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Transinfection reveals the crucial importance of *Wolbachia* genotypes in determining the type of reproductive alteration in the host

HIRONORI SAKAMOTO[1,2]*, YUKIO ISHIKAWA[2], TETSUHIKO SASAKI[3], SAKAE KIKUYAMA[1], SADAHIRO TATSUKI[2] AND SUGIHIKO HOSHIZAKI[2]
[1] *Department of Biology, School of Education, Waseda University, Nishiwaseda, Tokyo 169-0051, Japan*
[2] *Department of Agricultural and Environmental Biology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi, Tokyo 113-8657, Japan*
[3] *Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Hongo, Tokyo 113-0033, Japan*

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Summary

Wolbachia, a group of endosymbiotic bacteria in arthropods, alter the reproduction of their hosts in various ways. A *Wolbachia* strain (*w*Sca) naturally infecting the adzuki bean borer moth *Ostrinia scapulalis* induces male killing, while another strain (*w*Kue) infecting the Mediterranean flour moth *Ephestia kuehniella* induces cytoplasmic incompatibility (CI) in the resident host. Transinfection of *Wolbachia* can be a powerful tool to elucidate the relative importance of *Wolbachia* and the host in determining the type of reproductive alterations. Recently, male killing was shown to occur in *E. kuehniella* transinfected with *w*Sca. In the present study, we transferred *w*Kue to *O. scapulalis* by embryonic microinjection. In the *O. scapulalis* transinfected with *w*Kue, CI, but not male killing occurred. Thus, in addition to *w*Sca, *w*Kue was shown to induce the same type of alteration in a foreign host as in its natural host. These results demonstrate the crucial role of the *Wolbachia* genotype in determining the type of reproductive alteration. However, the present study also revealed the involvement of host factors. First, the degree of incompatibility induced by *w*Kue in *O. scapulalis* was stronger than that in *E. kuehniella*, indicating that host factors can affect the level of CI. Second, the vertical transmission rate of *w*Kue in *O. scapulalis* was generally low, suggesting that the host affects the dynamics of *Wolbachia* transmission.

1. Introduction

Wolbachia is a group of endosymbiotic bacteria found in arthropods and nematodes (reviewed by Werren, 1997; Bourtzis & O'Neill, 1998; Stouthamer *et al.*, 1999). *Wolbachia* often manipulates the reproduction of its arthropod hosts to promote the spread of infection into host populations. Thus, *Wolbachia* is considered a selfish genetic element in arthropods. The reproductive alterations caused by *Wolbachia* infection include cytoplasmic incompatibility (CI), parthenogenesis, feminization of genetic males, and male killing.

* Corresponding author. Current address: Laboratory of Applied Entomology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi, Tokyo 113-8657, Japan. Tel: +81 3 58415093. Fax: +81 3 58415060. e-mail: hirowol@yahoo.co.jp

Since the phenotype of reproductive alteration is a consequence of interactions between *Wolbachia* and the host, both *Wolbachia* and the host potentially affect the phenotype. To date, not much information has been accumulated on the relative importance of *Wolbachia* and its host in determining the type of reproductive alteration. Examination of the relative importance in various *Wolbachia*–host combinations would help us to understand the genetic system and the evolutionary basis underlying the *Wolbachia*–host interaction. In this regard, the exchange of *Wolbachia* strains between different host species that show different types of reproductive alteration can be very informative.

In the present study, we focused on two lepidopteran insects, the Mediterranean flour moth *Ephestia kuehniella* (Pyralidae) and the adzuki bean borer moth *Ostrinia scapulalis* (Crambidae), which express

different reproductive alterations when infected with *Wolbachia*. A *Wolbachia* strain (*w*Kue) that naturally infects *E. kuehniella* belongs to group A (Werren *et al*., 1995; Masui *et al*., 1997), and induces CI in the host (Sasaki & Ishikawa, 1999). CI is the most common of the reproductive alterations induced by *Wolbachia* in arthropod hosts. CI is a sperm–egg incompatibility that typically occurs when an infected male mates with an uninfected female, whereas the reciprocal cross and self-crosses are compatible. *Wolbachia*-induced CI is currently explained by a modification–rescue system as follows (O'Neill *et al*., 1997; Werren, 1997). *Wolbachia* modifies developing sperm in testes of infected males, and uninfected eggs fertilized with the modified sperm do not develop. On the other hand, eggs infected with the same *Wolbachia* strain as that infecting the male can rescue the modified sperm.

Another *Wolbachia* strain (*w*Sca) that naturally infects *O. scapulalis* belongs to group B, and the infected females produce all-female progeny (Kageyama *et al*., 2003*a*). Based on the finding that antibiotic treatment of infected *O. scapulalis* at the larval and adult stages led to the regular generation of all-male progeny and sexual mosaics (Kageyama *et al*., 2003*a*, *b*), the all-female production by *w*Sca-infected females was once interpreted as the feminization of genetic males (Kageyama *et al*., 1998, 2002; Fujii *et al*., 2001). However, a detailed analysis of the genotype (male = ZZ, female = ZW) of individuals based on observations of the sex chromatin (W) and the comparative genomic hybridization of sex chromosomes led Kageyama & Traut (2004) to conclude that the all-female production in infected *O. scapulalis* is the consequence of male-specific death provoked by the feminizing effect of *Wolbachia*. They also indicated that the all-male production after antibiotic treatment is caused by female-specific death, suggesting that *w*Sca is indispensable to the survival of infected females.

Thus, *w*Kue induces CI in *E. kuehniella*, while *w*Sca induces male killing in *O. scapulalis*. Fujii *et al*. (2001) transferred *w*Sca from *O. scapulalis* to uninfected *E. kuehniella*, and found the occurrence of male killing in the transinfected insect. This finding is consistent with the hypothesis that the *Wolbachia* strain determines the type of reproductive alteration, but this hypothesis is yet to be confirmed by transinfection in the reverse direction. If *w*Kue-transinfected *O. scapulalis* expresses a reproductive phenotype other than CI, it suggests a *Wolbachia* × host interaction in determining the type of alteration.

In the present study, we transinfected *Wolbachia* in the reverse direction, from *E. kuehniella* to *O. scapulalis*, with the aim of elucidating interactions between *Wolbachia* and the host in determining the type of reproductive alteration. We here show that CI occurs in *w*Kue-transinfected *O. scapulalis*, and that host factors can affect the level of CI and the maternal transmission rate of *Wolbachia*.

2. Materials and methods

(i) *Insects*

A strain of *E. kuehniella* that was collected at Tsuchiura, Japan (Tsuchiura strain) is infected with *w*Kue and shows CI (Sasaki & Ishikawa, 1999). The insects were maintained according to Sasaki & Ishikawa (1999).

O. scapulalis moths were collected in Matsudo, Japan. Since a small proportion of *O. scapulalis* females are infected with *w*Sca in the field (Kageyama *et al*., 2003*a*), the *Wolbachia*-free status of the moths used in the present study was confirmed by diagnostic polymerase chain reaction (PCR) using *Wolbachia*-specific primers: wsp81F and wsp641R for *wsp* gene (Zhou *et al*., 1998), and 99F and 994R for 16S rDNA (O'Neill *et al*., 1992). The offspring of the uninfected females were reared on an artificial diet (Silkmate 2M, Nosan) at 25 °C under a 16 h light and 8 h dark photoperiod (16L : 8D).

(ii) *Microinjection*

We injected the ooplasm of *E. kuehniella* into the eggs of uninfected *O. scapulalis*. The injection was performed at room temperature under a microscope (Nikon SMZ-U) equipped with a 3D micromanipulator (Narishige MMN-1) and a microinjector (Narishige IM-6), according to Sasaki & Ishikawa (2000). An injection needle was made using a glass capillary puller (Narishige PN-3), and the tip was manually cut with a razor to make an angled point with a diameter of about 5–10 μm. The ooplasm of the donor ovary was drawn into the microcapillary and injected into freshly laid eggs (< 30 min old) of the recipient insects. The injected eggs were placed in a plastic dish (9 cm in diameter) containing a piece of moist filter paper and maintained at 25 °C, 16L : 8D. At the pupal stage, insects were sexed on the basis of the morphology of the abdominal tip. Transinfected insects were maintained as matrilines by crossing females with uninfected males.

(iii) *Diagnostic PCR*

Genomic DNA was isolated from egg masses, larvae, pupae, testes of adult males and ovaries of adult females, using a QIAamp DNA Mini kit (Qiagen). The diagnostic PCR assay for *Wolbachia* was conducted using Ampli Taq Gold (PE Biosystems) and *Wolbachia*-specific primers for the *groE* gene (GroE415AF and GroE641AR; Ikeda *et al*., 2003).

PCR conditions were 94 °C for 10 min followed by 31 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with a final extension reaction at 72 °C for 10 min. Products were run on 2% agarose gels to observe DNA bands with the expected size (226 bp).

(iv) *Sequence of* groE *and* wsp *genes*

The *groE* and *wsp* genes were amplified using *Wolbachia*-specific primers (groEf1 and groEr1: Masui *et al*., 1997; wsp81F and wsp641R: Zhou *et al*., 1998). PCR conditions were the same as for the diagnostic PCR. The PCR products were purified from gels using the GENE CLEAN III kit (Bio101, La Jolla, CA), and directly sequenced using the ABI 377 DNA sequencer (PE Biosystems) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems).

(v) *Testing for cytoplasmic incompatibility*

Insects were sexed at the pupal stage, and crossing experiments were performed using virgin females and males that were ⩽4 days old after emergence. Crosses that included males from transinfected matrilines were performed in a single-pair mating: a female and a male were put in a mesh cage (10 cm in diameter, 10 cm in height). Single-pair mating was adopted in these crosses because it was necessary to identify each female's mate. After mating, the male mates were examined for the presence of *Wolbachia*, and those pairs proved to involve *Wolbachia*-free males were disregarded. On the other hand, crosses that included uninfected males were performed in mass matings: 10–30 females and 10–30 males were put in a mesh cage (25 cm × 25 cm × 25 cm). Mass mating was adopted in these crosses because it was highly efficient in obtaining many copulated females and there was no need to identify each female's mate.

After 3 days for mating, females were individually allowed to oviposit in plastic cups (10 cm in diameter, 4·5 cm in height) for 3 or 4 days. Most females laid more than 50 eggs, and females that laid fewer than 30 eggs were not used for analyses. Eggs (30–100 eggs per family) were incubated at 25 °C for 10 days, and then unhatched eggs were enumerated. Hatchability was analysed by the Mann–Whitney *U*-test with *P* values adjusted using the sequential Bonferroni method for planned comparisons. Females that laid more than 30 eggs were dissected to examine the presence or absence of spermatophores in copulatory bursae, and data from unmated females were excluded before analysis of the results. When the insects from the transinfected lines were used, total DNA was extracted from testes of males after mating, and from ovaries of females after oviposition. These DNAs were subjected to a diagnostic PCR assay to examine the infection status. When an insect from the transinfected lines proved to be free of *Wolbachia*, the pair that involved this insect was disregarded.

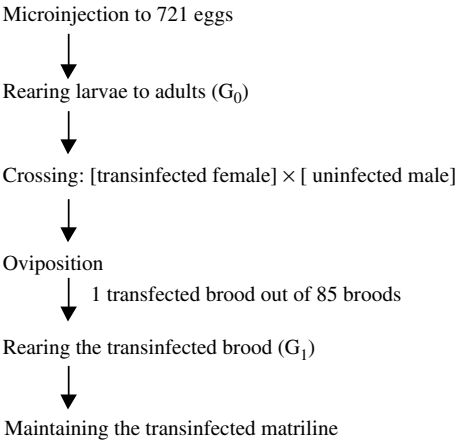

Fig. 1. Establishment of an *Ostrinia scapulalis* matriline transinfected with *Wolbachia* from *Ephestia kuehniella*.

3. Results

(i) *Establishment of the transinfected matriline*

We injected 721 eggs of *O. scapulalis* with the ooplasm of *w*Kue-infected *E. kuehniella* (Fig. 1). Insects that developed from the injected eggs were designated as generation 0 (G_0). Two or more G_1 egg masses were obtained respectively from 85 G_0 females. An egg mass from every G_1 brood was subjected to the diagnostic PCR, and *Wolbachia* infection was detected in one G_1 brood. The remaining eggs in this *Wolbachia*-positive G_1 brood were allowed to hatch. The hatched larvae were reared to adulthood, and 25 females and 16 males were obtained. The 25 G_1 females were crossed with uninfected males and allowed to lay eggs. *Wolbachia* infection was detected in 2 of the 25 G_2 broods. One of the two *Wolbachia*-positive broods developed into adults, while no adult was obtained from the other brood. For subsequent generations, one matriline of transinfected insects was maintained with the selection of broods that contained infected individuals. To confirm the presence of *w*Kue in the transinfected *O. scapulalis*, partial sequences of the *wsp* (461 bp) and *groE* (725 bp) genes of *Wolbachia* were determined. For both genes, the sequence determined was identical to the previously reported sequence for *w*Kue (DDBJ/EMBL/GenBank accession numbers AB024570 and AB002291 for *wsp* and *groE*, respectively).

(ii) *Maternal transmission rate*

The maternal transmission rate in the transinfected line was examined using G_5 and G_6 females. G_5

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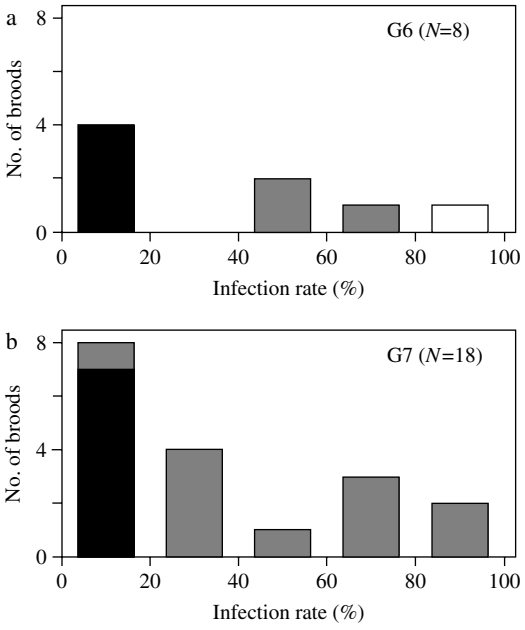

Fig. 2. The distribution of the infection rate of *Wolbachia* in the transinfected matriline of *O. scapulalis* in G_6 (*a*) and G_7 (*b*). All the G_7 broods examined were derived from one G_6 brood (white bar). *N* represents the number of broods. Four to 10 first-instar larvae per brood were diagnosed for *Wolbachia* infection by the PCR assay. Black bars represent broods in which all the larvae examined were uninfected.

females were separately allowed to produce offspring and then tested for infection with *Wolbachia* by diagnostic PCR. Infection was found in 8 G_5 females, and their offspring were tested for *Wolbachia* by diagnostic PCR (4–10 first-instar larvae per brood). Infection was not detected in 4 of the 8 broods, and the infection rate was 10–80% in the remaining 4 broods (Fig. 2). Females in one G_6 brood that showed the highest infection rate (8/10) among the 8 G_6 broods were separately allowed to produce offspring and then tested for infection. Infection was found in 18 fecund G_6 females, and their offspring were tested for *Wolbachia*, as in G_6 larvae. Of the 18 G_7 broods examined, 11 were positive for infection (Fig. 2). Distributions of the infection rate in G_6 (avg. 0·28, SD 0·10) and G_7 (avg. 0·30, SD 0·07) were similar.

(iii) *Sex ratio*

In the G_6 and G_7 generations, we selected a total of 6 broods that showed a high infection rate (>60%) in the first-instar samples, and examined the sex ratio at the pupal stage (⩾46 pupae per brood) (Table 1). The sex ratio was not significantly distorted from 0·5 for any of the 6 broods (chi-square test, $P>0{\cdot}05$), indicating that infection with *w*Kue did not affect the sex ratio in *O. scapulalis*.

Table 1. *Sex ratio in the* w*Kue-transinfected matriline of* Ostrinia scapulalis

	Infection rate (*n*)	Female : male	χ^2 test
G_6 brood 1	0·96 (83)	55 : 48	ns
G_6 brood 2	0·80 (30)	17 : 30	ns
G_7 brood 1	0·62 (114)	101 : 104	ns
G_7 brood 2	0·77 (29)	64 : 67	ns
G_7 brood 3	0·75 (16)	24 : 34	ns
G_7 brood 4	0·84 (22)	36 : 35	ns

The infection rates in six broods with >60% infection are shown. The infection rates in this table include the data for the first-instar larvae samples and the data for adult samples that were used in crossing experiments. *n* denotes the number of individuals examined. ns, $P>0{\cdot}05$.

Table 2. *Hatchability of eggs in the* w*Kue-transinfected matriline of* Ostrinia scapulalis

Crosses (female × male)	*N*	Hatchability (%) 0–20	20–40	40–60	60–80	80–100
T × T	20	14 (9)	6	0	0	0
T × U	82	0	1	8	7	66
U × T	31	31 (31)	0	0	0	0
U × U	48	0	1	4	2	41

The distribution of hatchability in crosses between *O. scapulalis* individuals transinfected (T) and those uninfected (U) with *Wolbachia*. Data from uninfected females and/or uninfected males from the transinfected matriline were excluded in the analysis of results. *N* represents the number of pairs. Thirty to 100 eggs were examined for hatchability per single pair. Numbers in parentheses indicate pairs in which hatched eggs were not found.

(iv) *Cytoplasmic incompatibility*

Crosses of transinfected and uninfected individuals were conducted in all four possible combinations using G_6 and G_7 insects (Table 2). In crosses of transinfected (T) females × uninfected (U) males (the number of families examined, *N*, is 82) and female U × male U (control) crosses ($N=48$), hatchability was higher than 80% in most cases. No significant difference in hatchability was found between the female T × male U and control crosses ($P>0{\cdot}05$). In female U × male T crosses ($N=31$), no eggs hatched. Female T × male T crosses ($N=20$) were predicted to be rescued from incompatibility, but no eggs hatched in 9 crosses, and the hatchability in the remaining 11 crosses was 2–36%. The hatchability in female T × male T crosses was significantly higher than that in the female U × male T cross ($P<0{\cdot}001$), and was significantly lower than that in the control cross ($P<0{\cdot}001$).

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4. Discussion

CI was induced in *w*Kue-infected *O. scapulalis*. Two rather unusual characteristics were found in the CI in *O. scapulalis*. First, the hatchability in the female T × male T cross was not completely restored. This is probably due to poor transmission of *w*Kue in *O. scapulalis*: Due to the low transmission rate, a large proportion of the eggs produced by most transinfected females are uninfected (Fig. 2). Hence, these eggs have no ability to rescue incompatible sperm. Moreover, the *w*Kue density in the infected eggs may not be sufficiently high to rescue incompatibility. Consequently, only a small proportion of fertilized eggs in the female T × male T cross could be saved from death. Second, although the level of vertical transmission of *w*Kue was low, the level of incompatibility was very high. To date, the incidence of strong CI caused by *Wolbachia* with a low transmission rate has not been reported. The density of *w*Kue in testes and ovaries may reach different levels in *O. scapulalis*: the density in testes was probably higher than that in ovaries, since most sperm from transinfected males appeared to be modified by *Wolbachia*. Differences in the *Wolbachia* density among tissues were found in other infected insects (e.g. Dobson *et al*., 1999; Ijichi *et al*., 2002).

(i) *Factors controlling the type of reproductive alteration*

The present finding, together with those of Fujii *et al*. (2001), clarified that *w*Sca and *w*Kue respectively causes the same type of reproductive alteration in foreign hosts as in their resident hosts. These results suggest that *Wolbachia* genotype is crucial in determining the type of alteration. Here, we should refer to the interpretation of Fujii *et al*.'s (2001) findings. These authors (2001) concluded that the type of reproductive alteration was affected by the host–*Wolbachia* interaction. This conclusion was drawn on the basis of the past hypothesis that the all-female trait in *w*Sca-infected *O. scapulalis* is caused by feminization of genetic males. Since the all-female trait in *w*Sca-infected *O. scapulalis* is now shown to be caused by male killing (Kageyama & Traut, 2004), this interpretation of Fujii *et al*.'s (2001) results needs to be revised as discussed in the present paper.

The relative importance of *Wolbachia* and the host in determining the type of reproductive alteration in other *Wolbachia*–host combinations has been examined using crustaceans and parasitic wasps. A *Wolbachia* strain that induces CI in the crustacean *Cylisticus convexus* and another strain that induces feminization in *Armadillidium vulgare* were reciprocally transinfected by microinjection, and it was suggested that the phenotype is determined by *Wolbachia* rather than by host nuclear genes (Bouchon *et al*., 1998; Moret *et al*., 2001). This case in Crustacea and the present case in Lepidoptera highlight the crucial importance of the *Wolbachia* genotype. However, Bordenstein *et al*. (2003) showed that *Wolbachia* is not always determinative. The incompatible CI cross in a wasp *Nasonia vitripennis* results in the conversion of diploid fertilized eggs into haploid males, while the incompatible cross in another wasp *N. giraulti* leads to embryonic death. Bordenstein *et al*. (2003) transferred *Wolbachia* from *N. vitripennis* to *N. giraulti* by serial backcrosses, and found that mortality-type CI occurred in the introgression line, indicating that the host nuclear genotype is the major determinant of CI type. Thus, differences in reproductive alterations found in *Wolbachia*-infected arthropods cannot simply be attributed to differences in *Wolbachia*. Studies on the interaction of *Wolbachia* and the host, along with comparative genomics of *Wolbachia* and studies on molecular mechanisms of the alteration, would help to elucidate the evolution of the phenotypic diversity of *Wolbachia*-induced reproductive alterations.

(ii) *Effect of host factors*

The present study demonstrates the effect of host factors on reproductive alteration in two ways. First, the level of incompatibility in the transinfected *O. scapulalis* was significantly higher than that in the donor strain of *E. kuehniella*: Complete incompatibility in the female U × male T cross in *O. scapulalis* (the present study) contrasts with the 60·8% hatchability in the female U × male T cross in the donor strain (Sasaki & Ishikawa, 1999). This indicates that host factors affect the level of incompatibility induced by *w*Kue. A similar host effect has been reported in several other *Wolbachia* strains (Boyle *et al*., 1993; Braig *et al*., 1994; Clancy & Hoffmann, 1997; Poinsot *et al*., 1998; Poinsot and Mercot, 2001; Bordenstein *et al*., 2003).

Second, the vertical transmission rate of *w*Kue was low in *O. scapulalis*, whereas it was nearly 100% in *E. kuehniella* (T. Sasaki, personal observation). This suggests that *w*Kue is physiologically not well adapted to the novel host *O. scapulalis*. Furthermore, because the transmission rate of *w*Sca is nearly 100% in its resident host *O. scapulalis* (H. Sakamoto, personal observation), *O. scapulalis* is not necessarily a bad vehicle for the vertical transmission of *Wolbachia* strains. Thus, both *Wolbachia* and the host affect the efficiency of maternal transmission. Reduction in the vertical transmission rate in a novel host species has also been found in other *Wolbachia* strains (Boyle *et al*., 1993; Clancy & Hoffman, 1997; Van Meer & Stouthamer, 1999; Pintureau *et al*., 2000; Poinsot & Mercot, 2001).

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