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PLANT SCIENCES

VIROLOGY

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SMALL RNA'S

Cucumber mosaic virus and its 2b RNA silencing suppressor modify plant-aphid interactions in tobacco

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Received
10 October 2011

Accepted
11 November 2011

Published
9 December 2011

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The cucumber mosaic virus (CMV) 2b protein not only inhibits anti-viral RNA silencing but also quenches transcriptional responses of plant genes to jasmonic acid, a key signalling molecule in defence against insects. This suggested that it might affect interactions between infected plants and aphids, insects that transmit CMV. We found that infection of tobacco with a 2b gene deletion mutant (CMVΔ2b) induced strong resistance to aphids (*Myzus persicae*) while CMV infection fostered aphid survival. Using electrical penetration graph methodology we found that higher proportions of aphids showed sustained phloem ingestion on CMV-infected plants than on CMVΔ2b-infected or mock-inoculated plants although this did not increase the rate of growth of individual aphids. This indicates that while CMV infection or certain viral gene products might elicit aphid resistance, the 2b protein normally counteracts this during a wild-type CMV infection. Our findings suggest that the 2b protein could indirectly affect aphid-mediated virus transmission.

Most viruses possess suppressor proteins that target one or more of the components of RNA silencing, an adaptive anti-viral mechanism occurring in plants and many other eukaryotes^{1,2}. One of the first suppressors to be identified was the 2b protein of cucumber mosaic virus (CMV)³. CMV is a positive-sense RNA virus with three genomic segments that between them encode five proteins⁴. The 2b protein is encoded by the 3'-proximal open reading frame of CMV RNA 2 and is expressed during infection from a sub-genomic mRNA, called RNA 4A⁵. It is a small (c. 12 kDa), multifunctional polypeptide that inhibits anti-viral RNA silencing by binding short-interfering (si)RNAs. It also disrupts micro (mi)RNA-regulated gene expression and DNA methylation through interactions with the host silencing proteins Argonautes 1 and 4, respectively^{6–11}. It also protects CMV to some extent from salicylic acid-induced anti-viral defences^{12,13} and it influences cell-to-cell and systemic movement of CMV^{14–16}. Experiments using mutant viruses lacking all or part of the 2b gene have revealed much concerning the biological functions of the 2b protein. Ryabov and colleagues¹⁷ developed one such deletion mutant from the Fny strain of CMV. In Fny-CMVΔ2b (hereafter referred to as CMVΔ2b) a sequence corresponding to the 2b gene and a small portion of the 2a gene was deleted. CMVΔ2b infects tobacco (*Nicotiana tabacum*), *N. benthamiana* and *Arabidopsis thaliana* (ecotype Col-0) but does not induce symptoms in these hosts^{10,16,18}.

Our group recently investigated the effects of the Fny-CMV 2b protein on the *A. thaliana* transcriptome using DNA microarrays. We found that in 2b-transgenic *A. thaliana* plants approximately 90% of genes that are regulated by jasmonic acid (JA) no longer respond following treatment with methyl-JA¹⁹. Consistent with this, infection of *A. thaliana* plants with CMV, but not with CMVΔ2b, inhibited the responses of JA-regulated genes to methyl-JA. As well as regulating resistance to certain microbes, JA regulates resistance to a number of insect pests, including insects that transmit viruses such as aphids and whiteflies^{20–25}. The effect of the 2b protein on JA-regulated defensive signalling could be significant since CMV is transmitted by aphids⁴. Interestingly, Mauck and



colleagues²⁶ found that CMV infection altered the attractiveness of squash plants to aphids, also suggesting that the virus was interfering with defensive signalling.

Aphids transmit CMV in a non-persistent manner. Non-persistently transmitted viruses are acquired, i.e. bind to the aphid mouthparts (stylets), after only seconds or minutes of contact with the plant, and transmission to new hosts is also rapid; occurring in the early stages of feeding on epidermal cells^{27,28}. Aphids do not internalise non-persistently transmitted viruses and they retain the ability to transmit the viruses for no more than a few hours^{27,29–31}. The coat protein, which is responsible for encapsidating the genomic RNAs of CMV into virus particles, is the sole factor required for binding of CMV to the stylet and it confers aphid transmissibility on this virus^{32–34}. However, because the CMV 2b protein disrupted JA-regulated gene expression we speculated that the 2b protein might indirectly influence CMV transmission by affecting the interactions of infected plants with aphids¹⁹. In this study we used virus-infected tobacco to investigate the effects of the 2b protein on aphid colonization, feeding behaviour and growth.

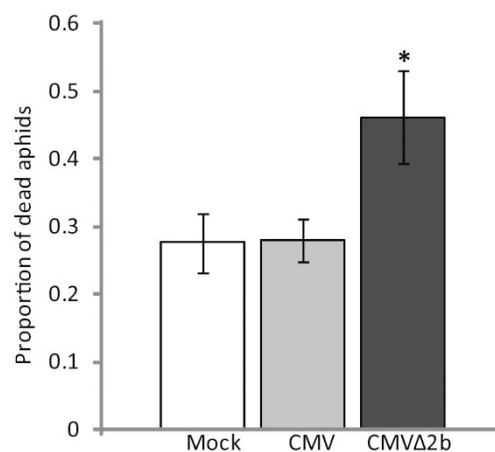
Results

Aphid survival is altered on tobacco infected with CMV and CMVΔ2b. Groups of wingless (apterous) aphids (*Myzus persicae*) comprising nymphs of the third and fourth developmental stage or instar were confined on virus-infected tobacco leaves using clip cages and 72 hours later the numbers of aphids that had died were counted. Fewer aphids survived on plants infected with CMVΔ2b than on mock-inoculated plants or plants infected with wild-type CMV (Fig. 1a). The proportion of dead aphids found at this time was similar on mock-inoculated and CMV-infected plants (27% and 28%, respectively; Fig. 1a). In contrast, a significantly higher proportion of aphids feeding on CMVΔ2b plants died over the course of the experiment (46%). An additional two experiments were carried out with similar results. Further statistical analysis was carried out to confirm that these results were significant and consistent between independent experiments. Thus, when all the data from the three biological replicates were pooled, an effect of both virus infection and experimental repetition was found while there was no interaction ($p < 0.001$ for virus infection, $p < 0.001$ experimental repetition, and $p = 0.34$ for interaction) showing that the results were consistent across the experiments. These analyses were followed up using the statistical method of contrasts to compare the proportion of aphids that died on CMVΔ2b-infected plants with the proportions that died on mock-inoculated plants or plants infected with CMV. This methodology confirmed that the effect of CMVΔ2b was significant ($p = 0.00002$, 80df). Thus, the numbers of dead aphids were higher on CMVΔ2b-infected than on CMV-infected or mock-inoculated plants.

Since the survival of aphids on tobacco was compromised on CMVΔ2b-infected plants, we investigated in more detail the developmental stages at which the aphids were being affected. Thus, groups of first instar nymphs were selected and confined on mock-inoculated, CMV-infected and CMVΔ2b-infected plants. Again, fewer aphids survived on plants infected with CMVΔ2b than on mock-inoculated plants; however, the period of confinement was extended from three to seven days to see this difference (Fig. 1b). Furthermore, aphid survival was enhanced on plants infected with CMV (Fig. 1b). The proportion of dead aphids found on mock-inoculated leaves was 16%, while the proportion of dead aphids on wild-type CMV-infected plants was significantly lower at less than 2% ($p = 0.0009$; Student's *t*-test). In contrast, the proportion of aphids that were dead on CMVΔ2b infected-plants was significantly higher at 37% ($p = 0.04$; Student's *t*-test).

These data indicated that tobacco plants infected with CMVΔ2b were more resistant to aphids than mock-inoculated plants. Furthermore,

a Age on transfer to clipcage = 3rd and 4th instar



b Age on transfer to clipcage = 1st instar

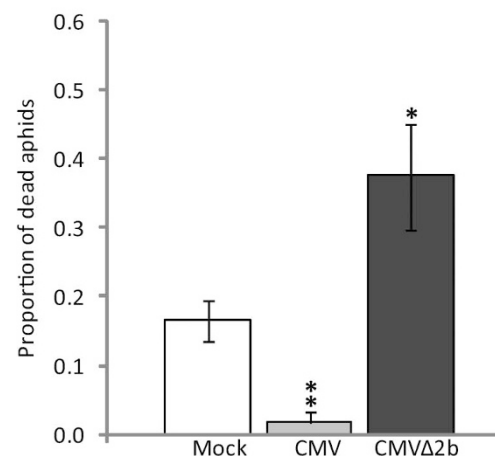


Figure 1 | Increased numbers of aphids die on tobacco plants infected with CMVΔ2b. (a) Ten third and fourth instar aphids were confined to clip cages attached to tobacco leaves systemically infected with either CMV or CMVΔ2b or that had been mock-inoculated. The proportion of aphids that died after 3 days was significantly higher on CMVΔ2b-infected plants (*) compared to CMV-infected or mock-inoculated plants (ANOVA, $p \leq 0.01$). (b) Ten first instar aphids were confined to clip cages on leaves systemically infected with either CMV, CMVΔ2b or that had been mock-inoculated. Significantly more aphids died on the CMVΔ2b-infected plants (*) compared to mock-inoculated plants (Student's *t* test $p \leq 0.05$). Additionally, aphid survival was significantly enhanced on CMV infected plants (**) compared to mock-inoculated (Student's *t* test $p \leq 0.0009$). Error bars represent the standard error of the mean.

aphids showed improved survival when transferred to CMV-infected plants at an early stage in their development.

Aphids on CMVΔ2b-infected tobacco spent less time phloem feeding. Aphid survival was compromised on tobacco plants systemically infected with CMVΔ2b and enhanced on CMV-infected tobacco. Therefore, we investigated the feeding behaviour of individual insects on virus-infected plants using the electrical penetration graph (EPG) technique. EPG is a continuous electronic monitoring technique that allows identification of the particular plant tissue that an aphid is probing as well as the feeding



activity it is engaged in (eg ingestion, salivation etc.)^{35–37}. This enables a full description of feeding behaviour over a given time period to be deduced. We investigated aphid feeding behaviour and stylet activity over a 12 h period on tobacco plants infected with CMV, CMV Δ 2b or on plants that had been mock-inoculated.

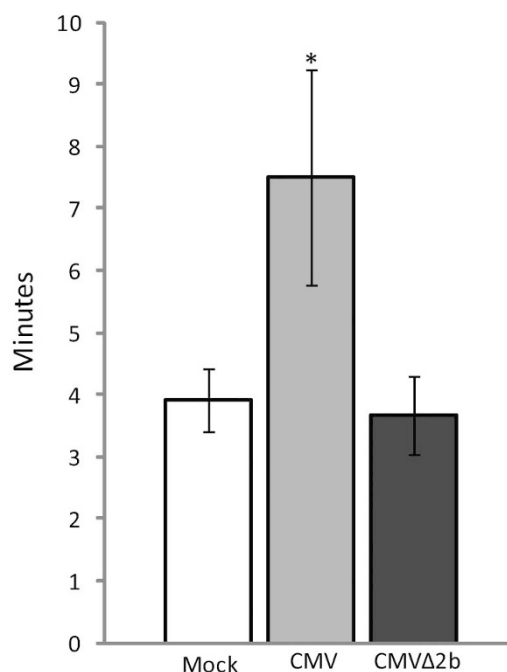
We examined seven key parameters of feeding behaviour (summarised in Supplementary Table 1). There were no significant differences in the total time spent on each feeding activity by aphids placed on mock-inoculated or virus-infected plants. However some marked trends were noticed (Supplementary Figure S1). For example, the total time spent ingesting phloem sap (the richest food source for aphids) was lowest for aphids on CMV Δ 2b-infected tobacco, correlating with the higher mortality rate of aphids on these plants. Also, the total time spent ingesting from the xylem (vessels containing few nutrients and also the conduits for nicotine translocation) was lowest for aphids on CMV-infected plants, which appeared to correlate with improved aphid survival.

The average duration of periods of phloem sap ingestion by aphids (calculated as the total time spent ingesting phloem divided by the number of phloem ingestion events) and the proportion of aphids able to carry out sustained phloem sap ingestion (classified as ingestion periods longer than 30 minutes) reflect an increased acceptance of the host plant by the aphid³⁷. We found that the average duration of periods of phloem ingestion (not including periods cut short by the end of the 12 hour recording period) was significantly longer in aphids on CMV-infected tobacco (7.5 minutes) compared to aphids on mock-inoculated or CMV Δ 2b-infected tobacco (3.9 and 3.7 minutes respectively; Fig. 2a). This indicated better host acceptance by aphids on CMV-infected plants. Furthermore, the proportion of aphids that could carry out sustained phloem feeding was highest on CMV-infected tobacco plants and lowest on CMV Δ 2b-infected plants (Fig. 2b). The difference in the total time spent on phloem ingestion between aphids that did or did not carry out sustained phloem feeding was large. Aphids that carried out sustained phloem-feeding spent about 300 minutes in total on this activity while aphids that did not carry out sustained phloem-feeding spent a total time of 40–60 minutes feeding (Supplementary Figure 2). Also, those aphids that did not display sustained phloem feeding spent more time inactive or ‘not probing’, i.e. their stylets were not engaged with the plant surface. This high proportion of aphids spending less time feeding on CMV Δ 2b-infected plants appeared to correlate with the decreased survival of aphids on these hosts.

The effects of virus infection on aphid colony growth. The analysis of feeding behaviour was followed up with an investigation of growth rate for individual first instar nymphs on virus-infected plants. Surprisingly, the mean relative growth rate (MRGR) of individual aphids over the five days after birth was significantly lower on CMV-infected plants compared to either mock-inoculated or CMV Δ 2b-infected plants (Fig. 3). The experiment was replicated three times with consistent results. The MRGR is mainly determined by food quality when the temperature is controlled and aphids are confined to clip cages³⁸. As adults spent more time phloem feeding on CMV-infected tobacco (Fig. 2), the lower growth rate of the nymphs indicated that CMV-infected plants are a poorer source of nutrition than either mock-inoculated or CMV Δ 2b-infected plants.

Because aphid nymphs had an enhanced survival rate on CMV-infected plants, yet showed poorer growth on these plants, we went on to investigate the outcome of these apparently contradictory effects on colony development. Nymphs that had been born on mock-inoculated or virus-infected tobacco plants were confined to clip cages and their survival and reproduction were monitored over 15 days (Fig. 4). The greatest total number of progeny was observed on the CMV-infected plants (Fig. 4a). This was largely the consequence of the increased numbers of founder aphids surviving on CMV-infected leaves (Fig. 4b), in line with our previous results

a Average duration of phloem ingestion



b Aphids showing sustained phloem ingestion

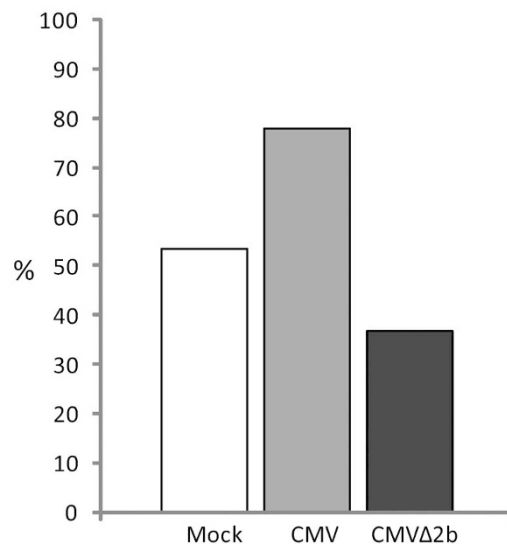


Figure 2 | Duration of phloem ingestion by aphids changes on virus-infected tobacco. Virus infection caused significant changes in aphid feeding behaviour, as measured by the electrical penetration graph (EPG) technique. Aphids were tethered to EPG probes and placed on individual plants. Their feeding behaviour was recorded for 12 hours. $n \geq 14$ for each plant group. (a) The average duration of phloem ingestion was significantly increased for aphids on tobacco plants systemically infected with CMV (*) compared to mock or CMV Δ 2b-infected plants (Kruskal Wallis test $p=0.0029$). The average duration phloem ingestion does not include any period that was cut short by the end point of the EPG recording. (b) The proportion of aphids showing sustained phloem ingestion for periods longer than 30 minutes is greatest on CMV-infected plants and lowest on CMV Δ 2b-infected plants (χ^2 , $P=0.042$).

(Fig. 1). Furthermore, it was noticed that the decline in the founder aphid population on mock-inoculated and CMV Δ 2b-infected tobacco occurred at the same time as the first progeny were deposited

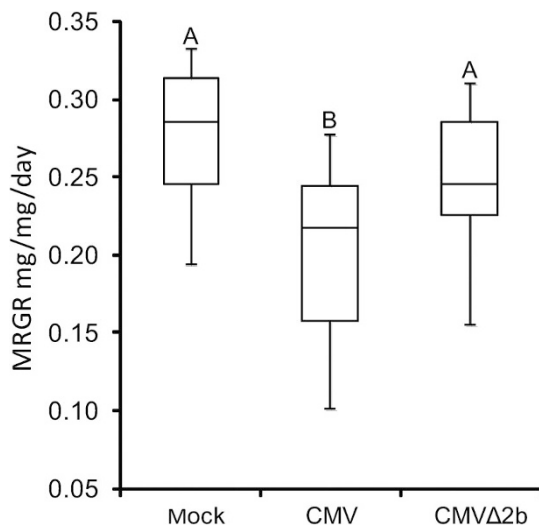


Figure 3 | Growth rates of aphids feeding on virus-infected and mock-inoculated plants. The mean relative growth rate (MRGR: mg/mg/day) of individual aphids feeding on CMV-infected plants was significantly lower compared to aphids feeding on CMVΔ2b-infected and mock-inoculated plants (ANOVA, $p=0.0003$). Different letters are assigned to significantly different results (post-hoc Tukey tests, $p<0.01$). Data is presented in a box plot where the horizontal line within the box represents the 50th percentile (median) and the top and bottom of the box represent the 75th and 25th percentile respectively, while the extent of the whiskers indicates the maximum and minimum of all the data.

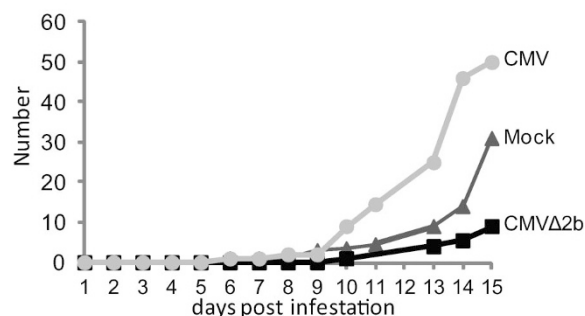
(at 7–8 days post infestation). This critical period in early adulthood and concomitant decline in aphid survival has also been observed for *M. persicae* on potato plants³⁹.

Taken together, these results show that aphid survival and colony establishment is enhanced on CMV-infected plants while plants infected with CMVΔ2b are more resistant to aphids. Thus the 2b protein is implicated in promoting aphid survival on CMV-infected tobacco despite the poorer MRGR of aphids on these plants. Recent research suggests that the signals controlling plant preference and initiation of reproduction are detected early during the stylet penetration process, i.e. before the phloem sap (nutrient supply) is sampled⁴⁰. It may be that changes in the CMV-infected cells peripheral to the vasculature play an important role in promoting aphid survival, despite the poorer MRGR of aphids on CMV-infected tobacco. It may also explain why only aphids reared from birth (or an early stage in their development) showed improved longevity on CMV-infected tobacco (Fig. 1b and Fig. 4b).

Virus infection did not affect nicotine accumulation in tobacco.

Plants of tobacco and other *Nicotiana* species produce the alkaloid, nicotine, which is toxic to aphids and contributes to resistance to herbivory²⁴. In *N. attenuata* RNA dependent RNA polymerase 1, a component of the RNA silencing machinery that is affected by the 2b protein⁴¹, regulates a range of JA-responsive processes including nicotine production⁴². Therefore, we investigated the potential role of nicotine in CMVΔ2b-induced aphid resistance in tobacco by determining whether infection with this viral mutant or with wild-type CMV affected nicotine accumulation. However, high performance liquid chromatography showed that nicotine levels were not elevated in CMVΔ2b-infected tobacco within the time-frame that aphid experiments were carried out, i.e. four weeks after virus inoculation (Fig. 5). Treatment with methyl-JA modestly enhanced nicotine levels in mock-inoculated plants and this was also seen in CMV and CMVΔ2b-infected plants. Thus, resistance to aphids induced by CMVΔ2b infection is unlikely to result from induction of increased nicotine accumulation and there is no reason to suppose that the 2b protein directly or indirectly affects the biosynthesis of this alkaloid.

a Colony development



b Founder aphid survival

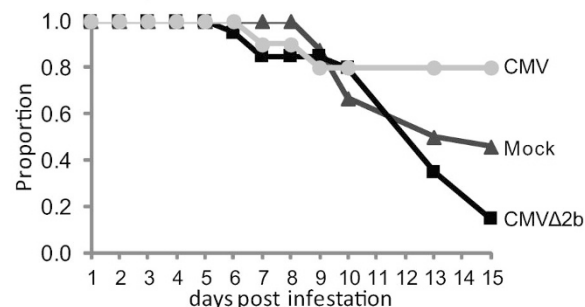


Figure 4 | Increased survival and colony development of aphids raised from birth on CMV-infected tobacco plants. (a) Aphid nymphs were confined from birth to 15 days old in clip cages attached to leaves of tobacco plants systemically infected with either CMV or CMVΔ2b or that had been mock-inoculated. The total number of progeny aphids was greater on leaves systemically infected with CMV. The combined data from 18 clip cages is presented. The experiments were carried out twice with similar results. (b) Survival of the founder aphid was lowest on leaves systemically infected with CMVΔ2b and enhanced on leaves systemically infected with CMV compared to the mock treatment.

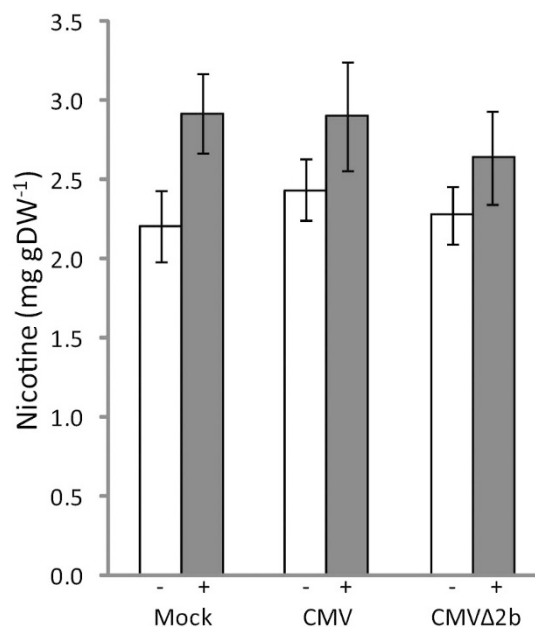


Figure 5 | Accumulation of nicotine in virus-infected tobacco plants. Nicotine levels in the upper leaves of plants 14 days after mock-inoculation or inoculation with CMV or CMVΔ2b, with (+) or without (-) methyl-JA treatment 24 h prior to harvest.



Discussion

Plants systemically infected with the mutant virus CMV Δ 2b exhibited strong resistance to *M. persicae* indicated by increased numbers of dead aphids on CMV Δ 2b-infected plants. In contrast, aphid survival and colony development was improved on CMV-infected relative to mock-inoculated plants. The results imply that in tobacco one or more CMV gene products other than the 2b protein (i.e., the 1a, 2a, movement or coat proteins) are elicitors of resistance to aphids. These observations further suggest that during an infection of tobacco with wild-type CMV the 2b protein somehow masks or inhibits the effects of the conjectured elicitor molecule and prevents the triggering of resistance to aphids. Our results indicate that in tobacco the CMV 2b protein fine-tunes resistance signalling to aid aphid infestation, which is a novel property for this multifunctional viral protein.

Infection of host plants altered the feeding behaviour of aphids. Although the average duration of periods of phloem feeding was significantly longer on CMV-infected tobacco, the rate of aphid nymph biomass increase was lowest on these plants. Fu et al.⁴³ also found that the mass of individual adult aphids was lower on CMV-infected tobacco compared to mock-inoculated controls. Thus, increased feeding from the phloem of CMV-infected plants may be a response to decreased nutritional quality of these plants. We did not find any differences in nicotine levels following four weeks of CMV or CMV Δ 2b infection indicating that nicotine was not responsible for the lower aphid mass or for the increased death of aphids placed on CMV Δ 2b-infected plants. We also found that the proportion of aphids indulging in sustained phloem feeding (an indication of the ease with which aphids can feed on a host) was highest on CMV-infected plants and lowest on CMV Δ 2b-infected plants. The difficulty in attaining sustained phloem feeding on CMV Δ 2b-infected plants could have contributed to the higher mortality rates of aphids on these hosts.

Virus infections are known to alter host plant attractiveness and suitability for aphid colonisation. Macias and Mink⁴⁴ observed that aphids were attracted to sugar beet plants infected with beet yellows virus and showing symptoms of chlorosis. Aphids are also attracted by volatiles released by virus-infected plants. This has been thought to be associated with the persistent mode of transmission, where long feeding periods are needed for successful virus acquisition^{39,45–48}. However, volatile signals can attract aphids to plants infected with non-persistently transmitted viruses like CMV²⁶. Virus infection alters host carbohydrate metabolism^{49–51}, which could conceivably influence the performance and fecundity of aphids on infected plants. Nevertheless, it is not always the case that this will encourage onward transmission of viruses⁵².

Powell²⁸ showed that viruses with a non-persistent mode of transmission are acquired very rapidly from epidermal cells of infected plants by aphids after only a short probing/feeding period. Aphids do not retain these viruses for long. CMV is retained for no more than four hours, providing the aphid does not lose the virus during subsequent feeds⁴. Our results show that in tobacco, CMV fostered the longevity of aphids thereby promoting colony development. In contrast, CMV Δ 2b induced resistance to aphids. While it should be remembered that CMV Δ 2b is an artificial construct that does not occur in nature, experiments with this mutant virus strongly suggest that in tobacco a potential role for the 2b protein is to inhibit the induction of resistance to aphids, caused either by CMV-encoded factors, or by the process of virus infection itself. Since virus transmission efficiency is substantially diminished on plants exhibiting strong resistance to aphids^{22,53}, it might be argued that the 2b protein indirectly aids transmission by inhibiting insect resistance. However, while inhibiting resistance to insects may aid aphid survival and favour the growth of aphid populations, it may not favour spread of virus-carrying aphids to new hosts.

An alternative possibility is that the action of the CMV 2b protein is mutually advantageous to the vector and virus by promoting the persistence of both within a plant community. For example, aphids over-winter on a variety of hardy plants that act as reservoirs of CMV and other viruses. When aphids become active in springtime, these plants are potential foci for renewed virus transmission⁵⁴. Thus, inhibition of aphid resistance in host plants may not accelerate the rate of CMV transmission but may help ensure the persistence of the virus and its seasonal re-emergence. Given the importance of insect transmission to most plant viruses, we suggest that manipulation of defensive signalling pathways to enhance vector survival may be a general role for viral silencing suppressor proteins.

Methods

Insect, plant and virus stocks and chemical treatments. Colonies of *M. persicae* (Sulzer) (Insecta: Hemiptera: Aphididae) clone US11⁵⁵ were maintained on tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants at 20°C and 70% relative humidity under artificial light with a photoperiod of 16 hours. Tobacco seeds were sown on Levington M3 compost (Scotts, Chilworth, Ipswich, UK) and seedlings transplanted into individual pots at the two-to-three leaf stage. Plants were cultivated in a growth room (Conviro, MB, Canada) at 21°C with a photoperiod of 16 hours light (200 μ E.m⁻².sec⁻¹). CMV strain Fny⁵⁶ and its 2b gene deletion mutant, CMV Δ 2b¹⁷, were propagated in tobacco and purified by the method of Ng and Perry⁵⁷.

Tobacco plants were inoculated with purified virions of CMV or CMV Δ 2b at the 3- to 4-leaf stage. Infection by CMV led to visible symptoms and did not require additional confirmation. Systemic infection of leaves by CMV Δ 2b (which is symptomless) was assessed either by DAS-ELISA (Bioreba AG, Reinach, Switzerland), or using Agdia 'Immunostrips' (Agdia Inc., Elkhart, IN, USA). All samples that tested positive for CMV Δ 2b were verified by a reverse-transcription polymerase chain reaction method as described previously¹⁸.

Aphid performance. For aphid survival experiments, ten aphids were selected based on instar and placed into clip cages on leaves of tobacco plants that were either mock-inoculated or infected with either CMV or CMV Δ 2b. Aphids were monitored daily over the course of experiments. Statistical significance within each experiment was assessed by ANOVA [with individual testing performed by introducing appropriate contrasts⁵⁸] or Student's *t*-test. For experiments in which individual 'founder' aphids were monitored for survival and production of progeny, a single adult aphid was confined in a clip cage for 24 hours to allow birth of a single nymph. The adult aphid was then removed, and the remaining 'founder' nymph was monitored over the following 15 days.

The mean relative growth rate (MRGR) of aphids in mg/mg/day was calculated using the formula $MRGR = (\log_e W_{final} - \log_e W_{initial})/t$, where *t* = time in days between initial and final measurements of aphid fresh weight (*W* = mg). One-day-old first instar nymphs were individually weighed on a microbalance (MX5, Mettler-Toledo Inc., Columbus, OH, USA) before being placed on test plants. The final weight of each aphid was taken five days after infestation. Twenty replicates per treatment group were used. The experiment was performed three times with similar results.

Monitoring of aphid feeding behaviour. The direct-current EPG method³⁵ was used to monitor aphid stylet probing activity on tobacco leaves. Individual aphids were pre-starved for 30–60 mins and tethered to 4 cm of 20 μ m diameter gold wire (EPG systems, Wageningen, The Netherlands) using conductive silver paint (Electrolube, Swadlincote, UK, or EPG systems). The gold wire was soldered to a 1 cm brass pin, connected to an amplifier with 1 G Ω resistance and 50–100X gain. Connected aphids were placed on individual plants inside a Faraday cage and signals received from the EPG monitor were displayed and analysed using PROBE 3.4 software (EPG systems). Waveforms were scored according to Tjallingii and Hogen Esch³⁷. Relevant aphid behaviour-related EPG parameters were calculated using Microsoft Excel-based spreadsheets developed by Dr. Edgar Schliephake (Julius Kühn Institut, Germany) and described by Sarria et al.⁵⁹.

Nicotine analyses. The lowest leaves of tobacco plants were inoculated with CMV or CMV Δ 2b or mock-inoculated. Four weeks post-inoculation, the upper leaves were harvested and confirmed as virus-infected by RT-PCR. Leaves were ground in liquid nitrogen and freeze-dried. Nicotine was extracted in methanol-water-concentrated HCl [40/59.9/0.1 (v/v/v)] and analysed by high performance liquid chromatography as described by Gonzalez-Rabade et al.⁶⁰ using a Luna C18 column (2 \times 10 mm, 3 μ m particle size, Phenomenex, Macclesfield, UK) and a Finnigan Surveyor system coupled to a Finnigan PDA detector (Thermo Fisher Scientific, Hemel Hempstead, UK). Quantification was carried out using the linear range of a standard curve constructed with known amounts of nicotine (Sigma-Aldrich, Poole, Dorset, UK) and Xcalibur software (Thermo Fisher Scientific).

1. Csorba, T., Pantaleo, V. & Burguán, J. RNA Silencing: An antiviral mechanism. *Adv. Virus Res.* 75, 35–71 (2009).



2. Palukaitis, P. & MacFarlane, S. Viral Counter-Defense Molecules. In *Natural Resistance Mechanisms of Plants to Viruses* (G. Loebenstein & J.P.Carr, Eds) pp 165–185 Springer (2006).
3. Brigneti, G., Voinnet, O., Li, W. X., Ji, L. H., Ding, S. W. & Baulcombe, D. C. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J.* **17**, 6739–6746 (1998).
4. Palukaitis, P. & García-Arenal, F. Cucumoviruses. *Adv. Virus Res.* **62**, 241–323 (2003).
5. Ding, S. W., Anderson, B. J., Haase, H. R. & Symons, R. H. New overlapping gene encoded by the cucumber mosaic virus genome. *Virology* **198**, 593–601 (1994).
6. Cillo, F., Mascia, T., Pasciuto, M. M. & Gallitelli, D. Differential effects of mild and severe *Cucumber mosaic virus* strains in the perturbation of microRNA-regulated gene expression map to the 3' sequence of RNA 2. *Molec. Plant-Microbe Interact.* **22**, 1239–1249 (2009).
7. González, I. et al. *Cucumber mosaic virus* 2b protein subcellular targets and interactions: Their significance to RNA silencing suppressor activity. *Mol. Plant-Microbe Interact.* **23**, 294–303 (2010).
8. Goto, K., Kobori, T., Kosaka, Y., Natsuaki, T., & Masuta, C. Characterization of silencing suppressor 2b of cucumber mosaic virus based on examination of its small RNA-binding abilities. *Plant Cell Physiol.* **48**, 1050–1060 (2007).
9. Lewsey, M., Robertson, F. C., Canto, T., Palukaitis, P. & Carr, J. P. Selective targeting of miRNA-regulated plant development by a viral counter-silencing protein. *Plant J.* **50**, 240–252 (2007).
10. Lewsey, M. et al. The role of the *Cucumber mosaic virus* 2b protein in viral movement and symptom induction. *Molec. Plant-Microbe Interact.* **22**, 642–654 (2009).
11. Ye, J., Qua, J., Zhang, J. F., Geng, Y. F. & Ye, J., Qua, J., Zhang, J. F., Geng, Y. F. & Fang, R. X. A critical domain of the *Cucumber mosaic virus* 2b protein for RNA silencing suppressor activity. *FEBS Lett.* **583**, 101–106 (2004).
12. Naylor, M., Murphy, A. M., Berry, J. O. & Carr, J. P. Salicylic acid can induce resistance to plant virus movement. *Molec. Plant-Microbe Interact.* **11**, 860–868 (1998).
13. Ji, L. H. & Ding, S. W. The suppressor of transgene RNA silencing encoded by cucumber mosaic virus interferes with salicylic acid-mediated virus resistance. *Molec. Plant-Microbe Interact.* **14**, 715–724 (2001).
14. Ding, S. W., Li, W. X. & Symons, R. H. A novel naturally occurring hybrid gene encoded by a plant RNA virus facilitates long distance virus movement. *EMBO J.* **14**, 5762–5772 (1995).
15. Shi, B.-J., Palukaitis, P. & Shi, B.-J., Palukaitis, P. & Symons, R. H. Differential virulence by strains of *Cucumber mosaic virus* is mediated by the 2b gene. *Molec. Plant-Microbe Interact.* **15**, 947–955 (2002).
16. Soards, A. J., Murphy, A. M., Palukaitis, P. & Carr, J. P. Virulence and differential local and systemic spread of *Cucumber mosaic virus* in tobacco are affected by the CMV 2b protein. *Molec. Plant-Microbe Interact.* **15**, 647–653 (2002).
17. Ryabov, E. V., Fraser, G., Mayo, M. A., Barker, H. & Taliany, M. Umbravirus gene expression helps *Potato leafroll virus* to invade mesophyll tissues and to be transmitted mechanically between plants. *Virology* **286**, 363–372 (2001).
18. Ziebell, H., Payne, T., Berry, J. O., Walsh, J. A. & Carr, J. P. A cucumber mosaic virus mutant lacking the 2b counter-defence protein gene provides protection against wild-type strains. *J. Gen. Virol.* **88**, 2862–2871 (2007).
19. Lewsey, M. G. et al. Disruption of two defensive signaling pathways by a viral RNA silencing suppressor. *Molec. Plant-Microbe Interact.* **23**, 835–845 (2010).
20. de Vos, M. et al. Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Molec. Plant-Microbe Interact.* **18**, 923–937 (2005).
21. Ellis, C., Karafyllidis, I. & Turner, J. G. Constitutive activation of jasmonate signaling in an *Arabidopsis* mutant correlates with enhanced resistance to *Erysiphe cichoracearum*, *Pseudomonas syringae*, and *Myzus persicae*. *Molec. Plant-Microbe Interact.* **15**, 1025–1030 (2002).
22. Howe, G. A. & Jander, G. Plant immunity to insect herbivores. *Annu. Rev. Plant Biol.* **59**, 41–66 (2008).
23. Koornneef, A. & Pieterse, C. M. J. Cross talk in defense signaling. *Plant Physiol.* **146**, 839–844 (2008).
24. Rohwer, C. L. & Erwin, J. E. Horticultural applications of jasmonates: A review. *J. Hortic. Sci. Biotechnol.* **83**, 283–304 (2008).
25. Zarate, S. I., Kempema, L. A. & Walling, L. L. Silverleaf whitefly induces salicylic acid and suppresses effectual jasmonic acid defenses. *Plant Physiol.* **143**, 866–875 (2007).
26. Mauck, K. E., De Moraes, C. M. & Mescher, M. C. Deceptive chemical signals induced by a plant virus attract insect vectors to inferior hosts. *Proc. Natl. Acad. Sci. USA* **107**, 3600–3605 (2010).
27. Hull, R. *Matthews' Plant Virology* 4th Edition. London and San Diego: Academic Press (2002).
28. Powell, G. Intracellular salivation is the aphid activity associated with inoculation of non-persistently transmitted viruses. *J. Gen. Virol.* **86**, 469–472 (2005).
29. Gray, S. M. & Banerjee, N. Mechanisms of arthropod transmission of plant and animal viruses. *Microbiol. Molec. Biol. Rev.* **63**, 128–148 (1999).
30. Nault, L. R. Arthropod transmission of plant viruses: A new synthesis. *Annals of the Entomological Society of America* **90**, 521–541 (1997).
31. Ng, J. C. K. & Falk, B. W. Virus-vector interactions mediating nonpersistent and semipersistent transmission of plant viruses. *Annu. Rev. Phytopathol.* **44**, 183–212 (2006).
32. Chen, B. & Francki, R. I. Cucumovirus transmission by the aphid *Myzus persicae* is determined solely by the viral coat protein. *J. Gen. Virol.* **71**, 939–944 (1990).
33. Gera, A., Loebenstein, G. & Raccah, B. Protein coats of two strains of cucumber mosaic virus affect transmission by *Aphis gossypii*. *Phytopathology* **69**, 396–399 (1979).
34. Ng, J. C. K. & Perry, K. L. Transmission of plant viruses by aphid vectors. *Mol. Plant Pathol.* **5**, 505–511 (2004).
35. Tjallingii, W. F. Electronic recording of penetration behaviour by aphids. *Entomol. Exp. Appl.* **24**, 721–730 (1978).
36. Tjallingii, W. F. Electrical nature of recorded signals during stylet penetration by aphids. *Entomol. Exp. Appl.* **38**, 177–186 (1985).
37. Tjallingii, W. F. & Hogen Esch, T. Fine structure of aphid stylet routes in plant tissues in correlation with EPG signals. *Physiol. Entomol.* **18**, 317–328 (1993).
38. Dixon, A. F. G. Parthenogenetic reproduction and the rate of increase in aphids. In *Aphids: Their Biology, Natural Enemies and Control*, Vol. A pp. 269–87 (Eds. A.K. Minks & P. Harrewijn) Amsterdam: Elsevier (1987).
39. Castle, S. J. & Berger, P. H. Rates of growth and increase of *Myzus persicae* on virus-infected potatoes according to type of virus-vector relationship. *Entomol. Exp. Appl.* **69**, 51–60 (1993).
40. Powell, G., Tosh, C. R. & Hardie, J. Host plant selection by aphids: Behavioral, evolutionary, and applied perspectives. *Annu. Rev. Entomol.* **51**, 309–30 (2006).
41. Diaz-Pendon, J. A., Li, F., Li, W. X. & Ding, S. W. Suppression of antiviral silencing by cucumber mosaic virus 2b protein in *Arabidopsis* is associated with drastically reduced accumulation of three classes of viral small interfering RNAs. *Plant Cell* **19**, 2053–2063 (2007).
42. Pandey, S. P., Shahi, S., Gase, K. & Baldwin, I. T. Herbivory-induced changes in the small-RNA transcriptome and phytohormone signaling in *Nicotiana attenuata*. *Proc. Natl. Acad. Sci. USA* **105**, 4559–4564 (2008).
43. Fu, X., Ye, L., Kang, L. & Ge, F. Elevated CO₂ shifts the focus of tobacco plant defences from cucumber mosaic virus to green peach aphid. *Plant Cell Environ.* **33**, 2056–2064 (2010).
44. Macias, W. & Mink, G. I. Preference of green peach aphids for virus-infected sugarbeet leaves. *J. Econ. Entomol.* **62**, 28–29 (1969).
45. Alvarez, A. E., Garzo, E., Verbeek, M., Vosman, B., Dicke, M. & Tjallingii, W. F. Infection of potato plants with potato leafroll virus changes attraction and feeding behaviour of *Myzus persicae*. *Entomol. Exp. Appl.* **125**, 135–144 (2007).
46. Castle, S. J., Mowry, T. M. & Berger, P. H. Differential settling by *Myzus persicae* (Homoptera: Aphididae) on various virus infected host plants. *Ann. Entomol. Soc. Amer.* **91**, 661–667 (1998).
47. Eigenbrode, S. D., Ding, H. J., Shiel, P. & Berger, P. H. Volatiles from potato plants infected with potato leafroll virus attract and arrest the virus vector, *Myzus persicae* (Homoptera : Aphididae). *Proc. Royal Soc. Lond. B* **269**, 455–460 (2002).
48. Medina-Ortega, K. J., Bosque-Pérez, N. A., Ngumbi, E., Jiménez-Martínez, E. S. & Eigenbrode, S. D. *Rhopalosiphum padi* (Hemiptera: Aphididae) responses to volatile cues from barely yellow dwarf virus-infected wheat. *Environ. Entomol.* **38**, 836–845 (2009).
49. Blua, M. J. & Perring, T. M. Alatae production and population increase of aphid vectors on virus-infected host plants. *Oecologia* **92**, 65–70 (1992).
50. Fiebig, M., Poehling, H.-M. & Borgemeister, C. Barley yellow dwarf virus, wheat, and *Sitobion avenae*: a case of trilateral interactions. *Entomol. Exp. Appl.* **110**, 11–21 (2004).
51. Handford, M. G. & Carr, J. P. Plant metabolism associated with resistance and susceptibility. In *Natural Resistance Mechanisms of Plants to Viruses* pp. 315–340 (Eds. G. Loebenstein and J.P.Carr) Springer (2006).
52. Hodge, S. & Powell, G. Do plant viruses facilitate their aphid vectors by inducing symptoms that alter behavior and performance? *Environ. Entomol.* **37**, 1573–1581 (2008).
53. van Emden, H. F. Host-plant resistance. In *Aphids as Crop Pests*, pp. 447–468. Edited by H. F. van Emden & R. Harrington. Wallingford Oxfordshire: CABI (2007).
54. Sacristán, S., Fraile, A. & García-Arenal, F. Population dynamics of cucumber mosaic virus in melon crops and in weeds in central Spain. *Phytopathol.* **94**, 992–998 (2004).
55. Devonshire, A. L. & Sawicki, R. M. Insecticide-resistant *Myzus persicae* as an example of evolution by gene duplication. *Nature* **280**, 140–141 (1979).
56. Roossinck, M. J. & Palukaitis, P. Rapid induction and severity of symptoms in zucchini squash (*Cucurbita pepo*) map to RNA-1 of cucumber mosaic virus. *Mol. Plant-Microbe Interact.* **3**, 188–192 (1990).
57. Ng, J. C. K. & Perry, K. L. Transmission of plant viruses by aphid vectors. *Mol. Plant Pathol.* **5**, 505–511 (2004).
58. Crawley, M. J. The R Book. John Wiley & Sons, Chichester, U.K. (2007).
59. Sarria, E., Cid, M., Garzob, E. & Fereres, A. Excel workbook for automatic parameter calculation of EPG data. *Comput. Electron. Agric.* **67**, 35–42 (2009).
60. Gonzalez-Rabade, N. et al. Immunogenicity of chloroplast-derived HIV-1 p24 and a p24-Nef fusion protein following subcutaneous and oral administration in mice. *Plant Biotech. J.* **9**, 1–10 (2011).

Acknowledgements

We thank Peter Palukaitis and John Walsh for valuable discussions. Research was funded by the Biotechnology and Biological Research Council (Grants BB/D008204/1 and BB/



D014376/1), The Leverhulme Trust (Grant F/09 741/F), the Cambridge University Newton Trust, and The Chadacre Trust. HZ was supported by a Walter Scott Fellowship from Trinity Hall, Cambridge, UK.

Author contributions

H.Z. and A.M.M. designed and performed experiments and wrote the paper; S.C.C., T.T., J.H.W., M.G.L., and M.M. performed experiments; A.K. analyzed the numerical data and contributed to experimental design; A.G.S., M.S., and G.P. devised experiments and contributed through discussion and revision of the paper; J.P.C. initiated the project, designed experiments and wrote the paper.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Ziebell, H. *et al.* Cucumber mosaic virus and its 2b RNA silencing suppressor modify plant-aphid interactions in tobacco. *Sci. Rep.* 1, 187; DOI:10.1038/srep00187 (2011).