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Catherine Martel
Western University

Vladimir Zhurov
Western University

Marie Navarro
Western University

Manuel Martinez
Universidad Politécnica de Madrid-Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria

Marc Cazaux
Western University

See next page for additional authors

Authors
Catherine Martel, Vladimir Zhurov, Marie Navarro, Manuel Martinez, Marc Cazaux, Philippe Auger, Alain Migeon, M. Estrella Santamaria, Nicky Wybouw, Isabel Diaz, Thomas Van Leeuwen, Maria Navajas, Miodrag Grbic, and Vojislava Grbic

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Tomato Whole Genome Transcriptional Response to *Tetranychus urticae* Identifies Divergence of Spider Mite-Induced Responses Between Tomato and *Arabidopsis*

Catherine Martel,[1] Vladimir Zhurov,[1] Marie Navarro,[1,2] Manuel Martinez,[3] Marc Cazaux,[1,2] Philippe Auger,[4] Alain Migeon,[4] M. Estrella Santamaria,[1,3] Nicky Wybouw,[5] Isabel Diaz,[3] Thomas Van Leeuwen,[5,6] Maria Navajas,[4] Miodrag Grbic,[1,2] and Vojislava Grbic[1,2]

[1]Department of Biology, The University of Western Ontario, London, ON, Canada N6A5B7; [2]Instituto de Ciencias de la Vid y el Vino, 26006 Logrono, Spain; [3]Centro de Biotecnologıa y Genomica de Plantas, Universidad Politécnica de Madrid-Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, 28223 Madrid, Spain; [4]INRA, UMR CBGP (INRA/IRD/Cirad/Montpellier SupAgro), 34998 Montferrier-sur-Lez Cedex, France; [5]Department of Crop Protection, Ghent University, B-9000, Ghent, Belgium; [6]Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, 1098 XH, Amsterdam, The Netherlands

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The two-spotted spider mite *Tetranychus urticae* is one of the most significant mite pests in agriculture, feeding on more than 1,100 plant hosts, including model plants *Arabidopsis thaliana* and tomato, *Solanum lycopersicum*. Here, we describe timecourse tomato transcriptional responses to spider mite feeding and compare them with *Arabidopsis* in order to determine conserved and divergent defense responses to this pest. To refine the involvement of jasmonic acid (JA) in mite-induced responses and to improve tomato Gene Ontology annotations, we analyzed transcriptional changes in the tomato JA-signaling mutant *defenseless1 (def-1)* upon JA treatment and spider mite herbivory. Overlay of differentially expressed genes (DEG) identified in *def-1* onto those from the timecourse experiment established that JA controls expression of the majority of genes differentially regulated by herbivory. Comparison of defense responses between tomato and *Arabidopsis* highlighted 96 orthologous genes (of 2,133 DEG) that were recruited for defense against spider mites in both species. These genes, involved in biosynthesis of JA, phenylpropanoids, flavonoids, and terpenoids, represent the conserved core of induced defenses. The remaining tomato DEG support the establishment of tomato-specific defenses, indicating profound divergence of spider mite–induced responses between tomato and *Arabidopsis*.

Plants and herbivores have co-evolved over millions of years, resulting in a myriad of plant-herbivore interactions. Herbivores have evolved various levels of specialization to their hosts and differ in types of feeding damage they cause, while plants range in their ability to restrict herbivore performance, contributing to the diversity and complexity of plant-herbivore interactions. Generally, plants employ two lines of defenses, constitutive and inducible, to deter herbivory. Constitutive defenses, such as trichomes and the presence of certain toxins, are a primary line of protection against a broad spectrum of potential attackers (Howe and Jander 2008). However, the lack of specificity and high energy cost of constitutive defenses are believed to have led to the evolution of inducible defenses that are triggered in response to the detection of a specific attacker (e.g., pathogen, fungus, herbivore) (Schwachtje and Baldwin 2008; Steppuhn et al. 2008). Inducible defenses against herbivores include the synthesis of a wide range of species-specific toxic plant secondary metabolites (e.g., phenylpropanoids, flavonoids, anthocyanins, alkaloids, terpenoids, glucosinolates), and anti-nutritive enzymes and proteins (e.g., proteinase inhibitors, amino acid catabolizing enzymes, polyphenol oxidases, and peroxidases) (Campos et al. 2014; Howe and Jander 2008; Mithofer and Boland 2012; Santamaria et al. 2013). In addition to these direct inducible defenses, plants also emit complex cocktails of volatiles that attract predators of herbivores as indirect defense responses (Clavijo McCormick et al. 2012; Mithofer and Boland 2012). Despite the diversity of plant-herbivore interactions, jasmonic acid (JA) has been identified as the major regulator of plant defense responses to herbivory (Campos et al. 2014).

The two-spotted spider mite *Tetranychus urticae* is a piercing-sucking herbivore that can feed on more than 1,100 plant species (Migeon and Dorkeld 2006-2014). In the last several years, *T. urticae* has become a model chelicerate herbivore, with its genome sequenced (Grbic et al. 2011) and a number of tools and protocols for genomic and genetic studies developed (Dearden et al. 2002; Dermauw et al. 2013; Khila and Grbic 2007; Grbic et al. 2007, 2011; Van Leeuwen et al. 2012, 2013). Taking advantage of these tools, we previously reported the reciprocal whole-genome responses between *Arabidopsis thaliana* and *T. urticae*. This study highlighted JA as a key regulator and JA-dependent biosynthesis of indole glucosinolates as

Transcript profiling data from this article are deposited at Gene Expression Omnibus (GEO) platform GPL16358 as GEO superseries GSE61076.

Catherine Martel and Vladimir Zhurov contributed equally to the paper.

Corresponding author: V. Grbic; E-mail: vgrbic@uwo.ca

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the main functional output of the *Arabidopsis* defenses induced by spider mite feeding (Zhurov et al. 2014). To understand the evolution and diversity of plant-herbivore interactions, we have expanded our analysis of plant–spider mite interactions to include tomato (*Solanum lycopersicum*) as a complementary system to study defense responses induced by spider mite feeding.

Spider mites are an economically important pest of cultivated tomatoes (Jeppson et al. 1975; Zhang 2003). Several studies of tomato–spider mite interactions highlighted the importance of constitutive defenses, such as chemical content and density of glandular trichomes, in defense against spider mites in wild tomato relatives (Glas et al. 2012). For example, acylsugars, present in trichomes of *Solanum pennellii* (Blauth et al. 1998; Mirnezhad et al. 2010; Mutschler et al. 1996; Resende et al. 2002; Salinas et al. 2013), methyl ketones from *Solanum hirsutum* (Antonious et al. 2014), and terpenoids from *Solanum habrochaites* (Bleeker et al. 2012) confer high levels of resistance against spider mites. Studies of induced tomato defenses to spider-mite herbivory identified the importance of JA and suggested roles for salicylic acid (SA) and ethylene (ET) in the regulation of tomato induced defenses (Ament et al. 2004; Kant et al. 2004; Li et al. 2002a; Li et al. 2004). Expression levels of the JA biosynthetic enzymes *lipoxygenase D (LOXD)* and *allene oxide synthase 1 (AOS1)*, proteinase inhibitors (*PI*), *leucine amino peptidase (LAP)*, *threonine deaminase (TD)*, and *polyphenol oxidases* (*PPO*) have been identified as highly reproducible markers of JA-dependent induced defense responses in tomato, pointing to the importance of defense proteins that reduce the quality of the plant diet or the activity of digestive enzymes in the herbivore gut (Chen et al. 2005; Chung and Felton 2011; Fowler et al. 2009; Gatehouse 2011; Gonzales-Vigil et al. 2011; Green and Ryan 1972; Kessler and Baldwin 2002; Zhu-Salzman et al. 2008). Other well-characterized tomato induced defenses include emission of volatiles, such as TMTT (E,E-4,8,12-trimethyl-1,3,7,11-tridecatetraene) and MeSA (methyl salicylate) (Kant et al. 2004, 2008), that play an important role in the attraction of the spider mite predator *Phytoseiulus persimilis* (Ament et al. 2004; Dicke et al. 1998). Despite being useful markers of herbivory, expression of these marker genes fails to provide a comprehensive understanding of tomato defense response to spider mite.

The completion of tomato genome sequencing (Sato et al. 2012) enables a genome-wide analysis of induced tomato transcriptome responses upon spider mite herbivory. We employed the newly developed EUTOM3 whole genome exon array to monitor early transcriptional changes occurring in tomato leaves in the first 24 h following spider mite attack and have compared them with defense responses triggered by spider mite feeding on *Arabidopsis*.

RESULTS AND DISCUSSION

Tomato response to spider mite feeding.

Induced transcriptional responses of tomato upon spider mite feeding. In order to understand genome-wide tomato transcriptional responses to spider mite herbivory, a microarray study was designed to capture early changes in gene expression following *T. urticae* attack in timecourse and feeding-site scenarios. We used reference tomato and mite strains whose genomes were sequenced as representatives of their species, 'Heinz 1706' tomato variety and a London strain of *T. urticae* (maintained on beans and, thus, nonadapted to tomato). In the timecourse experiment, the terminal leaflet of the third leaf of three-week-old tomato plants was infested with 100 adult female spider mites and tissue was collected at 0, 1, 3, 6, 12, and 24 h postinfestation (hpi), while, in the feeding site (FS) experiment, the terminal leaflet was saturated with hundreds of mites that were allowed to feed for 1 hpi, following an experimental design described by Zhurov and associates (2014). RNA extracted from these samples was hybridized to the Affymetrix EUTOM3 tomato exon array. We detected 2,133 differentially expressed genes (DEG) in at least one timepoint as compared with noninfested control plants at an absolute fold change (FC) > 2 and a Benjamini-Yekutieli (BY) false discovery rate (FDR) adjusted P value < 0.01, using the Bioconductor package limma (Benjamini and Yekutieli 2001; Smyth 2004) (Fig. 1; Supplementary Dataset S1). In the FS samples, we detected 1,936 DEG relative to the non-infested control at the cut-offs described above. Since the London spider mite strain was previously used for studies of *Arabidopsis*-mite interaction following the same timecourse and similar experimental design (Zhurov et al. 2014), we could perform a direct comparison between responses of these two plant species to the same herbivore. Approximately 50% of the DEG identified in the timecourse experiment and in the FS sample have putative bidirectional best hit (BBH) orthologues in *Arabidopsis* (the establishment of the BBH orthologues between tomato and *Arabidopsis* is discussed below and is available in Supplementary Dataset S2) (Overbeek et al. 1999). Of the 2,133 DEG, 1,062 were up-regulated and 1,047 were down-regulated in at least one timepoint, with an additional 24 genes showing both significant up- and downregulation during the course of the experiment (Fig. 1A and C). Equal distribution between up- and downregulated DEG in tomato contrasts with *Arabidopsis* responses that were largely represented by upregulation of gene expression (Zhurov et al. 2014). Tomato responses overlap considerably between different timepoints, with the greatest number of unique DEG being detected at 3 hpi (Fig. 1A). Validation of microarray results by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) indicates that our microarray analysis is reproducible in capturing gene expression changes induced by spider mite herbivory (Supplementary Fig. S1).

In a principal component analysis, the majority of variance in gene expression was due to the factor attributable to spider mite treatment and number of mites deposited on a plant (PC1, 20% of total variation). The factor attributable to time postinfestation (PC2) explained another 18% of the total variation in the data, reflecting a division between early and late responses, with early (1 and 3 hpi) and late timepoints (12 and 24 hpi) clustering together and the 6 hpi timepoint found midway between these clusters (Fig. 1B).

Gene Ontology (GO) analysis. To characterize differentially regulated programs upon spider mite feeding, we wanted to identify GO categories enriched in DEG but found that the International Tomato Annotation Group (ITAG) GO annotation (Sato et al. 2012) associated with the tomato genome was limited in scope. Thus, we performed complete GO re-annotation of tomato proteins, using the Blast2GO workflow (Conesa et al. 2005). The Blast2GO annotation of the EUTOM3 microarray platform increased the number of annotated genes to 22,966 (80% of genes interrogated by microarray) compared with 18,340 (64% of genes interrogated by microarray) annotated by ITAG v.2.4 GO. The number of unique terms associated with genes represented on the microarray increased from 1,965 to 5,668, and the mean number of GO terms associated with a gene increased to 6.1 from 1.3 terms per gene. Despite a substantial increase in the number of GO terms associated with genes, the mean average distance of term to the GO root only slightly decreased to 5.6 compared with 6.1 in the original annotation, indicating that the Blast2GO annotation also maintained the level of specificity of the original GO annotation. For example, terms pertinent for our study that were signifi-

cantly improved include: GO:0009753 'response to jasmonic acid'—136 genes associated in the current annotation; GO:0009751 'response to salicylic acid'—57 genes; and GO:0010466 'negative regulation of peptidase activity'—19 genes, in comparison with 0 genes associated with these terms in the original annotation. The GO:0009753 'response to jasmonic acid' category was further augmented using results of our analysis of the transcriptional response of *def-1* tomato

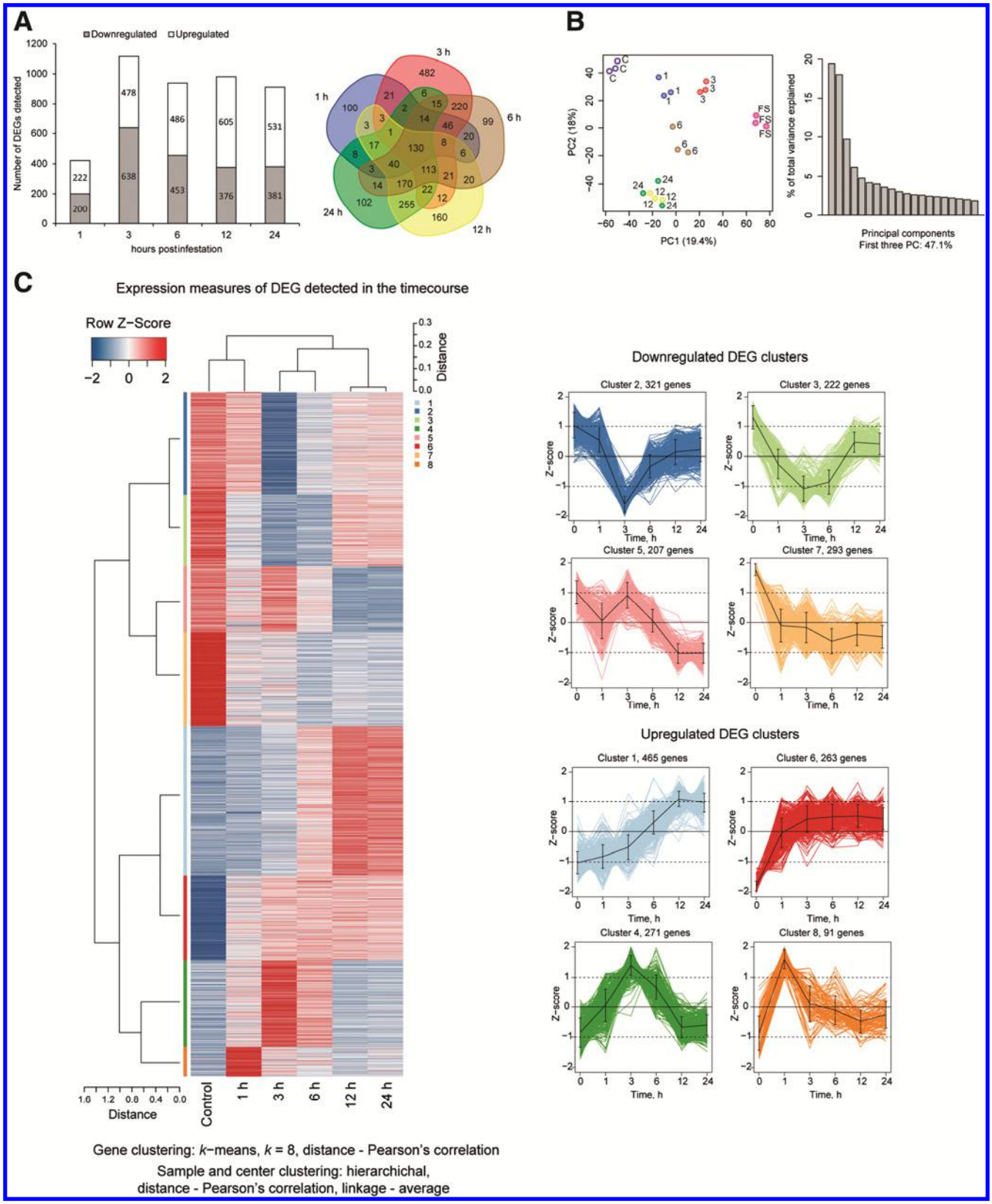


Fig. 1. Microarray analysis of tomato response to spider mite herbivory. **A,** Number and directionality of differentially expressed genes (DEG) in tomato upon spider mite herbivory in timecourse samples and Venn diagram of lists of DEG. **B,** Principal component analysis of microarray expression data for timecourse (1 to 24 h) and feeding-site (FS) samples. **C,** Clustering analysis and heat map of expression measures of DEG detected in timecourse samples and expression graphs of individual DEG clusters.

plants to JA treatment (discussed below) and ultimately included 274 tomato genes. The updated GO annotation is available as Supplementary Dataset S3.

GO analysis of biological processes (BP) revealed that DEG detected in the timecourse experiment samples were enriched in genes involved in defense responses common to many biotic and abiotic stresses, including categories such as 'response to jasmonic acid', 'response to wounding', 'negative regulation of peptidase activity', 'response to stress', and 'jasmonic acid biosynthetic process' (Supplementary Dataset S4 includes a list of the top 50 GO BP). Cluster analysis of DEG expression indicated that the transcriptional response developed in stages, starting with the perception of spider mite attack, followed by metabolic reprogramming, and ultimately resulting in the establishment and maintenance of a defense response (Fig. 1C).

Gene set enrichment analysis. In order to understand and visualize the dynamic development of tomato transcriptional response to spider mite attack, we performed gene set enrichment analysis (GSA) using a parametric analysis of gene set enrichment (PAGE) algorithm (Kim and Volsky 2005) of the complete list of 2,133 DEG detected in the timecourse experiment, using gene level statistics ($\log_2$ fold change, adjusted *P* value and *t* statistic) estimated by limma for each timepoint as an input. GO annotation was used to classify genes into sets with BP and cellular component (CC) ontologies treated separately. The distinct changes in gene set regulation were analyzed as described in Varemo and associates (2013).

A total of 60 gene sets based on the BP GO annotation were found to be significantly up- or downregulated in the timecourse samples (Fig. 2; Supplementary Fig. S2, for node labels for BP GO category terms can be found in). The identity of the gene sets at different timepoints highlights distinct stages of tomato responses to spider mite feeding.

At 1 hpi, 26 BP GO-based gene sets associated with perception of the attack were detected as differentially regulated (FDR adjusted *P* value < 0.05) with processes related to protein phosphorylation, cell signaling, and response to wounding being the most strongly upregulated and processes related to anabolism being suppressed. Closer examination of the identity of the kinases present in the 'protein phosphorylation' gene set reveals that they comprise mostly of receptor-like kinases (RLK). Certain gene sets showed transient upregulation exclusively at 1 hpi, including those associated with perception of herbivory and signal transduction, programmed cell death, and transport of metabolites and vesicles. In contrast, other gene sets remained differentially expressed throughout the full timecourse, such as 'response to wounding' and 'response to jasmonic acid stimulus' that were up-regulated and anabolism-related gene sets that were down-regulated.

At 3 hpi, only seven gene sets were detected as distinctly up- or downregulated, despite the highest number of DEG detected at this timepoint (1,166 DEG). Upregulated GO BP categories corresponding to responses to JA stimulus and wounding, and downregulated categories corresponding to chloroplast relocation and photosystem II assembly were similarly detected as differentially regulated in all other samples, indicating that these constitute the core defense programs. The low number of DEG sets relative to the high number of DEG detected at this timepoint is likely due to the shifting of transcriptional responses from initial perception and signaling at 1 hpi towards production of defense compounds against herbivore detected at 6 hpi onwards.

In later timepoints, distinct transcriptional reprogramming was established with 10, 41, and 32 gene sets detected as differentially regulated at 6, 12, and 24 hpi, respectively. The gene sets overlapped considerably; all 10 gene sets detected at 6 hpi were stably differentially regulated at later timepoints as well, and 12- and 24-hpi samples shared 29 gene sets. These results demonstrated that, while stable activation of responses to wounding and JA and suppression of anabolic processes occur in tomato very early upon spider mite herbivory, defense responses marked by secondary metabolite production and activation of proteinase inhibitors are established gradually at 6 to 12 hpi and are maintained at 24 hpi.

GSA based on CC GO classification identified 17 gene sets throughout the timecourse. Early transcriptional responses were associated with cellular components responsible for perception and transcriptional reprogramming at 1 and 3 hpi and were followed by gene sets associated with cellular components involved in defense responses in 6- to 24-hpi samples (e.g., endoplasmic reticulum, Golgi apparatus, vacuole), consistent with BP categories enriched at these timepoints (Supplementary Fig. S3).

Induced transcriptional response at the FS. In order to robustly capture early and local responses, we also performed a FS experiment as described above. A total of 1,936 DEG were detected relative to the non-infested control, and 758 DEG were detected relative to tomato response at 1 hpi in the timecourse scenario at the cut-offs described above (Supplementary Dataset S5). Transcriptional response in the FS sample demonstrated a considerable degree of overlap with both the response at 1 hpi and responses that were detected in the later timepoints (Fig. 3A). GSA based on BP GO terms implicated 45 DEG sets in the FS sample (Fig. 3B). Differentially regulated gene sets in the FS sample demonstrated significant overlap with gene sets enriched in the timecourse sample. In all pair-wise comparisons, a substantial number of gene sets detected at the individual timepoints overlapped with FS DEG sets (Fig. 3C). At 1 hpi, 16 of 25 gene sets overlapped with 45 FS gene sets; at 3 hpi, five of seven; at 6 hpi, six of 10; at 12 hpi, 11 of 41; and at 24 hpi, nine of 32, indicating that i) tomato defense responses to spider mite herbivory is robustly established as early as 1 hpi, and ii) tomato defense responses to spider mite herbivory at later timepoints capture early responses as well, probably due to continuous mite feeding. Further analysis of 758 DEG detected between 1 hpi and FS samples by GSA revealed that BP associated with responses to JA, wounding, chitin, and fungus were enhanced under the FS scenario, while processes associated with anabolism were downregulated to a greater extent (Fig. 3D). Hierarchical clustering analysis demonstrated an enhancement of the response for the majority of the 758 DEG detected between 1 h and FS samples (irrespective of the directionality of the response) (Fig. 3E; Supplementary Dataset S6).

Analysis of transcriptional responses of *def-1* plants to JA treatment and spider mite herbivory.

In tomato, JA signaling has been shown to be essential for the proper expression of a number of defense-related genes against spider mites (Ament et al. 2004; Kant et al. 2004; Li et al. 2002a; Li et al. 2004; Sarmento et al. 2011). Despite our improved GO annotation of the tomato genes by Blast2GO, well-known markers of JA responses such as *LOXD*, *PI-I*, *PI-II*, *LAP*, *TD*, and *PPO* were absent from the 'response to jasmonic acid stimulus' category, indicating that homology-based approaches for gene function prediction are biased towards establishing similarity with model organisms (e.g., *Arabidopsis*) and require additional functional experiments for determination of species-specific functions. Thus, to determine the extent of JA regulation of tomato defense responses to spider mite herbivory, we performed an assay using the JA signaling mutant *def-1* (cv. Castlemart). *def-1* has normal basal levels of JA but fails to induce its accumulation in response to wounding and herbivory (Howe et al. 1996). To identify genes

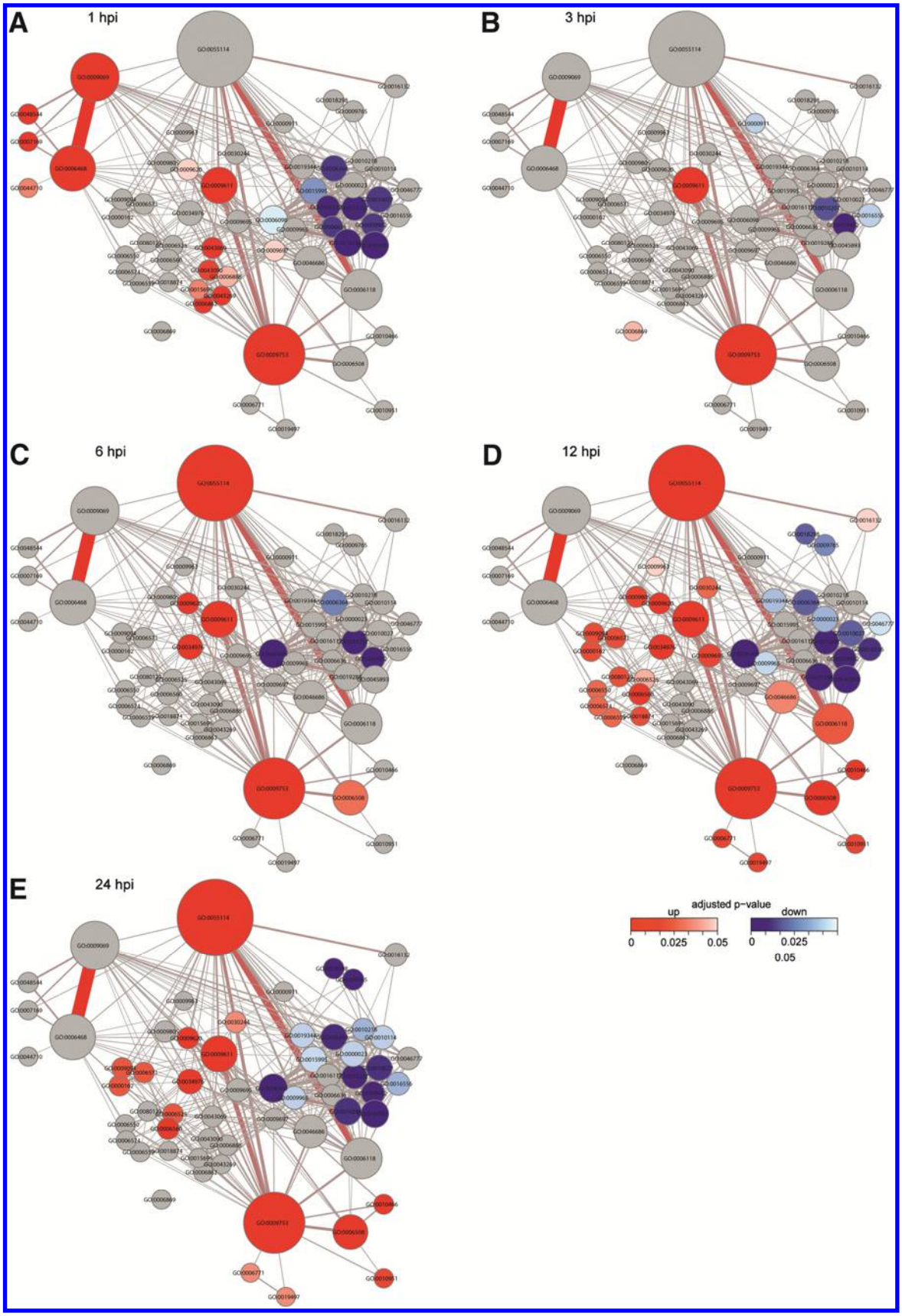
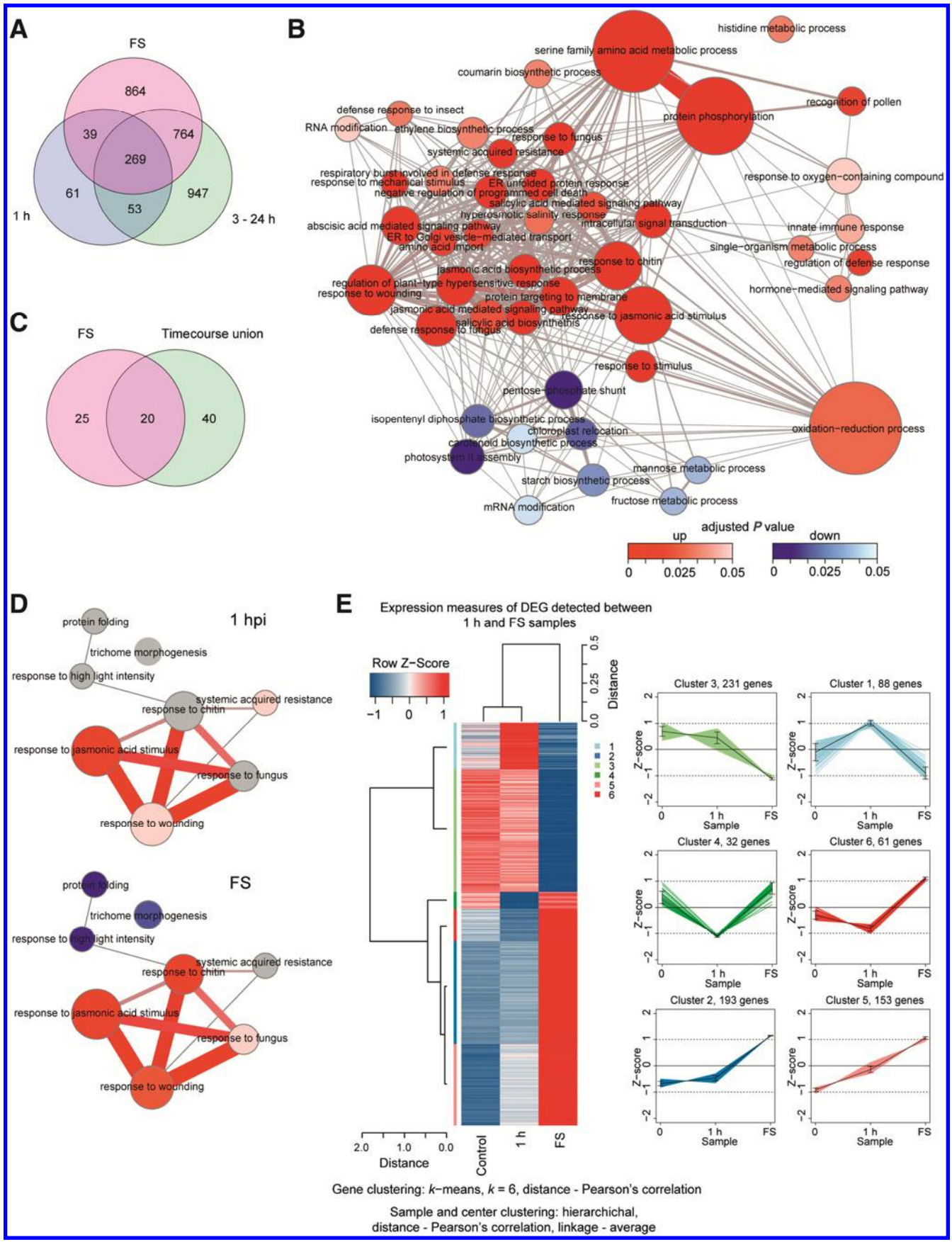


Fig. 2. Gene set enrichment analysis of biological processes for differentially expressed genes (DEG) detected in tomato timecourse samples upon spider mite herbivory. **A** to **E,** Union parametric analysis of gene set enrichment (PAGE) network based on Biological Processes (BP) Gene Ontology (GO) annotation with significantly enriched up- and downregulated gene sets in timecourse samples. Nodes represent gene sets, edges indicate the overlap in genes belonging to connected gene sets. Gene sets: blue = downregulated, red = upregulated, gray = not detected as differentially regulated. Size corresponds to number of genes in a given gene set (five to 186), labels indicate BP GO category identification. The color (gray to red) and width of the edges correspond to an overlap size (1 to 79).


A
FS
864
39
764
269
61
947
53
1 h
3 - 24 h
B
histidine metabolic process
serine family amino acid metabolic process
coumarin biosynthetic process
recognition of pollen
protein phosphorylation
defense response to insect
RNA modification
ethylene biosynthetic process
response to fungus
systemic acquired resistance
respiratory burst involved in defense response
response to mechanical stimulus
ER unfolded protein response
response to oxygen−containing compound
negative regulation of programmed cell death
salicylic acid mediated signaling pathway
hyperosmotic salinity response
abscisic acid mediated signaling pathway
intracellular signal transduction
innate immune response
ER to Golgi vesicle−mediated transport
amino acid import
single−organism metabolic process
response to chitin
regulation of defense response
jasmonic acid biosynthetic process
regulation of plant−type hypersensitive response
hormone−mediated signaling pathway
response to wounding
protein targeting to membrane
jasmonic acid mediated signaling pathway
response to jasmonic acid stimulus
salicylic acid biosynthetic
defense response to fungus
response to stimulus
pentose−phosphate shunt
isopentenyl diphosphate biosynthetic process
oxidation−reduction process
chloroplast relocation
carotenoid biosynthetic process
photosystem II assembly
mannose metabolic process
starch biosynthetic process
fructose metabolic process
mRNA modification
adjusted P value
up
down
0
0.025
0.05
0
0.025
0.05
C
FS
Timecourse union
25
20
40
D
1 hpi
protein folding
trichome morphogenesis
response to high light intensity
systemic acquired resistance
response to chitin
response to jasmonic acid stimulus
response to fungus
response to wounding
FS
protein folding
trichome morphogenesis
response to high light intensity
systemic acquired resistance
response to chitin
response to jasmonic acid stimulus
response to fungus
response to wounding
E
Expression measures of DEG detected between
1 h and FS samples
Row Z−Score
−1
0
1
Distance
0.5
0.25
0.0
2.0
1.0
0.0
Distance
Control
1 h
FS
Cluster 3, 231 genes
Cluster 1, 88 genes
Cluster 4, 32 genes
Cluster 6, 61 genes
Cluster 2, 193 genes
Cluster 5, 153 genes
Z−score
Sample
0
1 h
FS
Gene clustering: k−means, k = 6, distance - Pearson's correlation
Sample and center clustering: hierarchichal,
distance - Pearson's correlation, linkage - average

whose expression is regulated by JA, *def-1* plants were sprayed with exogenous JA. We tested a range of JA concentrations and determined that a 1 mM JA solution is sufficient to reproducibly induce several known JA markers (Supplementary Fig. S4). We also infested *def-1* plants with 100 adult female spider mites to identify genes induced by spider mite feeding independently of an increase in JA levels. For each experiment, tissue was collected 24 h post-treatment. We found 1,324 and 225 genes to be differentially expressed by 1 mM JA and spider mite treatments, respectively, at the absolute FC > 2 and Benjamini-Hochberg (BH) FDR (Benjamini and Hochberg 1995) adjusted $P < 0.05$ relative to mock-sprayed and non-infested plants, respectively (Supplementary Dataset S7). These experimentally derived JA-responsive genes were used to re-annotate the BP GO category GO:0009753 'response to jasmonic acid stimulus'. Based on limma estimated log-odds ratio (B) of being differentially expressed for known JA markers detected in JA-sprayed samples, an additional 129 DEG that demonstrated log-odds ratio (B) > 10 were included in this GO category, increasing the total number of tomato genes within the GO:0009753 'response to jasmonic acid stimulus' to 274.

DEG detected in *def-1* by exogenous JA application were approximately equally split between up- (770) and downregulated (572) 24 h after treatment (Supplementary Fig. S5). Overall, based on GO enrichment analysis, this treatment closely resembled tomato response to herbivore attack, reinforcing the fact that JA signaling was identified as a major regulator of defense responses (Ament et al. 2004; Li et al. 2002a; Li et al. 2004; Schweighofer et al. 2007; P. J. Zhang et al. 2009; Zheng et al. 2007; Zhurov et al. 2014). Biological processes associated with plant defense response were represented by upregulated DEG, and processes associated with anabolism and growth were represented by downregulated DEG (Supplementary Dataset S8). DEG detected in *def-1* after spider mite herbivory were mainly up- (207) rather than down-regulated (17). Based on GO analysis, these DEG represent a subset of genes inducible by JA signaling ('response to jasmonic acid' BP category), suggesting that some of these genes can be induced redundantly with or without an increase in JA concentration. In addition, the GO category 'salicylic acid biosynthetic process', reported to be activated later in the tomato defense response to spider mite herbivory (Kant et al. 2004), was also enriched, suggesting that evolutionary conserved antagonistic cross-talk between JA and SA signaling pathways (Thaler et al. 2012) may not be fully functional in *def-1* plants.

Comparison of the DEG identified in *def-1* with the DEG detected in the 24-hpi timecourse sample allowed us to classify the latter genes in different categories reflecting their dependency on JA. A total of 322 genes were differentially regulated by both spider mite feeding on 'Heinz 1706' and by JA treatment of *def-1*, indicating that JA is sufficient to regulate their expression. Additionally, 39 genes were differentially regulated by spider mite attack in both 'Heinz 1706' and *def-1* but not by JA treatment of *def-1*; thus, their expression is regulated by factors associated with mite herbivory independently of JA regulation. The majority of DEG, 503 genes, were differentially regulated by spider mite feeding on 'Heinz 1706' but not by either JA or spider mite treatments of *def-1* plants. These genes likely require the coordinated action of the JA and some other pathways activated upon spider mite feeding. Finally, 48 genes were differentially regulated by all three treatments. Thus, the expression of approximately 95% of DEG detected at 24 h upon spider mite feeding in 'Heinz 1706' are dependent on JA, indicating that this hormone has a pivotal role in establishing tomato defense responses against mite herbivory.

Overview of tomato defense responses.

Having determined genome-wide transcriptional responses in both tomato and *Arabidopsis* upon feeding by the same London strain of spider mites and within the same response time frame (this work; Zhurov et al. 2014), we can compare the complexity and conservation in spider mite–induced DEG between these plant species. Out of 2,133 tomato genes that are differentially expressed upon mite herbivory, 1,092 have *Arabidopsis* orthologues, and 360 of 841 *Arabidopsis* time-

Fig. 3. Analysis and comparison of FS and timecourse differentially expressed genes (DEG). **A,** Venn diagram of DEG detected in feeding site (FS) samples 1 h postinfestation (hpi) and 3 to 24 hpi. **B,** Gene set enrichment analysis of biological processes (BP) for DEG detected in tomato FS samples upon spider mite herbivory. Nodes represent gene sets, edges indicate overlap in genes belonging to connected gene sets. Gene sets: blue = downregulated, red = upregulated, gray = not detected as differentially regulated. Size corresponds to the number of genes in a given gene set (five to 131). Labels are the BP Gene Ontology (GO) category identification. Color (gray to red) and width of edges correspond to an overlap size (1 to 86). **C,** Venn diagram of BP GO categories detected as differentially regulated by gene set enrichment analysis in FS and timecourse samples. **D,** Gene set enrichment analysis of BP for DEG detected between tomato FS and 1-h samples upon spider mite herbivory. Nodes represent gene sets, edges inidicate overlap in genes belonging to connected gene sets. Color is as in B, size corresponds to number of genes in a given gene set (five to 38). Labels are BP GO category identification. Color (gray to red) and width of edges correspond to an overlap size (1 to 10). **E,** Clustering analysis and heat map of expression measures of DEG detected between tomato FS and 1-h samples upon spider mite herbivory and expression graphs of individual DEG clusters.

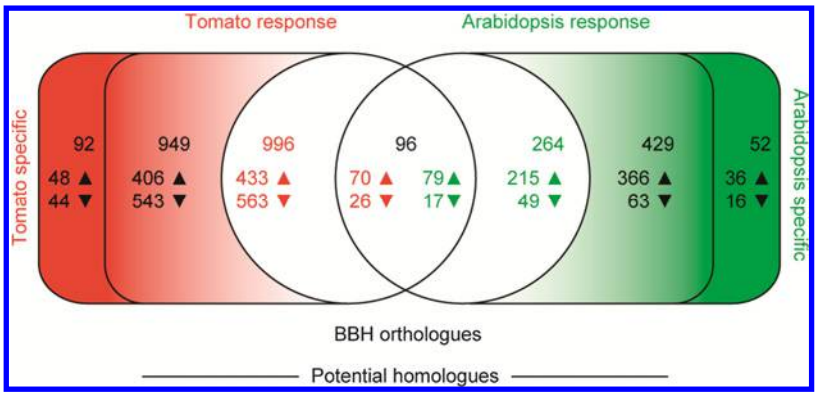


Fig. 4. Analysis of phylogenetic relationships of differentially expressed genes (DEG) detected in tomato and *Arabidopsis* transcriptional responses to spider mite herbivory.

course DEG (Zhurov et al. 2014) have tomato counterparts. Of these DEG, only 96 are induced by spider mite herbivory in both species (Fig. 4). The most prominent class of conserved core set of DEG is associated with JA biosynthesis and signaling (*LOX3*, *LOX6*, *AOS*, *OPR3*, *OPCL1*, *ACX1*, *JMT*, *JAZ1*, *JAZ8*, *MYC2*)

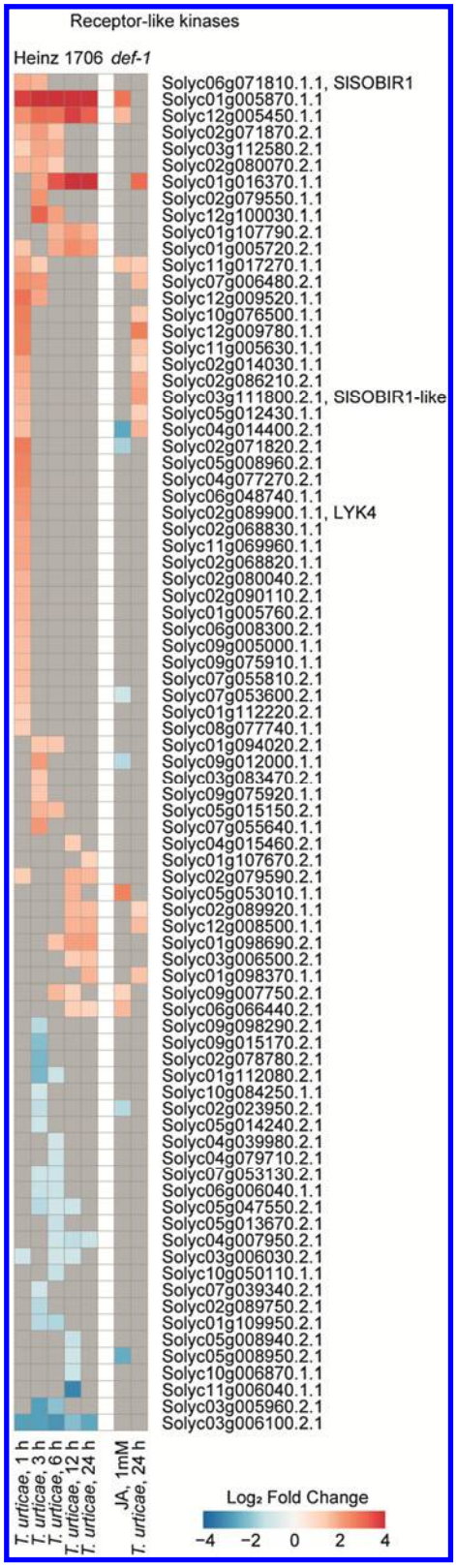


Fig. 5. Heat map of $\log_2$ fold changes of receptor-like kinases detected as differentially expressed in response to mite herbivory or jasmonic acid (JA) treatment in Heinz 1706 and *def-1* tomato plants.

(Supplementary Dataset S9). In addition, several genes involved in the perception (receptor kinases) and Ca^{2+}-signaling, biosynthesis of secondary metabolites (phenylpropanoids, flavonoids and isoprenoids), cell-wall modification, and endoplasmic reticulum (ER) stress are also present. It is striking that the remainder of DEG that represent one-to-one orthologous pairs and are, thus, assumed to have the same function in both species (996 differentially expressed in tomato and 264 in *Arabidopsis*) are induced in only one of the species, indicating profound divergence of species-specific transcriptional responses to spider mite herbivory. In tomato, a hallmark of this group of orthologous genes is a subset of downregulated DEG associated with anabolism processes. These genes potentially reflect a greater shift from growth to defense in tomato relative to *Arabidopsis*, which may be associated with different life history patterns between these species. The GO analysis of upregulated orthologous DEG (433 in tomato and 215 in *Arabidopsis*) demonstrates both conservation at the level of biological programs (such as further recruitment of genes to support the JA signaling cascade) and a divergence of responses (such as biosynthesis of various secondary metabolites). While the majority of DEG possess a degree of similarity (2,041 tomato DEG have *Arabidopsis* homologues and 789 *Arabidopsis* DEG have tomato homologues), there is a subset of DEG unique to respective responses (92 in tomato and 52 in *Arabidopsis* [Fig. 4]). About half of these species-specific DEG are uncharacterized. However, in *Arabidopsis*, four *defensin-like* (*DEFL*) genes are differentially expressed in response to spider mite. *DEFL* gene families are expanded in *Arabidopsis* and are known to be recruited for a multitude of biological functions, including defense (Nguyen et al. 2014; Penninckx et al. 1996; Silva et al. 2014; Silverstein et al. 2005, 2007). On the other hand, in tomato, five PI lacking *Arabidopsis* homologues are differentially expressed. Thus, although JA is a conserved signaling hormone mediating responses to spider mite herbivory, the majority of plant defenses against spider mites are ultimately manifested as species (or at least plant family) -specific.

In order to highlight specific pathways that underlie tomato responses to spider mite herbivory, we combined annotations from Blast2GO analysis, the GOMapMan (Ramsak et al. 2014) and relevant literature to associate DEG with individual defense-related pathways.

Defense responses conserved between tomato and *Arabidopsis*.

Perception of spider mite herbivory. RLK play a critical role in the establishment of defense responses, as they are involved in the initial perception of extracellular elicitors originating from spider mites (herbivory-associated molecular patterns [HAMPs]) or damaged tissue resulting from mite feeding (damage-associated molecular patterns [DAMPs]). The mechanism by which either spider mites, tissue damage, or both are recognized by the plant is currently unknown. Following the paradigm of plant-pathogen interaction in which RLK involved in the perception of pathogen derived elicitors (PAMPs) are transcriptionally induced early upon PAMP recognition (Postel et al. 2010; Yamaguchi and Huffaker 2011), we reasoned that our data might include potential receptors involved in detection of spider mite feeding. A total of 82 RLK (identified based on the GOMapMan annotation) were differentially expressed in a time-course sample upon spider mite attack (Fig. 5; Supplementary Dataset S10), a number of which were induced within the first 3 h of tomato response in a pattern expected from candidate receptors of spider mite feeding (including both HAMP and DAMP elicitors).

We further hypothesized that if plants perceive conserved elicitors associated with spider mite feeding, they will be rec-

ognized by RLK that are conserved across plant species and will be induced by spider mite herbivory in both tomato and *Arabidopsis*. We identified eight such RLK, six of which encode leucine-rich repeat (LRR)-RLK whose function has not been tested in either tomato or *Arabidopsis*. However, two of them encode characterized receptors *SUPPRESSOR OF BIR1-1 (SOBIR1*) and *LYSM-CONTAINING RECEPTOR-LIKE KINASE 4 (LYK4*). Tomato expresses two homologues of *AtSOBIR1*, named *SlSOBIR1* (Solyc06g071810, whose transcripts are elevated during the first 3 h of mite feeding) and *SlSOBIR1*-like (Solyc03g111800, transiently induced by spider mite feeding during the first hour) (Fig. 5). *SOBIR1* genes encode a LRR-RLK, proposed to act as a co-receptor in complexes containing LRR-receptor-like proteins (RLP) (Liebrand et al. 2013, 2014), suggesting that LRR-RLP may play an important role in the recognition of spider mite feeding. *LYK4*, on the other hand, encodes an RLK with a peptidoglycan-binding LysM extracellular domain, shown to be involved in chitin-triggered signaling (Wan et al. 2012). Intriguingly, the arthropod exoskeleton is composed of chitin, raising the possibility that carbohydrate patterns may be recognized as spider mite conserved elicitor.

Jasmonic acid. A prominent role of JA in regulating defenses against spider mites has been described for several plants (Ament et al. 2004; Li et al. 2002a; Li et al. 2004; Schweighofer et al. 2007; P. J. Zhang et al. 2009; Zheng et al. 2007; Zhurov et al. 2014), indicating that regulatory mechanisms leading to mite-induced defense programs are broadly conserved across plant species. Consistently, we found that genes encoding JA biosynthetic enzymes were induced by mite feeding in our dataset (Fig. 6). These biosynthetic enzymes are encoded by gene families and, in general, only some genes within these families were induced by mite herbivory. For example, out of 22 tomato *LOX* genes present in the GOMapMan annotation (Ramsak et al. 2014), only three were induced. The expression of *LOXD* was up-regulated throughout the timecourse, consistent with its previous characterization as an early herbivory responsive gene (Heitz et al. 1997; Yan et al. 2013). Interestingly, *LOXD* expression also increased when *def-1* plants were challenged with spider mites, suggesting that the initial increase in expression of some of the JA biosynthetic genes could be triggered in a JA-independent way. In contrast to *LOXD*, the expression of *LOXA* increased at later timepoints in both 'Heinz 1706' and *def-1* plants treated with JA, suggesting that its expression is controlled by a JA-regulated positive feedback loop consistent with a previous report (Beaudoin and Rothstein 1997)). Three *AOS* genes showed upregulation in our dataset. *AOS1* expression is rapidly and continuously upregulated from 1 to 24 h. The *def-1* microarray dataset indicates that expression of this gene is both JA sufficient and JA independent, as previously reported (Howe et al. 2000). *AOS2* is also transiently upregulated at 12 h, likely as a result of a JA positive feedback loop (our data; Howe et al. 2000). Unsurprisingly, the root-specific *AOS3* gene (Itoh et al. 2002) is not detected. *Allene oxide cyclase* expression shows significant upregulation starting at 6 h after spider mite attack. Three *OPDA (12-oxophytodienoate) reductase* genes were identified in tomato (*OPR1*, *OPR2* and *OPR3*); however, only *OPR3*, shown to participate in JA biosynthesis (Strassner et al. 2002), had increased expression at 12 hpi in our dataset.

Like JA biosynthesis, perception and JA signaling are dependent on conserved proteins that are part of the ubiquitin-proteasome system *(COI1*) and transcriptional regulators (*JAZ*) (Chini et al. 2007; Feys et al. 1994; Li et al. 2004; Sheard et al. 2010; Thines et al. 2007; Xie et al. 1998). Twelve putative *JAZ* genes have been identified in tomato (Ishiga et al. 2013), seven of which were up-regulated upon mite feeding in a JA-dependent way (e.g., being induced both by mite feeding on 'Heinz 1706' and upon JA treatment of *def-1* plants). In *Arabidopsis*, the COI1-JAZ pathway regulates the expression of *AtMYC2*, a bHLH transcription factor (Kazan and Manners 2013). Two *AtMYC2* homologues, *JAMYC2* and *JAMYC10* (Boter et al. 2004), were also induced by spider mite feeding (Fig. 6).

Expression levels of several genes have been used as markers of JA responses in tomato. These include the JA biosynthetic enzymes *LOXD* and *AOS1*, *PI-I* and *PI-II*, *LAP*, *TD*, and *PPO*. As expected, all of these marker genes were induced by spider mite feeding (in 'Heinz 1706') and JA (in *def-1* mutant plants). They belong to clusters 1 (*PI*, *LAP*, *TD*, *PPO*) and 6 (*LOXD*, *AOS1*) of expression patterns shown in Figure 1, which contain 465 and 263 genes with similar expression patterns, respectively. This extensive list of coexpressed genes that are stably up-regulated after 12 hpi supports the establishment of the tomato defense and will be an invaluable resource for future investigation of tomato-pest interaction.

Ethylene. ET is synthesized from *S*-adenosine methionine through the sequential action of enzymes 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO). ACS enzymes are often regulated at post-transcriptional levels (Chae et al. 2003; Oetiker et al. 1997); thus, it was not unexpected to find that only a single putative *ACS* gene (Solyc08g079750) showed altered expression out of the eight *ACS* genes annotated in the tomato genome (Lincoln et al. 1993; Nakatsuka et al. 1998; Olson et al. 1995; Rottmann et al. 1991; Shiu et al. 1998; Yip et al. 1992). Of the annotated 14 *ACO* genes, 11 were differentially regulated, including characterized *ACO1*, *ACO2*, and *ACO5* (Blume and Grierson 1997; Nakatsuka et al. 1998; Sell and Hehl 2005) that were up-regulated at various timepoints and duration during the initial 24 h of tomato response to spider mite feeding.

The signaling cascade downstream of ET synthesis involves perception by ER-localized receptors (Nr, ETR1-6), signaling by CTR1, and EIN2 leading to activation of the plant-specific transcription factor EIN3 (and other EIN3-like [EIL] transcription factors). EIN3 and other EIL transcription factors directly regulate a second tier of transcriptional regulators termed ET response factors (ERF) that regulate the expression of ET-responsive genes (Merchante et al. 2013). Several *ERF* genes have been characterized in tomato, including *Pti4*, *Pti5*, *Pti6* (Gu et al. 2002; Zhou et al. 1997), *Sl-ERF2* (Pirrello et al. 2006; Z. Zhang et al. 2009), *TERF1* (Huang et al. 2004), *TSRF1* (Zhang et al. 2008), *JERF1* (Zhang et al. 2004), *JERF3* (Wang et al. 2004), and *ERF1-4* (Tournier et al. 2003). The expression of most of the early ET signaling components (*CTR1*, *EIN2*, *EIN3*) did not change in our experiment. Of the 20 annotated tomato *AP2/ERF* genes, a subset was differentially regulated. However, as downstream ET components showed both up- and downregulation (Fig. 6B), it is difficult to assess the importance ET may have in the regulation of spider mite defense in tomato. Further experiments using ET mutants and ET treatments should help understand the importance of this hormone in tomato defense against spider mites.

Salicylic acid. SA is a master regulator of plant responses against biotrophic pathogens. Reciprocal antagonism between SA and JA has been described in at least 17 different species (Thaler et al. 2012) and is exploited by both pathogens and herbivores to manipulate plant defense responses (Bhavsar et al. 2007; Diezel et al. 2009; El Oirdi et al. 2011; Howe and Jander 2008; Musser et al. 2002). In tomato, exogenous application of SA has been shown to reduce JA biosynthesis and to inhibit defense responses against caterpillar herbivory (Chandok et al. 2004; Thaler et al. 2002, 2010). However, Kant and associates (2004) reported increased expression of

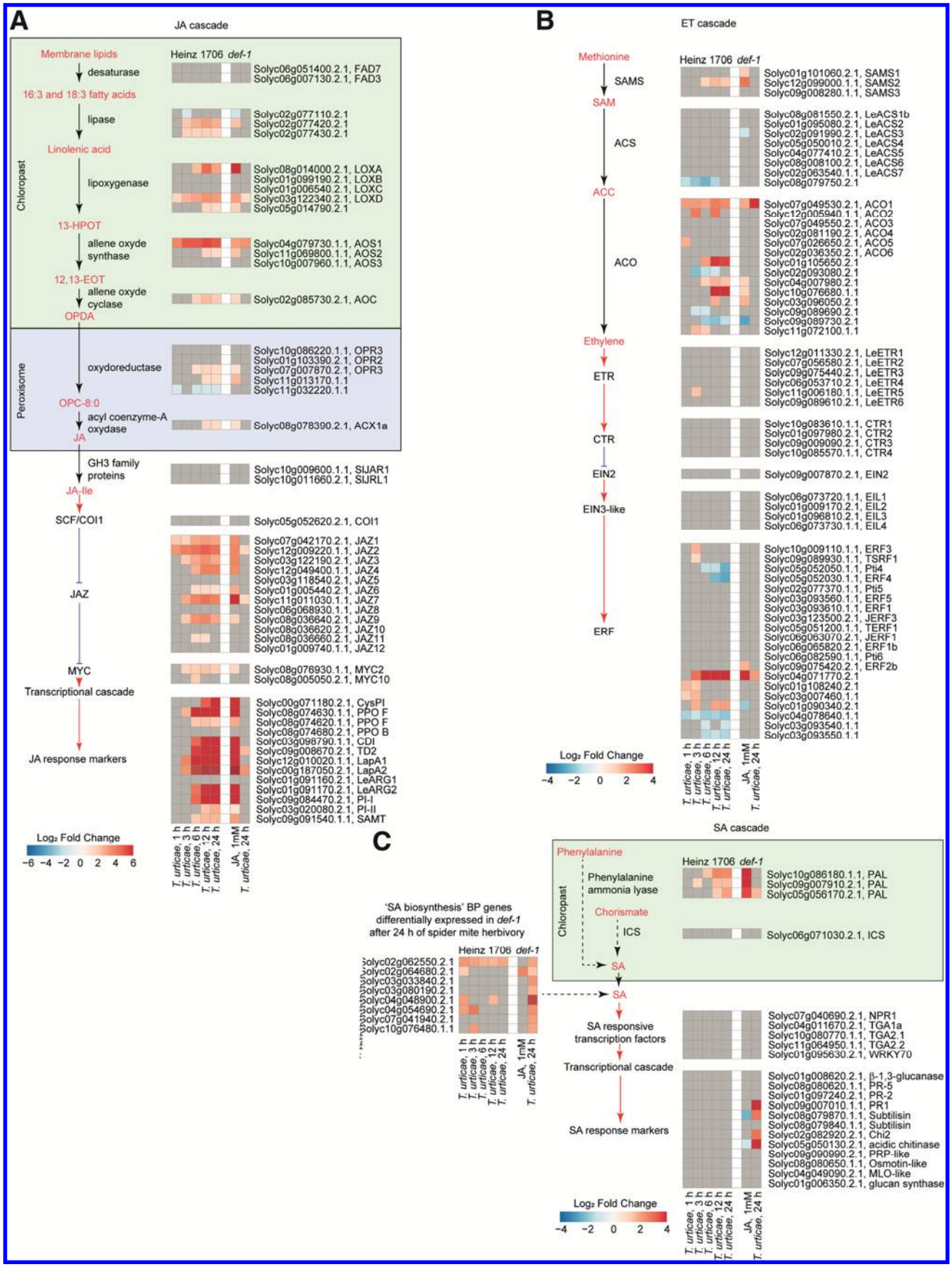


Fig. 6. Heat map of $\log_2$ fold changes of genes involved in plant hormone biosynthetic and signaling cascades and their downstream targets or markers in response to mite herbivory or jasmonic acid (JA) treatment in Heinz 1706 and *def-1* tomato plants. **A,** JA biosynthesis, signaling, and response. **B,** Ethylene (ET) biosynthesis, signaling, and response. **C,** Salicylic acid (SA) biosynthesis, signaling, and response. In schemes of cascades, compounds are shown in dark red, proteins and enzymes in black, black arrows represent direct (solid) or indirect (dashed) biochemical transformations, red arrows indicate activation, and blue arrows indicate inactivation.

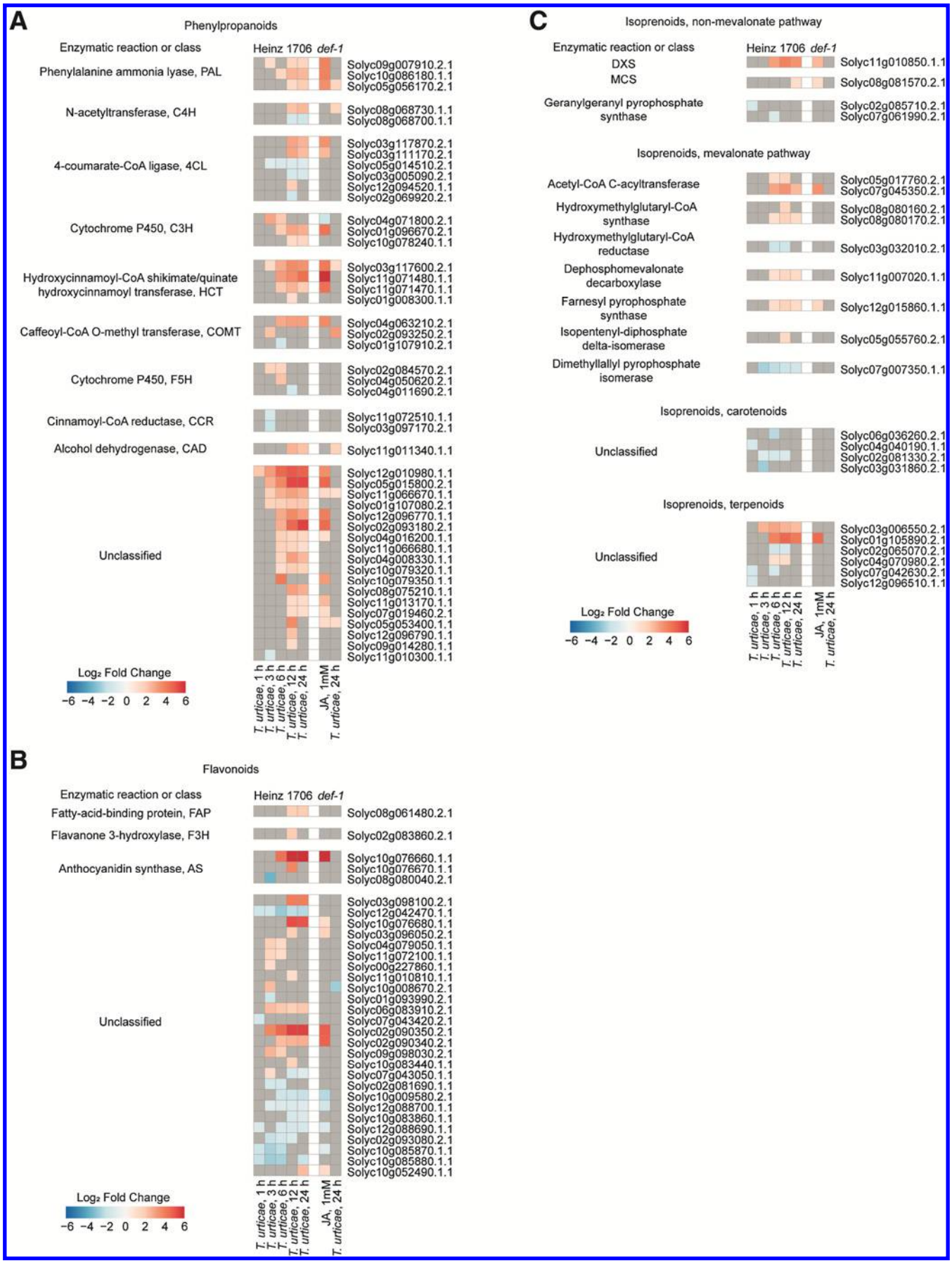


Fig. 7. Heat map of $\log_2$ fold changes of genes involved in biosynthesis of secondary metabolites such as **A,** phenylpropanoids, **B,** flavonoids, and **C,** isoprenoids detected as differentially expressed in response to mite herbivory or jasmonic acid (JA) treatment in Heinz 1706 and *def-1* tomato plants.

both JA and SA marker genes at 1 and 4 days following spider mite herbivory, suggesting that mites can trigger both SA and JA pathways simultaneously. However, of SA biosynthetic genes, only three *phenylalanine ammonia-lyase (PAL)* genes were induced by mite feeding (Fig. 6C). These genes encode enzymes that are not specific for SA biosynthesis, as they also support the biosynthesis of phenylpropanoids, metabolites expected to accumulate upon mite herbivory (Fig. 7A). Thus, induction of *PAL* gene expression may not be sufficient to predict accumulation of SA. In addition, none of the genes encoding SA signaling proteins nor commonly used SA markers (*PR1*, *PR2*, *PR5*, *NPR1*, *subtilisin*, *chitinases*) (Kant et al. 2004; Nachappa et al. 2013; Uehara et al. 2010) were differentially expressed when spider mites were feeding on 'Heinz 1706' plants, suggesting that mite feeding did not induce accumulation of SA within the first 24 hpi.

However, several genes associated with the GO category 'salicylic acid biosynthetic process', and a few SA markers (*PR1* [Solyc09g007010], *subtilisin-like protease* [Solyc08g079870], and two *chitinases* [Solyc05g050130, Solyc02g082920]) were induced upon mite feeding on *def-1* plants (Fig. 6C). Furthermore, some of the SA marker genes were down-regulated in *def-1* plants treated with JA, suggesting that JA-induced pathways in wild-type plants can suppress spider mite–dependent SA responses. Observed differences in tomato responses to spider mite feeding described in our study and those performed by Kant and associates (2004) could be due to different timing of the responses, origin of the spider mite strains, differences between tomato cultivars and experimental set-ups, individually or in combination, used in assays. While Kant and associates (2004) used a tomato-adapted strain of spider mites, our experiments were performed with tomato non-adapted mites (London strain).

Phenylpropanoids and flavonoids. Our microarray dataset predicts an increased production of phenylpropanoids and flavonoids following spider mite infestation, as genes encoding several key enzymes involved in phenylpropanoid biosynthesis, such as *PAL*, *cinnamate-4-hydroxylase*, and *4-coumarate-CoA* ligase, were up-regulated following mite attack (Fig. 7). A total of 76 DEG were predicted to encode enzymes involved in the biosynthesis of phenylpropanoids and flavonoids based on the GOMapMan annotation (Ramsak et al. 2014). These biosynthetic pathways are well conserved between different plant species. In *Arabidopsis*, 44 genes are associated with the phenylpropanoid pathway, each one of which has a predicted tomato homologue, including 24 one-to-one orthologues at all enzymatic steps.

Compounds involved in indirect defenses. The homoterpene TMTT and MeSA constitute the most abundant volatiles produced by tomato in response to spider mite herbivory (Ament et al. 2004, 2006). Isoprenoids are synthesized by two pathways. One is the mevalonate pathway, which operates in the cytosol of higher plants, and the other is the nonmevalonate pathway, which is localized in chloroplasts (Kuzuyama 2002). Genes encoding enzymes acting in both of these pathways have been induced in tomato upon spider mite feeding (Fig. 7). Even though *geranylgeranyl pyrophosphate synthase 1* has been suggested as the key regulator of TMTT accumulation (Ament et al. 2006), its expression levels did not change in response to spider mite attack. However, genes encoding other enzymes involved in terpenoid biosynthesis, such as *mevalonate diphosphate decarboxylase* (Solyc11g007020), *DOXP-synthase* (Solyc11g010850), and *geranyllinalool synthase* (Solyc03g006550) were up-regulated by spider mite attack, consistent with reported induced production of terpenoid-based volatiles (Ament et al. 2004, 2006). Similarly, upregulation of *salicylic acid carboxyl methyltransferase* genes following mite attack is also consistent with a predicted increase in MeSA production (Ament et al. 2004).

Other tomato defense responses.

JA-induced defense proteins that target herbivore digestive physiology. Commonly used markers of tomato induced defenses are *LAPA1* and *LAPA2*, *LeARG1* and *LeARG2*, *TD2*, and *PPO*, which encode proteins that act in the pest gut to reduce amino acid availability from ingested plant tissues. *Arabidopsis* does not have genes encoding PPO and TD2 (Chen et al. 2007; Tran et al. 2012) nor JA-inducible *LAPA* genes that have been recruited for defense in tomato (Bartling and Nosek 1994). These enzymes impact herbivores' digestive physiology within an alkaline pH range that is characteristic of lepidopteran midgut (Chen et al. 2004, 2007; Chung and Felton 2011; Fowler et al. 2009; Gonzales-Vigil et al. 2011; Gu et al. 1999) and have been shown to be ineffective against pests with acidic guts, such as the Colorado potato beetle (Felton et al. 1992; Gonzales-Vigil et al. 2011). Spider mites are expected to have acidic gut content (Carrillo et al. 2011; Erban and Hubert 2010), and thus, even though *LAPA1* and *LAPA2*, *LeARG1* and *LeARG2*, *TD2*, and *PPO* were used as useful markers of mite-induced tomato defenses, these defense compounds may have little or no effect on spider mite herbivory.

The PI gene family in tomato. PI act as antidigestive and defensive compounds by interacting with their target proteases in the arthropod gut (Benchabane et al. 2010; Bode et al. 2013; Carrillo et al. 2011; Ortego 2012; Santamaria et al. 2012; Schluter et al. 2010). In tomato, two serine *PI* (*PI-I* and *PI-II*) were shown to be consistently induced by spider mite attack (Kant et al. 2004, 2008; Li et al. 2002b). We identified a total of 95 *PI* genes in the tomato genome that can be classified into eight families based on their inhibition specificity to serine-, cysteine-, aspartyl-, and metalloproteases (Supplementary Dataset S11). This is in contrast to 38 PI that are annotated in *Arabidopsis*, demonstrating a great expansion of this class of proteins in the tomato genome. Of 95 tomato *PI* genes, 25 were differentially expressed upon spider mite feeding compared with only one in *Arabidopsis* (Fig. 8A; Supplementary Table S1). Tomato *PI* genes are among the most highly induced DEG in our dataset, suggesting that they represent one of the major tomato defense response outputs upon spider mite herbivory. These *PI* were also induced by JA in *def-1* plants, demonstrating that JA is sufficient to coordinately regulate their expression. Phylogenetic analysis of tomato and *Arabidopsis* PI showed that defense response to spider mite attack is limited to genes belonging to tomato-specific expansions in I3 and I13 families (Fig. 8B and C). All but one of the induced *PI* lack an *Arabidopsis* BBH orthologue, indicating that they define tomato-specific members within expanded families that have acquired novel transcriptional regulation by JA and have been recruited for defense.

Conclusions.

Our study, focused on early genome-wide transcriptional responses of tomato (cv. Heinz 1706) to herbivory by the two-spotted spider mite *Tetranychus urticae* (London strain), identified 2,133 DEG that defined gradual establishment of tomato responses to spider mite feeding within the first 24 h of interaction. In addition, the role of JA in the establishment of tomato defense responses against spider mites was tested by treating a tomato mutant defective in JA synthesis (*def-1*) with JA or by mites. The study highlighted the importance of JA as a regulator of mite-induced defenses, since differential expression of approximately 95% of DEG at 24 h required JA, reinforcing the conserved role of JA in regulating plant defenses against a broad spectrum of plant-associated organisms (Ament

et al. 2004; Campos et al. 2014; De Geyter et al. 2012; Li et al. 2002b; Li et al. 2004; Schweighofer et al. 2007; P. J. Zhang et al. 2009; Zheng et al. 2007; Zhurov et al. 2014). The prominent role of JA in regulation of plant defenses triggered by a myriad of herbivores results in majorly overlapping responses within a plant species, indicating that induced defenses may lack herbivore specificity. In particular, tomato defenses targeting herbivore digestive physiology are expected to vary in their effectiveness due to the heterogeneity of herbivore gut environments.

The previous study of *Arabidopsis* responses to spider mite attack in a similar timecourse experiment allowed us to compare tomato and *Arabidopsis* transcriptional changes upon spider mite feeding. Indole glucosinolates, secondary metabolites characteristic for cruciferous plants, were identified as major defense compounds in *Arabidopsis* against mites (Zhurov et al.

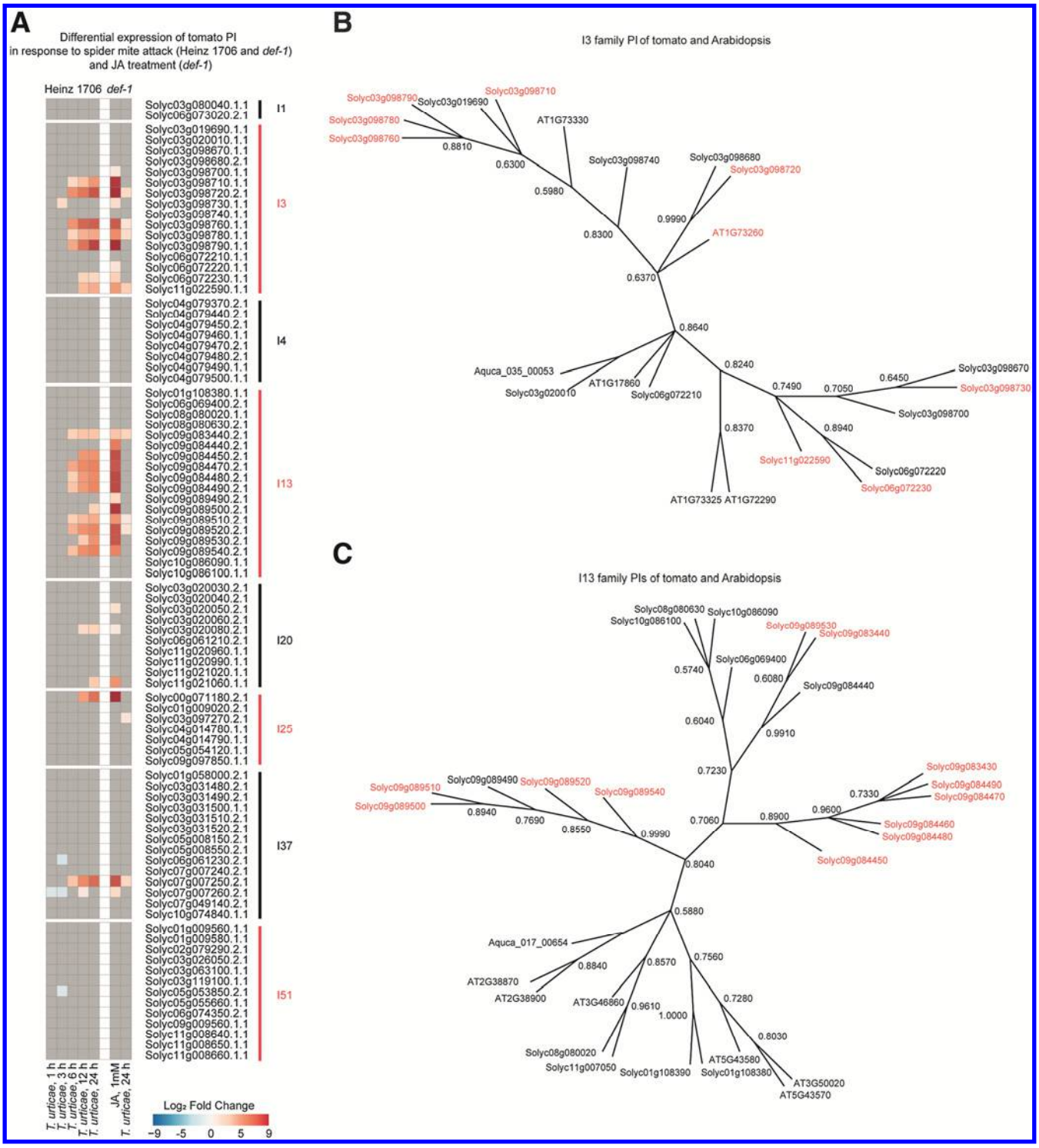


Fig. 8. Lineage-specific expansion of I3 and I13 peptidase inhibitor families in tomato is associated with antiherbivory function. **A,** Heat map of $\log_2$ fold changes of proteinase inhibitors (PI) genes in response to mite herbivory or jasmonic acid (JA) treatment in Heinz 1706 and *def-1* tomato plants. **B** and **C,** Unscaled cladograms of peptide sequences of I3 and I13 PI from tomato and *Arabidopsis*. PI highlighted in red are up-regulated in response to spider mite attack. Node labels are Shimodaira Hasegawa–like approximate likelihood ratio test (SH-aLRT) values. PI sequences from *Aquilegia coerulia* were used as outgroups.

2014), while the syntheses of defense proteins that target herbivore digestive physiology appear as a prominent part of tomato-induced defenses upon mite herbivory. Even though the concept of diversification of plant defenses against herbivores has been postulated (Agrawal 2007; Berenbaum and Zangerl 2008; Mithofer and Boland 2012), our study identified the striking magnitude of differential transcriptional responses of *Arabidopsis* and tomato to the same herbivore. Thus, despite the conservation of the JA core signaling pathway, this analysis points to the profound evolutionary divergence of JA-regulated downstream defense responses between tomato and *Arabidopsis* contributing to future analysis of the evolution of plant chemical diversity. Gene duplication is considered to be one of the major sources of plant chemical diversification (Kroymann 2011; Ober 2010). Several tomato defense genes against herbivory (e.g., *TD2*, *PPO*, *PI*) arose from gene duplication, followed by the acquisition of transcriptional regulation by JA. Systematic analysis of genes recruited for defense within the species-specific family expansions would determine how widespread this pattern might be. Identification of coexpressed gene clusters, integration of tomato metabolomic and transcriptomic responses, and functional analysis of individual DEG will, in the future, lead to the identification of tomato-specific defense compounds used to deter spider mite herbivory.

MATERIALS AND METHODS

Plant and mite rearing.

Tetranychus urticae (London strain) was mass reared on potted bean plants (*Phaseolus vulgaris* 'California Red Kidney Bean', Stokes) in a climate room with diurnal and night temperatures fluctuating between 26 and 20°C, 60% ± 20% relation humidity, and with a photoperiod of 16 h of light and 8 h of dark. To obtain cohorts of adult females of similar age, 100 female mites were placed on separated leaves two weeks before the experiment, allowing them to lay eggs for 24 h, after which they were removed. The offspring of the synchronized population was used for the infestation experiment. Potted tomato plants (cv. Heinz 1706 and *def-1* [cv. Castlemart]) were grown under growth-chamber conditions with a 25°C-light and 22°C-dark cycle, 50 to 70% relative humidity, and a photoperiod of 16 h of light and 8 h of dark.

Tomato response to spider mite attack microarray experiment.

In the spider mite feeding timecourse scenario, 100 *T. urticae* adult females were applied on a terminal leaflet of leaf 3 of 21- to 24-day-old tomato plants and were allowed to feed for 1, 3, 6, 12, or 24 h in an experimental design described previously (Zhurov et al. 2014). In the FS scenario, the terminal leaflets were covered with hundreds of mites that were allowed to feed for 1 h. Leaves whose terminal leaflet was inoculated by mites were harvested and used for RNA extraction. Three biological replicates representing two plants each were generated per treatment. Spider mites remained localized within the inoculated leaves without a need to restrain their movement. Experimental and control plants were kept in the same growth room. Total RNA was prepared using the RNeasy plant RNA extraction kit (Qiagen, Venlo, The Netherlands). RNA was hybridized to the EUTOM3 whole-genome exon microarray according to manufacturer's specifications (Affymetrix, Santa Clara, CA, U.S.A.). Analysis was performed using the Bioconductor framework (Gentleman et al. 2004). An initial data-quality assessment was conducted using arrayQualityMetrics (Kauffmann et al. 2009). Expression measures were computed using Robust Multi-array Average (RMA) on the complete data set (Irizarry et al. 2003). Detection of DEG was performed using limma with adjusted (BY) *P* values (Benjamini and Yekutieli 2001; Smyth 2004). Clustering of mean expression measures of DEG was performed using *k*-means clustering with $k = 8$, followed by ordering of gene clusters by hierarchical clustering with average linkage of *k*-means cluster centers. R session random seed was 25845159. Sample clustering was performed by hierarchical clustering with average linkage. Centered Pearson's correlation was used as a distance metric in all cases.

def-1 response to spider mite attack and JA treatment microarray experiment.

For the JA treatment, 24- to 28-day-old plants were sprayed with either a control (mock) 0.5% (vol/vol) ethanol and water or a 1 mM JA (Sigma-Aldrich, St. Louis) solution in 0.5% (vol/vol) ethanol and water. This concentration was chosen because it induced the most robust accumulation of the JA marker gene *TOMWIPII* (Sarmento et al. 2011) in 'Heinz 1706' plants treated with varying concentrations of JA. For mite infestation, 100 *Tetranychus urticae* (London strain) adult females were applied to the terminal leaflet of leaves 3 and 4 of 28-day-old plants. Three biological replicates representing two plants each were generated per treatment. Total RNA was prepared using the RNeasy plant RNA extraction kit (Qiagen). Analysis was performed using the Bioconductor framework (Gentleman et al. 2004). An initial data quality assessment was conducted using arrayQualityMetrics (Kauffmann et al. 2009). Expression measures were computed using RMA on the complete data set (Irizarry et al. 2003). Detection of DEG was performed using limma with adjusted (BH) *P* values (Benjamini and Hochberg 1995; Smyth 2004).

GO re-annotation of tomato genome.

GO re-annotation of tomato proteins using the Blast2GO workflow (Conesa et al. 2005) was performed as follows. We performed blastp (Altschul et al. 1997) searches of the ITAG v.2.3 release of tomato protein sequences against a local copy of the National Center for Biotechnology Information nonredundant database (release 2013-10-20). InterProScan v.5.1-44.0 (Hunter et al. 2012; Jones et al. 2014) was performed locally against PANTHER data v.8.1 (Mi et al. 2005). Results were integrated into the GO annotation using Blast2GO v.2.7.0 and local copy of the 2013-10 releases of Blast2GO and GO associations databases, using default stringency and Annex annotation augmentation (Myhre et al. 2006).

GO annotation of gene lists.

We have used topGO with the Fisher's test statistic and "weight01" algorithm (Alexa et al. 2006) to generate a list of the top 50 Biological Process GO annotations and annotation lists of genes that were detected as differentially expressed. The lists were further filtered by applying a cut-off of 0.05 to Fisher's weighted *P* values.

Gene set enrichment analysis.

Gene set enrichment analysis was performed using a custom version of Bioconductor package piano (Varemo et al. 2013). Log_2 fold changes, *P* and *t* values obtained using limma were used as input gene level statistics for the analysis. Following comparison of implemented gene set analysis methods, a PAGE algorithm (Kim and Volsky 2005) was utilized. GO annotation was used to classify genes into sets with biological process and molecular function and cellular component ontologies treated separately. We limited analysis to gene sets that had at least five genes associated with them and used an adjusted (BH) *P* value cut-off of 0.05 to determine significance of distinct up- or downregulation of a gene set.

Establishment of bidirectional best hit orthologues between tomato and *Arabidopsis*.

To determine one-to-one orthologues using the BBH approach (Overbeek et al. 1999), reciprocal blastp (Altschul et al. 1997) searches were conducted using the ITAG v.2.3 release of tomato and the TAIR10 release of *Arabidopsis* protein sequences. Output files were further processed to retain BBH pairs with $E < 10^{-4}$.

Genome-wide identification of tomato *PI* and phylogenetic analysis.

Initially, the MEROPS database (Rawlings et al. 2014) of proteinases and their inhibitors was used to establish the PI families present in plants by looking for the distribution of each family in the different groups and then, blastp (Altschul et al. 1997) searches for PI were performed in the publicly available tomato and *Arabidopsis* genome databases. Blast searches were made in a recurrent way. First, a complete amino acid plant sequence from data banks corresponding to a protein of the family was used. Then, the obtained tomato or *Arabidopsis* protein sequences were used to search for PI in the tomato or *Arabidopsis* genome, respectively.

The obtained I3 and I13 PI family amino acid sequences were aligned using MUSCLE v.3.8.31 with the default parameters (Edgar 2004). Alignments were further processed using the Gblocks v.0.91b server, allowing for smaller blocks and less strict flanking positions (Castresana 2000). The final block alignments contained 53 amino acid positions for I13 and 42 amino acid positions for I3 PI families. Phylogenetic tree reconstruction was performed using PhyML (v. 20120412) (Guindon et al. 2010) with the LG amino acid substitution model (Le and Gascuel 2008). The approximate likelihood-ratio test, based on a Shimodaira-Hasegawa-like procedure, was used as a statistical test for nonparametric branch support (Anisimova and Gascuel 2006). The resulting trees were visualized using Dendroscope (Huson et al. 2007), with *Aquilegia caerulea* used as an outgroup.

Real-time RT-qPCR and data analysis.

Total RNA was extracted using RNeasy plant mini kit, including DNase treatment (Qiagen). Total RNA (2 µg) was reverse transcribed using the Maxima first strand cDNA synthesis kit for RT-qPCR (Thermo Fisher Scientific, Waltham, MA, U.S.A.). qPCR reactions were performed in triplicate for each biological replicate, using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific). The RT-qPCR was performed on an Agilent Mx3005P qPCR instrument (Agilent Technologies, Santa Clara, CA, U.S.A.). Primer sequences and amplification efficiencies (E) are listed in Supplementary Table 2. *ACTIN* (Solyc03g078400.2.1) was used as a reference gene. Threshold cycle (C_t) values of technical replicates were averaged to generate the C_t of a biological replicate. For plotting, the expression value for each target gene (T) was normalized to the reference gene (R), and the normalized relative quantity (NRQ) was calculated as follows. $NRQ = (1 + E_R)^{CtR}/(1 + E_T)^{CtT}$. For statistical analysis, NRQ values were $\log_2$-transformed and analysis of variance (ANOVA) was used to assess the significance of the main effect (JA concentration) (Rieu and Powers 2009). ANOVA was followed by Tukey's honestly significant difference test.

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