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Title: **A densovirus induces winged morphs in asexual clones of the rosy apple aphid, *Dysaphis plantaginea***

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Abstract

Winged morphs of aphids are essential for their dispersal and survival. We discovered that the production of the winged morph in asexual clones of the rosy apple aphid, *Dysaphis plantaginea*, is dependent on their infection with a DNA virus, *Dysaphis plantaginea densovirus* (DpIDNV). Virus-free clones of the rosy apple aphid, or clones infected singly with an RNA virus, rosy apple aphid virus (RAAV), did not produce the winged morph in response to crowding and poor plant quality. DpIDNV infection results in a significant reduction in aphid reproduction rate but such aphids can produce the winged morph, even at low insect density, which can fly and colonize neighbouring plants. Aphids infected with DpIDNV produce a proportion of virus-free aphids, which enables production of virus-free clonal lines after colonization of a new plant. Our data suggest that a mutualistic relationship exists between the rosy apple aphid and its viruses. Despite the negative impact of DpIDNV on rosy apple aphid reproduction, this virus contributes to their survival by inducing wing development and promoting dispersal.

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Introduction

Polyphenism, the production of discrete phenotypes based on the same genome, plays a central role in biology. The life cycle of alternate, cyclically parthenogenetic aphid species includes both a sexual generation and a number of asexual generations (1). In asexually reproducing clones, genetically identical aphids are either wingless (apterae) or winged (alate). Apterae show maximum fecundity, allowing rapid colony growth during long day, warm conditions when resources are plentiful. Alates have lower fecundity, but are essential for dispersal and long-distance colonisation of new plants (2, 3). Alates are generally not produced during the asexual phase of reproduction unless there is stress resulting from crowding or poor nutritional resources. The wing development in asexual clones of aphids is influenced by interactions between environmental and intrinsic factors. Several cues are implicated, including temperature, population density (tactile stimulation), nutritional quality of the host plant, and interactions with natural enemies and ants, although these cues are not universal inducers for wing development in asexual clones of different lines of the same aphid species (4, 5, 6). Increased production of alates was observed in *Sitobion avenae* reared on oats infected with barley yellow dwarf virus (7), although infection of *Vicia faba* with pea enation mosaic virus, bean yellow mosaic virus or broad bean mottle virus did not increase production of alates in *A. pisum* (8). In addition, plant viruses have been reported to change aphid behaviour as a result of physiological changes in the infected plants, reviewed in (9).

Several viruses of aphids have been characterized including *Myzus persicae* densovirus (10); aphid lethal paralysis virus (11) and *Rhopalosiphum padi* virus (RhPV) (12), both members of the family Dicistroviridae; an iflavirus *Brevicoryne brassicae* virus (13) and the unclassified *Acyrtosiphon pisum* virus (APV) (14). Relatively little is known about the effect of these virus infections on aphid physiology, however a recent study reported a change in olfactory behaviour in response to RhPV infection (15). We discovered two viruses in the rosy apple aphid, *Dysaphis plantaginea*, which occur singly or as mixed infections. These are *Dysaphis plantaginea* densovirus (DplDNV) and rosy apple

aphid virus (RAAV). Here we report that the densovirus DplDNV plays a central role in induction of wing development and dispersal of asexual clones of the rosy apple aphid, which suggests that a mutualistic relationship exists between the rosy apple aphid and its viruses.

Results

Virus diversity in the rosy apple aphid. The original rosy apple aphid clones were established from single adults collected from apple trees at the end of summer 2002, in Warwickshire, United Kingdom. Clones were maintained as asexual lineages on plantain, the summer host of the rosy apple aphid, under long day conditions (16 h light / 8 h dark), at constant temperature $+20^{\circ}\text{C}\pm 1^{\circ}\text{C}$. Under these conditions the rosy apple aphid completes its lifecycle (from newborn nymph to reproducing adult) in approximately two weeks. Two of the clones, WS and 2-11, contained a proportion of a different phenotype, which was smaller, darker and had an ability to produce the winged morph. This was not observed in the other clones of rosy apple aphid.

Two approaches were used for virus discovery. Firstly, aphids were screened for viruses with high similarity to previously reported aphid (insect) viruses, using PCR and RT-PCR with specific and degenerate primers. Secondly, we used the method for the amplification of encapsidated RNA and DNA (13, 16). Both strategies resulted in the identification of cDNA fragments from an RNA virus showing high sequence similarity with a virus from the pea aphid, APV (14). Amplification of DNA also resulted in the identification of DNA fragments encoding peptides having sequence similarity with proteins of densoviruses, an insect-infecting group of the Parvoviridae family (17). Two types of spherical virus particles, 22.0 ± 1.5 nm and 32.0 ± 1.5 nm (mean \pm SD) with the buoyant densities of 1.35 - 1.45 g/cm³ in CsCl, were isolated from the aphids from clone WS [supporting information (SI) Fig. S1A]. The size of smaller particles was within the range reported for densoviruses (17), while the size of the larger particles was similar to that of APV (14). We determined the nucleotide sequences of the genomes of the novel viruses, which we named *Dysaphis plantaginea* densovirus, DplDNV (a DNA virus), and rosy apple aphid virus, RAAV (an RNA virus). The genome organisation of DplDNV

resembles that of the other members of the genus *Densovirus* (17) (Fig. S1B). The DplDENV ORF4 protein shows highest similarity with the coat protein of MpDENV (10) (37% aa identity). We identified densovirus sequences, MpDENV and putative *A. pisum* densovirus, in expressed sequence tags (ESTs) derived from aphid laboratory cultures of *M. persicae* and *A. pisum* (Tables S1, S2; Fig. S2), suggesting that densoviruses may occur in a range of aphid species. The positive-strand RNA genome of RAAV has the same organisation as the genome of APV (14) (Fig. S1C) and shows high similarity with it (87% aa identity).

Both RAAV and DplDENV infections were confirmed by (RT) PCR in clones WS and 2-11 of rosy apple aphid (Fig. S1 D,E). RAAV was present in all tested adult aphids and fourth instars from clone WS, while results from PCR showed evidence that DplDENV is less abundant in light aphids without wing buds compared to dark aphids with wing buds and winged aphids (Fig. S2F). Indeed qPCR showed that the levels of accumulation of DplDENV DNA in light aphids from the clone WS were significantly lower than those in the dark or winged aphids of clone WS (Table S3).

Transmission of rosy apple aphid viruses. Both RAAV and DplDENV nucleic acids were detected by (RT) PCR in plantain leaf tissue previously exposed to infected aphids. Replication of these viruses in plant cells is highly unlikely, since no increase in virus concentration was observed following the removal of the aphids from the plants. The majority of progeny nymphs by the aphids with high RAAV, reared on artificial diet, without exposure to plants, were RAAV-free; in total, only 1 out of 27 clones established from the individual progeny nymphs from by the aphids with high RAAV levels (from clones WS and R3) was RAAV-infected, the remaining 26 clones were RAAV-free (Table S4). When virus-free aphids were exposed to leaves previously contaminated by direct exposure to infected aphids they became RAAV positive. Aphids placed on leaves from distant unexposed parts of the same plant also became RAAV positive. It is likely that horizontal transmission of RAAV involves the plant vascular system, as in the case of the transmission of another aphid virus, RhPV (18, 19), and leafhopper A virus (20). Plant-mediated horizontal transmission of DplDENV also takes place but only from leaves

that have been in direct contact with the DpIDNV-infected aphids. Thus, both DpIDNV and RAAV associated with plant tissue are the source of inocula for their horizontal transmission. We observed also that there is efficient vertical transmission of DpIDNV from infected adults to nymphs, with the majority of nymphs produced by DpIDNV-infected aphids reared on artificial diet being DpIDNV-infected; 9 out of 10 clones established from individual progeny nymphs from aphids with high DpIDNV levels (from clone WS) were DpIDNV-positive (Table S4). Nevertheless, a proportion of the progeny from DpIDNV-infected aphids was DpIDNV-free.

Testing of Koch's postulates. Production of genetically identical rosy apple aphid clones infected with different virus combinations were required to complete of Koch's postulates. We established both the virus-free clone (clone 2D) and the DpIDNV infected clone (clone 10A) from clone WS (DpIDNV and RAAV infected) by propagation and selection on artificial diet. In addition, we established clone R3, which is infected only with RAAV, from clone WS by selection on plants (Fig. 1A). Aphid clones that are virus free or infected with only one of the viruses are susceptible to the other virus/viruses, namely RAAV and/or DpIDNV. The virus purification included homogenisation of aphids in 0.1 M sodium phosphate buffer (pH 7.5) followed by filtration through a 0.8/0.2 μm filter (Pall Gelman Laboratory), to exclude bacterial and fungal pathogens. An additional CsCl gradient centrifugation step was included in the preparation of virus inocula for microinjection and diet transmission experiments. The fraction with a buoyant density of 1.35 - 1.45 g/cm^3 in CsCl contained only DpIDNV and/or RAAV. It was diluted fivefold with 0.1M sodium phosphate buffer (pH 7.5) and the virus particles were pelleted by centrifugation (30,000 g, 3 h, 4°C). The virus concentration in the preparations was determined by real-time PCR. Aphids could be infected by: feeding on artificial diet containing a DpIDNV virus preparation (Table 1); by injection of a DpIDNV or RAAV virus preparation into the aphid haemolymph (Table 1); via plant tissue, either by rearing the recipient aphids on leaves previously exposed to virus-infected aphids, or by direct application of a virus preparation onto the leaf surface. Plant-mediated infection with DpIDNV or RAAV occurred in all of the triplicated experiments where groups of five virus-free

aphids were placed for seven days, either on the leaves previously exposed to the aphids infected with DpIDNV or RAAV or on leaves on to which virus preparations had been applied. No virus infections were resulted when leaves were exposed to the virus-free aphid cultures or were coated with a preparation from the virus-free aphids.

Characteristics of the aphid clones. Clone WS contained two phenotypes; dark with developing wing buds, and light (Fig. 1A), which were both smaller than the virus-free aphids of clone 2D. The levels of DpIDNV in the dark aphids were one thousand to ten thousand times higher than in the light aphids (Fig. 1B, 2G, S1F; Table S3). The low levels of DpIDNV observed in the light aphids, which were higher than the detection threshold, could have been the result of either early stage infection or surface contamination. It is also possible that some individuals can resist DpIDNV replication because of differences in immune response. Indeed, the DpIDNV-free clone R3 originated from a nymph of a light aphid adult from clone WS. When ten light aphids from clone WS with low DpIDNV levels were used to establish a colony, the resulting colony contained both dark and light aphids with high and low levels of DpIDNV, respectively, after two weeks of rearing (Table 2). Significantly, dark aphids with the high levels of DpIDNV produced nymphs, which developed into both dark (high DpIDNV levels) and light (low DpIDNV levels) phenotypes (Table 2). The proportion of dark and light aphids in individual colonies from clone WS was variable. Usually, the proportion of the dark aphids (with high levels of DpIDNV) was lower in younger colonies compared to more established colonies.

Aphids from clone 10A (infected with DpIDNV but RAAV-free) were smaller and darker than the virus-free aphids of the clone 2D (Fig 1A). All the darker aphids from clone 10A had developing wing buds, as well as high levels of DpIDNV; equivalent to those for the dark aphids in the clone WS. The remainder of the aphids from clone 10A had much lower levels of DpIDNV, similar to those in the light aphids of the clone WS (ANOVA, LSD test, $P < 0.05$) (Fig 1B). In aphids from clone Inj-9, originating from a single aphid from clone 2D after injection with a DpIDNV virus preparation, the levels of DpIDNV accumulation in dark aphids from the resulting progeny were similar to those in clone

10A (Fig. 1B). RAAV infection did not have a significant impact on the accumulation of DpIDNV (Fig. 1B) (ANOVA, LSD test, $P < 0.05$).

The levels of RAAV in both light and dark aphids from clone WS were similar (Fig. 1B). Interestingly, the levels of RAAV in the aphids from the DpIDNV-free clone R3 were slightly lower than in the clone WS (ANOVA, LSD test, $P < 0.05$) (Fig. 1B). Therefore, we cannot exclude the possibility, that DpIDNV may have a positive effect on RAAV replication.

DpIDNV infection resulted in increased movement and local dispersal of wingless aphids. While the DpIDNV-free aphids (clones 2D and R3) congregated at the plant base (Fig. 3A), the DpIDNV-infected aphids (clones WS and 10A) dispersed over the whole plant (Fig. 3B,C). In triplicate experiments 10 aphids from each clone were placed at the base of 15 cm long plantain leaves and allowed to establish colonies. The numbers of aphids were recorded for the upper and the lower (up to 5 cm from the leaf base) parts of the leaves from each plant. A significant difference was observed between aphid numbers for DpIDNV infected and DpIDNV-free clones found in the upper areas of the leaves (ANOVA, LSD test, $P < 0.05$). In the case of DpIDNV-free clones 2D and R3, 7.0 ± 2.08 and 6.0 ± 1.53 aphids (which was $2.67\% \pm 0.79\%$ and $2.40\% \pm 0.65\%$ of the total aphid number on a plant) were located in the upper areas, which was lower than in the case of DpIDNV-infected clones 10A and WS, 23 ± 2.65 and 20.3 ± 1.2 aphids ($45.18\% \pm 6.34\%$ and $27.48\% \pm 4.71\%$ of the total aphids on a plant) (Fig. S3).

Effect of virus infection on rosy apple aphid fecundity. The DpIDNV had a negative effect on fecundity in the case of both single DpIDNV infection and mixed infection (DpIDNV with RAAV). Plant aphid propagation experiments showed that there was a significant reduction in the offspring from clones infected with DpIDNV (10A and WS) compared with aphids from uninfected clone 2D and RAAV-infected clone R3, Table 2 and Table 3 (ANOVA, LSD test, $P < 0.05$). Such a reduction in the production of nymphs in DpIDNV-infected clones could be the result of either a pathological effect of DpIDNV infection on reproduction or a consequence of the high proportion of insects undergoing wing

development. Indeed, it has been reported that the number of nymphs produced by alates is lower than that of apterae (5).

DpIDNV induces development of the winged morph in asexual clones of the rosy apple aphid.

Winged morphs are essential for aphid dispersal and it is reported that they are produced in response to high population densities and poor plant quality (1). No winged morphs were observed in the case of the virus-free clone 2D and the RAAV-infected clone R3, even at high population densities and poor plant quality when reared under long day conditions. However, our rosy apple aphid clones, including 2D and R3, all produced sexual winged morphs under short day “autumn” conditions (8 h light/ 16 h dark, +15°C). Under long-day conditions, winged rosy apple aphids (Fig. 2C,F) and aphids with wing buds (Fig. 2B,E) were found only in clones, which were infected with DpIDNV, clones WS, 10A (Fig. 1B; Table S3). Also the infection of virus-free clone 2D with DpIDNV by injection, artificial feeding or via plant tissue resulted in the induction of the winged morph production, which was observed first approximately two weeks post-inoculation (Table 1, Fig. 1B). Regardless of how DpIDNV was introduced, aphids with high levels of DpIDNV from clones WS, 10A, Inj-9 reared under long day conditions were darker and showed the presence of developing wing buds (Fig. 2B,E,G) or had wings (Fig. 2C,F,G). There was no significant difference in DpIDNV DNA accumulation between these groups (ANOVA) (Fig. 2G). All aphids without wing buds or wings in the same clones were lighter and had low levels of DpIDNV (Fig. 1B, 2G). This was also observed in the DpIDNV-free clones 2D and R3 (Fig. 2A,D,G). The proportion of the aphids with high levels of DpIDNV (dark fourth instars with wing buds and the winged aphids) was significantly higher in clone 10A ($50.72\% \pm 4.14\%$) than in clone WS ($24.98\% \pm 3.99\%$) (ANOVA, LSD test, $P < 0.05$) (Table 3; Table S5). We suggest that the difference in DpIDNV infection rates in clones 10A and WS (affecting the proportion of dark and winged aphids in the population) may be attributed to the presence of RAAV in clone WS. It may be possible that RAAV reduces the infectivity of DpIDNV in mixed infections by reducing the rate of transmission and ultimately the proportion of dark and winged aphids in the population.

Wing development in DpIDNV-infected aphids is not induced by crowding but occurs in DpIDNV-infected aphids from the clones 10A and WS even when reared singly from the first instar on detached leaves. No wing development was observed in aphids from DpIDNV-free clones 2D and R3 under the same conditions (Table S6).

DpIDNV-induced dispersal of rosy apple aphid. The presence of the winged morph exclusively in DpIDNV-infected clones of rosy apple aphid prompted us to test whether these aphids had an increased ability to disperse and colonise neighbouring plants. Tests were carried out under controlled environmental conditions, as well as in the field. In the controlled environment experiments we tested the ability of aphids to fly from one plant to another inside an insect-proof chamber (Table 3, Fig. S4A). One plant was infested with ten aphids (adults or fourth instars) and acted as the aphid donor plant; the other plant, located 85 cm apart, was the recipient plant. Five replicate chambers were set up for each of the rosy apple aphid clones. After 11 days under long day conditions, colonisation of the recipient plants occurred in all five chambers where the donor plant was infested with clone 10A (DpIDNV-infected). In the case of clone WS (DpIDNV and RAAV infected) colonisation of the recipient plants was observed in three out of five chambers. No dispersal was observed in the five chambers where the donor plants were infested with aphids from virus-free clone 2D or the RAAV-infected clone R3 (Table 3, Table S5).

We also assessed the effect of plant quality on wing production under controlled environmental conditions. The experiment was set up using the same design, except that the donor plants were deprived of water after placing the aphids on the donor plant. By day 11 the majority of donor plants had died. We observed colonisation of 3 out of 5 recipient plants in the case of clones WS and 10A. No colonisation took place in the case of clones 2D and R3.

The field dispersal test involved four outdoor chambers. Each chamber contained a donor plant infested with one of the model clones (2D, 10A, R3, or WS). The donor and recipient plants were placed 2 m apart in each chamber (Fig. S4B, C). The recipient plants were colonised by aphids after 18 days in the case of the DpIDNV-infected clones 10A and WS, but not in the case of clones R3 and 2D, which

were free from DpIDNV. DpIDNV infection was detected in the aphids from the new colonies established on the colonised plants in the case of clones 10A and WS.

Discussion

There is limited information on the physiological and ecological impacts of viruses on insects, in particular those that cause sub-lethal infections, due to the paucity of studies on insect viral diversity. We used molecular screening to determine virus diversity in the rosy apple aphid, which resulted in the identification of two novel viruses, an RNA virus RAAV and a DNA virus, DpIDNV.

Asexual propagation in aphids is of the ameiotic type and does not involve endomeiosis or internal chromosomal recombination (21, 22, 23). Therefore, it is possible to produce and maintain rosy apple aphid clones with identical genotypes that are infected with different combinations of viruses. A surprising result was the difference in the development of the winged morph in virus-infected and virus-free cultures. Crowding and poor quality diet were reported as cues responsible for inducing wing development in asexual aphids as early as the 1920s (24). However, these cues are not universal inducers for wing development in all aphid species or even in different lines of the same species (4, 5). We found that winged morphs are not produced in clones that are DpIDNV-free (including RAAV-infected clone R3), even in response to crowding and poor plant quality under long day conditions. Conversely, winged morphs are produced by aphids with the same genetic background, even at low population density in the presence of DpIDNV, either as a single infection (clone 10A) or together with RAAV (clone WS). High levels of DpIDNV are detected in clones WS and 10A, exclusively in dark coloured aphids (fourth instars), which also have clearly developed wing buds and in winged adults. We found that while RAAV infection has no effect on the accumulation of DpIDNV in individual aphids, there is a possibility that RAAV may lessen the negative impact of DpIDNV for the whole colony by inhibiting the development of DpIDNV infection in some individuals. Indeed, a reduction in the proportion of dark aphids (all of which had wing buds and high levels of DpIDNV) was observed in the case of the clone WS compared to the clone 10A (Table 2). This suggests that the proportion of aphids

with wing buds is reduced in the presence of RAAV, which may account for the increased fecundity of clone WS. Alternatively, DpIDNV may have a direct effect on fecundity.

Winged morphs are essential for the dispersal of an aphid clone and, ultimately, for its survival. We hypothesise that despite the negative impact of DpIDNV on the fecundity of the rosy apple aphid, DpIDNV infection has a net positive effect in regard to dispersal. The glasshouse and the field dispersal experiments clearly showed that only the DpIDNV-infected aphids from clones WS and 10A were able to produce the winged morph, fly and colonize new plants (Table 3). In no cases were DpIDNV-free aphids found on recipient plants. The increased spread of aphids in DpIDNV-infected clones on the host plant (Fig. 3B,C) may be more advantageous to the virus rather than the aphid. Behavioural changes aiding virus dissemination have been reported for virus infections in other orders of insects (25). Even vertebrate viruses cause changes in behaviour, e.g. rabies virus, which are advantageous for the dissemination of the virus (26).

We propose the following model for the role of a virus in aphid dispersal. The progeny from a single winged aphid infected with both RAAV and DpIDNV includes insects with both high and low DpIDNV levels. The higher fecundity of the aphids with the lower levels of DpIDNV leads to an increase in the proportion of these aphids on a plant. However, since the rate of horizontal transmission for DpIDNV increases with the population density, the proportion of the aphids with high levels of DpIDNV also increases. These virus-infected aphids are likely to develop wings and colonise neighbouring plants. Density-dependent induction of the winged morph development has been reported for asexual clones of other aphid species (1, 2, 4) and may also be the result of the increased incidence of virus due to increased horizontal transmission under conditions of crowding. Densovirus ESTs were derived from aphid laboratory cultures with winged asexual females (Tables S1, S2; Fig. S2) suggesting that densovirus induced wing formation may occur in other aphid species.

The induction of winged morph development as a result of virus infection offers some advantages. All the offspring from an asexually reproducing aphid clone are genetically identical and will respond in a similar way to changes in external factors. However, if a virus infection is present in a

proportion of individuals in a colony, any resulting epigenetic changes may modify their response to environmental cues. Indeed, the non-structural proteins of vertebrate parvoviruses can activate transcription factors (27), as well as induce epigenetic modification through histone acetylation (28).

Our data suggests that a mutualistic relationship exists between the rosy apple aphid, RAAV and DpIDNV. The subliminal nature of the DpIDNV and RAAV infections ensures the survival of both viruses and the host. The DpIDNV induces the winged morph and increases mobility, which facilitates the dispersal of the host, as well as the viruses. The presence of RAAV decreases the DpIDNV-associated loss of the host fecundity. Such interdependence and existence of mutualistic relationships may indicate a long co-evolution of the three components of this system, which has resulted in the minimization of the virus-induced harm to the host and the development of dependence on a virus for some physiological processes (wing development). Indeed, recent reports have indicated that viruses may be beneficial to their hosts. For example, a latent herpesvirus infection confers resistance to harmful bacteria in mice and possibly humans by activating the immune system (29). Pathogens, including viruses, are an integral part of natural systems; therefore they are likely to play a role in “normal physiology”.

Materials and Methods

Aphid rearing. The clones of rosy apple aphid, *Dysaphis plantaginea* (Passerini) (Hemiptera: Aphididae), were reared on *Plantago longifolia* (plantain) in isolated growth chambers at 20°C±1°C under long day conditions, 16/8-h light/dark cycle. In artificial diet experiments, aphids were placed in the chambers containing artificial aphid diet (Bio-Serv Inc., Frenchtown, NJ) under stretched Parafilm.

Virus discovery and sequencing. Virus screening and sequencing were carried as described previously (6, 7). The amplifications of the full-length RAAV cDNA and the complete coding sequence of DpIDNV were carried out with Phusion DNA polymerase (Finnzyme), using cDNA to RNA and DNA, which were extracted from virus preparations from clone WS, and the primers 5'-GCTATAATACGACTCACTATAGGCGAAAATAAGTATATATTGCTTTTATTTTCG-3' and 5'-

CGGTGTTTAAAC(T)₂₇ATTTTGCCCAAATATGCTTTGCATAAACTATATAC-3' for RAAV or the primer 5'-GAACAAGTAACCAGTCGTAAGGTGC-3' for DplDNV. Mapping of the DplDNV transcripts included amplification of the fragments of the DplDNV mRNA with a series of primer pairs covering the DplDNV genome, using the cDNA to DNaseI-treated total RNA extracted from the DplDNV-infected aphids. The 3' termini of the DplDNV transcripts were mapped using a 3'-RACE RLM kit (Ambion).

Virus detection and quantification. The total RNA and DNA samples were isolated from the individual aphids, either fourth instars or adults, using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen). The cDNA to the total RNA was synthesised using the random hexanucleotides and Superscript II Reverse Transcriptase kit (Invitrogen). Detection of RAAV was carried out using the cDNA to total RNA with the primers 5'-ATGAGCGGCGCGCCAATGAATAGATCGGCTCCTAATAAC-3' and 5'-CACCATTGCTGAGGAAAAGTTTAAAGAATAACCTTTCTTTG-3'. Detection of DplDNV was carried out using total DNA extracts with the primers 5'-GAAAGCGGAGGTTCAAATGCAAGAC-3' and 5'-GAACCAGTTTGTGCGACAATTG-3', which flank the intron region in the non-structural protein gene of DplDNV. PCR was performed using *Taq* polymerase (Roche). Amplification included 5 min at 94°C and 35 cycles (94°C for 30 sec, 53°C for 1 min, 72°C for 1 min). Real-time quantitative PCR was performed in duplicates using the SYBR Green kit (Eurogentech). RAAV RNA was quantified using the cDNA with the primers 5'-AGAGAACGGAGTTGTTTATTACTACGAA-3' and 5'-TATGGAAATACCATCTTGGGAGTTG-3'. The DplDNV DNA was quantified from total DNA with the primers 5'-CGCCCGCGTAAATGGATATTATGGCG-3' and 5'-GATGGTCGTGACGCTGTTGTTT-3'. Amplification was performed in 20 µL reactions using ABI Prism 7900HT system (Applied Biosystems) and included 2 min at 50°C, 10 min at 95°C and 40 cycles (95°C for 15 sec, 60°C for 1 min). Viral DNA and RNA levels were determined using the comparative Ct analytical method with a cloned part of DplDNV genomic DNA and an *in vitro* RNA transcript

produced from the cloned cDNA to genomic RNA of RAAV as reference standards for qPCR detection of DplDNV and qRT-PCR detection of RAAV, respectively.

Microinjection. The virus and control preparations were resuspended in Schneider's cell culture medium and injected into the haemolymph of fifth instars of rosy apple aphid using the Femtojet (Eppendorf). Approximately 2.3 ± 0.2 nL of the virus suspension (DplDNV concentration 1 ng/ μ L or RAAV concentration 5 ng/ μ L) were administered using a capillary inserted in the lower side of insect abdomen using a compensatory pressure of 20 hPa, five 1s pulses and an injection pressure of 60 hPa.

Aphid dispersal. Dispersal tests in a controlled environment were carried out at $20^\circ\text{C} \pm 1^\circ\text{C}$ under 16/8-h light/dark cycle. Each pair of the donor and the recipient plantain plants, approximately 10 cm high, were located 85 cm apart within a separate insect-proof chamber, 100 cm long, 30 cm high and 20 cm wide, made from the transparent plastic with the nylon mesh-covered openings on two sides to ensure access and ventilation (Fig. S4A). Potted plants were placed in water traps to prevent movement of aphids from one plant to another by crawling. Watering of the plants and filling of the water traps were carried out through built-in piping.

The field dispersal tests were carried out in Warwickshire, UK from 13th June until 1st July 2008. The donor plantain plant with an aphid culture (which was developed for two weeks from 10 aphids) was placed at a distance of 2 m from the recipient plantain plant. The donor and recipient plants were placed inside an insect-proof tent-shaped chamber made of a nylon mesh, 2.5 m long, 1 m wide, 0.85 m high (Fig. S4B,C). The plants were placed inside water traps to exclude the crawling of the aphids between plants (Fig. S4C).

Microscopy. For transmission electron microscopy, the virus suspension deposited on a carbon-coated grid was negatively stained with 2% potassium phosphotungstate (pH7.0). For scanning electron microscopy, the anaesthetised aphids were attached to the stub with carbon paste, cryo-fixed and gold coated on a cryostage and viewed in the frozen hydrated state.

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References

1. Dixon AFG (1998) in *Aphid Ecology* (Chapman and Hall, London).
2. Zera AJ, Denno, RF (1997) Physiology and ecology of dispersal polymorphism in insects. *Annual Review of Entomology* 42:207-230.
3. Ishikawa A, Hongo S, Miura T (2008) Morphological and histological examination of polyphonic wing formation in the pea aphid *Acyrtosiphon pisum* (Hemiptera, Hexapoda). *Zoomorphology* 127:121-133.
4. Müller CB, Williams IS, Hardie J (2001) The role of nutrition, crowding and interspecific interactions in the development of winged aphids. *Ecological Entomology* 26: 330-340.
5. Braendle CG, Davis K, Brisson JA, Stern DL (2006) Wing dimorphism in aphids. *Heredity* 97:192–199.
6. Sloggett JJ, Weisser WW (2001) Parasitoids induce production of the dispersal morph of the pea aphid, *Acyrtosiphon pisum*. *Oikos* 98:323-333.
7. Gildow, FE (1980) Increased production of alate by aphids (Hemiptera, Aphididae) reared on oats infected with Barley yellow dwarf virus. *Ann. Entomol. Soc. Am.* 73: 343–347.
8. Hodge S, Powell G (2008) Do plant viruses facilitate their aphid vectors by inducing symptoms that alter behavior and performance? *Environ Entomol.* 37:1573-1581.
9. Fereres A, Moreno A (2009) Behavioural aspects influencing plant virus transmission by homopteran insects. *Virus Research* (Article in press) doi:10.1016/j.virusres.2008.10.020
10. van Munster M, *et al.* (2003) A new virus infecting *Myzus persicae* has a genome organization similar to the species of the genus *Densovirus*. *J Gen Virol* 84:165–172.

11. van Munster M, *et al.* (2002) Sequence analysis and genomic organization of Aphid lethal paralysis virus: a new member of the family Dicistroviridae. *J Gen Virol* 83:3131–3138.
12. Moon JS, Domier LL, McCoppin NK, D’Arcy CJ, Jin H (1998) Nucleotide sequence analysis shows that Rhopalosiphum padi virus is a member of a novel group of insect-infecting RNA viruses. *Virology* 243:54–65.
13. Ryabov EV (2007) A novel virus isolated from the aphid Brevicoryne brassicae with similarity to picorna-like viruses infecting Hymenoptera. *J Gen Virol* 88: 2590-2595.
14. Van der Wilk F, Dullemans AM, Verbeeck M, Van den Huevel, JFJM. (1997) Nucleotide sequence and genome organisation of *Acyrtosiphon pisum* virus. *Virology* 238: 353-362.
15. Ban L, Ahmed E, Ninkovic V, Delp G, Glinwood R (2008) Infection with an insect virus affects olfactory behaviour and interactions with host plant and natural enemies in an aphid. *Entomologia Experimentalis et Applicata* 127: 108-117
16. Allander T, *et al.* (2005) Cloning of a human parvovirus by molecular screening of respiratory tract samples. *Proc Natl Acad Sci USA* 102: 12891-12896.
17. Tattersall P, *et al.* (2005) Family Parvoviridae. In *Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses*, eds Fauquet, CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (London, Elsevier -Academic Press), pp. 353-369.
18. Ban L, Didon, A, Jonsson LMV, Glinwood R, Delp G (2007) An improved detection method for the Rhopalosiphum padi virus (RhPV) allows monitoring of its presence in aphids and movements within plants. *J Virol Meth* 142: 136-142.
19. Gildow FE, D’Arcy CJ (1988) Barley and oats as reservoirs for an aphid virus and the influence on barley yellow dwarf virus transmission. *Phytopathology* 78:811-816.
20. Ofori FA, Francki, RIB (1985) Transmission of leafhopper A virus, vertically through eggs and horizontally through maize in which it does not multiply. *Virology* 144:152-157.
21. Blackman RL (1979) Stability and variation in aphid clonal lineages. *Biological Journal of the Linnean Society* 11: 259 - 277.

22. Blackman RL (1987) Reproduction, cytogenetics and development. In *Aphids: Their Biology, Natural Enemies and Control*. eds Minks AK, Harrewijn P (Elsevier, Amsterdam) pp163-195.
23. Hales DF, *et al.* (2002) Lack of detectable genetic recombination on the X chromosome during the parthenogenetic production of female and male aphids. *Genetic Res. Cambridge* 79: 203-209.
24. Wadley FM (1923) Factors affecting the proportion of alate and apterous forms of aphids. *Annals of the Entomological Society of America* 16: 229-232.
25. Kamita SG, *et al.* (2005) A baculovirus-encoded protein tyrosine phosphatase gene induces enhanced locomotory activity in a lepidopteran host. *Proc Natl Acad Sci U S A* 102:2584-2589.
26. Fu ZF, Jackson AC (2005) Neuronal dysfunction and death in rabies virus infection. *Journal of NeuroVirology* 11: 101–106.
27. Duechting A, *et al.* (2008) Human parvovirus B19 NS1 protein modulates inflammatory signalling by activation of STAT3/PIAS3 in human endothelial cells. *J Virol* 82:7942-7952.
28. Iseki H, *et al.* (2005) Parvovirus nonstructural proteins induce an epigenetic modification through histone acetylation in host genes and revert tumor. *J Virol* 79: 8886–8893.
29. Barton ES, *et al.* (2007) Herpesvirus latency confers symbiotic protection from bacterial infection. *Nature* 447:326-329.

Figure legends

Figure 1. RAAV and DpIDNV in the rosy apple aphid. (A) Genetically identical aphids infected with different virus combinations. (B) Accumulation of RAAV and DpIDNV in individual aphids. Clone WS was originated from a single doubly infected aphid; clones 2D, R3 and 10A were produced by selecting progeny of clone WS. Clone Inj-9 was produced from a virus-free aphid of clone 2D following injection with a DpIDNV virus preparation from clone 10A.

Figure 2. DpIDNV-induced wing development in asexual clones of the rosy apple aphid. Scanning electron microscopy. (A) Adult aphid from clone 2D (virus-free). (D) Adult aphid from clone R3 (RAAV-infected). Aphids from clone 10A (DpIDNV-infected): (B) fourth instar with wing buds, (C) winged adult. Aphids from the clone WS (DpIDNV and RAAV infections): (E) fourth instar with wing buds, (F) winged adult. Wing buds are indicated with arrows. Bars are 1 mm. (G) Accumulation of DpIDNV DNA in individual light, dark (with wing buds) and winged aphids. ND - non detectable levels of DpIDNV DNA, n/a – not applicable. Clone Inj-9 was produced from a single aphid from clone 2D injected with a DpIDNV virus preparation; the progeny was sampled four weeks post injection. Bars depict the accumulation of DpIDNV DNA (mean \pm standard error) in individual aphids. Numbers of the sampled aphids are shown in parenthesis. Values without significant difference (ANOVA) are indicated with asterisks (*).

Figure 3. Dispersal of DpIDNV-infected aphids. Plantain two weeks post infestation with 10 aphids from clones (A) R3 (localised) and (B, C) WS (dispersed). Bars are 1 cm.



Clone 2D

Clone 10A

Clone R3

Clone WS
"Dark"

Clone WS
"Light"

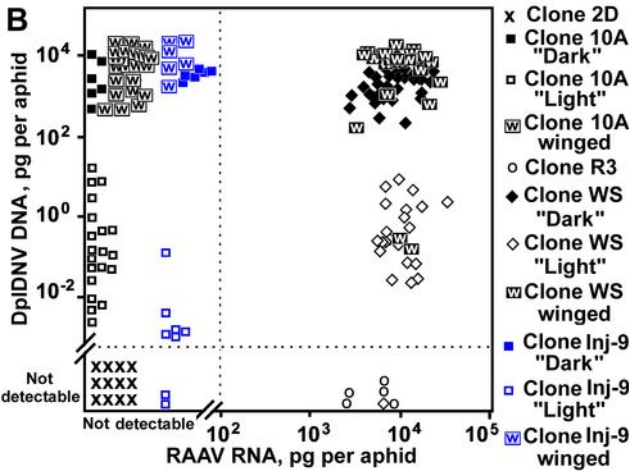
RAAV-free
DpIDNV-free

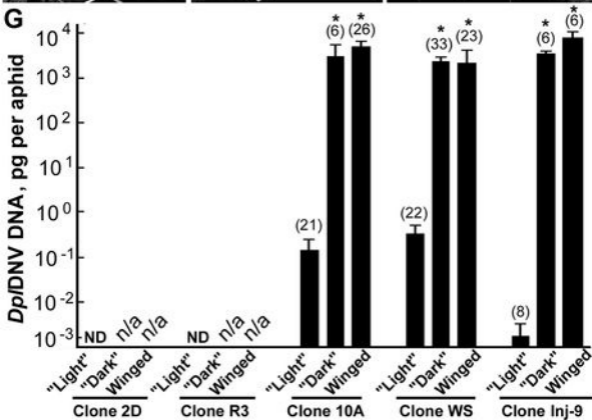
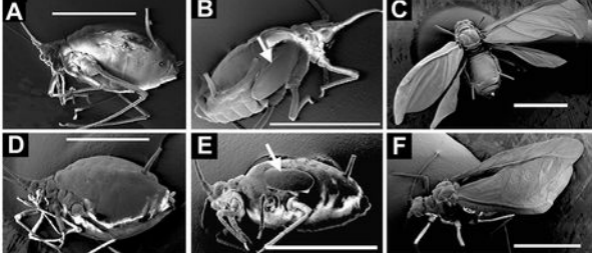
DpIDNV
RAAV-free

high RAAV
DpIDNV-free

high RAAV
high DpIDNV

high RAAV
low DpIDNV





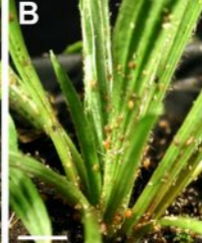


Table 1. Infection of the rosy apple aphids with RAAV and DpIDNV virus preparations*.

Inoculum [†]	Colonies with the winged morph [‡]			Colonies without the winged morph [§]		
	Virus-free	DpIDNV	RAAV	Virus-free	DpIDNV	RAAV
Artificial diet inoculation [¶]						
Control	0	0	0	12	0	0
DpIDNV	0	5	0	7	0	0
Inoculation by injection						
Control	0	0	0	11	0	0
DpIDNV	0	3	0	6	0	0
RAAV	0	0	0	4	0	6

* Fifteen fifth instars from virus-free clone 2D were used for infection for each group. Inoculation and progeny rearing on plantain plants was carried out at 20°C±1°C on a 16/8-h light/dark cycle. Virus accumulation was tested in the established colonies four weeks post inoculation (w.p.i.) by (RT) PCR in pooled samples extracted from twenty randomly selected fourth and fifth instars.

[†] Virus preparations from DpIDNV-infected aphids, clone 10A (DpIDNV), RAAV-infected aphids, clone R3 (RAAV), or DpIDNV-free aphids, clone 2D (Control).

[‡] Number of the established colonies with the winged morph, first appeared 2 w.p.i..

[§] Number of the established colonies without the winged morph, monitored up to 4 w.p.i..

[¶] Individual aphids were fed on artificial diet containing the DpIDNV virus preparation (20pg/μL of DpIDNV DNA) or a control virus-free preparation for 72 h. The surviving parent female aphid and progeny nymphs from each chamber were transferred to individual plantain plants to establish a colony.

^{||} Fifth instars aphid were injected with 2.3±0.2 nL of virus or control suspensions in Schneider's cell culture medium. The DpIDNV virus preparation contained 1ng/μL of DpIDNV DNA; the RAAV virus preparation contained 5 ng/μL of RAAV RNA, and the control virus-free suspension was isolated from virus-free aphids using the same method as that for virus isolation.

Table 2. Reproduction rate, wing development and DplDNV infection in the rosy apple aphid clones.

Clone*	Aphids [†]	Winged [‡]	DplDNV [§]	High DplDNV level [¶]	
				Wingless	Winged
2D	1550	0	-	0/20	n.a.
R3	1050	0	-	0/20	n.a.
10A	335	10	+	6/20	9/10
WS light	1382	39	+	5/20	4/10
WS dark	917	10	+	3/20	5/10

*Ten adult aphids were placed on a 20 cm-high plantain plants at 20°C±1°C on a 16/8-h light/dark cycle. The figures refer to the day 14 after the start of the experiment.

[†]Total number of parent and progeny aphids.

[‡]Numbers of aphids with fully developed wings.

[§]DplDNV DNV was tested by qPCR in the pooled samples extracted from 100 randomly selected wingless aphids.

[¶]Number of the aphids with more than 10 pg of DplDNV DNA per aphid per number of aphids tested.

Table 3. Effect of the DpIDNV and RAAV on the reproduction and dispersal of rosy apple aphids in a controlled environment*

Clone (Viruses)	Dispersal [†]	Donor plant [‡]	Recipient plant [§]	Winged [¶]	% dark aphids
2D (none)	0/5	215.8 ± 45.8	0	0	0
R3 (RAAV)	0/5	163.6 ± 49.5	0	0	0
10A (DpIDNV)	5/5	71.2 ± 14.3	11.4 ± 3.5	12.6 ± 3.6	35.4 ± 7.1
WS (DpIDNV, RAAV)	3/5	55.4 ± 25.0	9.6 ± 5.7	3.4 ± 0.7	11.1 ± 2.6

*Five cages were used for each aphid clone. Ten aphids were placed on donor plant in each cage and were reared at 20°C±1°C on a 16/8-h light/dark cycle. All the figures in the table refer to the day 11 after the start of the experiment.

[†]Number of cages where colonisation the recipient plant took place / total number of cages.

[‡]Number of aphids on the donor plants (mean ± standard error).

[§]Number of aphids on the recipient plants (mean ± standard error), only for the infested plants.

[¶]Number of winged aphids (including winged aphids on the both plants, and in the cage but not on the plants).

^{||}Percentage of dark-coloured aphids in cage, except the aphids with developed wings (mean ± standard error).