Cucumis melo MicroRNA Expression Profile During Aphid Herbivory in a Resistant and Susceptible Interaction

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Aphis gossypii resistance in melon (*Cucumis melo*) is due to the presence of a single dominant virus aphid transmission (*Vat*) gene belonging to the nucleotide-binding site leucine-rich repeat family of resistance genes. Significant transcriptional reprogramming occurs in *Vat*⁺ plants during aphid infestation as metabolism shifts to respond to this biotic stress. MicroRNAs (miRNAs) are involved in the regulation of many biotic stress responses. The role of miRNAs was investigated in response to aphid herbivory during both resistant and susceptible interactions. Small RNA (smRNA) libraries were constructed from bulked leaf tissues of a *Vat*⁺ melon line following early and late aphid infestations. Sequence analysis indicated that the expression profiles of conserved and newly identified miRNAs were altered during different stages of aphid herbivory. These results were verified by quantitative polymerase chain reaction experiments in both resistant *Vat*⁺ and susceptible *Vat*⁻ interactions. The comparative analyses revealed that most of the conserved miRNA families were differentially regulated during the early stages of aphid infestation in the resistant and susceptible interactions. Along with the conserved miRNA families, 18 cucurbit-specific miRNAs were expressed during the different stages of aphid herbivory. The comparison of the miRNA profiles in the resistant and susceptible interactions provides insight into the miRNA-dependent post-transcriptional gene regulation in *Vat*-mediated resistance.

MicroRNAs (miRNA) are a class of non-protein coding RNA that plays an important role in regulating gene expression of protein-coding genes at the RNA level. miRNAs are typically 18 to 22 nucleotides (nt) in size and are the most abundant class of endogenous small RNA (smRNA) in both plants and animals. In plants, miRNAs are known to regulate different biological processes, including development, metabolism, defense against pathogens, and various types of biotic and abiotic stress responses (Katiyar-Agarwal and Jin 2010; Sunkar 2010). The regulatory role of plant miRNAs is manifested by its ability to bind to the target mRNA with near-perfect complementarity, thereby, causing post-transcriptional regulation of gene expression (Jones-Rhoades et al. 2006). Biogenesis of

plant miRNA takes place in the nucleus (Zhu 2008). The primary miRNA (pri-miRNA) transcripts range in size from approximately 100 to 1,000 nucleotides (nt) and are processed by a double-stranded (ds)RNA specific RNaseIII enzyme Dicer-like 1 (DCL1) into a mature miRNA and miRNA* duplex. The duplex is methylated by Hua Enhancer 1 and exported to the cytoplasm where it is incorporated into the Argonaute 1-containing RNA-induced silencing effector complex (RISC). The mature miRNA guides RISC to the target mRNA, resulting in cleavage and repression of the target gene expression (Mallory and Vaucheret 2010).

Plant miRNAs respond to different types of biotic stress. In *Arabidopsis*, miR393 was shown to play a role in bacterial pathogenesis by negatively regulating the transport inhibitor responsive 1 (TIR1) gene, which is involved in auxin signaling (Navarro et al. 2006). The expression of miR160 and miR167 are also altered during bacterial infections, and these miRNAs target members of the auxin-response factor (ARF) family of transcription factors that are involved in the auxin signaling pathway (Fahlgren et al. 2007). Plant miRNAs have been implicated in other biotic interactions such as fungal infections (Lu et al. 2007b) and *Turnip mosaic virus* infection (He et al. 2008). Hewezi and coworkers (2008) found reduced susceptibility of *Arabidopsis* DCL and RNA-directed RNA polymerase (*RdR*) mutants to nematodes, implicating the involvement of smRNAs in plant-nematode interactions. In contrast, silencing *RdR1* makes the *Nicotiana attenuata* more susceptible to insect herbivory by chewing insects (Pandey et al. 2008). Altered expression levels of genes involved in jasmonic acid (JA) and ethylene (ET) biosynthesis and reduced accumulation of JA in *RdR1*-silenced *Nicotiana* plants indicates that smRNAs are involved in defense signaling in response to insect herbivory (Pandey et al. 2008).

Aphid herbivory induces differential expression of genes encoding proteins belonging to the phytohormone signaling pathways that are involved in defense responses (Smith and Boyko 2007). Aphids activate plant defense signaling pathways dependent on both salicylic acid (SA) and JA signaling molecules (Kaloshian 2004; Moran et al. 2002). Recently, Anstead and co-workers (2010) have shown that ET-related genes are activated in response to cotton-melon aphid (*Aphis gossypii*) feeding in resistant and susceptible melon (*Cucumis melo*) lines. *A. gossypii* resistance in melon is mediated by the single, dominant virus aphid transmission (*Vat*) gene, which is a member of the coiled-coil nucleotide-binding site leucine-rich repeat subfamily of plant resistance genes (Dogimont et al. 2008). *Vat*-mediated genetic resistance to aphids was identified in several sources of germplasm, including the Indian accession PI 371795

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that was subsequently introgressed into the susceptible aphid breeding line PMR 5 by eight successive backcrosses, followed by six generations of selfing to produce the nearly isogenic aphid resistant line AR 5 (McCreight et al 1984). *Vat* confers dual resistance against *A. gossypii* as well as several mosaic viruses transmitted by this aphid. Aphid resistance is exhibited as antixenosis (i.e., nonpreference of the aphids) and as antibiosis (i.e., reduction in aphid performance and fecundity) (Bohn et al. 1972), which significantly shortens the reproductive phase and life span of the aphids (Klingler et al. 1998). The *Vat*-mediated aphid resistance phenotype is expressed primarily in the phloem throughout all developmental stages in melon, although tissues along the pathway to the phloem could contribute to the resistance (Chen et al. 1996; Garzo et al. 2002; Kennedy and Kishaba 1977; Klingler et al. 1998).

A. gossypii infestation induces significant transcriptional reprogramming in melon plants with *Vat*-mediated resistance. Transcripts for several ET-responsive genes accumulate in response to *A. gossypii* feeding on a resistant melon line. Genes encoding transcription factors in a regulatory cascade associated with the ET signaling pathway are highly induced within the first 6 h after infestation during the resistant interaction (J. A. Anstead 2010). Several other transcription factor families that are associated with resistance such as NAC and bHLH are induced during aphid infestation in *Var*⁺ plants (J. Anstead, P. Samuel, N. Song, C. Wu, G. A. Thompson, and F. Goggin, unpublished data). Interestingly, many of these transcription factors are regulated by miRNAs: miR164 targets NAC transcription factors and miR393 regulates members of the bHLH family of transcription factors (Vidal et al. 2010). Expression profiles of smRNAs during different stages of *A. gossypii* infestation were studied to determine whether miRNAs are involved in regulating aphid-induced gene expression in nearly isogenic susceptible and resistant melon lines.

RESULTS

Sequence analysis of the *C. melo* smRNA population in *Var*⁺ lines.

Three smRNA libraries were generated from pooled leaf tissues of *Var*⁺ melon line AR 5 that were collected at different time points with and without aphid infestations and sequenced using an Illumina GAI analyzer. The control library yielded approximately 8 million raw reads, whereas the early and late library produced approximately 4.5 and 4.9 million reads, respectively. After removing the low-quality reads, adapter, and redundant sequences, there were about 7 million, 3.5 million, and 4.3 million unique sequences in the control, early, and late libraries, respectively (Table 1). Only those sequences of 18 to 30 nt with a count of 10 or more were retained as unique sequences (Supplementary Fig. S1). In the three libraries combined, 45 to 50% of the total smRNA were 24 nt, 11 to 15% were 23 nt, 8 to 9.5% were 22 nt, and 10 to 12% were 21 nt (Fig. 1). Endogenous siRNAs of 24 nt are the most abundant smRNAs in some plants such as *Arabi-*

dopsis, tomato, and, most recently, in another cucurbit, cucumber (*C. sativus*) (Martinez et al. 2011). In contrast, 24-nt smRNAs were not the predominant class identified from the melon smRNA transcriptome analyzed from *Watermelon mosaic virus* and *Melon necrotic spot virus* susceptible ('Tendril') and resistant ('T-111' and 'TGR-1551') cultivars (Gonzalez-Ibeas et al. 2011). The total number of smRNA sequences identified from the two aphid-infested libraries was larger in the early and late libraries compared with the control library (Table 1). Among the conserved miRNAs found in those libraries, 75% were 21 nt, with the remaining 25% comprising 19, 20, and 22 nt, and only a small fraction (2 to 5%) represented by 18- to 20-nt smRNAs.

Identification of aphid-induced conserved miRNAs in the resistant interaction.

To identify conserved miRNAs that accumulate in response to aphid herbivory on an aphid-resistant melon line, unique reads in the libraries were matched to the repository of plant miRNAs in miRBase using custom-written Perl script. Two mismatches were allowed during the sequence homology searches that resulted in the identification of 23 conserved miRNAs families in melon (Table 2). The total counts of conserved miRNAs were doubled in the late library when compared with the control, while the early library also showed a considerable increase (Table 1). Several conserved miRNAs were upregulated during aphid herbivory compared with the noninfested control library (Table 2). However, individual members of an miRNA family were not expressed uniformly in each of the libraries; some members were more abundant compared with others during the early and late stages of aphid infestation. The library counts of individual members in conserved miRNA families are documented in Supplementary Table S1.

Fig. 1. Size distribution of small RNA (smRNA) sequences in control, early, and late melon libraries.

Table 1. Statistics of small RNA (smRNA) sequences from control, early, and late libraries

Statistics ^a	Control	Early	Late
Raw reads	8,026,527	4,464,528	4,886,463
Adaptors removed	7,010,010	3,526,290	4,265,485
rRNA/tRNA exact matches removed	114,154	93,012	105,953
Match to known miRNAs	822	1,458	864
Match to known pumpkin smRNAs	15,149	19,936	14,512
Match to cucumber flower 454 EST database	13,506	14,914	10,217
Match to melon unigene database	19,455	17,991	20,371

^a miRNA = microRNA and EST = expressed sequence tag.

Identification of cucurbit specific miRNAs and novel miRNAs.

Following the identification of conserved miRNA, the remaining unique sequences in the library were searched for the presence of novel miRNAs. Melon whole-genome sequencing is still in progress; therefore, the melon expressed sequence tag (EST) collection (version 3.0) was used for this analysis. The closest relative of melon to have a complete genome available is cucumber, which was also used to identify cucurbit-specific miRNAs. The pumpkin (*Cucurbita maxima*) smRNA database was also used to search for smRNAs specific to this family. Unique sequences that did not match with conserved plant miRNAs were matched to these databases using the miRDeep algorithm. This algorithm uses the probabilistic model of the miRNA biogenesis to score the compatibility and the position of the sequence smRNA to the secondary structure of the miRNA precursor (Friedlander et al. 2008). Though miRDeep was originally described for identifying animal miRNAs, it has since been modified to successfully identify plant miRNAs (Yang et al. 2011). Animal miRNAs have a shorter precursor

length compared with plant miRNA precursors that vary from 100 to 1,000 bp. Precursor sequences of 300 bp were processed into stem-loop structures to identify novel candidate miRNAs in melon. This analysis identified 18 putative, cucurbit-specific miRNAs. Most of the miRNAs were 21 nt (42%), followed by 22 nt (26%), 23 nt (16%), 24 nt (10%), and 20 nt (5.2%). Of these, 5 (Cme-miR-2, Cme-miR-4, Cme-miR-5, Cme-miR-8, and Cme-miR-9) were melon specific, while the remaining 13 sequences were identified from both melon and pumpkin smRNA databases (Table 3). Interestingly, none of the novel miRNAs identified in cucumber (Martinez et al. 2011) were represented in any of the three melon libraries, indicating that those reported miRNAs are species specific. The secondary structures predicted for each of the novel candidate melon-specific miRNAs are shown in Figure 2. Some of the sequences from the three libraries matched to the sequences in the pumpkin smRNA database. Approximately 0.21% of the control library population, 0.56% of the early library, and 0.34% of the late library matched to the smRNA sequences from pumpkin (Table 1).

Table 2. Conserved microRNA (miRNA) families and their predicted target genes in melon

Family	Sequence	Normalized library frequency ^a			Target ^b	
		Control	Early	Late	Conserved	Putative
miR156	AUGACAGAAGAUAGAGAGCAC	30,006	16,255	22,676	SPB protein	MU29917 (SPB protein)
miR157	UGACAGCAGAUAGAGAGCAC	781.8	304.9	216.5	SPB protein	MU34102 (SPB protein)
miR159	AUUGGAUUGAAGGGAGCUCUA	231.2	1,056.2	100.8	MYB protein	MU39994, MU40740 (unknown)
miR160	AGCCUGGCUCUCCUGUAUGCCA	0	20.9	2.4	ARF	MU38981 (ARF)
miR162	UCGAUAAACCUCUGCAUCCAA	1.92	0	2.69	DCL-1	MU30859, MU41038 (unknown)
miR164	UGGAGAAGCAGGGCACGUGCA	30.71	225.5	312.5	NAC domain protein	MU22717 (NAC domain protein, SORBIDRAFT)
miR165	UCGGACCAGGCUUCAUCCCC	10.83	187.5	60.2	HD-Zip transcription factors	No target identified
miR166	AACGGACCAGGCUUCAUCCCC	9,735	178,641	33,844	HD Leucine Zipper III transcription factors	No target identified
miR167	ACAAGCUGCCAGCAUGAUCUAA	3,135.7	4019	11,826	ARF	MU26857 (ARF3)
miR168	ACGAUGGUGCAGGUCGGGAA	2,224.4	25,433	6,506.1	AGO1	No target identified
miR169	CAGCCAAGGAUGAAUUGCCGG	453.4	571.4	1,748.1	CCAAT binding factor–HAP2-like proteins	MU31362 (no matches in BLAST)
miR171	UGAUUGAGCCGCGCCAAUAUC	63.3	115.1	431.9	Scarecrow proteins–transcription factors	MU36464 (no matches in BLAST)
miR172	AGAAUCUUGAUGGUGCUGCAU	6,951.9	1,357.3	10,122	Apetala 2 (AP2) proteins	MU29652, MU31408, MU33447 (AP2 like protein)
miR2111	UAAUCUGCAUCCUGAGGUUUC	53.4	204.3	94.4	Unknown	MU34470 (Phospholipid/glycerol acyl transferase protein)
miR2911	UCCGGGGGACGGACUGGGA	0	7.9	3.1	Unknown	MU27177 (CytP450 like TBP, senescence associated protein)
miR390	CAGCUCAGGAGGGAUAGCGCC	0	43.4	9.78	Unknown	MU29926, MU39453 (no matches in BLAST)
miR393	UCCAAAGGGAUCGCAUUGAUC	23.59	10.1	22.2	F-box proteins and bHLH transcription factors	MU21869 (Transport inhibitor responsive factor, AFB2 [f-box protein])
miR394	UUGGCAUUCUGUCCACCUC	20.77	11.1	28.38	F-box proteins	No target identified
miR395	CUGAAGUGUUUGGGGAACUC	1.63	5.39	5.87	ATP sulphurylases	MU24817 (ATP sulphurylase)
miR396	AUCCACAGCUUUCUUGAACUC	623.4	605.3	1,367.8	Growth regulating factors	MU41399, MU21674 (no match, mitochondria import receptor subunit TOM20)
miR397	CAUUGAGUGCAGCGUUGAUGU	24.9	15.9	73.2	Laccases and β -6 tubulin	MU40742 (laccase-1a, diphenol oxidase)
miR398	UGUGUUCUCAGGUCGCCCCUG	1.78	7.61	5.1	Copper superoxide dismutases and cytochrome C-oxidase subunit	MU25081 (Uclacyanin-2)
miR408	CUGCACUGCCUCUCCUGGC	0	9.5	0	Stress-related miRNA	MU25436, MU24829 (predicted protein, 2CBP cucumber basic protein, chain A blue Cu protein)
miR894	CGAUUCACGUCGGGUUCAC	23.6	279.5	73.4	Unknown	No target identified

^a Normalized frequency = (number of reads/total clone counts in the library) $\times 10^6$.

^b Conserved target in *Arabidopsis* and putative target in melon and its annotation. SPB = Squamosa promoter binding and ARF = auxin response factor.

Expression profile of conserved miRNA during different stages of aphid herbivory in resistant and susceptible melon lines.

The bioinformatic analysis of the smRNA libraries revealed that the expression of most conserved miRNAs increased during the early and late stages of aphid herbivory. Quantitative real-time polymerase chain reaction (qPCR) experiments were

performed to validate the sequencing-based profiling data. The deep sequencing data revealed that individual members of a conserved family were differentially expressed during the early and late stages of aphid herbivory. The library data showed increases of miR160, miR164, miR165, miR166, miR167, miR169, miR2111, miR2911, miR390, miR393, miR396, miR397, miR398, miR408, and miR894 during early

Table 3. Cucurbit-specific microRNAs (miRNAs) and novel miRNAs identified from melon

Cme-miRNA ^a	Length	Novel miRNA sequence	Normalized library frequency			Target	Annotation
			Control	Early	Late		
miR-1	24 mer	GGAAUGUUGUCUGGCUCGAGGACA	10.2	0	0	No target	
miR-2	23 mer	AGUCAAUUCUAAACGAUCGUGUA	1.7	0	0	MU31433, MU35904	No hits
miR-3	21 mer	GCGUAUGAGGAGCCAUGCAUA	77.3	0	0	No target	
miR-4	23 mer	GUUGACAGAAGAGAGUGAGCACA	38.6	0	136	MU34102, MU29917	Liguleless-1 protein, POPTRDRAFT_576281
miR-5	22 mer	UGAGCCAAGAAGACUUGCCGGC	5.1	0	36.5	No target	
miR-6	21 mer	GCGUAUGAGGAGCCAAGCAUA	4.7	0	41	No target	
miR-7	20 mer	CGACGUCGCGAGAAGUCCAU	3.1	0	0	MU21621	Unknown protein
miR-8	24 mer	GUUUGGAAUGUGCGAGAUGUGUC	6.5	0	0	No target	
miR-9	22 mer	AGGCUUCUCUCCAUUGGCAGG	0	67.5	0	No target	
miR-10	22 mer	GCUCACUUCUCUUCUGUCAGU	0	24.4	0	No target	
miR-11	21 mer	GUGACAGAAGAGAGUGAGCAC	0	258.3	0	MU29917	POPTRDRAFT_755123, POPTDRAFT_576281
miR-12	21 mer	UUCCAUCUCUUGCACACUGGA	0	4.8	0	MU41094	F-box family protein, ubiquitin protein ligase
miR-13	21 mer	ACCGGUCGGCUCGUCCUUUU	0	3.7	0	No target	
miR-14	21 mer	AUCUCGGACCAGGCUUCAUUC	0	19.3	0	No target	
miR-15	22 mer	CGCAAGUCGUCCUUGGCUACC	0	0	251	No target	
miR-16	21 mer	GGGAUUAUUGGUGCGGUCAAU	0	0	5.9	No target	
miR-17	22 mer	GCUCACUUCUCUUCUGUCAGC	0	0	34.7	No target	
miR-18	23 mer	UGAAGCUGCCAGCAUGAUCUGAA	0	0	71	No target	

^a miRNAs in bold are novel miRNAs identified only in melon.

Fig. 2. Predicted secondary structure of primary microRNA (miRNA) transcripts of novel miRNAs.

stages of aphid feeding, and qPCR data showed a similar trend (Fig. 3A). Following Bonferroni corrections for multiple comparisons of the qPCR data, miR166 and miR2911 showed significant ($P \leq 0.002$) upregulation, providing strong statistical

support for their differential expression during late stages of aphid infestations in AR 5. Support for differential upregulation of the other miRNA families was not as strong, because these families showed significance at $P \leq 0.05$ without Bonfer-

Fig. 3. Expression profiling of conserved microRNA (miRNA) differentially expressed in melon leaf tissues at different stages of aphid infestation. Mature miRNA expression level was estimated by quantitative real-time polymerase chain reaction using RNA isolated from aphid-infested and uninfested leaf tissue collected at early (2, 4, and 6 h) and late (8, 10, and 12 h) post aphid infestation. Expression levels were normalized using *profilin* as an internal reference gene. Fold changes were calculated using Pfaffl's method and represent change in the expression level of miRNA family relative to control uninfested leaf. Data are averages of three independent replicates $\pm$ standard error of mean. Significance between control and aphid infestation was determined by Student's *t* test, and is represented by * ($P \leq 0.05$). Significance between the two time points post aphid infestation is represented as "a" ($P \leq 0.05$). **A**, Expression profile during resistant interaction in the virus aphid transmission (*Var*) gene *Var*⁺ melon line. **B**, Expression profile during susceptible interaction in *Var*⁻ melon line.

roni corrections. The statistical analysis, conducted to reduce the frequency of type I errors (false positives), revealed significance for two conserved miRNA families. However, the overall expression trends revealed by the qPCR data are useful to develop additional hypotheses for the potential involvement of a broader range of miRNA families in aphid resistance. At this point, hypothesis development is the most important aspect of this research and, therefore, type I errors (false positives) are more acceptable than type II errors (false negatives). The qPCR expression data for miR156/miR157, miR162, miR168, and miR171 did not correspond to the library counts. To validate the library data and qPCR data, smRNA blots were performed for some of these miRNAs. smRNA blot analysis confirmed the qPCR expression pattern for miR156, miR157, miR159, and miR162, showing upregulation during the early stages of aphid herbivory (Fig. 4). The expression of miR156, miR162, and miR159 was shown to be downregulated during late stages of aphid herbivory by smRNA blots in accordance with the library data. Although miR395 also appears to increase in response to aphid feeding as revealed by the counts in three different libraries, miR395 expression could not be detected by the qPCR assay. smRNA blots detected two different miR395 sizes with distinct signals at 21 and 24 nt (Fig. 4) that were induced following aphid feeding.

The design and construction of the smRNA libraries in the *Vat*⁺ melon line AR 5 only addresses the resistant interaction. Expression profiles of the conserved miRNAs between aphid-resistant and -susceptible melon lines were compared by qPCR during the early and late stages of aphid infestation in the nearly isogenic, susceptible melon line PMR 5. In the susceptible interaction, 22 conserved miRNAs were downregulated in the early response to aphid infestation, whereas only miR408 was upregulated during the early stage and eight miRNAs (miR156, miR157, miR166, miR168, miR169, miR171, miR172, and miR396) were upregulated during the late stages of aphid herbivory (Fig. 3B). Following Bonferroni corrections, five miRNA families (miR159, miR160, miR172, miR168, miR393, and miR397) showed significant ($P \leq 0.002$) downregulation during early stages and two families (miR408 and miR398) during the late stages of aphid infestation in PMR 5.

Expression pattern of novel melon-specific miRNAs in the resistant and susceptible line.

The expression profiles of novel melon-specific miRNAs identified by miRDeep were analyzed using low molecular weight (LMW) RNA isolated from AR 5 and PMR 5 by stem-loop qPCR. In the resistant melon line AR 5, the expression of Cme-miR-2, Cme-miR-4, Cme-miR-5, Cme-miR-8, and Cme-miR-9 did not show any significant change during both the early and late stages of aphid herbivory (Fig. 5A). The expression of these novel miRNAs was also studied in the susceptible line PMR 5, where Cme-miR-2, Cme-miR-8, and Cme-miR-9

were significantly ($P \leq 0.05$) downregulated during early stages of aphid infestation. Significant changes in the expression of these novel miRNAs between early and late stages of infestation were not observed.

Prediction of miRNA targets and their validation by 5' rapid amplification of cDNA ends (RACE).

Plant miRNAs, unlike their animal counterparts, bind to their targets with near-perfect complementarity (Jones-Rhoades and Bartel 2004; Rhoades et al. 2002). This unique characteristic of plant miRNAs has been used to predict their mRNA targets. The targets of the conserved and the cucurbit-specific miRNAs were predicted by in silico analysis using the melon smRNA target identification tool. The predicted targets in melon are summarized in Tables 2 and 3. Most of the conserved plant miRNA target genes have been validated in *Arabidopsis*, rice, and poplar. Several putative target genes were identified in this study (Table 2), and a new target gene for miR398 was identified in melon. In *Arabidopsis* and rice, miR398 targets members of the copper superoxide dismutase (CSD) gene family. In melon, miR398 is predicted to target the gene represented by the melon EST MU25081; the miRNA-guided cleavage of MU25081 was validated by 5' RACE (Fig. 6). MU25081 is annotated as a homolog of *Zea mays* Uclacyanin-2 (UCC2) and

Fig. 5. Expression profile of the candidate novel microRNAs (miRNAs) during a resistant interaction. Mature miRNA expression level was estimated by quantitative real-time polymerase chain reaction using RNA isolated from aphid-infested and uninfested leaf tissue collected at early (2, 4, and 6 h) and late (8, 10, and 12 h) post aphid infestation. Expression levels were normalized using *profilin* as an internal reference gene. Fold changes were calculated using Pfaffl's method and represent change in the expression level of miRNA family relative to aphid-uninfested leaf as control. Data are averages of three independent replicates $\pm$ standard error of mean. Significance between control and aphid infestation was determined by Student's *t* test, and is represented by * ($P \leq 0.05$). **A**, Novel miRNAs expressed in virus aphid transmission (*Vat*) gene *Vat*⁺ resistant line **B**, *Vat*⁻ susceptible line of *Cucumis melo*.

Fig. 4. Small RNA blots of microRNA (miRNA) families miR156, miR159, miR162, and miR395 expressed during early and late stages of aphid herbivory in resistant interaction. U6 was used for normalization.

Fig. 6. Target validation of miR398 in melon. A 5' rapid amplification of cDNA ends analysis was carried out for the predicted target gene. The 5' end of the cleavage product is indicated by arrow. Target expressed sequence tag sequence is shown at the top of the microRNA sequence.

the closest *Arabidopsis* homolog, AT2G26720.1, belongs to the same cupredoxin superfamily of proteins (Khan et al. 2007). Expression profiling in the resistant melon line revealed that miR398 was the most abundant miRNA during early stages of aphid feeding (Fig. 3A) and was downregulated in the susceptible interaction. Predicted targets could not be identified for miR159, miR165, miR166, miR168, miR394, and miR894, which is possibly due to the limited coverage of the melon EST library.

DISCUSSION

smRNAs are important gene regulators in controlling plant responses to biotic and abiotic stress. In this study, three smRNA libraries were generated from the aphid-resistant melon line AR 5 to compare smRNA expression in noninfested plants with responses that occur during two temporal stages during aphid herbivory. The rationale for choosing these two time points, representing early and late stages of aphid herbivory, is based on the distinctive pattern observed during the electrical penetration graph (EPG) studies in the nearly isogenic melon lines AR 5 and PMR 5. The EPG data revealed prolonged sieve-element probing and salivation accompanied by a significant inhibition of the sustained sap ingestion during the first 3 h of the interaction between *A. gossypii* and the resistant line AR 5 (Klingler et al. 1998). Sieve-tube sap ingestion quantified indirectly as rates of honeydew excretion decreased 10-fold on AR 5 and was delayed by 6 h compared with the susceptible plants during a 12-h period (Klingler et al. 1998). Transcriptional studies revealed that expression of genes in the ET signaling pathway were differentially regulated between 6 and 24 h, with gene induction occurring at 6 h compared with the 24-h time point. Thus, it appears that significant physiological events impacting resistance occur within the first 6 h of aphid infestation. In this study, pooled leaf tissues were collected from 2, 4, and 6 h post aphid infestation to represent the early stages. Similarly, leaf tissues collected from 8, 10, and 12 h were pooled together to represent the late stages of aphid infestation. Pooling of the leaf tissues provided maximum coverage for each stage of the interaction.

Twenty-three families of conserved plant miRNAs were identified from the three libraries (Table 2), and their expression patterns in response to aphid herbivory were studied in detail (Fig. 3). The numbers of conserved miRNAs in the libraries provided evidence that all of the conserved miRNA families were differentially regulated during the early and late phases of aphid herbivory in the resistant interaction (Table 2). These data are compatible with the results obtained from the EPG analyses of AR 5, where it was shown that aphids display prolonged probing behaviors prior to sustained sap ingestion (Klingler et al. 1998). Salivation that occurs during prolonged probing at the early stages of aphid infestation could initiate a resistance response by the plant and, in turn, trigger the induction of miRNAs and other smRNAs (Table 1). This agrees with the observations that *N. attenuata* mutants lacking *RdR1* produce decreased amounts of smRNAs and are more susceptible to chewing insect attack (Pandey et al. 2008), suggesting that defense responses are regulated by smRNAs. Similarly, the susceptible melon line (*Var*⁻) also showed an overall downregulation in the conserved miRNA population. This comparison yielded contrasting observations with respect to miRNAs expression or regulation in aphid resistant and susceptible lines during herbivory (Fig. 3). The *Var*⁺ interaction showed an overall trend toward enhanced miRNA expression, whereas the susceptible interaction showed downregulation of miRNAs. The opposite trends in these lines could be due to differences in miRNA transcription or biogenesis in the nearly isogenic

susceptible and resistant melon lines during aphid feeding. The key components of the miRNA and siRNA biogenesis pathway are DCL proteins. Recently, Ahmadovichbozorov and co-workers (2012) have reported the regulatory role of different DCL proteins during *Manduca sexta* herbivory in *N. attenuata*. It will be of interest to identify the melon orthologues of the enzymes involved in the smRNA biogenesis pathway and study their expression profile in the susceptible and resistant melon lines to verify their role in the resistance mechanism.

A snapshot of the total smRNA population revealed that smRNA biology of the resistant *Var*⁺ melon line was distinctive from the susceptible *Var*⁻ melon line. To understand the detailed role of these smRNAs in the resistance mechanism, specific miRNAs that are candidates for resistance were screened and further analyzed. Several stress-responsive miRNAs including miR160, miR164, miR167, miR393, miR395, miR398, and miR408, were identified in the melon libraries and their expression profile analyzed in the resistant and susceptible interactions (Fig. 3). The putative targets of these miRNAs were predicted by in silico analysis. Melon miR164 targets NAC6, which is a member of the NAC transcription factor family (Table 2). Barley NAC6 is a positive regulator of transcription involved in early defense against powdery mildews (Micali et al. 2008). Another group of miRNAs (miR160, miR167, and miR393) have well-established regulatory functions in bacterial resistance in *Arabidopsis* (Navarro et al. 2006). These miRNAs target the ARF family of genes and the TIR1 gene in *Arabidopsis* and are predicted to target ARF 2 and 3 and TIR1 genes in melon (Table 2). Kuśnierczyk and coworkers (2007) reported that several genes belonging to the auxin biosynthesis pathway were induced following green peach aphid (*Myzus persicae*) infestation in different eco-types of *Arabidopsis*. The significant downregulation of miR160, miR167, and miR393 in the susceptible melon line PMR 5 during both early and late stages of aphid feeding indicates a role for auxin-mediated gene regulation in the host-aphid interaction. Another stress responsive miRNA miR395 targets ATP-sulfurylases (ATPS) in *Arabidopsis* (Jones-Rhodes and Bartel 2004) and is predicted to have the same target in melon. The library data indicated accumulation of miR395 during aphid infestation during the resistant interaction. miR395 could not be detected by qPCR but two distinct signals were reported from the smRNA blots, which were differentially regulated during the early and later stages of the aphid infestation. ATPS is the first enzyme in the sulfate assimilation pathway and is sensitive to glutathione concentrations: ATPS activity is reduced in the presence of higher amounts of exogenous glutathione (Yi et al. 2010). Glutathione is an important antioxidant and plays a major role in plant defense by participating in reactive oxygen species signaling. Zechmann and associates (2009) have shown that the level of glutathione and its precursor cysteine is enhanced at the site of stylet penetration during black bean aphid (*A. fabae*) infestation in sugar beet. Glutathione synthase (GSH2) was also induced in *Arabidopsis* by green peach aphid adults and nymphs after 48 h of feeding (Kempema et al. 2007). It will be interesting to explore the role of miR395 in glutathione regulation via ATPS in response to *A. gossypii* infestation in melon.

Novel target genes were predicted for a number of miRNA families (miR169, miR171, miR2111, miR2911, miR396, miR398, and miR408) in melon (Table 2). A homolog of *Z. mays* UCC2 was confirmed as the target of melon miR398 by 5' RACE (Fig. 6). In *Arabidopsis*, miR398 targeted CSD1 and CSD2 genes and copper-chaperone for superoxide dismutase (Li et al. 2010; Sunkar et al. 2006). UCC2 is a glycosylphosphatidylinositol-anchored protein, which is a member

of the family of copper-binding proteins involved in single-electron transfer during redox reactions. A homolog of a putative senescence-associated gene (SAG) from *Pisum sativum* is the predicted target of miR2911. SAG are known to play a role in plant responses to aphid feeding. Increased SAG expression in response to *M. persicae* feeding in *Arabidopsis* was reported (Pegadaraju et al. 2005). Premature senescence of the aphid-infested leaf is induced in plants as part of the programmed degradation of cellular components and contributes to defense against the generalist insect *M. persicae* in *Arabidopsis* (Pegadaraju et al. 2005). It has been reported that aphid infestation changes the resource allocation in the host plant, leading to increased flow of nutrients to the insect-infested tissue due to the creation of a strong sink in those tissues (Girousse et al. 2005); however, Pegadaraju and co-workers (2005) have proposed that premature leaf senescence of the aphid-infested leaves inhibits the ability of the aphids to redirect the flow of resources and, thus, can be of benefit to the host plant. Upregulation of melon *SAG-21* was reported by Anstead and associates (2010) in both resistant and susceptible interactions during the early stages (6 h) of aphid infestation. Another predicted target of melon miR2911 is a cytochrome P450 gene. Although an extremely large and diverse family, cytochrome P450s have well-established roles in wound healing and insect resistance (Noordermeer et al. 2001). In addition to the conserved targets, conserved miRNAs appear to target additional genes in different plant species (Lindow and Krogh 2005). The completion and release of the melon genome sequence will aid in the validation of predicted targets and should lead to the identification of additional target genes.

Several novel miRNAs were identified from the melon libraries. Eighteen putative cucurbit-specific miRNAs were identified from the melon libraries, five of which were specific to melon. In contrast to the conserved miRNA families that have multiple family members, the cucurbit-specific miRNAs are represented by a single member. Potential precursors were identified and a predicted fold-back structure was generated for each of these miRNAs. In silico target prediction revealed targets for only a subset of these cucurbit-specific miRNAs (Table 3). Cme-miR-4 was predicted to target ligueless-1 protein, a transcription factor homologous to Squamosa promoter-binding protein and POPTDRAFT_576281 from poplar (*Populus tremuloides*). Cme-miR-10 is predicted to target a homolog of poplar genes POPTDRAFT_755123 and POPTDRAFT_576281 (Table 3). The predicted target for Cme-miR-11 shows homology to protein with an F-box-containing domain and a putative ubiquitin protein ligase from castor bean (*Ricinus communis*). Qiao and associates (2009) reported that the stability of *Arabidopsis* ET-insensitive 2 (EIN2), a central regulator of ET response in plants, is modulated by F-box proteins ETP1 and ETP2. EIN2 in *Var*⁺ melon plants is upregulated after 6 h of *A. gossypii* feeding (Anstead et al. 2010). This indicates that F-box domain-containing proteins play a crucial role during aphid herbivory by fine tuning the ET pathway, and the miRNAs that target these proteins could be important in regulating ET responses in melon.

In summary, this study shows that miRNAs respond differentially to aphid infestation during a resistant and susceptible interaction. An overall increase in the smRNA population was detected in the resistant interaction. Also, the expression of conserved miRNAs was upregulated during different stages of the resistant interaction. Eighteen putative cucurbit-specific miRNAs and five melon-specific miRNA were identified in the libraries. Some of the conserved miRNAs presented in the library were found to target genes involved in biotic and abiotic stress responses.

MATERIALS AND METHODS

Plant and insect growth conditions.

The nearly isogenic AR 5 (*Var*⁺, resistant) and PMR 5 (*Var*⁻, susceptible) melon lines were grown in controlled conditions of 23°C, 60% relative humidity, and a photoperiod of 16 h of light and 8 h of darkness for 4 weeks until they reached the four-leaf stage. *A. gossypii* were reared and maintained on susceptible honeydew melon plants in a controlled growth chamber at 21°C, with a photoperiod of 16 h of light and 8 h of darkness.

Plant treatments.

The third and fourth leaves from 4-week-old melon plants were used for aphid infestations. Each leaf was exposed to 50 apterous aphids confined in a 2.5-cm-diameter clip cage. A mix of aphids from different growth stages was used to model a natural crop infestation. Infestations were terminated after specific time periods: 2, 4, 6, 8, 10, and 12 h. After these periods, all aphids were gently brushed off the leaf tissue and a 2.5-cm leaf disk was excised. Leaf tissues collected from 2, 4, and 6 h of aphid infestation were pooled together (early-stage infestations), snap frozen in liquid nitrogen, and stored at -80°C. Similarly, leaf tissues collected from 8, 10, and 12 h of aphid infestation were combined (late-stage infestations) and stored at -80°C. Noninfested melon leaf tissues (clip cages without aphids) from identical time points were collected as controls and handled the same as the treatment tissues. All experiments were biologically independent and three independent replicates were used for each time point.

Cloning of smRNA and Illumina sequencing.

Total RNA was isolated by TRIzol reagent (Invitrogen) following the manufacturer's instructions for plants with high polysaccharide content. The RNA was enriched for LMW RNA by precipitation with 25% polyethylene glycol and 5 M NaCl (Lu et al. 2007a). The LMW RNA quality was evaluated using an RNA600 Nano LabChip kit with the Agilent Bioanalyzer system (Agilent Biotechnologies, Santa Clara, CA, U.S.A.) and the concentration was measured with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, U.S.A.).

LMW RNA (50 µg) from control, early, and late infestations was separated in a 15% denaturing polyacrylamide gel. Size fractions of 20 to 24 nt were excised and the RNA was extracted from the gel slices with 0.3 M NaCl, followed by ethanol precipitation. The RNA was ligated to a 26-bp 5' adapter, size selected on a polyacrylamide gel, and ligated to a 3' adapter. The adapter sequences and PCR primers used in smRNA library preparation are provided in Supplementary Table S2.

First-strand cDNA synthesis was performed with the adapter-ligated smRNA template using the Superscript II reverse-transcriptase (Invitrogen) and a primer complementary to the 3' adapter. The cDNA was used as a template for a low cycle PCR amplification (initial denaturation of 30 s at 98°C; 15 cycles of 98°C for 10 s, 60°C for 30 s, and 72°C for 15 s; followed by final extension at 72°C for 10 min) to generate sufficient template for deep sequencing. The size-fractionated cDNA of approximately 100 bp was gel eluted and subjected to Illumina GAII analyzer for deep sequencing.

Bioinformatic analysis of the smRNA transcriptome.

All Illumina sequencing data were initially converted to FASTA format from FASTQ. smRNA sequences were extracted from raw reads by matching them with the first 8 nt of the 3' adapter sequences. Based on the length of the mature miRNA and adapter length, sequences shorter than 18 nt and

greater than 30 nt in length were removed. At this stage, the data were screened for redundant sequences. The remaining sequences were queried against ribosomal and transfer RNAs from Rfam and the *Arabidopsis* tRNA database. The matches to the rRNA and tRNA were discarded. Sequences having 10 or more counts were assigned to the unique sequence library. These sequences were then queried against melon coding RNA regions for perfect matches. Those sequences that matched to the sense strand were discarded as degraded mRNA; however, those matching to the anti-sense strand were retained and aligned against the miRBASE to identify the conserved miRNA (two mismatches allowed). All bioinformatic analyses were performed using custom-written PERL script. Bowtie, which is an ultrafast memory efficient short-read aligner (Langmead et al. 2009), was used to match the sequences to the pumpkin smRNA database, as well as the melon EST and cucumber 454 genome sequence databases. Unaligned sequences were potential candidates for novel melon miRNAs. Novel melon miRNAs were identified using the algorithm miRDeep (Friedlander et al. 2008). Because the complete genome sequence of melon was not available during the processing of the dataset, melon EST version 3.0 was used to find novel miRNAs.

Expression profiling of conserved miRNAs in melon by qPCR.

LMW-enriched RNA was used to study the differential expression pattern of conserved miRNA in the early and late aphid infestations. The miRNA expression was measured using a two-step process. In the first step, a stem-loop (RT) primer designed according to Chen and associates (2005) was hybridized to the miRNA and reverse transcribed in a pulsed RT reaction (Varkonyi-Gasic et al. 2007). In the second step, the RT reaction product was PCR amplified using a miRNA-specific forward primer and a universal primer (Supplementary Table S3) in real time with SYBR green chemistry using ABI 7500. The different members of the miRNA families expressed in the three libraries were aligned and an identical region was chosen for the design of the stem-loop (RT) primer and the miRNA-specific forward primer, allowing representative members of the miRNA to be amplified with near equal efficiency. The melon *profilin* gene (GenBank accession number AY879597) was used as housekeeping control and showed consistent expression during different stages of aphid herbivory (Anstead et al. 2010). The cycle threshold (Ct) values for profilin amplified from LMW RNA were comparable with the Ct values for profilin amplified from total RNA. Quantification of the relative changes in miRNA expression was performed using the method described by Pfaffl (2001). The data for relative quantities were converted to fold differences by logarithmic transformation to express the data as a normal distribution. The data were represented as averages of three measurements $\pm$ standard error. The comparisons of the miRNA expression levels between the three groups were performed using student's *t* test ($P \leq 0.05$). Bonferroni corrections were used to adjust for multiple comparisons.

smRNA blots.

Some of the miRNAs identified from the Illumina deep sequencing could not be verified by qPCR experiments. smRNA blots were performed to detect these miRNA and to confirm any discrepancy between the library data and the qPCR expression profiles. LMW RNA (10 μ g) was separated on a 15% denaturing polyacrylamide gel electrophoresis gel, electrophoretically transferred to N⁺ hybond membrane (GE Healthcare, Piscataway, NJ, U.S.A.), and UV cross linked (Stratagene). Synthetic antisense DNA oligomers (IDT Inc.) were labeled at the 5' end using [γ^{32} P]-ATP (MP Biomedicals, Solon, OH,

U.S.A.) and T4 Polynucleotide kinase (New England Biolabs, Ipswich, MA, U.S.A.) and used as probes. Probes were purified on a Sephadex G-25 column. Prehybridizations were carried out for 1 h using PerfectHyb Plus buffer (Sigma-Aldrich, St. Louis) at 37°C and hybridization was done for over 16 h using the same buffer. Hybridized membranes were washed twice at 52°C with 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate for 15 min each. The hybridized blots were exposed to phosphorimager screens, viewed in TyphoonTrio (GE Healthcare), normalized with small nuclear RNA U6 oligonucleotide probe for smRNA gel blot analysis.

Target prediction and validation.

Conserved plant miRNA targets were previously validated in *Arabidopsis thaliana* and available on miRBASE. To verify whether the targets of the conserved miRNAs were also conserved in *Cucumis melo*, an in silico prediction analysis was performed using the sRNA target prediction tool at the Cucurbit Genomics Database. Some of the conserved miRNA had a related or completely new target gene. To further validate the results of the in silico analysis, an RNA ligase-mediated amplification of cDNA ends experiment was conducted for each putative cDNA target gene with the GeneRacer kit (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions.

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LITERATURE CITED

- Ahmadovichbozorov, T., Pandey, S. P., Dinh, S. T., Kim, S. G., Henrich, M., Gase, K., and Baldwin, I. T. 2012. Dicer-like proteins and plant herbivore interactions in *Nicotiana attenuate*. J. Integr. Plant Biol. 54:189-206.
- Anstead, J., Samuel, P., Song, N., Wu, C., Thompson, G. A., and Goggins, F. L. 2010. Activation of ethylene-related genes in response to aphid feeding on resistant and susceptible melon and tomato plants. Entomol. Exp. Appl. 134:170-181.
- Bohn, G. W., Kishaba, A. N., and Toba, H. H. 1972. Mechanisms of resistance to melon aphid in a muskmelon line. Hortic. Sci. 7:281-284.
- Chen, C., Ridzon, D. A., Broomer, A. J., Zhou, Z., Lee, D. H., Nguyen, J. T., and Barbisin, M. 2005. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 33:179-188.
- Chen, J. Q., Delobel, B., Rahbé, Y., and Sauvion, N. 1996. Biological and chemical characteristics of a genetic resistance of melon to the melon aphid. Entomol. Exp. Appl. 80:250-253.
- Dogimont, C., Chovelon, V., Tual, S., Boissot, N., Rittener, V., Giovannazzo, N., and Bendahmane, A. 2008. Molecular diversity at the Vat/Pm-W resistance locus in melon. Pages 219-227 in: Cucurbitaceae 2008. Proc. IXth EUCARPIA Meet. Genet. Breeding of Cucurbitaceae. M. Pitrat, ed. INRA, Avignon, France.
- Fahlgren, N., Howell, M. D., Kasschau, K. D., Chapman, E. J., Sullivan, C. M., Cumbie, J. S., Givan, S. A., Law, T. F., Grant, S. R., Dangler, J. L., and Carrington, J. C. 2007. High-throughput sequencing of *Arabidopsis* microRNAs: Evidence for frequent birth and death of MIRNA genes. PLoS ONE 2:e219. Published online.
- Friedlander, M. R., Chen, W., Adamidi, C., Maaskola, J., Einspanier, R., Knespel, S., and Rajewsky, N. 2008. Discovering microRNAs from deep sequencing data using miRDeep. Nat. Biotechnol. 26:407-415.
- Garzo, E., Soria, C., Gómez-Guillamón, M. L., and Feres, A. 2002. Feeding behavior of *Aphis gossypii* on resistant accessions of different melon genotypes (*Cucumis melo*). Phytoparasitica 30:129-140.
- Girousse, C., Moulia, B., Silk, W., and Bonnemain, J. L. 2005. Aphid infestation causes different changes in carbon and nitrogen allocation in alfalfa stems as well as different inhibitions of longitudinal and radial expansion. Plant Physiol. 137:1474-1484.

- Gonzalez-Ibeas, D., Blanca, J., Donaire, L., Saladie, M., Mascarell-Creus, A., Cano-Delgado, A., Garcia-Mas, J., Llave, C., and Aranda, M. 2011. Analysis of the melon (*Cucumis melo*) small RNAome by high-throughput pyrosequencing. *BMC Genomics* 12:e393.
- He, X. F., Fang, Y., Feng, L., and Guo, H. 2008. Characterization of conserved and novel microRNAs and their targets, including a TuMV-induced TIR-NBS-LRR class R gene derived novel miRNA in Brassica. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 582:2445-2452.
- Hewezi, T., Howe, P., Maier, T. R., and Baum, T. J. 2008. *Arabidopsis* small RNA and their targets during cyst nematode parasitism. *Mol. Plant-Microbe Interact.* 21:1622-1634.
- Jones-Rhoades, M. W., and Bartel, D. P. 2004. Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Mol. Cell.* 14:787-799.
- Jones-Rhoades, M. W., Bartel, D., and Bartel, B. 2006. MicroRNAs and their regulatory roles in plants. *Annu. Rev. Plant Biol.* 57:19-53.
- Kaloshian, I. 2004. Gene-for-gene disease resistance: Bridging insect pest and pathogen defense. *J. Chem. Ecol.* 30:2419-2438.
- Katiyar-Agarwal, S., and Jin, H. 2010. Role of small RNAs in host-microbe interactions. *Annu. Rev. Phytopathol.* 48:225-246.
- Kempema, L. A., Cui, X., Holzer, F. M., and Walling, L. L. 2007. *Arabidopsis* transcriptome changes in response to phloem-feeding silverleaf whitefly nymphs. Similarities and distinctions in responses to aphids. *Plant Physiol.* 143:849-865.
- Kennedy, G. G., and Kishaba, A. N. 1977. Response of alate melon aphids to resistant and susceptible muskmelon lines. *J. Econ. Entomol.* 70:407-410.
- Khan, J. A., Wang, Q., Sjölund, R. D., Schulz, A., and Thompson, G. A. 2007. An early nodulin like protein accumulates in the sieve element plasma membrane if *Arabidopsis*. *Plant Physiol.* 143:1576-1589.
- Klinger, J., Powell, G., Thompson, G. A., and Isaacs, R. 1998. Phloem specific aphid resistance in *Cucumis melo* line AR 5: Effects on feeding behavior and performance of *Aphis gossypii*. *Entomol. Exp. Appl.* 86:79-88.
- Kusnierczyk, A., Winge, P., Midelfart, H., Armbruster, W. S., Rossiter, J. T., and Bones, A. M. 2007. Transcriptional responses of *Arabidopsis thaliana* ecotypes with different glucosinolate profiles after attack by polyphagous *Myzus persicae* and oligophagous *Brevicoryne brassicae*. *J. Exp. Bot.* 58:2537-2552.
- Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. L. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 10:R25.
- Li, Y., Zheng, Y., Addo-Quaye, C., Zhang, L., Saini, A., Jagadeeswaran, G., Axtell, M. J., Zhang, W., and Sunkar, R. 2010. Transcriptome-wide identification of microRNA targets in rice. *Plant J.* 62:742-759.
- Lindow, M., and Krogh, A. 2005. Computational evidence for hundreds of non-conserved plant microRNAs. *BMC Genomics* 6:e119.
- Lu, C., Meyers, B. C., and Green, P. 2007a. Construction of small RNA cDNA libraries for deep sequencing. *Methods* 43:110-117.
- Lu, S. F., Sun, Y. H., Amerson, H., and Chiang, V. L., 2007b. MicroRNAs in loblolly pine (*Pinus taeda* L.) and their association with fusiform rust gall development. *Plant J.* 51:1077-1098.
- Mallory, A., and Vaucheret, H. 2010. Form, function and regulation of Agronaute proteins. *Plant Cell* 22:3879-3889.
- Martínez, G., Forment, J., Llave, C., Pallás, V., and Gómez, G. 2011. High-throughput sequencing, characterization and detection of new and conserved cucumber miRNAs. *PLoS One* 6:e19523. Published online.
- McCreight, J. D., Kishaba, A. N., and Bohn, G. W. 1984. AR Hale's Best Jumbo, AR 5, and AR Topmark: Melon aphid resistant muskmelon breeding lines. *Hortic. Sci.* 19:309-310.
- Micali, C., Göllner, K., Humphry, M., Consonni, C., and Panstruga, R. 2008. The Powdery Mildew Disease of *Arabidopsis*: A Paradigm for the Interaction Between Plants and Biotrophic Fungi. *The Arabidopsis Book* 6:e0115. The American Society of Plant Biologists, Rockville, MD, U.S.A. Published online.
- Moran, P. J., Cheng, Y., Cassell, J. L., and Thompson, G., A. 2002. Gene expression profiling of *Arabidopsis thaliana* in compatible plant-aphid interactions. *Arch. Insect Biochem.* 51:182-203.
- Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Vionnet, O., and Jones, J. D. G. 2006. A plant miRNA contributing to antibacterial resistance by repression auxin signaling. *Science* 321:436-439.
- Noordermeer, M. A., Veldink, G. A., and Vliegthart, J. F. G. 2001. Fatty acid hydroperoxide lyase: A plant cytochrome P450 enzyme involved in wound healing and pest resistance. *ChemBiochem* 2:494-504.
- Pandey, S. P., Shahi, P., Gase, K., and Baldwin, I. T. 2008. Herbivory induced small-RNA induced transcriptome and phytohormone signaling in *Nicotiana attenuata*. *Proc. Natl. Acad. Sci. U.S.A.* 105:4559-4564.
- Pegadaraju, V., Knepper, C., Reese, J and Shah, J. 2005. Premature leaf senescence modulated by the Arabidopsis PHYTOALEXIN DEFICIENT4 gene is associated with defense against the phloem-feeding green peach aphid. *Plant Physiol.* 139:1927-1934.
- Pfaffl, M. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:2002-2007.
- Qiao, H., Chang, K. N., Yazaki, J., and Ecker, J. R. 2009. Interplay between ethylene, ETP1/ETP2 F-box proteins, and degradation of EIN2 triggers ethylene responses in *Arabidopsis*. *Genes Dev.* 23:512.
- Rhoades, M. W., Reinhart, B. J., Lim, L. P., Burge, C. B., Bartel, B., and Bartel, D. P. 2002. Prediction of plant microRNA targets. *Cell* 110:513-520.
- Smith, C. M., and Boyko, E. V. 2007. The molecular basis of plant resistance and defense responses to aphid feeding: Current status. *Entomol. Exp. Appl.* 221:1-6.
- Sunkar, R. 2010. MicroRNAs with macro-effects on plant stress responses. *Semin. Cell Dev. Biol.* 21:805-811.
- Sunkar, R., Kapoor, A., and Zhu, J. 2006. Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by downregulation of miR398 and important for oxidative stress tolerance. *Plant Cell* 18:2051-2065.
- Varkonyi-Gasic, E., Wu, R., Wood, M., Walton, E. F., and Hellens, R. P. 2007. Protocol: A highly sensitive RT-PCR method for detection and quantification of microRNAs. *Plant Methods* 3:12.
- Vidal, E. A., Araus, V., Lu, C., Parry, G., Green, P. J., Coruzzi, G. M., and Gutiérrez, R. A. 2010. Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 107:4477-4482.
- Yang, X., Zhang, H., and Li, L. 2011. Global analysis of gene-level miRNA expression in *Arabidopsis* using deep sequencing data. *Genomics* 98:40-46.
- Yi, H., Galant, A., Ravilious, G. E., Preuss, M. L., and Jez, J. M. 2010. Sensing sulfur conditions: Simple to complex protein regulatory mechanisms in plant thiol metabolism. *Mol. Plant* 3:269-279.
- Zechmann, B., Zelený, F., Wonisch, A., and Zelená, E. 2009. Aphid infestation affects subcellular glutathione and cysteine contents in *Beta vulgaris*. *Belg. J. Bot.* 142:50-59.
- Zhu, J. 2008. Reconstituting plant miRNA biogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 105:9851-9852.

AUTHOR-RECOMMENDED INTERNET RESOURCES

Cucurbit genomics database:
www.icugi.org/cgi-bin/ICuGI/genome/cuke.cgi
 The Genomic tRNA database: lowelab.ucsc.edu/GtRNAdb/Athal
 miRBase: The microRNA database: www.mirbase.org
 Sanger Institute Rfam database: www.sanger.ac.uk/resources/databases