MPMI Vol. 27, No. 7, 2014, pp. 747–756. http://dx.doi.org/10.1094/MPMI-01-14-0018-R

e-Xtra*

Suppression of Plant Defenses by a *Myzus persicae* (Green Peach Aphid) Salivary Effector Protein

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Submitted 26 January 2014. Accepted 12 March 2014.

The complex interactions between aphids and their host plant are species-specific and involve multiple layers of recognition and defense. Aphid salivary proteins, which are released into the plant during phloem feeding, are a likely mediator of these interactions. In an approach to identify aphid effectors that facilitate feeding from host plants, eleven *Myzus persicae* (green peach aphid) salivary proteins and the GroEL protein of *Buchnera aphidicola*, a bacterial endosymbiont of this aphid species, were expressed transiently in *Nicotiana tabacum* (tobacco). Whereas two salivary proteins increased aphid reproduction, expression of three other aphid proteins and GroEL significantly decreased aphid reproduction on *N. tabacum*. These effects were recapitulated in stable transgenic *Arabidopsis thaliana* plants. Further experiments with *A. thaliana* expressing Mp55, a salivary protein that increased aphid reproduction, showed lower accumulation of 4-methoxyindol-3-ylmethylglucosinolate, callose and hydrogen peroxide in response to aphid feeding. Mp55-expressing plants also were more attractive for aphids in choice assays. Silencing Mp55 gene expression in *M. persicae* using RNA interference approaches reduced aphid reproduction on *N. tabacum*, *A. thaliana*, and *N. benthamiana*. Together, these results demonstrate a role for Mp55, a protein with as-yet-unknown molecular function, in the interaction of *M. persicae* with its host plants.

Aphids feed from host plants by inserting their stylets and navigating between cells to reach the phloem where they ingest phloem sap. During feeding, aphids produce two different types of saliva, gelling and watery (Tjallingii 2006). Gelling saliva forms a proteinaceous sheath around the stylets, protecting them as the aphids probe (Miles 1999). Watery saliva is injected into the phloem and is thought to influence aphid-host plant compatibility. Similar to bacterial pathogens, aphids secrete effector proteins into plant cells, thereby modulating cellular activities. Protein effectors in aphid watery saliva, which is discontinuously injected into the phloem during aphid feeding, are required to circumvent plant defenses but may also allow the plant to recognize the presence of aphid feeding (Moreno et al. 2011; Tjallingii 2006; Will et al. 2007).

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*The *e*-Xtra logo stands for "electronic extra" and indicates that three supplementary figures and one supplementary table are published online.

Several proteomic studies have identified potential effectors in aphid saliva and salivary glands (Carolan et al. 2009, 2011; Cooper et al. 2010, 2011; Cui et al. 2012; Harmel et al. 2008; Nicholson et al. 2012; Rao et al. 2013; Will et al. 2012). In addition to aphid-encoded proteins, proteins produced by *Buchnera aphidicola*, obligate bacterial endosymbionts of aphids, have been found in the secreted saliva (Filichkin et al. 1997; Vandermoten et al. 2014). The heat-shock protein GroEL, which is the most abundant of these bacterial proteins, also has been reported in the aphid hemolymph (van den Heuvel et al. 1994,1997), suggesting potential transport from the bacteriocytes to the salivary glands.

To date, only a few of the identified salivary proteins have been subjected to functional characterization. Calcium-binding proteins in aphid saliva can trigger the condensation of forisomes, protein bodies found in many members of the family Fabaceae that block phloem sieve elements in their dispersed form (Will et al. 2007, 2009). However, a more recent study showed that this forisome phase reversal may occur only in vitro and not when aphids are actually feeding from plants (Walker and Medina-Ortega 2012).

Transgenic expression of individual aphid salivary proteins in plants can affect aphid fecundity (Atamian et al. 2013; Pitino and Hogenhout 2013). C002, the currently best-studied salivary effector, is aphid-specific and facilitates feeding (Mutti et al. 2008). Silencing of C002 transcription reduces aphid fitness (Mutti et al. 2006; Pitino et al. 2011) and, conversely, C002 overexpression in planta increases aphid reproduction (Bos et al. 2010). The latter effect is species-specific; *Myzus persicae* (green peach aphid) C002 protein expression in transgenic plants promotes colonization by this aphid species, whereas the *Acyrthosiphon pisum* (pea aphid) homolog does not (Pitino and Hogenhout 2013). In planta expression of two other proteins from *M. persicae* salivary glands, Mp10 and Mp42, reduced aphid reproduction (Bos et al. 2010).

Plants have evolved to recognize specific herbivores and have multiple defenses against aphid colonization. Plant hormone and signaling pathways, including the jasmonic acid (JA) and salicylic acid (SA) pathways, are activated upon aphid feeding (Thompson and Goggin 2006). Induction of the JA pathway reduces aphid growth on *Arabidopsis thaliana* (Ellis et al. 2002). It is hypothesized that some phloem-feeding insects induce the SA-related signaling pathways, which negatively influences more effective defenses that are regulated by JA signaling (Dicke et al. 2009; Walling 2008).

Molecular interactions between *M. persicae* and *A. thaliana* have been studied extensively (Louis et al. 2012). In response to aphid feeding, *A. thaliana* specifically induces the production of indole glucosinolates and the conversion of indol-3-ylmethylglucosinolate (I3M) to 4-methoxyindol-3-ylmethylglucosinolate (4MI3M), which is a more effective feeding deterrent (Kim and

Jander 2007). Natural variation in the production of 4MI3M affects *A. thaliana* resistance to *M. persicae* (Pfalz et al. 2009). Another plant defense strategy against aphids is callose deposition at the feeding site and in the phloem sieve elements (Botha and Matsiliza 2004; Kusnierczyk et al. 2008). By increasing callose formation and plugging the sieve elements, plants are able to inhibit feeding. Reactive oxygen species, particularly hydrogen peroxide, which is observed specifically at the site of aphid feeding (Martinez de Ilarduya et al. 2003) also have been implicated in aphid defense (Kusnierczyk et al. 2008). A mutation in the *Arabidopsis respiratory burst oxidase homolog D (RbohD)* gene, resulting in decreased H_2O_2 accumulation, causes increased aphid sensitivity (Miller et al. 2009).

In order to identify and characterize additional aphid salivary effectors, we cloned individual genes encoding secreted *M. persicae* salivary proteins for expression in *A. thaliana*, *Nicotiana benthamiana*, and *N. tabacum* (tobacco). Aphid bioassays determined whether these salivary proteins promote aphid feeding or are recognized by plants to mount defense responses or both. Further functional studies were conducted with one aphid effector protein to determine its effects on plant responses to aphid feeding.

RESULTS

M. persicae effector proteins affect reproduction.

Proteins that have been identified in proteomic studies of secreted aphid saliva are likely to be effectors that mediate plant-aphid interactions. We used 12 of these secreted *M. persicae* proteins (Table 1) in further experiments to determine their role in plant-aphid interactions. Following a published nomenclature for *M. persicae* salivary proteins (Bos et al. 2010), the nine previously unstudied aphid salivary proteins have been named Mp55 to Mp63. Among the 12 proteins, seven are aphid-specific and have no known homologs in other species. No predicted functional domains were identified in these proteins with the National Center for Biotechnology Information protein BLAST program (blastp) and the simple modular architecture research tool (Letunic et al. 2012; Schultz et al. 1998). Of the remaining five proteins, Mp56 is a predicted retinol dehydrogenase, Mp59 a regucalcin (SMP-30), Mp62 an AMP-dependent CoA ligase, Mp63 a predicted hydroxyacyl dehydrogenase, and GroEL, a heat shock protein of *Buchnera aphidicola*, a bacterial endosymbiont of *M. persicae*.

Full-length sequences for the predicted salivary proteins were obtained from salivary gland cDNA sequences (Ramsey et al. 2007) and the *Buchnera* Mp genome sequence (Jiang et al. 2013). Genes were amplified from cDNA or genomic DNA (GroEL) by polymerase chain reaction (PCR) and were cloned into *Agrobacterium* plasmids for plant transformation. Although all twelve of the proteins were found in secreted aphid saliva, only six have predicted secretion signal sequences, as determined by SignalP v3 (Bendtsen et al. 2004) (Table 1). In initial experiments, three proteins (Mp1, Mp57, and Mp58) with their signal sequences intact were expressed transiently from the constitutive *Cauliflower mosaic virus* (CaMV) 35S promoter in *N. tabacum* for aphid bioassays (Fig. 1). In planta expression of the transgenes was verified by PCR (Supplementary Fig. 1A). A second *Agrobacterium* strain encoding a *Turnip crinkle virus* coat protein, p38, which suppresses native plant gene silencing (Thomas et al. 2003), was coinfiltrated with each aphid effector construct to promote gene expression. In control experiments, transient expression of p38 alone had no significant effect on aphid reproduction (Fig. 1). Mp1, Mp57, and Mp58 expression did not affect aphid fecundity (Fig. 1), suggesting that cleavage of the signal sequences during secretion into the saliva might be required for in planta function of these proteins.

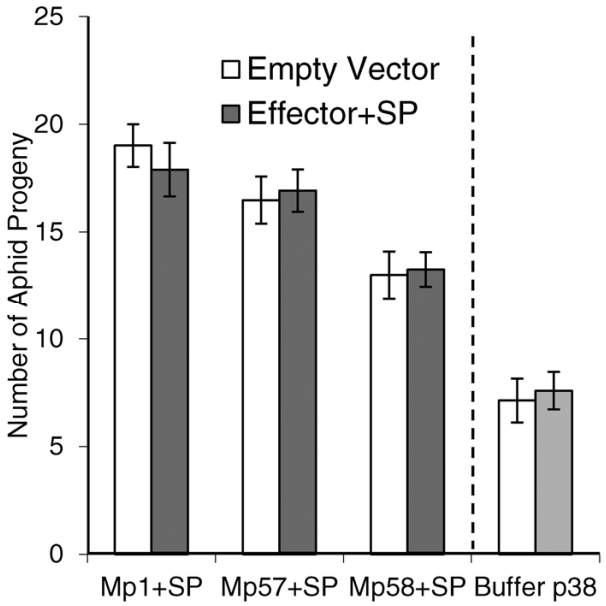

Fig. 1. Overexpression of *Myzus persicae* salivary effector proteins with signal peptides does not alter aphid fecundity. Effector proteins with their signal peptides and a coat protein of *Turnip crinkle virus* p38, which suppresses silencing, were expressed in *Nicotiana tabacum* using agroinfiltration. For each comparison, experimental and control samples were infiltrated into plants grown at the same time. The difference in aphid growth on control plants is due to environmental variation from one experiment to the next. After 3 days, a single adult aphid was caged on the infiltrated area, and its progeny were counted after 7 days, (mean ± standard error, n = 15 to 20) *$P < 0.05$, two-tailed Student's t-test comparing protein-expressing and empty-vector samples and p38 to a $MgCl_2$ control buffer. SP = signal peptide.

Table 1. *Myzus persicae* salivary effector proteins

Protein name	Genbank ID	Size (kD)	Predicted function	Signal sequence	Proteomic reference
MpC002	EC389503.1	28	Unknown	Yes	Harmel et al. 2008; Mutti et al. 2008
Mp1	EE571823.1	16	Unknown	Yes	Harmel et al. 2008; Carolan et al. 2009; Carolan et al. 2011
Mp55	EC389393.1	40	Unknown	Yes	Harmel et al. 2008
Mp56	EC388700.1	32	Retinol dehydrogenase	No	Harmel et al. 2008
Mp57	EC388952.1	44	Unknown	Yes	Harmel et al. 2008
Mp58	ES225976.1	17	Unknown	Yes	Harmel et al. 2008; Carolan et al. 2009; Carolan et al. 2011
Mp59	EC387947.1	36	Regucalcin SMP 30	No	Carolan et al. 2009; Carolan et al. 2011
Mp60	EC389958.1	12	Unknown	Yes	Harmel et al. 2008
Mp61	EC389075.1	13	Unknown	No	Harmel et al. 2008
Mp62	ES221969.1	19	AMP dependent CoA ligase	No	Harmel et al. 2008
Mp63	DW010534.1	27	Hydroxyacyl dehydrogenase	No	Harmel et al. 2008
GroEL	AF367248.1	58	Heat shock protein	No	Vandermoten et al. 2014

In further experiments, all salivary proteins with predicted signal sequences were cloned without these sequences for in planta expression and aphid bioassays. In planta expression of the transgenes was verified by PCR. Two effector proteins, Mp55 and MpC002, increased aphid fecundity on *N. tabacum* (Fig. 2A). Three *M. persicae* salivary proteins (Mp56, Mp57, and Mp58) and GroEL decreased aphid reproduction, likely through activation of plant defense responses. Six other *M. persicae* effector proteins, Mp1, Mp59, Mp60, Mp60, Mp61, Mp62, and Mp63 did not significantly affect aphid fecundity on *N. tabacum* (Fig. 2A).

To confirm the observed effects, salivary proteins that altered aphid reproduction on *N. tabacum* were expressed in stable transgenic *A. thaliana.* Mp1, a highly expressed salivary protein that was previously investigated (Pitino and Hogenhout 2013), also was included in this experiment. In planta expression of the transgenes was verified by PCR. For each salivary protein construct, three independent transgenic *A. thaliana* lines were created and tested with aphids, showing similar results in each case (Fig. 2B). For each tested salivary protein, the effect on aphid reproduction on *A. thaliana* was similar to that previously observed with transient expression in *N. tabacum* (Fig. 2A). Mp55 and MpC002 increased reproduction; Mp58, Mp56, Mp57, and GroEL decreased reproduction; and Mp1 caused no significant effect (Fig. 2B).

Aphids feed primarily from the plant phloem, suggesting that this could be where salivary proteins function in plant-aphid interactions. Therefore, we generated stable transgenic lines expressing MpC002 and Mp58 under the sucrose transporter promoter AtSuc2, which is phloem-specific (Gottwald et al. 2000). In planta expression of the transgenes was verified by PCR. As in the case of expression from the constitutive 35S promoter in *N. tabacum* and *A. thaliana*, expression of MpC002 and Mp58 from the AtSuc2 promoter increased and decreased aphid reproduction, respectively (Fig. 2C). To confirm protein accumulation in the vascular tissue, MpC002 was fused to

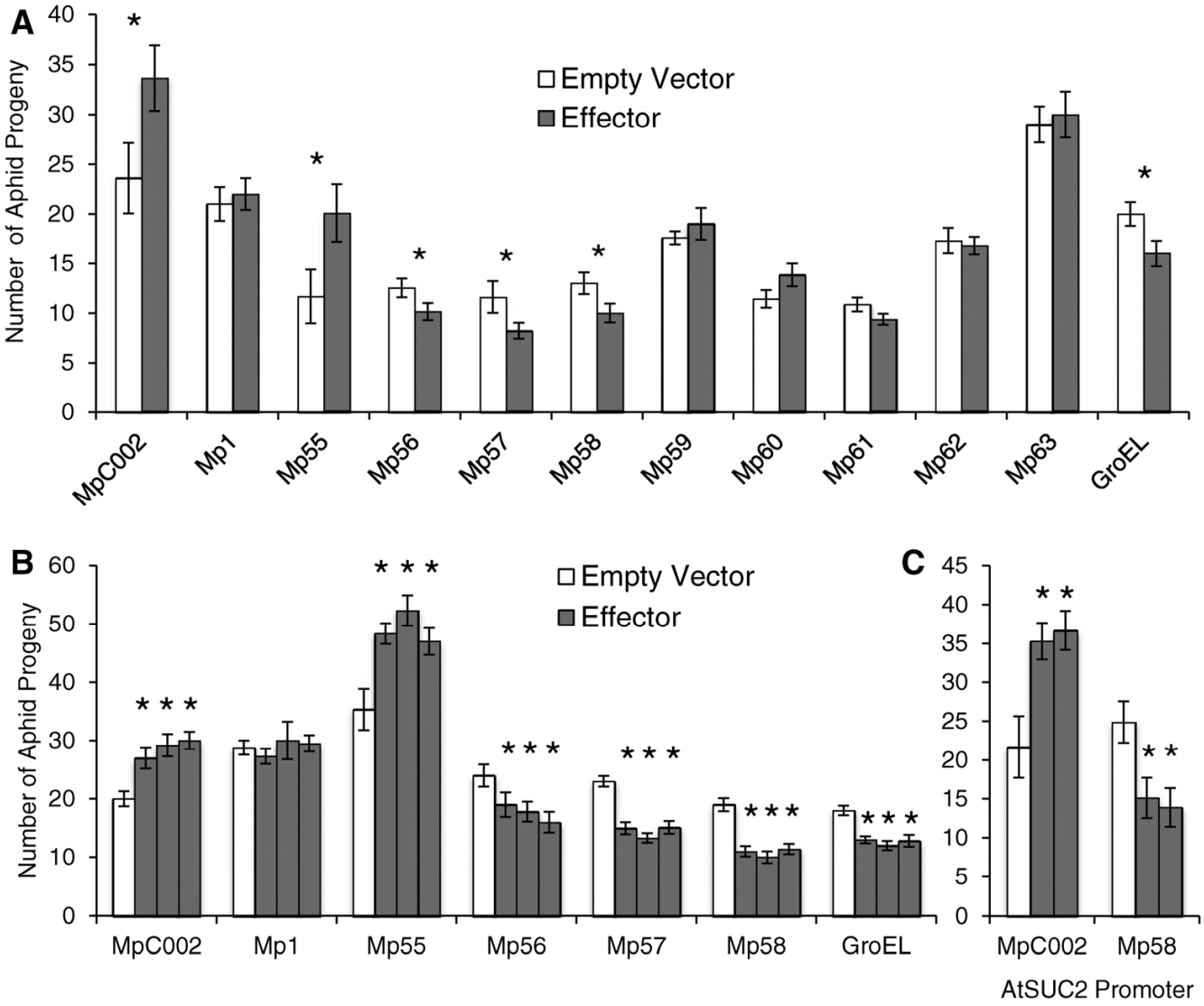

Fig. 2. Overexpression of *Myzus persicae* salivary effector proteins alters aphid fecundity. **A,** Effector proteins were expressed in *Nicotiana tabacum* using agroinfiltration. After 3 days, a single adult aphid was caged on the infiltrated area, and its progeny were counted after 7 days. For each comparison, experimental and control samples were infiltrated into plants grown at the same time. Difference in aphid growth on control plants is due to environmental variation from one experiment to the next. **B,** Independent stable *Arabidopsis thaliana* transgenic lines expressing *M. persicae* effector proteins under the constitutive 35S promoter. A single aphid was caged on a leaf of a three-week-old plant, and its progeny were counted after 7 days. **C,** Independent stable *A. thaliana* transgenic lines expressing *M. persicae* effector proteins under the phloem-specific promoter AtSuc2. A single aphid was caged on a leaf of a three-week-old plant, and its progeny were counted after 7 days (mean ± standard error, $n = 15$ to 20); $^*P < 0.05$, two-tailed Student's *t*-test comparing protein-expressing and empty-vector samples.

green fluorescent protein and the localization of the protein produced from the 35S and AtSuc2 promoters was visualized in tobacco (Supplementary Fig. 2).

GroEL is a highly conserved bacterial protein (Baumann et al. 1996; Humphreys and Douglas 1997) with over 80% sequence identity between the *Buchnera* Mp and *Escherichia coli* homologs (Hara and Ishikawa 1990). Thus, it is possible that plants would recognize this protein as an indicator of bacterial infection and mount a defense response. Induction of *PR1* gene expression by transgenic expression of GroEL in *A. thaliana* is consistent with this hypothesis of upregulated antimicrobial defense responses (Fig. 3).

M. persicae effector gene expression depends on the host plant.

Different effector proteins may be important for *M. persicae* feeding from multiple host plants. To determine whether this is the case, expression of the six previously identified aphid effector genes (Mp1, C002, Mp55, Mp56, Mp57, and Mp58) was measured in aphids feeding from *Brassica oleracea* (cabbage), *N. tabacum*, and *A. thaliana* (Fig. 4A). These host plants differentially affect Mp55 expression in the aphids, with the highest expression on *Brassica oleracea*, twice as high as when feeding from *N. tabacum*. In contrast to the host plant effects, expression of six tested *M. persicae* salivary genes was not affected by aphid age; no differences in salivary effector gene expression were seen when comparing adults and nymphs (Fig. 4B).

Mp55 expression promotes aphid fecundity.

Mp55, which increases aphid fecundity when overexpressed in both *N. tabacum* and *A. thaliana* (Fig. 2), was chosen for further analysis. The Mp55 gene sequence is identical in two sequenced *M. persicae* lineages (American clone G006 and British clone O, available AphidBase) and has a likely homolog in *Acyrthosiphon pisum* with 53% sequence identity at the amino acid level (Supplementary Fig. 3). When Mp55 gene expression was silenced in *M. persicae* through plant-mediated RNA interference (RNAi) in *N. tabacum* (Fig. 5A), *N. benthamiana* (Fig. 5B), and *A. thaliana* (Fig. 5C), there was decreased aphid reproduction in each case (Fig. 5D through F).

Suppression of defenses is a possible cause for the increased aphid reproduction on plants expressing Mp55. As aphid feeding induces conversion of I3M to 4MI3M, which is a more-deterrent glucosinolate for *M. persicae* (Kim and Jander 2007), leaf indole glucosinolates were measured in Mp55-expressing *A. thaliana* and control plants. The 4MI3M levels were significantly lower in Mp55-expressing plants compared with those transformed with the empty vector (Fig. 6A). Conversely, I3M was more abundant in Mp55-transgenic *A. thaliana*, suggesting a lower level of I3M to 4MI3M conversion.

Callose, which can plug phloem sieve elements, thereby inhibiting aphid feeding, is a common plant defense response against aphids (Botha and Matsiliza 2004). To determine whether this effect is reduced in plants expressing Mp55, aphids were fed for 48 h on Mp55-transgenic and control plants. Mp55-transgenic *A. thaliana* had fewer callose deposits than the empty vector controls (Fig. 6B), suggesting that aphids could feed more easily on the Mp55-expressing plants. Additionally, aphids feeding from Mp55-transgenic *A. thaliana* induced less hydrogen peroxide accumulation in the leaves (Fig. 6C), a further indication of suppressed defense responses.

M. persicae prefer Mp55 plants.

To determine whether *M. persicae* prefer plants overexpressing Mp55, *N. tabacum* leaf disks transformed with either Mp55 or an empty vector were placed in petri dishes with a single adult aphid in the middle. After 24 h, significantly more aphids chose leaf disks expressing Mp55 (Fig. 7A). In a similar experiment with detached leaves from Mp55- and empty vector–transgenic *A. thaliana*, aphids also chose the Mp55 leaves (Fig. 7B). In subsequent experiments, aphids were given a choice between entire *N. benthamiana* plants transiently expressing either Mp55 or the empty vector, growing together in the same pot. As in the case of leaf disks and whole leaves, aphids showed a significant preference for *N. benthamiana* expressing Mp55 as compared with the empty vector controls (Fig. 7C).

DISCUSSION

Aphids modulate plant cellular processes by injecting pulses of salivary proteins into the phloem as they are feeding. Among twelve tested *M. persicae* salivary proteins, two promote aphid reproduction, four decrease aphid progeny production, and six have no significant effects (Fig. 2). Our results showing no difference in aphid reproduction on plants expressing Mp1 are in agreement with a previous *M. persicae* effector screen in *N. benthamiana* (Bos et al. 2010). However, a follow-up study showed increased aphid reproduction on stable transgenic *A. thaliana* expressing Mp1. Although silencing Mp1 had no effect on aphid reproduction (Pitino and Hogenhout 2013), this could be explained by the incomplete reduction in the RNA levels of this abundantly expressed gene. Mp58 is homologous to Me10, a previously investigated *Macrosiphum euphorbiae* (potato aphid) effector protein. When overexpressed in *N. benthamiana* and *Solanum lycopersicum* (tomato), Me10 increased *M. persicae* and *M. euphorbiae* reproduction, respectively (Atamian et al. 2013). This is in contrast to our findings with Mp58 (Fig. 2), but it is known that aphid effectors can act in a plant and aphid species–specific manner (Pitino and Hogenhout 2013).

In cases in which salivary protein expression decreases aphid reproduction, this could be caused by plant recognition mechanisms and induction of defense responses. GroEL expression increases expression of the defense-related *PR1* gene

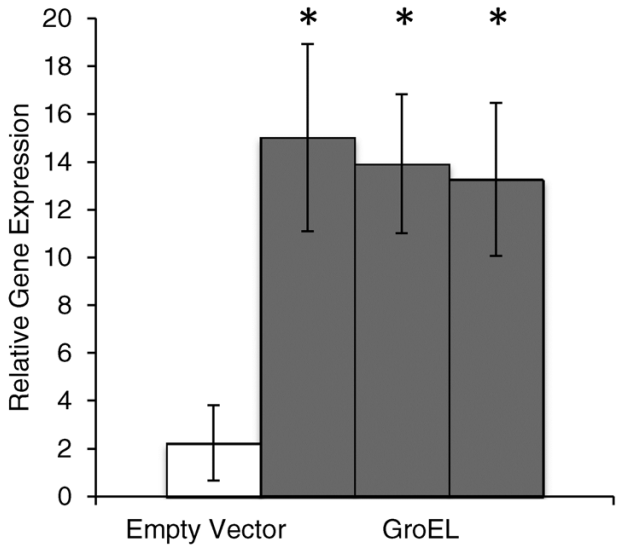

Fig. 3. *PR1* gene expression analysis of pathogenesis response gene *PR-1* in GroEL-expressing *Arabidopsis thaliana*. Gene expression was measured by quantitative reverse transcription-polymerase chain reaction relative to a control gene, *elongation factor a*, in 3 week-old *A. thaliana* stably expressing the *Buchnera aphidicola* GroEL gene. Three independent transgenic lines were tested in the absence of aphid feeding. Mean ± standad error, $n = 6$; $*P < 0.05$, two-tailed Student's *t*-test relative to the empty-vector control.

(Fig. 3). Although *PR1* is induced by aphid feeding (Divol et al. 2007; Kusnierczyk et al. 2007; Moran and Thompson 2001), this protein has not been associated with aphid resistance in plants and may not be a direct cause of aphid resistance in our experiments (Fig. 2). However, consistent with our observations, expression of GroEL from a bacterial endosymbiont of *Bemisia tabaci* (silverleaf whitefly) confers resistance to some viruses in *Solanum lycopersicum* and *N. benthamiana* (Akad et al. 2007; Edelbaum et al. 2009), suggesting a general upregulation of antimicrobial defenses.

Suppression of plant defense responses by *M. persicae* salivary protein Mp55 causes increased aphid reproduction (Fig. 2) and makes plants more attractive in choice assays (Fig. 7). The indole glucosinolate 4MI3M is an effective feeding deterrent for *M. persicae* (Kim and Jander 2007). Aphids feeding on Mp55-expressing *A. thaliana* induce lower levels of 4MI3M than on empty vector controls (Fig. 6A), suggesting reduced defense induction. In accordance with this, the level of the precursor indole glucosinolate I3M is increased in Mp55-transgenic plants. However, we cannot rule out the possibility that altered indole glucosinolate content in this experiment is an indirect effect of altered aphid feeding behavior or reproduction. Interestingly, it was previously shown that 4MI3M degradation is required for flg22-induced callose deposition in *A. thaliana* (Clay et al. 2009), suggesting that a 4MI3M breakdown product is a signaling intermediate in this pathway. Consistent with this, leaves of Mp55-expressing *A. thaliana* have less callose deposition in response to *M. persicae* feeding than the empty vector controls. Additionally, the defense signaling molecule hydrogen peroxide is less abundant in Mp55-expressing *A. thaliana*.

When feeding from different host plants, *M. persicae* modifies its expression of salivary effector proteins (Fig. 4A), presumably to avoid activation of defenses and facilitate feeding. If Mp55 reduces 4MI3M glucosinolate production and perhaps other crucifer-specific defenses, it would be advantageous for aphids to increase expression of this gene in a targeted manner. Consistent with this hypothesis, Mp55 gene expression is higher on *Brassica oleracea* and *A. thaliana* than on *N. tabacum* (Fig. 4A). Further research will be required to determine whether modulation of salivary protein expression contributes to the ability of *M. persicae* to feed from a wide variety of host plants.

Expression of aphid salivary genes in plants can serve as a useful tool for identifying candidate effectors for further experiments. However, there are also limitations to this approach. For instance, it is likely that in planta expression from the 35S promoter will produce higher protein amounts than are biolog-

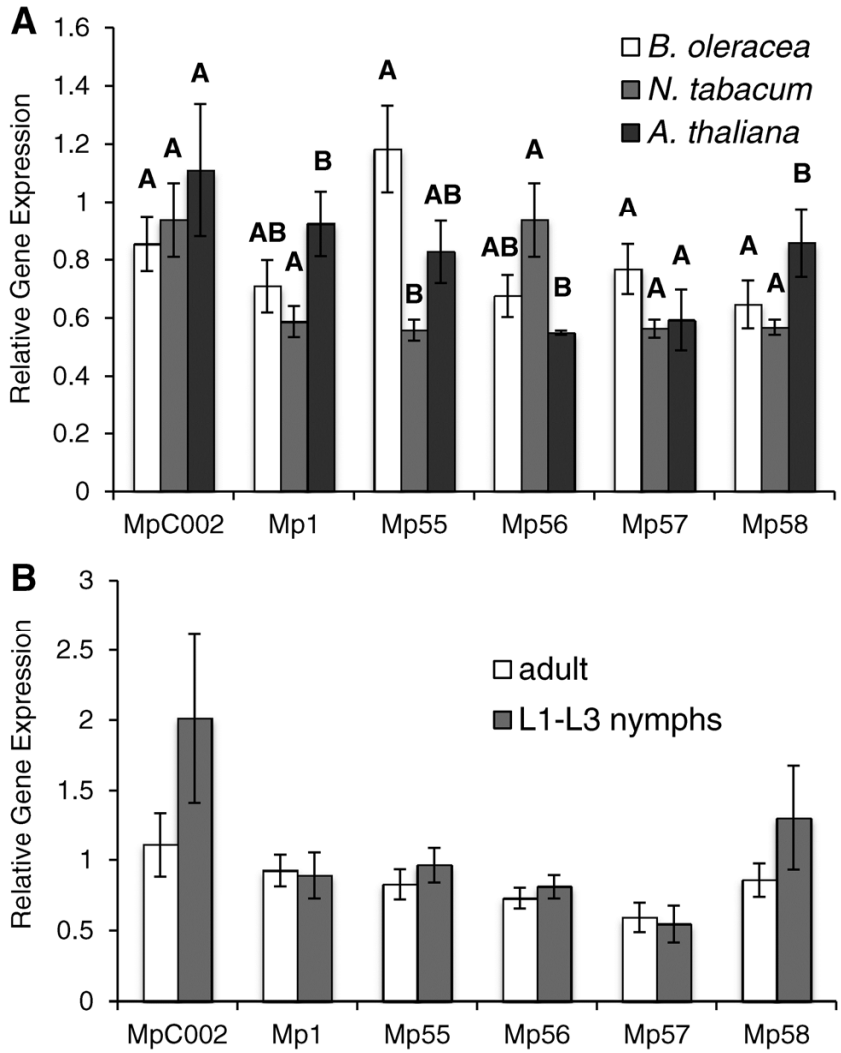

Fig. 4. *Myzus persicae* salivary effector gene expression is host plant dependant but is not influenced by age. Expression levels of *M. persicae* salivary effector genes were measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) relative to a control ribosomal gene (*RpL7*). **A,** Aphids were reared on cabbage (*Brassica oleracea*), tobacco (*Nicotiana tabacum*), and wild-type *Arabidopsis thaliana* (Col-0) L1-L3, (mean ± standard error [SE]; $n = 6$). Analysis of variance, Tukey honestly significant difference letters correspond to individual clusters only. **B,** Adult and L1-L3 nymphs were reared on wild-type *A. thaliana* (Col-0). Gene expression was measured by qRT-PCR, mean ± SE, $n = 6$, no significant differences, two-tailed Student's *t*-test.

ically relevant. Aphids appear to modulate their salivary gene expression on different host plants (Fig. 4A). Thus, a certain amount of salivary protein may be optimal for *M. persicae* feeding and overexpression could result in either false positive or false negative effects in our experiments. Correct subcellular localization also may not be achieved through in planta expression of salivary proteins. Although aphids feed primarily from the phloem, the identified effectors also could mediate plant-aphid interactions in other cell types or in intercellular spaces. Nevertheless, the similar aphid growth effects that we see with salivary effectors expressed from the constitutive 35S promoter (Fig. 2B) and the phloem-specific AtSuc2 promoter (Fig. 2C) suggest that the location of expression may not be critical for some aphid salivary effectors. Once the molecular functions of these salivary effectors have been identified, it will be possible to conduct more detailed experiments to localize their functions during natural aphid feeding.

In summary, our results show that specific *M. persicae* effector proteins can influence aphid fecundity on host plants. Mp56, Mp57, and Mp58 decrease aphid reproduction, likely by activating plant defense responses, whereas MpC002 and Mp55 increase aphid fecundity. *M. persicae* effector gene expression varies on different host plants, suggesting this as a mechanism of host plant adaptation. Mp55, a protein with as-yet-unknown molecular function, reduces accumulation of a toxic glucosinolate, inhibits callose deposition, and lowers accumulation of hydrogen peroxide in *A. thaliana*. These results indicate that *M. persicae* secretes this effector into the plant in order to suppress plant defense responses and improve aphid performance.

MATERIALS AND METHODS

Plants and growth conditions.

Wild-type *A. thaliana* landrace Columbia-0 (Col-0) was obtained from the Arabidopsis Biological Resource Center. Wild-type and transgenic Col-0 were grown in Cornell mix (by weight, 56% peat moss, 35% vermiculite, 4% lime, 4% Osmocoat slow-release fertilizer [Scotts, Marysville, OH, U.S.A., and 1% Unimix [Peters, Everris, The Netherlands) in Conviron growth chambers in 20 × 40-cm nursery flats with a photosynthetic photon flux density of 200 μmol m^{-2} s^{-1} and a 16-h photoperiod at 23°C and 50% relative humidity. Experiments were performed with three-week-old plants before flowering. Cabbage (*Brassica oleracea* var. Wisconsin Golden Acre; Seedway, Hall, NY, U.S.A.), *N. tabacum* (var. NC95), and *N. benthamiana* were germinated in Metro Mix 360 (Scotts), were transplanted after 2 weeks to Cornell mix, and were grown in Conviron chambers as described above. Experiments were performed using plants that were 5 to 7 weeks old.

Insect rearing.

Aphid experiments were conducted with a tobacco-adapted red lineage of *M. persicae* (Ramsey et al. 2007), which was obtained from S. Gray (United States Department of Agricul-

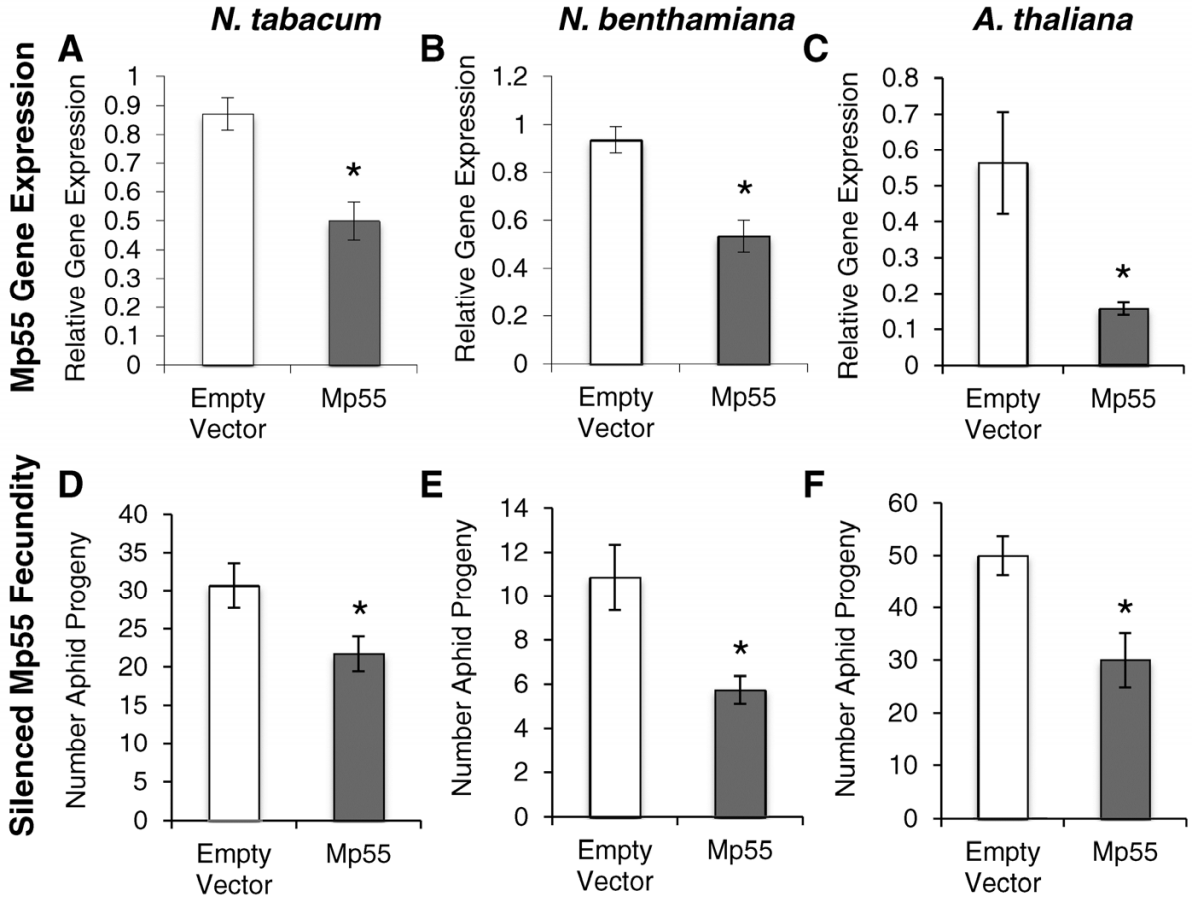

Fig. 5. *Myzus persicae* salivary effector protein Mp55 increases aphid fecundity. **A,** *M. persicace* expression levels of Mp55 relative to ribosomal control gene (RpL7) was measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) when expressing a hairpin RNAi knock-down construct in *Nicotiana tabacum* and in **B,** *N. benthamiana*, using agroinfiltration, and **C,** stably expressed in *Arabidopsis thaliana*. **D,** Knock-down of *M. persicae* Mp55 reduces aphid fecundity, using agroinfiltration in *N. tabacum* and **E,** *N. benthamiana* and **F,** stable transformation in *A. thaliana*. For agroinfiltration after 3 days, a single adult aphid was caged on the infiltrated area and, for stable *A. thaliana* transformants, a single adult aphid was caged on a three-week-old leaf. Aphid progeny were counted after 7 days (mean ± standard error; bioassays, $n = 20$; qRT-PCR, $n = 6$); $^*P < 0.05$, two-tailed Student's *t*-test.

ture Plant Soil and Nutrition Laboratory, Ithaca, NY, U.S.A.). Aphids were raised on *N. tabacum* unless otherwise noted, with a 16-h day and a photosynthetic photon flux density of 200 μmol m^{-2} s^{-1} at 23°C and 50% relative humidity.

Aphid cDNA synthesis.

RNA was extracted from groups of 10 to 50 aphids. Aphids were flash-frozen in liquid nitrogen and were ground to a fine powder using a paint shaker (Harbil, Wheeling, IL, U.S.A.) and 3-mm steel balls. Following homogenization, RNA was extracted using TRI Reagent (Sigma, St. Louis) and was purified with the SV total RNA isolation kit with on-column DNase treatment (Promega, Madison, WI, U.S.A.). From 1 μg of RNA, cDNA was reverse-transcribed using SMART MMLV reverse transcriptase (Clontech, Mountain View, CA, U.S.A.) and oligo-dT_{12-18} as a primer.

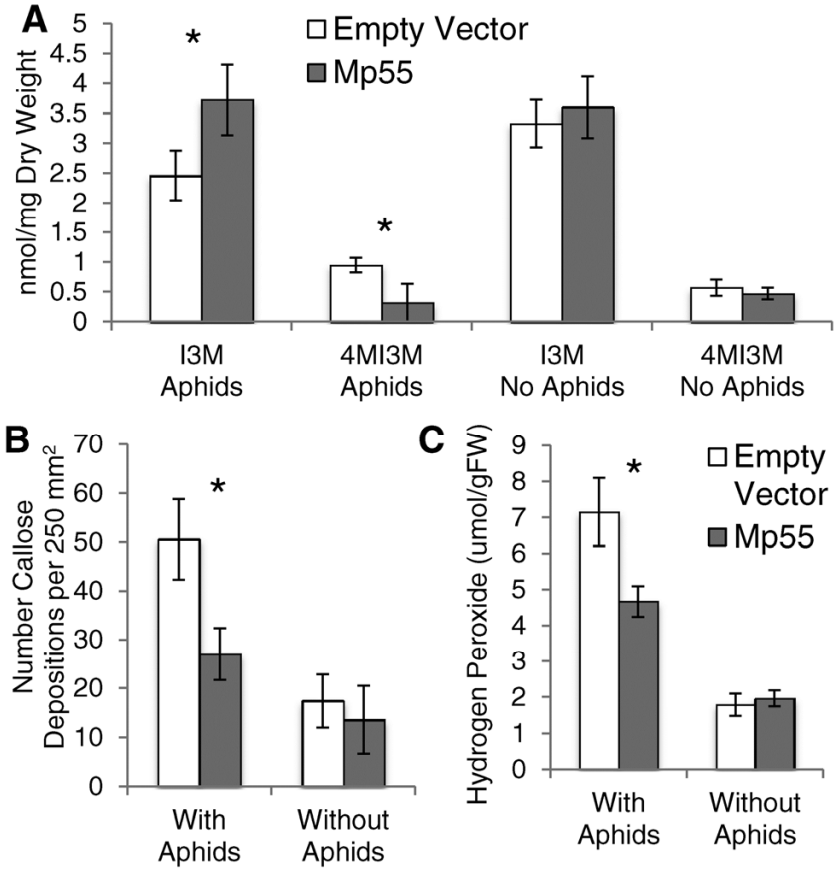

Fig. 6. Salivary protein Mp55 suppresses *Arabidopsis thaliana* defenses. Control plants and plants expressing the Mp55 protein were fed upon by aphids for 48 h. **A,** Indole glucosinolate profile of three-week-old *A. thaliana* leaves (mean ± standard error (SE), $n = 8$), $^*P < 0.05$, two-tailed student's *t*-test. **B,** Callose deposits in a 250 mm^2 area in three-week-old *A. thaliana* leaves (mean ± SE, $n = 48$), $^*P < 0.05$, two-tailed student's *t*-test. **C,** Hydrogen peroxide levels in three-week-old *A. thaliana* leaves (mean ± SE, $n = 5$), $^*P < 0.05$, two-tailed Student's *t*-test.

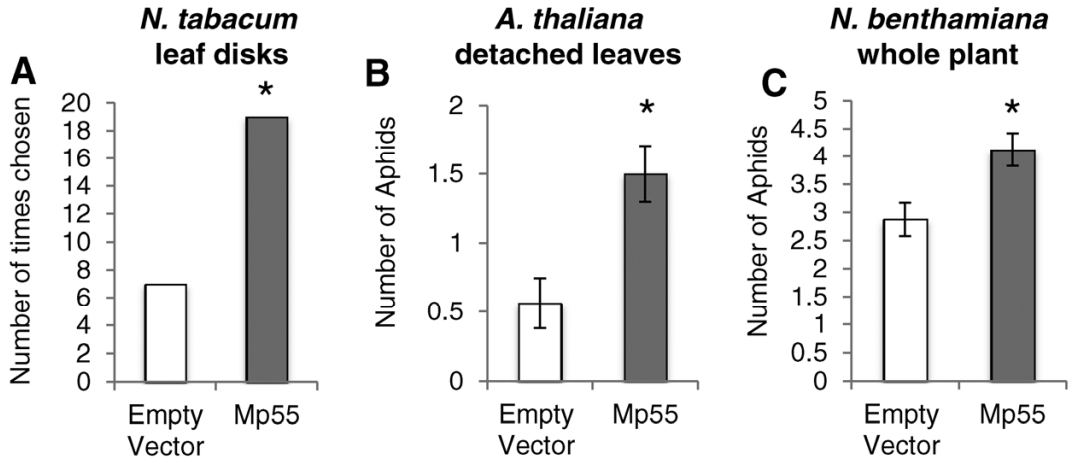

Fig. 7. *Myzus persicae* has a preference for plants expressing Mp55. **A,** Expression of Mp55 in *Nicotiana tabacum* leaf disks through agroinfiltration. A single aphid was given a choice between Mp55 and empty-vector disks placed in a petri dish; $^*P < 0.05$; χ^2-test, $n = 35$. **B,** Detached *Arabidopsis thaliana* leaves stably expressing Mp55. Three adult aphids were given a choice between Mp55 and empty-vector leaves placed in a petri dish (mean ± standard error [SE], $n = 30$); $^*P < 0.05$, two-tailed Student's *t*-test. **C,** Expression of Mp55 through agroinfiltration of four-week-old whole *N. benthamiana* plants. Five adult aphids were given a choice between Mp55 and empty vector–expressing plants in the same pot (mean ± SE, $n = 20$); $^*P < 0.05$, two-tailed Student's *t*-test.

Gene cloning.

Using cDNA sequences from *M. persicae* salivary glands (Ramsey et al. 2007), full-length sequences of salivary proteins were obtained. Only proteins that were verified to be found in *M. persicae* saliva through proteomics studies (Carolan et al. 2009, 2011; Harmel et al. 2008) were chosen. Using the Gateway cloning system (Invitrogen, Carlsbad, CA, U.S.A.), primers were designed corresponding to the coding regions with or without signal peptides (Supplementary Table 1). Using EX *Taq* DNA polymerase (Takara, Shiga, Japan) genes were cloned into entry vector pDONR207 using BP clonase (Invitrogen). Followed by an LR reaction with LR clonase (Invitrogen) into destination vector pMDC32 or pMDC85 (Curtis and Grossniklaus 2003) for overexpression, constructs driven by the 2× CaMV 35S promoter. Phloem specific constructs were also made by cutting out the 35S promoter and inserting the *A. thaliana* sucrose transporter promoter AtSuc2 (Gottwald et al. 2000). Restriction enzyme cut sites *hin*dIII and *kpn*I were used for the pMDC32 vector and *hin*dIII and *pac*I for pMDC85; primers were designed for the AtSuc2 promoter, including the corresponding restriction site.

For knock-down by RNAi in aphids, at least 900 bp of MpC002, Mp1, Mp55, Mp56, Mp57, Mp58, Mp59, Mp60, Mp61, Mp62, Mp63, and GroEL without the signal peptide were cloned into pDONR207 (Invitrogen), following an LR reaction into destination vector pANDA 35HK (Miki and Shimamoto 2004) driven by the *CaMV* 35S promoter. The final destination plasmids containing a specific salivary effector gene sequence were transformed into *Agrobacterium tumefaciens* GV3101.

Stable *A. thaliana* transformants.

Using an established protocol (Clough and Bent 1998), *A. thaliana* Col-0 was grown until flowering and was dipped in a sucrose solution containing *Agrobacterium tumefaciens* GV3101 containing an expression vector with a single effector gene. T1 seed was collected and hygromycin-resistant transformants were selected on Murashige and Skoog medium (Murashige and Skoog 1962) containing 50 μg of hygromycin per milliliter. Hygromycin-resistant plants were transferred to soil, and T2 seeds were collected. Plants showing hygromycin resistance and expression of the transformed effector gene in the T2 generation were used for further analysis. Reverse transcription (RT)-PCR was performed on all independent lines to verify effector gene expression.

Transient overexpression assays.

Recombinant *Agrobacterium tumefaciens* GV3101 cultures, at a final OD_{600} (optical density at 600 nm) of 0.2, expressing an empty vector, a single effector protein, or hairpin RNAi structure were pressure-infiltrated into *N. tabacum* or *N. benthamiana* using a 1-ml plastic syringe. A vector expressing the capsid protein of *Turnip crinkle virus* tomato (p38), which suppresses native plant protein silencing (Thomas et al. 2003), was coinfiltrated to increase the duration of protein expression. Experiments for each empty vector and effector comparison were conducted at the same time, two spots per leaf, two leaves per plant were infiltrated with the same construct and plants were randomized in the flats. Three days after infiltration, a single adult aphid was caged on the infiltrated area, and its progeny were counted after 7 days. Expression of the effector transcript was verified for each aphid bioassay.

***A. thaliana* bioassays.**

Transgenic *A. thaliana* was grown as described above, a single adult aphid was caged on a leaf, and its progeny were counted after 7 days. *A. thaliana* plants transformed with an empty vector were used as controls. For each effector vs. control comparison, plants were grown at the same time in the same pots.

Quantitative gene expression analysis.

Total RNA was extracted using the SV total RNA isolation kit with on-column DNase treatment (Promega). Abundance of all transcripts was analyzed by quantitative real-time RT-PCR, using ribosomal protein RpL7, ubiquitously expressed and likely a single-copy gene, as an internal standard. After extraction and DNAse treatment, 1 μg of RNA was reverse transcribed using SMART MMLV reverse transcriptase (Clontech) and oligo-$dT_{12\text{-}18}$ as a primer. Gene-specific primers were designed using Primer3. Reactions were performed with 5 μl of 2× Power SYBR Green (Applied Biosystems, Foster City, CA, U.S.A.) and 800 nM primer in the 7900HT instrument (Applied Biosystems) with an initial incubation at 95°C for 10 min. The following cycle was repeated 40 times: 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. The cycle threshold values were quantified and analyzed according to the standard curve method.

Glucosinolate assays.

Wild-type Col-0 and transgenic *A. thaliana* were grown as described above, and 30 aphids were caged for 48 h on individual leaves. Leaf tissue was collected, flash frozen in liquid nitrogen, and lyophilized. Extraction and preparation of desulfoglucosinolates from leaf tissue was described previously (Barth and Jander 2006; Kim et al 2008). Desulfoglucosinolates were separated using a Waters 2695 high-pressure liquid chromatography (HPLC) and were detected using a Waters 2996 photodiode array detector. For HPLC separation, the mobile phases were water (A) and 90% acetonitrile with a flow rate of 1 ml min^{-1} at 23°C. Column linear gradients for samples were as follows: 0 to 1 min, 98% A; 1 to 6 min, 94% A; 6 to 8 min, 92% A; 8 to 16 min, 77% A; 16 to 20 min, 60% A; 20 to 25 min, 0% A; 25 to 27 min, hold at 0% A; 27 to 28 min, 98% A; 28 to 37 min, 98% A.

Callose staining.

Leaves were destained in 95% ethanol for 24 h and were washed for 30 min three times with 0.07 M phosphate buffer and were then stained with 0.01% aniline blue in 0.07 M phosphate buffer for 2 h. After staining, the leaves were rinsed with 0.07 M phosphate buffer three times for 20 min. Callose deposition was visualized at 360 to 380 nm, and spots were counted.

Diaminobenzidine staining.

Wild-type Col-0 and stable transgenic *A. thaliana* were grown as described above, and 30 aphids were caged for 48 h on individual leaves. Following aphid feeding, leaves were vacuum-infiltrated and were stained with 3,3′diaminobenzidine (Sigma-Aldrich). After destaining and bleaching, the leaves were ground in liquid nitrogen, were homogenized in 0.2 M $HClO_4$, and were centrifuged at 12,000 × *g* for 10 min in an Eppendorf model 5424 centrifuge. Absorption of the supernatant was measured at 450 nm and was compared with a standard curve of known hydrogen peroxide levels.

Aphid choice assays.

Recombinant *Agrobacterium tumefaciens* GV3101 expressing an empty vector or a gene for a single *M. persicae* effector protein was pressure-infiltrated using a 1-ml syringe into *N. tabacum* at a final OD_{600} of 0.2. Two days after infiltration, leaf disks 2 cm in diameter of empty vector and effector protein expressing plants were placed in a petri dish with moist Whatman paper, a single adult aphid was released in the center, and its choice was recorded.

Transgenic *A. thaliana* was grown as described above for 3 weeks and a single leaf was detached and placed in a petri dish with moist Whatman paper. Mp55 overexpression and empty-vector control leaves were placed on opposite sides, a single adult aphid was released in the center, and its choice was recorded.

Recombinant *Agrobacterium tumefaciens* GV3101 expressing an empty vector or a single effector protein was pressure-infiltrated using a 1-ml syringe into *N. benthamiana*, at a final OD_{600} of 0.2. Pairs of experimental and control plants were grown together in the same pot. Three days after infiltration, five adult aphids were placed in the center of the pot and their choice of host plant was recorded.

Statistical analysis.

Aphid bioassays were compared, using unpaired two-tailed *t*-tests, with a significance cutoff of $\alpha = 0.05$. Aphid gene expression was compared using an unpaired *t*-test ($\alpha = 0.05$) and analysis of variance followed by Tukey's honestly significant difference test. Aphid choice assays were compared using a chi-squared test. All statistical comparisons were conducted using JMP (2010) for Windows (SAS Institute Inc., Cary, NC, U.S.A.). All sequence alignments were done using ClustalW2 (Larkin et al. 2007).

ACKNOWLEDGMENTS

This research was funded by the United States Department of Agriculture-National Institute of Food and Agriculture awards 2010-65105-20558 and 2012-67013-19350 to G. Jander. D. A. Elzinga was funded by National Institutes of Health/National Institute of General Medical Sciences grant 5T32GM008500. DNA sequence data were downloaded from AphidBase. Funding for *Myzus persicae* clone O genomic sequencing was provided by The Genome Analyses Centre Capacity and Capability Challenge programme project CCC-15 and BB/J004553/1 from the Biotechnology and Biological Sciences Research Council and the John Innes Foundation.

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AUTHOR RECOMMENDED INTERNET RESOURCES

AphidBase, the aphid genome database: www.aphidbase.com
The Arabidopsis Biological Resource Center: www.arabidopsis.org
Primer3 software: biotools.umassmed.edu/bioapps/primer3_www.cgi