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Horizontal transmission of the insect symbiont *Rickettsia* is plant-mediated

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Bacteria in the genus *Rickettsia*, best known as vertebrate pathogens vectored by blood-feeding arthropods, can also be found in phytophagous insects. The presence of closely related bacterial symbionts in evolutionarily distant arthropod hosts presupposes a means of horizontal transmission, but no mechanism for this transmission has been described. Using a combination of experiments with live insects, molecular analyses and microscopy, we found that *Rickettsia* were transferred from an insect host (the whitefly *Bemisia tabaci*) to a plant, moved inside the phloem, and could be acquired by other whiteflies. In one experiment, *Rickettsia* was transferred from the whitefly host to leaves of cotton, basil and black nightshade, where the bacteria were restricted to the phloem cells of the plant. In another experiment, *Rickettsia*-free adult whiteflies, physically segregated but sharing a cotton leaf with *Rickettsia*-plus individuals, acquired the *Rickettsia* at a high rate. Plants can serve as a reservoir for horizontal transmission of *Rickettsia*, a mechanism which may explain the occurrence of phylogenetically similar symbionts among unrelated phytophagous insect species. This plant-mediated transmission route may also exist in other insect–symbiont systems and, since symbionts may play a critical role in the ecology and evolution of their hosts, serve as an immediate and powerful tool for accelerated evolution.

Keywords: Aleyrodidae; bacteriocyte; *Bemisia tabaci*; horizontal transfer; whiteflies

1. INTRODUCTION

Maternally inherited symbionts have profound and diverse effects on their arthropod hosts [1,2]. The presence of closely related symbionts in evolutionarily distant hosts presupposes inter- and intra-specific mechanisms of horizontal transmission [2]; yet no general mechanism appears to explain the diffuse phylogenetic pattern of lineages among hosts. Natural shifts of symbionts from one insect host species to another have been previously hypothesized and supported by phylogenetic evidence [3,4]. Although recent studies provided experimental support for this hypothesis [5,6], current knowledge cannot account for the majority of host associations. Horizontal transmission of bacteria via mating or cannibalism has also been recorded [7,8], but these routes are generally limited to conspecifics. Several studies demonstrate the potential for symbiont lineages to infect multiple hosts through artificial procedures, such as microinjection [9,10], suggesting that the physiological host range for symbionts may be broader than the patterns observed.

One hint about possible infection routes may come from insects that vector plant pathogens among host plants. For example, the bacteria *Spiroplasma citri*, *Spiroplasma kunkellii* and *Phlomobacter* are phloem-restricted plant pathogens that are horizontally transferred from diseased to healthy plants by insect vectors [11–13]. However, insect symbionts, which are not known as plant pathogens, have not generally been shown to be transmitted to plants. Such plant-mediated horizontal transmission was suggested by Sintupachee *et al.* [14], who found very closely related *Wolbachia* in taxonomically diverse insects sharing the same pumpkin host, but the presence of that symbiont in the plant was not verified.

Rickettsia (Alphaproteobacteria) are best known as pathogens of vertebrate hosts, vectored by blood-feeding arthropods [15], but recent surveys have uncovered many *Rickettsia* in non-blood-feeding hosts [16,17]. Although *Rickettsia* species have been reported to function as primary nutritional symbionts in one host, reproductive manipulators in others, and as an insect-vectored plant pathogen [4,18–20], their role in the vast majority of hosts is unknown [16,21].

Rickettsia was found in the sweetpotato whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), a polyphagus insect that feeds on phloem sap [22,23]. The *Rickettsia* in *B. tabaci* is in the well-defined *bellii* clade [17], dominated by lineages in herbivorous arthropods, such as aphids, leafhoppers and mites [16,22]. The lack of concordance of host and *Rickettsia* phylogeny suggests that horizontal transmission among host species has occurred multiple times in this group [17]. Here, we show for the first time that horizontal transmission of *Rickettsia* between conspecific whiteflies can be mediated by a plant.

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Figure 1. Results of PCR amplification of *Rickettsia* DNA in cotton leaves, using *Rickettsia* Pgt primers (table 2), demonstrating its presence. Samples 1–8: leaves not exposed to whiteflies; 9–15, 17: leaves exposed to R^+ whiteflies; 16: NC, negative control—DNA from basil leaves not exposed to whiteflies; 18: NTC, no template control; 19: PC, positive control—DNA from R^+ whiteflies.

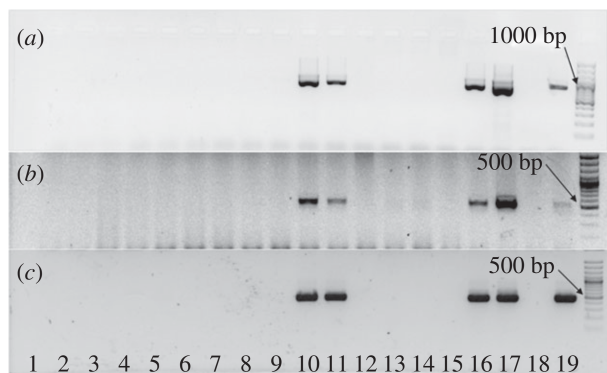

Figure 2. Results of PCR amplification of *Rickettsia* DNA in black nightshade and basil leaves demonstrating the presence of *Rickettsia* DNA. (*a*) PCR using *Rickettsia* GltA primers; (*b*) PCR using *Rickettsia* 16S rRNA primers; (*c*) PCR using *Rickettsia* Pgt primers (table 2). Samples 1–8: black nightshade leaves not exposed to whiteflies; 9–11: black nightshade leaves exposed to R^+ whiteflies; 12–15: basil leaves not exposed to whiteflies; 16, 17: basil leaves exposed to R^+ whiteflies; 18: NTC, no template control; 19: PC, positive control—DNA from leaves exposed to R^+ whiteflies.

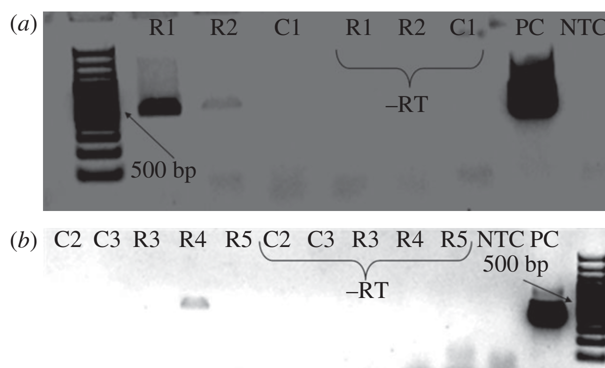

Figure 3. (*a*,*b*) PCR amplification of *Rickettsia* cDNA in cotton leaves using *Rickettsia* Pgt primers, demonstrating the presence of *Rickettsia* RNA in two runs. C—cDNA from leaves not exposed to whiteflies; R—cDNA from leaves of five different plants exposed to R^+ whiteflies; –RT, without the reverse transcriptase; NTC, no template control; PC, positive control—DNA from leaves exposed to R^+ whiteflies. The PCR on samples R1, R2 and R4 reveal the presence *Rickettsia* RNA, but the bacterium could not be seen in the absence of reverse transcriptase since DNA digestion was performed after RNA extraction.

2. RESULTS

(a) Rickettsia *in plant leaves*

(i) *Polymerase chain reaction tests*

When plants were exposed to whitefly adults carrying *Rickettsia* (R^+) for 20 days, the bacterium was detected by polymerase chain reaction (PCR) in most leaves of cotton (*Gossypium hirsutum* 'Acala'; six out of eight plants; figure 1), basil (*Ocimum basilicum*) and black nightshade (*Solanum nigrum*; both two out of three plants; figure 2). The *16S rRNA* and *Rickettsia Phosphoglycerol transferase gene* sequences retrieved from both cotton leaves and *B. tabaci* were 100 per cent identical. No *Rickettsia* was detected in leaves of the control plants. The presence of *Rickettsia* RNA was revealed in three out of five R^+-infested cotton leaf discs using RT-PCR (figure 3) while *Rickettsia* RNA was not found in either the no-whitefly or the reverse transcriptase enzyme-free controls. These results thus provide evidence for the transfer of a living, and presumably metabolically active, symbionts from an insect to a plant.

(b) *Visualization of* Rickettsia *using fluorescence* in situ *hybridization*

Once the presence of *Rickettsia* in leaves was established, its localization within the host plant was determined using fluorescence *in situ* hybridization (FISH) with a *Rickettsia*-specific probe. The symbiont was found in three out of 25 leaf-strips after exposure to R^+ whiteflies, always restricted to the phloem vessels (figure 4), but not in any of the control leaves.

The results suggest that once inside the plant, *Rickettsia* are restricted to the phloem, a localization pattern that reflects the phloem-limited feeding of the whitefly host and suggests an inability of the bacterium to invade neighbouring cells.

(c) *Horizontal transmission of* Rickettsia *between adult whiteflies*

To test whether *Rickettsia* found in the phloem cells of cotton can be acquired by another insect host, *Rickettsia*-free (R^-) *B. tabaci* adults were placed inside leaf cages and surrounded on the same leaf by R^+ whiteflies. PCR analysis showed that 10 days after exposure, the symbiont could be detected in adults from three of five cages (table 1) but not in a control experiment in which the surrounding whiteflies were *Rickettsia*-free (R^-).

3. DISCUSSION

Our results provide novel evidence for the presence of the insect symbiotic bacterium *Rickettsia* in plant leaves, where it establishes in phloem cells and can move from one whitefly host to the other. Importantly, the *Rickettsia* did not cause any visible pathogenic symptoms in the plant. These results are similar to those of Purcell *et al.* [24] who found by culture assays that the bacterial symbiont of the leafhopper *Euscelidius variegates* was transmitted from one insect to the other via feeding on the same plant. Their sap-sucking habit brings insect species of the order Hemiptera into close and constant contact with cells of the plant's circulatory system. This interaction is bidirectional since during the feeding process the insects both absorb nutrients from the plant and secrete various substances into it [25]. Our results suggest that this practice facilitates the transmission of their own symbionts. Within the body of *B. tabaci*, the symbionts *Rickettsia*, *Wolbachia* and *Cardinium* can be

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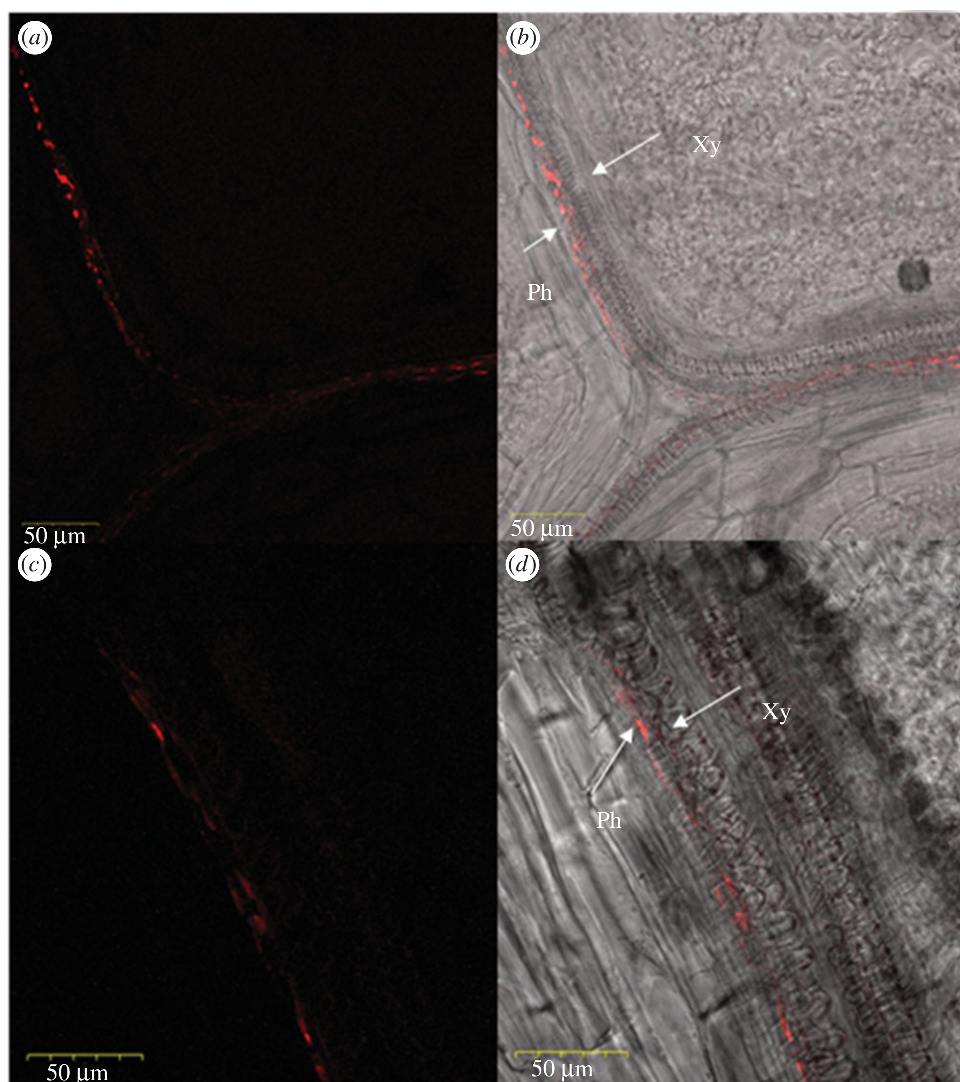

Figure 4. (*a-d*) FISH of two different cotton leaves with *Rickettsia*-specific probe for the Phosphoglycerol transferase gene. (*a*,*c*) *Rickettsia* channel; (*b*,*d*) overlay of *Rickettsia* on bright field channels; *Rickettsia* (red) can be seen in the phloem. Ph, phloem; Xy, xylem.

Table 1. *Rickettsia* frequency found in *Rickettsia*-free (R^-) whiteflies exposed to R^+ (treatment) or R^- (control) whiteflies. The exposure time was 10 days.

	treatment		control	
replicates	number of R^- females exposed to R^+	% *Rickettsia* in R^- females	number of R^- females exposed to R^-	% *Rickettsia* in R^- females
1	5	0	7	0
2	7	0	9	0
3	4	50	18	0
4	5	20	5	0
5	3	33	9	0
sum	24		48	

seen dispersed throughout the haemolymph, occasionally in very high numbers, in contrast to symbionts, such as *Portiera*, *Arsenophonus* and *Hamiltonella*, which are confined within specific bacteriocytes [22,26]. It can thus be speculated that symbionts which are free within the haemolymph can invade the insect salivary glands and consequently be injected via the stylets into the plant phloem, while bacteriocyte-confined bacteria are not likely to be transmitted through this route.

Although in their vertebrate and invertebrate hosts *Rickettsia* are obligate intracellular bacteria, they frequently move inside and between host cells and tissues by inducing the formation of a phagocytic vacuole [27]. Once inside the host cell, the spread of *Rickettsia* is facilitated by its ability to induce actin-tail formation by manipulating host cytoskeletal proteins [28]. This mode of active movement cannot occur in the plant phloem, which consists of tube-like, anucleate sieve elements

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Table 2. PCR primer sets used in this study.

expected size (bp)	nucleotide sequence 5′ to 3′	primer set	reference	gene
~800	AGGTTTAGGCTAGTCTACACG	Pgt-F1	this paper based on *Rickettsia bellii* genome	*Rickettsia* phosphoglycerol transferase
	GTCTACGCACGATTGATG	Pgt-R1		
~500	ACTCATGAAATTATCGGCACAG	Pgt-F2		
	GCATGAATTTGGCACTTAAGC	Pgt-R2		
~550	ACTAATCTAGAGTGTAGTAGGGGATGATGGG	528-F	Werren *et al.* [4]	*Rickettsia* 16S rRNA
	TTTTCTTATAGTTCCTGGCATTACCC	1044-R		
~800	TCCTATGGCTATTATGCTTG	GltA-F	this paper based on *Rickettsia bellii* genome	*Rickettsia* citrate synthase
	CCTACTGTTCTTGCTGTGG	GltA-R		

lacking a cytoskeleton in their mature stage [29]. In the absence of other evidence, it is therefore reasonable to assume that within its plant host the bacterium is moving with the phloem current, and in whitefly-free plants parts will be found downstream of feeding sites.

Although it is not yet known whether the symbiont reproduces within its plant host, the *Rickettsia* RNA recovered provides evidence that the bacteria are alive within cotton leaves. The only other evidence of *Rickettsia* in plants comes from papaya, where the bacterium has been reported to be pathogenic [19]. However, no disease symptoms were seen in cotton plants exposed to R^+ *B. tabaci* throughout our experiments. Nevertheless, the presence of the bacterial invader may have consequences for the plant. For example, whitefly feeding usually activates pathogenesis-related (PR) protein genes in plants that are associated with anti-pathogenic responses [30,31]. Our results suggest that the transfer of *Rickettsia* might contribute to this unique plant response to phloem-feeders. The interactions between whiteflies and their hosts are complex, and if plant responses are indeed mediated by the transferred symbionts, they could have a cascade of effects on the entire community, i.e. competitors and natural enemies [20].

A survey of field-collected *B. tabaci* in the southwestern USA recently revealed a dramatic sweep of *Rickettsia* into *B. tabaci* populations (from 1% infection in the year 2000 to 97% by 2006) [32]. In that study, horizontal transmission of *Rickettsia* was not found, and the different results may reflect differences in symbiont–host–plant interactions in the two regions, or simply differences in methods used to detect it. Many species of hemipteran insects feed on plant phloem, and that common feeding ground may serve as a route by which *Rickettsia* and other symbiotic micro-organisms can be acquired frequently enough and in high enough numbers to allow the establishment of new symbiotic associations. Indeed, the closest relative to the *Rickettsia* in *B. tabaci* is found in the phloem feeding pea aphid *Acyrthosiphon pisum* [17]. It should be noted that our results demonstrate only the uptake of *Rickettsia* from the plant by *B. tabaci*; whether the newly acquired bacterium is transmitted to the next generation remains to be tested. Determining the frequency of interspecific transmission will require an understanding of the spread of the bacterium in the plant, its persistence, the frequency of encounters of phloem-feeders with the bacterium, and the rate at which *Rickettsia* is able to establish and form a permanent association with a new host.

4. MATERIAL AND METHODS

(a) *Whitefly rearing*

Bemisia tabaci colonies were reared on cotton (*Gossypium hirsutum* 'Acala') under standard greenhouse conditions: $26 \pm 2°C$, 60 per cent relative humidity, and a photoperiod of 14 L : 10 D. The lines used in the experiments were B biotype *B. tabaci* harbouring either the scattered *Rickettsia* (R^+) [26] or no *Rickettsia* (R^-) [33], both originated from a laboratory population at the ARO, Israel.

(b) Rickettsia *in plant leaves*

(i) *PCR tests*

To verify the presence of *Rickettsia* in cotton leaves, and assess possible horizontal transmission through leaves of other plant genera, two leaf cages (5 cm diameter) sealed with a non-drying adhesive (Rimifoot, Rimi chemicals, Petach Tiqva, Israel) were placed on either cotton (*Gossypium hirsutum* 'Acala'), basil (*Ocimum basilicum*) or black nightshade (*Solanum nigrum*) plants. The plants were then placed in the R^+ *B. tabaci* rearing cage and 20 days later, the leaf discs that were protected from whiteflies under the leaf cages were excised. To detect the presence of *Rickettsia*, DNA was extracted from the leaf discs following the procedure described by Fulton *et al.* [34], and was consequently used as a template in a PCR using *Rickettsia*-specific Pgt, 16S rRNA and GltA primers (table 2). Whitefly-free plants (10 cotton, eight basil and 10 black nightshade) were used as negative controls. PCR reactions were performed in 25 µl volume containing 3 µl of the template DNA lysate, 10 pM of each primer, 0.2 mM dNTP's, 1X Red Taq buffer and one unit of Red Taq DNA polymerase (Sigma). PCR products were stained with ethidium bromide and visualized on a 1.2 per cent agarose gel. For detecting *Rickettsia* with Pgt gene in plants, we used nested PCR. The procedure is similar to the PCR routine described earlier. After amplification with Pgt-F1/Pgt-R1, the product was diluted 1 : 100 in water and 3 µl of the dilution was used for another PCR reaction with internal primers—Pgt-F2/Pgt-R2 (table 2). To sequence the products, bands were isolated and cloned into the pGEM T-easy plasmid vector (Promega), transformed into *Escherichia coli*, and for every band, two colonies were randomly picked and sequenced. Sequencing was done in three replicates for each colony, and all sequences obtained for each gene were assembled into a consensus sequence, which was compared with known sequences in databases using the BLAST algorithm in NCBI. Sequence has been deposited into the GENBANK database under accession number JN940922.

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To test whether *Rickettsia* in the plants is alive, RNA was extracted from leaf discs (5 cm diameter) of five different cotton plants exposed to whiteflies and from four plants not exposed to whiteflies (negative control), using the Aurum total RNA mini kit according to the instruction manual (Bio-Rad). DNase was added to remove DNA contamination. cDNA was synthesized using Verso cDNA kit (Thermo scientific) and the specific primer used Pgt-R1 (table 2).

The cycling program for the reverse transcription was: cDNA synthesis of 30 min in 42°C, following inactivation of 2 min in 95°C. Negative controls for the RT-PCR reaction were reactions without the reverse transcriptase enzyme (to exclude DNA contamination).

(ii) *Visualization of* Rickettsia *using fluorescence* in situ *hybridization*

In order to determine where *Rickettsia* is found in the plant, six fresh cotton leaves infested with R^+ whiteflies for six weeks were thinly sliced vertically into 25 strips with a razor blade. The FISH procedure generally followed Gottlieb *et al.* [22] and Ghanim *et al.* [35] (with slight modifications). Specimens were collected directly into Carnoy's fixative (chloroform : ethanol : glacial acetic acid, 6 : 3 : 1) and fixed overnight. After fixation, the samples were decolourized in 6 per cent H_2O_2 in ethanol for 2 days at dark conditions and then hybridized overnight in hybridization buffer (20 mM Tris-HCl (pH 8.0), 0.9 M NaCl, 0.01% sodium dodecyl sulphate, 30% formamide) containing fluorescent probes (10 pmol ml^{-1}). Preliminary experiments with the *Rickettsia* 16S rDNA probe [22] resulted in an extensive non-specific reaction with the plastids. To avoid this problem, a *Rickettsia*-specific DNA probe PGT-Cy3 (5-Cy3-GCATGAATTTGGCACTTAAGC) was designed based on the *Rickettsia bellii* phosphoglycerol transferase gene sequence. Stained samples were whole mounted and viewed under a 1 × 81 Olympus FluoView 500 confocal microscope (Olympus, Tokyo, Japan). Negative controls consisted of leaves infested with R^- whiteflies. Specificity of detection was confirmed using no-probe staining and whitefly-free leaves to exclude cross reaction of other bacteria with the probe. It should be noted, however, that the presence of additional endophytes, unrelated to the presence of whiteflies, cannot be ruled out.

(c) ***Horizontal transmission of* Rickettsia *among adult whiteflies***

Leaf cages were attached to cotton plants and sealed with Power-Tack (Ningbo Xinyinli Imp & Exp Trade Co., Ltd, China) to prevent nymphs from entering. The plants were exposed to R^+ whiteflies for 10 days, and then approximately 60 R^- whiteflies were inserted into the cages through a small hole in the covering net, and the hole was sealed with a cotton wool plug. After 10 more days live adults from the cage were collected and sexed. Females were placed alive in 96 per cent alcohol, grounded in lysis buffer as described by Frohlich *et al.* [36] and tested for the presence of *Rickettsia* by PCR using the *Rickettsia 16S rRNA*-specific primers (table 2). Negative controls followed the same protocol but the tested whiteflies (inside the cage) were surrounded by R^- whiteflies outside the cage.

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