Aphid Protein Effectors Promote Aphid Colonization in a Plant Species-Specific Manner

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Microbial pathogens and pests produce effectors to modulate host processes. Aphids are phloem-feeding insects, which introduce effectors via saliva into plant cells. However, it is not known if aphid effectors have adapted to modulate processes in specific plant species. *Myzus persicae* is a polyphagous insect that colonizes *Arabidopsis thaliana* and *Nicotiana benthamiana*, while the pea aphid *Acyrtosiphon pisum* specializes on colonizing plant species of the family Fabaceae. We found that *M. persicae* reproduction increased on transgenic *Arabidopsis*, producing the *M. persicae* effectors C002, PIntO1 (Mp1), and PIntO2 (Mp2), whereas reproduction of *M. persicae* did not increase on *Arabidopsis* producing the *A. pisum* orthologs of these three proteins. Plant-mediated RNA interference experiments showed that *c002*- and *PIntO2*-silenced *M. persicae* produce less progeny on *Arabidopsis* and *N. benthamiana* than nonsilenced aphids. Orthologs of *c002*, *PIntO1*, and *PIntO2* were identified in multiple aphid species with dissimilar plant host ranges. We revealed high nonsynonymous versus synonymous nucleotide substitution rates within the effector orthologs, indicating that the effectors are fast evolving. Application of maximum likelihood methods identified specific sites with high probabilities of being under positive selection in PIntO1, whereas those of C002 and PIntO2 may be located in alignment gaps. In support of the latter, a *M. persicae* *c002* mutant without the NDNQGEE repeat region, which overlaps with an alignment gap in C002, does not promote *M. persicae* colonization on *Arabidopsis*. Taken together, these results provide evidence that aphid effectors are under positive selection to promote aphid colonization on specific plant species.

Insect pests are often highly selective in their host choices for long-term feeding or reproduction. The majority of chewing herbivores such as lepidopterans and sap-feeding insects of the order Hemiptera are able to colonize single or a few related plant species and, as such, are described as oligophagous (Schoonhoven et al. 2005). Relatively few insect species are polyphagous, with wide host ranges consisting of plant species

of multiple distantly related plant families. There is a growing body of evidence that insects produce virulence factors (effectors) that modulate host processes to facilitate feeding and colonization (Hogenhout and Bos 2011). Given that the majority of insects are oligophagous (Schoonhoven et al. 2005), pest effectors are likely to act in a host-specific manner (Hogenhout and Bos 2011). However, few investigations have focused on the host-specific nature of insect effector action. This research is important, because targeting of pest effectors by vaccination of humans and animals and for selection for resistance in plants could limit insect colonization and insect-transmitted diseases.

The sap-feeding aphids (family Aphididae, order Hemiptera) are particularly amenable for studying the role of effectors in insect-host interactions (Dogimont et al. 2010; Hogenhout and Bos 2011). The genome of the legume-specialist pea aphid *Acyrtosiphon pisum* has been sequenced (IAGC 2010) and transcriptome data of a number of aphid species, including the polyphagous green peach aphid *Myzus persicae*, are available (Ollivier et al. 2012; Ramsey et al. 2007). Moreover, aphid effectors have been identified (Bos et al. 2010; Mutti et al. 2006, 2008; Pitino et al. 2011; Will et al. 2007). Because *M. persicae* utilizes the model plants *Nicotiana benthamiana* and *Arabidopsis thaliana* as feeding and reproductive hosts, the role of effectors in aphid-plant interactions can be studied directly on the plant host by expressing *M. persicae* effector genes transiently in *N. benthamiana* leaves and stably in *Arabidopsis* and by silencing effector-encoding genes in *M. persicae* by plant-mediated RNA interference (RNAi) (Bos et al. 2010; Pitino et al. 2011). In addition, aphids have a short generation time and can build up to large populations consisting of genetically identical asexually producing females, enabling the consistent and accurate monitoring of effector influence on aphid colonization ability in relatively short time frames.

Aphids most likely introduce effectors via their saliva during feeding (Cherqui and Tjallingii 2000; Miles 1999; Tjallingii 2006). Aphids have piercing-sucking mouthparts that consist of stylets which can navigate between plant cells to the vascular tissue, where they feed mainly from the phloem. Aphids produce several types of saliva (Tjallingii 2006). Gelling sheath saliva surrounds the aphid stylets, protects these aphid mouthparts from the apoplastic defenses, and reduces the influx of extracellular compounds into plant cells. Watery saliva is released into plant cells during probing and penetrations while navigating to the vascular system and during long-term feeding from the plant phloem. Mass spectrometry of proteins in aphid saliva and salivary glands revealed a diversity of proteins, and some of these proteins appear to have effector functions (Carolan et al. 2009; Harmel et al. 2008; Nicholson et al. 2012). Conserved proteins among insects, such as glucose oxidase (GOX), elicit plant defense responses (Tian et al. 2012).

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Furthermore, calcium-binding proteins prevent phloem sieve cell plugging upon mechanical damage by the aphid stylets (Will et al. 2007, 2009). In addition, the aphid-specific protein C002 aids aphid colonization. Silencing of *c002* by injection of double-stranded (ds)RNA into the *Acyrtosiphon pisum* hemolymph or by feeding *M. persicae* on transgenic plants expressing dsRNA corresponding to C002 by plant-mediated RNAi reduces survival or reproduction rates of these aphids (Mutti et al. 2006; Pitino et al. 2011). C002 was detected in *A. pisum*-exposed plants (Mutti et al. 2008) and expression of *M. persicae* C002 in plants promotes *M. persicae* reproduction rates (Bos et al. 2010). Finally, the *M. persicae* effector Mp10 suppresses basal immunity pathways but also triggers an SGT1-dependent chlorosis response in *N. benthamiana* (Bos et al. 2010). Together, these data provided evidence that aphid effectors are likely to suppress plant defense to aphids, modulate plant processes to aid aphid colonization, or may be recognized by specific plant resistance proteins, resulting in effector-triggered immunity (Hogenhout and Bos 2011).

In this study, we wished to elucidate whether effectors act in a plant-species-specific manner, thereby providing evidence that aphid effectors may play a role in determining aphid host range. We report here that *M. persicae* has more progeny on transgenic *Arabidopsis* that produce *M. persicae* C002 but not on those that produce the *A. pisum* C002 ortholog or an *M. persicae* C002 mutant in which the sequence corresponding to a polymorphic repeat was deleted. Similar results were obtained for two other *M. persicae* and *A. pisum* effector orthologs. These results indicate that *M. persicae* effectors modulate *Arabidopsis* responses to the benefit of *M. persicae*, whereas the *A. pisum* effectors do not. These data are consistent with *Arabidopsis* being a host for *M. persicae* but not for *A. pisum*. We determined that aphid effectors are fast evolving and identified sites under positive selection.

RESULTS

M. persicae C002 promotes *M. persicae* colonization on *Arabidopsis*.

Peptides corresponding to C002 were detected in the saliva and salivary glands of *M. persicae* (EC389283; Harmel et al. 2008) and *A. pisum* (Mutti et al. 2008) (ACYPI008617; Carolan et al. 2011). These proteins possess signal peptides with ≥ 0.925 probability values and predicted cleavage sites between amino acids 24 and 25 (Fig. 1A). We previously noted that *M. persicae* C002 (MpC002) contains five repeats with the sequence NDNQGEE in the N-terminal part of the protein that are lacking in *A. pisum* C002 (ApC002) (Bos et al. 2010) (Fig. 1A). To assess whether the sequence divergence between the two proteins determines effector specificity, we generated transgenic *Arabidopsis* Col-0 lines stably expressing transcripts corresponding to MpC002 and ApC002 under control of the *Arabidopsis* sucrose transporter promoter AtSUC2 for phloem-specific expression. We generated three independent transgenic lines for each protein, named AtSUC2:MpC002 lines 4, 6, and 7 and AtSUC2:ApC002 lines 3, 4, and 8. A transgenic line (AtSUC2:GFP) that stably expresses the green fluorescent protein (GFP) coding sequence and Col-0 were included as controls. Transgene transcripts were detected in all *Arabidopsis* lines (Supplementary Fig. S1A).

M. persicae produced approximately 20% more progeny on AtSUC2:MpC002 lines than on Col-0 and AtSUC2:GFP plants (generalized linear model [GLM], three biological replicates with $n = 4$ plants per replicate, $P < 0.05$) (Fig. 1B). In contrast, *M. persicae* reproduction did not increase on the AtSUC2:ApC002 lines (GLM, $P =$ not significant [ns]) (Fig. 1B) even though ApC002 transcript levels were similar to the MpC002 transcript levels of AtSUC2:MpC002 lines 4 and 6.

To assess whether the MpC002 NDNQGEE repeat (Fig. 1A, motifs I to V), which is lacking from ApC002 (Fig. 1A), is responsible for the *M. persicae* progeny increase, we also generated three transgenic Col-0 lines, which produce a MpC002 mutant without the five NDNQGEE repeats (MpC002Δ) (Fig. 1A) under control of the AtSUC2 promoter. The *M. persicae* reproduction rates on the AtSUC2:MpC002Δ lines did not increase and were similar to those of the aphids on AtSUC2:ApC002 plants (GLM, $P =$ ns) (Fig. 1B), even though the MpC002 and MpC002Δ transcript levels were similar. Together, these data indicate that the five NDNQGEE present in MpC002 are involved in promoting *M. persicae* reproduction on *Arabidopsis*.

Fig. 1. Sequence polymorphisms of C002 confer effector specificity. **A**, *Myzus persicae* (Mp) C002 contains a region with five times the NDNQGEE sequence lacking in *Acyrtosiphon pisum* (Ap) C002 and is deleted from the MpC002Δ mutant (MpΔ) of *M. persicae* C002. NDNQGEE repeats are numbered I to V and SP is signal peptide. **B**, *M. persicae* produces more progeny on *Arabidopsis* MpC002 transgenic lines but not on the ApC002 and MpC002Δ lines. Single plants were seeded with five nymphs, which started to produce offspring approximately on day 10. Progeny were counted on days 10, 13, and 16 and removed. Columns show the average nymph production plus standard deviations of three biological replicates, with each replicate consisting of the progeny produced by 20 aphids at 5 aphids per plant ($n = 4$ plants). Numbers under columns on x axis indicate independent transgenic lines. Asterisk (*) indicates generalized linear model, $P < 0.05$ compared with green fluorescent protein plants.

Fig. 2. MpPIntO1 (Mp1) but not ApPIntO1 promotes aphid colonization on *Arabidopsis*. **A**, Protein alignment of *Myzus persicae* and *Acyrtosiphon pisum* PIntO1. **B**, *M. persicae* produces more progeny on *Arabidopsis* MpPIntO1 (Mp1) transgenic lines but not on the ApPIntO1 lines. Single plants were seeded with five nymphs, which started to produce offspring approximately on day 10. Progeny were counted on days 10, 13, and 16 and removed. Columns show the average nymph production plus standard deviations of three biological replicates, with each replicate consisting of the progeny produced by 20 aphids at 5 aphids per plant ($n = 4$ plants). Numbers under columns on x axis indicate independent transgenic lines. Asterisk (*) indicates generalized linear model, $P < 0.05$ compared with green fluorescent protein plants.

Fig. 3. MpPIntO2 (Mp2) but not ApPIntO2 promotes aphid colonization on *Arabidopsis*. **A**, Protein alignment of *Myzus persicae* and *Acyrtosiphon pisum* PIntO2. **B**, *M. persicae* produces more progeny on *Arabidopsis* MpPIntO2 (Mp2) transgenic lines but not on the ApPIntO2 lines. Single plants were seeded with five nymphs, which started to produce offspring approximately on day 10. Progeny were counted on days 10, 13, and 16 and removed. Columns show the average nymph production plus standard deviations of three biological replicates, with each replicate consisting of the progeny produced by 20 aphids at 5 aphids per plant ($n = 4$ plants). Numbers under columns on x axis indicate independent transgenic lines. Asterisk (*) indicates generalized linear model, $P < 0.05$ compared with green fluorescent protein plants.

were higher (GLM, three biological replicates with $n = 4$ plants per replicate, $P < 0.05$) than on line 3, consistent with higher expression levels of the Mp2 transcripts in lines 1 and 2 compared with line 3. Hence, we named Mp2 “Progeny Increase to Overexpression 2” (PIntO2). We found a homolog of PIntO2 in *A. pisum* that has a signal peptide with probability score of 0.997 and a predicted cleavage site between amino acids 20 and 21 (Fig. 3A). However, *M. persicae* did not produce more progeny on three independent AtSUC2:ApPIntO2 transgenic lines (Fig. 3B), which all have transgene expression levels similar to the AtSUC2:ApPIntO2 lines. Thus, *M. persicae* PIntO1 and PIntO2 promote *M. persicae* reproduction on *Arabidopsis*, whereas *A. pisum* PIntO1 and PIntO2 do not.

Silencing of PIntO1 and PIntO2 reduces aphid reproduction on plants.

To obtain additional evidence that PIntO1 and PIntO2 have effector functions in *M. persicae*, we wished to silence the expression of corresponding genes in *M. persicae* using plant-mediated RNAi and assess whether this reduced *M. persicae* reproduction, as has been shown for MpC002-silenced aphids (Pitino et al. 2011). Nucleotide sequences corresponding to amino acids MEMIQP to IDRAIL of PIntO1 and amino acids EVTEL to SKNDSP of PIntO2 were cloned as inverted repeats into the *Agrobacterium tumefaciens* pJawohl8-RNAi binary silencing vector (I. E. Somssich) and transiently expressed in *N. benthamiana* leaves by *Agrobacterium*-mediated infiltration (agro-infiltration) (Pitino et al. 2011). Aphid reproduction rates were analyzed and silencing of aphid genes were assessed as previously described (Pitino et al. 2011). C002-silenced aphids produced fewer nymphs compared with control aphids fed on *N. benthamiana* producing double-stranded GFP (analysis of

variance [ANOVA], six biological replicates with $n = 4$ to 6 leaf disks per replicate, $P = 0.009$) (Fig. 4A), confirming previous results (Pitino et al. 2011). Silencing of PIntO2 in *M. persicae* also reduced nymph production (ANOVA, $P = 0.041$), while silencing of *M. persicae* PIntO1 did not (ANOVA, $P = 0.732$) (Fig. 4A). The expression levels of the effector genes were similarly reduced in the silenced aphids (Student's *t* test, three biological replicates, $P < 0.05$) (Fig. 4B).

We also assessed the effect of silencing of three effector genes on *Arabidopsis*. We used the published silencing lines

Fig. 4. Silencing of *Myzus persicae* C002 and PIntO2 on *Nicotiana benthamiana* reduces aphid fecundity. **A**, C002 and PIntO2-silenced *M. persicae* produce fewer progeny on *N. benthamiana*. Individual leaf disks were seeded with four nymphs, which started to produce offspring approximately on day 12. Progeny was counted on days 12, 14 and 17 and removed. Columns show the average nymph production plus standard deviation per aphid of 6 biological replicates with each replicate consisting of $n = 4$ –6 leaf disks. Asterisk (*) indicates analysis of variance, $P < 0.05$. **B**, *M. persicae* exposed to leaf disks in A have reduced C002, PIntO1, and PIntO2 expression levels. Columns indicate the average target gene (x axis) expression levels and standard deviations relative to tubulin and L27 reference genes (Pitino et al. 2011) of $n = 3$ biological replicates. In each biological replicate, the four nymph-producing adults on leaf disks of three of six experiments shown in A were collected at day 17 and pooled for RNA extraction and quantitative reverse-transcriptase polymerase chain reaction. Asterisk (*) indicates Student's *t* test, $P < 0.05$.

Fig. 5. Silencing of *Myzus persicae* C002 and PIntO2 on *Arabidopsis* reduces aphid fecundity. **A**, C002- and PIntO2-silenced *M. persicae* produce fewer progeny on *Arabidopsis*. Single plants were seeded with five nymphs, which started to produce offspring approximately on day 10. Progeny were counted on days 10, 13, and 16 and removed. Columns show the average nymph production plus standard deviations of three biological replicates, with each replicate consisting of the progeny produced by 20 aphids at 5 aphids per plant ($n = 4$ plants). Asterisk (*) indicates generalized linear model, $P < 0.05$ compared with aphids fed on double-stranded green fluorescent protein (dsGFP) plants. **B**, *M. persicae* exposed to transgenic plants in A have reduced C002, PIntO1, and PIntO2 expression levels. Target gene expression levels of experimental plants and dsGFP control plants were assayed independently for each line as shown. Columns indicate the average target gene (x axis) expression levels and standard deviations relative to tubulin and L27 reference genes (Pitino et al. 2011) of $n = 3$ biological replicates. In each biological replicate, the five nymph-producing adults kept on four plants for 16 days of the experiment shown in A were pooled for RNA extraction and quantitative reverse-transcriptase polymerase chain reaction. Asterisk (*) indicates Student's *t* test, $P < 0.05$ compared with aphids fed on dsGFP plants.

for MpC002 (Pitino et al. 2011) and generated three independent silencing lines for PIntO1 and PIntO2. Plant-mediated RNAi of both MpC002 and PIntO2 genes reduced *M. persicae* nymph production (GLM, three biological replicates with $n = 4$ plants per replicate, $P < 0.05$), whereas silencing of PIntO1 did not (GLM, $P > 0.05$) (Fig. 5A). With the exception of aphids fed on dsPIntO1 line 6, the expression levels of target genes in *M. persicae* were significantly reduced on all lines (Student's *t* test, three biological replicates, $P < 0.05$) (Fig. 5B). Together, these results show that plant-mediated silencing of the *M. persicae* C002 and PIntO2 genes on *N. benthamiana* and *Arabidopsis* reduced nymph production, whereas silencing of the PIntO1 gene did not.

Assessment of positively selected sites in C002, PIntO1, and PIntO2.

First, we searched the GenBank expressed sequence tags (EST) database for aphid EST corresponding to C002, PIntO1, and PIntO2 and selected full-length transcripts. C002 EST in-

We also examined the molecular evolution of PIntO1 and PIntO2 orthologs. Full-length PIntO1 were identified in several aphid species, including 44 in *M. persicae* and 80 in *Acyrtosiphon pisum*. The *A. pisum* EST group into PIntO1_a (36 EST) and PIntO1_b (44 EST) sequences. Of these, PIntO1_a was used for generating the transgenic plants and conducting *M. persicae* fecundity assays (Fig. 2). PIntO2 EST were identified in five aphid species, with 16 and 36 EST in *M. persicae* and *A. pisum*, respectively. Deduced protein sequences of PIntO1 and PIntO2 EST all harbor predicted N-terminal signal peptides with high probability scores.

The PIntO1 alignment showed two areas with gaps of 2 to 3 amino acids immediately after the signal peptide sequences (Fig. 7), whereas the PIntO2 alignment revealed several gaps in some sequences, including immediately after the signal peptide sequence and at the end of the proteins (Fig. 6B). The phylogenetic trees of these effectors were consistent with that of the aphid phylogeny based on 203 nuclear genes, indicating that they are orthologs (Ollivier et al. 2012). The pairwise d_N/d_S ratios of the PIntO1 and PIntO2 were higher overall than the 0.064 to 0.094 average (Ollivier et al. 2010), ranging from 0.375 to 1.038 for PIntO1 and 0.413 to 0.896 for PIntO2, though the *A. pisum* and *A. kondoi* pairwise comparison of PIntO2 was lower at 0.067. This indicates that both PIntO1 and PIntO2 are fast evolving among aphid species. The ML models of codon substitutions identified significant χ^2 distributions for PIntO1 in all models (P value ≤ 0.0069) (Supplementary Table S5). Positively selected sites were identified from 70% to greater than 99% (Fig. 7). On the other hand, no consistently significant χ^2 distributions among the models were identified for PIntO2 (Supplementary Table S6). However, positive selection may act on sites in alignment gaps that are excluded from the ML model analyses. Altogether, these data suggest that specific PIntO1 sites are subject to positive selection, whereas such sites were not identified in C002 and PIntO2, though positive selection may act on sites located in alignment gaps of the latter two effectors.

DISCUSSION

Aphids are phloem-feeding insects which release saliva that contains effectors into plant cells. Evidence that C002 is an effector in *M. persicae* and *A. pisum* has accumulated (Bos et al.

2010; Mutti et al. 2006, 2008; Pitino et al. 2011). This study is in agreement with these published findings, because we found that *M. persicae* produces more progeny on transgenic plants that produce MpC002 and less progeny on plants that induce silencing of *c002* in *M. persicae*. This indicates that C002 functions in the plant host upon release by the aphid consistent with effectors functioning in hosts rather than in the parasites in which they are synthesized (Hogenhout et al. 2009).

Although there is evidence that aphids produce effectors that modulate plant host processes to the benefit of aphids, it is not yet clear whether these effectors are involved in determining aphid compatibility with the plant host. *M. persicae* has a broad host range consisting of plant species in multiple plant families, and this includes *Arabidopsis* and *N. benthamiana*. However, the host range of *A. pisum* is restricted to plant species in the Fabaceae (Leguminosae) family. We observed that *M. persicae* and *A. pisum* effector orthologs harbor gap and single amino acid polymorphisms, which we hypothesized may reflect their function in different plant species. In this study, we have provided evidence for this hypothesis, because *M. persicae* generates more progeny on transgenic *Arabidopsis* plants that produce the *M. persicae* effectors C002, PIntO1, and PIntO2 but not on plants that produce the *A. pisum* orthologs of these proteins. Thus, the *M. persicae* protein effectors apparently modulate *Arabidopsis* processes, leading to increased progeny production by this aphid, whereas orthologs of these effectors of *A. pisum* do not. However, the *A. pisum* effectors may modulate processes to the benefit of *A. pisum* in Fabaceae species. Indeed, C002 and PIntO1 were detected in *A. pisum* saliva (Carolan et al. 2009). C002 was also detected in the *A. pisum* plant hosts upon feeding (Mutti et al. 2008). Moreover, silencing of C002 in *A. pisum* is lethal to the aphid when it feeds from its plant host but not when aphids feed from an artificial diet (Mutti et al. 2006). At this time, we cannot exclude the possibility that PIntO2 is not functional in *A. pisum*, albeit this seems unlikely, given that we identified multiple full-lengths EST encoding this protein in *A. pisum* and other aphid species and all have predicted signal peptide sequences with high probability scores.

The positive effects on aphid reproduction were particularly profound on *Arabidopsis* plant lines that have relatively high aphid effector transgene expression levels, indicating that ample amounts of protein effectors are required for a beneficial

Fig. 7. Specific sites of PIntO1 are subject to positive selection. PIntO1 orthologs of various aphid species were aligned in Muscle (v3.7). Sites with high probability ($P > 95\%$) of being under positive selection are highlighted in darkest gray, with ** indicating $P > 99\%$. Sites with lower probabilities are highlighted in gray ($70\% > P < 95\%$) and light gray ($50\% > P < 70\%$).

effect on aphid performance. This is in agreement with the abundance of these effectors in *M. persicae* and *A. pisum* saliva and the high expression levels of corresponding genes in these aphids. Whereas the expression levels of C002 and PIntO1 genes are higher than that of PIntO2, that of PIntO2 is nonetheless similar to that of β -tubulin, which is an abundant protein in aphids. C002, PIntO1, and PIntO2 transcripts were predominantly detected in *M. persicae* salivary glands (Bos et al. 2010) and C002 and PIntO1 have been identified as abundant proteins in *M. persicae* and *A. pisum* saliva (Carolan et al. 2009; Harmel et al. 2008). However, PIntO2 has not been identified in *M. persicae* and *A. pisum* saliva. Mass spectrometry may be biased toward the detection of the most abundant proteins. Furthermore, because saliva used for mass spectrometry is collected from aphids fed on diet, it is also possible that aphids produce PIntO2 more abundantly when feeding from plants but not when feeding from diet. Therefore, this study demonstrates that a functional genomics approach is complementary to other approaches used for the identification of aphid protein effectors, because it can result in the discovery of less abundant protein effectors in aphids or effectors that are specifically produced by aphids during plant feeding.

We previously demonstrated that silencing of *rack-1* and *C002* in *M. persicae* by plant-mediated RNAi leads to a reduction in *M. persicae* progeny on plants (Pitino et al. 2011). In this study, we demonstrated that plant-mediated RNAi of *PIntO2* also reduces *M. persicae* reproduction to a similar degree as *c002*-silenced aphids on both *N. benthamiana* and *Arabidopsis* hosts. On the other hand, we did not observe an effect on progeny production of *PIntO1*-silenced *M. persicae*. It is possible that PIntO1 is redundant in function. Effectors may act redundantly by modifying the same plant targets or by modulating different processes in the same plant pathway (Cunnac et al. 2012). Conversely, C002 and PIntO2 may target more unique plant processes. Silencing of PIntO1 may also cause another as-yet-unknown phenotype in the aphid.

The phylogenetic tree of aphid species based on 203 concatenated nuclear genes (Ollivier et al. 2012) and those of the three protein effectors are similar. Unlike some microbial effectors (Liu et al. 2005; Win and Kamoun 2008; Win et al. 2007), C002, PIntO1, and PIntO2 do not belong to protein families. Thus, the protein effectors shared among the species are orthologs that have most likely diverged approximately 140 million years ago, when the extant aphid species diversified at the same time as main branches of the angiosperms (Von Dohlen and Moran 2000). The average d_N versus d_S nucleotide substitution rate of 253 reciprocal genes shared by aphid species ranges from 0.064 to 0.094 (Ollivier et al. 2010), which is much lower than that of the three effectors, which have pairwise d_N/d_S values of 0.375 to 1.038, indicating that effectors are fast evolving. Protein effectors are often subject to positive selection because of pressure to adapt to their host targets that is under diversifying selection (Kaschani and Van der Hoorn 2011; Kaschani et al. 2010).

None of the ML models detected single sites under positive selection in C002. This is in agreement with Ollivier and associates (2010), who also found no significant evidence of positive selection of single sites in C002. However, the C002 alignment shows gap polymorphisms, which are excluded from model analyses to detect positively selected sites. We found that a mutant MpC002 in which the five NDNQGEE repeats are deleted does not promote *M. persicae* progeny production similarly to ApC002, which lacks the NDNQGEE repeat region, indicating that this region is essential for target specificity in *Arabidopsis* in relation to *M. persicae*. In addition, we found a second *M. persicae* C002 transcript encoding a protein with four NDNQGEE repeats instead of five, and the gap

region is also polymorphic in C002 ortholog of the melon and cotton aphid *Aphis gossypii*. This indicates that the positively selected sites conferring target specificity may be located in the alignment gaps of C002. The ML models identified at least three sites with high significance of being under positive selection in PIntO1, whereas none were detected in PIntO2. The PIntO1 alignment shows small gap polymorphisms of 2 to 3 amino acids, whereas the PIntO2 alignment has larger gap polymorphisms, particularly at the C-terminal ends of the proteins. Therefore, it is possible that sites in alignment gaps of PIntO2 are under positive selection, similarly to C002.

In summary, we have provided evidence that *M. persicae* effectors modulate *Arabidopsis* processes that are advantageous to *M. persicae* colonization ability. In contrast, the *Acyrtosiphon pisum* orthologs appear not functional in *Arabidopsis* in relation to *M. persicae* colonization. This is in agreement with the host ranges of these two aphid species, with *M. persicae* being able to readily feed and reproduce on *Arabidopsis* and *A. pisum* having a host range predominantly restricted to the Fabaceae family and incapable of colonizing *Arabidopsis*. We also provide evidence that aphid effectors are fast evolving, in which specific sites under selection were identified in PIntO1 and those in C002 and PIntO2 may be located in alignment gaps. Evidence for the latter is provided for C002, because an *M. persicae* C002 mutant without the NDNQGEE repeat region, which overlaps with an alignment gap in C002, does not promote *M. persicae* colonization on *Arabidopsis*. Taken together, these results provide evidence that aphid effectors contribute to aphid-plant compatibility.

MATERIALS AND METHODS

Insect rearing.

The aphid lineages used in this study are *M. persicae* RRes (Clone O) (Bos et al. 2010) and *A. pisum* DbS (collected from *Pisum sativum* field crop near Dickleborough, Suffolk in 2005). *M. persicae* were reared on *N. tabacum* or *N. benthamiana* plants for *N. benthamiana* leaf disk assays and on Chinese cabbage (*Brassica rapa*) for the fecundity assays on *Arabidopsis thaliana*. *A. pisum* were reared on *Vicia faba* plants. The insects were maintained in custom-built acrylic cages located in controlled-environment conditions at 18°C under 16 h of light.

Aphid cDNA synthesis.

Total RNA was prepared from 5 to 10 aphids. Liquid nitrogen frozen samples were homogenized for 60 s to a fine powder using a Mini-Bead-Beater-8TM (Biospec Products, Bartlesville, OK, U.S.A.) and 1.2-mm stainless steel beads. After the homogenization, the samples were used for RNA extraction with TRI Reagent (Sigma-Aldrich, St. Louis) and purified using Qiagen RNeasy columns (Qiagen Ltd., West Sussex, U.K.). cDNA was synthesized from 0.5 μ g of total RNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.). Samples were then stored at -80°C .

Generation of constructs

for phloem expression or dsRNA constructs.

To generate transgenic *Arabidopsis*, coding nucleotide sequences for PIntO1 and PIntO2 without the region corresponding to the signal peptides were codon optimized for efficient expression in *Arabidopsis thaliana* and cloned into pUC57 vector (Genscript, Piscataway, NJ, U.S.A.). These sequences were amplified with specific primers with additional attb1 and attb2 linker sites for cloning into the plasmid vector pDONR207 (Invitrogen) plasmid using Gateway BP reaction and trans-

formed into DH5 α . Coding sequences without a signal peptide sequence for C002 were directly amplified from aphid total RNAs by reverse-transcriptase polymerase chain reaction (RT-PCR) using specific primers containing additional attb1 and attb2 linker sites (Supplementary Table S7). MpC002 Δ was generated using an overlapping PCR approach using the MpC002_a sequence as template and with specific primers. The GFP open reading frame was amplified with specific primers. All clones were sequenced to verify correct size and sequence of inserts. Next, the inserts were introduced into the destination vector pB7WG2 carrying the AtSuc2 promoter sequence for phloem-specific expression (Gottwald et al. 2000) using the Gateway LR reaction procedure. The vectors were introduced into *Agrobacterium tumefaciens* GV3101 plasmid and used for generating transgenic *Arabidopsis thaliana* as described previously (Pitino et al. 2011).

To generate the dsRNA constructs, a 710-bp MpC002_a fragment corresponding to the entire mature *M. persicae* MpC002_a protein without the signal peptide (as described previously) (Pitino et al. 2011), a 263-bp MpPIntO1 fragment starting at nucleotide position +69 (CCGTCCG) and ending at nucleotide position +332 (CGTTGAT) of the *M. persicae* PIntO1 transcript sequence, a 254-bp PIntO2 fragment starting at nucleotide position +26 (CGTGATA) and ending at nucleotide position +280 (TCAC-CGG) of the *M. persicae* PIntO2 transcript sequence, and a 537-bp GFP fragment starting at nucleotide position +29 (GAGTGG) and ending at nucleotide position +566 (...TTAGCAG) of the GFP open reading frame (as described previously) (Pitino et al. 2011) were introduced into pDONR207 (Invitrogen) plasmid using Gateway BP reaction and transformed into DH5 α . Subsequent clones were sequenced to verify correct size and sequence of inserts. Subsequently, the inserts were introduced into the pJawohl8-RNAi binary silencing vector (I. E. Somssich, Max Planck Institute) using Gateway LR reaction generating plasmids pJMpC002 and pJGFP (Pitino et al. 2011) and pJAMPIntO1 and pJAMPIntO2 (this study). The four constructs were introduced into *Agrobacterium tumefaciens* GV3101 containing pMP90RK plasmid and used for transient assays in *N. benthamiana* leaves and transformation of *Arabidopsis thaliana*.

Generation of transgenic plants.

Arabidopsis thaliana Col-0 plants were grown in a growth chamber under long-day conditions until flowering. Flowers were dipped in an *Agrobacterium tumefaciens* (GV3101) suspension containing the appropriate plasmid. T1 seed were germinated in soil and the transformants were BASTA selected. T2 seed of BASTA-resistant lines were plated on Murashige-Skoog media containing phosphinothricin at 20 μ g/ml. The lines showing a single insertion based on a 3:1 segregation ratio of live/dead seedlings were selected, and homozygous progeny of these plants were used for further analyses.

Arabidopsis RNA extraction.

Total RNA was prepared from 3- to 6-week-old plant materials. Liquid-nitrogen-frozen leaves were homogenized for 60 s to a fine powder using a Mini-Bead-Beater-8TM (Biospec Products) and 1.2-mm stainless steel beads. After the homogenization, the samples were used for RNA extraction with TRI Reagent (Sigma-Aldrich) and purified using Qiagen RNeasy columns (Qiagen Ltd.). cDNA was synthesized from 0.5 μ g of total RNA using M-MLV reverse transcriptase (Invitrogen). Samples were then stored at -80°C .

RT-quantitative PCR analysis.

Total RNA was extracted from *Arabidopsis thaliana* leaves or aphids using TRIzol reagent. DNA contaminations were

removed by treating RNA extraction with RNase-free DNase (Qiagen Ltd.) and purified with QIAamp columns (Qiagen Ltd.). First-strand cDNA was synthesized at 37°C from total RNA using M-MLV (Invitrogen) reverse transcriptase according to the manufacturer's instructions and subjected to real-time PCR using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) in a DNA Engine Opticon 2 (Bio-Rad, Life Sciences) using gene-specific primers. For RT-quantitative (q)PCR of *Arabidopsis*, the cycle threshold (Ct) values were normalized using *Arabidopsis* actin as reference (Sugio et al. 2011). For RT-qPCR in aphids, the Ct values were normalized for difference in cDNA amount using tubulin and L27 transcripts as reference (Mutti et al. 2006; Shakesby et al. 2009). Fold changes were calculated by comparing the normalized transcript levels of C002, PIntO1, and PIntO2 of *M. persicae* fed on C002-dsRNA, PIntO1-dsRNA, and PIntO2-dsRNA transgenic plants to aphids fed on GFP-dsRNA transgenic plants, as previously described (Pitino et al. 2011).

M. persicae fecundity assay on *Arabidopsis* transgenic lines.

Three transgenic *Arabidopsis* T3 lines for each construct (AtSUC2-) were used for each experiment, with wild-type Col-0 plants and AtSUC2:GFP plants as controls. To measure the *M. persicae* fecundity, four plants each from independent transgenic and wild-type lines were grown for 4 weeks in a controlled environment room in pots with soil (type of soil: Levington F2), and maintained under conditions of 18°C (day) and 16°C (night), 8 h of light, and 60% humidity. The seed were cold treated for 2 days in a refrigerator (4°C) in order to synchronize germination. After 4 weeks, the plants were moved to new pots (one plant per pot) with soil (F2) and covered by a transparent plastic cover with mesh on the top to allow plant transpiration and prevent insect escape. For *M. persicae* fecundity assays, five adult aphids were transferred from the host Chinese cabbage (*B. rapa* subsp. *chinensis*) plants to 4-week-old *Arabidopsis* plants within the sealed experimental cages. Two days later, five nymphs were left on the plants and the adults were removed. Numbers of offspring produced on each plant at the 10th, 13th, and 16th day of the experiment were counted and removed.

N. benthamiana leaf infiltration and leaf disk assays.

Single *Agrobacterium* colonies harboring pJGFP, pJMpC002, pJAMPIntO1, and pJAMPIntO2 were inoculated into Luria broth containing kanamycin at 25 mg/liter, gentamicin at 25 mg/liter, rifampicin at 50 mg/liter, and carbenicillin at 25 mg/liter and grown (28°C at 225 rpm) until an optical density at 600 nm of 0.3 was reached (Eppendorf BioPhotometer; Eppendorf, Cambridge, U.K.). Cultures were resuspended in infiltration medium (10 mM MgCl_2 and 10 mM 2-(N-morpholino)ethanesulfonic acid, pH 5.6) with 150 mM acetosyringone to initiate expression. Each construct was infiltrated into the youngest fully expanded leaves of 4- to 6-week-old *N. benthamiana* plants. The plants were grown in a growth chamber with daily temperatures of 22 to 25°C under a short-day regime. One day after infiltration, leaves were harvested and used in leaf disk assays. The leaf disks were cut from the infiltrated areas using an 11-mm-diameter borer and placed in single wells of a 24-well plate on top of a plug consisting of 1 ml of solidified 1% distilled water agar. Four first-instar nymphs (1 to 2 days old) reared on *N. tabacum* were placed onto the leaf disks for a total of six leaves per construct. The wells were individually sealed with mesh lids and put upside down in controlled-environment conditions at 18°C under 16 h of light. The 24-well plate was replaced with freshly infiltrated (1 day post infiltration) leaf disks after 6 and 12 days. Aphid survival was counted at 6, 12, 14, and 17 days after initial transfer of

aphids to the first 24-well plate, and the numbers of nymphs produced by these aphids at 12, 14, and 17 days were also counted. The nymphs were removed after counting. This experiment was repeated six times to generate six independent biological replicates, each containing six leaf-disks per construct.

Statistical analysis

of *M. persicae* survival and reproduction data.

All statistical analyses were conducted using the GenStat 11 statistical package (VSNi Ltd., Hemel Hempstead, U.K.) as previously described (Pitino et al. 2011) and is here described for clarity. Data were checked for approximate normal distribution by visualizing residuals. Classical linear regression analysis using a GLM with Poisson distributions was applied to analyze the *M. persicae* fecundity on plants, with “nymphs” as a response variable. The aphid nymph production on four plants per treatment were used as independent data points in statistical analyses in which the biological replicate was used as a variable.

N. benthamiana leaf disk assay fecundity data were analyzed using an unbalanced one-way ANOVA design, with “construct” as the treatment and “repeat” as the block. In the *N. benthamiana* leaf disk assay, aphid fecundity was monitored on individual leaf disks at six disks per treatment. Numbers of aphid nymphs produced on each leaf disk were used as independent data points in statistical analyses in which the biological replicate was used as a variable. Leaf disks that dried up because of lack of humidity were excluded, giving four to six leaf disks per construct for each biological replicate.

Positive selection analysis.

M. persicae C002, PlntO1, and PlntO2 nucleotide sequences were searched against the EST databases of GenBank using blastn (Altschul et al. 1990). All the similar sequences were downloaded and were aligned using Muscle (Edgar 2004a and b) in CLC Main Workbench (Aarhus, Denmark). Incomplete EST were removed and remaining sequences were analyzed for start and stop codons to ensure their full lengths.

Nucleotide sequences of full-length EST were aligned in Muscle (v3.7) and saved in PHYLIP format. The phylogenetic tree was reconstructed with remove gaps enabled and using the ML method implemented in the PhyML program (v3.0 aLRT). The GTR substitution model was selected with 100 bootstrap replicates. Estimates of d_N versus d_S were computed pairwise between sequences using the approximate methods (Nei and Gojobori 1986). Maximum-likelihood (ML) analysis of codon substitutions were performed under different models with the program CODEML implemented in the PAML 4.4 software package (Goldman and Yang 1994; Yang and Bielawski 2000). The different models were compared using the likelihood ratio test (LRT), which compares twice the differences in log likelihood to a χ^2 distribution with the required number of degrees of freedom. The six models recommended by Yang and Bielawski (2000) were tested. They were null models M0 (one ratio), M1 (neutral), and M7 (β), corresponding to alternative models M3 (discrete), M2 (selection), and M8 ($\beta + \omega$), respectively. (Nielsen and Yang 1998; Yang and Bielawski 2000). Significant differences between the two models were evaluated by calculating the LRT following χ^2 distribution. We use an LRT comparing M0 (one ratio) with M3 (discrete) to test for variable selective pressure among sites, and LRT to test for sites evolving by positive selection, comparing M1 (neutral) versus M2 (selection) and M7 (β) against M8 ($\beta + \omega$). When LRT was significant, we used Bayes Empirical Bayes analysis for detection of positive sites (Nielsen and Yang 1998; Yang and Bielawski 2000).

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