In Planta Expression or Delivery of Potato Aphid *Macrosiphum euphorbiae* Effectors *Me10* and *Me23* Enhances Aphid Fecundity

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The interactions between aphids and their host plants seem to be analogous to those of plant-microbial pathogens. Unlike microbial pathogen effectors, little is known about aphid effectors and their ability to interfere with host immunity. To date, only three functional aphid effectors have been reported. To identify potato aphid (*Macrosiphum euphorbiae*) effectors, we developed a salivary gland transcriptome using Illumina technology. We generated 85 million Illumina reads from salivary glands and assembled them into 646 contigs. *Ab initio* sequence analysis predicted secretion signal peptides in 24% of these sequences, suggesting that they might be secreted into the plant during aphid feeding. Eight of these candidate effectors with secretion signal peptides were functionally characterized using *Agrobacterium tumefaciens*-mediated transient overexpression in *Nicotiana benthamiana*. Two candidate effectors, *Me10* and *Me23*, increased aphid fecundity, suggesting their ability to suppress *N. benthamiana* defenses. Five of these candidate effectors, including *Me10* and *Me23*, were also analyzed in tomato by delivering them through the *Pseudomonas syringae* type three secretion system. In tomato, only *Me10* increased aphid fecundity. This work identified two additional aphid effectors with ability to manipulate the host for their advantage.

Aphids (Hemiptera: Aphididae) are soft-bodied insects with piercing-sucking mouthparts that cause serious economic losses to cultivated crops. They damage plants directly, by depleting nutrients and altering plant development, and indirectly, by vectoring plant viruses and supporting growth of the sooty mold fungus (Blackman and Eastop 2000). Some aphid species are

globally distributed due to their polyphagous nature and ability to adapt to different environmental conditions (Margaritopoulos et al. 2009). The life cycle of aphids is somewhat complex, comprising both sexual and asexual (parthenogenetic) modes of reproduction, the latter giving rise to live progeny (Blackman and Eastop 2000). Sexual reproduction occurs only in the fall season when eggs are laid on perennial plants for overwintering. Being hemimetabolous insects, aphids have no morphologically distinct larval or pupal stages. During asexual reproduction, females lay nymphs which, after three successive molts become adults (Moran 1992; Van Emden and Harrington 2007).

The potato aphid (*Macrosiphum euphorbiae*) has a broad host range including plants in family Solanaceae, transmits a number of plant viruses, and represents an aphid species of worldwide significance (Chan et al. 1991; Moeller 1973; Radcliffe and Ragsdale 2002). Its abundance and propensity to develop alatae makes this aphid species very important in viral epidemiology (Cerato et al. 1994; Singh and Boiteau 1986). In tomato (*Solanum lycopersicum*), resistance to potato aphids is mediated by the *Mi-1* gene, which encodes a coiled-coil nucleotide-binding leucine-rich protein and requires the Somatic Embryogenesis Receptor Kinase 1 (*SISERK1*) (Mantelin et al. 2011; Rossi et al. 1998). The aphid effector recognized directly or indirectly by *Mi-1* remains elusive.

Aphids are phloem feeders that use a pair of slender stylets to mostly move between cells until they reach the sieve element, where they feed for a prolonged period of time. Although the stylet's path is mainly intercellular, stylets do also puncture cells and cause cell-wall disturbance and damage to the plasma membranes of mesophyll and parenchyma cells (Moran et al. 2002; Pollard 1973; Tjallingii and Hogen Esch 1993). Unlike chewing insects that cause extensive tissue damage, this specialized aphid feeding behavior and interaction with its host, avoiding extensive mechanical tissue damage, is analogous to plant-biotrophic pathogen interactions in which the pathogen is sustained in a localized area and is dependent on living host plant cells. Consistent with these observations, induction of plant genes associated with pathogen-induced response pathways have been reported as a result of aphid feeding (De Vos et al. 2005; Kaloshian and Walling 2005; Martinez de Ilarduya et al. 2003; Thompson and Goggin 2006).

Aphids release two types of saliva during feeding, soluble saliva and gelling saliva. The soluble saliva is in liquid form and is delivered along the penetration path and in the sieve element, whereas the gelling saliva forms a proteinaceous sheath around the stylets as soon as it exits the stylet tip (Miles 1999;

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Nucleotide sequence data have been deposited in the DDBJ, EMBL, and GenBank databases under the following accession numbers: *Me5* (JX134487), *Me10* (JX134488), *Me13* (JX134489), *Me14* (JX134490), *Me17* (JX134491), *Me20* (JX134492), *Me23* (JX134493), *Me25* (JX134494).

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Tjallingii 2006). It has been hypothesized that constituents of aphid salivary secretions play crucial roles in modulating plant responses. Aphid saliva consists of a suite of bio-reactive compounds, some of which may serve as cues to elicit plant defenses, while others are expected to function in suppressing or circumventing plant defenses (Harris et al. 2003; Hogenhout and Bos 2011; Miles 1999).

It is speculated that aphid-host interactions shadow the commonly accepted zig-zag model of plant-microbial pathogen defense evolution described by Jones and Dangl (2006). In pathosystems, conserved sets of molecular signatures called pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) or general elicitors are recognized by pattern recognition receptors (PRR) present at the host cell surface. PRR-mediated non-self recognition activates pattern-triggered immunity (PTI) in the host plant. PTI is associated with activation of downstream signaling pathways, including mitogen-activated protein kinases and WRKY transcription factors, induction of defense responses, including production of reactive oxygen species, accumulation of pathogen-related (PR) proteins, and callose deposition that collectively restrict microbial growth (Segonzac and Zipfel 2011). Pathogens can secrete small molecules or proteins known as effectors (Abramovitch et al. 2006; Birch et al. 2006; Davis et al. 2008; Kamoun 2006; van der Hoorn 2008) to counteract PTI and effectively parasitize and colonize the host plant. Extensive research using various approaches has led to the identification of hundreds of effectors secreted by bacterial, fungal, and oomycete plant pathogens that were essential for understanding pathogenesis.

Unlike PAMPs and pathogen effectors, which have been extensively studied during the past decade, not much is known about herbivore-associated molecular patterns or herbivore effectors (Hogenhout and Bos 2011; Miles 1999; Tjallingii 2006). One or more proteinaceous elicitors with a size between 3 and 10 kDa in the saliva of green peach aphid *Myzus persicae* have been shown to induce defense responses in *Arabidopsis* (De Vos and Jander 2009). Similarly, expressing the *Myzus persicae* proteins Mp10 and Mp42 in *Nicotiana benthamiana* resulted in a decrease in aphid fecundity (Bos et al. 2010). Moreover, induction of plant defense genes by feeding of piercing-sucking herbivores, including aphids, has been extensively demonstrated (Kempema et al. 2007; Martinez de Ilarduya et al. 2003; Rodriguez-Saona et al. 2010; Thompson and Goggin 2006). In contrast, little is known about how the salivary proteins of piercing-sucking herbivores may interfere with the plant immune system or manipulate host metabolites for their advantage. Aphids alter host primary metabolism and improve nutrient composition of the phloem sap to enhance their growth (Giordanengo et al. 2010; Wilson et al. 2011). However, it is not clear how aphids are able to cause these changes or the one or more salivary secretions responsible for these changes. A few aphid salivary proteins have been implicated in enhancing aphid performance. The salivary protein MpC002 has been shown to enhance *Myzus persicae* fecundity or nymph production on *N. benthamiana* (Bos et al. 2010). In vitro analysis of the effect of the vetch aphid *Megoura viciae* saliva on forisomes provided direct evidence that aphid saliva has the ability to counteract plant defenses and prevent sieve tube plugging, providing aphids with access to a continuous flow of phloem sap (Will et al. 2007). Moreover, some piercing-sucking insects have been shown to suppress the expression of plant defense genes and manipulate defense signaling pathways to their advantage (Zarate et al. 2007; Zhang et al. 2011). Taken together, the ultimate outcome of interactions of piercing-sucking herbivores with their host plant is likely to depend on the salivary secretion and effectors produced by the herbivore and the ability of the plant to perceive these effectors and respond appro-

priately as well as the ability of the salivary secretions to alter host metabolism.

A large number of pathogen effectors have been identified using homology-based searches, while others were likely missed as their sequences are unique. In aphids, candidate effector molecules have been identified using three approaches involving i) sequencing the aphid salivary gland transcriptome or ii) the salivary gland proteome, followed by prediction of the protein secretion signal, or iii) by direct sequencing the aphid salivary proteome (Bos et al. 2010; Carolan et al. 2009, 2011; Harmel et al. 2008). In this study, we sequenced the salivary gland transcriptome of *M. euphorbiae*, using the Illumina sequencing platform, and assembled the reads into 646 contigs. Data mining of the assembled contigs identified 159 predicted *M. euphorbiae*-secreted proteins. Eight candidate effectors with secretion-signal peptides identified in the salivary transcriptome were functionally characterized, using *Agrobacterium tumefaciens*-mediated transient overexpression in *N. benthamiana* or delivered by the *Pseudomonas syringae* type three secretion system (T3SS) into tomato plants. Using these assays, we identified two effectors, *Me10* and *Me23*, that enhanced aphid fecundity.

RESULTS AND DISCUSSION

Transcriptome sequencing, assembly, and annotation.

To analyze the *M. euphorbiae* salivary gland transcriptome, an RNA-Seq library was prepared from 200 dissected salivary glands of adult aphids. To determine the quality of the library, an aliquot was cloned and 10 clones were subjected to Sanger sequencing. TBLASTX analysis of the clones against the non-redundant (nr) database at the National Center for Biotechnology Information (NCBI) identified unique sequences in each clone, suggesting that the library was not biased for certain transcripts.

A total of 85 million reads were generated from this RNA-Seq library, of which 4 million were unique. Initially, the generated reads were assembled using the Velvet/Oases assembler (Schulz et al. 2012). Further improvement of the assembly was achieved by reducing the redundancy in the data set with the SEED program, novel clustering algorithm for next-generation sequencing data, which resulted in longer contigs (Bao et al. 2011). In total, 646 contigs were generated, with 62% of the assembled contigs longer than 500 bp.

To determine how many of the *M. euphorbiae* salivary gland contigs have putative orthologs in the closely related species *Acyrtosiphon pisum*, whose genome has been sequenced, reciprocal TBLASTX analysis was performed. A total of 551 *M. euphorbiae* contigs were identified with sequences orthologous to 460 *A. pisum* transcripts (Supplementary Table S1). Some of the *M. euphorbiae* contigs matched to non-overlapping regions of the same *A. pisum* transcript, explaining the larger number of *M. euphorbiae* orthologous contigs. Consequently, these contigs were annotated according to version 2 of the *A. pisum* annotation (International Aphid Genomics Consortium 2010). Out of the 460 *A. pisum* transcripts identified in this study, 155 were also identified in the *A. pisum* salivary glands by Carolan and associates (2011). Putative orthologs for an additional 41 contigs were identified by performing reciprocal BLASTX analysis against the Uniprot database and were annotated accordingly. Although these *M. euphorbiae* contigs did not have orthologs in *A. pisum*, we cannot exclude the possibility that they may be present in other aphid species. Orthologs could not be identified for 54 *M. euphorbiae* contigs. They could be the result of inaccurate assembly of the Illumina reads, although it is possible that some of these sequences are correctly assembled and consequently unique to *M. euphorbiae*.

To determine the putative functions of the *M. euphorbiae* contigs, we used the database containing gene ontology (GO) assignments of all the publicly available *A. pisum* expressed sequence tags (EST). The functional classification of the contigs based on GO terms showed enrichment for the classes “translation”, “metabolic process”, and “transport” in the GO category “biological process” (Fig. 1A). Aphids have a pair of salivary glands, each consisting of a principal gland and two accessory glands (Ponsen 1972; Weidemann 1968). Besides the expected cell maintenance processes, the cells of the salivary glands undergo cycles of secretory activities (Miles 1999). Thus, transcripts grouped under the “translation” category are likely to serve both functions. To fulfill their respective roles, those proteins destined to be delivered in the saliva and potentially having roles in the interactions with the plant host are expected to have secretion signals allowing them to cross cell membranes into the salivary canal. Consequently, it is not surprising that transcripts predicted to have a transport function were enriched in this organ. On the other hand, the GO terms in the “molecular function” category showed more distributed and diverse enrichments of various molecular activity categories (Fig. 1B).

Identification of sequences with secretion signal peptides.

The amino acid sequences of the putative full-length *A. pisum* orthologs of the identified *M. euphorbiae* contigs were analyzed with the SignalP 4.0 (Petersen et al. 2011) and TargetP (Emanuelsson et al. 2000) prediction software programs trained

to identify signal peptides. Of the 460 examined sequences, 125 and 159 were predicted to have putative signal peptides predicted by SignalP 4.0 (hidden Markov model scores higher than 0.45) and TargetP (predefined set of cutoffs that yields specificity >0.95 on the TargetP test sets), respectively. TargetP predicted a signal peptide in 121 sequences predicted by SignalP. Moreover, it predicted an additional 38 sequences that were not predicted by SignalP. In the *A. pisum* salivary gland transcriptome, 30% (324 of 1,074) of the transcripts were predicted to have signal peptides using SignalP 3.0 (Carolan et al. 2011). Using the version 4.0 of the same program we predicted 27% (125 of 460) of the salivary gland transcripts to have signal peptide. Around 42% of the sequences with predicted signal peptides in this study were also identified by Carolan and associates (2011). This low overlap of sequences with signal peptides between the two studies suggests that the salivary gland sequences from both species are incomplete.

Selection of clones for functional analysis and their tissue-specific expression.

An efficient way to investigate the roles of aphid candidate effectors in planta is to transiently overexpress them in *N. benthamiana*, using *Agrobacterium tumefaciens*, and assay with a population of aphids that are adapted to plants with nicotine, such as *Myzus persicae*. A similar approach, using *N. benthamiana* leaf disks, was successfully used to evaluate *Myzus persicae* effectors (Bos et al. 2010). In order to use this assay system,

Fig. 1. Classification of the *Macrosiphum euphorbiae* contigs using the *Acyrthosiphon pisum* Gene Ontology terms. **A**, The contigs were annotated according to the biological process that they are predicted or known to be part of or **B**, the known or predicted molecular function.

Table 1. *Macrosiphum euphorbiae* selected genes with predicted secretion peptide and their *Acyrthosiphon pisum* and *Myzus persicae* putative orthologs

Gene	ID ^a	Size ^b	Ortholog and E-value		Conserved domain	Annotation	Phenotype ^c	
			<i>A. pisum</i>	<i>Myzus persicae</i>			<i>N. b.</i>	Tomato
Me5	Me_SG098	1,713	ACYPI002298 (10 ⁻¹⁹³)	MpContig8632 (10 ⁻⁴⁵)	Bacterial alpha-L-rhamnosidase	Trehalase	No	NA
Me10	Me_SG525	387	ACYPI008224 (10 ⁻⁶⁹)	MpContig6453 (10 ⁻⁴⁹)	No conserved domain	Uncharacterized	Yes	Yes
Me13	Me_SG442	324	ACYPI56566 (10 ⁻⁶³)	MpContig11439 (10 ⁻⁴⁷)	Cytochrome P450, Spumavirus gag protein	Uncharacterized	No	NA
Me14	Me_SG355	954	ACYPI21663 (10 ⁻¹⁷⁸)	MpContig3168 (10 ⁻¹²⁹)	Lipase superfamily	Lipase	No	No
Me17	Me_SG427	1,014	ACYPI53825 (10 ⁻¹⁵²)	MpContig11230 (10 ⁻¹⁰³)	Tat binding protein 1-interacting protein	Hypothetical	No	No
Me20	Me_SG497	1,131	ACYPI21412 (10 ⁻¹⁶⁶)	MpContig1043 (10 ⁻⁵⁷)	No conserved domain	Hypothetical	No	NA
Me23	Me_SG130	636	ACYPI002439 (10 ⁻¹²²)	MpContig1685 (10 ⁻⁹³)	Glutathione peroxidase	Glutathione peroxidase	Yes	No
Me25	Me_SG229	918	ACYPI006300 (10 ⁻¹⁷⁷)	MpContig2442 (10 ⁻⁸¹)	Carbonic anhydrase alpha (vertebrate-like)	Carbonic anhydrase 2-like	No	No

^a *M. euphorbiae* expressed sequence tag identification number.

^b Given in bp, excluding the signal peptide.

^c *N. b.* = *Nicotiana benthamiana*; NA = not tested.

we chose *M. euphorbiae* effectors with secretion signals and putative orthologs in *Myzus persicae*. To identify the *Myzus persicae* orthologs of our set of salivary gland-expressed *M. euphorbiae* contigs, we reassembled the publicly available *Myzus persicae* EST and used them in reciprocal TBLASTX analysis with the *M. euphorbiae* salivary gland transcripts. We chose eight *M. euphorbiae* salivary gland contigs with putative orthologs in *Myzus persicae*, four with annotations, and four encoding yet-uncharacterized proteins (Table 1). We cloned the *M. euphorbiae* open reading frames (ORF) encoding the mature proteins corresponding to these sequences, excluding the signal peptide. These eight ORF (*Me5*, *Me10*, *Me13*, *Me14*, *Me17*, *Me20*, *Me23*, and *Me25*) have 72 to 85% amino acid-sequence identity to the corresponding *Myzus persicae* EST contig (Table 1). The relationship among the *Myzus persicae* and *A. pisum* orthologs of these eight *M. euphorbiae* clones was also demonstrated by reciprocal TBLASTX comparisons.

Four proteins were chosen for further study based on possible effector functions suggested by their annotation. Based on *A. pisum* annotation, *Me5* encodes a trehalase. Trehalose accumulation is associated with *Arabidopsis* defense against aphids (Singh et al. 2011). Therefore, trehalase secreted by the aphid may hydrolyze trehalose and counteract host defenses. *Me14* encodes a lipase; members of this superfamily of proteins have diverse roles including defense against oxidative stress (Horne et al. 2009). *Me23* encodes a glutathione peroxidase (GPX) with a potentially protective role against oxidative burst (Lamb and Dixon 1997), while *Me25* encodes a carbonic anhydrase 2, which may function in catalysis of aldehydes induced during aphid feeding (Gosset et al. 2009). Of the four uncharacterized proteins, conserved domain search identified known domains for two, *Me13* and *Me17* (Table 1).

To confirm that the eight genes were expressed in the salivary glands, we evaluated the accumulation of their transcripts in dissected head and glands or gut tissues of *M. euphorbiae* (Fig. 2). Seven genes were expressed in the head and glands

and not in the gut suggesting that their corresponding proteins are produced in the salivary glands, while *Me5* transcripts were detected in all the tissues tested suggesting either tissue-unspecific expression of this gene or expression of its paralogs in different tissues. As seven of the genes tested exhibited tissue-specific expression, it was unlikely that our tissue-specific cDNAs were contaminated. Orthologous transcripts of five of these genes, *Me10*, *Me13*, *Me17*, *Me20*, and *Me23*, were also identified in *A. pisum* salivary gland transcriptome, while peptides corresponding to *Me5*, *Me10*, and *Me23* orthologs were identified in the *A. pisum* salivary gland proteome (Carolan et al. 2011). The presence of transcripts to most of our selected salivary gland genes in both *A. pisum* and *Myzus persicae*, aphid species with narrow and broad host ranges, respectively, suggests a general rather than specialized roles for these genes.

Evaluating the role of candidate *M. euphorbiae* effectors in aphid defense in *N. benthamiana*.

To investigate the roles of *M. euphorbiae* candidate effectors in planta, we transiently expressed the selected *M. euphorbiae* proteins in *N. benthamiana* and assayed with a population of *Myzus persicae* adapted to feeding on tobacco (Kim and Jander 2007). For the transient expression assays, a large area of a *N. benthamiana* leaf was agroinfiltrated with the recombinant binary pEarleyGate100 vector expressing a candidate *M. euphorbiae* protein. Expression of green fluorescent protein (GFP) was used as control to monitor expression and aphid fecundity. At 24 h after infiltration, four *Myzus persicae* adults were caged on each infiltrated leaf exposing the infiltrated area to aphids. The following day, the adults and newly born nymphs were removed, leaving four first-instar nymphs. These remaining aphids were moved to a fresh plant with recently agroinfiltrated leaves on day 6, day 9, and day 12. This schedule was based on efficient GFP expression. These nymphs became adults on day 8, and their fecundity was evaluated on days 9, 12, and 15. The average nymph production per aphid was calculated and normalized to those on the GFP control. Two candidate effectors, *Me10* ($P = 0.004$) and *Me23* ($P = 0.01$), significantly increased *Myzus persicae* fecundity compared with that in the GFP control (Fig. 3). There was no significant difference in aphid fecundity on the plants expressing the remaining six effectors (Fig. 3). Although the increase in aphid fecundity on plants expressing *Me10* or *Me23* was modest, this increase in aphid performance was consistent in three independent experiments. This suggests that *Me10* and *Me23* altered *N. benthamiana* responses for the aphid's advantage. Contributions of a single effector in manipulating host responses could be minor and this effect could be amplified when combined with additional components of the cocktail of effectors secreted by the aphid. Therefore, simultaneous expression of a combination of effectors, with no effect individually, may also result in enhancement of aphid performance.

To date, only a single aphid effector, *MpC002*, has been identified to increase aphid fecundity (Bos et al. 2010). Although it remains unknown how *MpC002* alters *N. benthamiana* responses to *Myzus persicae*, the *A. pisum* ortholog *ApC002* has been implicated in aphid orientation and feeding (Mutti et al. 2008). *ApC002* is also secreted inside the plant host (Mutti et al. 2008). To date, nine and 17 salivary proteins have been identified from *A. pisum* and *Myzus persicae*, respectively (Carolan et al. 2009; Harmel et al. 2008). Interestingly, one of the *A. pisum* salivary proteins is the ortholog of *Me10*, the effector with an unknown function. The second *M. euphorbiae* effector gene we identified to increase aphid fecundity, *Me23*, is predicted to encode a GPX and could be involved in reducing H_2O_2 and to function as an antioxidant to enhance aphid virulence and reduce the effect of the oxidative burst triggered by aphid feeding (Martinez de Ilarduya et al. 2003).

Fig. 2. Tissue-specific expression analysis of the eight *Macrosiphum euphorbiae* candidate effectors. RNA isolated from whole aphids or dissected aphid heads and salivary glands or guts was used in reverse transcription and semiquantitative polymerase chain reaction (PCR) with gene-specific primers. PCR cycles are indicated to the right of the panel. Expression of *MeC002* and *MeSuc* were used as controls for salivary glands and gut, respectively. Ribosomal gene *L27* was used as an internal control for cDNA.

Evaluating the role of candidate *M. euphorbiae* effectors in aphid defense in tomato.

To feed, aphids probe the plant host tissue with a pair of stylets and secrete both watery and gelling saliva in this process. During probing and stylet penetration, cells are punctured and saliva is delivered inside the host cytoplasm. Therefore, aphid feeding can be compared with gram-negative plant pathogenic bacteria that possess a T3SS to invade and colonize the host cell by injecting virulence effectors (Buttner and He 2009; Cornelis and Van Gijsegem 2000). Therefore, we took the advantage of an existing bacterial system to deliver candidate *M. euphorbiae* effectors into the tomato cells. The T3SS of the bacterial pathogen *P. syringae* pv. *tomato* have been successfully utilized to deliver oomycete *Hyaloperonospora parasitica* effectors into *Arabidopsis* cells (Rentel et al. 2008; Sohn et al. 2007). We characterized the function of five of the eight candidate effectors tested in *N. benthamiana* by delivering them through the *P. syringae* pv. *tomato* T3SS into the tomato cell cytoplasm and assaying aphid performance on the plants. To deliver aphid effectors through the T3SS, we used the pVSP_PsSPdes expression vector, which has the promoter and secretion translocation signal of the *P. syringae* pv. *tomato* effector AvrRpm1 (Guttman and Greenberg 2001; Rentel et al. 2008). We introduced this vector in a less virulent strain of *P. syringae* pv. *tomato* DC3000, DC3000Δ*avrPto*/Δ*avrPtoB* (Nguyen et al. 2010), lacking the two strong virulent effectors *avrPto* and *avrPtoB*, to minimize the interference of bacterial effectors to better evaluate subtle differences in aphid performance. The roles of the five aphid effectors were evaluated in tomato by assessing their effect on *M. euphorbiae* fecundity. The *Myzus persicae* colony used in this study is not adapted to tomato and, therefore, was not used in the tomato assays. To perform the *M. euphorbiae* fecundity assays, 4- to 5-week-old tomato plants were vacuum-infiltrated with the recombinant *P. syringae* pv. *tomato* expressing the aphid constructs or β-glucuronidase (GUS) as control. Each plant was infested with nine 1-day-old, age-synchronized, adult aphids 24 h postinfiltration. Nymph production was evaluated

for the next 5 days. The average nymph production per aphid per day was calculated and compared with the GUS control. Of the five candidate effectors tested, only *Me10* significantly increased ($P = 0.021$) aphid fecundity on tomato (Fig. 4). Since *Me10* encodes a protein of 128 amino acids, it is likely that it was delivered into the host cell through the *P. syringae* pv. *tomato* T3SS and that the delivery of *Me10* into the host cells resulted in the enhanced aphid fecundity phenotype. Thus, *Me10* is able to manipulate tomato plant for the aphid's advantage, as it did in *N. benthamiana*. None of the remaining effectors affected aphid fecundity (Fig. 4). We did not find statistically significant differences in aphid performance in tomato plants infected with *P. syringae* pv. *tomato* expressing *Me23* compared with the GUS control, suggesting that *Me23* is not able to alter tomato responses to detectable levels. It is possible that the different mode of effector delivery, the altered aphid assay used for tomato compared with *N. benthamiana*, or the shorter time exposure of aphids to plant expressing the effector did not allow detection of subtle differences in plant responses. Alternatively, *N. benthamiana* may be less tolerant than tomato to aphid infestation and, therefore, more suitable to detect the weak effects of *Me23* (Goodin et al. 2008). Since *Me10* is uncharacterized and has no known functionally conserved domains, it is difficult to speculate how it manipulates plant responses. Future experiments should elucidate this role. Nevertheless, this experiment showed that *P. syringae* pv. *tomato* T3SS can be used for delivery of aphid effectors in planta to evaluate aphid performance. This approach will allow the evaluation of aphid effectors without the need to express them by developing stable transgenic plants in hosts in which *Agrobacterium*-mediated transient expression does not work consistently or is not feasible to perform.

Me10 and *Me23* and their putative *A. pisum* and *Myzus persicae* orthologs.

Two genes of the *A. pisum* genome (ACYPI002439 and ACYPI38240) are annotated as glutathione peroxidases. The amino acid sequence identity between *Me23* and the *A. pisum*

Fig. 3. *Myzus persicae* performance on *Nicotiana benthamiana* plants expressing *Macrosiphum euphorbiae* candidate effectors. *M. euphorbiae* candidate effectors were transiently overexpressed in *N. benthamiana* using *Agrobacterium tumefaciens*. One day after agroinfiltration, each leaf sample was caged with four adult *Myzus persicae*. The following day, adults were removed, leaving four first-instar nymphs. The four nymphs were moved to a freshly agroinfiltrated leaf expressing the candidate effector on days 6, 9, and 12. Nymph production was evaluated up to day 15. Green fluorescent protein (GFP) was used as expression and aphid assay control. Graphs show the average number of nymphs produced per adult from one experiment. Two leaves per plant and four plants per construct were used ($n = 8$). Data from one experiment are presented, with error bars indicating the standard error. Asterisks indicate statistical significance compared with the GFP control (*Me10* $P = 0.004$; *Me23* $P = 0.01$). This experiment was performed three times with essentially identical results.

Fig. 4. *Macrosiphum euphorbiae* performance on tomato plants expressing *M. euphorbiae* candidate effectors. Five-week-old tomato plants were vacuum-infiltrated with recombinant *Pseudomonas syringae* pv. *tomato* DC3000Δ*avrPto*/Δ*avrPtoB* delivering individual aphid effector or β-glucuronidase (GUS) used as control. Five plants per treatment were vacuum-infiltrated with recombinant *P. syringae* pv. *tomato* at 1×10^3 CFU/ml, and at 24 h postinfiltration, each plant was infested with nine age-synchronized 1-day-old adult potato aphids. Aphid progeny were evaluated for 5 days. Graphs show the average number of nymphs produced per adult aphid ($n = 5$). Data from one experiment are presented with error bars indicating the standard error. Asterisk indicates statistical significance compared with the GUS ($P = 0.021$). This experiment was performed three times with essentially identical results.

proteins ACYPI002439 and ACYPI38240 is 87 and 42%, respectively, while the amino acid sequence identity between *Me23* and a putative *Myzus persicae* ortholog is 70% (Table 1). It is not clear how many *Me23* orthologs are present in the *Myzus persicae* genome, as the genome of this aphid has not been sequenced. The alignment of the amino acid sequences from the three aphid species shows blocks of conserved regions (Fig. 5). Near the N terminus within one of these conserved regions, a deletion of eight amino acids is present in the *Myzus persicae* protein. The presence of such an insertion or deletion might alter the protein's function.

Me10 encodes an uncharacterized protein. BLAST analyses indicated that *Me10* is a single-copy gene in *A. pisum* (Table 1). The amino acid sequence of *Me10* has 76 and 88% sequence identity to *Myzus persicae* and *A. pisum* orthologs, respectively. Although these three aphids belong to the same tribe, Macrosiphini, within the aphid subfamily Aphidinae, it is not surprising that *M. euphorbiae* sequences are more similar to *A. pisum*, since *M. euphorbiae* is phylogenetically more closely related to *A. pisum* than *Myzus persicae* (Von Dohlen et al. 2006).

MATERIALS AND METHODS

Plant material and aphid colonies.

Tomato (*Solanum lycopersicum*) cultivars UC82B and Mon-eymaker, *Nicotiana benthamiana*, and tobacco (*Nicotiana tabacum*) NC-95 were used. Seedlings were transplanted into California mix II or sand. The plants were maintained in growth rooms at 24°C with a 16-h-light and 8-h-dark photoperiod and 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity and were fertilized weekly with MiracleGro (18-18-21; Stern's MiracleGro Products, Port Washington, NY, U.S.A.).

Colonies of the parthenogenetic potato aphid (*Macrosiphum euphorbiae*) and green peach aphid (*Myzus persicae*) were reared on tomato cultivar UC82B and tobacco NC-95 plants, respectively. The colonies were maintained in insect cages in a pesticide-free greenhouse at 22 to 26°C supplemented with light for a 16-h day length. Age-synchronized, 1-day-old, adult *M. euphorbiae* aphids were produced as described by Bhattarai and associates (2007).

RNA extraction and cDNA synthesis.

RNA was isolated from 200 dissected *M. euphorbiae* salivary glands and was used for RNA-Seq library preparation as described previously (Atamian and Kaloshian 2012). Similarly, RNA was isolated from whole aphids and 20 dissected heads and salivary glands or guts. cDNA was synthesized from 100 ng of DNase-treated whole body, head and salivary glands, or gut RNA using Superscript III (Invitrogen, Carlsbad, CA, U.S.A.)

reverse transcriptase enzyme and oligo-dT primers according to the manufacturer's recommendations.

Library construction, sequencing, and de novo assembly.

A detailed procedure of RNA-Seq library preparation from salivary gland tissues has been described previously (Atamian and Kaloshian 2012). One lane of single-end 75-nucleotide long sequencing was performed with an Illumina Genome Analyzer II instrument at the Institute for Integrative Genome Biology, University of California Riverside. Data from the Illumina sequencing run was processed using the Illumina standard pipeline version 1.4. The sequence data generated in this study have been deposited in NCBI's Short Read Archive, accession number SRR547988. Redundancies in the data set were removed with the SEED NGS clustering tool (Bao et al. 2011). The remaining sequences were assembled with the Velvet/Oases assembler (Bao et al. 2011; Schulz et al. 2012). This Transcriptome Shotgun Assembly project has been deposited in the DDBJ, EMBL, and GenBank databases under the accession GAAF00000000 or presented in Supplementary Table S2. The version described in this paper is the first version, GAAF01000000.

Annotation and secretion signal prediction.

Reciprocal TBLASTX analyses were performed between *M. euphorbiae* and *Acyrtosiphon pisum* sequences to identify the putative orthologs of the *M. euphorbiae* sequences generated in this study. These putative orthologs were annotated based on the latest *A. pisum* annotation (aphidbase_2.1_peptides). The *M. euphorbiae* sequences with no putative orthologs in *A. pisum* were annotated by performing reciprocal TBLASTX analysis against the NCBI nucleotide (nt/nr) database. The annotated sequences were assigned to different GO categories based on available GO analysis. The full-length putative *A. pisum* orthologs of the *M. euphorbiae* sequences were subjected to de novo signal peptide prediction analysis using SignalP 4.0 and TargetP 1.1 programs.

Cloning *M. euphorbiae* salivary gland EST sequences.

Eight *M. euphorbiae* salivary gland EST sequences with predicted secretion signal in their putative *A. pisum* orthologs were cloned. Gene-specific primers were designed, excluding the secretion signal peptide at the 5' end, based on the *A. pisum* full-length sequences, and a start codon was added (Supplementary Table S3). The polymerase chain reaction (PCR)-amplified products, obtained using Phusion high-fidelity polymerase (New England BioLabs, Beverly, MA, U.S.A.) were cloned into the pDONR207 (Invitrogen), were recombined into the binary vector pEarleyGate100 (Earley et al. 2006), and were sequenced using Sanger sequencing. The clones were trans-

Fig. 5. Alignment of deduced amino acid sequences of *Macrosiphum euphorbiae* effectors Me10 and Me23, with putative orthologs from *Acyrtosiphon pisum* (ACYP) and *Myzus persicae* (Mp). Black and gray shades indicate identical and highly conserved amino acids, respectively. Putative secretion signal peptide sequences are underlined.

formed into *Agrobacterium tumefaciens* GV3101 for transient overexpression in *N. benthamiana*. A subset of these sequences were also recombined into the pVSP_PsSPdes vector (Rentel et al. 2008) and were sequenced and transformed into DC3000/ Δ avrPto/ Δ avrPtoB (*P. syringae* pv. *tomato*) for the assay in tomato plants (Nguyen et al. 2010).

Aphid bioassay on *N. benthamiana*.

Recombinant *Agrobacterium tumefaciens* containing candidate effector or GFP were grown overnight in Luria Bertani media supplemented with rifampicin (25 μ g/ml), gentamycin (14 μ g/ml), and kanamycin (50 μ g/ml) at 28°C. The cultures were diluted to a final optical density at 600 nm of 0.3 in an induction buffer (10 mM MES, 10 mM MgCl₂, 150 μ M acetosyringone, pH = 5.6). Leaves of 4- to 5-week-old *N. benthamiana* plants were agroinfiltrated using a needleless syringe. Two leaves of four plants were infiltrated per construct. On day 2 after infiltration, four *Myzus persicae* adults were caged on each infiltrated leaf. The following day, the adults were removed leaving four first-instar nymphs. On day 6, the 4-day-old nymphs were moved to a leaf of a plant infiltrated 2 days earlier. On day 9, the adult aphids were moved again to a leaf of a plant infiltrated 2 days earlier. On day 12, the same adults were moved to a plant infiltrated 2 days earlier. Aphid survival was counted at 6, 9, 12, and 15 days after the start of the experiment, and the number of newly produced nymphs was counted on days 9, 12, and 15. The average number of nymphs produced per adult per day was calculated by dividing the number of nymphs produced by the number of live adult aphids on days 9, 12, and 15, divided by 2, 3, and 3, respectively, the number of days between counts.

Aphid bioassay on tomato.

The *P. syringae* pv. *tomato* culture was grown on King's B medium (KBM) plates containing rifampicin (25 μ g/ml), carbenicillin (100 μ g/ml), and kanamycin (50 μ g/ml) at 30°C for 2 days. A single colony was inoculated into 200 μ l of liquid KBM, was plated onto KBM, and was incubated for another day at 30°C. A good healthy lawn on the plate was resuspended in 10 mM MgCl₂. Whole plants were vacuum infiltrated with *P. syringae* pv. *tomato* (1×10^3 CFU/ml) containing a candidate effector or GUS in 1 mM MgCl₂ and 0.02% Silwet L-77. Plants were infested with nine 1-day-old adult *M. euphorbiae* 24 h after infiltration. Five plants were used per construct. Aphid fecundity was assessed by counting the number of nymphs and removing them daily for 5 days.

Gene expression analysis.

Semiquantitative reverse transcription PCR analysis was performed using cDNA prepared from whole aphids or dissected aphid heads and salivary glands or guts using gene-specific primer pairs (Supplementary Table S4). *C002* (Mutti et al. 2008) and *Sucrase* (Price et al. 2007) were used as tissue-specific markers and the ribosomal gene L27 (NM_001126221) was used as internal control. PCR was performed in 25 μ l with 40 ng of cDNA template, 1 $\times$ PCR buffer, 2.5 mM MgCl₂, 0.4 mM dNTPs, 2 U of *Taq* DNA polymerase, and 10 μ M of each of the forward and reverse primers. The PCR program was initialized at 94°C for 5 min, followed by 23 cycles of 94°C for 1 min, 58°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 10 min.

Statistical analyses.

Statistical analysis with aphid data from *N. benthamiana* plants was performed using the one-tailed Flinger-Policello test followed by Bonferroni adjustment (BenMamoun 2006). Student's *t*-test was used for aphid data from tomato plants.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The *Acyrtosiphon pisum* EST database: www.aphidests.org
The Aphid genome database: www.aphidbase.com/aphidbase
Center for Biological Sequence Analysis TargetP prediction server: www.cbs.dtu.dk/services/TargetP
NCBI Conserved Domain database: www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi
SignalP4.0 server: www.cbs.dtu.dk/services/SignalP