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Profiling transcriptional changes in *Citrus sinensis* (L.) Osbeck challenged by herbivory from the xylem-feeding leafhopper *Homalodisca coagulata* (Say) by cDNA macroarray analysis

Jerry Mozoruk [a,b], Laura E. Hunnicutt [a,c], Ronald D. Cave [b], Wayne B. Hunter [c], Michael G. Bausher [a,*]

[a] *Horticulture and Breeding Research Unit, USDA ARS U.S. Horticultural Research Laboratory Ft. Pierce, FL 34945, United States*
[b] *Indian River Research & Education Center, University of Florida, Ft. Pierce, FL 34945, United States*
[c] *Subtropical Insects Research Unit, USDA ARS U.S. Horticultural Research Laboratory Ft. Pierce, FL 34945, United States*

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Abstract

The molecular mechanisms underlying plant defense to sap-feeding insects are slowly being uncovered. In large part, past research has focused on interactions between phloem-feeding insects and their annual host plants with little emphasis on xylem-feeders or woody perennials—especially fruit trees. Using nylon filter cDNA arrays, we analyzed the transcriptional changes of 1731 non-redundant citrus transcripts that resulted from herbivory by a xylem-feeding leafhopper, the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say) (Hemiptera: Cicadellidae). In addition, herbivory-elicited changes were compared to those of mechanical damage to better identify GWSS-specific responses. GWSS feeding led to a significant expression change in 50 transcripts. Of these, 14 were also changed by mechanical damage; however, the magnitude was in many cases reduced, suggesting transcriptional modification by GWSS-derived elicitors. Sequence similarity searches with the public database GenBank indicated that the responsive transcripts broadly function in direct defense, defense signaling, ROS scavenging, transport, cell wall modification, photosynthesis and abiotic stress. In particular, GWSS feeding resulted in a transcript profile that resembled wounding, likely through jasmonic acid-independent pathways, as well as an association with dehydration stress. In contrast to similar studies with aphids, salicylic acid-dependent pathogenesis-elated genes were weakly induced. Interestingly, six of the GWSS-responsive transcripts failed to significantly match any public protein sequence signifying their potential as novel genes functioning in plant defense, wound response or abiotic stress.
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1. Introduction

The glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say), has recently become a serious pest of numerous economically important agricultural and ornamental plant species, principally for its ability to effectively vector the bacterial pathogen *Xylella fastidiosa* Wells [1–4]. While current attention is primarily focused on vectoring of the *X. fastidiosa* strain causing Pierce's disease in grapevines; there is concern that if the citrus variegated chlorosis (CVC) strain of *X. fastidiosa* were to enter North America, GWSS could rapidly vector this disease throughout the U.S. citrus production areas [5,6]. Much of the current focus surrounding GWSS management involves its association with Pierce's disease in grapevines. In addition to the CVC threat, it has been demonstrated that citrus is the major over-wintering and breeding host for the GWSS in California and it is thought that vineyards in close proximity to citrus groves are at higher risk of acquiring Pierce's disease [7].

Current control strategies for managing GWSS populations are based on insecticidal treatments and classical biological control [8]; however, little attention has been directed toward development of sharpshooter-resistant hybrids, particularly for citrus. For that reason, investigations into the molecular

* Corresponding author at: USDA ARS U.S. Horticultural Research Laboratory, 2001 S. Rock Road Fort Pierce, FL 34945, United States.
Tel.: +1 772 462 5918; fax: +1 772 462 5986.
E-mail address: mbausher@ushrl.ars.usda.gov (M.G. Bausher).

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mechanisms of the citrus defense response will assist in the elucidation of resistance traits that can provide direction for programs aimed at insect-resistant crops or alternate management strategies.

Plants are exposed to a wide range of abiotic and biotic stimuli for which effective, fitness-based responses must be applied. In addition to constitutive defenses, such as stored alleochemicals and physical barriers, plants respond to insect herbivory by activating an array of defensive/stress genes in an effort to counteract the attacker [9,10]. These genes encode transcriptional regulators, enzymes related to secondary metabolic pathways (e.g. leading to synthesis of phytoalexins), anti-nutritive proteins such as protease inhibitors (PI) or polyphenol oxidase (PPO) and pathogenesis-related proteins (PR). Much of this work has been established using chewing herbivores or wounding events that stimulate wound signal transduction pathways, and has shown that the response is principally mediated by the phytohormone jasmonic acid (JA) [9,11,12]. By contrast, considerably less is known about the molecular mechanisms of plant defense to sap-feeding insects, particularly xylem feeders.

While a large amount of data indicates that JA is the principle wound/defense signaling compound in plants [9,13–15], an ever-growing number of findings indicate that JA acts synergistically and antagonistically with other phytohormones in a complex network of signaling cross-talk that is proposed to define the most effective defense posture to counter a diverse range of attackers [16–19]. It has been demonstrated that insects from different feeding guilds elicit disparate defense responses [18,20] and that this transcriptional perception is likely modulated by compounds in insect salivary secretions or metabolites of insect-associated pathogens [9,11]. For example, herbivory or regurgitant from the tobacco hornworm, *Manduca sexta* (L.), induced novel genes in potato (*Solanum tuberosum* L.) not observed in mechanically damaged tissues [20]. Remarkably, Roda et al. [21] showed that the two most abundant fatty acid–amino conjugates (FAC) in *M. sexta* regurgitant were responsible for the complete induction of trypsin proteinase inhibitors (TPI), the endogenous JA burst and 89% of all transcriptional changes in tobacco. Although not as detailed, sap-feeders have also demonstrated elicitor modification. For instance, polygalacturonase (PG), a component of aphid saliva, is thought to enyzmatically release oligogalacturonides (OGA) from pectin in the cell wall during feeding, which subsequently trigger the initiation of defense signaling cascades [10,22]. While these findings provide direction for explaining the dissimilar defense profiles exhibited by herbivores from different feeding guilds, it emphasizes that wounding is merely one aspect of herbivory.

Whether plants recognize insect herbivores by elicitors, the amount of wounding inflicted during feeding or both remains to be fully elucidated; however, it is apparent that insects from different feeding guilds do elicit distinct transcript profiles both in defense genes and in genes with no apparent defensive role. For sap-feeding insects, evidence – primarily based on aphid studies – indicates these insects stimulate transcript profiles resembling pathogen attack, specifically strong induction of SA-dependent pathways and a weaker induction of JA/ethylene-dependent defense cascades [10,23,24]. It is thought that increased SA signaling reflects perception to an influx of insect-associated pathogens at the wound site [25] or perhaps similarities between intracellular fungal hyphal growth and stylet penetration [26]. The limited induction of JA/ethylene response genes by sap-feeding insects suggests that – at least for aphids – these insects evade JA-mediated wound-response pathways by way of limited tissue damage. Interestingly, more recent evidence has shown that sap-feeding insects and even some fungal pathogens do elicit expression of wound-induced PIs independent of JA-dependent signaling; intimating that stealthy feeders and some pathogens alike may elicit wound-responsive genes through mechanisms not yet known, but are clearly independent of JA-signaling [27].

Recently, more panoptic gene expression analyses using microarrays have revealed many diverse and subtle transcriptional changes in response to sap-feeding herbivores [28–30]. Moran et al. [23] showed that genes involved with oxidative stress, calcium-dependent signaling, PR response, and tryptophan biosynthesis were induced, while genes involved with aromatic biosynthesis were repressed in *Arabidopsis* after 72–96 h of herbivory by the green peach aphid, *Myzus persicae* (Sulzer). Greenbug, *Schizaphis graminum* (Thomas), feeding on sorghum (*Sorghum bicolor* (L.) Moench) induced the expression of genes involved with biosynthetic pathways for phenolics, abiotic-stress (drought, salt, temperature), nitrogen assimilation and photosynthesis [31]. These studies indicate that defense/stress gene expression make up only a subset of the total changes that occur as a result of herbivory and that many genes with no apparent defensive role can play important but indirect roles in the phenotypic plasticity of plant stress.

In this study, we examined the transcriptional response of citrus to feeding by the GWSS and compared these transcript profiles to those generated by mechanical wounding using a membrane-based macroarray. To our knowledge, this is the first array analysis targeting perennial fruit tree responses to herbivory by a xylem-feeding insect. Our findings show fundamental differences in transcript expression relating to damage caused by mechanical wounding and damage caused by GWSS feeding aligning with other reports of transcriptional modulation by insect-derived elicitors. Furthermore, we find that citrus responded to GWSS herbivory by increasing transcripts that would take part in JA-independent wounding, abiotic stress, pathogen invasion and resource allocation.

2. Materials and methods

2.1. Plant and Insect maintenance

Twenty-four *Citrus sinensis* (L.) Osbeck cv. 'Madam Vinous' plants (greenhouse-grown; age 24 months) were pruned to allow for emergence of new flush and subsequently placed in an insect-free plant growth chamber at 26 °C/16 h light, 22 °C dark, 70% RH; lighting: 400 W metal Halide, mean intensity 3669 lux. Plants were acclimated to growth chamber conditions for a minimum of 14 d prior to use in the

experiments. During this period, plants were irrigated daily and fertilized using a 14–14–14 standard fertilizer every 14 d.

GWSS were obtained by net collection from several sites in north Florida and consisted of a mixed-age population of adults. Collected insects were maintained on lemon basil, sweet orange, crepe myrtle, trifoliate orange, pigeon pea and several species of cotton (all from seed stock, greenhouse-grown) in a growth chamber under analogous conditions to that of the experimental plant material (see above). Only adult insects were used in the herbivory experiments.

2.2. Insect herbivory treatments

C. sinensis plants exhibiting ~15 cm of new flush were randomly assigned as treatment (GWSS-infested) or control (GWSS-free). For treatment plants, 10 mixed-gender adult GWSS were confined to the new flush using aluminum screen cages (18 × 16 mesh, 0.05 mm wire diameter, 12 cm length × 5 cm diameter). Control plants received the same insect cage minus insects. All plants were kept in a growth chamber under the same conditions used to maintain the insects and plant material. GWSS were allowed 4 h settling time to begin probing after which the feeding time was set to 0 h. At 24, 48 and 96 h of feeding (four replicate plants per time point), the plant material within the insect cage was excised and immediately submerged in liquid nitrogen. Frozen plant material was either processed for RNA extraction immediately or kept at −80 °C. Experiments were performed in three in-time biological replicates.

2.3. Mechanical damage treatments

Experiments for mechanical wounding were carried out in the same manner as the herbivory experiments except autoclaved minuten stainless steel insect pins (dia. 150 μm mm; GWSS stylet complex dia. ~70 μm [3]) were used to simulate probing by GWSS stylets. Ten pins were randomly inserted to a depth of ~1.5 mm into the 15 cm stem tissue of the treatment plants. Each penetration was preceded by 1–4 randomized punctures before settling into its final position in an effort to simulate multiple probings. Pins were kept in position for 24 h, after which they were repositioned. Control plants were handled in the same manner as the treatment without inflicting wounds. Plant tissue was harvested by the same method as the herbivory experiments.

2.4. RNA processing, probe preparation and membrane hybridization

Total RNA was extracted from frozen stem tissue using the RNeasy® plant mini kit following the manufacturer's instructions (Qiagen, Valencia, CA). Prior to array analysis, equal amounts of RNAs from four individual replicates were pooled (same treatment type and time point). Ten micrograms of total RNA was converted to first-strand cDNA in the following 40 μL reaction: 1× first strand buffer, 1 μg oligo $(dT)_{18}$, 0.625 mM dATP, dGTP and dTTP, 80 μCi [^{33}P]dCTP (3000 Ci/mmol; ICN, Irvine, CA), 0.01 M DTT (Invitrogen, Carlsbad, CA), 40 U RNasin (Promega, Madison WI), and 400 U Superscript II (Invitrogen). The mixture was incubated at 42 °C for 1 h after which the RNA template was removed by incubation at 65 °C for 45 min in the presence of 0.4 M NaOH. Labeled cDNAs were separated from unincorporated nucleotides by passage thru a ProbeQuant™ G-50 Micro-column (Amersham Pharmacia Biotech, Piscataway, NJ). Nylon arrays were prehybridized for 3 h at 65 °C in 25 mL of the following buffer: 6× SSPE, 1.0% SDS, 7× Denhardt's solution (Amresco, Solon, OH) and 100 μg/mL denatured salmon sperm DNA (Amresco). Citrus vascular tissue macroarrays (details describing array construction in Bausher et al., manuscript in preparation) were hybridized with probes at 65 °C for 16 h, then washed twice for 15 min in 5× SSC, 0.5% SDS at RT, twice for 15 min in 1× SSC, 1% SDS at 37 °C, then finally three times for 15 min in 0.1× SSC, 1% SDS at 65 °C. Washed arrays were exposed to a phosphor storage screen for 24 h at RT, then scanned at a resolution of 600 dpm using a Cyclone PhosphorImager (Perkin-Elmer, Boston, MA).

2.5. Data acquisition and analysis

The signal intensities for each spot were captured using ArrayVision™ v8.0 (Invitrogen). Following local background subtraction, the intensity values for each spot were globally normalized within and across all three biologically replicated experiments to compensate for non-linearity of intensity distributions and differences in RNA loading and labeling variations. This was accomplished by dividing each spot's intensity by the 50th percentile of all the spot intensities on that array, then dividing by the median of all its measurements from all arrays. Signals less than a value representing two standard deviations above the mean expression of the negative control spots were removed to further increase the significance of the results. Transcripts that responded to herbivory and mechanical damage were identified by analysis of variance (ANOVA; $P < 0.05$) using the three independent biological replicates. A Benjamini and Hochberg false discovery rate multiple testing correction was employed to reduce the number of false positives. Transcripts showing significant ($P < 0.05$) changes were further culled to select those that resulted in expression ratios ≥1.5-fold or ≤0.67-fold when compared to their respective non-treated controls. Fold-cutoffs for valid expression changes were established by a self versus self experiment in which two independent cDNA synthesis reactions were processed from the same RNA sample and hybridized to two different arrays. This resulted in a false positive rate of 1.1% using 1.5-fold change cutoffs. Normalization and statistical analyses were accomplished using GeneSpring (Silicon Graphics, Redwood City, CA) or Excel® (Microsoft, Redman, WA).

2.6. Semi-quantitative RT-PCR

The reliability of the quantitative array data was independently corroborated for 16 representative transcripts using semi-quantitative RT-PCR. RNA samples (pooled samples used

Table 1
PCR primers used in semi-quantitative RT-PCR confirmation analysis

Target gene	Accesion #[a]	Putative description[b]	Forward primer 5′ → 3′	Reverse primer 5′ → 3′
CsVc5	DR909408	Unknown	CTCCTGAAGATAGCAGTAGCAG	CTCCCATTTTGTGGGACAAC
CsVc581	DR911023	Unknown	CCTTGAAGAACATGGAAACTCC	GAAACTAACACACACATCCAG
CsV30D05	DR910512	Unknown	ATTAAACCTCCGCCTTCTCC	CGTGCCGCAGACAAAATAAC
CsV24E08	DR909238	Unnamed protein product	TTCAGTGCGTTCATGCTCTC	TCCAGTCTCGGTGCATAAATC
CsV20D09	DR909655	Ripening regulated protein	TGCCTGATGCTCAAGATGTG	GGTTTTCCTGCCTGCTTTAG
CsV24H01	DR910164	Drought-induced hydrophobic protein	AAGATGGCAGATGGAAGCAC	TAAACACATGATGCCCTTGG
CsV31D09	DR909787	Benzodiazepine receptor	CAACGAGGTCATCAAAGGTG	CCAACAAAGAAGGGAACCAG
CsV33B02	DR908158	Glutathione-*S*-transferase 13 type III	CACAACGGGAAACCAATCTC	TTGCTAGGCCAATAGCTTCC
CsV33D01	DR909742	Putative ABC transporter	TCTGTGCCCATGAAACAAAC	CCTCTTCCACCCATGAGAAC
CsVc12	DR908940	Potato type I proteinase inhibitor	TGTAAACGGGGAGATTGCTG	AATCCTGACCATCCATCCAG
CsVc23	DR910461	Myo-inositol 1-phosphate synthase	CCTTGGCTCCTGAGAACAAC	ACAAAGGCAATGGGCTACAC
CsVc184	DR909980	LEA5 protein	TCTTGCTCCTGTTGCTGATG	TCTGCTGGATCAATCTCGAC
CsVc452	DR908400	lipoxygenase (LOX2)	GATCCAAAAGCTCCTCATGG	CCACGGTTCGAATTTCAGTC
CsVc399	DR909146	Miraculin-like protein 3	ATGGAGGCACAAATGGAGAG	ACGATTCCTCCGGTTGTTAG
CsVc273	DR911044	Miraculin-like protein 2	TTGTCCATTGTCCGAGTGTC	CATTCATTACGCGGTCATTG
CsVc77	DR909103	Acidic chitinase class II	TACTTACGGTGCGGAGAAGG	CGTCATTTGCTGATGGTTTC
EF-1-α	DR908282	Elongation factor-1-alpha	AAGCCCATGGTTGTTGAGAC	CAACAGCAAACTGGTGGAAG

[a] For contiged cDNAs accesion # refers to a single EST within that contig.
[b] Putative descriptions are based on the most current BLASTX match from GenBank®.

in array analysis) were cleaned of contaminating DNA by DNase I treatment at 37 °C for 30 min (GeneHunter®, Nashville, TN). Reverse transcription was carried out on 2 μg of DNase treated RNA using the Superscript™ First-strand synthesis system following the manufacturer's directions (Invitrogen). No-RT controls for each RNA sample were run in parallel to control the presence of genomic template contamination.

PCR primers for each gene tested were designed using the software Primer3 [32] (Table 1). When possible, primers were designed based on the 3′ untranslated region (UTR) consensus sequence of multiple ESTs to avoid co-amplification of related gene family members. All primer pairs were assayed for amplicon specificity to ensure only a single product was generated. PCR was carried out in 15 μL reaction mixtures containing 13 μL of Platinum® PCR SuperMix (Invitrogen), 1 μL of diluted RT reaction (3:1) and 1 μL of primer mix (10 μM each). Cycling conditions consisted of 94 °C (1 min) followed by 24–32 cycles of 94 °C (15 s), 60 °C (15 s), 72 °C (30 s). The optimal number of PCR cycles for which product generation remained linear during amplification was predetermined empirically for each transcript tested by electrophoretic resolution of PCR products generated at increasing cycle numbers (Table 1). All reactions were run in triplicate using three independent biological replications. Amplicons were separated in a 2.0% TAE agarose gel stained with ethidium bromide and fluorescent images captured using a Kodak 440 Image station (Eastman Kodak Company, Rochester, NY). Normalization of cDNA loading was accomplished using primers for elongation factor-1-alpha (EF-1-α; Table 1). Quantitative data were obtained using Kodak 1D image analysis software (Eastman Kodak). Band intensities were normalized by dividing the target genes intensity value by the intensity value of the corresponding EF-1-α band amplified from the same cDNA sample. All

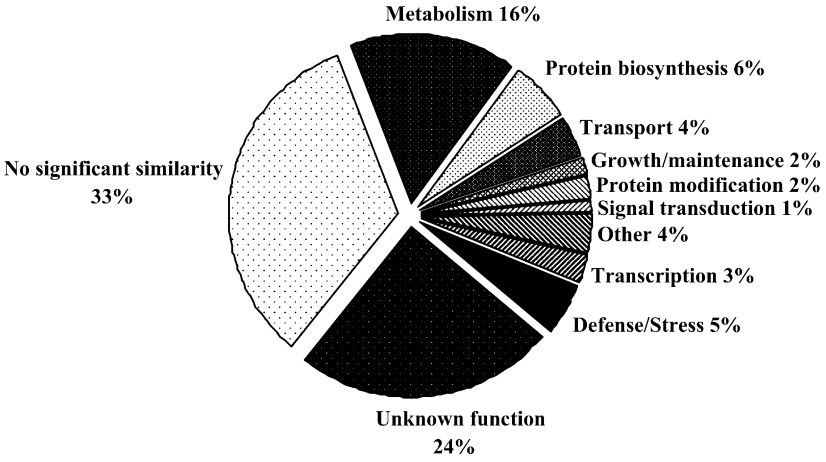


Fig. 1. Distribution of spotted cDNAs on the citrus array based on putative molecular function. BLASTX similarity searches to the public protein database GenBank® coupled with gene ontology (http://www.geneontology.org) molecular function data were used to categorize the citrus cDNAs. Percentages are based on non-redundant transcripts obtained by cluster analysis of ESTs.

amplicons were sequenced to determine primer specificity (data not shown).

3. Results

3.1. Description of the macroarray and experimental protocol

We employed a cDNA-based macroarray array constructed with 3425 randomly selected cDNAs from a *C. sinensis* (cv. Valencia) vascular tissue cDNA library. Based on cluster analysis, the spotted cDNAs correspond to ~1731 unique genes representing a diverse set of molecular functions (Fig. 1). A significant number of the genes (1025 or 55%) either have matches to proteins with functions that are unknown or have no significant match (BLASTX; $e > 10^{-10}$) to proteins currently deposited in the public database GenBank®, creating an environment rich in potential for discovery of novel defense/stress genes from woody perennial sources.

Experimental RNAs consisted of a pool of four independent plants subjected to the same treatment—a time course of 24, 48 and 96 h of GWSS herbivory or mechanical damage (see Section 2 for details). Untreated controls were included for each time point to provide a means of limiting the effect of time-induced plant variation unrelated to the treatments. Because of the stringent criteria used for data acceptance (see Section 2), it is likely that several important transcripts showing differential expression during our treatments were eliminated. For instance, small changes in expression (1.2–1.5-fold) of mRNAs coding for transcription factors can exert great changes in downstream gene expression leading to a substantial biological effect [33].

A self versus self hybridization experiment to assess systematic variation resulted in a calculated correlation coefficient of 0.97, suggesting a high degree of reproducibility between individual arrays (Fig. 2). Applying our fold-change selection criteria to this analysis resulted in 42 cDNAs with a greater than 1.5-fold difference in expression. This suggests that 1.1% of the differentially regulated cDNAs may be mis-categorized due to array systematic error. However, it must be noted many of the cDNAs spotted on the array are redundant clones of a unique transcript and it is likely that when extrapolated to clustered transcripts, the array systematic error reported here would be lower.

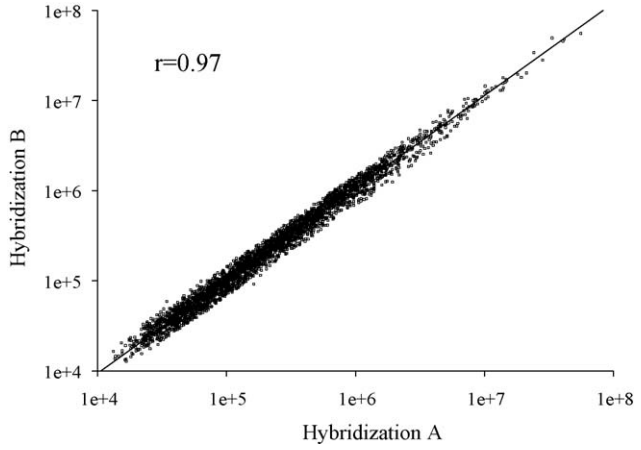


Fig. 2. Scatterplot analysis of systematic variability. Signal intensities of two hybridization events (A and B) whose probes were generated from the same RNA sample (untreated control) and hybridized to two independent citrus vascular arrays are plotted. The calculated correlation coefficient ($r = 0.97$) suggests minimal variability between arrays.

3.2. Differentially regulated transcripts

A total of 226 citrus cDNA clones, representing 50 unique transcripts based on cluster alignment, were found to be significantly ($P < 0.05$) induced or repressed ≥1.5-fold by GWSS feeding in at least one time point. In most cases, individual cDNA members of a responsive transcript cluster showed similar expression patterns, further confirming the significance of the analysis.

To separate GWSS specific responses from that of wounding, we compared the effects of mechanical damage to herbivory. Of the 50 GWSS-responsive transcripts, 14 (27%) were also differentially regulated by mechanical damage, though in most cases the degree of change was considerably less (Table 2). For genes induced by both treatments, at no time point tested was the expression higher in mechanical damaged plants than in herbivory plants. One exception was a transcript matching a ribosomal protein L2 (GenBank accession no. NP_084775; $e - 10^{-15}$) that showed greater repression at 96 h by mechanical damage. The Venn diagrams (Fig. 3) provide a graphical overview of the differential regulation across the three treatment time points for both GWSS-infested and mechanical damage treatments. Globally, the maximum level of responsiveness for most transcripts was detected after 96 h (Fig. 4); however, six showed the largest change at 48 h and two at 24 h. Of the 50 responsive transcripts, 30 were up-regulated and 20 were down-regulated in relation to untreated control plants. None of the 20 down-regulated genes showed significance ($P < 0.05$) at 24 h in either GWSS-infested or mechanical damaged tissues, whereas 14 of the 30 up-regulated genes were responsive at least 1.5-fold at the same time point.

3.3. Transcript categories

Putative functions for the 50 responsive transcripts were deduced by comparing translated nucleotide sequences from the corresponding ESTs (expressed sequence tag) to GenBank® using BLASTX [34]. This allowed us to consolidate the responsive transcripts into eight broad categories based on putative function: defense/stress related, signal pathway, radical scavenging, photosynthesis, transport, structural, other and proteins with unknown function or sequences with no significant similarity to the public protein databases.

3.3.1. Defense/stress related

Strong induction of six different putative trypsin proteinase inhibitors (PI) was observed in GWSS infested plants with a corresponding significant, but weaker response by mechanical damage treatments (Table 2). Three of the six putative PIs

Table 2
Transcripts responding to GWSS herbivory and/or mechanical damage

			Ratio of expression [d]					
			GWSS herbivory			Mechanical damage		
			24 h	48 h	96 h	24 h	48 h	96 h
Clone Id [a]	Accession # [b]	Putative description [c]	FC ± SE	FC ± SE	FC ± SE	FC ± SE	FC ± SE	FC ± SE
Defense/Stress								
CsVc148	DR909506	Miraculin-like protein	3.07 ± 0.44	7.59 ± 1.99	7.08 ± 1.60	1.87 ± 0.22	2.71 ± 0.37	2.47 ± 0.76
CsVc273	DR911044	Miraculin-like protein 2	2.55 ± 0.43	5.46 ± 0.90	5.27 ± 1.31	1.55 ± 0.17	2.61 ± 0.38	2.36 ± 0.73
CsVc399	DR909146	Miraculin-like protein 3	3.77 ± 0.48	6.27 ± 1.08	6.92 ± 3.25	2.28 ± 0.49	2.54 ± 0.57	2.35 ± 0.83
CsVc4	DR908110	Soybean trypsin inhibitor	0.97 ± 0.15	1.08 ± 0.08	2.47 ± 0.71	1.28 ± 0.10	1.46 ± 0.39	1.84 ± 0.62
CsV20A10	DR908252	Miraculin-like protein 2	3.19 ± 0.44	6.76 ± 1.86	6.70 ± 2.31	2.04 ± 0.24	3.83 ± 1.20	3.41 ± 1.75
CsVc12	DR908940	Potato type I proteinase inhibitor	1.70 ± 0.18	2.18 ± 0.23	5.29 ± 0.39	1.41 ± 0.12	1.91 ± 0.45	2.11 ± 0.50
CsVc77	DR909103	Acidic chitinase class II	2.32 ± 0.08	2.13 ± 0.44	3.46 ± 1.23	1.66 ± 0.29	1.67 ± 0.11	2.08 ± 0.74
CsV24H01	DR910164	Drought-induced hydrophobic protein	2.02 ± 0.54	1.72 ± 0.16	2.89 ± 0.26	1.29 ± 0.10	1.07 ± 0.17	0.92 ± 0.17
CsVc184	DR909980	LEA5 protein	1.37 ± 0.16	1.02 ± 0.11	2.41 ± 0.73	1.05 ± 0.26	0.89 ± 0.20	1.21 ± 0.10
CsV7A07	DR908351	Heat shock protien (HSP70)	1.38 ± 0.37	0.79 ± 0.07	0.42 ± 0.02	0.89 ± 0.01	0.82 ± 0.15	0.86 ± 0.13
CsV28B11	DR911002	Aspartic protease family protein	0.77 ± 0.03	0.66 ± 0.02	0.54 ± 0.08	0.98 ± 0.08	1.14 ± 0.07	0.85 ± 0.24
Signal Pathway								
CsV3G12	DR908475	Calmodulin	0.97 ± 0.15	1.57 ± 0.34	2.22 ± 0.60	1.35 ± 0.16	1.16 ± 0.07	1.37 ± 0.23
CsVc452	DR908400	Lipoxygenase (LOX2)	1.55 ± 0.13	1.59 ± 0.20	2.33 ± 0.56	1.19 ± 0.07	1.15 ± 0.07	1.30 ± 0.17
CsV31D09	DR909787	Peripheral-type benzodiazepine receptor	1.35 ± 0.19	1.88 ± 0.12	2.88 ± 0.25	1.23 ± 0.20	1.30 ± 0.07	1.35 ± 0.26
CsV17D04	DR909259	Ring zinc finger protein (C3HC4 type)	1.40 ± 0.16	1.70 ± 0.26	1.72 ± 0.09	0.92 ± 0.14	1.10 ± 0.14	1.31 ± 0.01
CsV14F08	DR909008	Scarecrow-like protein 6	1.34 ± 0.24	0.68 ± 0.05	0.54 ± 0.05	1.01 ± 0.03	0.83 ± 0.10	0.79 ± 0.14
Radical Scavenging								
CsV33B02	DR908158	Glutathione S-transferase 13 type III	1.40 ± 0.14	1.50 ± 0.11	2.44 ± 0.25	0.90 ± 0.19	0.84 ± 0.01	1.00 ± 0.10
CsVc30	DR908294	Dehydroascorbate reductase	1.15 ± 0.14	1.40 ± 0.02	1.88 ± 0.05	1.00 ± 0.06	1.16 ± 0.14	1.19 ± 0.14
Photosynthesis								
CsV33E11	DR908553	RuBisCO subunit binding protein	0.68 ± 0.04	0.66 ± 0.01	0.89 ± 0.07	0.92 ± 0.02	0.85 ± 0.03	0.71 ± 0.05
CsVc47	DR911533	Photosystem I assembly protein ycf3	0.86 ± 0.08	0.49 ± 0.04	0.66 ± 0.20	0.76 ± 0.06	0.88 ± 0.06	0.58 ± 0.13
Transport								
CsV33D01	DR909742	Putative ABC transporter	1.44 ± 0.12	1.52 ± 0.22	2.50 ± 0.59	1.11 ± 0.20	1.26 ± 0.20	1.22 ± 0.07
CsV26E11	DR909205	Oligopeptide transporter protein	0.99 ± 0.07	1.14 ± 0.06	1.81 ± 0.34	0.93 ± 0.11	1.05 ± 0.08	1.01 ± 0.03
CsVc335	DR909579	Aquaporin (PIP1C)	0.79 ± 0.12	0.73 ± 0.01	0.65 ± 0.09	1.02 ± 0.02	1.03 ± 0.13	0.81 ± 0.14
CsVc380	DR909695	Delta-tonoplast intrinsic protein (TIP)	0.97 ± 0.09	0.67 ± 0.05	0.58 ± 0.06	0.92 ± 0.06	1.07 ± 0.16	0.77 ± 0.13
Structural								
CsVc17	DR908476	Extensin precursor-like protein	1.55 ± 0.25	1.00 ± 0.06	2.26 ± 0.45	1.20 ± 0.09	1.36 ± 0.22	1.30 ± 0.17
CsV34H08	DR911055	Proline-rich protein	0.95 ± 0.11	0.58 ± 0.04	0.61 ± 0.21	0.88 ± 0.14	1.03 ± 0.13	0.76 ± 0.12
CsVc549	DR910878	Expansin	1.16 ± 0.08	0.74 ± 0.08	0.63 ± 0.08	1.17 ± 0.05	0.88 ± 0.09	0.81 ± 0.05
CsVc476	DR909987	Arabinogalactan protein	0.85 ± 0.05	0.63 ± 0.05	0.58 ± 0.14	0.96 ± 0.15	1.03 ± 0.18	0.78 ± 0.10
CsV30F11	DR909185	Ribosomal protein L2	0.89 ± 0.17	0.51 ± 0.07	0.64 ± 0.16	0.74 ± 0.07	0.77 ± 0.07	0.51 ± 0.11
CsVc512	DR908987	Ribosomal protein S19	0.98 ± 0.14	0.56 ± 0.08	0.74 ± 0.25	0.83 ± 0.09	0.98 ± 0.16	0.78 ± 0.10
Other								
CsV20D09	DR909655	Ripening regulated protein	3.26 ± 0.98	5.51 ± 1.09	7.11 ± 2.49	1.56 ± 0.07	2.53 ± 0.41	2.50 ± 0.82
CsVc23	DR910461	myo-inositol 1-phosphate synthase	1.30 ± 0.40	2.17 ± 0.42	1.51 ± 0.30	1.29 ± 0.31	1.67 ± 0.23	1.38 ± 0.16
CsV22G09	DR910509	Putative gamma-glutamyl hydrolase	1.40 ± 0.14	1.43 ± 0.04	1.63 ± 0.23	1.17 ± 0.11	1.20 ± 0.18	1.12 ± 0.10
CsV26C11	DR908270	Plastid development protein	0.73 ± 0.03	0.61 ± 0.05	0.76 ± 0.09	0.87 ± 0.01	0.98 ± 0.04	0.75 ± 0.03
Unknown function/NSS								
CsV22A08	DR911226	Unknown Protein	1.40 ± 0.23	1.54 ± 0.15	1.83 ± 0.26	1.16 ± 0.12	1.09 ± 0.17	1.03 ± 0.17
CsV31G10	DR908425	Unknown Protein	2.17 ± 0.36	1.99 ± 0.43	1.62 ± 0.34	0.89 ± 0.12	0.90 ± 0.09	1.01 ± 0.07
CsVc581	DR911023	Unknown Protein	1.40 ± 0.13	2.60 ± 0.12	3.96 ± 1.02	1.08 ± 0.07	1.28 ± 0.07	1.66 ± 0.33
CsVc3	DR910209	Unknown Protein	1.92 ± 0.30	2.44 ± 0.26	2.30 ± 0.80	1.73 ± 0.40	1.35 ± 0.08	1.73 ± 0.23
CsVc5	DR909408	Unknown Protein	1.07 ± 0.10	1.51 ± 0.20	3.51 ± 1.29	1.02 ± 0.02	1.31 ± 0.10	1.34 ± 0.25
CsV28F04	DT660123	Unknown protein	1.09 ± 0.32	1.61 ± 0.29	2.28 ± 0.69	1.27 ± 0.11	1.59 ± 0.67	1.71 ± 0.49
CsV24E08	DR909238	Unknown Protein	1.13 ± 0.21	1.29 ± 0.07	2.39 ± 0.73	1.28 ± 0.21	1.02 ± 0.28	0.95 ± 0.16
CsVc297	DR909207	Unknown Protein	0.82 ± 0.04	0.73 ± 0.05	0.63 ± 0.18	1.07 ± 0.12	1.08 ± 0.07	0.95 ± 0.06
CsVc69	DR908549	Unknown Protein	1.13 ± 0.26	0.61 ± 0.02	0.34 ± 0.07	1.18 ± 0.07	0.98 ± 0.15	0.77 ± 0.24
CsV30D05	DR910512	NSS	1.18 ± 0.27	1.53 ± 0.17	2.48 ± 0.68	1.09 ± 0.05	1.28 ± 0.26	1.26 ± 0.07
CsVc11	DR910082	NSS	1.16 ± 0.14	0.81 ± 0.06	1.77 ± 0.27	0.97 ± 0.24	0.69 ± 0.07	1.17 ± 0.07
CsVc54	DR908941	NSS	1.15 ± 0.23	0.82 ± 0.15	0.82 ± 0.16	1.06 ± 0.08	0.66 ± 0.16	0.57 ± 0.16
CsVc8	DR908967	NSS	1.47 ± 0.34	0.52 ± 0.05	0.43 ± 0.10	0.94 ± 0.03	0.86 ± 0.09	0.90 ± 0.22
CsV28C08	DT660122	NSS	1.14 ± 0.05	0.62 ± 0.05	0.83 ± 0.10	1.00 ± 0.04	0.80 ± 0.01	0.89 ± 0.11
CsV8F09	DR911197	NSS	1.24 ± 0.28	0.62 ± 0.08	0.20 ± 0.05	0.86 ± 0.21	0.69 ± 0.10	0.70 ± 0.18
			Color key					
			>5.0	5.0-1.5	1.5-0.67	0.67-0.2	?0.20	

FC-fold change. [a]cDNAs used for semi-quantitative RT-PCR confirmation experiments are underlined. [b]For clustered cDNAs the accession # refers to a single EST within that cluster. [c]Putative function is based on the most current BLASTX match to the protein database GenBank; NSS-no significant similarity. [d]Shown are the mean ± standard error (S.E.) of signal intensity ratios between treated and untreated RNAs based on three independent biological replications as described in Section 2.

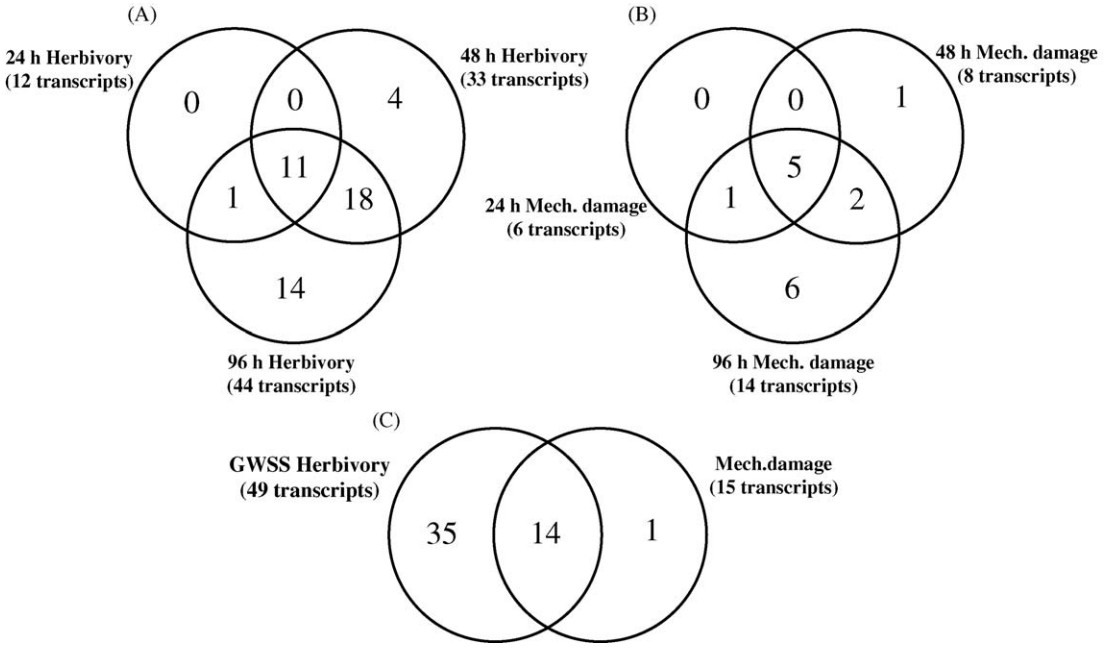


Fig. 3. Venn diagrams comparing responsive transcripts. (A) Diagram showing the distribution of responsive transcripts following GWSS herbivory for the indicated time course 24, 48 and 96 h. (B) Transcripts responding to mechanical damage treatments for the same time course 24, 48 and 96 h. (C) Overlap between herbivory and mechanical damage. The numbers in the overlapping areas indicate the shared number of genes in the comparisons and numbers outside the overlapping area represent transcripts specific to that treatment.

exhibited strong similarity to the miraculin-like proteins (Legume Kunitz inhibitors) type 1 (GenBank Accession no. AAG38517; $e-128$), type 2 (GenBank Accession no. AAG38518; $e-86$) and type 3 (GenBank Accession no. AAG38519; $e-88$) from *Citrus paradisi* Macf. [35]. Together they showed three-fold increases beginning at 24 h, reaching 5–7-fold by 96 h of herbivory. One additional PI transcript showing weak similarity again to miraculin-like protein type 2 ($e-19$), was also induced strongly by GWSS herbivory, modeling that of the previous three. In mechanically damaged plants, these same transcripts also showed induction; however, expression ratios were in most cases half that of herbivory treatments. Interestingly, not all PIs were induced during the 24 and 48 h treatments as evidenced by the expression of a transcript once again with similarity ($e-40$) to the miraculin-like type 2 proteins. It was up-regulated only after 96 h of treatment by both herbivory and mechanical damage, suggesting an alternative role for this family member that is disparate from the PIs described early.

Neither GWSS feeding nor mechanical damage changed the expression of the acidic form of the PR proteins β-1, 3-glucanase (PR-2; GenBank accession no. XP_483019; $e-44$), or thaumatin-like protein (PR-5; P50699; $e-61$) (data not show); however, acid chitinase (PR-3; GenBank accession no. CAA93847 $e-133$) did show greater than two-fold repeatable induction across all time points in GWSS-infested plants and a corresponding, but weaker increase by mechanical damage. Increased PR protein expression is regarded as a signature marker for SA-dependent signaling and is commonly observed in plants attacked by sap-feeders and pathogens [10,24].

GWSS Herbivory induced several abiotic stress genes and reduced others. A transcript with similarity to a drought-

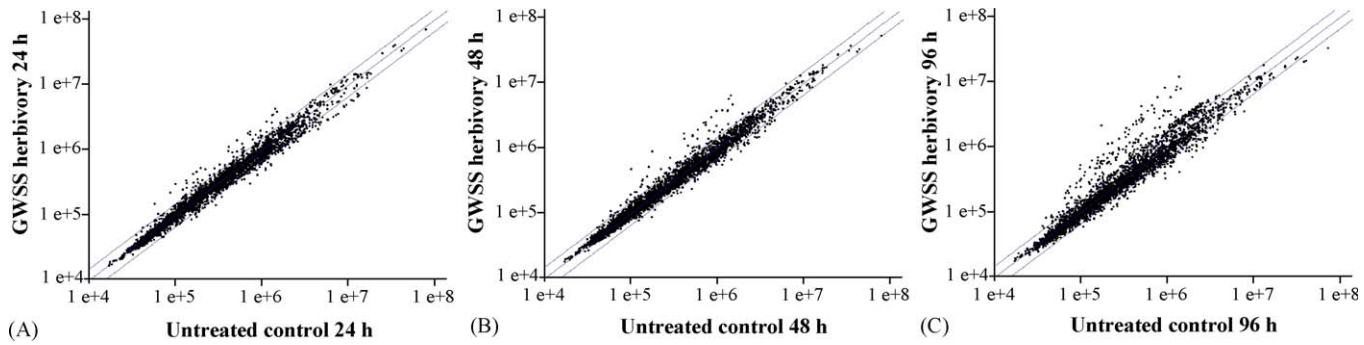


Fig. 4. Scatterplot analyses of signal intensity for herbivory treatments. Shown are mean normalized signal intensities for three independent biological replications. GWSS herbivory treatments are plotted against untreated control for (A) 24 h, (B) 48 h and (C) 96 h time points. For comparison of signal variation, guidelines were added to indicate 1.5-fold increase or decrease in expression.

induced hydrophobic protein from *Poncirus trifoliata* (L.) (GenBank accession no. AAS72306; $e-20$) was induced > 2-fold throughout all time points, but showed no regulation in mechanically damaged plants. Also specific to GWSS feeding was the induction of a late embryogenesis abundant protein 5 (LEA5; GenBank accession no. CAA86851; $e-47$) that showed two-fold increase in expression after 96 h. The heat shock protein HSP70 (GenBank accession NM_124369; $e-48$) was moderately repressed at 96 h of GWSS feeding as was a transcript matching an aspartic protease (GenBank accession no. AJ313385; $e-45$) at 48 and 96 h. Neither showed expression changes in plants challenged by mechanical damage.

3.3.2. Signaling pathways

Specific to GWSS feeding, lipoxygenase (LOX; BAB84352; $e-106$) showed a weak increase in transcript accumulation at 24 and 48 h, with a marked rise to 2.3-fold by 96 h. Interestingly, a concomitant increase in several other enzyme members of the JA biosynthetic pathway was not observed (data not shown). Transcripts matching the Ca^{2+} binding protein calmodulin (GenBank accession no. AAM81202; $e-79$) showed induction after 48 h increasing linearly through 96 h in infested plants, but not mechanically damaged plants as did transcripts matching a peripheral-type benzodiazepine receptor (PBR; GenBank accession no. CAH10765; $e-31$). The expression of two transcription factors was also changed by GWSS herbivory. A transcript matching a putative RING zinc finger C3HC4 type protein (GenBank accession no. AAM19707; $e-20$) was weakly induced (1.7-fold), while a transcript matching a scarecrow-like protein 6 (SCL6; GenBank accession no. AAD24406; $e-42$) was repressed. Neither was responsive to mechanical damage.

3.3.3. Radical scavenging

Transcript levels of the oxidative stress genes glutathione-*S*-transferase (GST13 type III; GenBank accession no. AAG16758; $e-65$) and GSH-dependant dehydroascorbate reductase (DHAR; GenBank accession no. AAL71857; $e-93$) increased modestly as a result of herbivory at 96 h, but remained unchanged by mechanical damage, suggesting that accumulation and detoxification of reactive oxygen species (ROS) is actively occurring in GWSS-infested plants. Interestingly, four other GST homologs represented on the array were unresponsive to our treatments (data not shown), suggesting defined roles for GST family members in defense/stress.

3.3.4. Photosynthesis-related

Repression of transcripts for photosynthesis-related proteins by insect attack, mechanical wounding, pathogen infection and exogenous hormone application has been previously reported [36–38]. Consistent with these findings, our results show a transient down-regulation of the α-subunit of RuBisCO (GenBank accession no. P08926; $e-97$) and the photosystem I assembly protein ycf3 (GenBank accession no. AAZ03964; $e-17$) following GWSS feeding. Only ycf3 was similarly repressed in mechanically damaged plants. It is presumed that this regulation allows reallocation of energy towards defense induction allowing for a more aggressive response directed at the attacker.

3.3.5. Transport

GWSS herbivory induced several proteins with putative function in biochemical and water transport. A putative ATP-binding cassette (ABC) transporter (GenBank accession no. XP_450985; $e-17$) and an oligopeptide transport protein (GenBank accession no. AAM10330; $e-53$) increased modestly after 96 h; however, neither transcript was changed by mechanical damage. In contrast, two transcripts matching aquaporins (GenBank accession nos. CAE53882, AAC39480; $e-85$, $e-64$) were repressed by GWSS herbivory at 96 h, but not by mechanical damage, implying shifts in water homeostasis specific to GWSS feeding.

3.3.6. Structural

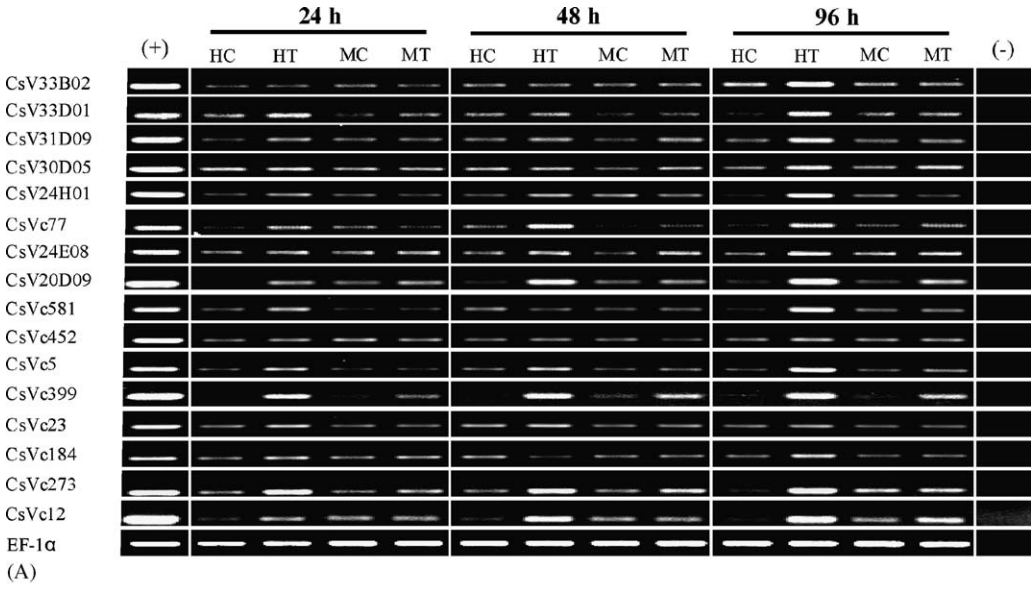
Transcripts matching expansion (GenBank accession no. AAP48989; $e-24$) and an arabinogalactan protein (GenBank accession no. CAC16734; $e-40$), both of which are implicated in cell growth and development and more recently in cell-to-cell signaling [39], were weakly repressed in GWSS infested plants. Additionally, herbivory reduced the transcript level of a proline-rich protein (PRP; GenBank accession no. AAD03487; $e-14$) also involved with the cell wall mechanics and development. A transcript weakly matching the hydroxyproline-rich glycoprotein (HRPG) extension (GenBank accession no. S54157; $e-24$) was increased slightly at 24 h and moderately at 96 h, but remained unchanged at 48 h, suggesting a biphasic response. At no time was the expression of these transcripts changed in plants subjected to mechanical damage.

3.3.7. Transcripts with no defined category

A transcript matching a ripening regulated protein from tomato (GenBank accession no. AAG49030; $e-54$) was strongly induced by herbivory and also by mechanical damage though at a reduced magnitude. The expression pattern for this gene is strikingly similar to that observed for the PI transcripts, suggesting an association with wounding or defense pathways. GWSS herbivory induced a minor accumulation of a transcript weakly matching a putative γ-glutamyl hydrolase (GGH; GenBank accession no. XP_475718; $e-10$), after 96 h of feeding. Both herbivory and mechanical damage induced a transcript matching a myo-inositol 1-phosphate synthase (MIPS; GenBank accession CAA83565; $e-21$) that peaked after 48 h of treatment and was only slightly increased after 96 h. MIPS catalyze the rate regulating step in the conversion of glucose 6-phosphate to L-myo-inositol 1-phosphate by cyclization [40] and has been implicated in signaling as well as in the synthesis of D-glucuronate used in the production of cell wall components, glycoproteins, gums, and mucilage [41].

3.3.8. Genes with unknown function or no significant match

We were unable to assign function to 15 GWSS-responsive transcripts as they matched proteins with unknown functions



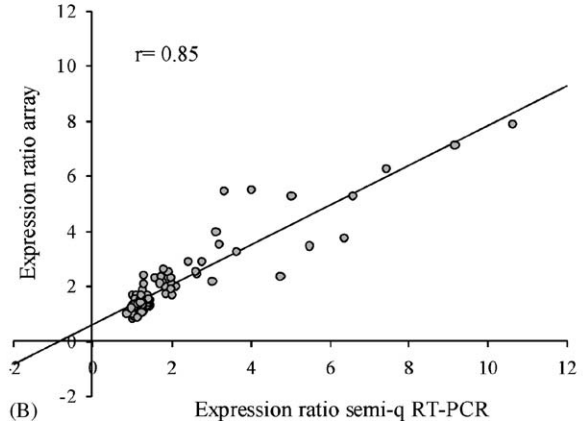


Fig. 5. Semi-quantitative RT-PCR confirmation for 16 selected transcripts responsive to GWSS herbivory and/or mechanical damage. (A) Total RNAs isolated from *C. sinensis* stems subjected to 24, 48 or 96 h of GWSS herbivory (HT) or mechanical damage (MT) and their respective untreated controls (HC, MC) were used as templates for RT-PCR. For each transcript, the optimum cycle number for analysis was predetermined empirically and reflected maintenance of linear amplification. Elongation factor-1α (EF-1α) was used to control RNA loading. Vertical white lines are to simplify viewing, all bands within a horizontal gel image were electrophoresized together. (B) Scatterplot showing the correlation ($r = 0.85$) of the mean expression ratios (3 independent biological replicates) obtained by array analysis (y-axis) to those obtained by semi-quantitative RT-PCR confirmation analysis (x-axis) for the 16 transcripts subjected to secondary confirmation. For each transcript comparisons were made at each time point (24, 48 and 96 h) for both herbivory and mechanical damage treatments.

or failed to match any protein sequence in the public database GenBank®. Six were down-regulated and nine were induced by at least one of the treatments. Of the 16, twelve were GWSS-specific, whereas three responded to both treatments. One gene responded negatively to mechanical damage treatments but not herbivory, indicating a potential role in abiotic stress.

3.4. Semi-quantitative RT-PCR confirmed array data

Sixteen of the 50 responsive transcripts (31%) were selected to independently confirm the reliability of array results by semi-quantitative RT-PCR (Fig. 5A). In most cases (13 of 16), the expression patterns obtained with RT-PCR were consistent to those obtained by array screening. Also, the RT-PCR results showed similarity to the array data with respect to the time course of expression. To determine the overall correlation between RT-PCR and array results, the expression ratios for array data were plotted against expression ratios of RT-PCR data. The calculated correlation coefficient of 0.85 (Fig. 5B) confirms the overall utility of our array-based strategy to identify genes whose expression is affected by GWSS herbivory.

4. Discussion

When comparing mechanical damage to GWSS herbivory, it was apparent that for transcripts responsive to both treatments the magnitude of change was, in most cases, larger in GWSS challenges (Table 2). In addition, considerably more responsive

transcripts were specific to herbivory. While we considered our mechanical wounding treatments to be more physically damaging than GWSS stylet insertion (i.e. pin size 2× larger than the GWSS stylet complex; non-targeted pin insertion), it is possible that GWSS probings occur more frequently than we set out to mimic and that the cumulative damage from this process elicited a stronger transcriptional response. Otherwise, it is conceivable and perhaps more likely that this stronger response was the effect of GWSS-derived elicitors in combination with the physical damage associated with feeding. Modulation of transcription by insect-derived elicitors has been well documented for chewing herbivores and more recent evidence suggests a similar mechanism by sap-feeding insects. For example, Moran and Thompson [24] reported that the transcriptional responses to aphids are distinct from those of mechanical damage in *Arabidopsis* and that this perception is likely through saliva components or metabolites of endosymbiotic bacteria. Similarly, in whitefly–plant interactions, van de Venn et al. [42] reported two genes, SLW1 and SLW3 that appear to respond to species-specific signals or even different life stages of the same species. It should be noted that because the GWSS used in this study were field-collected, we cannot be certain as to the level of microbial contamination present on or within the insects, and therefore it is possible that some of the responses observed here reflect changes resulting from elicitors of these associated pathogens.

GWSS herbivory induced transcriptional responses characteristic of wounding, specifically up-regulation of PIs and repression of photosynthetic proteins. However, changes in transcripts matching several JA biosynthetic enzymes (i.e. allene oxide synthase, allene oxide cyclase, phospholipase D), or the JA/ethylene-regulated antimicrobial peptide defensin (PDF1.2), which are considered among the signature genes of JA-dependant signaling in a number of other plant species, were not altered (data not shown). This implies that the wound/defense response which occurred here may be independent of JA. This is consistent with what has been reported for other sap-feeding herbivores [28,30,31], bolstering the proposal that limited physical damage, characteristic of this feeding method, avoids the activation of JA-dependant wound signaling. Interestingly, GWSS feeding did induce transcripts of LOX-isoforms of which are implicated in JA biosynthesis [13,43]. However, it is unclear if the citrus LOX homolog (CsVc452) induced here participates in this role of another role unrelated to JA biosynthesis. In tomato, for example, antisense depletion of a LOX homolog decreased expression of PI genes, but not through its involvement with JA as JA levels were similar in both antisense and wild-type plants [44].

While large amount of data, principally related to model plant systems, indicates that JA is the principle defense-signaling molecule, it is possible that for situations of minimal wounding such as sap-feeding herbivory, other signaling compounds may orchestrate defense cascades. For example, endogenous ethylene not only has been shown to act as a cross-talk regulator with JA [13,45,46], it has also been shown to induce defense gene expression independent of JA. Enhanced production of ethylene has been reported in aphid-infested barley, which indicates active biosynthesis for this phytohormone in events of minimal wounding [47]. It is possible that ethylene is active in GWSS-infested citrus tissues. For example, we found transcript accumulation of extensin, homologs of which has been shown to be regulated by ethylene in tobacco, tomato and *Arabidopsis* [47,48]. Also a transcript with homology to ripening-regulated protein DDRT8 from tomato was strongly induced in GWSS-infested tissues. However, at this stage, further research is needed to better define a possible role for ethylene in defense transcript regulation in GWSS-infested citrus.

The induction of two enzymes involved with radical scavenging suggested that reactive oxygen species (ROS) were accumulating in GWSS-infested citrus tissue. These inductions may be specific to GWSS feeding as we did not observe a corresponding increase in mechanically damaged plants. ROS are generated in the initial steps of the response of plants to pathogen attack, inducing the so-called hypersensitive response. Additionally, ROS can initiate the biosynthesis of ethylene and other signaling molecules [47,49]. Calcium ion influx has also been implicated in initial steps of oxidative signaling [50,51]. GWSS feeding induced a transcript matching calmodulin, a calcium binding protein, which supports the idea of calcium ion participation possibly playing a part in initiation of stress signaling in GWSS infested citrus. Interestingly, some ROS may act alone to induce defense gene expression directly. For example, plants supplied with a biochemical H_2O_2 generation system were found to up-regulate defense genes, while genes coding for signaling pathways were unchanged [52]. Similarly, inhibitors of H_2O_2 production blocked induction of PIs, but not JA signaling pathway genes [53]. We postulate that induction of GST13 and DHAR may be an attempt to limit the damage by ROS or perhaps, more intriguingly, a method that regulates the level of signal transduction initiated by these compounds.

It has been proposed that aphids and whiteflies are perceived as pathogens due to the similarities between stylet insertion and fungal hyphal growth [10]. More recent evidence indicates that pathogens associated with the feeding insect may also take part in a plants' perception [25]. Consequently, plants responding to herbivory from these insects by display transcript profiles that resemble pathogen attack, characterized with increased transcription of PR proteins mediated by SA-dependant signaling [10]. Interestingly, GWSS feeding did not change expression levels of citrus homologs to PR-2 and PR-5 (data not shown; PR-1 was not tested) contrasting our expectation. However, GWSS did significantly increase the levels of acidic PR-3 in all three time points tested. Whether this indicates active SA signaling in GWSS-infested citrus is not clear. Active SA-signaling has been shown to suppress JA-signaling in numerous species [19,54,55], which could also explain the lack of JA-dependent responses in GWSS-infested citrus.

It is possible that several isoforms for both PR-2 and PR-5 exist in the citrus genome and that transcripts tested here play other roles that are distinct from stress/defense. It is interesting to note that in mechanically damaged plants, PR-3 transcripts showed a delayed induction (96 h) suggesting that minimal

wounding alone may be sufficient to stimulate active SA-signaling in citrus, although signaling is likely increased by GWSS-elicitors associated with feeding.

Often, insect herbivory elicits transcript profiles that partially overlap with those responsive to abiotic stresses such as drought, low temperatures, heat shock and others. While the overlap with drought stress is particularly evident in studies examining chewing insect herbivory, sap-feeding insects can also cause substantial water loss even though they generate little mechanical damage. For GWSS, feeding rates have been calculated at 2.2 mL/d/insect [2]. At this rate, heavy infestations will significantly affect water homeostasis. Therefore, it is not unexpected that in GWSS challenged citrus, we observed transcriptional changes in several genes previously shown to respond to drought-stress. For instance, a transcript matching a drought-induced hydrophobic protein (DRT) was up-regulated. In *Arabidopsis*, homologs of DRT maintain membrane integrity during drought-stress challenges and are transcriptionally induced by ABA-dependent and -independent pathways [56]. Zhu-salzman et al. [31], working with *S. bicolor*, reported the induction of a DRT homolog following aphid herbivory, but not by exogenous application of JA or SA, suggesting that DRT regulation is distinct from typical defense signaling pathways and is most likely prompted by aphid elicitors.

GWSS feeding also increased a LEA5 that was not responsive to mechanical damage, contrasting previous reports that show transcriptional modification of LEA homologs by wounding [57,58]. LEA proteins cover a number of loosely related groups based on sequence similarity and are generally associated with desiccation, though several groups have no defined function [59,56]. LEA group 5 proteins are atypical in that this family displays a distinctive hydropathic amino-terminal and a hydrophilic carboxy terminal, in addition to a 12 amino acid motif that shares no similarity with other LEA classes [59]. Recent evidence has suggested that a LEA5 homolog in rice acts as zinc finger transcription factor involved in the GA-induced expression of the α-amylase gene [60]. In *Arabidopsis*, a LEA5 protein has been shown to be responsive to Nep1, a fungal protein, suggesting its potential as a pathogen-responsive gene-signaling factor [61]. Based on this newer evidence, we are tempted to hypothesize that the citrus LEA5 homolog induced here may, in addition to other civilian roles, function in defense signaling that is disparate from abiotic stress. Further examination will be required to better define its role in plant response to stress.

Aquaporin expression was repressed by GWSS feeding but not mechanical damage, further suggesting a response to a decrease in water potential within GWSS challenged tissues. Aquaporins facilitate osmosis by forming water-specific pores through which passage is dependent on the water potential gradient outside cells [62]. While it is intuitive to surmise that plants under water-stress would decrease aquaporin expression this is in contrast to several herbivory studies. For instance, both *M. sexta* feeding on tobacco [63] and spider mite (*Tetrayicus urticae* Koch) herbivory on lima bean [64] induce expression of aquaporin homologs. While the reason for this difference is unclear, one report in tobacco suggests that aquaporin expression may depend on active JA-dependent signaling. The authors found that plants expressing antisense-LOX repressed aquaporin expression, implying dependence for octadecanoid-mediated signaling for their elicitation [65]. Based on this more recent data, it is possible that the inconsistency between our findings and other herbivory studies may depend on the presence of active JA signaling or possibly other signaling cascades for which an association with aquaporin expression has yet to be revealed.

When attacked by herbivores, plants re-route and store resources, remobilizing them when conditions are more favorable. Our analysis showed the induction of two citrus transcripts matching an ATP-binding cassette (ABC) transporter and an oligopeptide transporter that may play a role in these resource logistics. ABC transporters act as membrane pumps and have been implicated in the efflux of a wide variety of cytotoxic compounds [66]. In *Arabidopsis*, JA- and SA-dependent signaling as well as ethylene have been shown to increase ABC transporter homolog [67], which indicates more of a generalized defense role, rather than a specificity to a particular type of stress or herbivore feeding guild. Although induction of ABC transporters has not been previously reported by sap-feeding insects, it has been for chewing insects [63] and pathogens [68], further implying a role in plant defense. For oligopeptide transporters (OPT), the existing evidence points to a role involving the transport of small peptides consisting of more than three amino acids [69]. Higgins and Payne [70] suggested that the transport of peptides in vascular tissue is a more efficient means of nitrogen distribution than free amino acids. Perhaps citrus is utilizing this mechanism to transport nitrogen from degraded protein products to sites of active defense gene translation via the vascular system and that GWSS modulates their expression in order to take advantage of this increased nitrogen loading in the vascular system. More interestingly, it is possible that OPT are being used to transport small hormonal peptides within the vascular tissue of citrus to systemically propagate the defense response.

GWSS herbivory elicited transcriptional changes of four cell wall associated genes suggesting activity in wall modification (Table 2). Of the four, extensin is by far the most studied in terms of stress/defense. Once secreted into the wall, extensin monomers are rapidly insolubilized – most likely by peroxidase and H_2O_2 activity – resulting in substantially increased wall tensile strength [71]. Increased expression of extensin transcripts can be triggered by various biotic and abiotic stresses including wounding and pathogen attack (q.v. ethylene signaling), where it has been suggested to function as part of a plants' primary defense, impeding pathogen ingress until secondary defense genes can be mobilized [71–73]. Likewise, strengthening of the wall may provide a barrier to insect stylets, although this has yet to be demonstrated to effectively reduce herbivory.

In summary, we have successfully identified citrus genes preferentially expressed in response to GWSS herbivory by comparison to mechanical damage treatments. Our results show a fundamental difference in the magnitude of transcript expression in addition to treatment specificity suggesting

elicitor modulation of citrus transcription by GWSS feeding. Several of the GWSS-specific genes have not yet been characterized in detail, or are currently unknown to the public protein databases. Others, although characterized, have not been reported previously as insect-induced. Furthermore, attack by the GWSS results in transcriptional changes that are not representative of either chewing insect (JA-dependent) or sap-feeding insect (SA-dependent) herbivory. This noteworthy difference implies that many novel genes and mechanisms involving plant perception, tolerance and resistance to herbivorous insects remain to be discovered. Research with model organisms like *Arabidopsis* and tobacco have started to unravel these complex processes; however, it is clear that the existing genetic diversity found in non-model species, such as citrus, can result in the discovery of novel signaling and defensive compounds.

Acknowledgments

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