RESEARCH PAPER

Combined transcriptome and metabolome analysis identifies defence responses in spider mite-infested pepper (*Capsicum annuum*)

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Abstract

Plants regulate responses towards herbivory through fine-tuning of defence-related hormone production, expression of defence genes, and production of secondary metabolites. Jasmonic acid (JA) plays a key role in plant–herbivorous arthropod interactions. To understand how pepper *(Capsicum annuum)* responds to herbivory, leaf transcriptomes and metabolomes of two genotypes different in their susceptibility to spider mites were studied. Mites induced both JA and salicylic acid (SA) signalling. However, mite infestation and exogenous JA resulted in distinct transcriptome profiles. Compared with JA, mites induced fewer differentially expressed genes involved in metabolic processes (except for genes involved in the phenylpropanoid pathway) and lipid metabolic processes. Furthermore, pathogen-related defence responses including WRKY transcription factors were more strongly induced upon mite infestation, probably as a result of induced SA signalling. Untargeted analysis of secondary metabolites confirmed that JA treatment induced larger changes in metabolism than spider mite infestation, resulting in higher terpenoid and flavonoid production. The more resistant genotype exhibited a larger increase in endogenous JA and volatile and non-volatile secondary metabolites upon infestation, which could explain its stronger defence. Reasoning that in JA–SA antagonizing crosstalk, SA defences are prioritized over JA defences, we hypothesize that lack of SA-mediated repression of JA-induced defences could result in gain of resistance towards spider mites in pepper.

Keywords: *Capsicum annuum*, JA/SA crosstalk, plant–arthropod interactions, specialized metabolites, transcriptional changes, two-spotted spider mites.

Introduction

Plants have evolved broad phenotypic plasticity to defend themselves against herbivores and pathogens. Constitutively present leaf surface waxes, morphological structures including trichomes, and various secondary metabolites form a first barrier discouraging herbivore attack (Furstenberg-Hagg *et al.*, 2013). Should these barriers not be sufficient, plants can

produce defensive proteins and secondary metabolites in response to feeding to affect the herbivore's growth and reproduction directly. In addition to these induced direct defences, arthropod herbivory also results in release of volatiles that attract natural enemies of the herbivores, protecting the plant indirectly (Kappers *et al.*, 2005; War *et al.*, 2012; Gols, 2014) Ubiquitous or species-specific plant secondary metabolites belonging to different biochemical classes including phenolics, flavonoids, terpenoids, and green leaf volatiles (GLVs) play a key role in these direct and indirect defences (Snoeren *et al.*, 2010; Kappers *et al.*, 2011; Cheynier *et al.*, 2013; Scala *et al.*, 2013; Tholl, 2015).

Plants can distinguish herbivore presence from mechanical damage by recognizing herbivore oral secretions or oviposition fluids (Little *et al.*, 2007; Bandoly *et al.*, 2015). These elicitors, together with the physical damage caused by the herbivore, trigger electrical signals and change Ca^{2+} homeostasis subsequently, followed by downstream defence responses regulated through hormone signalling pathways of which jasmonic acid (JA), ethylene (ET), and salicylic acid (SA) are the major players involved (Bari and Jones, 2009). Both the individual hormones and the crosstalk between them play an essential role in fine-tuning defence responses to specific attackers (Kawazu *et al.*, 2012; Proietti *et al.*, 2018).

Jasmonates (JAs) mediate multiple aspects of plant development including root and trichome formation, flower development, and leaf senescence, as well as plant responses to various biotic and abiotic stresses (Wasternack and Hause, 2013). Biosynthesis of JA starts with oxygenation of α-linolenic acid released from chloroplast membranes via the octadecanoid pathway (Turner *et al.*, 2002). The isoleucine-conjugated form of JA (JA-Ile) accumulates in response to wounding or herbivory and is considered to be the active form (Staswick and Tiryaki, 2004). In unstimulated conditions, basic helix–loop–helix (bHLH) transcription factor genes including *MYC2*, *MYC3*, and *MYC4* are repressed, while upon stress *MYC2* binds to JA-responsive elements (G-boxes) in the promoters of JA-regulated genes (Pauwels *et al.*, 2010; Fernandez-Calvo *et al.*, 2011). In Arabidopsis, *MYC2* positively regulates expression of wound-responsive genes such as *VEGETATIVE STORAGE PROTEIN* (*VSP*) and *LIPOXYGENASE* (*LOX*) (Lorenzo *et al.*, 2004). *MYC2* enhances the production of flavonoids in *Medicago truncatula* (Adolfsson *et al.*, 2017) and is required for JA-mediated tolerance to the generalist herbivore *Helicoverpa armigera* (Dombrecht *et al.*, 2007). JA has been identified as the hormone responsible for the accumulation of several different classes of bioactive secondary metabolites (Memelink *et al.*, 2001).

SA is synthesized from phenylalanine via phenylalanine ammonia lyase (PAL) and from chorismate through isochorismate synthase (ICS) action. SA is generally known to accumulate upon pathogen infection but also upon herbivory by phloem stylet-feeding whitefly (Zarate *et al.*, 2007) and aphids (Kloth *et al.*, 2016). The volatile conversion product of SA, methyl-SA, can be detected in the headspace of lima bean, Arabidopsis, tomato, and cucumber within hours after the onset of herbivory (Ament *et al.*, 2004; De Boer and Dicke, 2004; Kappers *et al.*, 2005, 2011). Methyl-SA alone or in combination with other

volatile compounds is known to be attractive to predators of herbivores, including mites predaceous to spider mites (De Boer and Dicke, 2004). *NON-EXPRESSOR OF PR GENES1* (*NPR1*), a master transcriptional co-regulator in the SA signalling pathway, is regulated by the cellular redox state (Fu and Dong, 2013) and activates SA-dependent gene transcription by binding to transcription factors of the TGACG sequence-specific binding protein (TGA) family (Wang *et al.*, 2006).

SA acts antagonistically with JA signalling (Bari and Jones, 2009; Erb *et al.*, 2012; Furstenberg-Hagg *et al.*, 2013). Increasing SA accumulation in tomato and Arabidopsis resulted in inhibition of JA-induced responses (Doherty *et al.*, 1988). This supports the antagonistic relationship between JA and SA pathways, but also suggests that SA-dependent defences are being prioritized over JA-related defences (Spoel *et al.*, 2003). Bypassing JA biosynthesis by exogenous application of methyl-JA in a JA biosynthesis-deficient mutant *aos/dde2* did not affect SA-mediated suppression of JA-responsive genes *PDF1.2* and *VSP* in Arabidopsis (Leon-Reyes *et al.*, 2010). In Arabidopsis, WRKY transcription factors were found to be essential for mediating crosstalk between JA and SA (Spoel *et al.*, 2003). Overexpression of the *NPR1*-independent transcription factor gene *WRKY70* resulted in constitutive expression of SA-induced pathogenesis-related genes and increased resistance to virulent pathogens, while antisense suppression of *WRKY70* activated JA-responsive/COI-independent genes (Li *et al.*, 2004). Several other WRKY transcription factors are also involved in downstream transcriptional responses to SA (Eulgem and Somssich, 2007; Kloth *et al.*, 2016).

Capsicum annuum, including sweet and hot peppers, belongs to the nightshade family *Solanaceae*, and is a worldwide grown and economically important vegetable crop (Carrizo García *et al.*, 2016; http://www.fao.org). *Tetranychus urticae* (two-spotted spider mite, TSSM) is a generalist herbivorous arthropod reported to infest >150 crop species, especially within the *Solanaceae* family (Fasulo and Denmark, 2016), including pepper. TSSMs feed through their stylet, penetrating leaf tissue via stomatal openings or by the intercellular space in between epidermal pavement cells, thus maintaining the integrity of the leaf epidermis (Bensoussan *et al.*, 2016). Intriguingly, closure of plant stomata is one of the first plant physiological responses to TSSM feeding, leading to reduced photosynthetic rates and crop yields (De Freitas Bueno *et al.*, 2009), along with down-regulation of photosynthetic gene expression (Mercke *et al.*, 2004). Overall transcriptome responses to TSSM feeding have been studied in, for example, Arabidopsis (Zhurov *et al.*, 2014), tomato (Martel *et al.*, 2015), and grape (Díaz-Riquelme *et al.*, 2016), and suggest JA to be the dominant player in plant defence responses. As TSSMs quickly develop resistance towards chemical acaricides (Van Leeuwen *et al.*, 2010), the use of predaceous arthropods as biological control agents has become of increasing interest. For example, the predatory mites *Phytoseiulus persimilis* feed on both TSSM adults and their eggs, and use the odour emitted by infested plants as a cue to locate their prey (van den Boom *et al.*, 2004; Kappers *et al.*, 2005, 2011).

To obtain a comprehensive insight into the defence responses of pepper against TSSM and how stress-related hormones, especially JA, play a role in TSSM-induced defences, we performed comparative transcriptome and metabolome analysis upon TSSM infestation and JA treatment in two *C. annuum* genotypes that differ in TSSM susceptibility.

Materials and methods

Plants, arthropods, and experimental set-up

Two-spotted spider mites (*Tetranychus urticae*) were propagated on lima bean plants (*Phaseolus lunatus*) for many generations. Seeds of *Capsicum annuum* varieties 'Vania' (bell pepper inbred line originally from INRA, Genotype 8, G8) and 'Ta Pien Chiao' [hot pepper, land race originating from China, obtained via CGN (Wageningen University), Genotype 29, G29] were germinated at 23 °C and seedlings were cultivated in potting soil (Lentse potgrond, Katwijk, The Netherlands) in a greenhouse [16 h day (23 °C)/8 h night (18 °C), 50–60% relative humidity]. Plants were watered daily and received half-strength Hoagland nutrient solution once a week. Five-week week-old vegetative plants were treated with JA (100 μM, 0.001% Tween-80) for either 6 h or 24 h prior to harvest, or infested with ~300 adult TSSMs for 3 d, or were left untreated. TSSMs were collected by washing lima bean leaves in water. Adults of different ages were checked for aliveness and transferred to a Petri dish using a fine brush before transferring to the pepper leaves. JA was applied by spraying leaves until drops fell off the leaves. For each treatment, three independent biological replicates were generated. For each individual sample, leaves of two different plants were combined. RNA isolations, and metabolite and phytohormone extractions were performed using the same sample. To evaluate susceptibility of both pepper genotypes to TSSMs, additional plants were placed in between TSSM-infested lima bean plants for 3 weeks and the number of adults, nymphs, and eggs, and chlorotic spots on the leaves, were counted using a binocular microscope. To evaluate predator preferences, female *P. persimilis* mites were tested towards the odours of TSSM-infested pepper plants using a Y-shaped olfactometer as previously described (Kappers *et al.*, 2005). For analysis of WRKY gene expression, plants were sprayed with SA (0.1 μM, 0.001% Tween-80) similar to as described for JA, or infested with 100 TSSMs for 3 d.

RNA isolation, library preparation, sequencing, and validation

Total RNA was extracted from leaf tissue using TriPure (Roche, Mannheim, Germany), cleaned with the RNeasy Plant Mini Kit (Qiagen, USA), and the DNA was digested using RNase-free DNase (Qiagen, USA). RNA integrity was evaluated by 1.0% agarose gel electrophoresis. Total RNA for RNA sequencing (RNA-Seq) was quantified and, for each condition, two samples were sent for cDNA library construction and sequencing (Bioscience, WUR, Wageningen, The Netherlands). Transcriptome libraries were constructed using the TruSeq™ RNA sample Prep Kit (Illumina, CA, USA) and sequenced using the Illumina HiSeq™2500 (Illumina, CA, USA) platform which produced 9–20 million bp paired-end reads per sample. Trimmomatic (Bolger *et al.*, 2014) was used to remove adaptor sequences, empty reads, short reads (<25 bp), reads with an N-ratio >10%, and low quality sequences. For quantitative reverse transcription–PCR (RT–qPCR), 1 μg of DNA-free RNA was reverse transcribed using iScript Reverse Transcriptase (BioRad, USA). Gene-specific primers were designed based on sequences obtained by BLAST search in the pepper genome database. RT–qPCR analyses were performed in biological triplicates and technical duplicates, using iQ SYBR Green Supermix (BioRad, USA) and the following PCR program: 3 min at 95 °C; 40 cycles of 15 s at 95 °C, and 30 s at 58.0 °C. Relative expression values were obtained using the 2–ΔΔCt method with *ACTIN* and *RPL2* as reference genes.

Quality control, mapping, and functional annotation of sequences

FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used for quality control and to generate clean reads in FASTQ format. Clean reads were mapped to the Pepper Zunla-1 reference genome (http://peppersequence.genomics.cn/page/species/index.jsp). Transcript assembly, quantification, normalization, and differential expression analysis were performed using the CLC Genomics Server 7.0.3 (Mortazavi *et al.*, 2008) with default settings for RNA-Seq mapping and analysis, using quantile normalization. Gene sequences were downloaded from the Pepper Genome Database (release 2.0, Pepper Institute, Zunyi Academy of Agricultural Science). Assembled sequences were putatively annotated using NCBI-blast-2.2 in the case of >90% identity and an E-value of <0.00001.

Gene expression and enrichment analysis

RNA-Seq reads were mapped to assembled sequences to calculate read counts for each unigene. Transcript levels of each unigene were calculated and normalized as reads per kilobase of transcript per million mapped reads (RPKM). Differently expressed genes (DEGs) between different experimental conditions were filtered using a Benjamini and Hochberg (1995) false discovery rate (FDR) of 0.05 and a threshold of log2-transformed fold changes (treatment/control) >|1.5|. *Capsicum* genes were blasted against the Arabidopsis genome using the CLC Workbench (QIAGEN Bioinformatics). DEGs were mapped to Gene Ontology (GO) terms in the general GO database (Du *et al.*, 2010). Gene numbers were calculated for every GO term and enrichments analysed by agriGO (http://bioinfo.cau.edu.cn/agriGO/analysis.php) using an FDR of 0.05 to find significant enrichments.

Stress-related phytohormones

Endogenous stress-related hormones, namely SA, JA, its biosynthetic precursor *cis*-12-oxo-phytodienoic acid (OPDA), and the biologically active metabolite JA-Ile, were extracted according to Floková *et al.* (2014) with modifications as described in Supplementary Protocol S1 at *JXB* online and analysed using ultra-performance liquid chromatography (UPLC)-MS/MS analysis on a Acquity UPLC® System (Waters, USA) coupled to a triple quadrupole mass spectrometer Xevo™ TQ-S (Waters, UK). For detailed description of analytical conditions, see Supplementary Protocol S1.

Volatile metabolites

Headspace of TSSM-infested JA-treated and non-treated plants was collected on Tenax absorbent using dynamic headspace sampling in a climate-controlled cabinet for 2 h and analysed on a Thermal Desorber TD100-xr (Markes, UK) connected to a GC-quadrupole time of flight (QToF; Agilent Technologies, USA). Experimental conditions and GC-QToF operating conditions can be found in Supplementary Protocol S1. Chromatograms were analysed for the presence of plant-derived compounds using MassHunter Unknown Analysis deconvolution software (Agilent Techologies, USA), in combination with NIST98 and Adams (2017) spectral libraries. Data files generated from the GC-MS platform were processed with MZmine V2.23 (mzmine.github.io/) for baseline correction, mass spectra extraction, and mass signal alignment.

Endogenous semi-polar metabolites

For untargeted analysis of semi-polar metabolites, 20 mg of leaf tissue was extracted with 2 ml of 75% MeOH (0.125% formic acid) according to De Vos *et al.* (2007). Samples were analysed using UPLC (Waters Alliance HPLC2695) coupled to a QToF mass spectrometer (Synapt G25 QToFMS Ultima, Waters, US). LC-MS operating conditions can be found in Supplementary Protocol S1. Data files generated from the LC-MS platform were processed with Metalign (Lommen, 2009) for baseline correction, mass spectra extraction, and mass signal alignment.

MSClust (Tikunov *et al*., 2012) was used for data reduction by unsupervised clustering and extracting putative metabolites from mass spectra from ion-wise chromatographic alignment data. Selected endogenous metabolites were putatively annotated based on their molecular weight using the knapsack database (http://kanaya.naist.jp/knapsack_jsp/top.html) taking possible adduct formation into account.

Multivariate analysis

Multidimensional analyses of log2-transformed data of DEGs and metabolites were performed using MetaboAnalyst 3.0 (Xia *et al*., 2016). Hierarchical clustering analysis (HCA) distances were calculated using Pearson correlation and summarized using UPGMA. Both unsupervised principal component analysis (PCA) and supervised partial least squares-discriminant analysis (PLS-DA) algorithms were used to identify clustering among samples. Cluster IDs that define class separation on PLS-DA were obtained to identify genes and metabolites. Individual metabolites and plant hormones were analysed for significant changes upon infestation using Student *t*-tests.

Results

Spider mite performance on two Capsicum *genotypes*

Selection of genotypes used in this study was based on previously found differences in relative resistance to TSSMs as well as in relative attractiveness towards predatory mites (unpublished). In the current study, the inbred line 'Vania' (G8) was confirmed to be more susceptible to TSSMs compared with the land race 'Ta Pien Chiao' (G29) as more offspring were found on G8 3 d after introducing female adults on whole plants (Fig. 1). In particular, more eggs developed into young nymphs on leaves of G8 compared with G29. Damage inflicted by TSSMs was assessed by observing chlorotic spots on leaves after 3 weeks of infestation, and this occurred to a greater extent in G8 than in G29 (6.9 ± 0.3 and 3.2 ± 0.4 spots cm^{-2}, respectively).
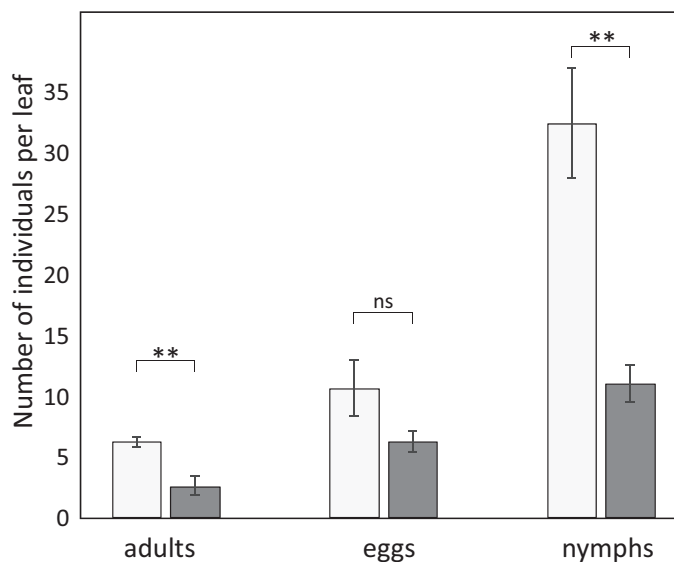
Fig. 1. Spider mite population developing on *Capsicum*. Individuals including adults and offspring (eggs and nymphs) found on *Capsicum annuum* inbred line 'Vania' (G8, light grey bars) and land race 'Ta Pien Chiao' (G29, dark grey bars), 4 d after introduction of adult mites. Data are means ($\pm$SE) of five replicate plants and were tested for significance using a two-tailed Student *t*-test. ns, not significant, **$P<0.01$.

Induced responses to spider mite feeding in Capsicum

Global transcriptome changes

To characterize the mechanism of TSSM-induced defence responses in *Capsicum* and disentangle the role that plant hormones in general and JA in particular play in this process, TSSM-induced transcriptome responses were compared with those induced by JA. As visible TSSM damage appears first after 4–5 d on the susceptible genotype, early mite-induced responses were captured after 3 d of infestation. To cover the relatively transient response induced by JA, two time points (6 h and 24 h) of JA induction were analysed.

Unsupervised PCA of all detected transcripts (log2 values, normalized to RPKM), showed that the first two PCs explain 32% of the total variation (Fig. 2A). PCA shows that genotypes different in their susceptibility towards TSSM infestation have different transcriptome profiles regardless of whether they are infested or not. Upon TSSM feeding, transcriptional profiles change in the same direction in both genotypes, while they changes in opposite directions, in both genotypes, upon application of JA.

To reduce the complexity of the data set, 1955 DEGs between treated and non-treated conditions were selected. In all experimental conditions and in both genotypes, the number of up-regulated DEGs was higher than that of down-regulated genes (Fig. 2B). DEGs partly overlap between TSSM-infested and JA-treated plants, but are mostly unique for both treatments (Table S1; Figs S1, S2, available at the Dryad Digital Repository, https://doi:10.5061/dryad.n34h180). RT–qPCR analysis for a selection of genes validates our RNA-seq data as reproducible (Table S2 at Dryad). HCA classified DEGs into seven main groups of transcripts with similar expression profiles over different genotypes and treatments (Fig. 2C). GO enrichment for each of these groups showed that JA predominantly induced genes associated with metabolic processes, response to jasmonic acid, response to fungus, osmotic stress, salt stress, water deprivation, wounding, oxidative stress, and response to cadmium ion, while TSSM infestation resulted in over-representation of DEGs in processes associated with responses to fungus, wounding, response to jasmonic acid, and response to salicylic acid, as well as protein phosphorylation (Table S3 at Dryad).

Less than one-fifth of TSSM-induced DEGs were in common with those induced by JA (13.5% in G8, 19.5% in G29), of which genes involved in lipid metabolic process and stress-related processes were over-represented based on GO enrichment (Table S3 at Dryad). Genes repressed by TSSMs as well as JA include those with homology to a tomato abscisic acid (ABA) and environmental stress-inducible protein-encoding gene *TAS14* (Parra *et al*., 1996) and a JA-dependent disease-resistant zinc finger CCCH domain-containing protein-encoding gene *C3H12* (Deng *et al*., 2012).

Defence-related plant hormones

TSSM infestation significantly increased *cis*-OPDA, JA, and SA in both genotypes, and JA–Ile in G29 (Fig. 3A), suggesting that TSSMs induced the production of both JA and SA metabolites in *Capsicum*. In contrast, a slight but consistent decrease in
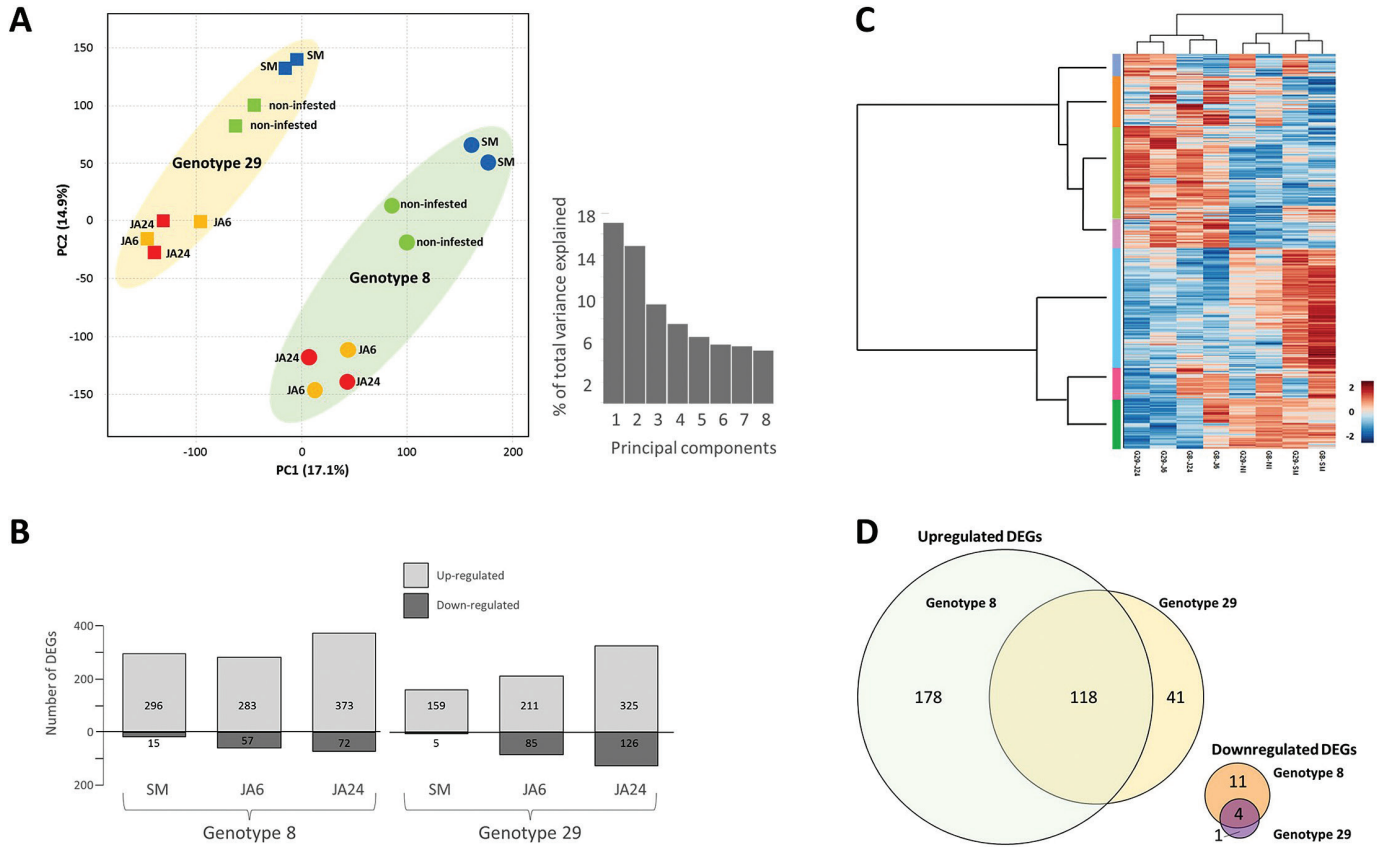
Fig. 2. Transcriptional changes upon spider-mite infestation or JA application. (A) Principal component analysis (score plot) of all transcripts (RPKM values) detected in leaves. Data points represent the different samples. SM, leaves infested with TSSMs for 3 d; JA6, leaves treated with jasmonic acid for 6 h; JA24, leaves treated with jasmonic acid for 24 h. The first three PCs explain 41.4% of total variation. (B) Number of differentially expressed genes (DEGs) that are up- and down-regulated in each of the experimental conditions. (C) Clustering analysis and heat map of expression measures of DEGs detected in each of the experimental conditions. Colour coding represents the range of log2 fold induction (red) or repression (blue). (D) Number of TSSM-induced DEGs characteristic for or overlapping in both genotypes.

ABA was found as a result of TSSM infestation (Table S4 at Dryad). In non-infested plants, basal concentrations of JA and SA were 242-fold (*P*=0.023) and 1.64-fold (*P*=0.007) higher in G8 than in G29, while *cis*-OPDA was 2-fold lower in G8 (*P*=0.006) and JA-Ile, and ABA did not differ. Accumulation of JA and JA–Ile in response to TSSMs was higher in G29 [log2(infested:non–infested) 9.82 and 2.16, respectively] compared with those in G8 [log2(infested:non–infested) 2.57 and 0.08]. Accumulation of SA in response to TSSMs was similar in both genotypes.

To zoom in on how TSSM influences expression of genes involved in JA and SA biosynthesis and signalling cascades, we compared *Capsicum* transcripts with their tomato homologues involved in these processes using the lay-out as presented by Martel *et al.* (2015). TSSM infestation resulted in differential expression of multiple genes putatively involved in the biosynthesis of JA and SA (Fig. 3B, C). As the specific genes involved in these pathways are mostly not yet characterized for *Capsicum*, we depicted the most likely gene candidates. Genes involved in the formation of α-linoleic acid, an early intermediate in JA biosynthesis, include desaturases and lipases that play a role in the breakdown of membrane lipids. Several of these genes were differentially regulated in both genotypes upon TSSM infestation. Multiple 13-OH

LOX-encoding genes, involved in the conversion of linoleic acid into 13(*S*)-hydroperoxylinolenic acid (13-HPOT), were up-regulated upon TSSM infestation. In contrast, most of the *ALLENE OXIDE SYNTHASE* (*AOS*) and *ALLENE OXIDE CYCLASE* gene homologues show repression of transcription, with the exception of Capana08G002494 that is up-regulated upon TSSM feeding, indicating that this *AOS* might be one that is associated with induced JA biosynthesis in *Capsicum*.

Interestingly, application of JA resulted in up-regulation of multiple genes in the biosynthetic pathway upstream of JA, including gene candidates encoding desaturases and lipases. Also 13-OH *LOX* genes including *LOX6* and *LOX7* (Sarde *et al.*, 2018), multiple *AOS* homologues, *12-OXOPHYTODIENOATE REDUCTASE*, *ACYL COENZYME-A OXIDASE*, and *JASMONIC ACID CARBOXYL METHYLTRANSFERASE* gene candidates were up-regulated upon JA treatment (Fig. 3B). Transcripts of genes related to JA signalling including *JASMONATE ZIM DOMAIN* (*JAZ*) and *MYC2* homologues were induced by JA, while in response to TSSMs only minor changes in transcription of these genes were detected. In addition, multiple *Capsicum* homologues to known JA/ ET-responsive genes including *PATHOGENESIS-RELATED 4* (*PR4*) and *CHIB* (Table S3 at Dryad) indicate that TSSMs induced the JA signalling pathway in *Capsicum*.
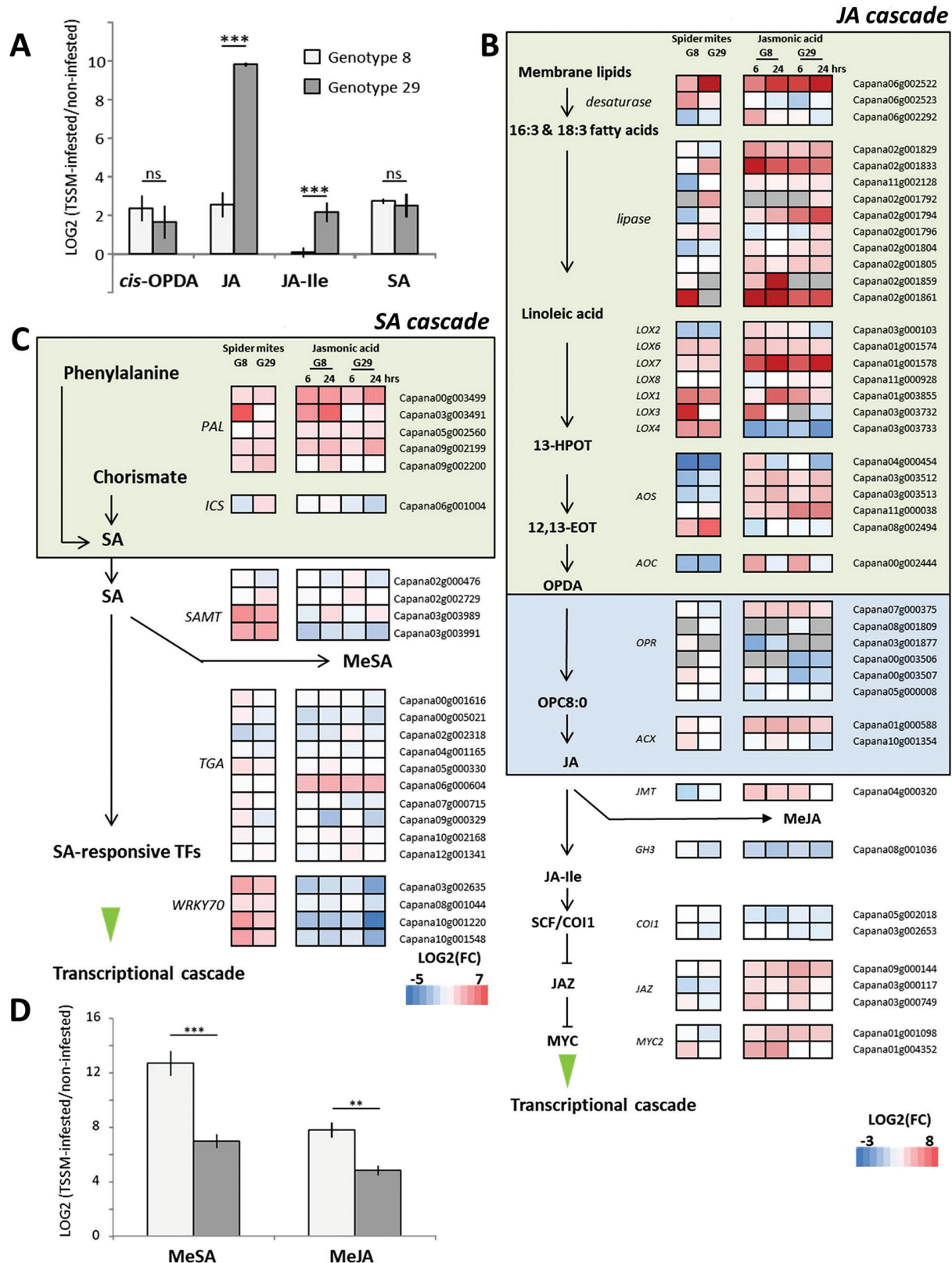
Fig. 3. Spider mite-induced responses in JA and SA pathways. (A) Log2 fold changes in stress-related hormones upon TSSM infestation. (B) Heat map of log2 fold changes of genes involved in the jasmonic acid (JA) biosynthesis and signalling cascade. (C) Heat map of log2 fold changes of genes involved in salicylic acid (SA) biosynthesis and signalling cascade. In schemes of cascades, compounds are shown in bold and enzymes in italics. *cis*-OPDA, *cis*-(+)-12-oxo-phytodienoic acid; JA-Ile, JA-isoleucine conjugate; *LOX*, lipoxygenase; *AOS*, allene oxide synthase; *AOC*, allene oxide cyclase; *OPR*, oxidoreductase; *ACX*, acyl coenzyme-A oxidase; *GH3*, GH3 family proteins; *COI1*, coronatine-insensitive protein 1; *JAZ*, Jasmonate ZIM domain; *MYC2*, bHLH transcription factor; *PAL*, phenylalanine ammonia lyase; *ICS*, isochorismate synthase, *SAMT*, salicylate-*O*-methyl transferase; *TGA*, TGACG sequence-specific binding proteins; *WRKY70*, (T)TGAC(C/T) sequence-specific binding proteins. Colour coding represents the range of log2 fold changes. (D) Log2 fold changes of methyl salicylate (MeSA) and methyl jasmonate (MeJA), volatile metabolites of SA and JA, respectively, as a result of TSSM infestation.

Both TSSM- and JA-induced genes were related to the conversion of phenylalanine to SA, while transcript changes in a homologue of *ICS* were only minor and different in both genotypes.

TSSM infestation induced genes associated with SA signalling, including genes with homology to *WRKY40* and *WRKY70*, while JA treatment repressed their expression. Furthermore, TSSM infestation, but not JA, resulted in induction of *SALICYLATE O-METHYLTRANSFERASE* candidates, responsible for the conversion of SA into methyl-SA. SA-inducible genes, including *GLUTAREDOXIN* (Ndamukong *et al.*, 2007), the SA positive regulator *RECEPTOR LECTIN KINASE* (Luo *et al.*, 2017), *PR4*, and several fungal defence-related genes which are important components of insect and pathogen defences especially in the *Solanaceae* family (Kim *et al.*, 2009), were also induced by TSSMs. Emission of the volatile methyl esters of SA and JA strongly increased upon TSSM infestation and was higher in G8 than in G29 (Fig. 3D).

Volatile and non-volatile specialized metabolism
PLS–DA representation of non-volatile metabolites explains 40% of the data set variation in the first two components (Fig. 4A). Cross-validation indicates the first three components relevant for classification of the variation, illustrative for different directions of response to JA and TSSMs in both genotypes. After 3 d of TSSM infestation, prior to visible damage, 14 metabolites were significantly up-regulated in G29 when compared with non-infested plants, while seven were suppressed (Fig. 4B). The number of metabolite features altered by TSSMs was smaller in G8 (four induced, three repressed). Most of the metabolites induced upon TSSM feeding are glycosylated terpenoids, including several di- and triterpene glucosides. Furthermore, a flavonoid-*O*-glucoside strongly increased upon TSSM infestation in G8, specifically. Only two metabolites, a putative alkaloid and an aromatic compound, were repressed in both genotypes.

JA induced larger changes in more metabolites than did infestation with TSSMs, including terpenoids, flavonoids, and alkaloids (Fig. 4B; Table S5 at Dryad). In addition, JA significantly repressed the abundance of the carotenoids β-cryptoxanthin (mol. wt 552.4331 Da) and zeaxanthin (mol. wt 568.4280 Da) in both genotypes, while TSSMs only repressed β-cryptoxanthin in G29 and had no effect on carotenoids in G8. Xanthophylls are accessory pigments for photosynthesis and assist chlorophylls in light harvesting (Xu *et al.*, 2015), and their lower abundance is consistent with the JA-repressed transcripts of photosynthesis-related genes found in the transcriptome analysis of both genotypes (Table S6 at Dryad).

With regard to volatile metabolites, TSSMs induced changes in the emitted blend of both genotypes. The first two components of the PCA explained 44.3% of the total variation and, interestingly, the direction of the response differs between the two genotypes (Fig. 4D). Within the top 20 most distinctive features contributing to the altered volatile blend, at least 12 of them are terpenoids, including the monoterpenes linalool and (*E*)-β-ocimene, six sesquiterpenes, and the homoterpenes

(3*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) (Table S7 at Dryad). Most of these compounds are volatiles detected in the blend of multiple herbivore-infested plant species. (*E*)-2,4-Hexadiene was the most distinct induced compound present in the volatile blend of TSSM-infested G8 plants, while other GLVs were more strongly induced in TSSM-infested G29. Furthermore, the induced volatile blend of G29 plants predominantly consisted of sesquiterpenes while methyl-SA was more strongly induced in G8 compared with G29 (Fig. 3D). Except for methyl-SA, benzenoid compounds are the most distinctive group of compounds whose emission was repressed in both genotypes. As cinnamoyl-CoA and benzoyl-CoA are involved in the biosynthesis of both SA and benzenoids in, for example, petunia flowers (Klempien *et al.*, 2012), this suggests that upon TSSM infestation common precursors are converted to SA and methyl-SA instead of benzenoids.

While JA induced multiple genes involved in primary metabolic processes including biosynthesis and metabolism of isopentenyl diphosphate, fatty acids, glutamine, and aromatic amino acids, TSSM infestation did not have any effect on transcription of these genes. Also genes related to photosynthesis were repressed by JA, but not by TSSMs (Table S6 at Dryad). JA induced genes involved in the biosynthesis of several secondary metabolites, including terpenoids, phenylpropanoids, and flavonoids (Fig. 4C; Table S3 at Dryad). TSSM infestation for 3 d did not result in visible chlorotic spots, and only a limited number of metabolic processes showed up-regulated gene expression, including lipid and fatty acid metabolism, and, in G8 only, phenylpropanoid metabolism (Table S3 at Dryad). Nevertheless, a number of JA-induced *TERPENE SYNTHASE* genes were induced by early TSSM infestation, including multiple putative sesquiterpene synthase genes (Fig. 4C). In contrast, a geranylgeranyl diphosphate synthase, providing the precursor for diterpenes and TMTT, was found to be repressed upon TSSM infestation, while it was induced by JA (Fig. 4D). Also, *GERANYL LINALOOL SYNTHASE* was found to be induced upon JA treatment, and, based on the expression profiles of all samples, this gene strongly correlated (Pearson correlation >0.90) with 34 genes, including a number of *CYTOCHROME P450 MONOOXYGENASES* (*P450*) genes (Table S8 at Dryad). Further research will elucidate which of these *P450* genes are responsible for the formation of volatile TMTT and hydroxylated geranyl linalool, which is the precursor for non-volatile diterpene glycosides.

Next to terpenoid-related metabolism, genes involved in the biosynthesis of flavonoids, including those with homology to *CAFFEIC ACID 3-O-METHYLTRANSFERASE*, whose product catalyses multistep methylations in the lignin and flavonoid biosynthetic pathway (Do *et al.*, 2007), and *CHALCONE SYNTHASE* whose product catalyses the condensation of 4-coumaroyl-CoA and malonyl-CoA to form the flavonoid naringenin (Coburn *et al.*, 2015), were found to be induced upon JA treatment, while transcriptional differences were minute in TSSM-infested leaves (Fig. 4C). In contrast, transcripts of *4CL3* and *TT7* were induced upon TSSM infestation, while they were repressed by JA.
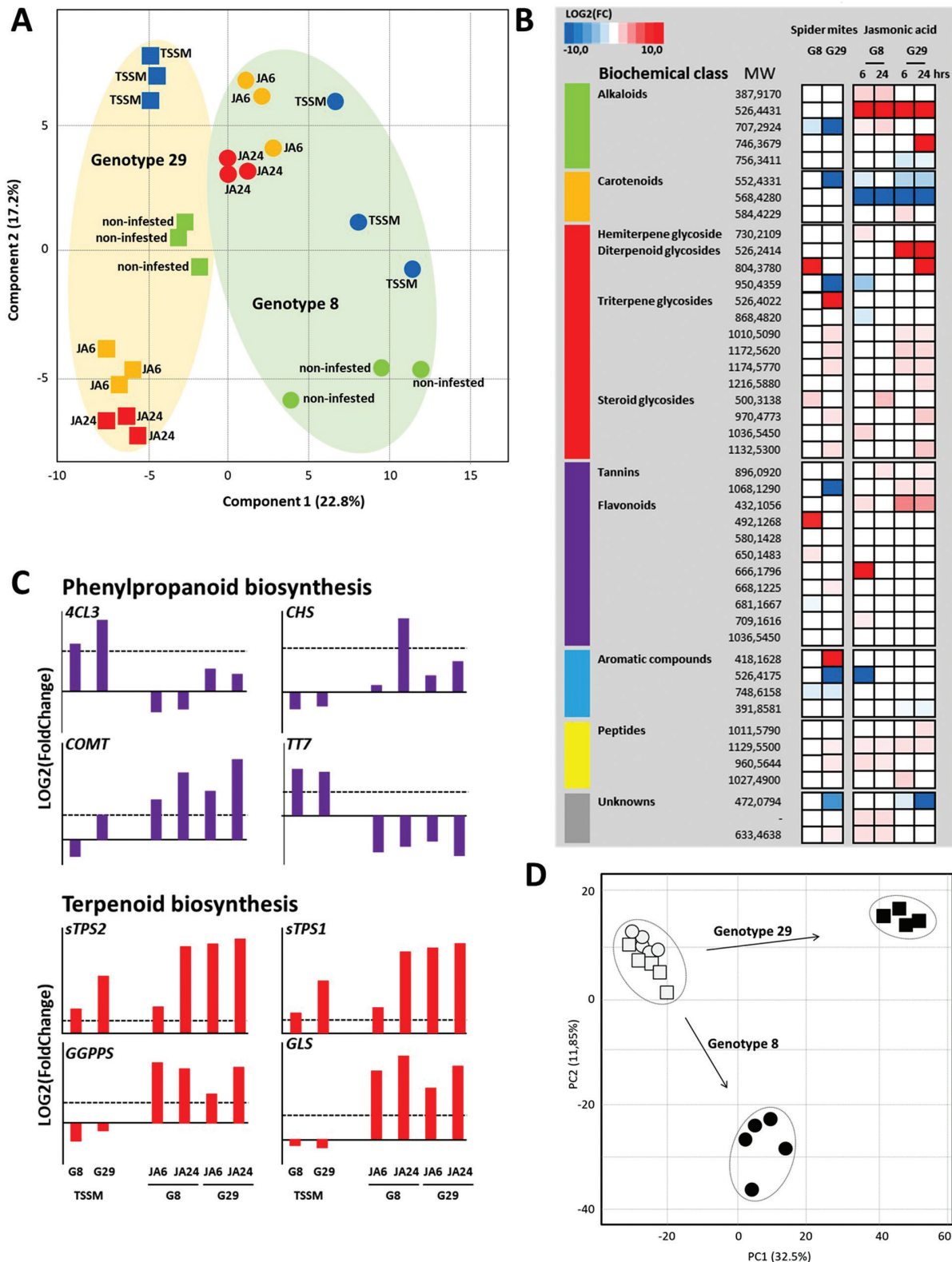
Fig. 4. Changes in specialized metabolism upon spider mite infestation or JA application. (A) Partial least squares discriminant analysis (score plot) of endogenous semi-polar metabolites detected in leaves. Data points represent the different samples. TSSM, leaves infested with spider mites for 3 d; JA6, leaves treated with jasmonic acid for 6 h; JA24, leaves treated with jasmonic acid for 24 h. The first two components explain 40% of the total variation. (B) Metabolic features (accurate masses and putative biochemical class) that were found to be significantly induced or repressed in each of the experimental conditions. Colour coding indicates log2(FC) of induction (red) or repression (blue). (C) Log2(FC) of selected genes involved in biosynthesis of specialized metabolites. The dotted line indicates log2(FC)=1. *4CL3*, 4-coumarate-CoA ligase; *CHS*, chalcone synthase; *COMT*, caffeic acid 3-*O*-methyltransferase; *TT7*, cytochrome P450 flavonoid biosynthesis related; *sTPS*, sesquiterpene synthase; *GGPPS*, geranylgeranyl diphosphate synthase; *GLS*, geranyl linalool synthase. For gene identifiers, see Supplementary Table S9 at Dryad. (D) Principal component analysis (score plot) of volatile metabolites emitted by non-infested and TSSM-infested *Capsicum* plants. Open circles indicate non-infested G8, open squares indicate non-infested G29, and filled symbols indicate TSSM-infested samples. Arrows indicate the direction of response within the first and second PC for both genotypes.

Across the different treatments, the changes in the metabolome are more or less consistent with the changes in the transcriptome. Together they suggest that JA induces extensive changes in specialized metabolism while early TSSM infestation predominantly induces genes involved in the lipid metabolic process and the phenylpropanoid pathway.

Both genotypes exhibited enhanced attractiveness to predatory mites upon TSSM infestation, but the odour blend of G8 was more attractive than that of G29 (Fig. 5). G29 had higher induced emission of multiple GLVs and terpenoids, including (*E*)-β-ocimene, (*E,E*)-α-farnesene, TMTT, and (*E*)-3-hexenol, which are compounds suggested to be involved in the attraction of natural enemies in many plant species (Schnee *et al.*, 2006; Zhang *et al.*, 2009; Kappers *et al.*, 2011). Intriguingly, only a few volatile metabolites were induced to a higher level in the relatively susceptible genotype G8, including (*E*)-2,4-hexadiene and methyl-SA. Methyl-SA has been extensively reported to be attractive to predatory mites in many plant species (De Boer and Dicke, 2004; Ishiwari *et al.*, 2007; Shimoda, 2010; Kappers *et al.*, 2011). Although the attractiveness to predatory mites is most likely to be the result of the blend of volatile metabolites emitted, our data seem to confirm that methyl-SA is an important compound determining predatory mite preferences, also in pepper.

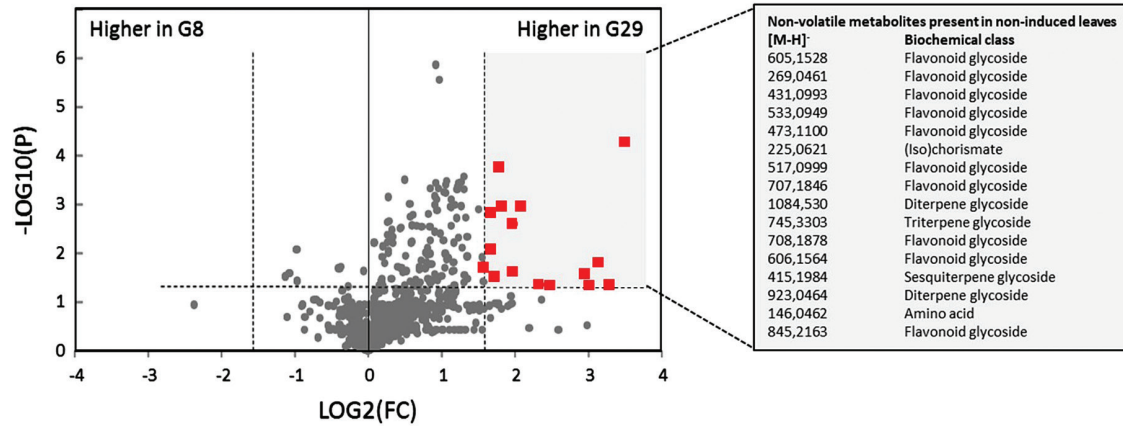*Constitutive defences of both genotypes are comparable*
The final level of defence towards herbivores is determined by the magnitude of induced defences upon infestation as well as the basal defence level. We therefore compared transcriptomes of both genotypes under non-infested/treated conditions. In the relatively resistant G29 plants, no GO term was found significantly (FDR <0.05) enriched in genes with higher expression levels compared with those in G8. In contrast, the GO terms response to biotic stimulus and response to stress were enriched in genes with higher expression in G8. Apparently these constitutively expressed genes do not play an important role in defence as G8 is more susceptible. In contrast, when comparing metabolite profiles of non-induced plants, no metabolite features were found to be more than 3-fold [log2(1.58)] higher in G8 plants, while in G29 a total of 16 metabolite features were significantly (*P*<0.05) more abundant in non-infested plants compared with G8 (Fig. 6). The majority of these metabolites are flavonoids and there are also two diterpene glycosides.

Gain of herbivore resistance by lack of SA-induced suppression of JA defences?
Upon TSSM feeding, G8 displayed about twice more transcriptional changes than G29 (Fig. 2D). Interestingly, a relatively large portion of DEGs was specific for each genotype, with 60% of up-regulated DEGs specifically induced in G8 while 25% of up-regulated DEGs found in G29 were not detected in G8. To compare transcriptional changes quantitatively, we compared transcriptomes of TSSM-infested leaves between both genotypes directly. Upon TSSM infestation, more genes were found enriched in response to SA in G8, including genes with homology to the SA-responsive transcription factor gene *WRKY70* and several other SA-inducible genes (Table S3 at Dryad) while G29 showed stronger enrichment in secondary metabolic processes including the phenylpropanoid biosynthetic pathway. With the hypothesis that TSSMs induced SA signalling, which in turn repressed JA signalling, we examined JA-induced genes that were not induced or even repressed by TSSMs. In G29, genes enriched in amino acid metabolic process and wound response were detected, while in G8, genes enriched in lipid metabolic process and various secondary metabolic processes including isoprenoid biosynthetic pathway, phenylpropanoid biosynthetic pathway, lignin metabolic process, terpenoid metabolic process, amino acid metabolic process, and wound responses were found. In G8, more genes responsive to JA were not induced or even repressed upon TSSM infestation than in G29, which could support the hypothesis that there is stronger suppression of JA responses in G8. Indeed, TSSM-induced accumulation of methyl-SA was larger in G8 (Fig. 3), suggesting a higher induction of SA-related defences. *WRKY70* transcription factor genes are known to have a regulatory role in the crosstalk between SA- and JA-induced defences, and *Capsicum WRKY70* homologues were more strongly induced by TSSMs in G8 (Fig. 3C). When plants of both genotypes were equally induced by SA, or infested with an equal number of TSSMs, two out of three analysed *WRKY70* genes were more strongly induced in G8 compared with G29 (Fig. 7), suggesting that the higher relative resistance of G29 to TSSMs could be partly due to a lack of SA-induced suppression of JA-related defences, possibly via *WRKY70*.
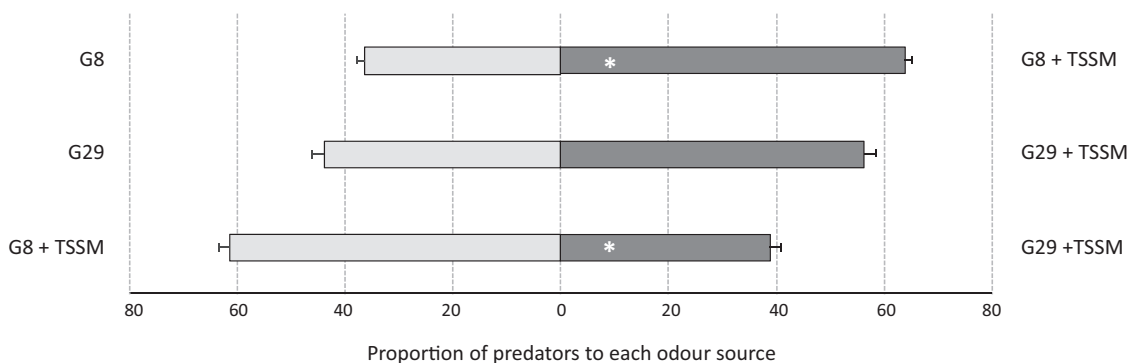
Fig. 5. Attraction of predatory mites. Percentage of *Phytoseiulus persimilis* adult females that were attracted to the odours of TSSM-infested *C. annuum* plants in a two-choice olfactometer. Experiments were repeated four times for comparisons between TSSM-infested and non-infested plants in each genotype, and eight times in the comparison between TSSM-infested plants of both genotypes. In each experiment, 20 individual adult predatory mites were tested (χ^2 test, *P<0.05).

Fig. 6. Constitutive defences. Volcano plot indicating endogenous semi-polar metabolites in non-induced plants. Indicated as red squares are those compounds that significantly [–log10(*P*)>1.30] differ between both genotypes with a log2(FC)>2.3. Data are based on three repetitions for each genotype.
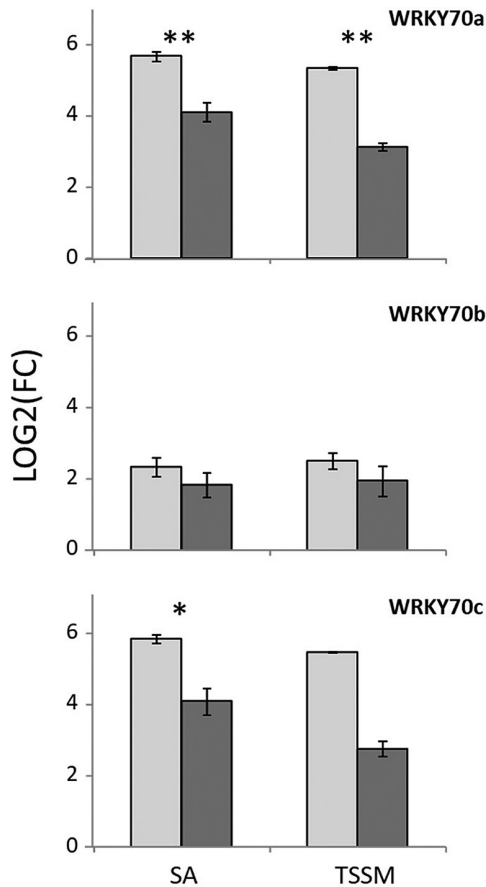
Fig. 7. WRKY70 induced by spider mites. Induction of *WRKY70* homologue genes upon spraying with SA or infestation with spider mites. Transcript levels are calculated as log2(FC), $2^{-\Delta\Delta Ct}$ relative to control treatment and reference genes. Light grey bars indicate G8 and dark grey bars indicate G29. Data are means of three biological replicates ±SE.

Discussion

Many studies have shown that JA plays a prominent role in plant defence against herbivores (Bari and Jones, 2009) and induces production of metabolites including terpenoids, alkaloids, and proteins deterring herbivorous attackers directly or indirectly (Aerts *et al.*, 1994; Van der Fits and Memelink, 2000; Ament *et al.*, 2004; Chen *et al.*, 2005). Here, we found that in *C. annuum*, spider mites induce distinctive transcriptional changes different from those in response to JA. JA treatment

induced genes enriched in wound responses and JA biosynthesis genes. In multiple plant species it has been described that wound responses are mediated by *COI1*-dependent JA signalling (Wasternack *et al.*, 2006; Wang *et al.*, 2008) and that JA regulates JA biosynthesis via a positive feedback loop (Wasternack and Hause, 2013). However, *COI1*-independent induction of numerous genes and processes via OPDA have also been described in multiple plant species (Wasternack and Hause, 2016).

The expression of *Capsicum* genes related to different secondary metabolite biosynthesis pathways including those of terpenoids, flavonoids, and phenylpropanoids was induced by JA. In addition, multiple terpenoid, flavonoid, and alkaloid metabolites were found in higher abundance upon JA treatment. JA enhances flavonoid production probably via MYB transcription factors (An *et al.*, 2015; Pireyre and Burow, 2015) in which *MYC2* was shown to have a positive role (Dombrecht *et al.*, 2007). Several terpenoids and alkaloids have been confirmed to be JA induced, and multiple biosynthetic genes and regulators in these pathways were identified in tomato (Ament *et al.*, 2004), *Artemisia annua* (Yu *et al.*, 2012), and *Catharanthus roseus* (Zhu *et al.*, 2015). Although due to the complexity of different metabolic biosynthetic pathways and limited data sets of the current study, it is difficult to precisely connect metabolite production with gene expression, our data provide comprehensive evidence suggesting that JA induces multiple secondary metabolite biosynthetic pathways in pepper and is responsible for a burst of secondary metabolite production.

It is noteworthy that JA repressed genes functional in the tyrosine biosynthesis pathway. Tyrosine is generated from chorismate, an intermediate in the biosynthesis of phenylalanine, which is the starting point of the phenylpropanoid biosynthesis pathway (Vogt, 2010; Fraser and Chapple, 2011). In contrast to tyrosine biosynthesis, multiple transcripts related to the phenylpropanoid pathway were significantly induced by JA, suggesting that JA represses the production of tyrosine in favour of up-regulation of the phenylpropanoid pathway.

JA-induced secondary metabolites are important for Capsicum *defence against spider mites*

Based on the numbers of TSSMs that developed from eggs to nymphs and adults in the short time span of the bioassay, G8

is more susceptible than G29. Upon infestation, the flux in SA metabolism increased in G8, corresponding to the higher expression of SA-responsive genes found in this genotype. In contrast, JA production increased more strongly, and more endogenous metabolites increased upon TSSM feeding in G29. Transcriptome comparison between both genotypes in non-infested samples suggests that some stress-related genes are more highly expressed in G8 under unchallenged conditions, consistent with higher constitutive concentrations of SA and JA in G8. However, TSSM infestation leads to a stronger JA response in G29, resulting in higher expression of JA-responsive genes and the accumulation of various secondary metabolites, eventually resulting in a stronger induced defence correlating with the observed higher resistance for G29.

Spider mites induce both the SA and JA signalling pathways, but not all JA-induced metabolic processes

JA biosynthetic genes up-regulated by TSSM feeding caused the accumulation of *cis*-OPDA, JA, and JA-Ile, and up-regulated JA-responsive genes. Together, this confirms that TSSMs induced both JA biosynthesis and signalling in *Capsicum*. However, compared with large chewing herbivores such as caterpillars (Kawazu *et al.*, 2012), TSSMs cause relatively less mechanical damage and, potentially via saliva-associated microorganisms, also induced the SA pathway, resulting in an increased SA flux and up-regulation of SA-responsive genes. This result is consistent with studies on TSSM-induced responses in tomato (Martel *et al.*, 2015) and in grape (Díaz-Riquelme *et al.*, 2016).

Our study shows that early TSSM infestation induces only a limited number of metabolite pathways compared with JA treatment. As a result, plants accumulated less secondary metabolites upon infestation than plants treated with JA, and distinctive metabolic profiles were found for JA-induced plants compared with TSSM-infested and control plants. However, both JA and TSSMs up-regulated the expression of genes encoding 4-coumarate-CoA ligase 2, in both genotypes. This enzyme is involved in the final step of the phenylpropanoid pathway, which precedes the biosynthesis of both SA (Chen *et al.*, 2009) and flavonoids (Vogt, 2010). JA induced multiple genes in the flavonoid pathway, downstream of the phenylpropanoid pathway, and produced more flavonoid metabolites compared with non-treated plants. In contrast, TSSMs induced the SA pathway, but fewer flavonoids compared with JA treatment. This suggests that TSSMs induce the phenylpropanoid pathway only, and do not up-regulate the flavonoid pathway as JA does, resulting in the production of SA, but not flavonoids. In accordance with our results, overproduction of SA in tobacco plants by bacterial transgenes strongly inhibited the accumulation of the flavonoids quercetin, kaempferol, and rutin (Nugroho *et al.*, 2002). However, exogenous SA application has been reported to boost the production of flavonoids in several plant species (Xu *et al.*, 2009; Gondor *et al.*, 2016), and SA induced by pathogens resulted in accumulation of flavonoid phytoalexins in a range of crops in the *Brassicaceae*, *Fabaceae*, *Solanaceae*, *Vitaceae*, and *Poaceae* (Ahuja *et al.*, 2012). Altogether, we hypothesize that JA-induced flavonoid biosynthesis is restricted upon spider mite herbivory because the production of

SA induced by the mites diverts a substantial part of the precursors available to SA biosynthesis.

Even though no GO terms related to terpenoid pathway metabolic processes were found enriched upon TSSM infestation, a number of terpene biosynthetic genes were induced, and multiple volatile and non-volatile terpenoids were found to be present in higher amounts after TSSM infestation. Our results indicate that the terpenoid biosynthetic pathway is mainly induced via JA signalling, while the SA signalling pathway activated by TSSM does not repress this biosynthetic pathway.

JA–SA crosstalk in spider mite-induced defence

In addition to multiple secondary metabolic pathway genes, we found many other genes responsive to exogenous JA but not induced by TSSMs even though JA signalling is induced by TSSM feeding. JA–SA antagonism has been demonstrated in many plant species (Thaler *et al.*, 2012) and we propose that in *Capsicum* the SA pathway suppresses part of spider mite-induced JA responses. To verify this hypothesis and demonstrate a possible mechanism of antagonizing crosstalk, we identified transcription factors induced by JA and TSSMs, respectively, as well as genes whose expression strongly correlates with that of these transcription factors.

MYC2 is well known as the master regulator of JA signalling. Two homologues are identified in the *C. annuum* genome, Capana01g004352 and Capana01g001098. Since only Capana01g001098 shows strong correlations (Pearson correlation >0.85) with multiple DEGs, we predict that Capana01g001098 is the functional homologue of *MYC2* in *Capsicum*. This *Capsicum MYC2* homologue is induced by exogenous JA in both genotypes, though not statistically significantly (Tables S1, S3 at Dryad). Based on correlation analysis, 40 DEGs positively correlate with *MYC2*, and 24 and 25 of those 40 genes are induced by JA in G8 and G29, respectively, while none of them was induced by TSSMs in either of the genotypes. In Arabidopsis, protein levels of MYC2, MYC3, and MYC4 were strongly diminished by *Pieris brassicae* egg extract in an SA-dependent manner, suggesting that MYC transcription factors are targets of SA–JA antagonism (Schmiesing *et al.*, 2016). Apart from the MYC2 branch of the JA signalling pathway, exogenous JA application also induced transcription factors from the JA/ET branch, including three *ERF* genes in G8, and *ERF1* and *MYB3* in G29. In total, 107 DEGs positively correlated with these JA-induced transcription factors in G8 and 51 of these DEGs were induced by JA while two were also induced by TSSMs. In G29, fewer DEGs (28) positively correlated with these JA-induced transcription factors, of which 25 were induced by JA and five also by TSSMs. Interestingly, TSSM feeding resulted in the up-regulation of an *ERF*-related gene (Capana12g000990) in both genotypes, annotated as *TIFY10B* which encodes the MYC repressor, *JAZ*, suggesting repression of the MYC branch in the JA signalling pathway by TSSMs. In our current work, we aim to elucidate the importance of this MYC branch in spider mite-induced defences.

As discussed, JA signalling was suppressed by spider mite-induced SA, but our data also show that exogenous JA

application represses SA signalling. JA application represses the expression of *WRKY70* homologues [Capana10g001548, log2(FC) −3.46 in G8; Capana10g001220, log2(FC) −60.16 in G29]. *WRKY70* mediates the SA-dependent signalling pathway in multiple species (Pandey and Somssich, 2009). Overexpression of *WRKY70* results in constitutive expression of SA-induced pathogenesis–related genes and enhances resistance to *Erysiphe cichoracearum* but represses JA responses. Conversely, antisense suppression of WRKY70 activates JA-responsive/COI1-dependent genes in Arabidopsis (Li *et al.*, 2004, 2006).

In summary, this study gives a comprehensive overview of spider mite- and JA–induced responses in *C. annuum* by comparing transcriptome changes and corresponding metabolome changes, and provides insight into the antagonistic JA–SA crosstalk. JA induces extensive metabolic processes and represses photosynthesis, while spider mites induce SA biosynthesis and hence SA signalling as well as the JA biosynthesis pathway, resulting in the expression of SA- and some JA-responsive genes. Our data suggest that exogenous application of JA may suppress SA signalling by up-regulating the flavonoid biosynthetic pathway which may compete with SA biosynthesis and repress the SA-responsive regulator *WRKY70*. On the other hand, SA signalling induced upon TSSM infestation suppresses the *MYC*-regulated branch in the JA signalling pathway possibly by inducing expression of a *MYC* repressor. In our further studies, we aim to investigate the details of this mutual antagonizing JA–SA crosstalk in pepper and its consequences for specialized metabolism and direct and indirect defences towards cell-feeding chelicerate spider mites.

Supplementary data

Supplementary data are available at *JXB* online.

Protocol S1. Technical details on operating conditions of mass spectrometry platforms.

Data deposition

The following tables and figures are available at Dryad Data Repository: https://doi:10.5061/dryad.n34h180

Table S1. Differentially expressed genes (DEGs).

Table S2. RT–qPCR validation of selected gene transcripts.

Table S3. Gene Ontology enrichment of DEGs.

Table S4. Stress-related hormones.

Table S5. Non-volatile metabolites.

Table S6. Genes related to photosynthesis repressed by JA, but not by TSSMs.

Table S7. Volatile metabolites.

Table S8. Pearson correlation values between DEGs.

Table S9. Identifiers of genes discussed.

Fig. S1. GO enrichment of JA-induced genes not induced by TSSMs.

Fig. S2. Number of DEGs in both genotypes under TSSM infestation and early or late JA induction.

Author contributions

HB and IK conceived and designed the experiments; YZ performed the experiments; YZ and IK analysed the data; and all authors contributed to the writing of the manuscript. The authors declare no conflicts of interest

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