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

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An aphid effector targets trafficking protein VPS52 in a host-specific manner to promote virulence

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One sentence summary: A secreted salivary protein from an herbivorous insect targets a protein in host plants to promote infestation

[1]

[1] **List of author contributions**
P.R. and J.B. designed the experiments. P.R., C.E-M, and J.B. performed the experiments and analyzed the data. P.R. and J.B. wrote the manuscript. J.B. directed the project. All authors read and approved the final manuscript.

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Abstract

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 Protein 52 (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 *Myzus persicae*-host interactions. Also, co-expression 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.

Introduction

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 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[1]. 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., 2010). These include proteins with predicted functions, such as metalloproteases, disulfide isomerases, calreticulins, ARMET proteins, glutathione peroxidases, and CLP-domain serine 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 though of these proteins in aphid saliva/salivary glands, suggests they exhibit an important effector activity towards promoting aphid virulence.

Upon interaction with plant pathogen microbes, most plants are able to defend themselves by recognizing conserved parasite molecules, or PAMPs (Pathogen-Associated-Molecular-Pattern), to activate PTI (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 an ubiquitin E3 ligase (Bos et al., 2010), 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 proteins involved in JA-signalling, 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; Ustun et al., 2013), extracellular proteases (Song et al., 2009) as well as 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 defence responses (Bos et al., 2010; 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 (ETS) (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 and co-authors (Zheng et al., 2014) identified 8 *Phytophthora infestans* effectors that were able to suppress flg22-actived reporters, of which 5 were only able to do so in host tomato but not in nonhost *Arabidospis* 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* over-expression assays as well as RNAi in aphids (Mutti et al., 2008; Bos et al., 2010; 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 over-expression, including Mp10, Mp42, Mp56, Mp57, and Mp58 (Bos et al., 2010; Elzinga et al., 2014). Importantly, several aphid effectors have been identified to date that contribute to aphid virulence. Effector C002, first identified in the *Acyrthosiphum pisum* (pea aphid), 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. Other effectors found to enhance aphid virulence upon over-expression in host plants are *Me*10 and *Me*23 from *Macrosiphum euphorbiae* (potato aphid), 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; Carolan et al., 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, 2014). While Mp1 promotes *M. persicae* virulence upon over-expression 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 Protein 52 (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 *Solanum tuberosum* library to a depth of 7.6 x 10^6 yeast transformants. Yeast prey constructs were isolated from colonies recovered from selection plates, sequenced, and subjected to co-transformation 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 (Vacuolar Protein Sorting-Associated Protein 52) (XP_006338692.1, 3 independent clones), FLX-like 2 (XP_015160018.1, 3 independent clones), phytochrome B (XP_006355734.1, 1 clone) and an uncharacterized protein (XP_006340727.1, 1 clone). Independent co-transformation experiments showed that yeast co-expressing 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). Since 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. 1). 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 into expression vectors with N-terminal epitope tags, as well as Ap1, an Mp1-like effector from the aphid species *A. pisum* (pea aphid). Co-expression 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. 1B 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 *Rhopalosiphum padi* (bird cherry-oat aphid), and *Myzus cerasi* (black cherry aphid) 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. 2). Co-immunoprecipitations 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 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 co-expression experiments of GFP-VPS52s and FLAG-Mp1 in *Nicotiana benthamiana* followed by co-immunoprecipitation. 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).

Since *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. Co-expression in *N. benthamiana* followed by co-immunoprecipitation showed that GFP-HvVPS52 and -MtVPS52 did not pull down any of the Mp1-like proteins in these experiments (Supplemental Fig. 3SA and B). This suggests that the Mp1-VPS52 association may be specific to *M. persicae* and its host plants.

193

194 **Co-expression of Mp1 with AtVPS52 or StVPS52 results in its relocalization to**
195 **vesicle-like structures that associate with prevacuolar compartments**

196

197 In plants, VPS52 has been previously identified and characterized in *Arabidopsis* (Lobstein
198 et al., 2004; Guermonprez et al., 2008), where this protein was shown to localize partially
199 to post-Golgi and prevacuolar compartments suggesting it may have similar functions in
200 trafficking as in other eukaryotes. We further investigated the (co-)localisation of VPS52
201 and Mp1 upon overexpression in *N. benthamiana* and found that StVPS52, similar to GFP-
202 AtVPS52, localized in mobile vesicle-like structures, which partially co-localized with the
203 prevacuolar compartment marker PS1 (Kotzer et al., 2004) (Fig. 3A and B). Limited co-
204 localization was observed with the endomembrane trafficking compartment markers Ara6
205 and Ara7 (Supplemental Fig. 4). In the absence of over-expressed VPS52, GFP-Mp1
206 (±35kDa) predominantly localized to the cytoplasm and nucleus similar to the free GFP
207 control (Fig. 3C). However, in the presence of over-expressed mRFP-AtVPS52 or -
208 StVPS52, Mp1 re-localised to vesicle-like structures that were associated with RFP-VPS52
209 (Figure 3C). Similar to StVPS52 and AtVPS52, MtVPS52 and HvVPS52 localized to
210 vesicle-like structures upon over-expression in *N. benthamiana* (Supplemental Fig. 5 and
211 6). However, no Mp1-relocalization was observed upon co-expression with HvVPS52 or
212 MtVPS52 (Supplemental Fig. 5), indicating that relocalization only takes place when Mp1
213 is co-expressed with VPS52s that are able to associate with it.
214 In addition, we performed co-localization of the Mp1-like effectors from *A. pisum* (Ap1), *R.*
215 *padi* (Rp1) and *M. cerasi* (Mc1) with the VPS52s cloned from the 4 different plant species.
216 GFP-Ap1, -Mc1 and -Rp1 were only detected in the cytoplasm and nucleus, similar to free
217 GFP, in the absence of over-expressed VPS52 (Supplemental Fig. 6). Western blotting
218 confirmed the different GFP-Mp1-like fusions were expressed as full-length proteins
219 (Supplemental Fig. 7). No relocalization was observed with any of the Mp1-like effector-
220 VPS52 combinations. These data are in line with the co-immunoprecipitation data and
221 provide further evidence that the Mp1-VPS52 association may be species specific.

222

223 **Phloem-specific over-epxression of Mp1, but not Mp1-like effectors from other**
224 **species, promotes *Myzus persicae* virulence in *Nicotiana benthamiana***

225

226 Whilst Mp1, but not Ap1, promotes virulence when expressed in the phloem companion
227 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., 2010). 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* 1st-instar nymphs. Virulence, as measured by nymph production, was monitored over a 14-day period, with aphids being moved to fresh infiltration sites every 6 days. 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 Fig. 6, Supplemental Fig. 7). Importantly, the lack of virulence activity of the Mp1-like effectors from different aphid species towards *M. persicae* correlates with the inability to associate with StVPS52 or AtVPS52.

Phloem-specific over-expression StVPS52 reduces *Myzus 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 *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 over-express StVPS52 in a solanaceous host, *N. benthamiana*, under a phloem-specific promoter to determine whether this affected aphid virulence. Over-expression of StVPS52 reduced nymph production by about 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 harbours a T-DNA GUS insertion in the 10^{th} 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 transcript levels indicating that there is no reduction of expression in this line (Supplemental Fig. 8A). 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. 8B). 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 post-translational level (Supplemental Fig. 8C).

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 post-translational 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 defence response. Co-expression assays of VPS52 with Mp1 (Fig. 6B, and Fig. 1B, C, and 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. Since phloem-specific over-expression 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 4 flowering plants. We

monitored aphid numbers after 12 days 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 around 6 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. Over-expression 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. Since 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 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) (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 over-expression 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 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; Frohlich et al., 2012). Also, VPS51, another component of the GARP complex, is expressed in the plant vasculature as well as 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 re-allocation 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

368 mammalian systems (Conibear and Stevens, 2000; Conibear et al., 2003; Reggiori et al.,
369 2003). Schindler *et a*l. (Schindler et al., 2015) recently showed that several components of
370 this complex, including VPS52, also take part in a complex involved in endosome to
371 plasma membrane trafficking, pointing to different functions of GARP-complex proteins in
372 cellular trafficking. Although the role of VPS52 in plant cellular trafficking is not well
373 understood, this protein was previously shown to localize to post-Golgi and prevacuolar
374 compartments suggesting it may have similar functions in trafficking as in other systems
375 (Lobstein et al., 2004; Guermonprez et al., 2008). VPS52 interacts with at least one other
376 component of the GARP-complex in plants, VPS51, which is involved in the maintenance
377 of vacuolar morphology as well as leaf shape and vein patterning (Pahari et al., 2014). A
378 recent proteomics study to identify proteins associated with endosomal and secretory
379 pathways in *Arabidopsis* revealed the enrichment of VPS52 in pull-downs of RABF1/ARA6,
380 RABG3f, CLC2, RABD2a/ARA5, RABF2b/ARA7 (Heard et al., 2015). This suggests that
381 VPS52 may associate with a variety of endomembrane vesicles, perhaps through binding
382 GTPases (Heard et al., 2015). How exactly aphids impact vesicle trafficking via VPS52
383 remains to be investigated and will require a better understanding of VPS52 function
384 inside plant cells.
385
386 Our work provides important evidence that the existing plant pathogenic microbe effector
387 paradigm can be extended to herbivorous insects in that effector-target interactions inside
388 host cells modify critical host processes to promote plant susceptibility. The negative
389 impact of VPS52 on aphid virulence suggests this host protein either contributes to plant
390 immunity to aphids, or perhaps negatively regulates nutrient availability in the phloem. In
391 recent years it has become evident that host vesicle trafficking regulates plant defence
392 responses to pathogenic microbes (Teh and Hofius, 2014; Ben Khaled et al., 2015). For
393 example, receptor activation, and activation of defence signalling, as well as redirecting
394 cargo to infection sites all require host trafficking machinery. Also, plant pathogenic
395 microbes target components of this machinery to promote disease (Nomura et al., 2006;
396 Bozkurt et al., 2011; Gu and Innes, 2012). We showed that VPS52 degradation was
397 specific to aphid infestation and was not observed upon activation of plant immunity and
398 plant microbe infection. Therefore, an important next step will be to dissect how vesicle
399 trafficking pathways mediated by VPS52 are impacted by aphid infestation and how this
400 promotes aphid virulence. And as evidenced from our work, this would need to take into
401 consideration activities in specific plant tissues. Ultimately, the identification and
402 characterization of aphid effectors and their host targets will provide us with 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 up new avenues for crop improvement.

Materials and Methods

Plants and growth conditions

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

Plasmid construction

The sequences of the *M. cerasi* and *R. padi* effector variants were identified by transcriptome sequencing of the different aphid species(Thorpe et al., 2016). The Mp1 (GenBank accession KY273521), Ap1 (Genbank accession KY273522), Mc1 (Genbank accession KY273524), and Rp1 (Genbank accession KY273523) coding sequences without signal peptide encoding sequences were amplified from *M. persicae*, *A. pisum, M. cerasi* and *R. padi* cDNA, respectively. The StVPS52 and AtVPS52 coding sequences were amplified from *S. tuberosum* and *A. thaliana*, respectively. Amplicons were cloned into the pDONR207 vector (Invitrogen, Carlsbad, CA, U.S.A) using Gateway technology. BP recombination reactions were transformed in *Escherichia coli* JM109 (Promega). Constructs were verified by sequencing. MtVPS52 (NCBI Reference Sequence: XM_003590877.2) and HvVPS52 (GenBank: AK361277.1) were synthesized and cloned into pUC57 vector and provided including suitable Gateway recombination sites by GenScript (New Jersey, USA). 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 10xMyc tag)(Nakagawa et al., 2007) as destination vectors for transient over expression in *N. 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 (LB) medium with above-mentioned antibiotics, harvested, and resuspended in infiltration buffer (acetosyringone 125 µM and $MgCl_2$ 10mM) to reach an optical density at 600nm (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-immunoprecipitation assays

Plant tissue for co-immunoprecipitation (CO-IP) experiments was extracted with GTEN lysis buffer (10% Glycerol, 25mM Tris pH 7.5, 1mM EDTA, 150mM NaCl, 0.1% NP-40, with freshly added 10mM DTT and 1x protease inhibitor cocktail (Sigma, # P9599)). Samples were incubated for 15 min in lysis buffer at 4ºC. Lysate was centrifuged at 10,000g for 10 min and the supernatant was subjected to CO-IP with GFP-Trap®_M agarose beads (Chromotek, # gtm-20) for affinity binding of GFP-fused proteins. Western blotting was performed with the antibodies anti-GFP (# sc-8334, Santa Cruz Biotechnology Inc, USA), anti-FLAG (# sc-166384, Santa Cruz Biotechnology Inc, USA), and anti-c-Myc (# sc-40, Santa Cruz Biotechnology Inc, USA) followed by anti-rabbit-HRP (# sc-2004, Santa Cruz Biotechnology Inc, USA), or anti-mouse-HRP (# sc-2005, Santa Cruz Biotechnology Inc, USA) to detect the corresponding epitopes. Each CO-IP 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, 120mM Tris-Cl (pH 6.8) and 0.02% (w/v) bromophenol blue) and then subjected to western blotting procedures.

flg22 and *P. capsici* infection assays

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

***Myzus persicae* rearing**

Aphid experiments were done with *M. persicae* individuals, genotype O, reared on oil seed rape (*Brassica napus*) plants, with a long day (12h) day light and a photosynthetic photon flux density of 200 µml m^{-2} s^{-1} at 18ºC 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 one day after agroinfiltration using clip cages. The next day, adult aphids were removed and 3 1-st instar nymphs were left per infiltration site inside of a clip cage. Seven days after agroinfiltration, nymphs were transferred to newly infiltrated leaves, and aphid progeny was counted after 14 days 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 (MS) medium containing kanamycin. Seedlings were moved and potted into arabidopsis mix, and after 2 weeks under long-day conditions, aphid assays were performed. Two adult *M. persicae* aphids were placed on plants, and one day later adults were removed and 3 1-st instar nymphs were left on the rossette leaves. The total aphid number was counted 14 days after.

Aphid tissue preference test

Thirty alate aphids reared on oil seed rape plants were released inside a cage containing 4 flowering *Arabidopsis* plants of the ecotype Col-0. The aphid release point was approximately 8cm from the cage bottom so that it was positioned at about half the plant height. Twelve days after aphid release, total numbers of aphids were counted in rossette leaves and in stems and flower tissues. This experiment was repeated twice with similar results.

Histochemical localisation VPS52-GUS

Arabidopsis pok mutants were selected as described above and grown under long-day conditions. Flowering plants were placed in individual cages and challenged or unchallenged (control) with aphids for 12 days. Above ground plant tissues were collected and stained with 1mg/ml of 5-bromo-4-chloro-3-indolyl-B-D-glucuronic acid (X-gluc) (# R0852, Thermo Scientific, USA) in X-gluc buffer containing 100mM sodium phosphate buffer pH 7.0, 0.1% Triton X-100, 2mM potassium ferricyanide and 2mM 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 light microscopy. 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 (# 74104, Qiagen, Germany) following manufacturer instructions. cDNA was generated with M-MLV Reverse transcriptase (# M1701, Promega, USA) and samples analyzed by real-time PCR using QuantiTect SYBR® Green kit (# 204143, Qiagen, Germany). The primers were designed and analysed 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. EF1alpha was used as housekeeping gene to calculate relative expression using double delta Ct analysis.

Confocal imaging

Imaging was performed on a Leica TCS-SP2 AOBS (Leica Microsystems) using and HCX PL APO L x40/0.8 water dipping objective and on a Zeiss 710 confocal microscope using a Zeiss PL APO 40x/1.0 water dipping objective. Images were at 1024x1024 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. ImageJ (NIH) was used for post-acquisition image processing. The excitation wavelength for mRFP was 561nm, its emission was collected from 600 to 630nm. 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. Co-expressed mRFP and GFP as well as co-expressed 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 ProQuest™ Two-Hybrid system protocol (Invitrogen, USA). Transformants were plated onto yeast synthetic drop-out medium (Sigma) lacking Leu and Trp (-LW) and incubated at 30°C for 2 to 3 days. 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 uL 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 days before photographing. Positive interactions between the expressed proteins resulted in yeast growth and the activation of β-galactosidase in the X-gal membrane assay.

Competing interests

The authors declare that they have no competing interests.

Supplemental Material

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 colocalisation of VPS52 with Ara6 and Ara7.

Supplemental Figure S5. Mp1 does not relocalise to vesicle-like compartments upon co-expression with HvVPS52 or MtVPS52.

Supplemental Figure S6. Mp1-like effectors Ap1, Mc1 and Rp1 do not re-localise upon co-expression 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 *Nicotiana benthamiana*.

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

575 Supplemental Figure S9. Uncropped western blot images corresponding to figures in the
576 main manuscript.
577
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583 mutant line.
584
585 **Figure Legends**
586
587 **Figure 1. *Myzus persicae* effector Mp1 associates with host protein VPS52.** (A)
588 Confirmation of specific two-hybrid interactions in yeast between Mp1 and StVPS52 or
589 AtVPS52 through activation of various reporter genes. Control A represents a negative
590 control, and Control C represents a positive control. -LW represents double drop-out
591 medium lacking Leucine and Tryptophan; -LWH represents triple drop-out medium lacking
592 Leucine, Tryptophan and Histidine; 3AT is the abbreviation of 3-amino-1,2,4-triazole,
593 added to suppress the self-activation of HIS3 gene; X-Gal assay was used to asses the
594 activation of lacZ gene. (B) Co-immunoprecipitation of FLAG-Mp1 and FLAG-Ap1 with
595 GFP-StVP52 shows that Mp1, but not Ap1, interacts with StVPS52. Leaves of *Nicotiana*
596 *benthamiana* were infiltrated with *Agrobacterium* strains expressing different combinations
597 of GFP-VPS52 or GFP vector control (GFP-EV) with FLAG-Mp1 or FLAG-Ap1. Three days
598 after infiltration proteins were extracted and subjected to immunoprecipitation with GFP-
599 magnetic beads (IP:GFP) for western blotting with GFP or FLAG antibodies. Lower panel
600 indicates Rubisco stained with Ponceau S (PS) to show equal loading. - indicates absence
601 and + indicates presence of treatment according to upper left panel. This experiment was
602 repeated two times with similar results. The original blots from which images were cropped
603 are shown in Figure S9. (C) Co-immunoprecitations of FLAG-Mp1 and FLAG-Ap1 with
604 GFP-AtVPS52 shows that Mp1, but not Ap1, interacts with AtVPS52. Leaves of *N.*
605 *benthamiana* were infiltrated with *Agrobacterium* strains expressing different combinations
606 of GFP-AtVPS52 or GFP vector control (GFP-EV) with FLAG-Mp1 or FLAG-Ap1. Three
607 days after infiltration proteins were extracted and subjected to immunoprecipitation with
608 GFP-magnetic beads (IP:GFP) for western blotting with GFP or FLAG antibodies. Lower
609 panel indicates Rubisco stained with Ponceau S (PS) 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 Figure S9.

Figure 2. *Myzus persicae* effector Mp1 specifically associates with host but not poor-host VPS52s

(A) Co-immunoprecitations 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 *Nicotiana benthamiana* were infiltrated with *Agrobacterium* strains expressing different combinations of GFP-StVPS52 with Mp1variants or GFP vector control (GFP-EV). Three days after infiltration proteins were extracted and subjected to immunoprecipitation with GFP-magnetic beads (IP:GFP) for western blotting with GFP or FLAG antibodies. Lower panel indicates Rubisco stained with Ponceau S (PS) 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 Figure S8. (B) Co-immunoprecitations of FLAG-Mp1 with VPS52 variants St- (*Solanum tuberosum*), At- (*Arabidopsis thaliana*),Hv- (*Hordeum vulgare*) andMt-StVPS52 (*Medicago 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 (GFP-EV). Three days after infiltration proteins were extracted and subjected to immunoprecipitation with GFP-magnetic beads (IP:GFP) for western blotting with GFP or FLAG antibodies. Lower panel indicates Rubisco stained with Ponceau S (PS) 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 Figure S9.

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 *Nicotiana benthamiana* leaves transiently expressing GFP-*At*VPS52 in combination with subcellular marker CFP-PS1. Images were taken 3 days after agroinfiltration. Merged figures represent the overlay images of the GFP with CFP channels. Spatial plot profiles represent the colocalisation level across a defined region of interest (ROI) depicted in the merged images. Plot profiles were done with Fiji software (NHI). (B) Confocal microscopy images of *N. benthamiana* leaves transiently expressing GFP-*St*VPS52 in combination with subcellular marker CFP-PS1. Images were taken 3

days after agroinfiltration Merged figures represent the overlay images of the GFP with CFP channels. Spatial plot profiles represent the colocalisation level across a defined region of interest (ROI) depicted in the merged images. Plot profiles were done with Fiji software (NHI). (C) Colocalization of GFP-Mp1 with mRFP-StVPS52, mRFP-AtVPS52, or mRFP by confocal microscopy. Images were taken 3 days after agroinfiltration of *N. benthamiana* leaves. Merged figures represent the overlay images of the GFP and mRFP channels. Scale bars= 20 µm

Figure 4. *Myzus persicae* effector Mp1, but not Mp1-like effectors from other aphid species, promotes virulence on *Nicotiana benthamiana* upon phloem-specific overexpression

Leaves of *Nicotiana 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-day period. Ap1, Rp1, and Mc1, are the Mp1-like effectors from *Acyrthosiphon pisum*, *Rhopalosiphum padi*, and *Myzus cerasi*, respectively. Empty vector (EV) was used as a control. The graph shows the means of 4 independent replicates, where *n*=12 represent 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 standard error of the means and asterisks (***) indicates significant differences between treatments versus vector control (P<0.01).

Figure 5. StVPS52 reduce *Myzus persicae* virulence on *Nicotiana 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-day period. Empty vector (EV) was used as a control. Graphs represent the means of 3 independent replicates, where *n*=12 represents the number of samples per treatment in each replicate, and error bars represent the standard error of the means. Asterisks (*) indicate significant differences between treatment and the control (*t* test, P<0.05).

Figure 6. Aphid infestation causes degradation of VPS52. (*A*) *pok* mutant plants were infested with *Myzus persicae* for 5-7 days and collected for GUS-staining. Images were taken with a light microscopy. The experiment was repeated three times with similar

results. (B) *N. benthamiana* leaves transiently over-expressing GFP vector control (GFP-EV),GFP-StVPS52, GFP-AtVPS52 in combination with FLAG-Mp1 or infested with *M. persicae* for 3 days were collected for protein extraction and western blotting with anti-GFP and anti-FLAG antibodies. Lower panel indicates Rubisco stained with Ponceau S (PS) to show equal loading. - indicates absence and + indicates presence of treatment according to upper left panel. (C) *N. benthamiana* leaves transiently over-expressing GFP vector control (GFP-EV) and GFP-StVPS52 were challenged with *Phythophthora capsici* zoospores prior to protein extraction and western blotting with GFP antibodies. *N. benthamiana* leaves transiently over-expressing GFP vector control (GFP-EV) and GFP-StVPS52 were challenged with the PAMP flg22 prior to protein extraction and western blotting with GFP antibodies. Lower panel indicates Rubisco stained with Ponceau S (PS) to show equal loading. - indicates absence and + indicates presence of treatment according to upper left panel.

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 days after the release of alate adults. Graph represents the means of 2 independent replicates, where n=4 represents the number of plants in each replicate, and error bars represent the standard error of the means. Asterisks (*) indicate significant differences between rosette leaves and stems plus flowers tissues (paired t test, $P<0.05$).

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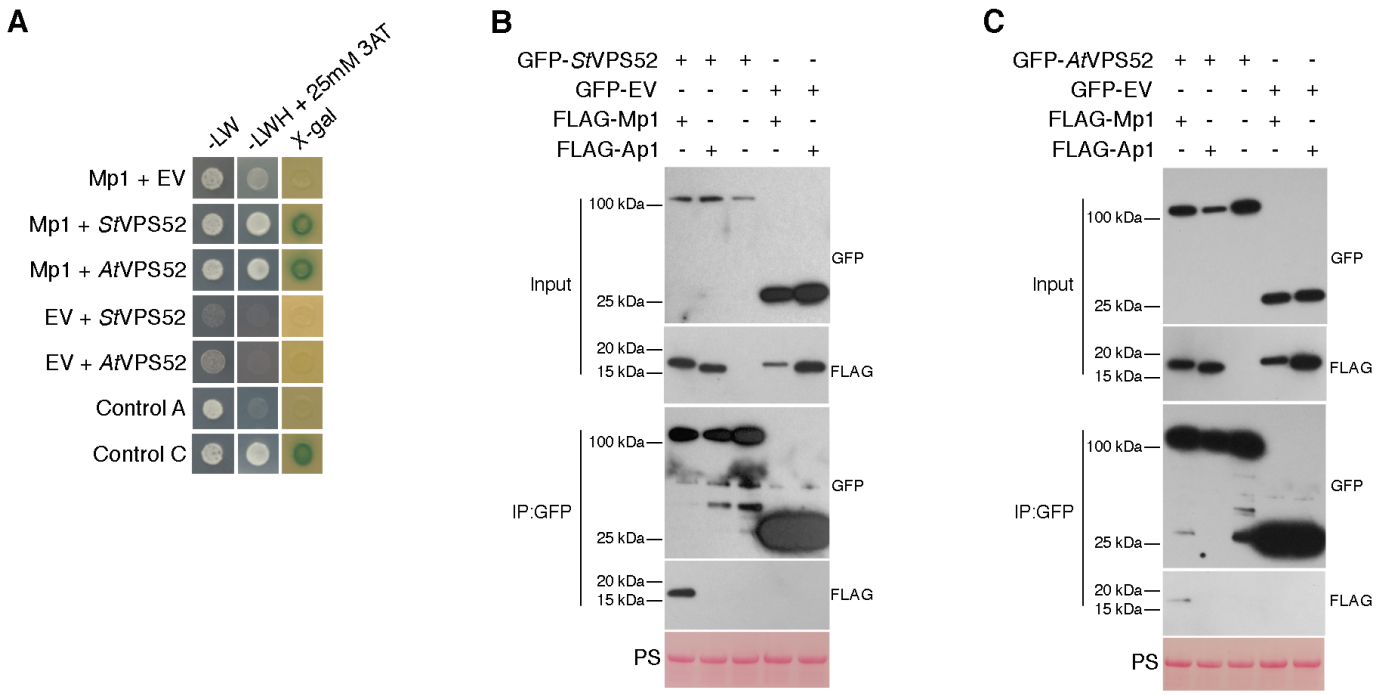

Figure 1.*Myzus persicae* effector Mp1 associates with host protein VPS52. (A) Confirmation of specific two-hybrid interactions in yeast between Mp1 and StVPS52 or AtVPS52 through activation of various reporter genes. Control A represents a negative control,and Control C represents a positive control. -LW represents double drop-out medium lacking Leucine and Tryptophan; -LWH represents triple drop-out medium lacking Leucine, Tryptophan and Histidine; 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 asses the activation of *lacZ* gene. (B) Co-immunoprecipitation of FLAG-Mp1 and FLAG-Ap1 with GFP-StVP52 shows that Mp1, but not Ap1, interacts with StVPS52. Leaves of *Nicotiana benthamiana* were infiltrated with *Agrobacterium* strains expressing different combinations of GFP-VPS52 or GFP vector control (GFP-EV) with FLAG-Mp1 or FLAG-Ap1. Three days after infiltration proteins were extracted and subjected to immunoprecipitation with GFP-magnetic beads (IP:GFP) for western blotting with GFP or FLAG antibodies. Lower panel indicates Rubisco stained with Ponceau S (PS) 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 Figure S8. (C) Co-immunoprecitations 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 (GFP-EV) with FLAG-Mp1 or FLAG-Ap1. Three days after infiltration proteins were extracted and subjected to immunoprecipitation with GFP-magnetic beads (IP:GFP) for western blotting with GFP or FLAG antibodies. Lower panel indicates Rubisco stained with Ponceau S (PS) 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 Figure S9

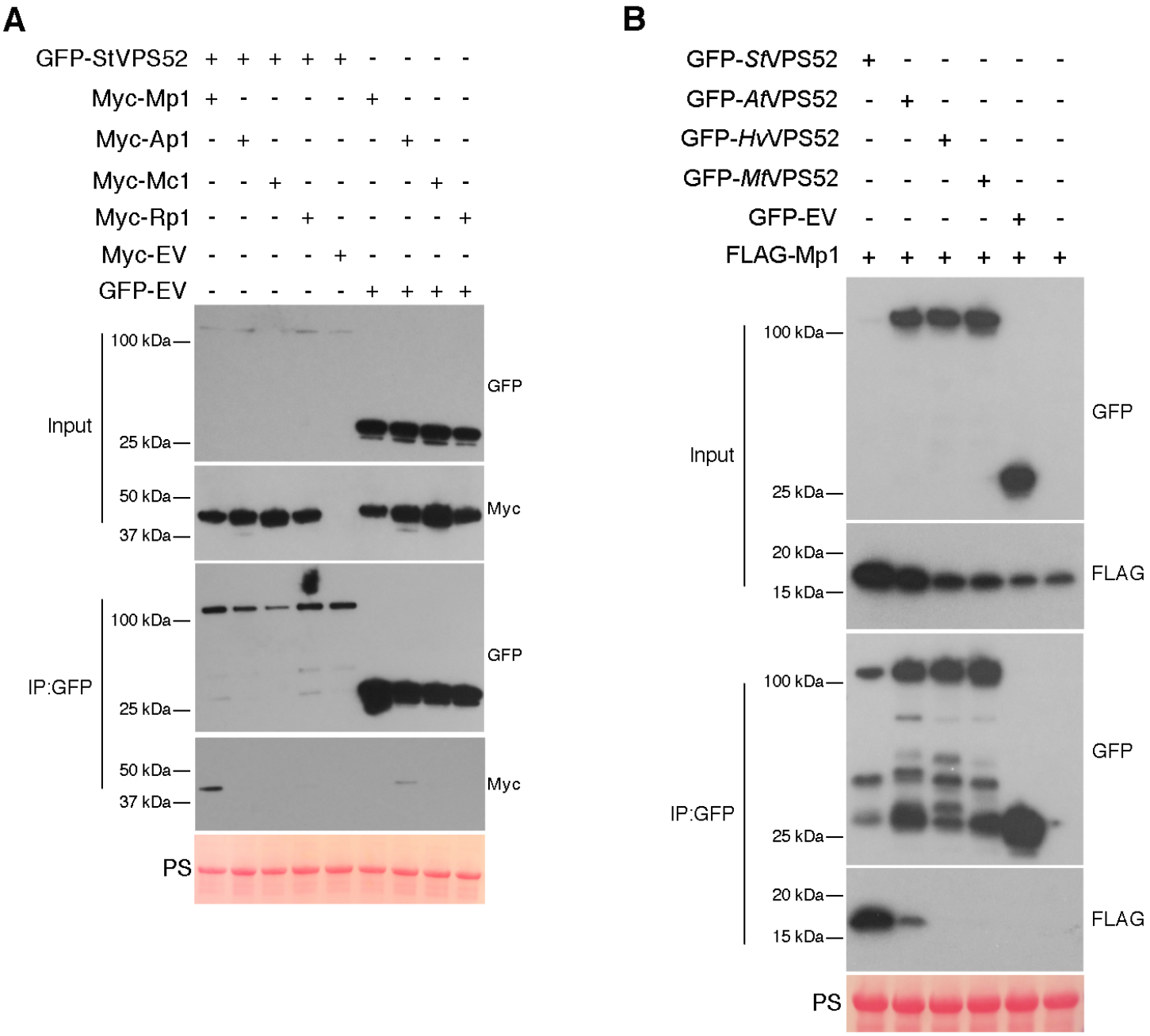

Figure 2. *Myzus persicae* effector Mp1 specifically associates with host but not poor-host VPS52s (A) Co-immunoprecitations of GFP-StVPS52 with Myc-Mp1, Myc-Ap1, Myc-Mc1 or Myc-Rp1 show that Mp1, but not Mp1-like variants, interacts with StVPS52 Leaves of *Nicotiana benthamiana* were infiltrated with Agrobacterium strains expressing different combinations of GFP-StVPS52 with Mp1variants or GFP vector control (GFP-EV). Three days after infiltration proteins were extracted and subjected to immunoprecipitation with GFP-magnetic beads (IP:GFP) for western blotting with GFP or FLAG antibodies. Lower panel indicates Rubisco stained with Ponceau S (PS) 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 Figure S8. (B) Co-immunoprecitations of FLAG-Mp1 with VPS52 variants St- (potato), At- (*Arabidopsis thaliana*),Hv- (*Hordeum vulgare*) and Mt-StVPS52 (*Medicago truncatula*) show that Mp1 interacts only with StVPS52 and AtVPS52. Leaves of *Nicotiana benthamiana* were infiltrated with Agrobacterium strains expressing different combinations of GFP-StVPS52 with FLAG-Mp1 or GFP vector control (GFP-EV). Three days after infiltration proteins were extracted and subjected to immunoprecipitation with GFP-magnetic beads (IP:GFP) for western blotting with GFP or FLAG antibodies. Lower panel indicates Rubisco stained with Ponceau S (PS) 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 Figure S9.

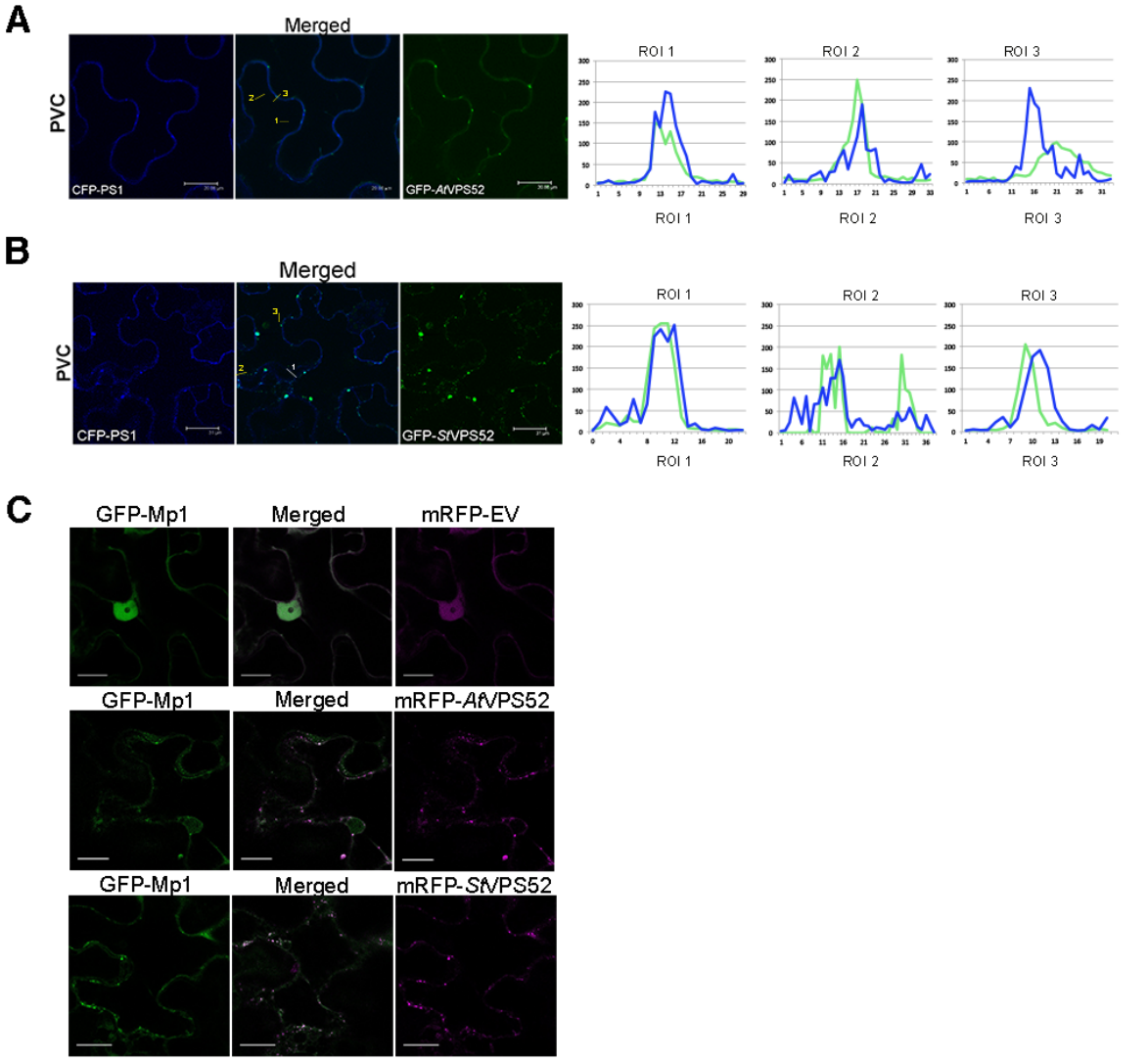

Figure 3. Overexpression of host VPS52s causes relocalization of Mp1 to vesicle-like structures that associate with the prevacuolar compartment (A) Confocal microscopy images of *Nicotiana benthamiana* leaves transiently expressing GFP-AtVPS52 in combination with subcellular marker CFP-PS1. Images were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

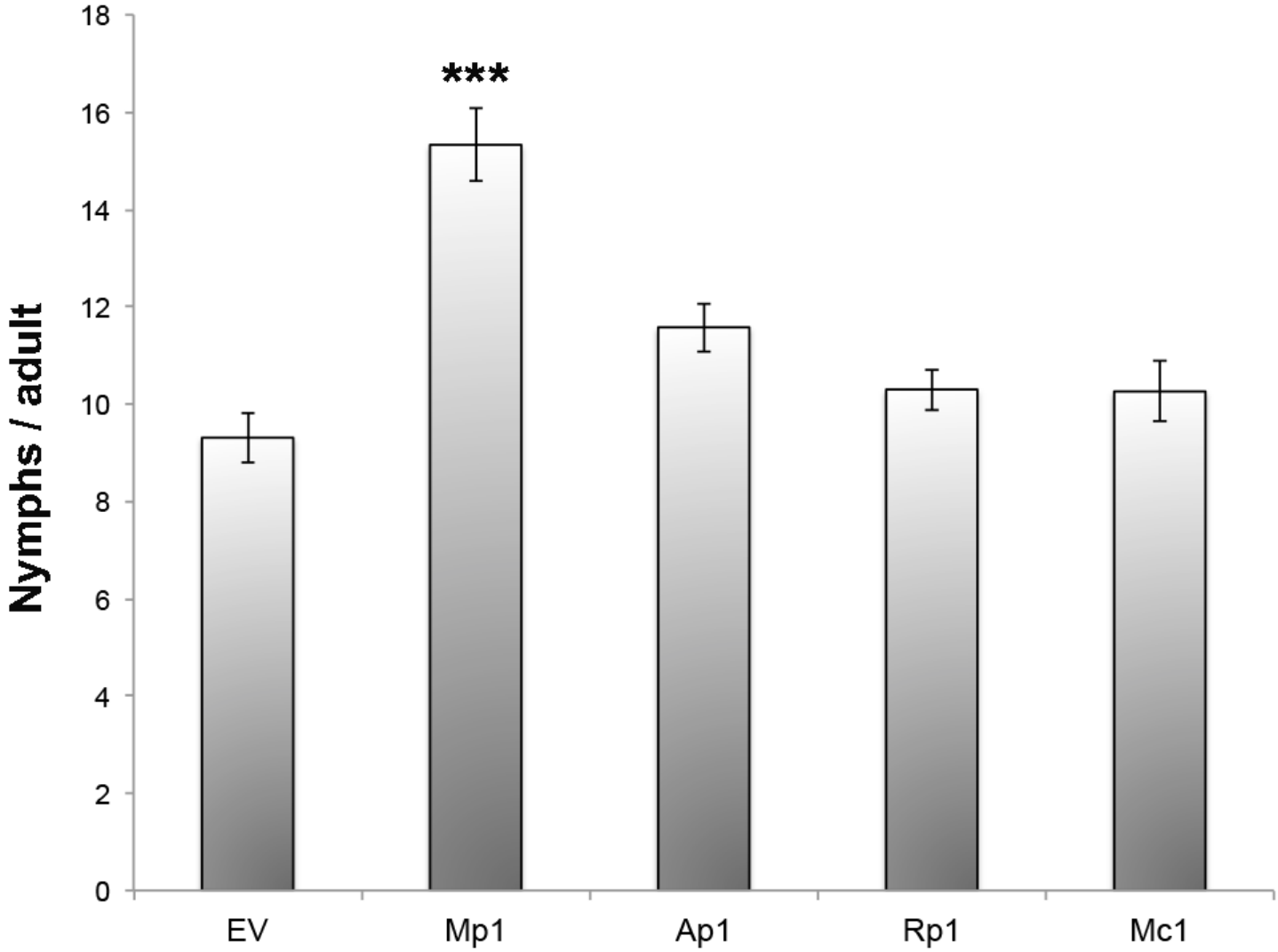

Figure 4. *Myzus persicae* effector Mp1, but not Mp1-like effectors from other aphid species, promotes virulence on *Nicotiana benthamiana* upon phloem-specific overexpression.

Leaves of *Nicotiana 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-day period. Ap1, Rp1, and Mc1, are the Mp1-like effectors from *Acyrthosiphon pisum*, *Rhopalosiphum padi*, and *Myzus cerasi*, respectively. Empty vector (EV) was used as a control. The graph shows the means of 4 independent replicates, where n=12 represent 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 standard error of the means and asterisks (***) indicates significant differences between treatments and the vector control (P<0.01).

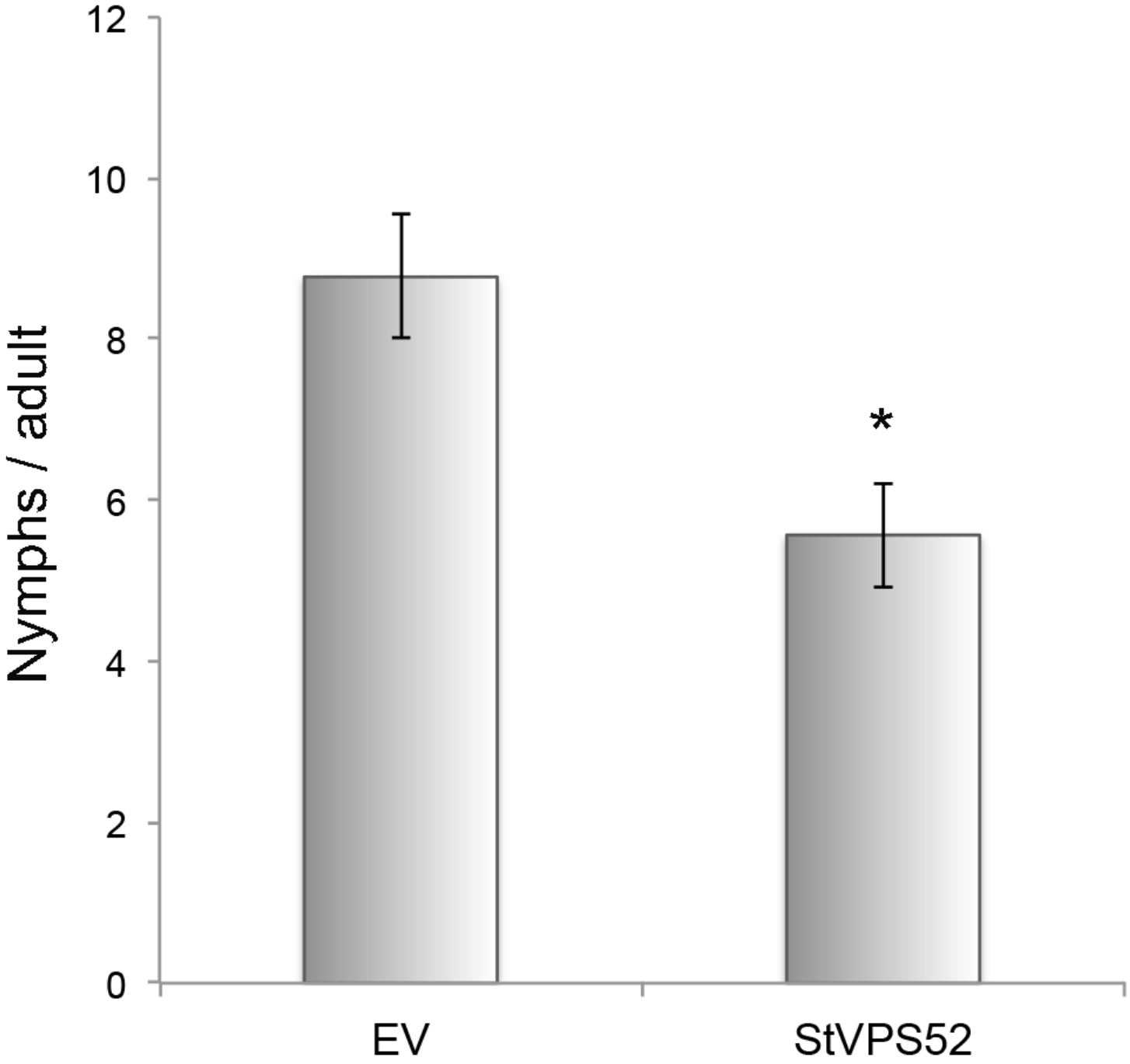

Figure 5. StVPS52 reduce *Myzus persicae* virulence on *Nicotiana benthamiana* upon phloem-specific overexpression.
Leaves of *N. benthamiana* transiently expressing StVPS52 (potato) under the phloem-specific AtSUC2 promoter were challenged with *M. persicae*. Aphid fecundity was assessed over a 14-day period. Empty vector (EV) was used as a control. Graphs represent the means of 3 independent replicates, where n=12 represents the number of samples per treatment in each replicate, and error bars represent the standard error of the means. Asterisks (*) indicate significant differences between treatment and the control (t test, $P < 0.05$).

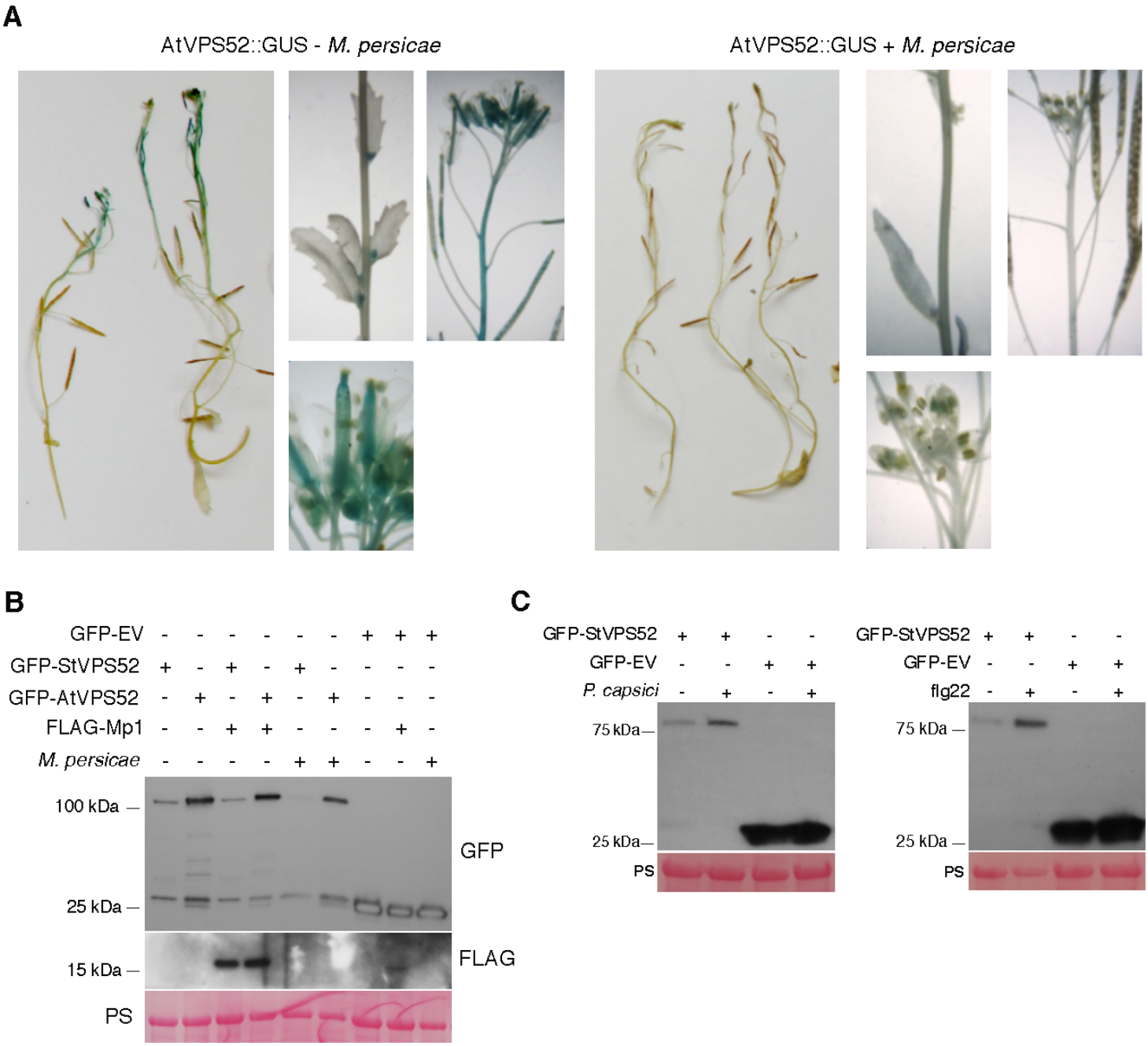

Figure 6. Aphid infestation causes degradation of VPS52. (A) pok mutant plants were infested with *Myzus persicae* for 5-7 days and collected for GUS-staining. Images were taken with a light microscopy. The experiment was repeated three times with similar results. (B) *Nicotiana benthamiana* leaves transiently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

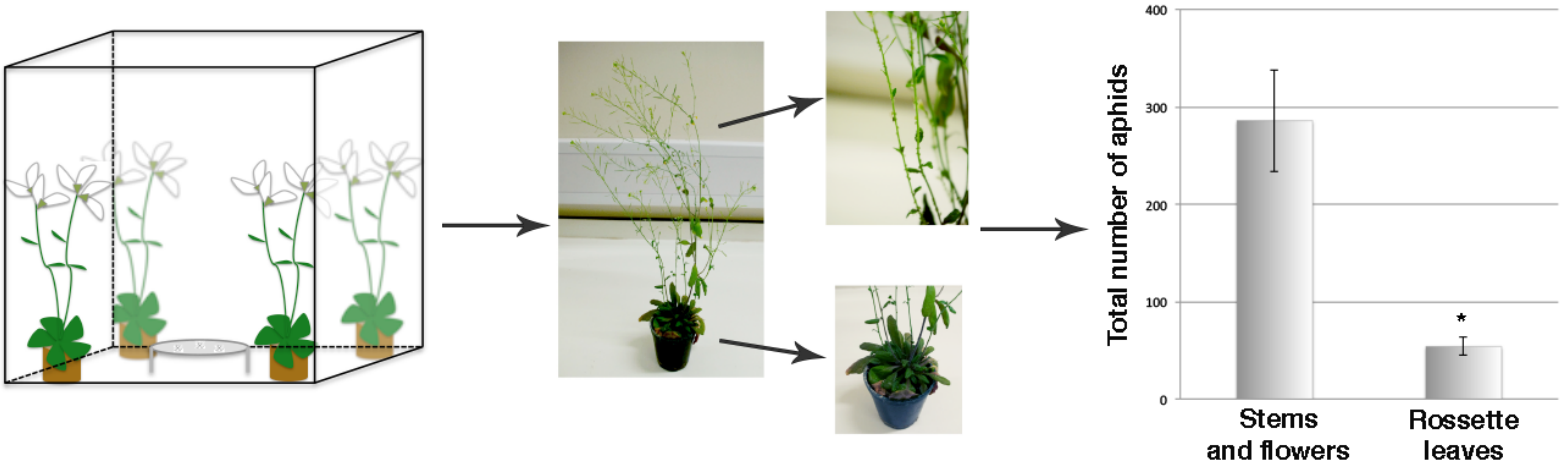

Figure 7. Aphids preferentially colonize Arabidopsis stems and flower tissues over rosette leaves. Aphid numbers on rosette leaves or stem and flower tissues of flowering plants 12 days after the release of alate adults. Graph represents the means of 2 independent replicates, where n=4 represents the number of plants in each replicate, and error bars represent the standard error of the means. Asterisks (*) indicate significant differences between rosette leaves and stems plus flowers tissues (paired t test, $P<0.05$).

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