Maternal transmission is the main transmission pathway of facultative bacterial endosymbionts, but phylogenetically distant insect hosts harbor closely related endosymbionts, suggesting that horizontal transmission occurs in nature. Here we report the first case of plant-mediated horizontal transmission of Wolbachia between infected and uninfected Bemisia tabaci AsiaI7 whiteflies. After infected whiteflies fed on cotton leaves, Wolbachia was visualized, both in the phloem vessels and in some novel 'reservoir' spherules along the phloem by fluorescence in situ hybridization using Wolbachia-specific 16S rRNA probes and transmission electron microscopy. Wolbachia persisted in the plant leaves for at least 50 days. When the Wolbachia-free whiteflies fed on the infected plant leaves, the majority of them became infected with the symbiont and vertically transmitted it to their progeny. Multilocus sequence typing and sequencing of the wsp (Wolbachia surface protein) gene confirmed that the sequence type of Wolbachia in the donor whiteflies, cotton phloem and the recipient whiteflies are all identical (sequence type 388). These results were replicated using cowpea and cucumber plants, suggesting that horizontal transmission is also possible through other plant species. Our findings may help explain why Wolbachia bacteria are so abundant in arthropods, and suggest that in some species, Wolbachia may be maintained in populations by horizontal transmission.

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Introduction

Offspring vertically inherit both nuclear and non-nuclear genetic material from their mothers. Intracellular bacteria are the non-nuclear materials inherited vertically from mother to offspring (Oliver et al., 2010). There has been an increasing interest in intracellular bacteria over the past two decades, because of their widespread distribution in nature and their significance to the ecology, evolution and reproductive biology of their hosts (Gotth et al., 2007; Himler et al., 2011; Segoli et al., 2013; Baldini et al., 2014). Innumerable species of insects and other arthropods are associated with various intracellular bacteria (Hilgenboecker et al., 2008; Watts et al., 2009; Jaenike and Brekke, 2011). These bacteria often live in symbioses with their hosts (Oliver et al., 2010), and may be obligate (that is, primary endosymbionts essential for host survival) or facultative (that is, secondary endosymbionts that can increase or decrease host fitness; Himler et al., 2011; Jiggins and Hurst, 2011). The obligate symbionts are found within specialized cells and typically share a long evolutionary history with their hosts (Buchner, 1965), whereas the facultative symbionts tend to have more recently formed associations with their hosts. Wolbachia (Alphaproteobacteria: Rickettsiales) is a genus of facultative endosymbionts common among arthropods and is estimated to have infected the majority of arthropods and filarial nematodes. In arthropods, Wolbachia most commonly interact with their hosts via a parasitic manipulation of the reproductive system (Werren et al., 2008). As with other facultative endosymbionts, Wolbachia have been thought to undergo primarily vertical transmission from mother to offspring with high fidelity. The horizontal transmission pathway is thought to be the most likely explanation for closely related symbionts occurring in phylogenetically distant insects (Werren et al., 1995; Vavre et al., 1999; Noda et al.,...
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2001; Baldo et al., 2008; Ahmed et al., 2013). Over the past two decades, there have been multiple phylogenetic and transinfection studies reporting evidence of Wolbachia transmission between both phylogenetically close and phylogenetically distant species (Boyle et al., 1993; Heath et al., 1999; Vavre et al., 1999). Thus, it is probable that Wolbachia horizontal transmission is occurring between some arthropod taxa (Ahmed et al., 2015). Although Wolbachia has been shown to undergo extensive horizontal transmission between several host taxa (Werren et al., 1995; Baldo et al., 2006; Chiel et al., 2009; Raychoudhury et al., 2009; Oliver et al., 2010; Ahmed et al., 2015), the mechanisms for this are poorly understood.

There is growing evidence for common horizontal transmission of Wolbachia from one species to another (Ahmed et al., 2013; Gerth et al., 2013; Brown and Lloyd, 2015), and the transmission route is becoming a hotspot of research, given its importance in ecological and evolutionary biology (Vavre et al., 1999; Sintupachee et al., 2006; Caspi-Fluger et al., 2012; Gehrer and Vorbuerger, 2012). Recently, Wolbachia horizontal transmission through invertebrate predators and parasitoids has been revealed (Hugens et al., 2000, 2004; Le Clech et al., 2013; Ahmed et al., 2015). However, the presence of identical strains of Wolbachia among species that do not share predators and parasitoids but have similar habitats, such as shared host plants or food sources, suggests that plants or food may be involved in Wolbachia horizontal transmission (Sintupachee et al., 2006; Caspi-Fluger et al., 2012; Weinert et al., 2015).

The whitefly Bemisia tabaci (Hemiptera: Aleyrodidae) is a small hemipterous insect that feeds on phloem sap of numerous host plants; it has a very wide host plant range of over 500 species worldwide (Stansly and Naranjo, 2010). Bemisia tabaci is a complex of distinct cryptic species, harboring various bacterial symbionts such as Wolbachia, Arsenophonus, Cardinium, Hamiltonella and Rickettsia, but endosymbionts vary largely among the different whitefly species (Chiel et al., 2007; Ahmed et al., 2010; Skaljac et al., 2013). Here, we investigated if host plant had a role in the horizontal transmission of Wolbachia between whiteflies. We also studied the transfer dynamics of Wolbachia during this plant-mediated transmission.

Materials and methods

Plants and insects
Cotton plants (Gossypium hirsutum L. var. Lumianyan no. 32) were used in this study. Cotton seeds were sown in 15-cm-diameter plastic pots containing a soil–sand mixture (10% sand, 5% clay and 85% peat) in a greenhouse at ambient temperature and photoperiod. Plants were watered as necessary before being used in experiments at the 6–8 expanded leaf stage.

The whiteflies used in the study were Bemisia tabaci AsiaII7 (formerly known as Cv biotype), which is an indigenous cryptic species in South China (De Barro and Ahmed, 2011). Details regarding the collection, rearing and Wolbachia infection monitoring of AsiaII7 whiteflies are shown in the Supplementary Method S1.

Wolbachia transmission from whiteflies to cotton plants
We investigated the effects of the number and feeding time of Wolbachia-positive AsiaII7 on the efficiency of Wolbachia transmission from whiteflies to cotton plants. We collected AsiaII7 adults 24–48 h after they emerged from the pupal stage, using a hand aspirator from the Wolbachia-positive subcolony. In AsiaII7, Wolbachia created the scattered infection pattern described by Ahmed et al. (2015). Whiteflies were released into leaf cages (2 cm high, 3 cm diameter) covered on the undersurface of clean, healthy cotton leaves (as shown in Supplementary Figure S5). We studied three treatments, each with a different amount of whiteflies per cage (1 pair, 5 pairs and 10 pairs), and performed 10 replicates per treatment.

Two days after the AsiaII7 B. tabaci were released into the leaf cages, we recorded which leaves were being fed upon by the whiteflies (herein referred to as ‘fed leaves’). We then began cutting ~0.01 g of leaf material (equivalent to leaf surface area of ~50 mm²) every 24 h from both the fed leaf and from the leaf immediately below it on the same stem (~2 cm distance between the bases of the two leaves). The presence of Wolbachia in the cotton leaves was detected by PCR using the Wolbachia-specific primers of Wolbachia surface protein (wsp) gene and 16S rRNA genes (O’Neill et al., 1992; Braig et al., 1998); the primers and protocols for PCR detection are shown in Supplementary Table S1 and Supplementary Method S2. The initial time when Wolbachia was positively detected in cotton leaves was recorded for each replicate. For negative controls, the same procedure was followed using whiteflies that were collected from the Wolbachia-free subcolony.

Visualization of Wolbachia in plants
Fluorescence in situ hybridization (FISH) was used to identify the location of Wolbachia in cotton plants. Ten pairs of AsiaII7 were released onto a single leaf of each plant and were allowed to feed for 25–28 days. Then, a 50 mm² leaf section (10 mm × 5 mm) was removed by cutting longitudinally along the leaf vein; an equally sized section was also removed from the leaf immediately below the fed leaf. These leaf samples were placed in Carnoy’s fixative. FISH detection was then performed following the method of Sakurai et al. (2003; see Supplementary Method S3), using a Wolbachia-specific 16S rRNA probe (W2-Cy3:
5′-CTTCTGTGAGTACGTCATTATC-3′) that had its specificity tested with the Ribosomal Database Project II ‘probe match’ analysis tool (http://rdp.cme.msu.edu) by Gottlieb et al. (2008). The stained leaf samples were mounted and viewed under a Nikon eclipse Ti-U FluoView inverted microscope (Nikon Instruments Inc., Tokyo, Japan). Specificity of Wolbachia detection was confirmed using two controls: (1) Wolbachia-infected cotton leaves without the Wolbachia 16S rRNA probe and (2) Wolbachia-free cotton leaves with the Wolbachia 16S rRNA probe. FISH visualization experiments were also performed on cowpea and cucumber plants, with the same protocol and primers used in the cotton plant experiment.

Transmission electron microscopy was used to confirm the location of Wolbachia in the cotton leaves. Samples of Wolbachia-deposited cotton leaves (1.0 mm × 0.5 mm) were fixed in 4% glutaraldehyde in cacodylate buffer (pH 7.4) at 4 °C for 24 h, and then overnight in 1% osmium tetroxide. The fixed leaf samples were dehydrated through an alcohol series and embedded in Spurr's resin. Ultrathin sections were collected on copper grids with a single slot, stained with 1% uranyl acetate and lead citrate, and finally examined under a Transmission electron microscopy (JEOL, Tokyo, Japan).

**Persistence of Wolbachia in cotton plants**

Two experiments, each with three replicates, were performed to study the persistence of Wolbachia in the cotton leaves. In the first experiment, 10 two-day-old pairs of Wolbachia-positive AsiaII7 adults were released into a leaf cage to feed on the cotton leaves for 24 days (we have already shown that the 24th day is approximately the earliest day in which Wolbachia can be detected in plant leaves). On day 25th, the adult whiteflies and their immature progeny were collected for DNA extraction and the detection of Wolbachia presence using PCR and quantitative real-time PCR (q-PCR) with the wsp primers. For the next 50 days, additional 0.01 g leaf segments were cut every 5 days and tested for the presence of Wolbachia; a total of 11 sets of leaf samples were tested in this experiment. The second experiment was run with a nearly identical protocol, except the 10 pairs of AsiaII7 whiteflies and their offspring were not collected on day 25th. Instead, they were allowed to feed continuously on the cotton leaves during the experiment. The wsp primers used in PCR detection were wsp 81F and wsp 691R (Supplementary Table S1) and wsp primers used in q-PCR were wsp-QF and wsp-QR (Supplementary File Supplementary Method S2). A UBQ7 gene of cotton fiber (DQ116441) was used as an internal control for data normalization and quantification (Tu et al., 2007). Detailed procedures for PCR and q-PCR Wolbachia detection are shown in Supplementary Method S2.

To test whether the Wolbachia detected in the plants was still alive, RNA was extracted from leaf discs (2 cm diameter) of five cotton plants that had each been exposed to Wolbachia-positive AsiaII7 whiteflies for a different amount of time (24, 34, 44, 54 or 64 days). RNA was also extracted from a cotton plant that had not been exposed to whiteflies, as a negative control. Extractions were performed using the Trizol RNA Extraction Kit (Omega, Stamford, CT, USA) following the protocol in the instruction manual. The RNA was then reverse transcribed to cDNA using Moloney murine leukemia virus (Invitrogen, Carlsbad, CA, USA) and the specific 16S rRNA primers (Supplementary Table S1).

**Wolbachia transmission from cotton to whitefly and its subsequent vertical transmission**

To detect the presence of Wolbachia on the cotton leaves, 10 newly emerged adults collected from the Wolbachia-negative colony were released into 10 different leaf cages. They were allowed to feed on the Wolbachia-positive cotton leaves for 20 days, and then collected for Wolbachia PCR detection. This experiment was repeated 10 times.

To evaluate the Wolbachia acquisition efficiency of recipient whiteflies, 100 pairs of AsiaII7 adults from the Wolbachia-negative colony were introduced into 20 leaf cages (five pairs per cage) covered with the Wolbachia-positive cotton leaves. Additional Wolbachia-negative whitefly adults were released into a leaf cage with Wolbachia-free leaves, as a negative control. Every 2 days, five randomly selected pairs of adults were collected for Wolbachia PCR and q-PCR using the wsp primers (see Supplementary Table S1 and Supplementary Method S4, respectively). A β-actin gene of B. tabaci was used as an internal control for data normalization and quantification in q-PCR (Ghanim and Kontsedalov, 2009); the detailed procedure for q-PCR is described in Supplementary Method S4. This q-PCR experiment was repeated three times.

In the instances where Wolbachia was detected in a recipient whitefly, RNA was extracted from the whitefly to test whether the Wolbachia was still alive. Extractions were performed using the Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA) following the procedure in the instruction manual. DNase was added to remove DNA contamination, and cDNA was synthesized using a Verso cDNA Kit (Thermo Scientific, Waltham, MA, USA). Reactions without the reverse transcriptase enzyme (to exclude DNA contamination) were used as negative controls for the RT-PCR.

To test if the Wolbachia acquired by AsiaII7 during feeding can be vertically transferred in subsequent generations of AsiaII7, we randomly selected 10 pairs of adults that had been feeding on Wolbachia-positive leaves for 15 days (Wolbachia can be initially detected in a recipient whitefly after...
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10 days). Each pair was then introduced into a separate leaf cage containing new healthy cotton leaves to encourage oviposition. After 24 h, the adult whiteflies were removed from the cages and used for Wolbachia PCR detection; all eggs (the F1 generation) were left in the cages and allowed to fully mature. Twenty randomly selected specimens of the offspring adults were then used to examine the Wolbachia distribution pattern, and the others were used to determine the percentage of F1 whiteflies that retained Wolbachia. This experiment was repeated 10 times.

Multilocus sequence typing and phylogenetic analysis of Wolbachia
Multilocus sequence typing (MLST) was used to identify Wolbachia strains in (1) donor Wolbachia-positive whiteflies, (2) recipient Wolbachia-negative whiteflies, (3) the F1 generation progeny of the newly infected whiteflies and (4) cotton leaves. Five MLST genes (gatB, coxA, hcpA, ftsZ and fbpA) as well as the wsp gene were sequenced following the methods of Baldo et al. (2006). The five MLST genes were concatenated using Geneious (version R8; Kearse et al., 2012). MLST loci were blasted against the Wolbachia MLST database (http://pubmlst.org/Wolbachia). Two MLST loci from supergroup A, four from supergroup B, one from supergroup D and our MLST loci were analyzed, using maximum likelihood (ML) in RAxML (Stamatakis, 2006), to construct a phylogenetic tree (Supplementary Table S2 and Supplementary Method S5).

Transmission of Wolbachia through other plant species
To examine Wolbachia transmission through other plant species, we repeated the Wolbachia horizontal transmission and FISH visualization experiments using cucumber, Cucumis sativus L. (var. Xiayou168), and cowpea, Vigna unguiculata (L.) Walp (var. Kefeng), instead of cotton. Wolbachia was transmitted from Wolbachia-positive AsiaII7 to cucumber and cowpea plants, and then to the Wolbachia-negative AsiaII7 individuals and their F1 generation offspring. PCR detection of Wolbachia in the new host plant species, determinations of the strain genotypes and phylogenetic analyses were all conducted using the same methodology as in the cotton plant experiments.

Results
Wolbachia can be transmitted to cotton plants by whiteflies
Results of the Wolbachia PCR detection using wsp and 16S rRNA gene primers (Supplementary Table S1) revealed that this endosymbiont could be detected in cotton plants after several weeks of Wolbachia-positive whitefly feeding. In samples taken from leaves that the whiteflies were directly feeding on (that is, the fed leaves), the time interval from when the whiteflies began feeding to when Wolbachia was initially detected in the leaf was inversely correlated with the number of infected whiteflies in the leaf cage (Figure 1, 35.8 ± 0.6, 27.4 ± 0.8 and 23.4 ± 0.7 days for the groups of 1, 5 and 10 pairs of insects, respectively, mean ± s.e.). Wolbachia was also detected in samples taken from leaves immediately below the fed leaves; that is, in each treatment, Wolbachia was positively detected 1–3 days after Wolbachia was detectable on the corresponding fed leaf (Figure 1). In the negative control treatments, which only involved Wolbachia-free whiteflies, Wolbachia was not detected on any of the cotton leaves.

The localization of Wolbachia in cotton leaves
Using a cyanine3-labeled Wolbachia-specific 16S rRNA probe (Supplementary Method S3), the FISH visualization of the fed leaves revealed that Wolbachia could be found in most parts of the phloem sieve tube, which is usually 5–8 cm from the whitefly feeding site. Wolbachia was unexpectedly also found in some novel globular regions along the phloem sieve tube (Figure 2a and Supplementary Figure S1). Wolbachia was also found in the phloem of the leaves immediately below the fed leaves (Figure 2b). This presence of Wolbachia in cotton leaves that had not been directly fed on by whiteflies suggests that Wolbachia can move between leaves after the initial transmission. As with the cotton leaves, spherules of Wolbachia were also in the phloem of the cowpea and cucumber leaves that had been exposed to Wolbachia-positive whiteflies (Supplementary Figure S2). However, the quantities and sizes of these spherules were different from those found in the cotton leaves (Supplementary Figure S2). Wolbachia was not observed in any leaves from the negative controls (Supplementary Figure S3).

Figure 1 Correlation between the number of Wolbachia-infected whiteflies (AsiaII7 Bemisia tabaci) feeding on cotton plants and the amount of time [as of the initial whitefly introduction] before Wolbachia was initially detected in the cotton leaves. The column and error bars are the mean ± s.e. of time (in days) and 10 replicated were repeated in each column.

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Transmission electron microscopy images were used to visualize *Wolbachia* morphology in the bacteriocytes located in the adult whitefly abdomen and in the cotton leaf phloem. In the whitefly bacteriocytes, most individual *Wolbachia* have a small (0.5–1 μm), irregular coccoid form with a double membrane (Figures 3a and b). In the phloem, *Wolbachia* was found in the vacuole of a plant cell and was morphologically similar to those in whitefly adult abdomens (Figures 3c and d).

**Changes in the amount of Wolbachia in cotton leaves over time**

In this study, two plant groups were exposed to *Wolbachia*-infected AsiaII7 whiteflies. In one group, the whiteflies were removed after the first 24 days, whereas in the other group, the whiteflies were left on the plants for the entire duration of the experiment (74 days). *Wolbachia* persisted in cotton leaves for at least 50 days after its first detection (that is, 74 days after the *Wolbachia*-positive whiteflies first fed on the leaf) in both of the plant groups, regardless of whether the whiteflies had been removed (Figure 4). The relative quantity of *Wolbachia* in all treatments increased to its highest amount during the first 5–10 days after it was positively detected (that is, days 25th–34th), and during this period the leaves that were continuously fed on by whiteflies had significantly higher quantities of *Wolbachia* than the leaves that had the whiteflies removed. After reaching this peak quantity, the amount of *Wolbachia* reduced gradually in leaves from both groups.

**Wolbachia transmission from cotton to whiteflies and its subsequent vertical transmission**

When uninfected adult whiteflies continuously fed on the *Wolbachia*-infected cotton leaves, *Wolbachia* was initially detected in the recipient whiteflies after 10.0 ± 0.3 days. After 20 days of feeding, *Wolbachia* was detected in 62.0 ± 5.5% of the recipient female whiteflies. In the more sensitive q-PCR evaluation, *Wolbachia* was initially detected in the recipient whiteflies after 4 days of feeding on the *Wolbachia*-infected cotton leaves, and the relative quantity of *Wolbachia* in female adults greatly increased as the feeding time increased (Figure 5). We also found that 95.0 ± 1.67% of the F1 whitefly adults tested positive for *Wolbachia*, indicating that *Wolbachia* can be vertically transmitted from newly infected female whiteflies to their offspring (Supplementary Figure S4).

**Multilocus sequence typing and phylogenetic analysis of Wolbachia from plants and whiteflies**

Our genetic analysis of the *Wolbachia* strain being transmitted between cotton plants and AsiaII7
whiteflies revealed that all of the tested Wolbachia samples were identical. The Wolbachia from the donor whiteflies, the infected cotton leaves, the recipient AsiaII7 whiteflies and the recipient whitefly offspring are all Wolbachia ST388 (Figure 6a and Supplementary Table S3). These results were confirmed by DNA sequencing and by our phylogenetic analysis (Figure 6b). Furthermore, all of the tested Wolbachia endosymbionts belong to the Con group within Supergroup B (Figure 6). No sequence variation was found in the wsp gene of these Wolbachia endosymbionts.

Transmission of Wolbachia through different plant species

After repeating our Wolbachia transmission and detection experiments with two additional types of plants, cucumber and cowpea, we found evidence that the whiteflies’ choice of host plant does not have any effect on the sequence type or the fidelity of Wolbachia during horizontal transmission (Figure 6). This demonstrates that this particular Wolbachia strain (ST388) remains highly conservative during its plant-mediated horizontal transmission.

Discussion

There has been an increased interest in studying the horizontal transmission of intracellular bacterial endosymbionts over the past two decades, particularly Wolbachia and Rickettsia because of their widespread distribution and significant role in the ecology and evolution of their hosts (Vavre et al., 1999; Sintupachee et al., 2006; Gotoh et al., 2007; Himler et al., 2011; Caspi-Fluger et al., 2012). The vector-mediated interspecific transmission of intracellular bacterial endosymbionts was observed through shared food sources (in Wolbachia, Spiroplasma, Hamiltonella defensa; Rigaud and Juchault, 1995; Oliver et al., 2010; Caspi-Fluger et al., 2012), ectoparasitic mites (Jaenike et al., 2007; Gehrer and Vorburger, 2012), host plants (in Rickettsia, Arsenophonus; Caspi-Fluger et al., 2012; Bressan, 2014) and parasitoids (in Arsenophonus, Wolbachia; Duron et al., 2010; Ahmed et al., 2015). The hypothesis that Wolbachia can be horizontally
transmitted between two insect species has been supported by both phylogenetic and experimental analyses (Vavre et al., 1999; Huigens et al., 2000, 2004; Sintupachee et al., 2006; Yang et al., 2006; Yang et al., 2013). Ahmed et al. (2013) compared the phylogeny of different Bemisia species and their endosymbionts, revealing the incongruence of Wolbachia with their whitefly hosts and suggesting a host shift of Wolbachia through horizontal transmission. Recently, Ahmed et al. (2015) revealed that parasitoids can transmit Wolbachia by feeding and probing their uninfected hosts. Both the phylogenetic analysis and transmission experiments demonstrated that parasitoids could serve as potential routes for horizontal transmission of Wolbachia between different hosts. Sintupachee et al. (2006) discovered a potential route for lateral transmission of Wolbachia between different insects that share the same leaf substrate in pumpkin plants. Huigens et al. (2000, 2004) discovered a frequent horizontal transmission of Wolbachia from infected to uninfected wasp larvae (Trichogramma kaykai) when they feed on a common food source: eggs of the butterfly Apodemia mormo deserti. After the wasps matured, the females then vertically transmitted Wolbachia to their offspring. Sintupachee et al. (2006) showed that four taxonomically diverse insects feeding on the same host plant contained very closely related Wolbachia, suggesting the potential role of host plants in Wolbachia horizontal transmission (Sintupachee et al., 2006). Work by Yang et al. (2013) also showed that identical strains of Wolbachia can be shared by two species that live in the same plant tissue: the gall wasp Andricus mukaiga- wae and its inquiline wasp Synergus japonicas. Stahlhut et al. (2010) used a multigene approach to provide evidence that ecological associations can facilitate horizontal transmission of Wolbachia within mycophagous fly communities. Our current study supplements the findings on Wolbachia transmission by providing direct evidence of plant-mediated transmission of Wolbachia, including the transmission efficiency, distribution pattern and persistence of Wolbachia in both plant tissues and in the progeny of its recipient host.

The distribution patterns of endosymbionts in their hosts may have important influences on their transmission ability. Caspi-Fluger et al. (2011) found that another endosymbiont, Rickettsia, can have two distribution patterns in its whitefly host: a scattered pattern localized in the whitefly hemocoel and a confined pattern restricted to the bacteriocytes. Moreover, Chiel et al. (2009) found that the scattered pattern of Rickettsia facilitates its transmission to whitefly parasitoids. Previous work by Ahmed et al. (2015) showed that, like Rickettsia, Wolbachia can also be found in scattered and confined distribution patterns. The scattered Wolbachia have the potential to be transmitted horizontally between whiteflies through the feeding or oviposition probing of an Eretmocerus parasitoid. In the current study, we found that Wolbachia-positive AsiaII7 whiteflies with a scattered Wolbachia pattern can transmit Wolbachia to cotton leaves, and Wolbachia-negative AsiaII7 whiteflies can become infected with scattered-pattern Wolbachia after feeding on Wolbachia-positive cotton leaves for at least 10 days. This suggests similarities between the plant-mediated transmission routes of Wolbachia and Rickettsia.

The distribution pattern of Wolbachia in plant tissues has not been previously demonstrated. Wolbachia could not be detected in plant tissue until the Wolbachia-positive whiteflies had been feeding for 24 days, at which point the amount of Wolbachia noticeably increased for the ensuing 5 days. We surmise that Wolbachia requires 24 days to accumulate of a titer, with the subsequent amplification being a barrier break in the interaction
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between invasive Wolbachia and the physiological contents of the plant leaf. Here, for the first time, we have shown that Wolbachia can persist in cotton leaves for more than 50 days; our RNA RT experiments revealed that all of the Wolbachia endosymbionts present in the cotton leaves for 24–64 days were alive. In order for Wolbachia to live that long in the plant, there should be some sort of interaction effect or nutritional support. This requires further investigation. Interestingly, we found that some Wolbachia cluster in an irregular globular shape along the leaf phloem. The biological role of these globular clusters is unclear, but we suspect that these may be the reservoirs of Wolbachia in plant leaves. Purcell et al. (1994) found that a bacterial parasite of the leafhopper Euscelidius variegatus can be horizontally transmitted between different individuals of E. variegatus that feed on the same plant leaves. This particular bacterium did not multiply or move within the plant. In contrast, we found that Wolbachia can move within its plant after transmission; FISH visualization showed the presence of Wolbachia in leaves that had not been fed on by whiteflies.

Facultative endosymbionts have already been shown to change host fitness or biology for multiple reasons, including host protection against entomopathogenic fungi and parasitic wasps, amelioration of the detrimental effects of heat and influence on host plant suitability (Oliver et al., 2003, 2005, 2010; Scarborough et al., 2005). One main consequence of Wolbachia horizontal transmission is the induction of unknown phenotypes in the novel host (Werren et al., 2008; Ahmed et al., 2015). Wolbachia can confer positive fitness benefits by increasing the resistance against natural pathogens in fruit flies (Teixeira et al., 2008). Hornett et al. (2006) revealed that Wolbachia, in some host species, do not currently induce any phenotype but may have done so in the past, implying that more species have had their biology affected by Wolbachia than previously estimated. In other situations, after transinfection of Wolbachia, the newly induced phenotype can be suppressed by its novel host (Hornett et al., 2008) and can also be changed into a completely different phenotype (Sasaki et al., 2002). It is therefore necessary to investigate each strain’s genotype and phenotype in its natural host, as well as other possible hosts in which it may have been transferred through shared host plants.

Plant-mediated transmission might explain the widespread abundance of Wolbachia infection in phytophagous arthropods and the presence of its identical strains in evolutionarily distant species. Overall, plant-mediated transmission might be having a crucial unknown role in ecological and evolutionary biology. In this study, some novel ‘reservoir’ spherules were found along the cotton leaf phloem using FISH, but this kind of spherule was not found when the Rickettsia-infected B. tabaci MEAM1 species fed upon cotton leaves while contaminating the phloem with Rickettsia (An et al., 2015). The biological roles of the Wolbachia ‘reservoir’ in plant leaves, and the consequent plant-mediated transmission, need to be further investigated to fully understand the dynamics of Wolbachia infection.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

BLQ and SJL designed the study; SJL, MZA, NL, PQS and JLH performed the experiments; BLQ, SJL, XMW and MZA analyzed the data; BLQ and MZA wrote the paper; BLQ supported the grants.

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