A viral protease relocalizes in the presence of the vector to promote vector performance

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Vector-borne pathogens influence host characteristics relevant to host–vector contact, increasing pathogen transmission and survival. Previously, we demonstrated that infection with *Turnip mosaic virus*, a member of one of the largest families of plant-infecting viruses, increases vector attraction and reproduction on infected hosts. These changes were due to a single viral protein, Nla-Pro. Here we show that Nla-Pro responds to the presence of the aphid vector during infection by relocalizing to the vacuole. Remarkably, vacuolar localization is required for Nla-Pro’s ability to enhance aphid reproduction on host plants, vacuole localization disappears when aphids are removed, and this phenomenon occurs for another potyvirus, *Potato virus Y*, suggesting a conserved role for the protein in vector–host interactions. Taken together, these results suggest that potyviruses dynamically respond to the presence of their vectors, promoting insect performance and transmission only when needed.
Viruses dominate our planet, parasitizing all forms of life including other viruses. Because viruses are obligate parasites, they must encounter and then infect susceptible hosts to survive. After successful infection, transmission of a virus to the next host is a critical step for survival. Due to their limited mobility, many viruses, including ∼80% of all plant-infecting viruses, rely on other organisms for transmission. The specific organism that transmits a virus is known as a vector. Vectors are found among many different taxa, but for plant-infecting viruses they most often are hemipteran arthropods, and in particular aphids.

Because of their agricultural importance, plant viruses and their transmission have been the subject of research for more than a century. It has been documented that the yellow leaf colour and specific volatiles emitted by diseased plants modulate attractiveness for vectors (for review, see refs 5,6). In parallel, several studies have shown that viruses can suppress plant defences and thus vectors feeding on virus-infected plants often possess greater fitness than those residing on healthy plants (for review, see refs 5,7–10). These findings suggest that viruses may facilitate their own transmission by modulating host characteristics. Recently, it has been demonstrated that Cauliflower mosaic virus (CaMV) responds actively to the presence of its vector, changing transmission body morphs and increasing the potential for transmission. This new concept in virology has been called ‘perceptive viral behaviour’ and relies on the fact that viruses respond directly or via the host to potential vectors.

Turnip mosaic virus (TuMV) is considered to be one of the most damaging viruses of vegetable crops worldwide, infecting >300 species of dicotyledonous plants. TuMV is a member of the Potyviridae family and is non-persistently transmitted by >80 aphid species including Myzus persicae (green peach aphid). Its genome is a single ∼10 kb RNA molecule that codes for a large polyprotein plus the pipo frameshift protein. The polyprotein is cleaved into 1 coat protein and 10 non-structural products via 3 virus-encoded proteinases (VPg:NIa-Pro) during infection. In our study, NIa-Pro exists as a fully processed form and as a fusion with another TuMV protein, the viral genome-linked protein (VPg) during infection. NIa-Pro at the C terminus and is known as ‘NIa’ (Fig. 1a).

Previously, we demonstrated that TuMV NIa-Pro promotes aphid performance and suppresses plant defences. To confirm that the NIa-Pro protein versus its RNA sequence mediates increased aphid fecundity on host plants, we made a new construct that contains the full sequence of the NIa-Pro open reading frame but prevents production of the functional protein in a green fluorescent protein (GFP)-expressing vector. The construct introduced one extra thymine immediately after the first codon of the NIa-Pro open reading frame, resulting in a frameshift mutation that generates a stop codon directly after the first codon, thus preventing production of the functional protein (GFP:NIa-Pro:Fs; Supplementary Fig. 1a–c). To determine whether NIa-Pro protein production is critical for enhancing aphid performance, we performed fecundity tests on Nicotiana benthamiana transiently expressing GFP:NIa-Pro, GFP:NIa-Pro:Fs or the empty expression vector (free GFP). Consistent with our previous findings, expression of NIa-Pro increased aphid fecundity.

Results

The NIa-Pro protein is required to increase aphid fecundity. Previously we demonstrated that TuMV NIa-Pro promotes aphid performance and suppresses plant defences. To confirm that the NIa-Pro protein versus its RNA sequence mediates increased aphid fecundity on host plants, we made a new construct that contains the full sequence of the NIa-Pro open reading frame but prevents production of the functional protein in a green fluorescent protein (GFP)-expressing vector. The construct introduced one extra thymine immediately after the first codon of the NIa-Pro open reading frame, resulting in a frameshift mutation that generates a stop codon directly after the first codon, thus preventing production of the functional protein (GFP:NIa-Pro:Fs; Supplementary Fig. 1a–c). To determine whether NIa-Pro protein production is critical for enhancing aphid performance, we performed fecundity tests on Nicotiana benthamiana transiently expressing GFP:NIa-Pro, GFP:NIa-Pro:Fs or the empty expression vector (free GFP). Consistent with our previous findings, expression of NIa-Pro increased aphid fecundity.

Figure 1 | NIa-Pro relocates in the cell with aphids present.
(a) NIa-Pro can exist as a complex with another TuMV protein, the viral genome-linked protein (VPg) or in isolation during infection. In our study, only NIa-Pro was used. Images show confocal projections of agroinfiltrated N. benthamiana leaves with free GFP and GFP:NIa-Pro (b) without and (c) with aphids present. The first panel on the left shows GFP and GFP:NIa-Pro fluorescence in green. The second panel in the middle shows the nucleus of the cell in blue that was counterstained with DAPI. The third panel represents a merge of the first two panels, demonstrating GFP and GFP:NIa-Pro localization to the nucleus. The small insert panels represent single confocal sections. With the presence of aphids, GFP:NIa-Pro relocates in the cell, whereas free GFP does not. The nuclei are indicated with white arrows. Scale bars, 20 μm.
fecundity compared with aphids feeding on plants expressing the free GFP. Inhibiting production of the NIa-Pro protein in the NIa-Pro:Fs mutant led to no increase in aphid fecundity (Supplementary Fig. 1d). This result demonstrates that host plant changes mediating increased aphid fecundity are conferred by the NIa-Pro protein and not by its RNA sequence.

**NIa-Pro relocalizes in the cell with aphids present.** To analyse the intracellular localization of NIa-Pro, we transiently expressed NIa-Pro fused to GFP under the control of the 35S promoter (GFP:NIa-Pro) in *N. benthamiana* plants (Fig. 1a). As a control we used the same construct without NIa-Pro in transient expression experiments (free GFP). GFP:NIa-Pro localized to the nucleus and to the cytoplasm of the cell, similar to the free GFP (Fig. 1b). Nuclear localization was verified using 4,6-diamidino-2-phenylindole (DAPI) staining and by examining single sections on the confocal microscope (Fig. 1b, single sections shown in small panels). We next wanted to see if the presence of aphids on leaves alters NIa-Pro cellular localization because TuMV and NIa-Pro often occur with insect vectors in nature. To assess this, we allowed aphids to feed for 24 h on leaves transiently expressing free GFP or GFP:NIa-Pro. Surprisingly, aphids induced a relocalization of GFP:NIa-Pro in the cell, whereas free GFP localization did not change in the presence of the aphids (Fig. 1c).

**NIa-Pro relocalizes to the vacuole with aphids present.** GFP:NIa-Pro appeared to relocalize to the lumen of the vacuole in the presence of aphids. To confirm vacuolar localization, we stained the leaves with SNARF-1, a marker for the vacuolar lumen. As expected, when aphids were present GFP:NIa-Pro co-localized with the vacuole, whereas the free GFP control did not (see single sections; Fig. 2a,b). To further confirm localization to the lumen of the vacuole, we used a second marker, the spRFP:AFVY fusion protein. Using this construct, GFP:NIa-Pro also colocalized with the vacuole when aphids were present, whereas the free GFP did not (see single sections; Fig. 2c,d). We next wanted to confirm that the phenotypes observed were not due to autofluorescence of dead cells using time-lapse imaging. We captured active trafficking of the free GFP control (cytoplasmic streaming) and the GFP:NIa-Pro (intra-vacuolar trafficking) verifying the cells were still alive (Supplementary Fig. 2). Previous studies have demonstrated that the GFP protein can be visualized in the plant vacuole. Nevertheless, in a last control, we excited the GFP:NIa-Pro at 488 nm and performed a scan across 380–800 nm. We observed that the phenotype observed plus or minus aphids were characteristic of the GFP emission (Supplementary Fig. 3). The scan revealed some chloroplast autofluorescence around 650 nm of emission.

**Vacuolar localization disappears when aphids are removed.** To determine the kinetics of NIa-Pro relocalization, we next allowed aphids to feed for different durations of time (2, 6 and 24 h) on leaves transiently expressing free GFP or GFP:NIa-Pro. After 2 h of aphid feeding, no relocalization was observed in either treatment, suggesting that insect probing may not be enough to induce the response (Fig. 3a, second panel). However, after 6 h of aphid feeding, cytoplasmic vesicles were observed in plants expressing GFP:NIa-Pro, while there was no change in phenotype for free GFP (Fig. 3a, third panel). After 24 h of aphid feeding, ~60% of the visible epidermal cells exhibited relocalization of NIa-Pro throughout the vacuole lumen (Fig. 3a, fourth panel; Fig. 3b). The large numbers of cells exhibiting the phenotype after 24 h of feeding suggest that the response is not restricted to cells probed by the aphids, but is instead mediated by a systemic signal. In parallel experiments, aphids were allowed to feed for 24 h and then removed. Leaves were allowed to recover for 2 h before microscopic imaging. Surprisingly, vacuolar localization of GFP:NIa-Pro was no longer observed 2 h after aphid removal (Fig. 3a, right panel), suggesting that GFP:NIa-Pro was able to relocalize to the nucleus or was degraded in the vacuole after aphids were removed. Quantification of three independent experiments established that aphid presence significantly modifies localization of GFP:NIa-Pro and nuclear localization is restored when aphids are removed (Fig. 3b).

Because free GFP is small enough to freely diffuse through the nuclear pore, we could not discount the fact that the GFP observed in the nucleus in the GFP:NIa-Pro experiments could be GFP cleaved from NIa-Pro. To verify that the GFP from the GFP:NIa-Pro fusion is not cleaved, we performed a western blot with a GFP antibody using the agroinfiltrated *N. benthamiana* leaves expressing free GFP, GFP:NIa-Pro with and without aphid feeding and after reversion (Fig. 3c). We found that GFP:NIa-Pro is not cleaved (Fig. 3c). Because GFP:NIa-Pro is ~54 kD and too large to diffuse into the nucleus, our data suggest that the nuclear accumulation of GFP:NIa-Pro is due to a property of NIa-Pro and not just because of its small size, like the free GFP.

**Relocalization is required to increase aphid fecundity.** To determine the functional significance of NIa-Pro relocalization in the presence of aphid vectors, we created two NIa-Pro localization mutants. For one mutant, we confined NIa-Pro to the nucleus by adding a strong nuclear localization signal (NLS; PKKRRKVV) and for the other, we inhibited nuclear localization by adding a nuclear export signal (NES; NELALKLAGLDINK) to the C-terminal end of NIa-Pro. Localization
was verified using transient expression, confocal microscopy and DAPI staining (Fig. 4a–d; Supplementary Fig. 4). Localization of the GFP control and GFP:NIa-Pro was consistent with previous experiments with and without aphids (Fig. 4a,b). In contrast, GFP:NIa-Pro-NLS localized exclusively to the nucleus with and without aphids (Fig. 4c). For the GFP:NIa-Pro:NLS construct, some GFP:NIa-Pro:NLS still localized to the nucleus before aphid presence, yet in the presence of the aphids there was a greater number of cells with GFP:NIa-Pro:NES in the vacuoles compared with plants expressing GFP:NIa-Pro (Fig. 4d).

Next, we allowed aphids to feed and recorded callose deposition on plants transiently expressing the empty vector (free GFP), GFP:NIa-Pro, GFP:NIa-Pro-NLS and GFP:NIa-Pro:NES. Consistent with our previous findings, callose induction by aphids was significantly reduced in the leaves expressing NIa-Pro compared with free GFP (Fig. 4e). Interestingly, aphid-induced callose was not reduced in plants expressing GFP:NIa-Pro-NLS, whereas in plants expressing GFP:NIa-Pro:NES, aphid-induced callose deposition was still inhibited (Fig. 4e). These results suggest that the presence of NIa-Pro outside of the nucleus is required to decrease plant defenses. We also recorded aphid fecundity on plants expressing the NIa-Pro localization mutants and controls. Similar to our previous findings, aphid fecundity was significantly higher on plants transiently expressing GFP:NIa-Pro compared with the free GFP (Fig. 4f; refs 9,22). In contrast, aphids were not more fecund on plants expressing GFP:NIa-Pro confined to the nucleus as compared with the control (Fig. 4f; GFP:NIa-Pro-NLS).

NIa-Pro relocalizes during actual viral infection. To test whether NIa-Pro behaves similarly during actual viral infection, we expressed the GFP:NIa-Pro fusion in the context of the TuMV genome using an infectious clone. To do this, we used the TuMV backbone pCambia/TuMV/6k2:GFP33 and replaced the 6k2:GFP with GFP:NIa-Pro (Fig. 5a). To verify that the new construct also increases aphid fecundity similar to the unmodified TuMV/GFP9, we performed aphid fecundity experiments with the TuMV/GFP:NIa-Pro. Consistent with our previous findings, TuMV/GFP:NIa-Pro also increased aphid fecundity compared with controls (Fig. 5b; refs 9,22). A western blot analyses was performed with an antibody specific to the TuMV coat protein to verify the infectivity of the new mutant, TuMV/GFP:NIa-Pro (Fig. 5c). We next examined subcellular localization in the viral context using confocal microscopy. During infection, GFP:NIa-Pro also localized to the nucleus and cytoplasm without aphids and relocalized to the vacuole in the presence of the aphid vectors (Fig. 5d). Quantification of three independent experiments using TuMV/GFP:NIa-Pro established that aphid presence significantly induces GFP:NIa-Pro relocalization in the viral context (Fig. 5e).

Next, we wanted to further confirm vacuole localization using the TuMV/GFP:NIa-Pro infectious clone, vacuole purification and western blotting. We first purified vacuoles from leaves expressing the empty vector or TuMV/GFP:NIa-Pro with or without aphids present. Next, total protein isolated from vacuoles was analysed by western blot using a GFP antibody. Consistent with the microscopy work, GFP:NIa-Pro was only found in vacuoles purified from leaves with aphids present (Fig. 5f). As a

Figure 3 | NIa-Pro localization to the vacuole occurs only when aphids are given access to plants. (a) Images show confocal projections of N. benthamiana agroinfiltrated leaves with free GFP (top panel) or GFP:NIa-Pro (lower panel). Separate leaves were infested with aphids for 2, 6 or 24 h (middle panels) and a set of leaves were left uninfested as a control (left panel). For the reversion (right panel), all aphids were removed after 24 h of infestation, and the leaves were observed after a 2 h recovery period. GFP:NIa-Pro relocalizes from the nucleus (white arrows) to form cytoplasmic aggregates after 6 h (dashed arrows) and then diffuses into the vacuole after 24 h (dashed arrow). When the aphids were removed, GFP:NIa-Pro was observed in the nucleus again (white arrow). The free GFP control does not show relocalization with any treatments. (b) The percentage of cells with GFP in the cytoplasm for each of the treatments. (c) The image represents a western blot from leaf extracts expressing free GFP or GFP:NIa-Pro before aphid infestation, after 24 h of aphid infestation or after the 2 h recovery period (reversion), and incubated with a GFP antibody. The western blot shows that the phenotype in the presence of aphids and after reversion is not due to the cleaved GFP. (mean ± s.e. of N = 6, letters represent significant differences, ANOVA and Tukey’s honest significant difference post hoc, P < 0.05). Scale bars, 20 µm.
NIa-Pro relocalization is insect dependent. We next wanted to determine whether GFP:NIa-Pro also relocalizes in the presence of other piercing-sucking insects. To address this, we examined GFP:NIa-Pro relocalization in the presence of two distinct phloem-feeding insects: the beet leafhopper *Circulifer tenellus* and the silverleaf whitefly, *Bemisia tabaci* biotype A. Like aphids, whiteflies feed by moving their stylets intercellularly on the way to the phloem causing minimal cell damage. However, whiteflies generally make fewer intracellular probes before reaching the phloem and thus feeding is not equivalent to aphids. Leafhoppers, on the other hand, have larger mouthparts compared with aphids and whiteflies. Because of this, leafhoppers generally damage many cells on their intracellular pathway to the phloem. Some relocalization of GFP:NIa-Pro to the vacuole was observed in the presence of leaffoppers and whiteflies (Fig. 6e). This suggests relocalization is not correlated with feeding damage, as whiteflies cause less damage and leaffoppers cause more damage during feeding as compared with aphids. Although previous studies have demonstrated that both whiteflies and leaffoppers can transmit viruses to *N. benthamiana* plants, we next wanted to confirm insects were probing in our experiments. To address this, we collected the leaves that aphids, whiteflies and leaffoppers were confined to and stained them with acid fuchsin. Staining with acid fuchsin allowed us to visualize the saliva sheaths and verify plants were probed (Supplementary Fig. 5).

The function of NIa-Pro is conserved. As all potyvirus genomes encode NIa-Pro, we next wanted to determine whether this is a more generalized phenomenon. To address this, we examined the impact of another potyvirus, *Potato virus Y* (PVY), and expression of its NIa-Pro in host plants on aphid vector performance. Plants infected with PVY increased aphid fecundity compared with controls, similar to TuMV infection (Fig. 6a; refs 9,22). Perhaps, more interesting plants transiently expressing GFP:NIa-ProPVY also increased aphid fecundity compared with aphids feeding on controls (Fig. 6b). Next, we examined NIa-ProPVY localization using GFP and confocal microscopy. Similar to the phenomenon observed with GFP:NIa-ProTuMV, GFP:NIa-ProPVY also localized to the nucleus without aphids and to the vacuole with aphids present (Fig. 6c). Quantification of three independent experiments demonstrated that aphid presence significantly modifies GFP:NIa-ProPVY localization (Fig. 6d). This observation suggests NIa-Pro may have a conserved function for the genus *Potyvirus*, representing over 30% of all described plant viruses.

NIa-Pro relocalization is insect dependent. Although numerous studies have shown viruses can manipulate plant metabolism and promote vector performance, there is wide variation in response both within and across plant species. Previous studies have shown that NIa-Pro increases aphid performance on *Arabidopsis thaliana* in addition to *N. benthamiana*9,22, suggesting that this

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**Figure 4 | Relocalization of NIa-Pro is required to increase aphid fecundity.** Images show confocal projection of *N. benthamiana* leaves agroinfiltrated with (a) free GFP, (b) GFP:NIa-Pro, (c) GFP:NIa-Pro:NLS (NLS, nuclear localization signal) and (d) GFP:NIa-Pro:NES (NES, nuclear export signal) with or without aphids. (e) Callose deposition in agroinfiltrated *N. benthamiana* leaves with free GFP, GFP:NIa-Pro, GFP:NIa-Pro:NLS and GFP:NIa-Pro:NES with or without aphids infestation. The NLS mutant does not reduce aphid-induced callose accumulation. (f) Number of progeny produced by aphids on *N. benthamiana* agroinfiltrated leaves with free GFP, GFP:NIa-Pro, GFP:NIa-Pro:NLS and GFP:NIa-Pro:NES. (mean ± s.e. of N = 10 for e and N = 8-12 for f; letters represent significant differences, ANOVA and Tukey’s honest significant difference post hoc, P < 0.05). Results from one of three independent experiments are displayed. Scale bars, 20 μm.
Figure 5 | Nla-Pro relocalizes to the vacuole in the presence of aphids during virus infection. (a) Schematic representation of the recombinant TuMV-expressing GFP:Nla-Pro. Each rectangle represents individual proteins of the TuMV polyprotein. (b) Number of progeny produced by aphids on N. benthamiana infiltrated with empty agrobacterium (EV), TuMV/GFP or TuMV/GFP:Nla-Pro. (c) The western blot depicts a western blot from leaf extracts expressing empty vector (EV), TuMV/GFP and TuMV/GFP:Nla-Pro incubated with an antibody that binds to the coat protein (CP) of TuMV. (d) Images show confocal projection and a single section (on the right) of N. benthamiana leaves agroinfiltrated with TuMV/GFP:Nla-Pro with or without aphids for 24 h. GFP:Nla-Pro relocalizes from the nucleus (white arrow) to the vacuole (V; dashed arrow) in the presence of aphids. (e) The percentage of cells with GFP in the vacuole for N. benthamiana infected with TuMV/GFP:Nla-Pro with and without aphids. (f) The image represents a western blot from vacuole purification derived from leaves expressing EV or TuMV/GFP:Nla-Pro before or after aphid presence and incubated with a GFP antibody and an anti TuMV-CP antibody. The western blot shows that GFP is only visible in the vacuoles from leaves expressing TuMV/GFP:Nla-Pro and after aphid presence. (mean ± s.e. of N = 12–20 for b and N = 6 for e; letters and stars represent significant differences, ANOVA and Tukey’s honest significant difference post hoc for b or Student’s t-test for e, P < 0.05). Scale bars, 20 μm.

Figure 6 | The function of Nla-Pro in virus-plant-aphid interactions is conserved. (a) The number of progeny produced by aphids on healthy (mock) or PVY-infected N. benthamiana. (b) The number of progeny produced by aphids on N. benthamiana agroinfiltrated with empty agrobacterium (EV) or expressing GFP:Nla-Pro from PVY. (c) Images show confocal projection and single section (on the right) of N. benthamiana leaves agroinfiltrated with GFP:Nla-Pro from PVY with or without aphids for 24 h. GFP:Nla-Pro relocalizes from the nucleus (white arrow) to the vacuole (dashed arrow) in the presence of aphids. (d) The percentage of cells with GFP in the vacuole for N. benthamiana expressing GFP:Nla-Pro from PVY with and without aphids. (e) The percentage of cells with GFP:Nla-Pro in the vacuole for each of the treatments: agroinfiltrated with PVY with or without aphids. (mean ± s.e. of N = 24–27 for a, N = 6–9 for b, N = 6 for d and N = 3 for e; stars and letters represent significant differences, Student’s t-test, P < 0.05 for a–d, and ANOVA and Tukey’s honest significant difference post hoc for e, P < 0.05). Results from one out of two independent experiments are displayed in a, b, and from three independent experiments in d. Scale bars, 20 μm.

GFP:Nla-Pro was observed in the vacuole, whereas for free GFP there was no relocalization (Fig. 7a).

Next, we examined insect performance on Nicotiana tabacum plants infected with TuMV, PVY or expressing the corresponding Nla-Pro. In contrast to N. benthamiana and Arabidopsis plants22, aphids were not more fecund on infected N. tabacum plants compared with controls (Fig. 7b). Furthermore, aphids did not have enhanced fecundity on N. tabacum plants expressing GFP:Nla-ProTuMV or GFP:Nla-ProPVY compared with the appropriate controls (Fig. 7b). We next conducted confocal microscopy experiments using transient expression in N. tabacum to determine whether GFP:Nla-Pro is still able to relocalize to the vacuole. In contrast to the other host plants, GFP:Nla-ProTuMV may be a general response across plants. To determine whether Nla-Pro also relocalizes in Arabidopsis in the presence of aphids, we generated transgenic plants expressing free GFP or GFP:Nla-Pro as previously described40. Consistent with the N. benthamiana experiments, after 24 h of aphids infestation
and GFP:Nla-Pro\textsubscript{PVY} did not relocalize in \textit{N. tabacum} with aphids present (Fig. 7c,d), further supporting the role of relocalization in increased aphid fecundity and changes in plant metabolism. A western blot demonstrated that the expression level was similar for free GFP, GFP:Nla-Pro\textsubscript{TuMV} or GFP:Nla-Pro\textsubscript{PVY} in \textit{N. tabacum} and in \textit{N. benthamiana} (Fig. 7e). These observations suggest Nla-Pro must respond to the presence of the vector using a host plant-derived signal.

**Discussion**

Our results demonstrate that TuMV initiates host changes in response to vector presence, promoting insect performance and the likelihood of transmission at the most opportune moment. We show that Nla-Pro from TuMV relocalizes to the vacuole in the presence of insect vectors on infected hosts (Figs 1c,2,3a,b and 5d–f). Further, we demonstrate that relocalization is essential for Nla-Pro’s ability to inhibit plant defenses and increase vector fecundity on host plants (Fig. 4e,f). The large number of cells observed with the relocalization phenotype suggests Nla-Pro responds to a systemic signal that moves throughout the leaf from the cells the aphid come in contact with. Almost twice as much Nla-Pro relocalization was observed in the presence of aphid vectors as compared with non-vector insects (Fig. 6e). Because Nla-Pro relocalization did not correlate with insect damage (that is, whiteflies cause less damage and leafhoppers cause more damage compared with aphids; Fig. 6e), the systemic signal may be only partially due to the physical damage caused during insect probing. This suggests vector-specific factors may also contribute to the systemic signal recognized by Nla-Pro, such as salivary secretions, or other aphid-derived material. Our results also demonstrate that Nla-Pro from PVY, another potyvirus, has the
same properties (Fig. 6), suggesting a conserved role for the protein in vector–host interactions. Finally, we demonstrate that the nature of the host is very important for relocalization of NLa-Pro and the impact of viral infection on host–vector interactions, shedding light on new aspects that may determine viral host range and epidemiology in nature.

Previous studies have reported that animal and plant viruses can alter host physiology and host–vector interactions in ways that are predictive of enhanced transmission. In these studies, aphid-induced changes in CaMV transmission morphs increased the likelihood of transmission by the insect vector. Our findings are unique compared with previous studies with CaMV and viral 'recognition' of aphid vectors as CaMV proteins only responded in cells that were directly damaged by the aphids during the probing punctures. Our study may also show a slightly different mechanism, where it is not the virus recognizing the vector and initiating host changes for enhanced transmission but possibly the aphid manipulating the viral protein to suppress aphid defenses. Although the exact mechanisms are still unknown, it may be a 'perceptive insect behaviour' instead of a 'perceptive viral behaviour'.

Aphids move their needle-like mouthpart, the stylect, intercellularly to the phloem and puncture multiple cells before feeding (for review, see ref. 6). Because most plant cells have one large vacuole that occupies up to 90% of the cell volume, aphids may encounter vacuoles often during probing. The vacuole has numerous functions, including storage of metabolic products known to be involved in plant defence against microbial pathogens and herbivores. Vacular localization of NLa-Pro during insect presence might be important for inhibition of aphid-induced plant signals or defenses known to be stored in the vacuole. Previous studies have shown that NLa-Pro has protease activity and DNAse activity. As a protease, NLa-Pro could degrade proteins in the vacuole directly involved in plant defence or known to activate other molecule in defence pathways. Recently, a study has shown that TuMV particles and HC-Pro, another TuMV protein, also accumulate in the vacuole. HC-Pro is involved in aphid transmission, although the role of vacuolar localization in transmission remains unknown.

This is also the first evidence that NLa-Pro can localize into the nucleus without VPg. Previous studies have demonstrated that VPg encodes a NLS (Fig. 1a), mediating VPg-Nla-Pro accumulation in the nucleus, although its function in the nucleus remains unclear. It was previously suggested that once NLa-Pro is liberated from Nla, it might diffuse into the cytoplasm, as it is only 27 kD (ref. 20). Here we demonstrate that VPg is not essential for nuclear localization. Interestingly, NLa-Pro does not have any known NLSs, suggesting an unknown signal exists or that it uses a different mechanism to move in and out of the nucleus.

Vector-borne pathogens account for >17% of all infectious diseases worldwide, contributing to reductions in agricultural productivity, disrupted ecosystem services and over 1 million deaths each year. Viral pathogens specifically are the aetiologic agents of 5 of the 10 most important vector-borne diseases worldwide, in terms of impact on mortality. Surprisingly, very few studies using animal or plant viruses have examined the impact of viruses in combination with their vectors on hosts. The phenomenon described here not only opens up new areas of research for plant virology but also poses new questions for vector-borne viruses of humans and other animals. For example, mosquito vectors preferentially feed on hens infected with an arbovirus compared with control hens and lambs infected with Rift Valley fever virus are fed on more frequently by the mosquito vectors compared with uninfected animals. These studies suggest host traits that influence transmission dynamics also change in animals systems after infection, but the mechanisms mediating these changes remain unknown. These studies and ours are contributing to a rapidly changing image of virus transmission and in particular the host–virus–vector relationship, which is more complex than expected.

Methods

Plants and growth conditions. Nicotiana benthamiana and Arabidopsis seeds were originally obtained from Georg Jander (Boyece Thompson Institute, Ithaca, NY). Nicotiana tabacum cv. Glurck were obtained from Bryce Falk (University of California, Davis, CA). Plants were grown in growth chambers under controlled conditions (25/20 °C day/night with a photoperiod of 12/12 h day/night).

Aphids. Non-viruliferous aphid clones of a tobacco-adapted red strain of Myzus persicæ were reared under controlled conditions (25/20 °C day/night with a photoperiod of 12/12 h day/night) on N. tabacum. Aphids were originally obtained from Georg Jander (Boyece Thompson Institute, Ithaca, NY).

Virus infection. TuMV/GFP was propagated from the infectious clone p35:TuMV/GFPF3 by agroinfiltration as described in the 'Protein expression in Nicotiana spp' section below. PVY-O was obtained from Stewart Gray at Cornell University, Ithaca, NY and propagated by rub-inoculation. To prepare inoculum, fully infected N. benthamiana leaves were collected 3 weeks after inoculation and weighed. Leaves were then ground in two volumes 20 mM phosphate buffer (pH 7.2). For experiments, two leaves per plant were dusted with carborundum (Sigma-Aldrich, St Louis, MO) and rub-inoculated with the virus sap using a cotton-stick applicator. A corresponding set of control plants were dusted with carborundum and mock-inoculated with a cotton-stick applicator that was soaked in uninfected N. benthamiana sap in 20 mM phosphate buffer.

Aphid fecundity. One aperiodic adult aphid was placed on the underside of a fully infected or agroinfiltrated N. benthamiana leaf and confined in a plastic clip cage (2 cm diameter). After 24 h, all aphids except one nymph were removed. After 7–9 days, depending on the experiment, the progeny of this single nymph were counted to determine fecundity.

Plasmid constructs. The constructs were produced using the Gateway cloning kit (Invitrogen, Carlsbad, CA, USA, http://www.lifetechologies.com) following the manufacturers instructions. In brief, PCR products were amplified using gene-specific primers for each construct flanked by the attB1 and attB2 universal primers (Supplementary Table 1). The PCR products were cloned into the pDONR207 vector using BP clonase and then they were re-cloned into the pSITE:GFP destination vector or the pTA7001destination vector, using LR clonase. To construct the localization mutants, the NLS from SV40 (PKKKRKV) and the consensus NES (NELALKLAGLDINK) sequence were added in the primers. The TuMV/GFP:NLa-Pro construct was created by amplifying a PCR product with pSITE-GFP:Nla-Pro as the template using oligonucleotides containing a SacII restriction site in TuMV/6K2:GFP after removal of the corresponding restriction endonuclease site in TuMV/6K2:GFP after removal of 6K2:GFP and dephosphorylation.

Protein expression in Nicotiana spp. Constructs containing the coding sequence for the proteins fused to gfp were introduced into Agrobacterium tumefaciens GV3101 by heat shock and selected on LB plus 10 µg ml−1 of rifampicin and 50 µg ml−1 of spectinomycin for the pSITE plasmid or plus 10 µg ml−1 of rifampicin and 50 µg ml−1 of kanamycin for PCAMBIA and spRF EffPVY. One fresh colony was selected and grown overnight in liquid culture. The pellet of the culture was resuspended in 10 mM MES pH 5.85; 10 mM MgCl₂ and 150 mM acetosyringone and left at room temperature for 2 h. The solution was then diluted to an optical density of 0.1 at 600 nm for transient expression experiments and at 0.3 for the infectious clones. Single leaves from 3-week-old N. benthamiana on N. tabacum plants were then agroinfiltrated with the solution. For N. benthamiana, entire leaves were infiltrated. For N. tabacum, a circle of ∼5 cm diameter was infiltrated for each leaf. Plants were used in experiments 2 days after infiltration.
Expression was verified by microscopy, PCR and/or western blot as previously described. 

Transgenic Arabidopsis. The coding sequence from GFP or GFP-Nla-Pro was inserted into pTAT301 plasmid, containing the glucocorticoid-inducible system as described above. Wild-type Columbia (Col-0) Arabidopsis plants were transformed using the floral dip method as previously described. The seeds were then plated on MS plate plus 20 μg/mL of hygromycin. Resistant seedlings were selected after 2 weeks and moved to pots with soil. One month later, the plants were sprayed with 3 μM of dexamethasone and 24 h after plants were used in microscopy experiments with and without aphids.

Insect presence experiments. Approximately 50 aphids, 100 whiteflies or 50 leafhoppers were confined in a plastic clip cage with a 2 cm diameter and allowed to feed for 2, 6 or 24 h on the underside of a N. benthamiana or N. tabacum leaf, 2 days post agroinfiltration. The number of insects used for each species was selected to completely cover the surface of the leaf exposed in each cage. A plastic clip cage without insects was used as a control in all experiments. At least one leaf from two different plants was used for each treatment in each experiment and each experiment was repeated three times.

Stains. Two days after infiltration or immediately after aphid treatment, leaves were cut into small pieces and emerged in 10 μg/mL of DAPI to stain the nucleus (Sigma-Aldrich, St. Louis, MO) or with 10 μM of SNARF-1 (Invitrogen, Carlsbad, CA, USA; http://www.lifetechnologies.com) to stain the vacuole for 2 h at room temperature.

Salivary sheath staining. Insects were caged to N. benthamiana leaves as described above. After 24 h, insects were removed and leaves were cleared in 70% ethanol for at least 48 h. Following clearing, leaves were soaked in an acid fuchsin solution (0.035% in acetic acid/water, 1:3 V) for 2 min at room temperature and then immediately rinsed with water. Next leaves were mounted on a glass slide in 50% glycerol and analysed directly using a light microscope.

Microscopy. Leaves were cut into small pieces and placed on glass slides with a drop of perfluorocedalin (Sigma-Aldrich, St Louis, MO) and observed with a Leica TCS SP5 confocal laser scanning microscope system (Leica Micro- systems, Bannockburn, IL, USA). GFP fluorescence was detected with excitation at 488 nm and emission capture at 500–530 nm. The DAPI was excited with a 405 nm laser, and emission was collected from 405–500 nm. SNARF-1 was excited at 514 nm and emission was collected from 550–700 nm. Images were captured at 2,361 μm intervals using a ×20 objective Final figures were prepared using ImageJ software (image.nih.gov/ij/).

Nla-Pro localization quantification. Five microscopy fields were randomly selected for each leaf and visualized. For each field, the number of cells showing GFP inside the nucleus or inside the vacuole was quantified. Two leaves from separate plants were examined for each treatment and three independent experiments were conducted.

Vacuole purification. The vacuole isolation was performed as previously described. Briefly, N. benthamiana leaves agroinfiltrated with TuMV/GFP-Nla-Pro or the empty vector were sliced into 1 mm stripes with a razor blade. The cut leaves were then placed in protoplastation solution (0.4 M mannitol, 20 mM Mes, pH 5.7. 20 mM KCl, 1.5% (w/v) Cellulase R-10, 0.2% (w/v) Macerozyme R-10, 0.1% (w/v) were then placed in protoplastation solution (0.4 M mannitol, 20 mM Mes, pH 5.7). The gradient was centrifuged at 1,500 g for 5 min. Protoplasts were washed two times in 0.1% (w/v) antiprotease) and then boiled in Laemmeli buffer. The plant extract was then centrifuged for 5 min at 8,000 g. The supernatant was fractionated by a 12% SDS-polyacrylamide gel electrophoresis gel under reducing conditions and transferred to a nitrocellulose membrane using a transfer apparatus according to the manufacturer’s protocols (Bio-Rad, Hercules, CA). After incubation with 5% nonfat milk in PBST (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 2 mM KH2PO4, 0.3% Tween 20) for 30 min, the membrane was incubated with an antibody against GFP (1:2,000 dilution) for 2 h at room temperature. The GFP antibody was already conjugated to the horseradish peroxidase (Anti-GFP-HRP, http://www.milenyi-biotec.com, #130-091-833). For a loading control, the membrane was also incubated with an antibody specific to the coat protein of TuMV (provided by J-F Laliberté) (1:1,000 dilution) for 2 h at room temperature and then with a horseradish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad, #1706515, 1:10,000). Blots were washed with PBST three times for 10 min each and developed with an enhanced chemiluminescence system according to the manufacturer (Bio-Rad, Hercules, CA). All uncropped gel/blot images are available in Supplementary Figs 7 and 8.

Statistical analysis. The statistical analyses were performed using JMP 8 software (SAS Institute, Cary, NC, USA) and data were analysed by Student’s t-tests or analysis of variance followed by a Tukey’s honest significant difference post hoc test. All experiments were repeated at least three times. For fecundity, each experiment had at least 12 experimental units per repetition depending of the experiment. For localization experiments, at least two plants were used for each treatment and three repetitions were performed.

Data availability. The authors declare that all data supporting the findings of this study are available in the manuscript and its Supplementary Information files or are available from the corresponding author upon request.

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