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Biotype-dependent secondary symbiont communities in sympatric populations of *Bemisia tabaci*

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Abstract

The sweet potato whitefly, *Bemisia tabaci,* harbors *Portiera aleyrodidarum*, an obligatory symbiotic bacterium, as well as several secondary symbionts including *Rickettsia*, *Hamiltonella*, *Wolbachia*, *Arsenophonus*, *Cardinium* and *Fritschea*, the function of which is unknown. *Bemisia tabaci* is a species complex composed of numerous biotypes, which may differ from each other both genetically and biologically. Only the B and Q biotypes have been reported from Israel. Secondary symbiont infection frequencies of Israeli laboratory and field populations of *B. tabaci* from various host plants were determined by PCR, in order to test for correlation between bacterial composition to biotype and host plant. *Hamiltonella* was detected only in populations of the B biotype, while *Wolbachia* and *Arsenophonus* were found only in the Q biotype (33% and 87% infection, respectively). *Rickettsia* was abundant in both biotypes. *Cardinium* and *Fritschea* were not found in any of the populations. No differences in secondary symbionts were found among host plants within the B biotype; but within the Q biotype, all whiteflies collected from sage harboured both *Rickettsia* and *Arsenophonus*, an infection frequency which was significantly higher than those found in association with all other host plants. The association found between whitefly biotypes and secondary symbionts suggests a possible contribution of these bacteria to host characteristics such as insecticide resistance, host range, virus transmission and speciation.

Keywords: *Arsenophonus*, *Hamiltonella*, host plant, *Rickettsia*, whitefly, *Wolbachia*

Introduction

The sweet potato whitefly, *Bemisia tabaci* (Gennadius), is an exceedingly polyphagous pest capable of developing on hundreds of plant species, including many agricultural crops (Oliveira *et al*., 2001). Its broad host range has been the subject of many studies aimed at understanding the whitefly's host selection preferences, as well as the influence of host plants on whitefly fitness (Van Lenteren & Noldus, 1990; Gerling & Mayer, 1996).

The taxonomic status of *B. tabaci* is a complex issue; different populations, while morphologically indistinguishable, display a high degree of genetic and biological variability, and this has led to the definition of *B. tabaci* as a species complex. To date, more than 20 biotypes, differing genetically and in host range, insecticide resistance, and ability to transmit plant viruses and cause plant disorders, have been designated (Brown *et al*., 1995; Perring, 2001).

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While some of the biotypes have a limited host range and local distribution, others, particularly biotype B, are widely distributed and polyphagous. Because biotypes can not be distinguished by morphological characters, their identification is dependent on molecular techniques.

De Barro *et al*. (2005) recently proposed grouping *B. tabaci* populations into six geographical races based on COI and ITS1 gene sequences, but the common nomenclature used is still that of biotypes. In Israel, two biotypes have been reported: B (also referred to as the silverleaf whitefly, *B. argentifolii* Bellows & Perring); and Q. According to the classification of De Barro *et al*. (2005), these two biotypes belong to two closely related sister groups: the Q biotype, which originated in the Mediterranean Basin; and the B biotype, which arose in the Middle East and Asia Minor. Despite their close geographical origins, these two biotypes rarely interbreed. Females have been reported to reject the mating attempts made by males, and the rare inter-biotype crosses observed resulted in either sterile F_1 or a hybrid breakdown at the F_2 (Ma *et al*., 2004; Shai Morin, personal communication). Both pre- and post-zygotic reproductive barriers, thus, seem to exist between the Q and B biotypes tested. The most prominent phenotypic differences between the two biotypes are the higher fecundity and competitive abilities of the B biotype and the greater degree of pesticide resistance of the Q biotype (Pascual & Callejas, 2004; Horowitz *et al*., 2005).

Like other phloem-feeders, *B. tabaci* harbors primary symbionts, obligatory bacteria which are essential for its survival and development. The symbiont, *Portiera aleyrodidarum*, is confined to specialized cells (bactriocytes) and is vertically transmitted (reviewed in Baumann, 2005). In addition to this primary symbiont, *B. tabaci* is host to a diverse array of secondary symbionts (SS). Although SS are facultatively associated with their hosts, i.e. they are not essential for host survival, they may play important roles in their host's ecology and evolution. SS currently described from *B. tabaci* are *Arsenophonus*, *Hamiltonella*, *Wolbachia*, *Cardinium*, *Fritschea* and *Rickettsia* (reviewed in Baumann, 2005; Gottlieb *et al*., 2006), but only *Hamiltonella* and *Rickettsia* have been detected in Israeli *B. tabaci* populations (Gottlieb *et al*., 2006). The function of these SS in the whitefly is unknown, but other insect-SS systems reveal assorted and intriguing phenomena. In the pea aphid, *Acyrthosiphon pisum*, for example, five different SS have been identified and studied. Two of them, *Hamiltonella defensa* and *Serratia symbiotica*, increase resistance to parasitoids of the genus *Aphidius* (Oliver *et al*., 2003; Ferrari *et al*., 2004). *S. symbiotica* also improves the aphid's heat tolerance (Montllor *et al*., 2002) and compensates for the absence of the primary symbiont (Koga *et al*., 2003). Another SS, *Regiella insecticola*, is strongly correlated with host plant range, susceptibility to environmental factors, resistance to entomophthogenic fungi and the alteration of dispersal and mating (Tsuchida *et al*., 2002, 2004; Scarborough *et al*., 2005; Leonardo & Mondor, 2006).

Bacteria of the genus, *Rickettsia* (α-Proteobacteria), are mostly known as obligate intracellular symbionts of blood-feeding arthropods and as causative agents of many vertebrate diseases such as typhus. They are evidently associated with various phenomena in non-blood feeding arthropods as well, including sex ratio disorders (reviewed in Perlman *et al*., 2006). *Wolbachia* (α-Proteobacteria) is a well-known 'reproductive manipulator', owing to its ability to alter many arthropods' reproduction processes (Stouthamer *et al*., 1999). *Wolbachia* was traced in previous surveys of *B. tabaci* (Zchori-Fein & Brown, 2002; Nirgianaki *et al*., 2003; De Barro, 2005), and it has been cited as the possible cause of cytoplasmic incompatibility among some *B. tabaci* biotypes (De-Barro & Hart, 2000). *Arsenophonus* spp. (γ-Proteobacteria) also are distributed among a variety of insects including whiteflies, aphids, psyllids and a louse fly (Baumann, 2005; Dale *et al*., 2006). *A. nasoniae* causes male embryo mortality in the parasitoid wasp *Nasonia vitripennis* (Gherna *et al*., 1991), but its effect on other hosts is unknown. *Cardinium* (Bacteroidetes) is also known as a reproductive manipulator (Weeks *et al*., 2003). Bacteria of the order, Chlamydiales, are often pathogenic to humans and vertebrates, and some are vectored by arthropods (http://www.chlamydiae.com). However, nothing is known about the phenotype of *Fritschea* (Chlamydiales) in *B. tabaci* (Everett *et al*., 2005).

This study was initiated in order to determine if SS associations and infection frequencies are correlated with the biotype and host plant of *B. tabaci*, in both field and laboratory populations. We see this as a first step towards understanding the phenotypical characteristics of these symbionts in this insect species complex.

Methods and materials

Whitefly origin and rearing

Samples of *B. tabaci* were obtained from the laboratory collection of Dr R. Horowitz (Beit Dagan, Israel). These whiteflies were originally collected in various agricultural crops, during various years and seasons, at different locations in Israel (table 1) and were maintained in the laboratory as separate populations on cotton seedlings ('Acala') (26 ± 2°C, 60% RH, 14L:10D). In order to screen field populations, live *B. tabaci* adults were collected from various crops at 18 different locations throughout Israel (table 2) during September and October 2005 and were immediately preserved in 96% ethanol.

Screening for the presence of secondary symbionts

Laboratory populations were first screened in bulk to determine the presence of the various SS. Twenty to fifty adult whiteflies from each lab population were ground together in a lysis buffer as described by Frohlich *et al*. (1999). This was followed by incubation at 65°C for 15 min, and then at 95°C for 10 min. The presence of *Rickettsia*, *Hamiltonella*, *Wolbachia*, *Cardinium*, *Arsenophonus* and *Fritschea* in the samples was determined using genus-specific primers for amplifying 16S or 23S rDNA gene fragments (table 3). Reactions were carried out in 25 μl volumes each containing 2 μl of the template DNA lysate, 10 pM of each primer, 0.2 mM dNTP's, 1× RedTaq buffer and one unit of RedTaq DNA polymerase (Sigma). PCR products were visualized on 1.2% agarose gel containing ethidium-bromide.

In order to verify the products' identity, bands were eluted, cloned into the pGEM T-Easy plasmid vector (Promega) and transformed into *E. coli*. For each bacterium, two colonies were randomly picked and sequenced (ABI 3700 DNA analyzer, Macrogen Inc., Korea). The resulting

Table 1. Presence (+) or absence (–) of secondary symbionts in *Bemisia tabaci* laboratory populations.

Laboratory population number	Plant	Biotype	SS presence			
			H	A	W	R
1	Abutilon	B	+	–	–	–
2	Cotton	B	+	–	–	+
3	Cotton	B	+	–	–	+
4	Cotton	B	+	–	–	+
5	Cotton	B	+	–	–	+
6	Cotton	B	+	–	–	–
7	Cotton	B	+	–	–	+
8	Cotton	B	+	–	–	+
9	Cotton	B	+	–	–	+
10	Cotton	B	+	–	–	+
11	Eggplant	B	+	–	–	+
12	Eggplant	B	+	–	–	+
13	Melon	B	+	–	–	+
14	Pepper	B	+	–	–	+
15	Potato	B	+	–	–	–
16	Potato	B	+	–	–	+
17	Watermelon	B	+	–	–	–
18	Zucchini	B	+	–	–	–
19	Basil	Q	–	+	–	+
20	Cotton	Q	–	+	+	–
21	Cotton	Q	–	+	+	+
22	Cotton	Q	–	+	+	–
23	Cotton	Q	–	+	–	+
24	Cotton	Q	–	+	+	+
25	Gerbera	Q	–	+	–	+
26	Roses	Q	–	+	+	+
27	Sunflower	Q	–	+	–	+
28	Sunflower	Q	–	+	–	+

R, *Rickettsia*; H, *Hamiltonella*; W, *Wolbachia*; A, *Arsenophonus*.

Table 2. Infection frequencies of different secondary symbiont combinations in *Bemisia tabaci* field populations.

Field population number	Host plant	*n**	SS combination (%)										
			B biotype			Q biotype							
			RH	H	R	RWA	RW	RA	R	WA	W	A	None
1	Cotton	20	10	–	–	5	5	30	15	–	10	10	15
2		19	5	–	–	–	5	53	16	16	–	5	–
3		20	10	10	–	20	10	20	–	5	–	20	5
4		20	–	–	–	25	5	40	5	10	–	15	–
5	Cucumber	20	45	30	–	15	–	5	–	–	–	5	–
6		20	50	50	–	–	–	–	–	–	–	–	–
7	Eggplant	20	75	20	–	5	–	–	–	–	–	–	–
8	Lettuce	19	26	5	–	11	–	26	–	16	–	16	–
9		20	50	50	–	–	–	–	–	–	–	–	–
10	Sage	20	–	–	–	30	–	70	–	–	–	–	–
11		20	10	–	–	35	–	55	–	–	–	–	–
12	Squash	20	40	40	–	–	–	5	–	–	–	15	–
13	Sweet pepper	20	60	40	–	–	–	–	–	–	–	–	–
14		20	95	5	–	–	–	–	–	–	–	–	–
15		19	37	58	–	–	–	5	–	–	–	–	–
16		20	60	35	5	–	–	–	–	–	–	–	–
17	Tomato	20	75	25	–	–	–	–	–	–	–	–	–
18		18	11	–	–	17	–	33	–	6	6	22	6

n = total number of individuals tested from each population (including both biotypes).
R, *Rickettsia*; H, *Hamiltonella*; W, *Wolbachia*; A, *Arsenophonus*; –, zero.

Table 3. PCR primers and conditions used in the study.

Targeted gene	Primers' name	Primers sequence (5'→3')	Annealing temp. (°C)/ Product size (bp)	Reference
Rickettsia 16S rDNA	Rb-F Rb-R	GCTCAGAACGAACGCTATC GAAGGAAAGCATCTCTGC	60°/~900	Gottlieb *et al.*, 2006
Hamiltonella 16S rDNA	Ham-F Ham-R	TGAGTAAAGTCTGGAATCTGG AGTTCAAGACCGCAACCTC	60°/~700	Zchori-Fein & Brown, 2002
Wolbachia 16S rDNA	Wol16S-F Wol16S-R	CGGGGGAAAAATTTATTGCT AGCTGTAATACAGAAAGTAAA	55°/~700	Heddi *et al.*, 1999
Arsenophonus 23S rDNA	Ars23S-1 Ars23S-2	CGTTTGATGAATTCATAGTCAAA GGTCCTCCAGTTAGTGTTACCCAAC	60°/~600	Thao & Baumann, 2004
Cardinium 16S rDNA	CFB-F CFB-R	GCGGTGTAAAATGAGCGTG ACCTMTTCTTAACTCAAGCCT	58°/~400	Weeks *et al.*, 2003
Fritschea 23S rDNA	U23F 23SIGR	GATGCCTTGGCATTGATAGGCGATGAAGGA TGGCTCATCATGCAAAAGGCA	60°/~600	Everett *et al.*, 2005
B. tabaci MtCOI	C1-J-2195 L2-N-3014	TTGATTTTTTGGTCATCCAGAAGT TCCAATGCACTAATCTGCCATATTA	52°/~800	Khasdan *et al.*, 2005

sequences were compared to known sequences in the databases using the BLAST algorithm in NCBI.

In order to assess within- and between-population infection rates of the bacteria found in the laboratory screening, 20 adults were taken from each field population and ground individually according to the method described above. They were screened with the relevant primers for the presence of the SS identified by the bulk screening of laboratory populations (table 3).

Identification of B. tabaci *biotypes*

Biotypes were identified using a method described by Khasdan *et al.* (2005). Briefly, in this method a fragment of the mtCOI gene is amplified by PCR (Frohlich *et al.*, 1999), followed by restriction endonuclease digestion with *Vsp*I, which generates a clear polymorphism between biotypes B and Q. PCR reactions were carried out as described above with the addition of 0.2 mM $MgCl_2$. The PCR products were incubated with 0.1 units of *Vsp*I (Fermentas) at 37°C for 16 h before loading onto agarose gel.

Statistical analysis

SS frequencies among biotypes and/or plants were compared by a Pearson non-parametric test using the χ^2 distribution. JMP 5.0.1 software (SAS Institute) was used for all statistical analyses.

Results

Screening for the presence of secondary symbionts

Laboratory populations

Hamiltonella was detected in all 18 B biotype populations, but was not found in any of the ten Q biotype populations. *Wolbachia* and *Arsenophonus* were found only in Q biotype populations (5/10 and 10/10, respectively). *Rickettsia* was found in both Q biotype (80%) and B biotype (72%) populations ($\chi^2_1 = 0.21$, $P = 0.65$). Two Q populations were double-infected with *Arsenophonus* and *Wolbachia*, five were infected with both *Arsenophonus* and *Rickettsia*, and three

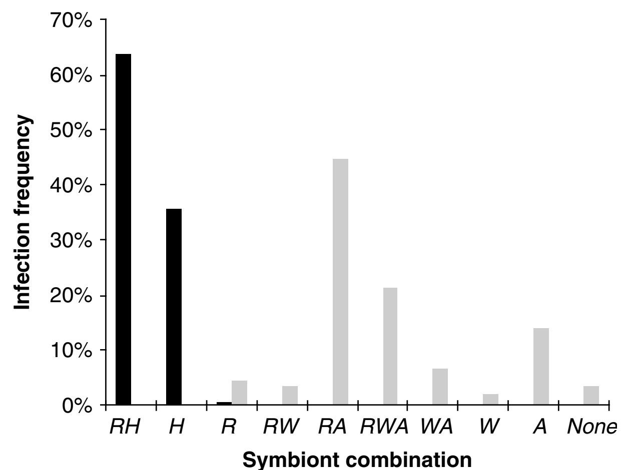

Fig. 1. Frequency of secondary symbiont combinations within field-collected individuals of B and Q biotypes of *Bemisia tabaci*. R, *Rickettsia*; H, *Hamiltonella*; W, *Wolbachia*; A, *Arsenophonus*; ■, B ($n = 205$); □, Q ($n = 150$).

harbored all three SS (table 1). None of the populations screened tested positive for the presence of either *Cardinium* or *Fritschea*.

Field populations

Since neither *Cardinium* nor *Fritschea* were detected in the mass laboratory screening, field populations were not screened for these two SS. As in the lab populations, *Hamiltonella* was detected in virtually all B biotype individuals, whereas none of the Q biotype whiteflies were infected with this bacterium (fig. 1). *Wolbachia* and *Arsenophonus* were detected only in Q biotype individuals (33% and 87% infection, respectively). Both biotypes harbored *Rickettsia*, with a somewhat higher infection percentage in the Q biotype (74% and 64%, respectively, $\chi^2_1 = 3.7$, $P = 0.054$). All possible combinations of *Rickettsia*, *Wolbachia* and *Arsenophonus* were found among the Q biotype individuals

(fig. 1); five of these individuals (3.3%) did not carry any of the SS, while none of the B individuals was SS-free ($\chi^2_1 = 6.93$, $P < 0.01$). The infection categories distribution was significantly different across the two biotypes ($\chi^2_9 = 351.4$, $P < 0.0001$).

The B biotype was dominant in field populations sampled from sweet pepper, cucumber, squash and eggplant (75–100%), while the Q biotype dominated in cotton and sage plots (80–100%) ($\chi^2_7 = 208.6$, $P < 0.0001$). In tomato and lettuce, the results were not uniform (table 2).

Infection status of SS did not differ among host plants within the B biotype ($\chi^2_{12} = 8.6$, $P = 0.73$) but did vary within the Q biotype ($\chi^2_{21} = 42$, $P < 0.01$). The differences among host plants within the Q biotype were mostly associated with individuals collected from sage plots ($n = 38$), all of which carried both *Rickettsia* and *Arsenophonus*. About one-third of these individuals also carried *Wolbachia*. Among individuals from other host plants, only ~38% carried both *Rickettsia* and *Arsenophonus* and 14–19% were triple infected (table 2). Infection with *Rickettsia* alone and with the combination of *Rickettsia* plus *Wolbachia* was found only in individuals sampled from cotton plots (seven and five individuals, respectively). *Wolbachia* infection rates were similar, 31–38% across samples from all host plants ($\chi^2_3 = 0.26$, $P = 0.96$).

Discussion

Biotype and SS

Our study demonstrates a clear association between certain SS and *B. tabaci* biotypes. All Israeli populations of the B biotype harbor *Hamiltonella*, whereas *Wolbachia* and *Arsenophonus* are found exclusively in the Q biotype (fig. 1). Variability of symbiont combinations has been reported in the past for the A and B biotypes (Costa *et al*., 1995), although at that time the identity of the bacteria was not established. It thus appears that biotype-dependent differences of SS composition can be of use in differentiating between *B. tabaci* biotypes.

Secondary symbionts are usually considered non-essential to their hosts, hence their presence between and within populations can be variable. *Hamiltonella*, for example, was previously reported from 40% of *B. tabaci* populations (Zchori-Fein & Brown, 2002) and from 0--46% of screened pea aphid populations. The incidence of *Rickettsia* in *A. pisum* also ranged from 1–48% in various reports (Chen *et al*., 2000; Darby *et al*., 2001; Tsuchida *et al*., 2002; Darby *et al*., 2003; Haynes *et al*., 2003; Ferrari *et al*., 2004). Exceptionally, all 40 clones of the aphid *Uroleucon ambrosiae* collected throughout the USA were found to carry *Hamiltonella* (Sandstrom *et al*., 2001), a result similar to the virtually 100% occurrence of *Hamiltonella* in the B biotype of *B. tabaci* reported here. Such a high incidence may indicate an obligatory or even mutualistic interaction between *Hamiltonella* and its host. There are many ways in which *Hamiltonella* may contribute to the whitefly. Two possibilities include complementing the primary symbiont, *Portiera*, as reported recently from a sharpshooter (Wu *et al*., 2006) and facilitating the higher fecundity of the B biotype, as compared to the Q and other biotypes (Brown *et al*., 1995; Nombela *et al*., 2001; Pascual & Callejas, 2004), thereby enhancing its own transmission. *Hamiltonella* might also be involved in plant physiological disorders that are exclusively caused by B biotype (e.g. squash silver-leafing). These hypotheses can be tested if *Hamiltonella*-free B biotype populations can be successfully established.

Wolbachia and *Arsenophonus* were harbored only by the Q biotype of *B. tabaci*. *Wolbachia* can be found in all major insect orders in varying frequencies (Stouthamer *et al*., 1999; Werren & Windsor, 2000). The ~30% infection proportion of *Wolbachia* found in our study is in agreement with previous reports (Zchori-Fein & Brown, 2002; Nirgianaki *et al*., 2003). Nirgianaki *et al*. (2003) reported that 11 out of 39 *B. tabaci* populations collected worldwide were infected with *Wolbachia*; of those, ten were 'non-silverleafing' biotypes, i.e. non-B biotype, as in our study. *Rickettsia* is the only SS that was found in both biotypes, a fact that might hint at a horizontal transmission pathway of this bacterium, possibly via the host plant.

The biotype-based divergence of SS resembles the strong association found between host races of *A. pisum* and their SS by Simon *et al*. (2003). In that study, *Hamiltonella* was found only in the alfalfa race of the aphid, while *Rickettsia* and *Serratia* were abundant in the pea race and rare on alfalfa and clover races. Double infections were also quite common in that study, as in ours. Other studies have revealed that *Rickettsia* and *Serratia* can suppress *Buchnera*, the primary symbiont of *A. pisum*, but *Serratia* can compensate for the essential role of *Buchnera* when the latter is absent (Koga *et al*., 2003; Sakurai *et al*., 2005). A quantitative PCR study can clarify if similar interactions exist in the *B. tabaci*-SS system.

There are several ways in which the biotype-dependence of SS communities can be explained:

1. Allopatric divergence. It has been suggested that historic geographic isolation has led to separation of races (De Barro *et al*., 2005); accordingly, the B and Q biotypes acquired different sets of SS when they were isolated in the past, and only later became sympatric.
2. Sympatric divergence. (i) B and Q biotypes may have acquired different SS while in sympatry due to the genetic differences between them; or (ii) a SS (e.g. *Hamiltonella* or *Arsenophonus*) may have invaded one population, thus erecting a reproductive barrier between this population and others, resulting in the development of a new biotype.

Wolbachia, *Rickettsia* and *Arsenophonus* are known to cause reproductive manipulations in arthropods, but their role in *B. tabaci* is unknown. The possibility that these symbionts and others may cause reproductive isolation among biotypes via cytoplasmic incompatibility or other mechanisms should be carefully explored. Understanding the role of SS in the reproductive isolation of biotypes may help resolve the long-standing debate surrounding the taxonomic status of members of the *B. tabaci* complex; if the B and Q biotypes are reproductively isolated merely because of transient SS infections, they can be regarded as biotypes of the same species, while the lack of such influence will support their division into two distinct species. However, it should be noted that the ability of biotypes to interbreed may have been lost due to molecular distance, as proposed by De Barro *et al*. (2005).

Biotype and host plant

In field populations, *B. tabaci* biotypes were distributed differently among different host plants: the Q biotype was

dominant on cotton and sage in this study; and the B biotype was dominant on sweet pepper, cucumber, squash and eggplant (table 2). While such biotype-plant affinities may suggest specific adaptations, they can also reflect the insecticidal history of the crops. For instance, Israeli cotton fields are frequently treated with pesticides against *B. tabaci* during September, and this may favour the establishment of the insecticide-resistant Q biotype on that crop (Horowitz *et al*., 2005). Firm conclusions regarding the nature of possible biotype-plant associations await further investigation. Interestingly, all whiteflies sampled in the sage plots proved to be of the Q biotype and carried *Rickettsia* and *Arsenophonus*, a significantly higher infection rate than was found on other host plants. Although these SS may be advantageous for *B. tabaci* on this plant, a bias resulting from insecticide exposure cannot be ruled out. Controlled experiments with both biotypes on sage will be necessary to gain insight into the sage-Q biotype-*Rickettsia* and *Arsenophonus* tri-trophic interactions.

The importance of SS in the biology, ecology and evolution of *B. tabaci* biotypes will be clarified by further information regarding the phenotype of each SS.

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