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Molecular, Biochemical, and Organismal Analyses of Tomato Plants Simultaneously Attacked by Herbivores from Two Feeding Guilds

Cesar R. Rodriguez-Saona · Richard O. Musser · Heiko Vogel · Sue M. Hum-Musser · Jennifer S. Thaler

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Abstract Previous work identified aphids and caterpillars as having distinct effects on plant responses to herbivory. We sought to decipher these interactions across different levels of biological organization, i.e., molecular, biochemical, and organismal, with tomato plants either damaged by one 3rd-instar beet armyworm caterpillar (*Spodoptera exigua*), damaged by 40 adult potato aphids (*Macrosiphum euphorbiae*), simultaneous damaged by both herbivores, or left undamaged (controls). After placing insects on plants, plants were transferred to a growth chamber for 5 d to induce a systemic response. Subsequently, individual leaflets from non-damaged parts of plants were excised and used for gene expression analysis (microarrays and quantitative real-time PCR), C/N analysis, total protein analysis, proteinase inhibitor (PI) analysis, and for performance assays. At the molecular level, caterpillars up-regulated 56 and down-regulated 29 genes systemically, while aphids up-regulated 93 and down-regulated 146 genes, compared to controls. Although aphids induced more genes than caterpillars, the magnitude of caterpillar-induced gene accumulation, particularly for those associated with plant defenses, was often greater. In dual-damaged plants, aphids suppressed 27% of the genes regulated by caterpillars, while caterpillars suppressed 66% of the genes regulated by aphids. At the biochemical level, caterpillars induced three-fold higher PI activity compared to controls, while aphids had no effects on PIs either alone or when paired with caterpillars. Aphid feeding alone reduced the foliar C/N ratio, but not when caterpillars also fed on the plants. Aphid and caterpillar feeding alone had no effect on the amount of protein in systemic leaves; however, both herbivores feeding on the plant reduced the amount of protein compared to aphid-damaged plants. At the organismal level, *S. exigua* neonate performance was negatively affected by prior caterpillar feeding, regardless of whether aphids were present or absent. This study highlights areas of concordance and disjunction between molecular, biochemical, and organismal measures of induced plant resistance when plants are attacked by multiple herbivores. In general, our data produced consistent results when considering each herbivore separately but not when considering them together.

Key Words Inducible responses · Herbivore performance · C/N · Proteinase inhibitors · Microarrays · qRT-PCR · Decoy hypothesis · Jasmonic acid · Salicylic acid · Pathogen defense

Cesar R. Rodriguez-Saona and Richard O. Musser contributed equally to this work

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C. R. Rodriguez-Saona (✉)
Department of Entomology,
Philip E. Marucci Center for Blueberry and Cranberry Research & Extension, Rutgers University,
Chatsworth, NJ 08019, USA
e-mail: crodriguez@aesop.rutgers.edu

R. O. Musser · S. M. Hum-Musser
Department of Biological Sciences, Western Illinois University,
Macomb, IL 61455, USA

H. Vogel
Department of Entomology,
Max Planck Institute for Chemical Ecology,
Beutenberg Campus,
Jena 07745, Germany

J. S. Thaler
Departments of Entomology and Ecology & Evolutionary Biology, Cornell University,
Ithaca, NY 14853, USA

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Introduction

Plants frequently are attacked by more than one herbivore species at the same time (e.g., Strauss, 1991; Vos et al., 2001; Hufbauer and Root, 2002). Specific plant responses can be related to feeding by insects from different feeding guilds (Walling, 2000), and can manifest themselves at different levels of biological integration, including transcriptional, biochemical, and organismal levels. Feeding by chewing caterpillars causes severe damage to plant tissues, most often accompanied by severe tissue loss. In addition, elicitors of plant defenses, including various fatty acid-amino acid conjugates, have been isolated from caterpillar regurgitant (Alborn et al., 1997; Pohnert et al., 1999; Halitschke et al., 2001). Mechanical wounding and elicitors in the caterpillar's oral secretions activate the jasmonate pathway, leading to the production of jasmonic acid (JA) and induction of defenses against many challengers (Farmer and Ryan, 1992; Karban and Baldwin, 1997; McCloud and Baldwin, 1997; Ohnmeiss et al., 1997; Moura and Ryan, 2001).

Compared to chewing caterpillars, phloem feeders cause limited tissue damage (Walling, 2000). Phloem-feeding insects, such as whiteflies and aphids, frequently activate the salicylic acid (SA) signaling pathway (Kempema et al., 2007; Zarate et al., 2007), and can manipulate resources within a plant by acting as sinks, causing increased translocation of nutrients to the attacked tissue (Walling, 2008). In addition, aphids secrete various, potentially signaling hydrolytic enzymes into the phloem during feeding including pectinases, glucosidases, peroxidases, and lipases (Miles, 1999; Mutti et al., 2008; De Vos and Jander, 2009). Aphids can not only alter localized expression of genes associated with the SA-dependent pathway, like pathogenesis-related (PR) proteins (Moran and Thompson, 2001; Martinez de Ilarduya et al., 2003; Zhu-Salzman et al., 2004), but also JA-dependent genes, such as proteinase inhibitors (PI) (Martinez de Ilarduya et al., 2003; Voelckel et al., 2004). Aphid-dependent regulation of gene expression also can act systemically on JA- and SA-associated genes (Martinez de Ilarduya et al., 2003; Heidel and Baldwin, 2004; Voelckel et al., 2004), as well as on several housekeeping genes (Divol et al., 2005). However, some studies have not found significant systemic induction of PI (Heidel and Baldwin, 2004) or PR proteins (Divol et al., 2005) by aphids.

How plants cope with multiple co-occurring species of herbivores can be critical in insect-plant interactions (Shiojiri et al., 2001; Vos et al., 2001; Rodriguez-Saona et al., 2003, 2005; Dicke et al., 2009; Zhang et al., 2009). Plant responses to attack by multiple species of herbivores may result in three possible outcomes: 1) an additive response due to a lack of response specificity to different herbivores; 2) Specificity in the plant's response with no trade-offs. In this scenario, the plant responds to each herbivore differently but induces a full response to each one when the plant is damaged by both herbivores; and, 3) specificity in the plant's response with trade-offs. Here, the plant responds to each herbivore differently, and there is an attenuation of the responses to each one when the plant is dual-damaged (e.g., Zhang et al. 2009). Given that plant responses to different herbivores are the result of coordinated up- and down-regulation of multiple defensive genes via signaling pathways, each of these outcomes is possible (Schenk et al., 2000; Heidel and Baldwin, 2004; Zhu-Salzman et al., 2004). In addition, the coordination of responses may vary temporally, spatially within the plant, and for different traits so that the plants response to multiple attackers may be dramatically different from the response to a single attacker.

Trade-offs in plant response can occur when different defense pathways are induced simultaneously, and there is increasing evidence that specifically the SA and JA signaling pathways can mutually affect each other. For instance, SA suppresses JA-dependent defense gene expression, either through inhibiting JA synthesis or its action (Doares et al., 1995). Similarly, induction of the JA pathway often inhibits the induction of the SA pathway (Stout et al., 1998; Preston et al., 1999; Felton et al., 1999; Paul et al., 2000; Thaler et al., 2002; Mur et al., 2006). Stout et al. (1998) showed that the negative effects of methyl jasmonate, a volatile derivative of JA, on beet armyworm caterpillar growth were eliminated by exposure of treated plants to SA. These trade-offs can occur as a result of simultaneous attack by herbivores and pathogens, and potentially when attacked by herbivores with different feeding habits (Stout et al., 2006). For example, growth rates of beet armyworm caterpillars increased when feeding on tomato plants previously fed upon by potato aphids compared to control plants (Stout et al., 1998).

In tomatoes (*Solanum lycopersicum* Mill.), caterpillars and aphids induce different plant responses. The beet armyworm, *Spodoptera exigua* (Hübner), induces the production of a variety of plant defenses including PIs (Broadway et al., 1986) via the jasmonate-signaling pathway (Thaler et al., 2002). In contrast, Fidantsef et al. (1999) found that feeding by the potato aphid *Macrosiphum euphorbiae* (Thomas) and the green peach aphid *Myzus persicae* Sulzer on tomato plants induces local expression of lipoxygenase and PR protein P4, but does not induce PI II. In a previous study, we investigated how single attack or attack by both beet armyworm caterpillars and potato aphids influence the adult preference and caterpillar performance of beet armyworm and its parasitoid *Cotesia marginiventris* (Cresson) in tomato plants (Rodriguez-Saona et al., 2005). We demonstrated that caterpillars and aphids induce different levels of plant resistance, but also

that dual-damaged plants were phenotypically distinct from plants damaged by either herbivore alone. For example, relative to undamaged plants, oviposition by moths was lower on caterpillar-damaged plants and higher on aphid-damaged plants compared to undamaged controls. Plants damaged by both herbivores, however, received equal oviposition as control plants. The widespread evidence across systems that aphids induce the salicylate pathway (e.g., Martinez de Ilarduya et al., 2003; Zhu-Salzman et al., 2004) suggests that aphid feeding may compromise resistance to caterpillars. Thus, we hypothesized that the plant's defensive response to caterpillars was weakened when aphids also fed on the plant.

In this study, we tested this hypothesis by employing an approach that integrates gene expression (microarray analysis and quantitative real-time PCR (qRT-PCR)) and biochemical measures (leaf carbon: nitrogen, protein quantification, and PI activity) with insect performance assays. Consistent with our hypotheses, we find a high level of attenuation and suppression of genes in dual-damaged plants compared to those that are damaged by a single herbivore.

Methods and Materials

Plants and Insect Colonies

Seeds of tomato plants (*S. lycopersicum* var. Castlemart) were planted in 500 mL pots filled with soil mix and 5–10 pellets of Nutricote (13-13-13 N-P-K). Plants were grown in a greenhouse between January and July under natural light supplemented with 400-W sodium halide lamps, watered daily, and fertilized weekly with a 15-20-15 N-P-K fertilizer. Plants, 25−27 d after planting, with two fully expanded leaves were used for experiments.

Two generalist insect herbivores were used to damage the tomato plants: the beet armyworm (*S. exigua*) and the potato aphid (*M. euphorbiae*). Beet armyworm caterpillars were obtained from the USDA Laboratory, Stoneville, MS, USA, and reared on an artificial diet (Southland Products, Lake Village, AR, USA) at room temperature (25°C). Potato aphids were collected in Southern Ontario from tomato plants and maintained on tomato plants in an environmental chamber (23°C, 14:10 L:D, and 60% relative humidity).

Experimental Treatments

Treatment procedures followed those described in Rodriguez-Saona et al. (2005). Plants (N=128) were randomly assigned to one of the following four treatments: (1) undamaged (control) plants; (2) plants damaged by 40 adult aphids on leaf 1; (3) plants damaged by one 3rd instar *S. exigua* caterpillar on leaf 2; and (4) simultaneous attack by both herbivores as in treatments 2 and 3. Leaves were numbered from the bottom of the plant, with the oldest leaf designated as "leaf 1." Please note: i) leaves 1 and 2 are strongly connected to leaf 3, but both leaves are only weakly connected to each other (Stout et al., 1996; Rodriguez-Saona et al., 2005); ii) the location of feeding, i.e., whether aphids or caterpillars fed on leaf 1 or 2, had no effect on the way leaf 3 responded to damage by these two herbivores (Rodriguez-Saona et al., 2005); and iii) there was no difference in the amount of leaf damage in the caterpillar only and dual damage treatments (Rodriguez-Saona et al., 2005). Both herbivores were confined to leaves 1 and 2 using spun polyester sleeves (35 cm wide×45 cm long) (Rockingham Opportunities Corporation, Reidsville, NC, USA). Control plants had sleeves but no herbivores.

Immediately after placing the insects on the plants, plants were transferred to a growth chamber (23°C, 14:10 h L:D, and 60% relative humidity, and a light intensity of 430 μmol/m^2/sec) for 5 d, a duration sufficient to induce systemic plant defenses (Rodriguez-Saona et al., 2005). After 5 d, plants had at least 4 fully-expanded leaves. Individual leaflets from leaf 3 of each plant (N=6, 8, 8, and 10 per treatment for trials 1, 2, 3, and 4, respectively) then were excised and used for either carbon and nitrogen analysis, total protein analysis, PI analysis, or gene expression analysis with cDNA tomato microarrays and quantitative RT-PCR. Leaflets from the same position of leaf 3 were used for each analysis. In addition, a leaflet from leaf 4 from each plant was used to conduct performance assays; similar to leaf 3, leaf 4 shares vascular connections with leaves 1 and 2 (Orians et al., 2000; C.R-S. unpublished data). The entire experiment was replicated 4 times (except for protein and PI which were replicated three times). Because aphids and caterpillars were placed on leaves 1 or 2 and the molecular, biochemical, and organismal measures were done on leaves 3 and 4, all the effects and interactions reported herein are systemic.

Gene Expression

Leaflets from leaf 3, taken from 5 different plants of the same treatment, were combined for each replicate (N=4 per treatment) and flash-frozen in liquid nitrogen for gene expression analyses. Tomato microarray chips (TOM 1 array) were purchased from the Boyce Thompson Institute (http://ted.bti.cornell.edu). The microarray target preparation and hybridization methods followed Dr. David Galbraith's (University of Arizona) protocols located at websites: http://www.maizearray.org/ and http://ag.arizona.edu/~dgalbrai/, and the instructions are paraphrased below.

Purification of tRNA and mRNA

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) from 5 g of powdered tomato leaf tissue for each treatment following the manufacturer's procedures. mRNA was purified from the total RNA samples using the Oligo (dT) Dynabead approach (Dynal Biotech, Inc., Lake Success, NY, USA) according to the manufacturer's protocol. We purified a minimum of 3 μg of mRNA in 4 rounds of purification of the total RNA through Oligo (dT) Dynabeads. Purified mRNA was stored at –70°C.

Direct Labeling of mRNA

Three μg of mRNA from each sample were reverse transcribed with Superscript II (Invitrogen) to produce one of two fluorescence-labeled targets, Cy3-dUTP or Cy5-dUTP (1 mM) (Amersham Biosciences, Piscataway, NJ, USA). Labeled cDNA was purified with Microcon YM30 columns (Millipore, Billerica, MA, USA). As an additional control, we included a dye-swap reversal for each treatment.

Hybridization of the Labeled cDNA to the Microarray Slides

The Cy3- and Cy5-labeled cDNA targets were denatured and subsequently hybridized onto preheated cDNA tomato microarray slides for 10 h at 60°C. The slides were washed according to the microarray hybridization protocol and dried prior to being scanned. A loop design was used for the hybridization scheme such that each sample was hybridized with every other as well as dye reversals for each of the samples. Eight true biological replicates of each treatment, including dye-swaps, were hybridized onto the microarrays. Ultimately, 6, 6, 7, and 3 samples of undamaged, aphid-damaged, caterpillar-damaged, and dual-damaged plants, respectively, were represented by the successful hybridizations.

Microarray Scanning and Analyses

The dry slides were scanned with a Gene Pix 4100 A (Axon Molecular Devices, Union City, CA, USA) at Western Illinois University. The laser intensity of the microarray scanner was set by the manufacturer. The photomultiplier detector was set such that the overall intensities of the scanned features for the two scanned images on a single microarray slide were close to equal. In addition, the detector was set at the highest level while maintaining the overall intensity below saturation. The scanned images were further normalized using the ratio of medians following the procedures outlined in the Acuity® 4.0 software (Axon Molecular Devices).

Each microarray slide was scaled to the slide with the highest signal intensity using Microsoft Excel. We ln-transformed the data and used the Acuity® 4.0 software to perform a one-way ANOVA for Multiple Groups, and then a 2-tailed *t-test* after testing the variance for each gene. The statistical cut-off used to assay significant genes was a *P*-value of ≤0.05 and at least a two-fold change in expression. The raw data are available in Table S1 (Supplemental Material). Genes that were statistically significant were organized further by hierarchal clustering using Acuity® 4.0 software. Genes were classified into biologically significant groups by using information about their known or expected function obtained though several search engines, gene databases, and protein databases. These protein databases included UniProt—Swiss-Prot Protein Knowledgebase (http://ca.expasy.org/sprot/) through The ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB). We also searched the Entrez Gene through the National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov, which provided Gene References Into Function (GeneRIFs).

Quantitative Real-time PCR (qRT-PCR)

To validate the microarray results, we conducted qRT-PCR for the following herbivore defense-related genes: arginase, wound-induced PI II CEV 157, threonine deaminase, wound-induced PI I and II, lipoxygenase, and polyphenol oxidase D and F. Total RNA isolated during the RNA purification procedure described above was treated with TURBO DNase (Ambion, TX, USA) to remove any remaining DNA. DNase was inactivated and removed, and RNA was further purified by the RNeasy RNA cleanup MinElute columns (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. RNA quantity was analyzed photospectrometrically, and RNA integrity was determined with RNA 6000 Nano Chips run on an Agilent 2100 Bioanalyzer (Agilent, Waldbronn, Germany). Subsequently, 400 ng of DNA-free total RNA were converted into single-stranded cDNA by using a mix of random and Oligo-dT primers according to the ABgene protocol (ABgene, Epsom, UK). Gene-specific primers were designed with the Primer3 software (http://frodo.wi.mit.edu/primer3) on the basis of sequences obtained for the genes of interest and 4 potential housekeeping genes (RPS18A, actin, beta-tubulin, eIF4A-2) to serve as the endogenous control ('normalizer'). qRT-PCR was done in optical 96-well plates on a MX3000P Real-Time PCR Detection System (Stratagene, La Jolla, CA, USA) using the Absolute™ QPCR SYBR® green Mix (ABgene) to monitor double-stranded DNA synthesis in combination with ROX as a passive reference dye included in the PCR master mix. A dissociation curve analysis was performed

for all primer/probe pairs, and all experimental samples yielded a single sharp peak at the amplicon's melting temperature. Furthermore, we tested the four housekeeping genes as invariant endogenous controls in the assay to correct for sample-to-sample variation in qRT-PCR efficiency and errors in sample quantitation, and found that both actin and eIF4A-2 performed best as endogenous controls ('normalizer'). The dynamic range of a given primer/probe system and its normalizer was examined by running triplicate reactions of five different RNA concentrations. Since the target and normalizer had similar dynamic ranges, the comparative quantitation method ($\Delta\Delta$Ct) was used, and data were transformed to absolute values with $2^{-\Delta\Delta Ct}$ for obtaining fold changes between treatments.

In addition, 5 stress-related genes (dehydrin, ethylene precursor 1-aminocyclopropane-1-carboxylate oxidase, allene oxide cyclase, peroxidase, and acidic endochitinase) and 3 pathogenesis-related genes (glutathione S-transferase, PR4, and PR-1A1) were validated by qRT-PCR. The same protocol described above was used, except that 400 ng of total RNA were converted into single-stranded cDNA by using a mix of random and Oligo-dT primers following the Verso™ SYBR® Green 2-Step qRT-PCR Low Rox Kit protocol (Thermo Fisher Scientific, Rockford, IL, USA). Then qRT-PCR was done in optical 96-well plates on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Quantification was performed on three biological replicates for each treatment.

Relative fold changes for each gene were compared to the aphid treatment, which was set to one. qRT-PCR fold change (relative to controls) was analyzed by using a one-way ANOVA with JMP (Sall and Lehman, 1996), followed by a Fisher's Least Significant Difference (LSD) test to assess separation of fold differences.

Carbon and Nitrogen

Leaflets for total leaf carbon and nitrogen analyses were oven-dried at 60°C for 48 h. Carbon and nitrogen concentrations were measured with 5 mg of dried ground leaf material by micro-combustion in an Elemental Combustion System 4010, CHNS-O analyzer (Costech Analytical Technologies, Valencia, CA, USA).

Total Protein

To determine total protein content, we followed methods modified from Jones et al. (1989). Buffer-soluble protein was extracted by homogenizing weighed leaf samples in 0.5 ml of 0.1 M NaOH (pH 11.8). The samples were vortexed for 3 sec and incubated at room temperature for 30 min. After incubation, samples were vortexed again and centrifuged at 11,000 rpm for 10 min at room temperature. Five µl of supernatant were mixed with 250 µl of Bradford reagent (Coomassie brilliant blue) in a cuvette, and the absorbance was measured at 595 nm. Total protein (mg of protein per gram of tissue sample) was calculated using a standard of bovine serum albumin in 0.1 M NaOH.

Proteinase Inhibitors

We measured the plant's ability to inhibit the hydrolysis of the artificial substrate azocasein by commercial trypsin in our four treatments. The procedure followed methods described in Rodriguez-Saona et al. (2005). Samples were ground in Tris HCl (pH 7.8) extraction buffer (3 µl/mg fresh weight), vortexed for 3 min, and centrifuged at 11,000 rpm for 10 min at 10°C. Sixty µl of the supernatant were added to 20 µl of Tris buffer, 50 µl of 2% azocasein in Tris buffer, and 20 µl of a 0.001 M HCl solution containing 200 ng of trypsin. A set of controls with no sample also was prepared for each sample using an identical procedure and adjusting the total volume with the Tris buffer. After incubation for 20 min at 28°C, 100 µl of trichloroacetate (100% w/v) were added to denature the substrate and stop the reaction. Samples then were centrifuged for 10 min at 8,000 rpm. One hundred µl of 1 M NaOH were added to 100 µl of the supernatant from each sample, and absorbance was measured at 450 nm. The activity of trypsin inhibitor is reported as one minus the percent ratio of sample to control absorbance (Orians et al., 2000).

Performance Assays

No-choice bioassays were conducted to test whether prior damage by aphids, caterpillars, or both herbivores differentially affected the performance of beet armyworm caterpillars compared to undamaged plants. Neonates were placed individually in 90-mm Petri dishes lined with moist filter paper and allowed to feed for 5 d on leaflets from one of the four treatments described above. All leaflets were excised from leaf 4. Petri dishes were kept at room temperature. Each trial had a total of 10 caterpillars per treatment (N=40) and was repeated 4 times. On day 5, all live caterpillars were weighed and mortality was recorded.

Statistical Analyses of Biochemical and Organismal Assays

The effects of aphids, caterpillars, and trial, and their interactions, on total leaf carbon:nitrogen ratio, total protein, PI activity, and caterpillar mass were analyzed using ANOVA (Systat 1998; SPSS Science, Chicago, IL, USA). *Post-hoc* pair-wise comparisons between treatments were performed using LSD tests. Percent data were arcsin-square root transformed and caterpillar mass were ln-

transformed prior to analysis to meet assumptions of normality and homogeneity of variance. Mortality data were analyzed using *G*-tests.

Results and Discussion

Gene Expression: Specificity of Induction

Specificity in the tomato responses to aphids and caterpillars at the transcriptomic level was detectable, and is represented in the microarray analysis where beet armyworm caterpillars induced the systemic expression of different genes than potato aphids (Fig. 1; Table 1). Herbivory systemically up- or down-regulated approx. 2% of the genes on the Cornell array (277 out of 13440) (Table S1). Eighty percent of the genes (222 out of 277) were up- or down-regulated by only one of the herbivores, confirming substantial specificity of the plant responses. Aphid feeding changed expression of 2.8 times more genes compared to caterpillar feeding (239 vs. 85, respectively) (Fig. 1). Ninety-three genes were up-regulated by aphid feeding, while 56 genes were up-regulated by caterpillar feeding compared to controls; twenty-five of these genes were up-regulated by both aphids and caterpillars (Fig. 1). One hundred and forty six genes were down-regulated by aphids, while 29 genes were down-regulated by caterpillars compared to controls; twenty-two of these genes were down-regulated by both herbivores (Fig. 1). Dual-damage induced changes in 11 genes; 8 of them were also induced by caterpillars alone, while the other 3 genes were induced by both caterpillars and aphids when feeding alone, and the expression of none of these genes was enhanced compared to when damaged by a single herbivore (Table 1).

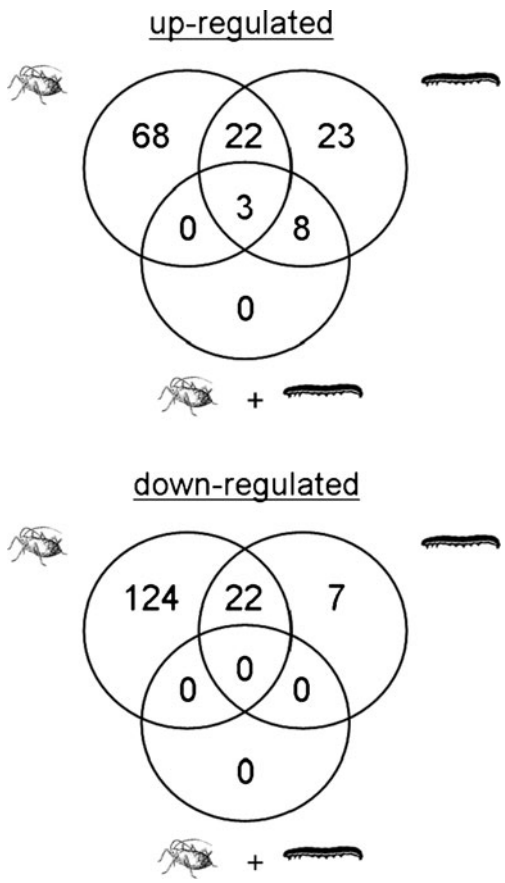


Fig. 1 Venn diagrams of the numbers of overlapping and non-overlapping transcriptional responses of tomato genes up-and down-regulated by aphids, caterpillars, or aphids and caterpillars. Numbers are up- or down-regulated genes that met our combined criteria of a minimum of 2-fold change in gene expression and a significant ANOVA (*P* values≤0.05)

Of all of the genes assayed, the most strongly systemically induced by herbivory were those related to herbivore defense, but gene expression was altered in many functional categories (Table 1). Seven herbivore defense-related genes were systemically induced by caterpillar feeding >10-fold greater than controls; including genes encoding for polyphenol oxidases, threonine deaminase, and an array of different protease inhibitors (Table 1A). These defense genes often are associated with the JA pathway (Fidantsef et al., 1999). In contrast, aphid feeding increased expression of fewer herbivore defense-related genes and to a much lesser extent than following caterpillar feeding (<6-fold induction).

Induction of pathogenesis-related proteins such as PR-1A1 and glutathione S-transferase was not specific to aphids (Table 1B). Similarly, salicylic acid methyltransferase that catalyzes the formation of methyl salicylate, the volatile derivative of SA, was induced by both herbivores (Table 1C). The lack of specificity in plant responses to aphid and caterpillar feeding in this category is surprising given previous results that aphids, but not caterpillars, are strong inducers of salicylate pathway-regulated genes (Walling, 2000). It is possible, however, for caterpillars to induce the SA pathway as shown for *S. exigua* caterpillars feeding on tobacco plants (Diezel et al., 2009). It is also possible for genes in the pathogen defense category to be regulated by JA (Li et al., 2004). An example is the translation-inhibitor protein that was strongly up-regulated by caterpillar feeding (Table 1B). Another possibility is that some of the SA pathway genes might have been missed because they may have been induced locally or soon after herbivore infestation.

Our findings are similar to a study by De Vos et al., (2005) who reported that the aphid *M. persicae* induces more *Arabidopsis* genes than the caterpillar *Pieris rapae* (L.), and a study in tobacco by Voelckel et al. (2004) that found that the magnitude of induction by *Myzus nicotianae* Blackman aphids was smaller than by the caterpillar *Manduca sexta* L.; although more genes were induced by

Table 1 Relative ratios of selected genes up- or down-regulated by aphid, caterpillar, and dual herbivory on tomato plants compared to non-wounded plants. For each gene, ratios with different letters indicate significant differences among treatments ($P \leq 0.05$)

Putative annotation+function	Gene name	Control	Aphid	Caterpillar	Caterpillar +Aphid
A. Herbivore defense related					
acid phosphatase	SGN-U145331	1.00(a)	1.80(a)	4.86(b)	1.80(a)
acid phosphatase	SGN-U145330	1.00(a)	2.08(a,b)	5.11(b)	1.85(a)
arginase	SGN-U145219	1.00(a)	1.06(a)	8.41(b)	4.79(a,b)
aspartic protease inhibitor 1 precursor	SGN-U143342	1.00(a)	3.31(a,b)	17.05(b)	10.79(b)
ethylene-responsive proteinase inhibitor I precursor	SGN-U144127	1.00(a)	2.05(a,c)	14.61(b,c)	9.03(b,c)
leucine-rich repeat resistance protein-like protein	SGN-U144588	1.00(a)	5.88(b)	1.58(a)	1.03(a)
polyphenol oxidase F (PPO) (Catechol oxidase)	SGN-U143365	1.00(a)	-1.63(a)	10.72(b)	2.04(a,b)
proteinase Inhibitor TYPE II TR8 Precursor	SGN-U143905	1.00(a)	4.60(b)	7.12(b)	8.35(b)
threonine deaminase	SGN-U143321	1.00(a)	2.18(a,b)	17.39(b)	10.23(a,b)
wound-induced proteinase inhibitor I precursor	SGN-U143552	1.00(a)	2.59(a,b)	18.49(b)	8.79(a,b)
wound-induced proteinase inhibitor I precursor	SGN-U143556	1.00(a)	3.80(b)	37.66(c)	14.33(b,c)
wound-induced proteinase inhibitor II precursor	SGN-U143329	1.00(a)	2.63(a,b)	20.35(c)	10.58(b,c)
wound-inducible carboxypeptidase	SGN-U148185	1.00(a)	4.21(b)	3.55(b,c)	1.16(a,c)
B. Pathogenesis related proteins					
catalase isozyme 1	SGN-U143191	1.00(a)	-2.67(a,b)	-2.29(b,c)	−1.17(a,c)
hypersensitive response assisting protein	SGN-U146564	1.00(a)	−1.14(a)	2.91(b,c)	1.94(a,c)
pathogenesis-related protein 1A1 precursor (PR-1A1)	SGN-U144656	1.00(a)	2.72(b)	2.03(a,b)	−1.08(a)
probable glutathione-S-transferase (Pathogenesis-related protein 1)	SGN-U143280	1.00(a)	3.79(b)	2.17(a,b)	−1.12(a)
remorin-like protein	SGN-U146116	1.00(a)	−2.53(b)	−2.05(a,b)	1.29(a,b)
translation-inhibitor protein	SGN-U143744	1.00(a)	1.80(a)	10.78(b)	7.62(b)
pto-responsive gene protein	SGN-U144888	1.00(a)	1.20(a)	4.12(b,c)	1.71(a,c)
C. Signaling related					
1-aminocyclopropane-1-carboxylate oxidase homolog	SGN-U143387	1.00(a)	11.41(c)	32.03(b,c)	2.03(a,b)
kinase interacting protein 1 (Petunia integrifolia)	SGN-U156478	1.00(a)	17.05(b)	6.65(a)	2.08(a)
lipase (class 3) family	SGN-U159040	1.00(a)	−4.57(b)	−2.84(a)	1.25(a)
MAP kinase	SGN-U146866	1.00(a)	−2.79(b)	−1.71(a)	−1.19(a)
S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase	SGN-U146660	1.00(a)	2.04(b)	2.03(b)	1.00(a)
WIZZ [Nicotiana tabacum]	SGN-U143779	1.00(a)	−6.75(b)	−2.88(a)	1.72(a)
WRKY family transcription factor [Arabidopsis thaliana]	SGN-U145810	1.00(a)	3.33(b)	2.27(a,b)	1.39(a)
WRKY family transcription factor [Arabidopsis thaliana]	SGN-U144503	1.00(a)	−1.03(a)	2.11(b)	−1.23(a)
D. Photosynthesis related					
phytoene synthase	SGN-U143396	1.00(a)	−3.92(b)	−2.43(a,b)	1.43(a)
ribulose bisphospate carboxylase small chain 2A, chloroplast precursor	SGN-U143665	1.00(a)	−2.18(c)	−2.19(b,c)	−1.02(a,b)
triose phosphate chloroplast precursor	SGN-U143665	1.00(a)	−2.05(b)	−2.49(b)	−1.17(a)

caterpillars in the later study than by aphids. A higher number of genes were systemically down-regulated by potato aphid feeding than up-regulated in our study (146 vs. 93; Fig. 1). This is consistent with De Vos et al. (2005) who found a total of 1349 being down-regulated and 832 up-regulated by *M. persicae*. Although we did not find genes expressed only in the dual-damage treatment, we found a different pattern of gene expression in this treatment compared to aphid- and caterpillar-damage treatments (Fig. 2). In fact, our cluster analysis shows greater similarities in gene expression between dual-damaged and control plants than to those damaged by only aphids or caterpillars. Voelckel and Baldwin (2004) also found specificity in gene expression of tobacco plants singly or simultaneously attacked by a piercing-sucking mirid bug and the chewing caterpillar, *M. sexta*. In plants damaged by both herbivores simultaneously, they found a distinct transcriptional pattern from either herbivore alone. In

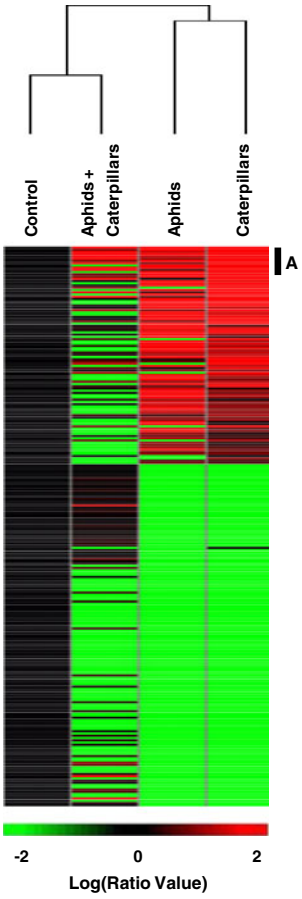


Fig. 2 Cluster analysis of the tomato genes up- and down-regulated in response to feeding by aphids, caterpillars, or aphids and caterpillars. The dendrogram on top of the cluster illustrates the relationship among the four treatments. Ratio values are calculated between herbivore-infested and non-infested control plants (set to 1). Genes up-regulated by treatment appear in red (positive log-ratio values), while those that were down-regulated appear in green (negative log-ratio values). Each row in the column corresponds to a single gene, and a color scale is presented below the figure. Letter A indicates a cluster of many of the plant defense genes associated with Table 1A

Fig. 3 Relative fold differences of arginase (**a**), wound induced proteinase inhibitor II CEV 157 (**b**), threonine deaminase (**c**), wound induced proteinase inhibitor I (**d**), wound induced proteinase inhibitor II (**e**), lipoxygenase (**f**), polyphenol oxidase D (**g**), polyphenol oxidase F (H), dehydrin (**i**), 1-aminocyclopropane-1-carboxylate oxidase (**j**), allene oxide cyclase (**k**), acidic endochitinase (**l**), peroxidase (**m**), glutathione S-transferase (**n**), pathogenesis-related PR4 (**o**), and pathogenesis-related PR-1A1 (**p**) determined with quantitative real time-PCR (qRT-PCR). Tomato plants were damaged by aphids, by caterpillars, or by both aphids and caterpillars. All of the qRT-PCR values are normalized to the aphid treatment (set to 1). Bars indicate means±SE. Different letters represent statistically significant differences between treatments ($P \leq 0.05$). $N=3$

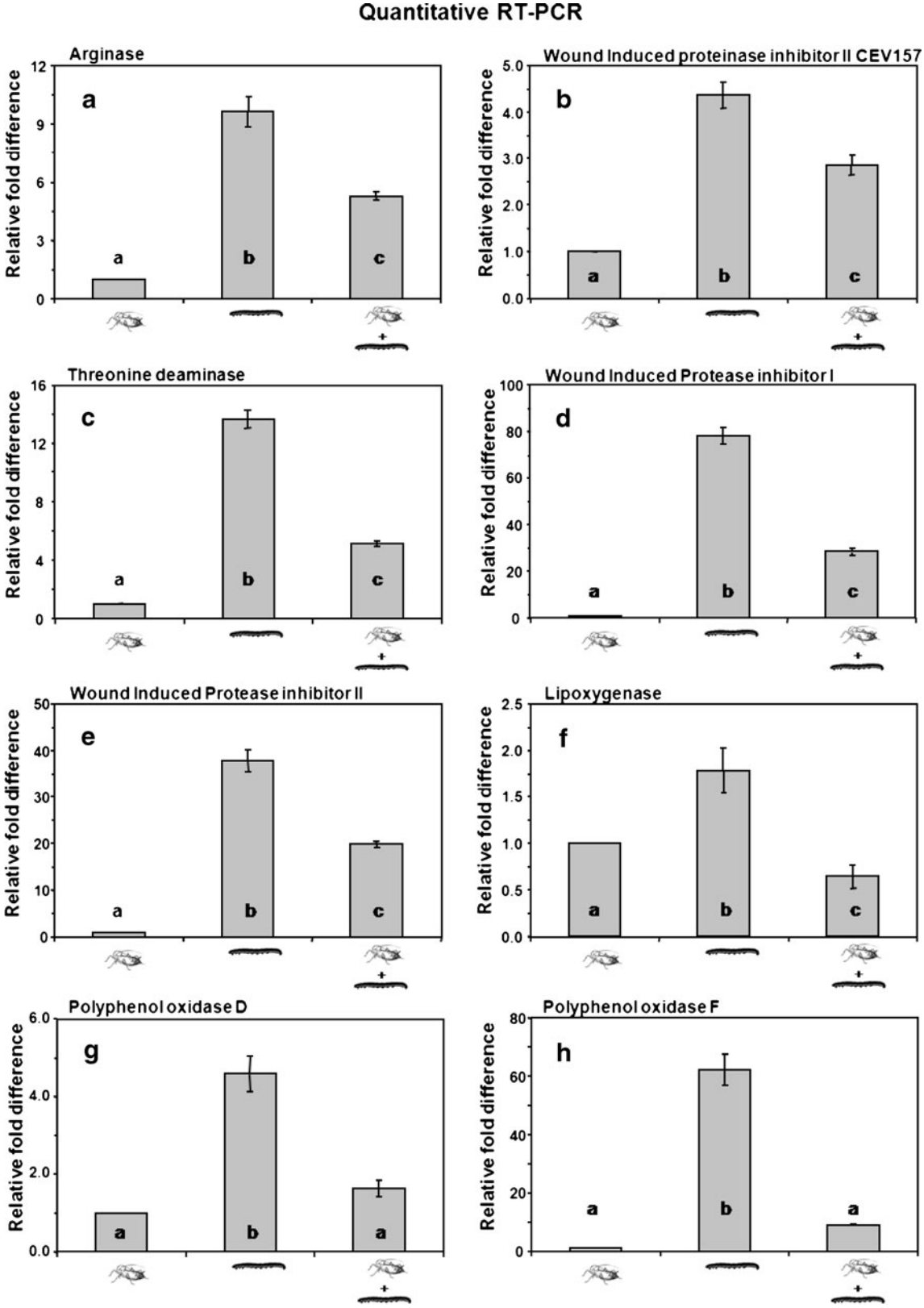
contrast to our finding, however, Voelckel and Baldwin's study found only specificity after the first day of feeding, not in later days, whereas our study shows specificity five days after attack. These two studies thus show distinct genetic outcomes for the integration of specific plant responses when damaged by multiple herbivores.

Gene Expression: Bi-Directional Trade-offs

Suppression of systemic gene expression in the dual-damage treatment, where expression is changed to levels no longer different from controls, was found in 58% of the genes up- or down-regulated by one herbivore (135 out of 222), indicating a high degree of trade-offs in the expression of specific responses (Table 1). These trade-offs were reciprocal and occurred across functional categories. Caterpillar feeding suppressed up- or down-regulation of 66% (127 out of 192) aphid regulated genes, whereas aphid feeding only prevented up- or down- regulation of 8 out of 30 (27%) of the genes regulated by caterpillar feeding (Table 1). The expression of 12 caterpillar-induced genes was confirmed by qRT-PCR (Fig. 3a–l). Of these 12 genes, 5 showed attenuated expression in the dual damage treatment (i.e., expression in the dual-damage treatment was less than in the caterpillar-only treatment but more than in the aphid-only treatment), while 7 showed suppression (i.e., expression in the dual-damage treatment was different from the caterpillar-only treatment but not from the aphid-only treatment).

The pattern of attenuation of expression falls into two categories: 1) in some cases, aphids had no effect on the genes independently (e.g., threonine deaminase), but reduced the response of those genes to caterpillar damage (Tables 1 and 2) more remarkably, some genes (e.g., proteinase inhibitor gene I—SGN-U143556) that are strongly up-regulated by caterpillar feeding, and weakly induced by aphid feeding, the expression in dual-damaged plants was still reduced compared to the caterpillar-only treatment (Table 1). The same was true for some genes that showed aphid-dependent regulation: caterpillar alone had no effect (e.g., MAP kinase; Table 1), but attenuated the down-regulation of these genes to aphid damage in the dual attack and some genes that were strongly down-regulated by aphid feeding and weakly suppressed by caterpillar feeding (e.g., lipase; Table 1), still were attenuated in the dual-damage treatment compared to the aphid only treatment.

Thus, although aphids may minimize the magnitude of induction of plant responses by causing little cellular damage and avoiding detection, they also reduce the plant's ability to induce the responses to caterpillar feeding. This is consistent with the "decoy hypothesis," which states that by inducing the salicylate pathway aphids can suppress the jasmonate pathway defenses to their own benefit (Zhu-Salzman et al., 2004; Thompson and Goggin, 2006; Zarate et al., 2007). Our evidence shows that aphid feeding can suppress or attenuate induction of many jasmonate pathway-regulated genes, but we do not have evidence that this occurs due to induction of the salicylate pathway as the pathogen defense-related genes were induced similarly in caterpillar and aphid treatments (Table 1B; Fig. 3n–p). The genes in our "pathogen defense-related" category may or may not be regulated by the salicylate pathway. However, based on the microarray data even the two genes known to be salicylate-regulated PR proteins 1 and 1A1 were only weakly up-regulated by aphids (Table 1B), while qRT-PCR analyses detected no differences in PR protein induction


Quantitative RT-PCR
Arginase
a
Relative fold difference
12
9
6
3
0
a
b
c
Wound Induced proteinase inhibitor II CEV157
b
Relative fold difference
5.0
4.0
3.0
2.0
1.0
0.0
a
b
c
Threonine deaminase
c
Relative fold difference
16
12
8
4
0
a
b
c
Wound Induced Protease inhibitor I
d
Relative fold difference
100
80
60
40
20
0
a
b
c
Wound Induced Protease inhibitor II
e
Relative fold difference
50
40
30
20
10
0
a
b
c
Lipoxygenase
f
Relative fold difference
2.5
2.0
1.5
1.0
0.5
0.0
a
b
c
Polyphenol oxidase D
g
Relative fold difference
6.0
4.0
2.0
0.0
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Polyphenol oxidase F
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Relative fold difference
80
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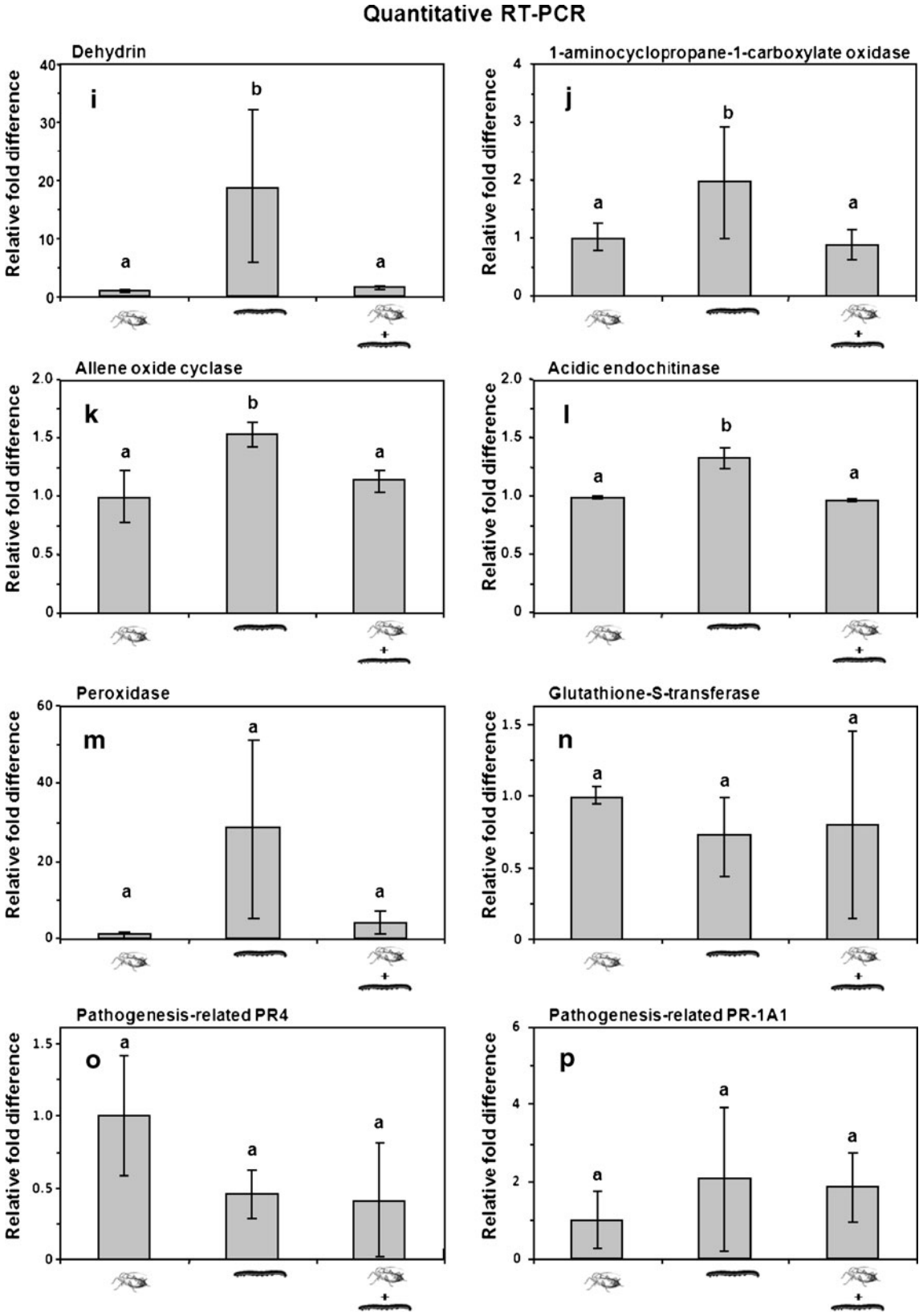

Fig. 3 (continued)

Table 2 Summary of 3-way ANOVAs for the effects of aphids, caterpillars, and trial on amounts of carbon, nitrogen, protein, and proteinase inhibitors in tomato plants, as well as the effects on *Spodoptera exigua* caterpillar mass. numbers indicate *F* values

Source	C/N	Protein	PI	Caterpillar mass
Aphids (A)	1.38 ns	0.13 ns	2.38 ns	0.06 ns
Caterpillar (C)	2.08 ns	15.06**	58.21**	38.93**
Trial (T)	7.68**	158.43**	3.34*	0.36 ns
A x C	5.11*	4.33*	0.09 ns	3.57[a]
A x T	0.44 ns	0.27 ns	1.78 ns	0.87 ns
C x T	2.15 ns	0.91 ns	6.26**	1.81 ns
A x C x T	2.03 ns	0.92 ns	0.57 ns	1.00 ns

[a] $0.07 \geq P > 0.05$; *=$0.05 \geq P > 0.01$; **=$P \leq 0.01$

between single and dual damaged treatments (Fig. 3o–p). In addition, we found attenuation of genes in many categories, not just those involved in defense (Table 1C–D). However it is achieved, attenuation of the jasmonate pathway expression may benefit the caterpillars and aphids, as both have lower performance on tomato plants treated with JA (Thaler et al., 2001; Cooper and Goggin, 2005).

Gene Expression: Lack of Specificity

There was an overlap of gene regulation in the case of 44 genes (out of the 277 genes influenced by herbivory), where both aphids and caterpillars, when feeding alone, up-regulated or down-regulated the systemic expression of the same genes (22 and 22 genes, respectively). Interestingly, however, for all 44 genes that lacked specificity the expression of these genes in the dual-damage treatment was not different from the controls. These included herbivore defense related genes (e.g., wound-inducible carboxypeptidase), photosynthesis related genes (e.g., triose phosphate chloroplast precursor), and signaling related genes (e.g., 1-aminocyclopropane-1- carboxylate oxidase homolog) (Table 1). For example, aphids and caterpillars down-regulated several genes associated with photosynthesis when feeding alone but not when feeding together on the same plant (Table 1C). A possible explanation for this scenario is that almost no gene is inducible only by a single transcription factor or signaling pathway. If two insects that induce different signaling pathways/transcription factors feed simultaneously on a plant, then these pathways/factors might interfere with each other. This could result in negative feedback or inhibition leading to a zero sum transcription of the respective subset of genes.

Ultimately the translation of these genetic changes to impacts on herbivores is what is critical, and such data from our system are discussed below.

Chemistry and Bioassays: Foliar Chemicals

Interactions between plant responses to caterpillar and aphid feeding impacted plant nutritional quality, as shown by the significant aphid×caterpillar effects on C/N and protein (Table 2). Aphid feeding decreased the C/N ratio in systemic leaves while caterpillar feeding had no effect (Fig. 4a). When both herbivores fed on the plant, however, the C/N ratio was equal to the control treatment, indicating that caterpillar feeding affected the plant's response to aphids. This effect is consistent with aphids manipulating the nutritional content of plants for their own benefit by increasing the proportion of nitrogen in leaves (e.g., Flynn et al., 2006), which is critical considering that leaf nitrogen is often a limiting resource for herbivorous insects (Mattson, 1980). This likely explains our previous finding that caterpillar consumption (in the first 7 days of development) increases on aphid-damaged plants compared to control plants, and also that the amount of caterpillar consumption on caterpillar-damaged plants was not affected by aphids (Rodriguez-Saona et al., 2005). Stout et al., (1998) also reported increased growth rates of beet armyworm caterpillars on tomato plants previously damaged by aphids compared to undamaged plants. Aphid and caterpillar feeding alone had no effect on the amount of protein in systemic leaves. However, both herbivores feeding on the plant reduced the amount of protein compared to aphid-damaged plants (Fig. 4b).

Our data show that caterpillars induced systemically high PI enzymatic activity, but this was not affected by aphids. Caterpillar-damaged plants had three times higher PI enzyme activity compared to control plants (Fig. 4c; Table 2), while aphids had no effect on PI activity, even though they did weakly induce PI gene expression (see results above). This is consistent with Stout et al. (1998) who found that local feeding by *Helicoverpa zea* (Boddie) increased PI activity, whereas feeding by potato aphids had no effect. However, the presence of aphids did not affect the PI activity induced in response to caterpillar damage even though our transcriptomic data indicated that aphid feeding strongly interfered with PI gene expression. Thus, although the results of the current study indicate that aphid feeding could both weakly induce expression of several PI genes and cause attenuation of PI genes induced by caterpillars, neither of these effects on gene expression led to a change in PI activity. Gene expression may not translate directly to protein activity due to a level of

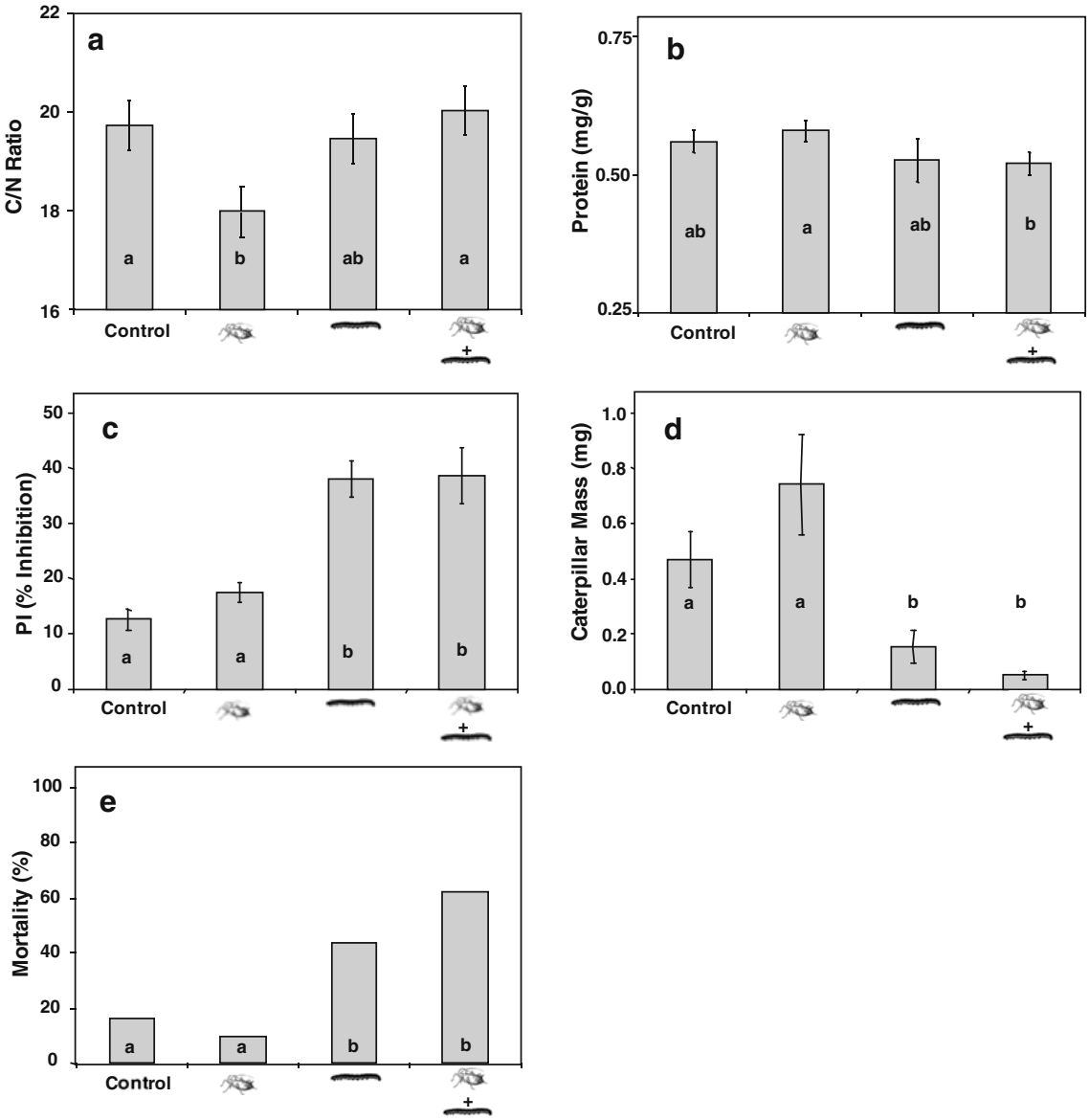

Fig. 4 Effects of damage singly by aphids or caterpillars or simultaneous damage by aphids and caterpillars on amounts of carbon/nitrogen (C/N) ratio (**a**), protein (**b**), and proteinase inhibitors (**c**), as well as on *Spodoptera exigua* caterpillar mass (**d**) and mortality (**e**). Bars indicate means±SE. Different letters represent statistically significant differences between treatments ($P \leq 0.05$). N=3–4

transcript above which no more protein is made, post-transcriptional regulation, or interactions between gene products. Alternatively, the PI assay used simply may not be sensitive enough. In fact, in our previous study (Rodriguez-Saona et al., 2005) PI activity was about 20% lower in the dual-damage treatment than in the caterpillar only treatment, although this difference was not significant and not observed in the present study.

Chemistry and Bioassays: Caterpillar Performance

Prior caterpillar feeding decreased the mass of bioassay caterpillars by 54% (Fig. 4d) and increased caterpillar mortality by 28% (Fig. 4e). Bioassay caterpillars were 46% heavier and had 5% lower mortality on aphid-damaged plants compared to control plants, but these differences were not significant (Fig. 4d–e; G=0.27, P=0.6). Caterpillar performance on dual-damaged plants was similar to that on caterpillar-damaged plants (Table 2).

Therefore, caterpillar damage had a strong negative effect on subsequent caterpillar growth and survivorship, whereas aphid damage had only a weak and non-significant effect on caterpillar performance, and we found no attenuation of resistance in dual-damaged plants. These bioassays reveal the complicated relationship between gene expression, biochemical activity, and impacts on herbivores. In a previous paper (Rodriguez-Saona et al., 2005), we demonstrated that aphid feeding does influence compo-

nents of *S. exigua*'s ecology. For example, moth host acceptance was substantially lower on caterpillar-damaged plants, higher on aphid-damaged plants, and intermediate on dual-damaged plants. This finding is concordant with gene expression results from the current study and others that find conflict between some JA and SA regulated genes and synergisms between others (Schenk et al., 2000; Bodenhausen and Reymond, 2007). In our previous study, we further concluded that beet armyworm moth preference correlates with caterpillar performance only when plants were damaged by a single herbivore (aphids or caterpillars), but not in the dual-damaged treatment (Rodriguez-Saona et al., 2005). Insect preference and performance often are not correlated in part because different plant traits influence these aspects of herbivore biology (e.g., Prudic et al., 2005). Thus, some components of the plant's response to caterpillars that are influenced by aphids have impacts on other herbivores. Because interactions with multiple herbivores on the same plant are common under more natural conditions (Strauss, 1991; Hufbauer and Root, 2002) and because aphid densities are often reported to be very high in the field (Dixon, 1977), it is likely that under these conditions the interference by aphids in direct plant defenses against chewing herbivores can be even stronger than observed in our experiments.

In summary, we employed a combination of molecular, biochemical, and organismal approaches to investigate the interactions between tomato plants, the beet armyworm caterpillar, and the potato aphid. At the molecular level, both attacking insects influenced each other. At the biochemical level, the presence of aphids was too weak to change the PI activity (caterpillar-induced proteins), but the presence of caterpillars did alter the aphid influence on C/N ratios. Finally, at the organismal level, aphids did not overwrite the negative effects of caterpillars on the caterpillar's performance. However, our previous study (Rodriguez-Saona et al., 2005) shows that aphids do overwrite the repellency effects of caterpillars on the moth's oviposition preference.

When comparing across all three biological levels of organization, our data produced consistent results when considering each herbivore separately: caterpillars induced high expression of several JA-regulated defensive genes, resulting in high PI activity and reduced caterpillar performance, whereas aphids induced weak expression of only a few defensive genes associated with the JA pathway and did not induce PI activity or affect herbivore performance; but not when considering them together: when plants were attacked simultaneously by caterpillars and aphids, strong interactions exhibited at the molecular level translated into interactive effects on some chemicals, i.e., C/N ratio, but not PI activity, and some components of host suitability for herbivores, i.e., adult preference, but not caterpillar performance. Taken together, our study shows that caterpillar performance on plants could not be explained simply by the levels of defenses but by their balance with the nutritional content as a result of single or multiple herbivory. This study is a reminder that predictions by global gene expression studies need further verification across different levels of biological integration. Future studies will explore the mechanisms for attenuation in the dual treatment, characterize the transcriptional responses of JA and SA signaling genes in a time course experiment, and investigate aphid performance on single- and dual-damaged plants.

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