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Asaia, a transformable bacterium, associated with *Scaphoideus titanus*, the vector of "flavescence dorée"

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Abstract

A recent survey on the microflora associated with the leafhopper *Scaphoideus titanus* Ball (Hemiptera Cicadellidae), the vector of "flavescence dorée" (FD), showed the presence of bacteria belonging to the genus *Asaia*. These bacteria are symbionts of insects of the genus *Anopheles*, malarial mosquito vectors. We focused on the association between *Asaia* sp. and *S. titanus* with the aim to evaluate a possible role of this microorganism as a symbiotic control agent. After development of artificial feeding systems for *S. titanus*, quantitative PCR on insects or feeds DNA was performed with specific primers. Results underlined that *Asaia* sp. was dominant in the insect microflora. A strain of *Asaia* expressing a green fluorescent protein was used to perform recolonization of the body of *S. titanus*, showing the colonization by the bacterium of salivary glands, guts, female and male reproductive organs of the insect. In situ hybridization with specific probes was performed on insect dissections, confirming the presence of the bacterium in spermatic bundles and in Malpighian tubules. Easy cultivability, dominance within the bacterial population in the insect body, cryogenic preservability and easiness for genetic manipulation demonstrated by the overall data make *Asaia* sp. an optimal candidate for carrying factors for FD control.

Key words: *Scaphoideus titanus*, "flavescence dorée" Phytoplasma, *Asaia*, symbiotic control, *Anopheles*.

Introduction

The microflora associated with the leafhopper *Scaphoideus titanus* Ball (Hemiptera Cicadellidae), the vector of "flavescence dorée" (FD), has been recently investigated using cultivation-independent methods. Among the microorganisms recognized by DGGE fingerprinting, bacteria belonging to the genus *Asaia* were found (Marzorati *et al*., 2006). Included in the family of Acetobacteraceae in 2000 (Yamada *et al*.) bacteria of the genus *Asaia* were indicated only as epiphytic bacteria and correlated to different plant species (Katsura *et al*., 2001; Yukphan *et al*., 2004); while Favia *et al.* (2007) described these bacteria as symbionts of insects belonging to the genus *Anopheles*, in particular *A. stephensi* Liston, *A. gambiae* Giles, malarial mosquito vectors, and *A. maculipennis* Meigen (Diptera Culicidae). In order to develop a symbiotic control approach important requirements are the cultivability and the easy genetical manipulation, besides the stable association with the insect vector, characteristics owned by *Asaia* sp.. Here we focused on *Asaia* sp. and the insect *S. titanus* with the aim to understand the bacterial association with the leafhopper and to evaluate a possible role of this microorganism as a symbiotic control agent.

Materials and methods

S. titanus adult individuals were collected between July and September 2004 in vineyards with heavy symptoms of FD from different areas in the Piedmont region (NW Italy). In order to simulate the natural situation of insects feeding on plant, artificial feeding systems were developed and filled with 300 µl of sucrose 5% in TE pH 8 with or without rifampicin (200 µg ml^{-1}). In each feeding system one insect was fitted and obliged to feed on it. DNA extractions from insects and feeds were performed according to a method previously described by Doyle and Doyle (1990). Quantitative real-time PCR on insects or feeds DNA was performed by using primers Asafor and Asarev as reported by Favia *et al*. (2007) with Brilliant Sybr green qPCR Master Mix (Stratagene, La Jolla, CA). Furthermore, *Asaia* SF2.1 (Gfp), a strain of *Asaia* isolated from *A. stephensi* and able to express a green fluorescent protein was used to perform recolonization of the body of *S. titanus* as previously described (Favia *et al*., 2007). After different times of exposure to the feeding solutions supplemented with *Asaia* sp. SF2.1 (Gfp), insects were dissected in PBS and guts, salivary glands and reproductive organs were observed with a IX71 fluorescent microscope (Olympus, Melville, NY) and a MRC600 laser scanning confocal microscope (Bio-Rad, Hercules, CA). Tissues were fixed as described previously (Favia *et al*., 2007). Field-collected insects were also employed for in situ hybridization. ISH was performed on paraffin-embedded sections of *S. titanus* organs as described elsewhere (Beninati *et al*., 2004; Favia *et al*., 2007). Probe EUB338 was used as a bacterial positive control, while for specific detection of *Asaia* cells probes called Asaia1 and Asaia2 were utilized.

Results and discussion

To evaluate the relative abundance of *Asaia* sp. in different *S. titanus* individuals and in their respective artificial feeding systems with or without antibiotic, we measured *Asaia* sp. to total bacteria 16S rRNA gene copy ratio (ABR) with quantitative real-time PCR. *Asaia* sp. 16S rRNA gene copies represented a mean of 4.9% and 1.2% of the total 16S rRNA gene copies in insects and feeds without antibiotic, respectively, while they constituted a mean of 0.3% and 4.2% of the total 16S rRNA gene copies in insects and feeds with antibiotic. These data underlined that *Asaia* sp. was a significant part of the insect microflora and that it was also released inside the feeding solutions as demonstrated through the detection of *Asaia* 16S rRNA gene copies in the artificial feeding solutions. The distribution pattern of this microorganism inside the insect body was investigated through the use of *Asaia* expressing a green fluorescent protein. *Asaia* strain SF2.1 tagged with Gfp was able to colonize efficiently salivary glands, gut, and female (ovary duct) and male reproductive organs (testes, seminal vesicles, gonoducts and accessory glands) of *S. titanus* individuals. *In situ* hybridization analysis performed with specific probes for *Asaia* sp. on insects not exposed to *Asaia* SF2.1 (Gfp) suspensions confirmed the presence of the wild bacterium in the Malpighian tubules between the brochosomes and within the spermatic bundles. Detection of *Asaia* in male and female reproductive organs was of particular interest, suggesting a possible bacterial transmission to the offspring, a fundamental requirement for an efficient symbiotic control approach.

Easy cultivability, dominance within the bacterial population in the insect body, the cryogenic preservability and the easiness for genetic manipulation make *Asaia* sp. an optimal candidate in order to vehiculate anti-Phytoplasma or anti-leafhopper factors for FD control, by the way of a natural symbiotic control approach or, as an alternative, by a paratransgenic approach (Bextine *et al.*, 2004; Beard *et al.*, 1998).

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