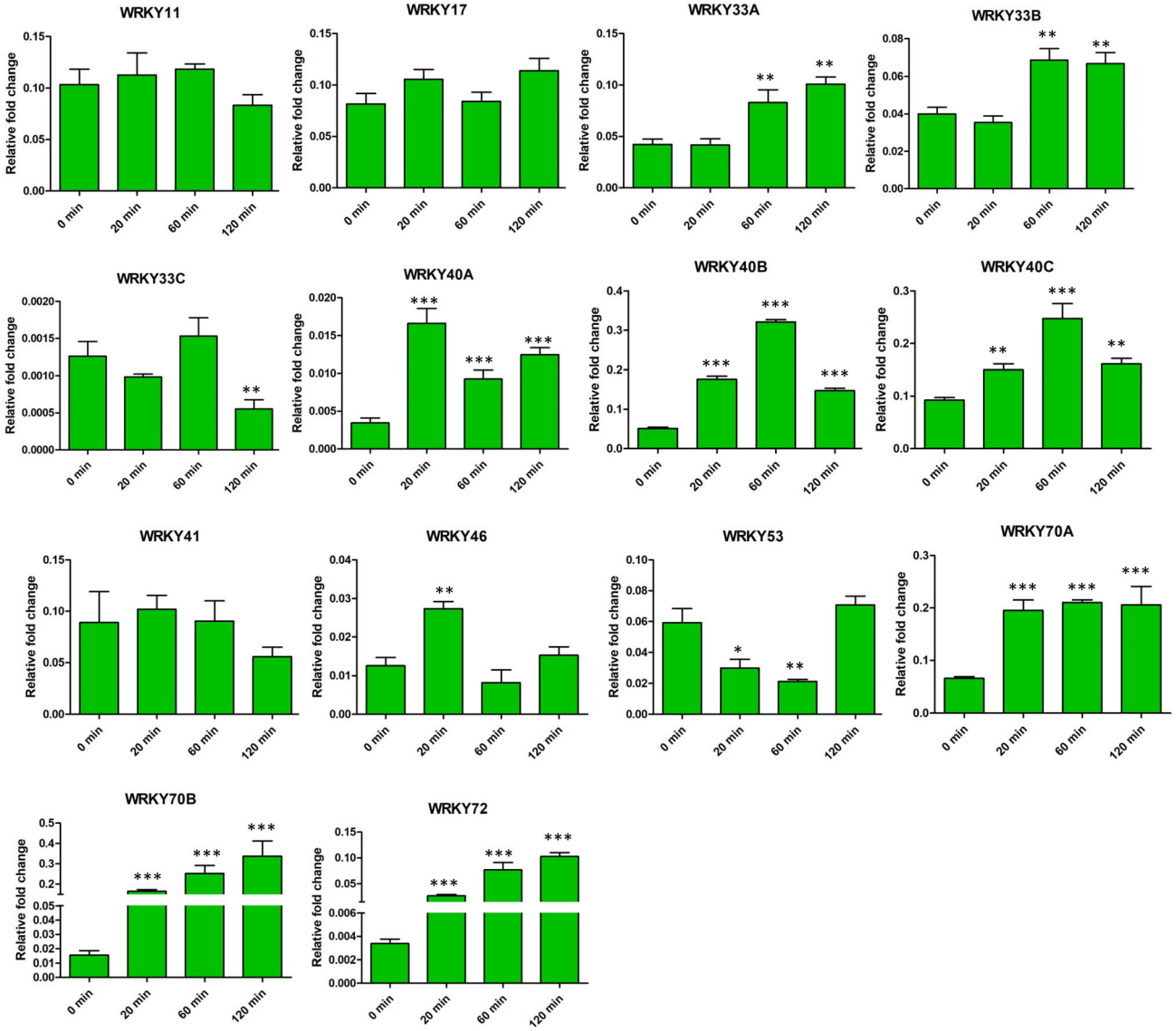

Fig. 4 Expression profiles of WRKY genes in response to ethylene. Leaves of eight-week-old unwounded chickpea plants were treated with 10 µl L^{-1}ethylene. Samples were collected at 0, 20, 60 and 120 min. Reactions were run and analyzed as described in Fig. 1

among the earliest signals produced within minutes after wounding. Since the rapid and strong increase of most WRKY genes in response to simulated herbivory could neither be explained by JA nor ethylene, we investigated the role of H_2O_2 as one of the possible inducers in their regulation.

Indeed, majority of the *WRKY* genes (12 out of 14) were rapidly and strongly induced by H_2O_2 treatment (Fig. 6 a) and peaked within 20 min of H_2O_2 exposure while two others, *WRKY53* and *WRKY70B,* showed a peak at 60 min. While the majority showed a 2–3 fold change in response to H_2O_2 treatment, three genes namely, *WRKY17, WRKY33B* and *WRKY41* showed a 5–10 fold increase at the 20 min time point that matched the scale of induction observed upon simulated herbivory. *WRKY72* was an exception in that H_2O_2 treatment induced a rapid decrease in transcript levels to less than 50% of the control values within 20 min of exposure and remained low thereafter. Transcript levels of *WRKY40C* also decreased in response to H_2O_2 treatment but only after 1 h. For most genes (with the exception of *WRKY70B*) the effect of H_2O_2 treatment on transcript levels was transient and no longer visible after 2 h of treatment. For *WRKY41,* the dynamics of induction in response to H_2O_2 differed from simulated herbivory as its induction was not as high as wounding. These expression analyses suggested that early wound responses were probably only partly dependent on H_2O_2 (for *WRKY33B*, *WRKY17* and *WRKY11*) and could not be explained solely through induction by H_2O_2.

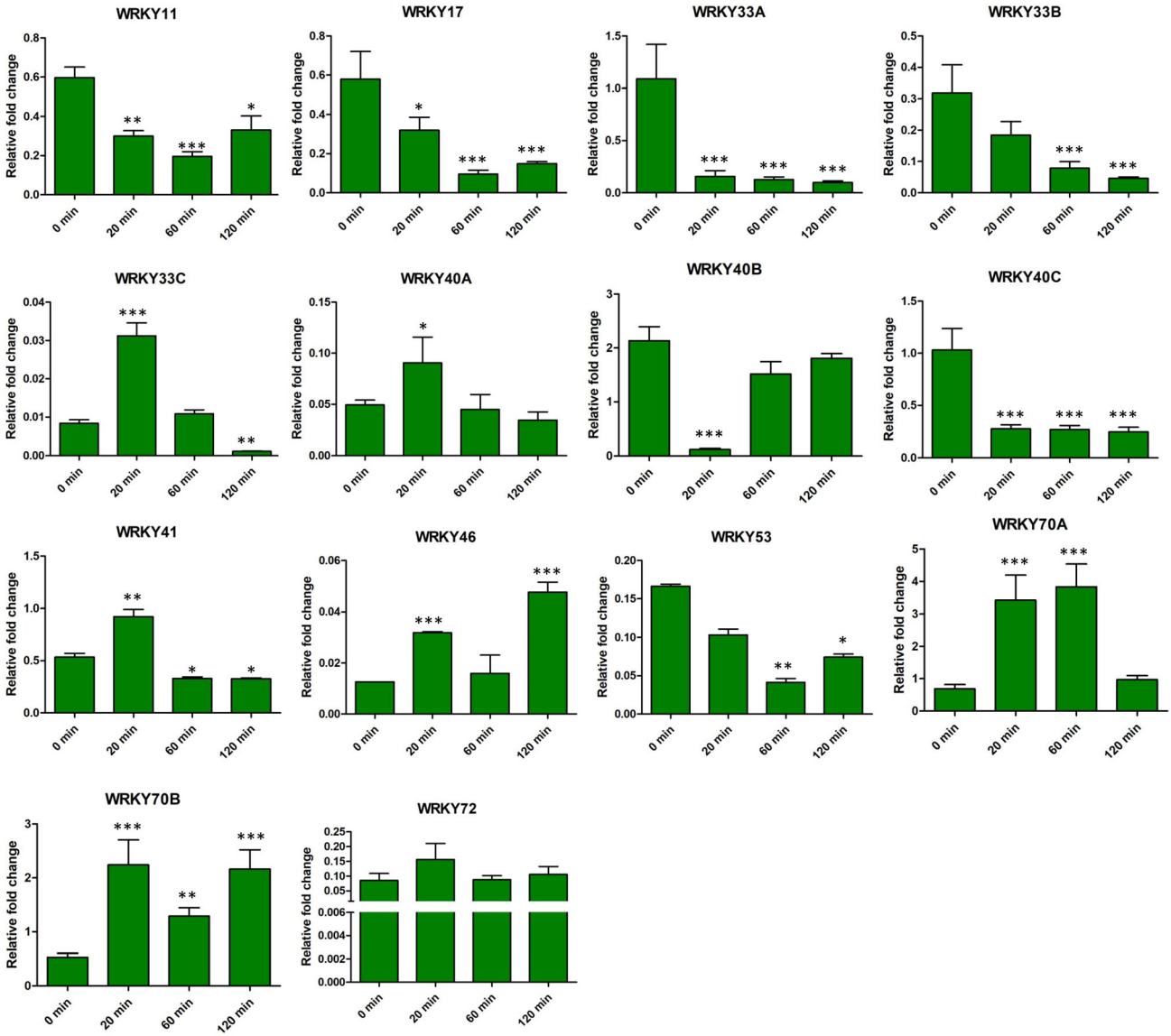

Fig. 5 Expression profiles of WRKY genes in response to salicylic SA. Leaves of eight-week-old unwounded chickpea plants were treated with 2 mM SA. Samples were collected at 0, 20, 60 and 120 min. Reactions were run and analyzed as described in Fig. 1

A heat map summarizing the effects of all treatments on WRKY gene expression is shown in Fig. 7.

In silico sequence analysis of WRKY promoters for identification of putative *cis* regulatory elements

For identifying the putative *cis* regulatory elements of WRKY genes of chickpea, a 2 kb region upstream of the translational initiation codon of all genes was extracted using the chickpea genome database of ICC4958. The *cis* acting elements in all these promoters were analyzed using the PlantCare database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al. 2002). Various elements (Fig. 8a; Supplementary Table S3) involved in responses to phytohormone stimuli (JA, SA, ABA, ethylene), light responses, defense responses, cell and tissue specificity and those functioning as binding sites for known transcription factors were identified within the 2 kb region on the positive as well as negative strands since some elements are likely to function as enhancers. The most prevalent elements identified in the WRKY gene promoters were the BOX4 site (all 14 promoters), MYB, MYC and ethylene response element (ERE) (present in 13 promoters each), the anaerobic response element (ARE) (12 promoters), the light responsive elements G-box and GT1 motif (10 promoters) followed by defense response elements such as W box, TC-rich elements, Wun element and WRE (present in 7–9 WRKY genes). Also abundant (found in

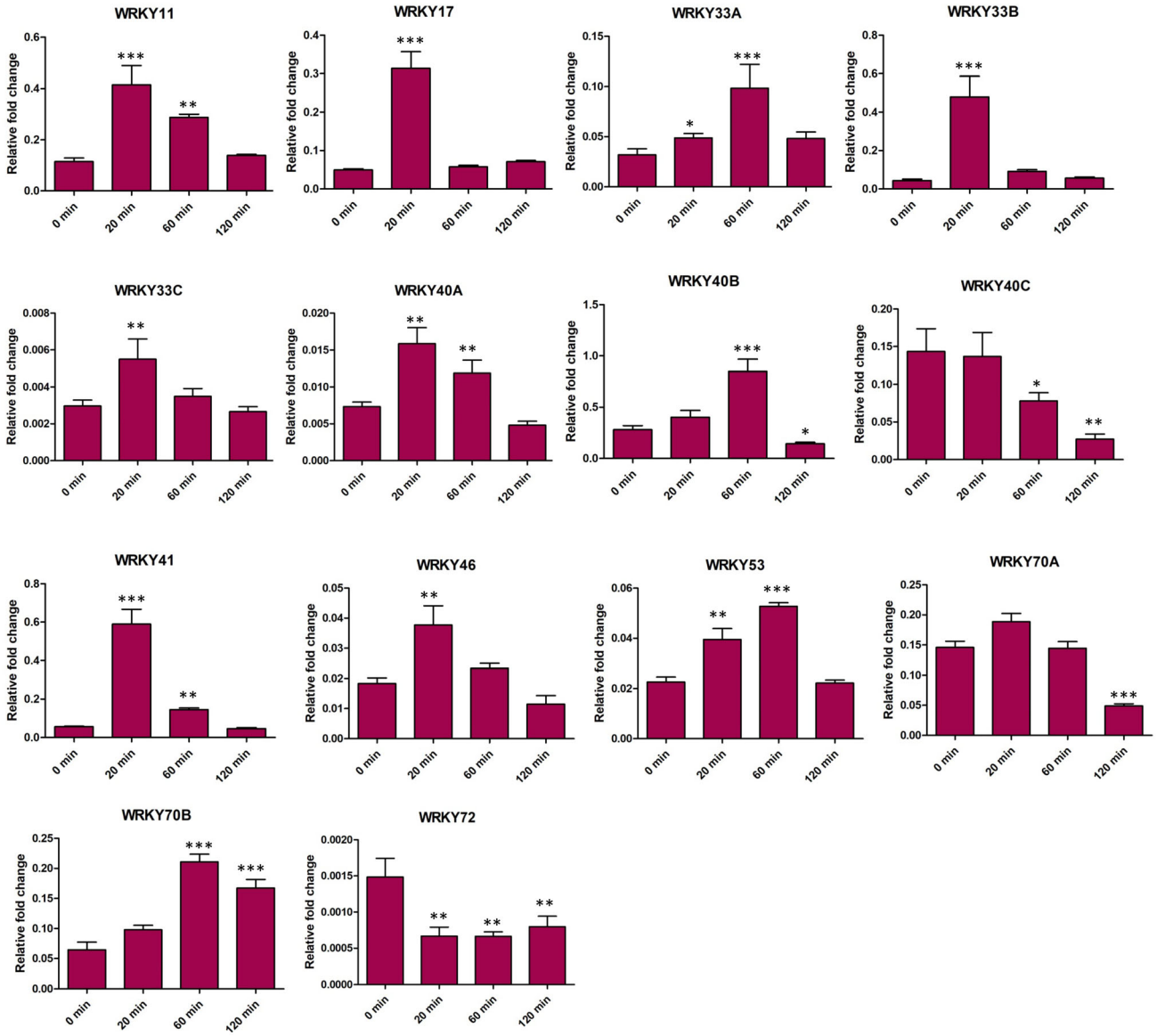

Fig. 6 Expression profiles of WRKY genes in response to H_2O_2. Leaves of eight-week-old unwounded chickpea plants were treated with 5 mM H_2O_2. Samples were collected at 0, 20, 60 and 120 min. Reactions were run and analyzed as described in Fig. 1

7–11 promoters) were JA responsive elements, ABREs (responsive to ABA) and TCA elements (involved in SA response).

The *WRKY41* promoter shows wound-inducible and SA-responsive GUS expression

Most *CaWRKY* genes showed rapid wound–inducible expression and also possessed an array of *cis* elements responding to different cues including defense and defense hormones. To study this *in vivo,* the promoter of one of the wound-responsive *WRKY* genes, *WRKY41* (labeled as *WRKY50* by Kumar et al., 2016), was studied in detail. A region of 931 nt upstream of the translation initiation codon of *WRKY41* (including 5' UTR) was introduced into *Arabidopsis* in fusion with GUS (Supplementary Fig S1a and S1b). As shown (Fig. 8b), transgenic plants expressing *WRKY41pro*:*GUS* showed a strong and specific GUS expression only at the site of wounding but not in the absence of wounding (except where the leaf was excised in the unwounded plants). Interestingly, pre-treatment of transgenic plants with neomycin (which suppresses JA-Ile formation) did not seemingly affect the wound-induced expression of *CaWRKY41pro.*

Since, JA, SA and ethylene influence the transcription of defense-related genes during wound signaling, transgenic *Arabidopsis* plants expressing *CaWRKY41pro*::*GUS* were also treated with JA (100 μM), SA (2 mM) and ethylene

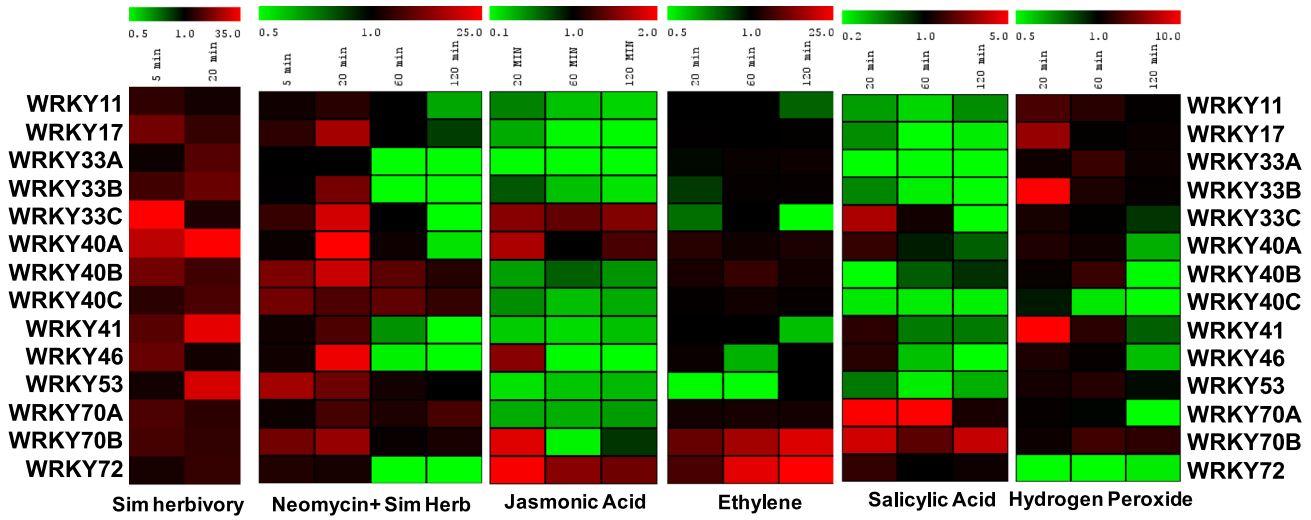

Fig. 7 Heat map summarizing the expression changes in different WRKYs in response to various treatments. (Sim Herbivory = simulated herbivory; neomycin + Sim herb = simulated herbivory after neomycin pretreatment)

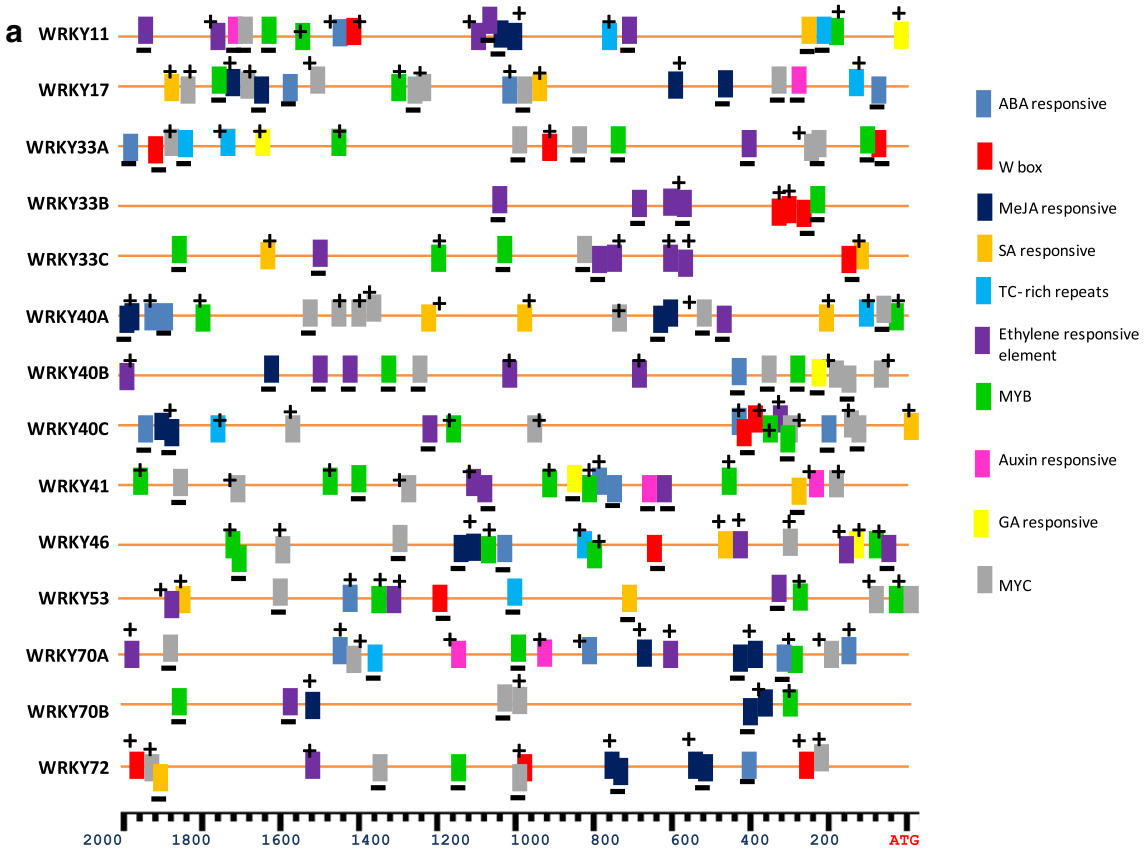

Fig. 8 *In silico* analysis of *CaWRKY* promoters and functional validation of the *CaWRKY41* promoter. **a** Schematic representation of the common *cis* elements present on the selected WRKY gene promoters**.** The various predicted *cis* regulatory elements with significant similarity to previously identified elements on the positive and negative strands are shaded with different color bars. Length of promoters is indicated with bars on scale that represent nucleotide number from the initiation codon. **b–d** Histochemical GUS assay in transgenic *Arabidopsis* plants expressing *CaWRKY41pro* under different conditions. GUS activity in transgenic *Arabidopsis* harboring *WRKY41pro::GUS* expression cassette after **(b)** mechanical wounding in the Arabidopsis Col-0 background, **c** upon treatment with JA, ethylene and SA (treatments as described in methods) and **(d)** in the *ein2*, *coi1* and *myc2myc3myc4* triple mutant backgrounds

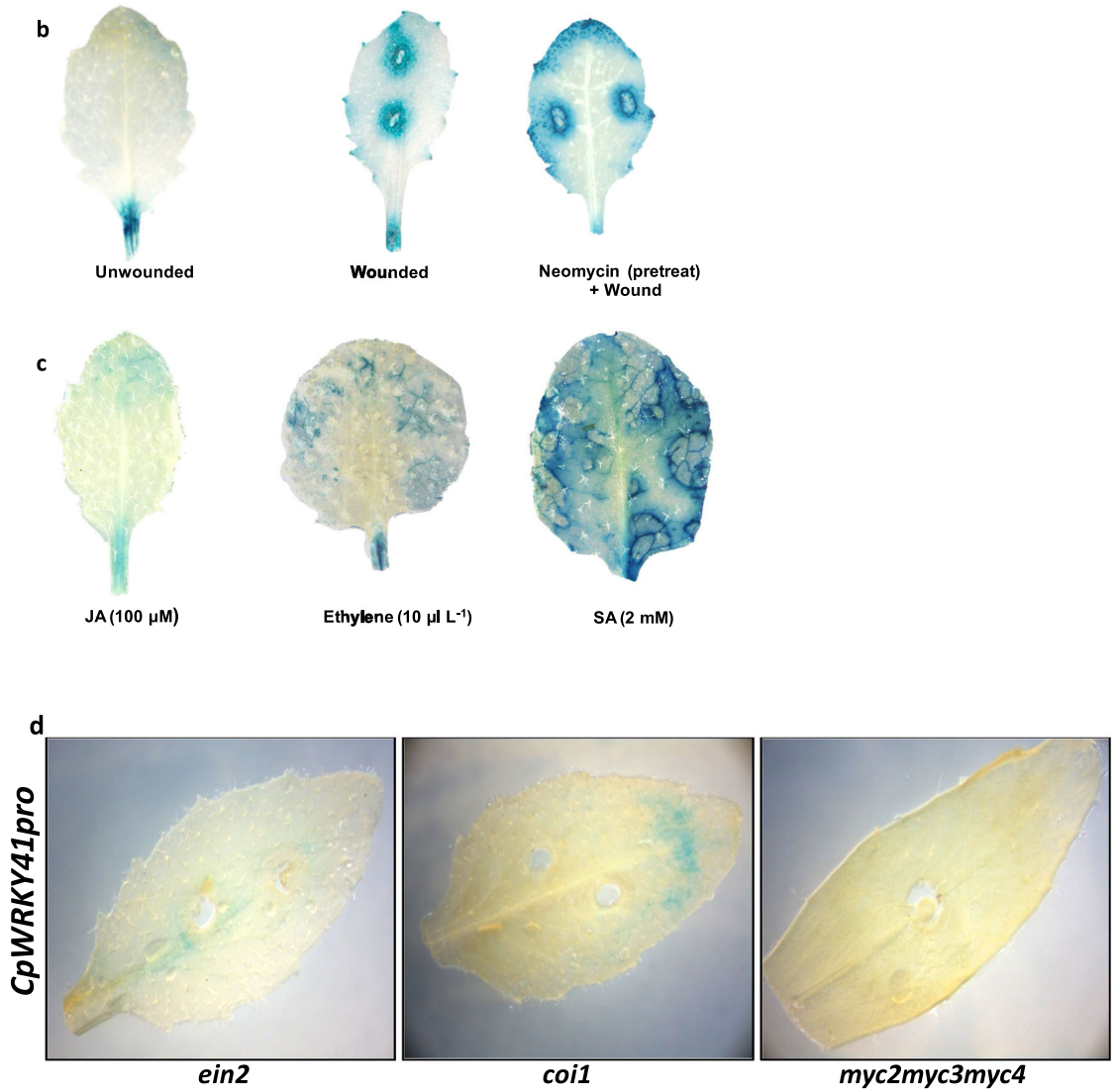

Fig. 8 continued

(10 µl L^{-1}) for 2 h (Fig. 8c). While the *WRKY41* promoter region used for study lacked known JA and SA responsive elements (as per the PlantCare database) it did possess the ERE element and elements for MYB and MYC binding. Surprisingly, SA was able to activate the promoter as observed by blue color in unwounded SA-treated transgenic leaves. However, minimal change in *GUS* expression could be seen in unwounded transgenic leaves treated with JA and ethylene. The results suggested that as yet undiscovered SA responsive elements may drive the expression of the promoter upon exposure to SA.

To obtain further insight into the wound-responsive expression of the promoter, the *WRKY41pro-GUS* construct was introduced into different hormone response mutants namely *coi1* (defective in JA responses), *ein2* (defective in ethylene responses) and the *myc2myc3myc4* triple mutant (defective in multiple MYC functions involved in JA signaling). Surprisingly, the wound-responsive expression was completely lost in all the three mutant backgrounds indicating that the wound-responsive GUS expression driven by *WRKY41pro* in *Arabidopsis* required a functional ethylene pathway as well as a functional JA pathway that depended on MYC2/3/4 (Fig. 8d).

Discussion

Wounding by chewing pests such as *Helicoverpa* (a major pest of chickpea) elicits a large number of defense responses in a plant, both early and late, for protection from further damage. Insect wound-induced chemical signals include Ca^{++} changes, glutamate, ROS, JA, ethylene, SA,

etc. that function collectively to modulate gene expression within minutes to hours, leading to the final response that either deters the insect or allows it to feed (Erb et al. 2012). The contribution of individual chemical cues to the final expression of the target, and the extent of their crosstalk with different chemical cues, is still not very clear. How these signals influence gene expression in absence of wounding is also important since it can shed light on the changes in signal machinery that occurs as cells shift from an unwounded to a wound-responsive state. During our studies on the early events upon simulated *H. armigera* herbivory in chickpea, we identified the WRKY family as one of the most prominently up-regulated groups wherein at least 14 genes showed 5–50 fold increase in expression within 20 min of wounding (Pandey et al. 2017). Although these WRKY genes may be involved in different processes, their strong up-regulation upon simulated herbivory suggested commonalities in their regulation by wound-responsive cues. In order to unravel the complexities of the early wound response, this group was therefore chosen for a comparative study of the contribution of different wound response cues when provided individually (in absence of wounding) versus that when observed upon wounding.

Our results show that contrary to the strong induction observed upon wounding, different chemical cues, individually, show distinct and often opposite effects on gene expression in absence of wounding. Strikingly, jasmonic acid, known to be a key factor in early wound responses (Pieterse et al. 2012), reduces transcript levels in 10/14 genes by 50–75% within 20–60 min of treatment in absence of wounding and these remain low, while none of the rest shows a greater than two-fold increase. Although a decrease in some of the Group III chickpea WRKYs by JA had also been noted in a previous study (Kumar et al. 2016) the strong inhibition observed in the current context was counter-intuitive. It suggested that the early wound response of most of the WRKY genes was either independent of JA or, more likely, that the regulation by JA was altered depending on the presence or absence of wound-responsive factors. In absence of these factors under unwounded conditions, JA exerted an inhibitory effect. Wound responses are governed by both JA-dependent and JA-independent pathways (Titarenko et al. 1997). To identify the extent of contribution by JA, leaves were pretreated with neomycin prior to simulated herbivory. Neomycin inhibits the release of Ca^{++} upon wounding and blocks the conversion of JA to its active form, JA-Ile, thereby affecting JA-dependent processes across dicot and monocot plants (Vadassery et al. 2014, 2019). Any reduction in transcript levels in presence of neomycin would indicate the involvement of JA while any wound-induced up-regulation in its presence would indicate JA-independent regulation. Indeed, neomycin treatment substantially reduced the rapid wound-responsive up-regulation at 5 min by 50–70% in 9/14 WRKY genes with levels rising again by 20 min in most cases. For genes like *WRKYs 11, 41, 53* and *72*, peak wound-inducible expression post-neomycin treatment was only about 30–50% of that in absence of neomycin. Since the transcriptional response had slowed down and shifted (delayed) from 5 to 20 min, it indicated that JA biosynthesis contributed partly to substantially for the up-regulation of most WRKY genes upon wounding. The wound-induced increase of *WRKY33A* transcript levels appeared to be entirely dependent on JA since it was completely blocked by neomycin at all time points. In contrast, WRKYs *40B, 40C* and *70B,* showed no reduction in the transcript levels upon neomycin treatment compared to unwounded, neomycin-untreated samples indicating that their wound-responsive transcription was independent of JA. The results are interesting since they show that although JA can activate the transcript levels of the WRKY genes upon wounding, it reduces these under unwounded conditions. The reason why treatment with JA reduces transcript levels below basal levels under unwounded conditions will require further studies. The studies reveal a context-dependent role for JA wherein wounding may change the transcriptional dynamics of the WRKY genes and their responses towards JA in a way that facilitates their transcription upon wounding but a reduction in absence of wounding. Such context-dependent changes in regulation introduce an added dimension to the regulatory roles of hormones like JA. The transcriptional dynamics that undergo such a change (from the unwounded state to the wounded state) due to interaction of the JA pathway components with wound-responsive cues, within the first few seconds/minutes, begs further studies.

Treatment with SA also reduces transcript levels of several genes in absence of wounding. In fact, six of the ten genes that were suppressed by JA under unwounded conditions, namely *WRKYs 11, 17, 33A, 33B, 40C, 53* and to some extent *41,* also showed a strong reduction upon treatment with SA. Since JA contributes at least partially to the wound-induced up-regulation of most of the above WRKYs, their suppression by SA is not entirely surprising since JA and SA largely antagonize each other functionally (Thomma et al. 1998; Glazebrook 2005; Thaler et al. 2012). Herbivory usually leads to suppression of the SA pathway because of its negative effect on the JA pathway and insect defenses (Thaler et al. 2012). In fact, oral secretions of some insects like *Schistocerca gregaria, Spodoptera littoralis* and *Spodoptera exigua* (but not *H. armigera*) elicit SA responses in plants and these probably help in survival of the insects through suppression of the JA pathway (Diezel et al. 2009; Rajendran et al. 2014; Schäfer et al. 2011). SA is, however, an important player in

biotrophic defenses, many of which are modulated through regulation of WRKY genes. In chickpea, WRKYs *33C, 70A* and *70B* were up-regulated 3–6 fold by SA even under unwounded conditions in a manner similar to the SA up-regulation of Grp III WRKYs (Kumar et al. 2016) suggesting involvement in SA-governed functions. Of these, chickpea *WRKY70* (labeled as *WRKY70A* in this study) was recently shown to be activated by SA and reported to suppress multiple defense responses including ROS production thereby increasing susceptibility to *Fusarium oxysporum* (Chakraborty et al. 2020). The homologue of these genes in *Arabidopsis, AtWRKY70,* is also known to be induced by SA during pathogenesis (Li et al. 2004) and is a key TF that suppresses JA responses in *Arabidopsis*. *SlWRKY70,* a homologue of *AtWRKY70,* is also induced by SA and suppressed by JA and mediates resistance to aphids and the root knot nematode *Meloidogyne javanica* (Atamian et al. 2012). Since insect wounding exposes the plant to infection by opportunistic bacteria on the leaf surface as well as those in oral secretions, it is likely that the transcription of some of these WRKYs induced by JA under wounded conditions, and involved in SA responses, may be necessary to protect the plant from biotrophic infections caused by bacterial entry at the wound site.

Compared to JA, ethylene has more specific effects and is believed to function as a modulator of signaling by fine-tuning sensitivity to other cues (von Dahl et al. 2006; Diezel et al. 2009; Erb et al. 2012). Not surprisingly, it induced a moderate increase in 6/14 genes that was limited to 1.5–5 folds compared to the 5–50 fold increase observed upon simulated herbivory. The two exceptions were *WRKY70B* and *WKRY72* where a 10–20 fold increase was observed that matched in scale with simulated herbivory. In most others, ethylene did not induce much of a change despite the presence of the ERE element in most promoters. With the exception of *WRKY53,* no other gene showed any reduction in transcript levels upon ethylene treatment. As in case of JA, it is likely that a combination of ethylene with wound-responsive factors may alter the regulation of the WRKY genes compared to that of ethylene alone under unwounded conditions.

Reactive oxygen species such as hydrogen peroxide are also produced in response to herbivory (Maffei et al. 2006) and are amongst the earliest cues that activate wound responses and gene expression. Different ROS can activate different pathways and possibly gene expression through changes in oxidation states of TFs (Desikan et al. 2001; Gadjev et al. 2006; Møller et al. 2007). Many ROS responsive genes possess distinct *cis* motifs (Petrov et al. 2012). Hydrogen peroxide is one of the most prominent ROS due to its stability and ability to move through membranes (Petrov and van Breusegem, 2012) and can strongly influence gene expression (Vandenabeele et al. 2003; He et al. 2018). Unlike the three hormones which largely failed to activate the genes much in absence of wounding, treatment with H_2O_2 strongly but transiently up-regulated 11/14 WRKY genes by 3–8 fold even under unwounded conditions within 20–60 min. For *WRKYs 11, 17, 33B* and *41,* the increase appeared close to the levels observed upon simulated herbivory and early enough. Compared to any other hormone, the response to H_2O_2 in absence of wounding was stronger, suggesting that unlike JA, ethylene or SA, responses to H_2O_2 were probably not as dependent on other wound-responsive cues. Thus, early wound-induced transcription of at least some of the WRKY genes appeared to be partly dependent on and could be attributed to ROS like H_2O_2.

As is clear from these studies, the summation of the individual effects of the primary chemical cues under unwounded conditions does not explain the large wound-responsive increase in transcript levels since these (especially JA) actually reduce transcript levels under unwounded conditions. One inference is that although wounding may activate several different signals near simultaneously, their action on target genes is probably not independent of the other signals and not mutually exclusive. Instead, these may require the interaction of specific wound-responsive factors and other changes in the cell to alter the sensitivity of the genes to the chemical cues. A combinatorial action by different signals on the promoters of the wound-responsive WRKYs may synergistically alter the transcriptional state or the chromatin. How these factors enable activation of gene expression from an inhibited state to a highly activated state will require further studies at the chromatin level. Such detailed chromatin level studies have been performed for SA under biotrophic infections (Jin et al. 2018; Singh et al. 2015). Another possibility is that gene transcription and transcript stability may undergo dynamic changes depending on local hormone concentrations. Rising levels of JA, ethylene and H_2O_2 may activate the genes while their increase beyond a certain level may induce transcript instability. Hormones like auxin and SA are known to have concentration-dependent effects that can be inhibitory as well as activating (Kubeš et al. 2012; Caarls et al. 2015; Pasternak et al. 2019).

We have tried to validate the observations through the study of the *WRKY41* promoter. Although studied in a heterologous system like *Arabidopsis*, the strong wound-responsive GUS expression driven by the 931 bp region (encompassing the promoter and 5'UTR of *WRKY41*) shows that wound-responsive *cis* elements on the *CaWRKY41* promoter respond to and are recognized even in *Arabidopsis* suggesting conservation of the basic wound response machinery across families. The wound-responsive expression of *CaWRKY41* is seen within minutes of wounding and is also apparent in presence of neomycin

indicating that much of the *WKRY41* expression may be JA-independent. Nevertheless, the promoter is neither activated in the *coi1* mutant background nor in *myc2myc3myc4* background. This indicates that transactivation of the promoter requires a functional JA pathway and is dependent on at least one of the MYC factors that are known to drive JA-dependent responses (Van Moerkercke et al. 2019). Indeed the *CaWRKY41* promoter contains a site for MYC binding which may govern this expression. While the results seem contrary to those showing activation of the promoter in *Arabidopsis* even upon neomycin treatment, one inference could be that at least some of the wound-responsive changes in *Arabidopsis* may actually bypass JA biosynthesis and activate JA signaling downstream via COI1 and MYCs. The inability of *CaWRKY41pro* to get activated in the *ein2* mutant background is also interesting and suggests requirement of a functional ethylene pathway for wound-responsive activation at least in *Arabidopsis*. Thus both the JA and ethylene pathways need to be functional for activation of the *CaWRKY41* promoter in *Arabidopsis*. The promoter also shows SA responsiveness in *Arabidopsis* which is in tune with its activation by SA in chickpea. Despite these results, the 931 bp region does not show any known SA-responsive element indicating that novel SA-responsive *cis* elements within this region may drive its expression. The studies highlight the complexity of the interactions that lead to *CaWRKY41* activation upon wounding. Like the strong, early wound-inducible *RbPCD1* promoter that was recently described (Pandey et al. 2019), the *CaWRKY41* promoter also possesses biotechnological value due to its early wound-responsive nature and is currently being characterized for further tests.

In conclusion, our studies show that individual wound-responsive chemical cues like JA, ethylene, SA, H_2O_2 have different and often opposite effects under unwounded conditions compared to their collective action under wounded conditions. Their action during wound responses is not a summation of the individual effects in isolation but probably requires interaction with several other wound-responsive compounds that function coordinately to induce gene expression. The study sheds light on the complexity of regulation of genes as they shift from the unwounded state to the wounded state.

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Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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