Plant-mediated interspecific horizontal transmission of an intracellular symbiont in insects

Elena Gonella1, Massimo Pajoro1,†, Massimo Marzorati1‡, Elena Crotti2, Mauro Mandrioli3, Marianna Pontini1, Daniela Bulgari4, Ilaria Negri3, Luciano Sacchi5, Bessem Chouaia2, Daniele Daffonchio2,6 & Alberto Alma1

Intracellular reproductive manipulators, such as Candidatus Cardinium and Wolbachia, are vertically transmitted to progeny but rarely show co-speciation with the host. In sap-feeding insects, plant tissues have been proposed as alternative horizontal routes of interspecific transmission, but experimental evidence is limited. Here we report results from experiments that show that Cardinium is horizontally transmitted between different phloem sap-feeding insect species through plants. Quantitative PCR and in situ hybridization experiments indicated that the leafhopper Scaphoideus titanus releases Cardinium from its salivary glands during feeding on both artificial media and grapevine leaves. Successional time-course feeding experiments with S. titanus initially fed sugar solutions or small areas of grapevine leaves followed by feeding by the phytoplasm vector Macrosteles quadripunctulatus or the grapevine feeder Empoasca vitis revealed that the symbionts were transmitted to both species. Explaining interspecific horizontal transmission through plants improves our understanding of how symbionts spread, their lifestyle and the symbiont-host intermixed evolutionary pattern.

The intracellular bacterium “Ca. Cardinium hertigii”1 (hereafter Cardinium) is an intriguing arthropod symbiont capable of reproductive manipulation2–5 or of determining effects ranging from stimulation of the host’s immune response6, to fecundity benefits5,7, or even to reduction of the host’s fitness8. Cardinium is a common symbiont of insects9,10, mites11, spiders12,13, and opilionids14. Cardinium is maintained in a given host population via maternal transmission to progeny through the egg cytoplasm. However, vertical transmission is not sufficient to explain the observed co-speciation patterns with insect hosts. Moreover, various Cardinium lineages are shared with different and distantly related host species9,15. Such a patchy evolutionary pattern indirectly suggests that Cardinium may also exchange hosts via horizontal transmission patterns16.

Horizontal transmission has been observed in several host models. Aphid symbionts may be transferred orally when individuals share the same food source17–19. Environmentally acquired symbiotic Burkholderia are transferred among individuals of the broad-headed insect Riptortus clavatus20. The actinobacterial
symbiont *Coriobacterium glomerans* of the red firebug *Pyrrhocoris apterus* and the symbionts of the firebrat *Thermobia domestica* are acquired horizontally by juveniles through symbiont-containing eggshells, faeces, or adult bugs21,22. *Rickettsia* symbionts of the whitefly *Bemisia tabaci* can be acquired by its parasitoids23 or by other whiteflies feeding on the same plant24. Phyllogenetic analyses of *Wolbachia*, a reproductive manipulator, and its associated hosts showed horizontal transmission between prey and predator mites25 and between hosts and parasitoids26, as well as among insects sharing the same food source or parasites27-28. Direct demonstrations of intra- or inter-specific horizontal transmission of *Wolbachia* from the host to a parasitoid29, or in parasitoids sharing the same host eggs30-31, have been reported in few host models. Horizontal transmission has also been directly or indirectly observed in *Arsenophonus nasoniae*, which is transferred among parasitic wasps sharing the same hosts32. On the other hand, in the case of *Cardinium*, there has only been speculation on possible horizontal transmission33-35.

Here we demonstrate that an inter-specific horizontal transmission of *Cardinium* can take place in leafhoppers and that such a transmission occurs through the plant tissue pierced by the insect host. By using the cicadellid *Scaphoideus titanus*, a *Cardinium*-holder strictly feeding on grapevine leaves, as a model, we show that *Cardinium* is released from the insect's salivary glands to the plant tissues and then horizontally acquired by other grapevine feeder and non-grapevine feeder insects.

**Results**

**Cardinium release in artificial feeding media and plant tissue.** The first set of experiments was conducted to assess the capability of *S. titanus* to release *Cardinium* during feeding on an artificial substrate (a feeding medium consisting of a sterile sugar solution) or on grapevine leaves. *S. titanus* individuals used in the inoculation experiments without antibiotic treatment were found to be highly colonized by *Cardinium*. The infection rate and the concentration of *Cardinium* cells were very similar in insects fed on the artificial media and the grapevine leaves (Table 1). In rifampicin-treated insects fed on both substrates, *Cardinium* was found as well, and the average infection rate and density of symbiont cells per leafhopper were not significantly lower than those in untreated specimens. However, according to the *Cardinium*-to-bacteria ratio (CBR), the percentage of *Cardinium* in the whole leafhopper bacterial community after the rifampicin treatment was drastically decreased in both experimental settings (Table 1).

*Cardinium* was consistently found to colonize *S. titanus* individuals, which were found to be able to inoculate symbiont cells in either feeding substrate and in either the absence or the presence of antibiotic treatment. However, both inoculation/infection rates and titres decreased in the insects treated with rifampicin compared with those that were untreated regardless of the feeding substrate. These values consistently declined after antibiotic treatment in the sugar diet and the grapevine diet, and a statistical significance was detected between samples with or without rifampicin. The CBR calculated for the diet samples followed a similar decrease (Table 1).

While grapevine leaves collected from *S. titanus*-free plants were always negative for the bacterium, 34.3% of grapevine leaves collected from plants in the field infested by the leafhopper were *Cardinium*-positive (Table 1). Even so, the average infection rate and titre were not significantly higher than the average infection rate and titre of grapevine leaves exposed to antibiotic-treated *S. titanus*.

We tested some of the salivary glands of *S. titanus* and the artificial feeding media and grape leaves using *in situ* hybridization (ISH) and fluorescence *in situ* hybridization (FISH). Hybridization signals related to the *Cardinium* probes were detected (Figs 1E–H, 2). TEM observations of the salivary glands of *S. titanus* confirmed the presence of bacterial cells with microtubular-like complexes typical of *Cardinium*36 (Fig. 1D). Moreover, FISH experiments on grapevine leaves collected in *S. titanus*-infested vineyards in the field also indicated the presence of *Cardinium* bacterial cells (Fig. 2B). On the other hand, when antibiotic-treated leafhoppers and their respective feeding substrates were tested by FISH, no hybridization signal was detected for *Cardinium*. Negative controls exhibited a lack of hybridization as well (Fig. S3).

**Cardinium transmission experiments.** Following the demonstration of the capability of *S. titanus* to inoculate *Cardinium* cells in its feeding medium, we tested if other insects could then acquire *Cardinium* from the feeding medium. We utilized the leafhoppers *Macrosteles quadripunctulatus* and *Empoasca vitis* as receiver hosts. Prior to the experiment, we assessed the *Cardinium* infection status of the recipient species. We did not detect the bacterium neither in *M. quadripunctulatus* nor in *E. vitis* individuals that had never been exposed to *S. titanus’s* feeding media. Transmission experiments via artificial feeding media (*M. quadripunctulatus*) or grapevine leaves (*E. vitis*) were then conducted. *Cardinium* was initially detected by qPCR in all *S. titanus* donor individuals. Moreover, in both cases, a transfer of *Cardinium* cells to *M. quadripunctulatus* and *E. vitis* was detected either by qPCR or by FISH experiments (Table 2; Fig. 3).

In the *M. quadripunctulatus* experiments, the *Cardinium* infection rate in recipients was initially about 50% (after one and three days of co-feeding) and then increased after one week. However, the average titres of *Cardinium* in positive individuals were modest with respect to donors in all experiments (with a peak after three days of co-feeding). The CBR in the recipient *M. quadripunctulatus* was low as well, following a similar trend of the titres (Table 2).
Table 1. Cardinium infection in S. titanus fed on antibiotic-treated or untreated artificial media or grapevine leaves. The average Cardinium titre is expressed as Cardinium cells [=Cardinium 16S rRNA gene copies/sample]. Samples are defined as follows: single insect, single diet unit (300 μl sugar solution), 100 mg of grapevine leaf. Values below the detection limit (1.66 × 10^1 Cardinium cells/sample) were considered negative. For grapevine leaves, three controls are indicated: seedlings are vines employed for inoculation trials before use; healthy plants are grapevines never exposed to S. titanus; field plants were considered negative. For grapevine leaves, three controls are indicated: seedlings are vines employed for inoculation trials before use; healthy plants are grapevines never exposed to S. titanus; field plants were considered negative. For grapevine leaves, three controls are indicated: seedlings are vines employed for inoculation trials before use; healthy plants are grapevines never exposed to S. titanus; field plants were considered negative.

| Samples                      | Avg Cardinium infection rate and (SE)* | Avg. Cardinium cell number and (SE) * | Range of Cardinium cell number | CBR Avg and (SE)* | CBR Range |
|------------------------------|----------------------------------------|---------------------------------------|---------------------------------|-------------------|-----------|
| Without antibiotic           |                                        |                                       |                                 |                   |           |
| Insect (on diet)             | 94.3 (5.7) a                           | 6.41 × 10^6                            | <1.66 × 10^1–1.92 × 10^9         | 0.0638 (0.0243)   | 0.0–0.7253 |
| Diet                         | 62.9 (13.4) abc                        | 3.20 × 10^6                            | <1.66 × 10^1–5.33 × 10^9         | 0.0402 (0.0237)   | 0.0–0.4709 |
| Insect (on leaves)           | 97.1 (2.9) a                           | 7.74 × 10^6                            | <1.66 × 10^1–4.68 × 10^9         | 0.1115 (0.0515)   | 0.0–0.7257 |
| Grapevine                    | 68.6 (7.4) abc                         | 8.70 × 10^6                            | <1.66 × 10^1–8.06 × 10^9         | ND*               | ND*       |
| With antibiotic              |                                        |                                       |                                 |                   |           |
| Insect (on diet)             | 94.3 (5.7) a                           | 4.94 × 10^6                            | <1.66 × 10^1–3.35 × 10^9         | 0.0463 (0.0247)   | 0.0–0.4895 |
| Diet                         | 22.9 (6.8) d                           | 3.54 × 10^6                            | <1.66 × 10^1–1.50 × 10^9         | 0.0018 (0.0014)   | 0.0–0.0059 |
| Insect (on leaves)           | 80.7 (10.9) ab                         | 2.06 × 10^6                            | <1.66 × 10^1–1.92 × 10^9         | 0.0383 (0.0211)   | 0.0–0.147  |
| Grapevine                    | 45.7 (12.1) bcd                        | 2.23 × 10^6                            | <1.66 × 10^1–9.82 × 10^9         | ND*               | ND*       |
| Grapevine controls           |                                        |                                       |                                 |                   |           |
| Seedlings                    | Nd*                                    | <1.66 × 10^1 d                         | Nd*                             | Nd*               | Nd*       |
| Healthy plants               | Nd*                                    | <1.66 × 10^1 d                         | Nd*                             | Nd*               | Nd*       |
| Field plants                 | 34.3 (9.5) cd                          | 3.16 × 10^6                            | <1.66 × 10^1–5.83 × 10^9         | ND*               | ND*       |

In the E. vitis experiments, both infection rates and concentrations were quite variable, with a peak after one day of co-feeding, a decrease after three days and a new increment after seven days. Interestingly, although the Cardinium concentration decreased in the seven-day trial, the CBR was higher for the longer co-feeding time; furthermore, Cardinium remained above 1% of total bacteria and always represented a higher percentage of the microbial community in E. vitis compared with M. quadripunctulatus (Table 2).

The transmission was confirmed by FISH and sequencing. Indeed, a massive hybridization signal correlated with Cardinium was detected in the midguts of M. quadripunctulatus and E. vitis after three days of co-feeding (Fig. 3A,C), whereas the negative controls were free of fluorescence signals related to probe hybridization (Fig. S4). Moreover, 835 bp amplicons were sequenced after the Cardinium-specific PCR was performed on both M. quadripunctulatus and E. vitis used in the co-feeding experiments. The sequences were identical to the deposited sequences of the Cardinium symbiont of S. titanus (AM042540), clustering in the A-group1 (Fig. S5).

Discussion

This study sought to verify if S. titanus is able to inoculate Cardinium into the surrounding feeding substrate during its feeding activity and then if Cardinium in the feeding substrate can be taken up by other insects. By means of qPCR and FISH results, we firstly demonstrated that S. titanus injects the symbiont into its feeding substrates, both under controlled conditions (artificial diet) and in a semi-natural environment (grapevine leaves) (Table 1, Fig. 2). High concentration values of Cardinium were observed in the leaffoppers and in their artificial diet, in agreement with previous data16. Additionally, the detection of Cardinium in grapevine leaves, used for feeding by insects infected by the symbiont, suggests that Cardinium can spend part of its life cycle in grapevine leaf tissues, exploiting the plant as a means to be transmitted among S. titanus populations, as previously observed in other plant sap-feeders17,18,24. The observation of the symbiont in grapevine plants in the field where S. titanus is naturally present...
suggests that the leafhopper regularly releases Cardinium into the plant tissues, although infection rates and symbiont densities observed in the field were lower than those detected in grapevine leaves experimentally exposed to the leafhopper. This finding opens questions on the nature of the interaction between Cardinium and grapevine leaves, as well as the occurrence of the symbiont in other plants hosting sap-feeding insects, in light of the wide diffusion of this bacterial symbiont among arthropods\textsuperscript{9,37}.

However, when S. titanus individuals were treated with rifampicin, we were not able to obtain completely Cardinium-free specimens. Moreover, these antibiotic-treated insects were still able to inoculate Cardinium into their feeding substrates (Table 1). The use of antibiotics was demonstrated to inhibit the occurrence of many symbionts completely\textsuperscript{38–40} even after a few days of administration\textsuperscript{41,42}. On the contrary, symbiotic bacteria in some cases could still be found in their hosts after antibiotic treatment\textsuperscript{42,43}. A reduction of the Cardinium titre by about one order of magnitude after rifampicin treatment was not as high as previously reported by Pajoro et al.\textsuperscript{16}, while it was in the same range of the results obtained by Boucias and colleagues\textsuperscript{43} for Burkholderia in the heteropteran Blissus insularis Barber. Due to the impossibility of obtaining Cardinium-cured S. titanus, we employed different Cardinium-free leafhopper species as recipients to demonstrate the symbiont acquisition step. Both M. quadripunctulatus and E. vitis were able to acquire Cardinium that then persisted in the two recipient species (Table 2, Fig. 3).

Figure 1. Morphology of the S. titanus head (including salivary glands) and occurrence of Cardinium in the salivary glands. (A) Section of the head of S. titanus, stained with hematoxylin/eosin. b = brain; e = eye; sg = salivary glands; opl = optical lobe; s = stylet. Bar = 0.25 mm. (B) Stylet, particularly showing cibarial pump muscles. (C) Salivary glands, particularly showing the lumen (arrows). b = brain; m = medulla; sg = salivary gland. Bar = 100\textmu m. (D) Micrograph of Cardinium showing the microtubular-like complex with numerous tubules (asterisks) and the Gram-negative architecture of the bacterium cell wall. m = mitochondrion. (E,F)ISH of the head (E, bar = 0.25 mm), showing positive staining (red) for Cardinium probes (arrow) in the salivary gland (F, particular of the framed part in E. Bar = 50\textmu m). e = eye; l = salivary gland lumen; opl = optical lobe; sg = salivary gland. (G,H) Confocal laser scanning microscopy images of salivary glands after hybridization with Cy5-labelled Cardinium-specific probes (G, green) and Texas Red-labelled eubacterial probes (H, red) probes. Blue spots indicate DAPI-stained gland nuclei. Bar = 50\textmu m.
longer periods may be necessary to achieve stable colonization. It would be of great interest to elucidate whether Cardinium is able to persist in the new host and finally to be maternally transmitted. Moreover, because M. quadripunctulatus is known to be a very efficient phytoplasma vector, this species may be particularly suitable for bacterial invasion of the salivary glands, leading to high transmission competence. The observation of the acquisition of Cardinium by M. quadripunctulatus raises a question about

Figure 2. FISH experiments on feeding substrates after exposure to S. titanus. (A, B) Video-confocal micrographs of a grapevine leaf midrib after it was used by the leafhopper for feeding (A) and a field vine leaf portion (B) after hybridization with the Cy5-labelled Cardinium probes. Arrows indicate Cardinium cells within the phloem tissues (x = xylem). (C) FISH of a sugar diet exposed to S. titanus. Signals corresponding to both the FITC-labelled eubacterial probe (green) and the Texas Red-labelled Cardinium-specific probe (red) are visible. Bars = 50 μm (A) and (B); 2.5 μm (C).

Table 2. Results of Cardinium transmission experiments to M. quadripunctulatus and E. vitis. The average Cardinium titre determined by qPCR is expressed as Cardinium cells [Cardinium 16S rRNA gene copies] per insect. Values below the detection limit (1.66 × 10^3) were considered negative. The infection rate is expressed as percentage Cardinium-positive individuals related to the total tested specimens. Donor = S. titanus (ST); recipient = M. quadripunctulatus (MQ) or E. vitis (EV). The controls are M. quadripunctulatus or E. vitis individuals never submitted to co-feeding experiments. ANOVA tests were carried out comparing Cardinium infection rates and concentrations for M. quadripunctulatus and E. vitis separately. For each experiment, different letters indicate significantly different values (p < 0.05). *Avg: average values; SE: Standard Error; Nd: Not Detectable (Cardinium below detection limit 1.66 × 10^3).
the capability of newly colonized insect individuals to transmit the acquired symbiont subsequently in the feeding medium and possibly to other insects.

Varying results were obtained both in terms of infection rates and density in the *E. vitis* experiments. After one day of co-feeding, a high percentage of the recipient leafhoppers exhibited considerable amounts of *Cardinium* uptake. However, after three days, the infection rates and concentrations declined and then increased again after seven days (Table 2). This variability suggests that the success of *E. vitis* colonization by *Cardinium* may be due to chance more than to a solid capability of the symbiont to invade the tissues of this insect. Hence, even though we demonstrated that at least in some cases the ingestion of *Cardinium* can be followed by successful colonization, the symbiont is likely to be infrequently established in *E. vitis*. Nonetheless, the symbiont concentration in *Cardinium*-positive *E. vitis* was always higher than that observed in *M. quadripunctulatus*, and it even reached values as high as those detected for *S. titanus* (Table 2). This evidence indicates that the tissues of grapevine leaves are suitable media for the acquisition of high titres of this bacterium, possibly because *Cardinium* is concentrated in the phloem of leaves and is more likely to be ingested than *Cardinium* from the artificial diet in which the bacteria are dispersed throughout the sugar solution. Since we never found *Cardinium*-positive field-collected *E. vitis*, the transmission from *S. titanus* to this leafhopper via grapevine leaves is probably not persistent, and the bacterium may not find optimal conditions in the new host to be then vertically transferred to its progeny. However, given that *Cardinium* was detected in grapevine leaves in the field, it is apparent

Figure 3. FISH experiments on midguts of leafhoppers sharing food substrates with *S. titanus*. (A,B) Midgut portion of *M. quadripunctulatus* after a three-day co-feeding on the artificial diet. The cyan signal corresponds to hybridization with the *Cardinium*-specific probes (A), while the red signal corresponds to the eubacterial probe (B,C,D) Midgut segment of *E. vitis* after sharing a grapevine leaf with *S. titanus* for 3 days. The hybridization with the *Cardinium*-specific probe is marked in yellow (C), whereas the eubacterial probe signal is stained in red (D). Intestine tissues are coloured with DAPI (blue). Bars = 75 μm.
that this plant may be a continuous reservoir of the symbiont, at least for S. titanus, and acquisition of this bacterium by other phloem-feeding insects residing on grapevine leaves cannot be excluded.

The overall results provide direct demonstration of the horizontal transmission of Cardinium in phloem-feeding leafhoppers via co-feeding, previously widely hypothesized\(^9,24,35\) but never demonstrated for this symbiont. The data presented demonstrate and support previous molecular evidence that horizontal transfer of symbionts does actually occur and may contribute to symbiont spread in natural insect lineages\(^9,24,35\). Even though maternal transmission is the main diffusion system for reproductive manipulators such as Cardinium, we showed that horizontal transmission through plants provides an alternative route of spread of these symbionts. This finding has major implications for our understanding of the evolutionary history of Cardinium symbionts and suggests that these bacteria may have adapted to living in plant tissues outside the insect host. The genome of the Cardinium endosymbiont of the whitely Bemisia tabaci (a phloem feeder like S. titanus) showed peculiar traits like genes associated with gliding motility, which may be responsible for the colonization of new hosts\(^46\), possibly including plants. Further clues to the adaptation to life in the plant tissues should be sought in the available genomes of Cardinium and should suggest the possibility of a dual lifestyle of this ubiquitous symbiont.

**Methods**

**Insect rearing.** Third- and fourth-instar nymphs of S. titanus and E. vitis were collected during the early summer from vineyards in the Piedmont region of northwestern Italy (Fig. S1) between 2007 and 2012 and reared on healthy grapevine plants in laboratory cages at the Dipartimento di Scienze Agrarie, Forestali e Alimentari (DISAFA) in growth chambers at 25 °C and a photoperiod of 16:8 (L:D) h until adult emergence. Macrosteles quadripunctatus individuals were obtained from laboratory lines reared on oats (Avena sativa) in growth chambers with the same temperature and photoperiod conditions.

All experiments were conducted in accordance with the legislation and guidelines of the European Union for the protection of animals used for scientific purposes (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm). All experimental protocols using animals were approved by the ad-hoc Committee of DISAFA of the University of Turin. In addition, all necessary permits were in hand when the research was conducted.

**Cardinium inoculation experiments through artificial and plant diets.** To observe—under controlled conditions—the injection of bacterial cells by S. titanus while feeding, artificial feeding systems were set up (Fig. S2A). A total of 100 newly emerged adults were maintained on the artificial diet for three days according to\(^16\) (see also SI material and methods). Half of the specimens (50 insects) were reared on the artificial diet supplemented with 300 μg ml\(^{-1}\) rifampicin, known to be active against Cardinium\(^9,16\).

To confirm bacterial release under semi-natural conditions, a cage system on the grapevine leaves was set up\(^16,47\) with small plastic insect chambers placed on the surfaces of the leaves (Fig. S2B) to force a total of 90 S. titanus to feed on small areas of the plants for three days (see also SI material and methods). Half of the leaves were supplied with 300 μg ml\(^{-1}\) rifampicin together with a nutritive solution, and the leafhoppers were exposed to these treated leaves for three days.

A total of 35 leaves from grapevine seedlings never exposed to S. titanus were used as the control in the molecular analyses. Furthermore, to check for grapevine infection by Cardinium in the field, 45 leaves (35 for qPCR and 10 leaf midribs for FISH) from different Barbera plants in Piedmont vineyards with high S. titanus incidence were also tested.

**Cardinium transmission tests.** Two cicadellid species, the phytoplasma vector M. quadripunctatus and the grapevine feeder E. vitis, were used to evaluate the actual transfer of the symbiont to recipient insects after the inoculation by S. titanus during feeding events (see also SI material and methods). Twenty-five M. quadripunctatus and 25 E. vitis were firstly checked by PCR and qPCR (with Card192F/1069R and EndoF1/R3 primer pairs, respectively, as described in SI material and methods) for the actual absence of Cardinium in the used populations to verify whether these species were suitable for transmission experiments.

The horizontal transfer of Cardinium to M. quadripunctatus was assessed via feeding experiments in artificial media chambers (Fig. S2C). Adult donor individuals of S. titanus were maintained for three days in groups of 15 insects on sugar solutions in suitable feeding chambers to allow the symbiont to be released in the supplied substrate. They were then replaced by groups of 15 M. quadripunctatus adults maintained for different acquisition periods: one day, three days, and seven days.

The transmission of Cardinium to E. vitis occurred via the grapevine leaves. Single donor S. titanus specimens were forced to feed for three days on portions of Barbera grapevine leaves for the release of bacterial cells into the plant tissues and then removed and preserved for subsequent analyses. The same leaf portions were then exposed to E. vitis individuals to allow bacterium acquisition for one day, three days, and seven days.

**DNA extraction and PCR-based analyses.** Subsequent to the inoculation experiments, total DNA was extracted from the leafhoppers and the respective sugar diets and the leaf portions for molecular analyses. Nucleic acid extraction from the insects and artificial diet was performed as previously
reported\textsuperscript{47,48}, whereas plant DNA was extracted from the leaf portions previously ground with liquid nitrogen in a sterile mortar, according to DNeasy Plant Mini Kit protocol (Qiagen, Italy) instructions.

Quantitative real-time PCR was performed to measure the presence and concentration of Cardinium cells in the insects, artificial diet and leaves. In all samples, reactions targeting the 16S rRNA gene of Cardinium were carried out. In addition, insect DNA was submitted to qPCR targeting the insect's 18S rRNA gene to normalize the absolute Cardinium density. Furthermore, insect and artificial diet DNA (but not the grapevine DNA due to a non-specific reaction with the plastids of bacterial universal primers) was used in reactions with universal bacterial primers to define the overall bacterial concentration in each sample. The CBR was then calculated to estimate the relative abundances of Cardinium in the bacterial community associated with the leafhoppers or with colonizing the diet. qPCR conditions are reported in the SI material and methods.

Subsequent to qPCR screening, 10 Cardinium-positive M. quadripunctatus and E. viti individuals were used for PCR screening (SI materials and methods). The obtained PCR products were purified and sequenced (Genechron, Rome, Italy), and the resulting sequences were compared with those in the National Center for Biotechnology Information (NCBI) sequence database by using BLAST (http://www.ncbi.nlm.nih.gov/blast). A phylogenetic analysis was performed using the MEGA 6 software by the neighbor joining method.

Transmission electron microscopy and in situ hybridization. Transmission electron microscopy was carried out on S. titanus individuals after dissection in saline solution, as previously described\textsuperscript{19}. ISH and FISH analyses were performed on the salivary glands of S. titanus, whereas the artificial feeding media, grapevine leaves, and Cardinium-recipient M. quadripunctatus and E. viti individuals were analyzed by FISH only.

ISH experiments were carried out by using specific oligonucleotide probes, Card172 and Card1069\textsuperscript{50}, 5' labelled with digoxigenin (DIG). FISH experiments performed on insect tissues and feeding media were carried out with Cardinium-specific probes along with a universal bacterial probe, which was employed as a positive control for the hybridization experiment. Conversely, for experiments on plant tissues only, Cardinium-specific probes were employed, due to non-specific hybridization of the eubacterial probe with the plastids. For the complete procedures, see also SI materials and methods.

References
1. Nakamura, Y. et al. Prevalence of Cardinium in planthoppers and spider mites and taxonomic revision of ‘Cardiulus’ Cardinium hortigai’ based on detection of a new Cardinium group from biting midges. Appl. Environ. Microbiol. 75, 6757–6763 (2009).
2. Zhori-Fein, E. et al. A newly discovered bacterium associated with parthenogenensis and a change in host selection behavior in parasitoid wasps. Proc. Natl. Acad. Sci. USA 98, 12555–12560 (2001).
3. Weeks, A. R., Marec, F. & Breeuwer, J. A Mitogen species that consists entirely of haploid females. Science 292, 2479–2482 (2001).
4. Hunter, M. S., Perlman, S. J. & Kelly, S. E. A bacterial symbiont in the Bacteroidetes induces cytoplasmic incompatibility in the parasitoid wasp Encarsia pergandei. Proc. Biol. Sci. 270, 2185–2190 (2003).
5. Weeks, A. R. & Southamer, R. Increased fecundity associated with infection by a Cytophaga-like intracellular bacterium in the predatory mite, Metaseiulus occidentalis. Proc. R. Soc. Lond. B Biol. Sci. 271, S193–S195 (2004).
6. Nakamura, Y. et al. Differentially expressed genes in silkworm cell cultures in response to infection by Wolbachia and Cardinium endosymbionts. Insect Mol. Biol. 20, 279–289 (2011).
7. Giorgini, M. et al. Feminization and the collapse of haplodiploidy in an asexual parasitoid wasp harboring the bacterial symbiont Cardinium. Heredity 102, 365–371 (2009).
8. Fang, Y.-W., Liu, L.-Y., Zhang, H.-L., Jiang, D.-F. & Chu, D. Competitive ability and fitness differences between two introduced populations of the invasive whitefly Bemisia tabaci Q in China. PLOS ONE 9, e100423 (2014).
9. Zhori-Fein, E. & Perlman, S. J. Distribution of the bacterial symbiont Cardinium in arthropods. Mol. Ecol. 13, 2009–2016 (2004).
10. Perlman, S. J., Kelly, S. E. & Hunter, M. S. Population biology of cytoplasmic incompatibility: maintenance and spread of Cardinium symbionts in a parasitic wasp. Genetics 178, 1003–1011 (2008).
11. Liu, Y., Miao, H. & Hong, X.-Y. Distribution of the endosymbiotic bacterium Cardinium in Chinese populations of the carmine spider mite Tetranychus cinnabarinus (Acari: Tetranychidae). J. Appl. Entomol. 130, 523–529 (2006).
12. Duron, O., Hurst, G. D. D., Hornett, E. A., Josling, J. A. & Engelstädter, J. High incidence of the maternally inherited bacterium Cardinium in spiders. Mol. Ecol. 17, 1427–1437 (2008).
13. Perlman, S. J., Magnus, S. A. A. & Copley, R. Pervasive associations between Cybaeus spiders and the bacterial symbiont Cardinium. J. Invertebr. Pathol. 103, 150–155 (2010).
14. Zhang, L., Masters, A., Avery, A. & Werren, J. H. A divergent Cardinium found in daddy long-legs (Arachnida: Opiliones). J. Invertebr. Pathol. 105, 220–227 (2010).
15. Breeuwer, H., Ros, V. I. D. & Groot, T. V. M. Cardinium: the next addition to the family of reproductive parasites. In Manipulative tenants: bacteria associated with arthropods, (eds Zchori-Fein, E. & Bouritzis, K.), 207–224 (CRC Press Taylor & Francis, 2012).
16. Pajoro, M. et al. Investigation on the life cycle of ST1-C, the endosymbiont of Scaphoideus titanus. Bull. Insectol. 61, 217–218 (2008).
17. Darby, A. C., Birkle, L. M., Turner, S. L. & Douglas, A. E. An aphid-borne bacterium allied to the secondary symbionts of whitefly. FEMS Microbiol. Ecol. 36, 43–50 (2001).
18. Darby, A. C. & Douglas, A. E. Elucidation of the transmission patterns of an insect-borne bacterium. Appl. Environ. Microbiol. 69, 4403–4407 (2003).
19. Russell, J. A., Latorre, A., Sabater-Muñoz, B., Moya, A. & Moran, N. A. Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. Mol. Ecol. 12, 1061–1075 (2003).
20. Kikuchi, Y., Hosokawa, T. & Fukatsu, T. Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. Appl. Environ. Microbiol. 73, 4308–4316 (2007).
21. Kaltenpoth, M., Winter, S. A. & Kleinhämmer. A. Localization and transmission route of Coriobacterium glomerans, the endosymbiont of phycorhodoid bugs. FEMS Microbiol. Ecol. 69, 373–383 (2009).
Acknowledgements
The authors are grateful to Federico Lessio for sample collection and insect rearing. This work was funded by the Italian Ministry for Research (MIUR), under project PRIN 2009 "Interazioni tra insetti vettori e microrganismi simbionti: nuove prospettive per il biocontrollo dei patogeni trasmessi alle piante, agli animali e all’uomo." DD acknowledges support from the King Abdullah University of Science and Technology.

Author Contributions
E.G., M.P., M.M., D.D. and A.A. designed the study; E.G., M.P., E.C., M.M., M.P., D.B., I.N., L.S. and B.C. performed the experiments; E.G. analyzed the data; and E.G., D.D. and A.A. wrote the paper.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.
How to cite this article: Gonella, E. et al. Plant-mediated interspecific horizontal transmission of an intracellular symbiont in insects. Sci. Rep. 5, 15811; doi: 10.1038/srep15811 (2015).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/