An Aphid Effector Targets Trafficking Protein VPS52 in a Host-Specific Manner to Promote Virulence[1][OPEN]

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Plant- and animal-feeding insects secrete saliva inside their hosts, containing effectors, which may promote nutrient release and suppress immunity. Although for plant pathogenic microbes it is well established that effectors target host proteins to modulate host cell processes and promote disease, the host cell targets of herbivorous insects remain elusive. Here, we show that the existing plant pathogenic microbe effector paradigm can be extended to herbivorous insects in that effector-target interactions inside host cells modify critical host processes to promote plant susceptibility. We showed that the effector Mp1 from *Myzus persicae* associates with the host Vacuolar Protein Sorting Associated Protein52 (VPS52). Using natural variants, we provide a strong link between effector virulence activity and association with VPS52, and show that the association is highly specific to *M. persicae*-host interactions. Also, coexpression of Mp1, but not Mp1-like variants, specifically with host VPS52s resulted in effector relocalization to vesicle-like structures that associate with prevacuolar compartments. We show that high VPS52 levels negatively impact virulence, and that aphids are able to reduce VPS52 levels during infestation, indicating that VPS52 is an important virulence target. Our work is an important step forward in understanding, at the molecular level, how a major agricultural pest promotes susceptibility during infestation of crop plants. We give evidence that an herbivorous insect employs effectors that interact with host proteins as part of an effective virulence strategy, and that these effectors likely function in a species-specific manner.

Many insect species secrete saliva, containing various proteins, inside their host to enable feeding. The identification and characterization of insect salivary molecules over recent years has contributed, to our knowledge, to novel insights into suppression of host immune responses. Aphids need to form a close association with their host to enable feeding and infestation. These insects use specialized mouthparts, or stylets, to penetrate the leaf surface and establish a feeding site. While probing and feeding, aphids secrete saliva into different host cell types as well as the apoplast. Advances in genomics and proteomics have facilitated the identification of a diverse array of proteins in aphid saliva as well as aphid salivary glands (Harmel et al., 2008; Carolan et al., 2009; Bos et al., 2010a, 2010b). These include proteins with predicted functions, such as metalloproteases, disulfide isomerases, calreticulins, ARMET proteins, glutathione peroxidases, and CLP-domain Ser proteases. However, nearly half of the predicted aphid salivary proteins described have no functional annotation and/or similarity to proteins in other organisms (Rodriguez and Bos, 2013). The abundance of these proteins in aphid saliva/salivary glands suggests they exhibit an important effector activity toward promoting aphid virulence.

Upon interaction with plant pathogen microbes, most plants are able to defend themselves by recognizing conserved parasite molecules, or pathogen-associated molecular patterns (PAMPs), to activate PAMP-triggered immunity. However, successful plant pathogenic microbes deliver effectors inside their hosts to suppress this and other types of plant defenses by interacting with and altering the mode of action of important plant defense signaling components. Over the past decade, studies on plant microbe effectors and their activities has revealed exciting insight into the host cell processes targeted to enable infection. For example, effectors from oomycete plant pathogens have been shown to target a ubiquitin E3 ligase (Bos et al., 2010a), a MAPKKK (King et al., 2014), a host autophagosome protein (Dagdas et al., 2016), and a host phosphatase (Boevink et al., 2016) to suppress host immunity. Other host targets include JAZ

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P.A.R. and J.I.B.B. designed the experiments; P.A.R., C.E.-M., and J.I.B.B. performed the experiments and analyzed the data; P.A.R. and J.I.B.B. wrote the manuscript; J.I.B.B. directed the project; all authors read and approved the final manuscript.

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proteins involved in JA-signaling, which are targeted by both bacterial and fungal effectors (Jiang et al., 2013; Plett et al., 2014), but also the host proteasome (Groll et al., 2008; Üstün et al., 2013), extracellular proteases (Song et al., 2009), and the cytoskeleton and secretion pathways (Bozkurt et al., 2011; Lee et al., 2012). Also, aphids alter host physiology as reflected by their ability to affect nutrient allocation (Sandström et al., 2000; Girousse et al., 2005) and to suppress defense responses (Bos et al., 2010b; Elzinga and Jander, 2013; Rodriguez et al., 2014). With parallels emerging between plant microbe infection and aphid infestation strategies, it is likely that aphid effectors also target important host cell processes to promote virulence- or effector-triggered susceptibility (Rodriguez and Bos, 2013).

Although there is limited insight into the molecular basis of plant pathogen and pest host range, effectors and their plant targets are predicted to be involved. For example, an effector may only be able to interact with a specific plant protein in host but not nonhost plants to suppress defenses as a consequence of target diversification (Schulze-Lefert and Panstruga, 2011). Indeed, Zheng et al. (2014) identified eight *Phytophthora infestans* effectors that were able to suppress flg22-actived reporters, of which five were only able to do so in host tomato (*Solanum lycopersicum*) but not in nonhost Arabidopsis (*Arabidopsis thaliana*) protoplasts. Among the different aphid species, *Myzus persicae*, is one of the major pests, which is partly due to its broad host range, which includes plants in over 40 families. How this species is able to infest such a wide range of plant species and whether this involves secretion of effectors remains to be elucidated.

To date, a number of aphid effectors has been identified that affect aphid virulence as determined by in planta overexpression assays as well as RNAi in aphids (Mutti et al., 2008; Bos et al., 2010b; Atamian et al., 2013; Pitino and Hogenhout, 2013; Elzinga et al., 2014). This includes effectors from the broad host range aphid species *M. persicae* that reduce aphid virulence upon overexpression, including Mp10, Mp42, Mp56, Mp57, and Mp58 (Bos et al., 2010b; Elzinga et al., 2014). Importantly, several aphid effectors have been identified to date that contribute to aphid virulence. Effector C002, first identified in the pea aphid (*Acyrthosiphum pisum*), contributes to aphid survival as evidenced by RNAi experiments that resulted in a reduction of C002 transcript levels and reduced aphid virulence (Mutti et al., 2008; Pitino and Hogenhout, 2013). Moreover, overexpression of *M. persicae* C002 in Arabidopsis and *Nicotiana benthamiana* enhances *M. persicae* virulence (Bos et al., 2010b; Pitino and Hogenhout, 2013). Other effectors found to enhance aphid virulence upon overexpression in host plants are *Me*10 and *Me*23 from potato aphid (*Macrosiphum euphorbiae*), as well as Mp1 (PIntO1), Mp2 (PIntO2), and Mp55 from *M. persicae* (Atamian et al., 2013; Pitino and Hogenhout, 2013; Elzinga et al., 2014). For several of these effectors, including Mp1, there is strong evidence for secretion into the plant-host interface, as proteomics-based approaches revealed their presence in aphid saliva (Harmel et al., 2008; Carolan et al., 2009, 2011). Another interesting observation is that there is specificity with regards to activity of similar effectors from different species (Pitino and Hogenhout, 2013; Elzinga and Jander, 2013). While Mp1 promotes *M. persicae* virulence upon overexpression in the phloem of Arabidopsis transgenic lines, Ap1, an Mp1-like sequence from *A. pisum*, does not. With increasing numbers of aphid effectors being identified, the next step is to investigate their function and understand the cellular processes they target.

We performed yeast-two-hybrid (Y2H) screens against a potato library to identify aphid effector host targets and gain insight into host cellular reprogramming by aphids. We found that *M. persicae* effector Mp1 associated with Vacuolar Protein Sorting Associated Protein52 (VPS52), a component of the Golgi-Associated Retrograde Protein (GARP) complex specifically from host but not poor-host plant species. Moreover, we implicate both the effector and host protein in host susceptibility to *M. persicae*. Our data support a model wherein aphids target a host cell trafficking pathway protein to promote infestation.

RESULTS

Aphid Effector Mp1 Associates with Arabidopsis and Potato VPS52

With several aphid effectors identified to date that promote virulence, including Mp1, we aimed to gain insight into the molecular mechanisms underlying virulence activity. We performed an Y2H screen against a potato (*Solanum tuberosum*) library to a depth of 7.6×10^6 yeast transformants. Yeast prey constructs were isolated from colonies recovered from selection plates, sequenced, and subjected to cotransformation with the Mp1-bait construct to identify sequences and verify interactions with individual prey plasmids. Among the prey-constructs we identified sequences corresponding to VPS52 (XP_006338692.1, three independent clones), FLX-like 2 (XP_015160018.1, three independent clones), phytochrome B (XP_006355734.1, one clone), and an uncharacterized protein (XP_006340727.1, 1 clone). Independent cotransformation experiments showed that yeast coexpressing a bait-Mp1 construct with a prey-StVPS52 (potato VPS52) construct was able to grow on –His medium and exhibited β-galactosidase activity (Fig. 1A). Because VPS52 has been previously identified and characterized in Arabidopsis (Lobstein et al., 2004; Guermonprez et al., 2008), also a host species of *M. persicae*, we tested whether Mp1 was able to interact with Arabidopsis VPS52 (AtVPS52) as well. AtVPS52 and StVPS52 share about 80% identity and the VPS52 sequence overall is highly conserved among plant species (Supplemental Fig. S1). Yeast reporter assays showed that Mp1 was able to interact with both AtVPS52 and StVPS52 (Fig. 1A).

To independently confirm the association of VPS52 with Mp1 in planta, we cloned StVPS52 and AtVPS52

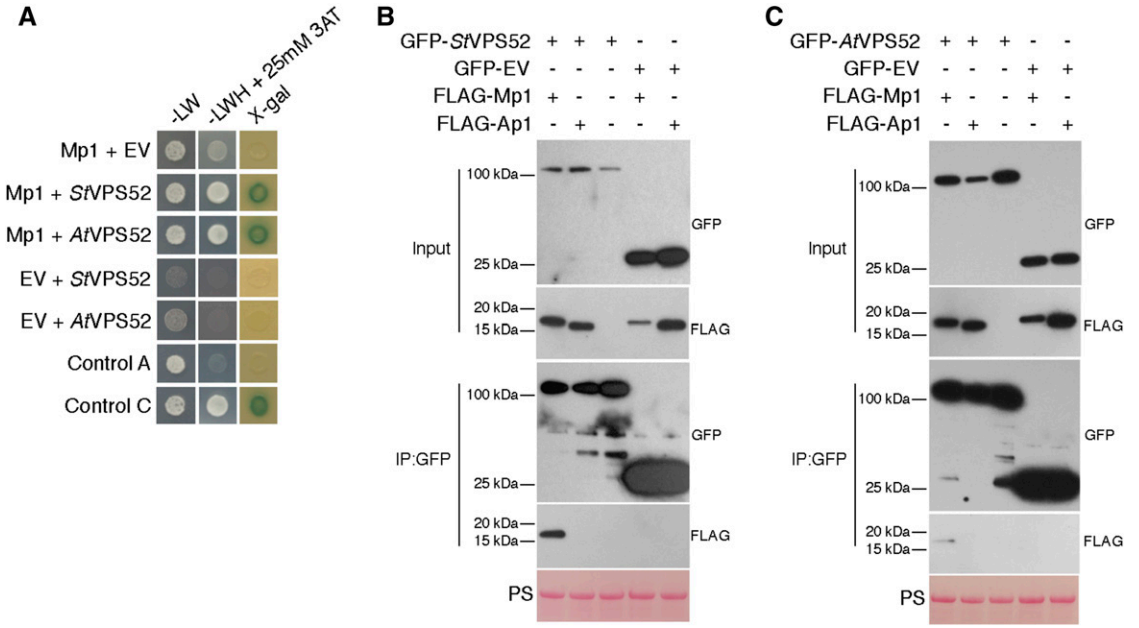

Figure 1. *M. persicae* effector Mp1 associates with host protein VPS52. A, Confirmation of specific two-hybrid interactions in yeast between Mp1 and StVPS52 or AtVPS52 through activation of various reporter genes. Control A represents a negative control, and Control C represents a positive control. −LW represents double drop-out medium lacking Leu and Trp; −LWH represents triple drop-out medium lacking Leu, Trp, and His; 3AT is the abbreviation of 3-amino-1,2,4-triazole, added to suppress the self-activation of HIS3 gene; X-Gal assay was used to assess the activation of lacZ gene. B, Co-IP of FLAG-Mp1 and FLAG-Ap1 with GFP-StVP52 shows that Mp1, but not Ap1, interacts with StVPS52. Leaves of *N. benthamiana* were infiltrated with *Agrobacterium* strains expressing different combinations of GFP-VPS52 or GFP vector control with FLAG-Mp1 or FLAG-Ap1. Three days after infiltration, proteins were extracted and subjected to immunoprecipitation with GFP-magnetic beads for western blotting with GFP or FLAG antibodies. Lower panel indicates Rubisco stained with Ponceau S to show equal loading. − indicates absence and + indicates presence of treatment according to upper-left panel. This experiment was repeated two times with similar results. The original blots from which images were cropped are shown in Supplemental Figure S9. C, Co-IPs of FLAG-Mp1 and FLAG-Ap1 with GFP-AtVPS52 shows that Mp1, but not Ap1, interacts with AtVPS52. Leaves of *N. benthamiana* were infiltrated with *Agrobacterium* strains expressing different combinations of GFP-AtVPS52 or GFP vector control with FLAG-Mp1 or FLAG-Ap1. Three days after infiltration, proteins were extracted and subjected to immunoprecipitation with GFP-magnetic beads or western blotting with GFP or FLAG antibodies. Lower panel indicates Rubisco stained with Ponceau S to show equal loading. − indicates absence and + indicates presence of treatment according to upper-left panel. This experiment was repeated two times with similar results. The original blots from which images were cropped are shown in Supplemental Figure S9. GFP-EV, GFP vector control; IP:GFP, GFP magnetic beads; PS, Ponceau S.

into expression vectors with N-terminal epitope tags, as well as Ap1, an Mp1-like effector from the aphid species *A. pisum* (pea aphid). Coexpression of GFP-StVPS52 or GFP-AtVPS52 with FLAG-Mp1 or FLAG-Ap1 in *N. benthamiana*, followed by immunoprecipitation using GFP-trap beads, confirmed that only Mp1 associated with both AtVPS52 and StVPS52 inside plant cells (Fig. 1, B and C). This correlates with the ability of Mp1, but not Ap1, to promote *M. persicae* virulence on Arabidopsis (Pitino and Hogenhout, 2013).

We cloned a set of additional Mp1-like sequences from the aphid species bird cherry-oat aphid (*Rhopalosiphum padi*), and black cherry aphid (*Myzus cerasi*) to determine whether Mp1-like effectors from additional species limited in their ability/unable to infest potato or Arabidopsis, were also able to associate with AtVPS52 or StVPS52 (Supplemental Fig. S2). Coimmunoprecipitations (Co-IPs) with these Mp1-like effectors were performed in parallel with Mp1 to test for association with VPS52. Although we occasionally detected faint bands for Mp1-like variants on western blots in the GFP or GFP-VPS52 pull-downs, which likely reflects nonspecific binding to the GFP-magnetic beads, we only consistently detected a strong band corresponding to Mp1 in the GFP-VPS52 pull-downs (Fig. 2A). Based on this, we conclude that none of the Mp1-like effectors were pulled down by GFP-VPS52 despite showing comparable expression levels to Mp1 (Fig. 2A). This provides further evidence that the association of Mp1 with VPS52 is specific to Mp1, but not Mp1-like effectors.

The Mp1-VPS52 Association Takes Place in a Host-Specific Manner

Although *M. persicae* has a broad host range, this aphid shows poor performance on *Medicago truncatula* and barley (*Hordeum vulgare*) under controlled conditions, and does not cause significant infestations on these plants in a natural environment (Gao et al., 2007; Davis and Radcliffe, 2008). To determine whether Mp1 is able

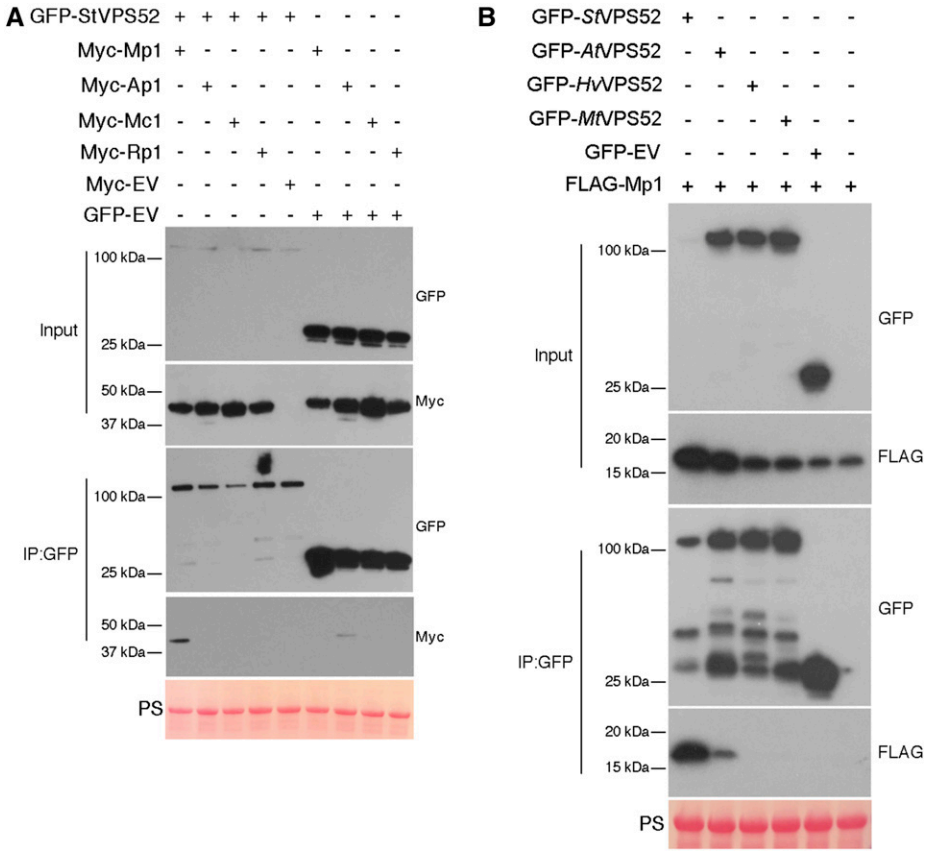

Figure 2. *M. persicae* effector Mp1 specifically associates with host but not poor-host VPS52s. A, Co-IPs of GFP-StVPS52 with Myc-Mp1, Myc-Ap1, Myc-Mc1, or Myc-Rp1 show that Mp1, but not Mp1-like variants, interacts with StVPS52. Leaves of *N. benthamiana* were infiltrated with *Agrobacterium* strains expressing different combinations of GFP-StVPS52 with Mp1variants or GFP vector control. Three days after infiltration, proteins were extracted and subjected to immunoprecipitation with GFP-magnetic beads for western blotting with GFP or FLAG antibodies. Lower panel indicates Rubisco stained with Ponceau S to show equal loading. − indicates absence and + indicates presence of treatment according to upper-left panel. The original blots from which images were cropped are shown in Supplemental Figure S8. B, Co-IPs of FLAG-Mp1 with VPS52 variants St- (*S. tuberosum*), At- (Arabidopsis), Hv- (*H. vulgare*), and Mt-StVPS52 (*M. truncatula*) show that Mp1 interacts only with StVPS52 and AtVPS52. Leaves of *N. benthamiana* were infiltrated with *Agrobacterium* strains expressing different combinations of GFP-StVPS52 with FLAG-Mp1 or GFP vector control. Three days after infiltration, proteins were extracted and subjected to immunoprecipitation with GFP-magnetic beads for western blotting with GFP or FLAG antibodies. Lower panel indicates Rubisco stained with Ponceau S to show equal loading. − indicates absence and + indicates presence of treatment according to upper-left panel. The original blots from which images were cropped are shown in Supplemental Figure S9. GFP-EV, GFP vector control; IP:GFP, GFP magnetic beads; PS, Ponceau S.

to associate with VPS52 proteins from poor-host plant species of *M. persicae*, we cloned VPS52 sequences from barley and *M. truncatula* into plant expression vectors. We performed coexpression experiments of GFP-VPS52s and FLAG-Mp1 in *N. benthamiana* followed by Co-IP. Of the different VPS52 proteins, StVPS52 showed the lowest expression level in the input as determined by western blotting. Despite this, only StVPS52 and AtVPS52, but not HvVPS52 and MtVPS52, were able to pull-down Mp1 (Fig. 2B). Also, we noted that Mp1 protein levels in the input were more abundant in the presence of StVPS52 and AtVPS52, but not HvVPS52 and MtVPS52, indicating that the association may stabilize the effector protein (Fig. 2B).

Because *R. padi* and *A. pisum* are able to infest barley and *M. truncatula*, respectively, we tested whether the Mp1-like proteins from these aphid species were able to associate with HvVPS52 and MtVPS52. Coexpression in *N. benthamiana* followed by Co-IP showed that GFP-HvVPS52 and -MtVPS52 did not pull down any of the Mp1-like proteins in these experiments (Supplemental Fig. S3, A and B). This suggests that the Mp1-VPS52 association may be specific to *M. persicae* and its host plants.

Coexpression of Mp1 with AtVPS52 or StVPS52 Results in Its Relocalization to Vesicle-Like Structures That Associate with Prevacuolar Compartments

In plants, VPS52 has been previously identified and characterized in Arabidopsis (Lobstein et al., 2004; Guermonprez et al., 2008), where this protein was

shown to localize partially to post-Golgi and prevacuolar compartments, suggesting it may have similar functions in trafficking as in other eukaryotes. We further investigated the (co)localization of VPS52 and Mp1 upon overexpression in *N. benthamiana* and found that StVPS52, similar to GFP-AtVPS52, localized in mobile vesicle-like structures, which partially colocalized with the prevacuolar compartment marker PS1 (Kotzer et al., 2004; Fig. 3, A and B). Limited colocalization was observed with the endomembrane trafficking compartment markers Ara6 and Ara7 (Supplemental Fig. S4). In the absence of overexpressed VPS52, GFP-Mp1 (±35 kDa) predominantly localized to the cytoplasm and nucleus similar to the free GFP control (Fig. 3C). However, in the presence of overexpressed mRFP-AtVPS52 or -StVPS52, Mp1 relocalized to vesicle-like structures that were associated with RFP-VPS52 (Fig. 3C). Similar to StVPS52 and AtVPS52, MtVPS52, and HvVPS52 localized to vesicle-like structures upon overexpression in *N. benthamiana* (Supplemental Figs.

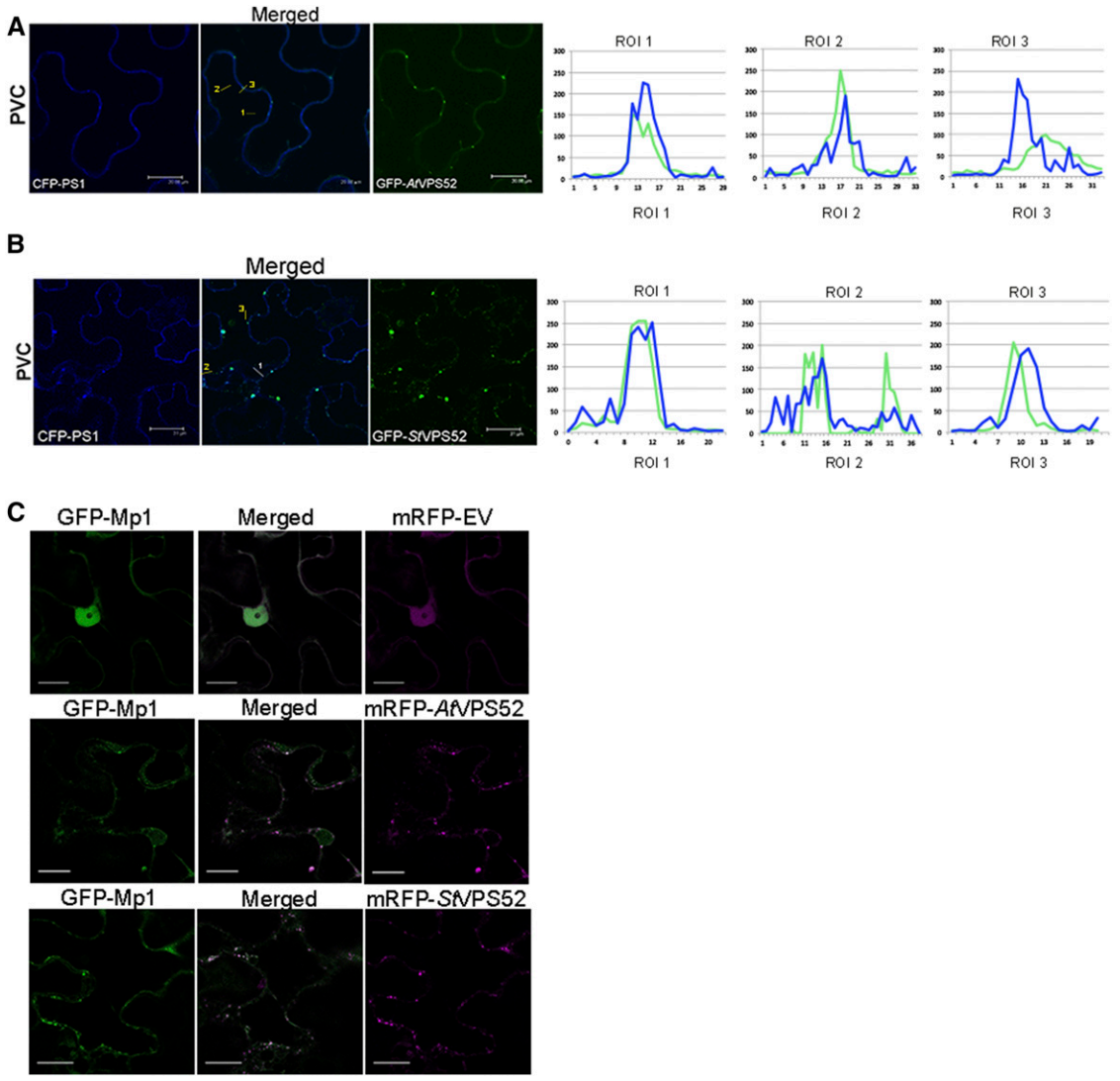

Figure 3. Overexpression of host VPS52s causes relocalization of Mp1 to vesicle-like structures that associate with the prevacuolar compartment. A, Confocal microscopy images of *N. benthamiana* leaves transiently expressing GFP-AtVPS52 in combination with subcellular marker CFP-PS1. Images were taken 3 d after agroinfiltration. Merged figures represent the overlay images of the GFP with CFP channels. Spatial plot profiles represent the colocalization level across a defined region of interest depicted in the merged images. Plot profiles were done with Fiji software (https://fiji.sc/). B, Confocal microscopy images of *N. benthamiana* leaves transiently expressing GFP-StVPS52 in combination with subcellular marker CFP-PS1. Images were taken 3 d after agroinfiltration. Merged figures represent the overlay images of the GFP with CFP channels. Spatial plot profiles represent the colocalization level across a defined region of interest depicted in the merged images. Plot profiles were done with Fiji software (https://fiji.sc/). C, Colocalization of GFP-Mp1 with mRFP-StVPS52, mRFP-AtVPS52, or mRFP by confocal microscopy. Images were taken 3 d after agroinfiltration of *N. benthamiana* leaves. Merged figures represent the overlay images of the GFP and mRFP channels. Scale bars = 20 μm. ROI, Region of interest.

S5 and S6). However, no Mp1-relocalization was observed upon coexpression with HvVPS52 or MtVPS52 (Supplemental Fig. S5), indicating that relocalization only takes place when Mp1 is coexpressed with the VPS52s that are able to associate with it.

In addition, we performed colocalization of the Mp1-like effectors from *A. pisum* (Ap1), *R. padi* (Rp1), and *M. cerasi* (Mc1) with the VPS52s cloned from the four different plant species. GFP-Ap1, -Mc1, and -Rp1 were only detected in the cytoplasm and nucleus, similar to free GFP, in the absence of overexpressed VPS52 (Supplemental Fig. S6). Western blotting confirmed the different GFP-Mp1-like fusions were expressed as full-length proteins (Supplemental Fig. S7). No relocalization was observed with any of the Mp1-like effector-VPS52 combinations. These data are in line with the Co-IP data, and provide further evidence that the Mp1-VPS52 association may be species-specific.

Phloem-Specific Overexpression of Mp1, But Not Mp1-Like Effectors from Other Species, Promotes *M. persicae* Virulence in *N. benthamiana*

While Mp1, but not Ap1, promotes virulence when expressed in the phloem companion cells of Arabidopsis transgenic lines (Pitino and Hogenhout, 2013), no virulence activity was found in *N. benthamiana* using 35S-based expression in leaf discs (Bos et al., 2010a, 2010b). We used transient assays in intact *N. benthamiana* plants, similar to Elzinga et al. (2014), to determine whether Mp1 and Mp1-like effectors impact aphid virulence when expressed in the phloem companion cells of a solanaceous host. We expressed Mp1, Ap1, Rp1, and Mc1 under the control of the AtSUC2 phloem-specific promoter and challenged infiltrated leaf areas with *M. persicae* first-instar nymphs. Virulence, as measured by nymph production, was monitored over a 14-d period, with aphids being moved to fresh infiltration sites every 6 d. While expression of Mp1 resulted in a 64% increase of *M. persicae* nymph production compared to the vector control (Fig. 4), none of the Mp1-like effectors showed any significant effect on virulence. The different Mp1-like effectors were all equally stable in planta when expressed as GFP-, FLAG-, and Myc-fusion proteins, suggesting that the observed differences in virulence activity are not due to differences in protein stability (Fig. 1; Supplemental Figs. S6 and S7). Importantly, the lack of virulence activity of the Mp1-like effectors from different aphid species toward *M. persicae* correlates with the inability to associate with StVPS52 or AtVPS52.

Phloem-Specific Overexpression StVPS52 Reduces *M. persicae* Virulence

We were interested to determine the impact of VPS52 on aphid virulence and tested whether we could use virus-induced gene silencing (VIGS) of VPS52 in

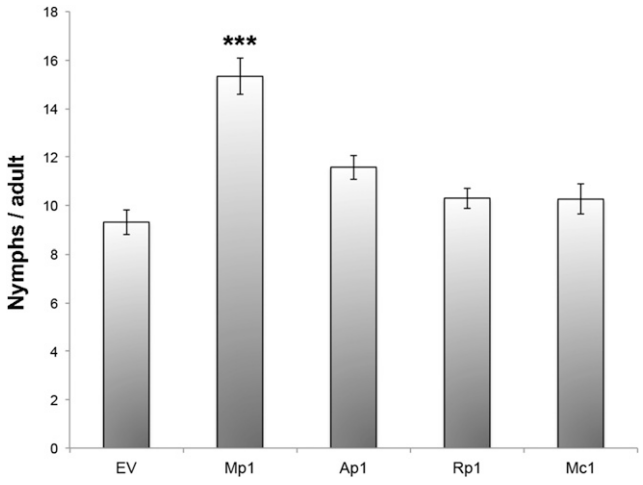

Figure 4. *M. persicae* effector Mp1, but not Mp1-like effectors from other aphid species, promotes virulence on *N. benthamiana* upon phloem-specific overexpression. Leaves of *N. benthamiana* transiently expressing different Mp1-like effectors under the phloem-specific AtSUC2 promoter were challenged with *M. persicae*. Aphid fecundity was assessed over a 14-d period. Ap1, Rp1, and Mc1 are the Mp1-like effectors from *A. pisum*, *R padi*, and *M. cerasi*, respectively. Empty vector was used as a control. The graph shows the means of four independent replicates, where $n = 12$ represents the maximum number of samples per treatment in each replicate. The normally distributed data set was treated with one-way ANOVA Welch and Brown-Forsythe tests for unequal variances, and Scheffe Post-Hoc test for unequal group size. Error bars represent the mean ± SE and asterisks (***) indicates significant differences between treatments versus vector control ($P < 0.01$). EV, Empty vector.

N. benthamiana in combination with *M. persicae* performance assays. Aphids were unable to survive on control plants in any attempted VIGS experiment, suggesting that, in our hands, VIGS was incompatible with *M. persicae* performance assays. We then decided to overexpress StVPS52 in a solanaceous host, *N. benthamiana*, under a phloem-specific promoter to determine whether this affected aphid virulence. Overexpression of StVPS52 reduced nymph production by approximately 40% compared to the vector control (Fig. 5). This suggests that high levels of host StVPS52 in phloem companion cells negatively impact *M. persicae* virulence.

AtVPS52 and StVPS52 Protein Levels Are Reduced upon Aphid Infestation

To investigate whether VPS52 is targeted by aphids during infestation, we made use of an available Arabidopsis mutant line, *pok* (Lobstein et al., 2004), which harbors a T-DNA GUS insertion in the 10th exon resulting in an active translational fusion. Homozygous plants cannot be obtained for this mutant due to a defect in male gametophyte formation (Lobstein et al., 2004). qRT-PCR analyses of *AtVPS52* transcripts in the hemizygous mutant did not show any differences in

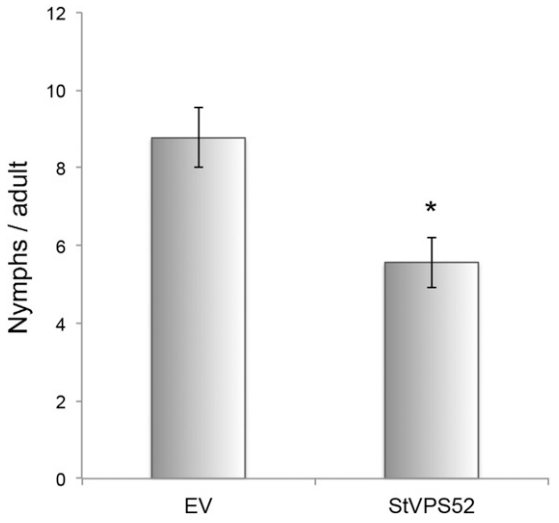

Figure 5. StVPS52 reduce *M. persicae* virulence on *N. benthamiana* upon phloem-specific overexpression. Leaves of *N. benthamiana* transiently expressing StVPS52 (potato) under the phloem-specific AtSUC2 promoter were challenged with *M. persicae*. Aphid fecundity was assessed over a 14-d period. Empty vector was used as a control. Graphs represent the means of three independent replicates, where $n = 12$ represents the number of samples per treatment in each replicate, and error bars represent the mean ± SE. Asterisks (*) indicate significant differences between treatment and the control (*t* test, $P < 0.05$). EV, Empty vector.

transcript levels, indicating that there is no reduction of expression in this line (Supplemental Fig. S8A). Also, aphid fecundity experiments did not show any significant difference in aphid susceptibility of this line compared to Ws-4 wild-type plants (control; Supplemental Fig. S8B). Using GUS-staining, we assessed expression of the translational VPS52-GUS fusion in both uninfested and aphid-infested plants. Although no VPS52-GUS expression was detected in rosette leaves, we found high levels of AtVPS52-GUS in apical stem tissues of flowering plants (Fig. 6A, left panels) and, as previously described, in flower tissues (Guermonprez et al., 2008). The high level of VPS52 in these tissues may reflect increased vesicle trafficking taking place to allow for growth and development. Interestingly, aphid infestation strongly reduced AtVPS52-GUS in the stem and flower tissues (Fig. 6A, right panels). However, no reduction of *AtVPS52* transcript levels was detected by qRT-PCR upon aphid infestation, suggesting the aphids target VPS52 at the posttranslational level (Supplemental Fig. S8C).

Using transient-overexpression of GFP-StVPS52 or GFP-AtVPS52 in *N. benthamiana* followed by *M. persicae* challenge, we independently confirmed the aphid-mediated reduction of VPS52, but not free GFP, at the posttranslational level (Fig. 6B). In parallel, we tested whether PAMP treatment or infection with a plant pathogen similarly affected GFP-VPS52 stability in *N. benthamiana*. We found that neither the bacterial PAMP flg22 nor the oomycete plant pathogen *Phytophthora capsici* reduced GFP-StVPS52 protein levels (Fig. 6C). In contrast, a slight increase of StVPS52 was detected when infiltrated tissues were exposed to flg22 or *P. capsici*. Therefore, the strong reduction of VPS52 levels specifically by *M. persicae* is unlikely the result of activation or suppression of a general plant defense response. Coexpression assays of VPS52 with Mp1 (Figs. 6B, and 1, B to D) did not show any evidence of a role for Mp1 in VPS52 degradation, suggesting that additional effectors in aphid saliva may be involved in the targeting and degradation of VPS52. Because phloem-specific overexpression of VPS52 negatively impacted aphid virulence, aphid-mediated degradation of this protein may be an important step during infestation.

During aphid infestation experiments on flowering Arabidopsis plants, we noticed that aphids were more abundant on stems and flower tissues than on rosette leaves of flowering plants. We therefore, followed this up with aphid choice-experiments, where 30 alate *M. persicae* aphids were released in a cage containing four flowering plants. We monitored aphid numbers after 12 d on rosette leaves or stems plus flowers. This showed that aphid colonization predominantly occurred on the stems and flowers as opposed to the rosette leaves, with approximately six times more aphids being present in these tissues (Fig. 7). The high level of VPS52 expression in these aphid-preferred tissues suggests that aphids indeed need to target this protein as part of an effective infestation strategy.

DISCUSSION

Here, we report the targeting of a host cell trafficking protein, VPS52, by an herbivorous insect. We used an aphid effector, Mp1, which promotes virulence, as a probe to identify potential aphid targets in host plants. We showed that Mp1 specifically associates with VPS52 from several host but not poor-host plant species. Moreover, we were unable to detect associations of Mp1-like effectors from other aphid species with host VPS52s, suggesting that the Mp1-VPS52 association is specific to *M. persicae* and its hosts. Overexpression of StVPS52 in a solanaceous host reduced *M. persicae* performance, indicating VPS52 is likely an important virulence target. Overall, our work provides important evidence that herbivorous insects, similar to plant pathogenic microbes, secrete effectors inside host cells that interact with host proteins and modify their activity to facilitate infestation.

The association of Mp1 with VPS52 was highly specific in that only Mp1, but not Mp1-like effectors from other aphid species, interacted with VPS52 from *M. persicae* hosts. Because the host species Arabidopsis and potato are in different families, *M. persicae* effector Mp1 may have evolved to associate with the same host protein in at least two distantly related plant species. The VPS52 family in plants is highly conserved, suggesting that perhaps minor structural differences among its members have a significant impact on the

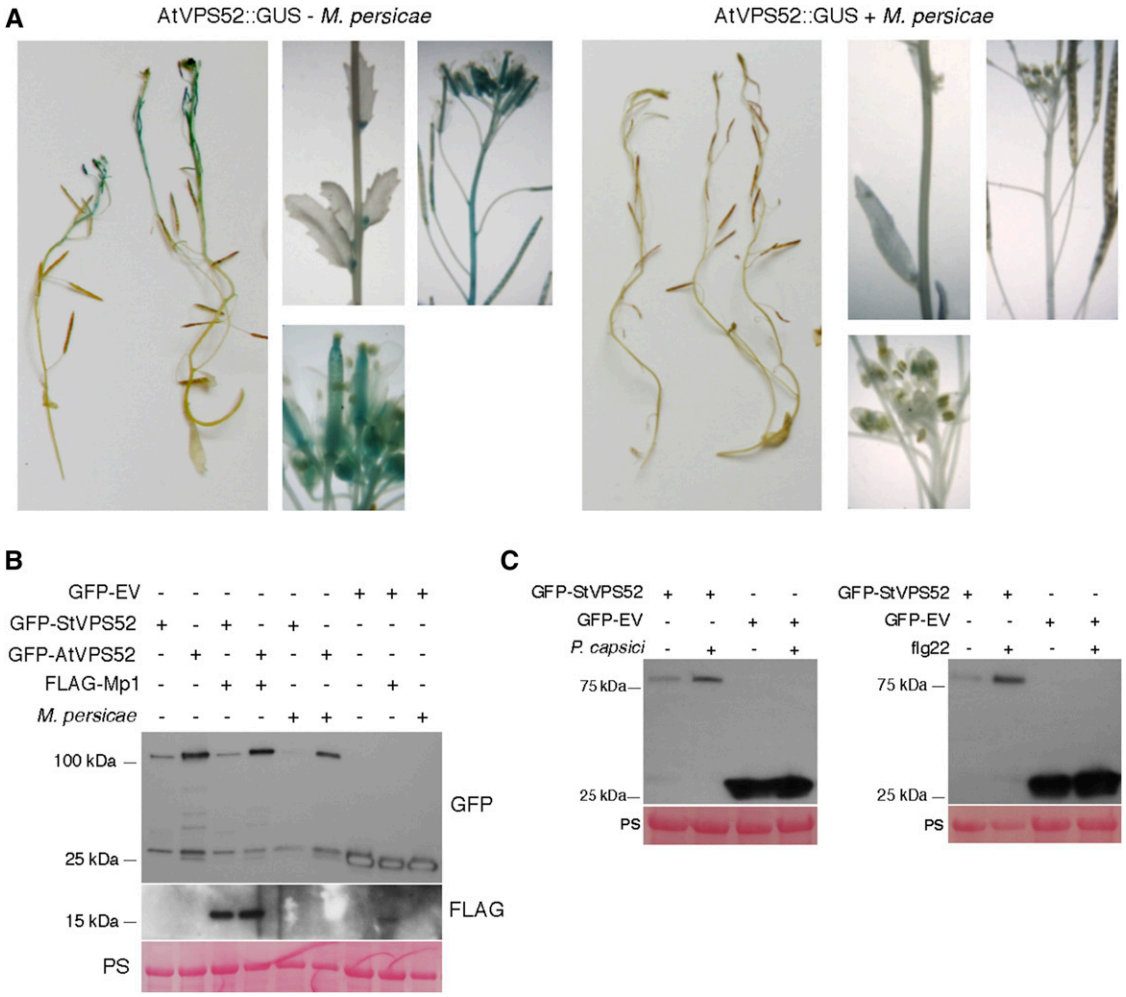

Figure 6. Aphid infestation causes degradation of VPS52. A, *pok* mutant plants were infested with *M. persicae* for 5 to 7 d and collected for GUS-staining. Images were taken with a lighted microscope. The experiment was repeated three times with similar results. B, *N. benthamiana* leaves transiently overexpressing GFP vector control, GFP-StVPS52, and GFP-AtVPS52 in combination with FLAG-Mp1 or infested with *M. persicae* for 3 d were collected for protein extraction and western blotting with anti-GFP and anti-FLAG antibodies. Lower panel indicates Rubisco stained with Ponceau S to show equal loading. − indicates absence and + indicates presence of treatment according to upper-left panel. C, *N. benthamiana* leaves transiently overexpressing GFP vector control and GFP-StVPS52 were challenged with *P. capsici* zoospores before protein extraction and western blotting with GFP antibodies. *N. benthamiana* leaves transiently overexpressing GFP vector control and GFP-StVPS52 were challenged with the PAMP flg22 before protein extraction and western blotting with GFP antibodies. Lower panel indicates Rubisco stained with Ponceau S to show equal loading. − indicates absence and + indicates presence of treatment according to upper-left panel. GFP-EV, GFP vector control; PS, Ponceau S.

association with Mp1. By including both different variants of Mp1 and VPS52 from different aphid and plant species, we were able to show that Mp1 association with VPS52 is linked to virulence activity, indicating this effector functions in a host species-specific manner. Host-specific effector functions have been proposed by Pitino and Hogenhout (2013), who showed that *M. persicae* effectors C002, PIntO1 (Mp1), and PIntO2 (Mp2), but not their putative orthologs from the pea aphid, promoted *M. persicae* virulence in Arabidopsis. Our results are in line with this, and provide evidence that difference in such virulence activities are not due to differences in effector protein stability. The lack of interaction between any of the Mp1-like effectors and VPS52s from corresponding host plant species suggests these effectors may have evolved to exhibit different activities.

Mp1 and VPS52 virulence effects were observed upon companion-cell-specific overexpression of these proteins, suggesting they possibly are involved in phloem-specific processes during plant-aphid interactions. When GFP is expressed under the AtSUC2 promoter, it can pass into sieve elements and reach sink tissues, including flowers (Imlau et al., 1999). Although Mp1 and VPS52 were specifically expressed in companion cells, this site is not necessarily the site of

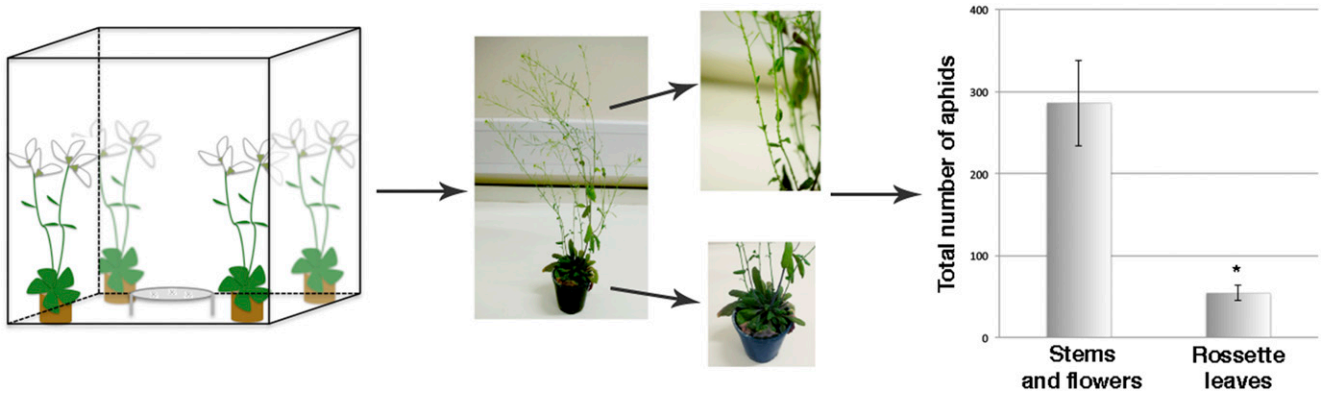

Figure 7. Aphids preferentially colonize Arabidopsis stems and flower tissues over rosette leaves. Aphid numbers on rosette leaves or stem and flower tissues of flowering plants 12 d after the release of alate adults. Graph represents the means of two independent replicates, where $n = 4$ represents the number of plants in each replicate, and error bars represent the mean ± SE. Asterisks (*) indicate significant differences between rosette leaves and stems plus flowers tissues (paired t test, $P < 0.05$).

activity as these proteins may be localized to, for example, the sieve elements similar to GFP. VPS52, although detected in leaves, was predominantly expressed in the inflorescence stems and specific flower organs. Moreover, VPS52, together with other vesicle trafficking proteins, has been detected in pumpkin phloem sap by proteomics, indicating that this protein functions in the phloem (Lin et al., 2009). Recent evidence suggests that membrane systems, including the Golgi apparatus and the ER, may be present inside sieve elements (Lin et al., 2009; Fröhlich et al., 2012). Also, VPS51, another component of the GARP complex, is expressed in the plant vasculature as well as in the flowers (Pahari et al., 2014). It is therefore possible that aphids may need to manipulate host processes in specific tissues, such as the phloem.

High expression of VPS52 was detected in tissues where cell elongation takes place (i.e. flower organs and apical stem), which may reflect increased vesicle trafficking to allow for growth and development. Interestingly, our results show that in the case of the Arabidopsis-*M. persicae* interaction, aphids prefer to feed on tissues with high levels of VPS52 expression (i.e. inflorescence stems and flowers) and thus on tissues with high growth or development rates. Preferred infestation by aphids, including *M. persicae*, of stems, flower buds, and flowers of certain host species has been reported previously (Guldemond et al., 1998; Ashouri et al., 2001), and is possibly triggered by nutrient reallocation to flowers and buds during plant developmental stages. Our finding that VPS52 is highly expressed in these tissues and that VPS52 phloem-specific overexpression negatively impacts aphid virulence suggests that aphid-mediated degradation of VPS52 is important for successful infestation.

VPS52 is a component of the GARP complex, which is involved in the transport from endosomes to the trans-Golgi network and has been mainly characterized in yeast and mammalian systems (Conibear and Stevens, 2000; Conibear et al., 2003; Reggiori et al., 2003). Schindler et al. (2015) recently showed that several components of this complex, including VPS52, also take part in a complex involved in endosome to plasma membrane trafficking, pointing to different functions of GARP-complex proteins in cellular trafficking. Although the role of VPS52 in plant cellular trafficking is not well understood, this protein was previously shown to localize to post-Golgi and prevacuolar compartments, suggesting it may have similar functions in trafficking as in other systems (Lobstein et al., 2004; Guermonprez et al., 2008). VPS52 interacts with at least one other component of the GARP-complex in plants, VPS51, which is involved in the maintenance of vacuolar morphology as well as leaf shape and vein patterning (Pahari et al., 2014). A recent proteomics study to identify proteins associated with endosomal and secretory pathways in Arabidopsis revealed the enrichment of VPS52 in pull-downs of RABF1/ARA6, RABG3f, CLC2, RABD2a/ARA5, and RABF2b/ARA7 (Heard et al., 2015). This suggests that VPS52 may associate with a variety of endomembrane vesicles, perhaps through binding GTPases (Heard et al., 2015). How exactly aphids impact vesicle trafficking via VPS52 remains to be investigated and will require a better understanding of VPS52 function inside plant cells.

Our work provides important evidence that the existing plant pathogenic microbe effector paradigm can be extended to herbivorous insects in that effector-target interactions inside host cells modify critical host processes to promote plant susceptibility. The negative impact of VPS52 on aphid virulence suggests this host protein either contributes to plant immunity to aphids, or perhaps negatively regulates nutrient availability in the phloem. In recent years, it has become evident that host vesicle trafficking regulates plant defense responses to pathogenic microbes (Teh and Hofius, 2014; Ben Khaled et al., 2015). For example, receptor

activation, and activation of defense signaling, as well as redirecting cargo to infection sites, all require host trafficking machinery. Also, plant pathogenic microbes target components of this machinery to promote disease (Nomura et al., 2006; Bozkurt et al., 2011; Gu and Innes, 2012). We showed that VPS52 degradation was specific to aphid infestation and was not observed upon activation of plant immunity and plant microbe infection. Therefore, an important next step will be to dissect how vesicle trafficking pathways mediated by VPS52 are impacted by aphid infestation and how this promotes aphid virulence. And, as evidenced from our work, this would need to take into consideration activities in specific plant tissues. Ultimately, the identification and characterization of aphid effectors and their host targets will provide us with, to our knowledge, novel insights into the virulence strategies employed by agricultural pests. With limited sources of genetic resistances available in crops, a detailed understanding of these virulence strategies promises to open new avenues for crop improvement.

MATERIALS AND METHODS

Plants and Growth Conditions

Wild-type Arabidopsis (*Arabidopsis thaliana*) landrace Columbia-0 (Col-0), *pok* mutants (after selection), and Wassilewskija-4 (Ws-4) were grown in Levington's M2 compost with 4 mm grit (8:1) in growth chambers after seed underwent a stratification period of 48 h at 4°C. Plants were grown under 12 h of light per d, with a photosynthetic photon flux density of 200 μm L m^{-2} s^{-1}, at 18°C and 50% relative humidity.

Plasmid Construction

The sequences of the *Myzus cerasi* and *Rhopalosiphum padi* effector variants were identified by transcriptome sequencing of the different aphid species (Thorpe et al., 2016). The Mp1 (GenBank: KY273521), Ap1 (GenBank: KY273522), Mc1 (GenBank: KY273524), and Rp1 (GenBank: KY273523) coding sequences without signal peptide encoding sequences were amplified from *Myzus persicae*, *Acyrthosiphum pisum*, *M. cerasi*, and *R. padi* cDNA, respectively. The StVPS52 and AtVPS52 coding sequences were amplified from *Solanum tuberosum* and Arabidopsis, respectively. Amplicons were cloned into the pDONR207 vector (Invitrogen) using Gateway technology. BP recombination reactions were transformed in *Escherichia coli* JM109 (Promega). Constructs were verified by sequencing. MtVPS52 (NCBI: XM_003590877.2) and HvVPS52 (GenBank: AK361277.1) were synthesized and cloned into pUC57 vector and provided including suitable Gateway recombination sites by GenScript (www.genscript.com). Subsequently, LR recombination reactions were performed using pB7WGF2 (N-terminal GFP tag), pK7WGR2 (N-terminal mRFP tag; Karimi et al., 2002), pGWB12 (N-terminal FLAG tag), and pGWB21 (N-terminal 10×Myc tag; Nakagawa et al., 2007) as destination vectors for transient overexpression in *Nicotiana benthamiana*.

Agrobacterium tumefaciens Infiltration Assays

Constructs were introduced into *A. tumefaciens strain* GV3101 or AGL1 by electroporation. Transformants were selected using gentamycin (12.5 μg/mL), rifampicin (50 μg/mL), and spectinomycin (50 μg/mL) for transformation into pB7WGF2, pB7WGR2, and gentamycin (12.5 μg/mL), rifampicin (50 μg/mL), and kanamycin (50 μg/mL) for transformation into pGWB12 and pGWB21. For infiltration into leaves, recombinant strains were grown in Luria-Bertani medium with abovementioned antibiotics, then harvested and resuspended in infiltration buffer (acetosyringone 125 μM and $MgCl_2$ 10 mM) to reach an optical density at 600 nm (OD_{600}) = 0.3 for western-blot experiments and OD_{600} = 0.1 for aphid virulence assays and confocal imaging.

Total Protein Extractions and Co-IP Assays

Plant tissue for Co-IP experiments was extracted with GTEN lysis buffer [10% Glycerol, 25 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1% NP-40, with freshly added 10 mM DTT and 1× protease inhibitor cocktail (no. P9599; Sigma-Aldrich)]. Samples were incubated for 15 min in lysis buffer at 4°C. Lysate was centrifuged at 10,000*g* for 10 min and the supernatant was subjected to CoIP with GFP-Trap_M agarose beads (gtm-20; Chromotek) for affinity binding of GFP-fused proteins. Western blotting was performed with the antibodies anti-GFP (sc-8334; Santa Cruz Biotechnology), anti-FLAG (sc-166384; Santa Cruz Biotechnology), and antic-Myc (sc-40; Santa Cruz Biotechnology) followed by antirabbit-HRP (sc-2004; Santa Cruz Biotechnology), or anti-mouse-HRP (sc-2005; Santa Cruz Biotechnology) to detect the corresponding epitopes. Each CoIP experiment was repeated at least two times. Plant tissues for detecting levels of expression in total extracts were extracted using Laemmli sample buffer [4% (w/v) SDS, 20% Glycerol, 120 mM Tris-Cl (pH 6.8) and 0.02% (w/v) bromophenol blue] and then subjected to western-blotting procedures.

flg22 and *Phytophthora capsici* Infection Assays

N. benthamiana leaves expressing GFP-*St*VPS52 2 d after agroinfiltration were drop-inoculated with two 10 μL droplets of *P. capsici* LT1534 zoospores in water (500,000 spores/mL) or water (control). Tissue was collected 24 h later and was used for western-blot experiments. For flg22 elicitor assay, *N. benthamiana* leaves expressing GFP-*St*VPS52 2 d after agroinfiltration were syringe-infiltrated with 1 mM flg22 and tissue was harvested after 3 h of elicitor treatment.

M. persicae Rearing

Aphid experiments were done with *M. persicae* individuals, genotype O, reared on oil seed rape (*Brassica napus*) plants, with a long d (12 h) daylight and a photosynthetic photon flux density of 200 μm L m^{-2} s^{-1} at 18°C and 50% relative humidity.

Aphid Virulence Assays on Whole Plants

For whole plant assays, *N. benthamiana* leaves were infiltrated with recombinant *A. tumefaciens* GV3101-carrying constructs to express the effectors (Mp1, Ap1, Mc1, and Rp1) or StVPS52 under the AtSUC2-promoter (Gottwald et al., 2000) at an OD_{600} of 0.1. Adult aphids were placed on the underside of leaves 1 d after agroinfiltration using clip cages. The next day, adult aphids were removed and three 1-st instar nymphs were left per infiltration site inside of a clip cage. Seven d after agroinfiltration, nymphs were transferred to newly infiltrated leaves, and aphid progeny was counted after 14 d of initial agroinfiltration. For aphid reproduction assays on *Arabidopsis pok* mutants and Ws-4 wild-type plants, mutant plants were firstly selected on Murashige-Skoog medium containing kanamycin. Seedlings were moved and potted into Arabidopsis mix, and after 2 weeks under long-d conditions, aphid assays were performed. Two adult *M. persicae* aphids were placed on plants, and 1 d later, adults were removed and three 1-st instar nymphs were left on the rosette leaves. The total aphid number was counted 14 d after.

Aphid Tissue Preference Test

Thirty alate aphids reared on oil seed rape plants were released inside a cage containing four flowering Arabidopsis plants of the ecotype Col-0. The aphid release point was approximately 8 cm from the cage bottom so that it was positioned at about half the plant height. Twelve d after aphid release, total numbers of aphids were counted in rosette leaves and in stems and flower tissues. This experiment was repeated twice with similar results.

Histochemical Localization VPS52-GUS

Arabidopsis pok mutants were selected as described above and grown under long-d conditions. Flowering plants were placed in individual cages and challenged or unchallenged (control) with aphids for 12 d. Above-ground plant tissues were collected and stained with 1 mg/mL of 5-bromo-4-chloro-3-indolyl-B-D-GlcA (X-gluc; R0852; Thermo Scientific) in X-gluc buffer containing 100 mM sodium P buffer pH 7.0, 0.1% Triton X-100, 2 mM potassium ferricyanide, and 2 mM potassium ferrocyanide. Tissues were vacuum-infiltrated for

10 min and incubated in darkness overnight at 37°C. Chlorophyll was removed by soaking in ethanol. The photographs were taken with a lighted microscope. Experiments were repeated three times with similar results.

Quantitative RT-PCR

For the expression analysis of *AtVPS52* in Arabidopsis, total RNA was purified using RNeasy Plant Mini Kit (Cat. no. 74104; Qiagen) following manufacturer instructions. cDNA was generated with M-MLV Reverse transcriptase (Cat. no. M1701; Promega) and samples analyzed by real-time PCR using QuantiTect SYBR Green Kit (Cat. no. 204143; Qiagen). The primers were designed and analyzed with Primer3. Real-time PCR primer sequences were as follows: AtVPS52_F2, 5′-AGGAGCCTGCACAAGCTACTTA-3′; AtVPS52_R2, 5′-ATGACAGAAAATGGACCCGCA-3′. These primers amplify a fragment of 116 bp. EF1α was used as a housekeeping gene to calculate relative expression using $\Delta\Delta$Ct analysis.

Confocal Imaging

Imaging was performed on a TCS-SP2 AOBS confocal system (Leica Microsystems) using an HCX PL APO 40×/0.85 water dipping objective (Leica Microsystems) and on a 710 confocal microscope (Zeiss) using a PL APO 40×/1.0 water dipping objective (Zeiss). Images were at 1024 × 1024 resolution and taken using line-by-line sequential scanning. The optimal pinhole diameter and the same gain level for the photomultiplier tube was maintained at all times. The software ImageJ (National Institutes of Health) was used for post-acquisition image processing. The excitation wavelength for mRFP was 561 nm, its emission was collected from 600 to 630 nm. GFP was imaged using 488 nm excitation, and its emission was collected from 500 to 530 nm. CFP was imaged using 405 nm excitation and its emission was collected from 455 to 490 nm. Coexpressed mRFP and GFP as well as coexpressed mRFP and CFP were imaged sequentially using a line-by-line mode.

Y2H Reporter Assays

Bait-protein encoding vector pDEST32 expressing Mp1 and the prey-encoding vector pDEST22 expressing *At*VPS52 and *St*VPS52 were transformed into the yeast strain MaV203 according to the ProQuest Two-Hybrid system protocol (Invitrogen). Transformants were plated onto yeast synthetic drop-out medium (Sigma-Aldrich) lacking Leu and Trp (−LW) and incubated at 30°C for 2 to 3 d. Colonies were picked and cultivated overnight in 5 mL of double drop-out medium (−LW). Dilution series were prepared (10^{-3}) of each suspension and 5 μL were dropped onto double drop-out medium (−LW), triple drop-out medium lacking Leu, Trp, and His (−LWH) plus 3AT (*HIS3* gene inhibitor), and onto a nylon membrane for the X-gal assay along with positive and negative controls according to the ProQuest Two-Hybrid system manufacturer's protocol. Plates were incubated at 30°C for 3 to 4 d before photographing. Positive interactions between the expressed proteins resulted in yeast growth and the activation of β-galactosidase in the X-gal membrane assay.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Protein sequence alignment of StVPS52 with AtVPS52, MtVPS52, and HvVPS52.

Supplemental Figure S2. Protein sequence alignment of Mp1 with Ap1, Mc1, and Rp1.

Supplemental Figure S3. Rp1 and Ap1 do not associate with any VPS52 variant proteins.

Supplemental Figure S4. Limited to no colocalization of VPS52 with Ara6 and Ara7.

Supplemental Figure S5. Mp1 does not relocalize to vesicle-like compartments upon coexpression with HvVPS52 or MtVPS52.

Supplemental Figure S6. Mp1-like effectors Ap1, Mc1, and Rp1 do not relocalize upon coexpression with any VPS52 variants.

Supplemental Figure S7. Western blot showing expression of GFP-Mp1, GFP-Ap1, GFP-Mc1, RFP-Rp1, and GFP upon agroinfiltration in *N. benthamiana*.

Supplemental Figure S8. Aphid performance on the *Arabidopsis pok* hemizygous mutant and transcript analyses.

Supplemental Figure S9. Uncropped western-blot images corresponding to figures in the main article.

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