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Investigation over the life cycle of ST1-C the endosymbiont of *Scaphoideus titanus*

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Abstract

Scaphoideus titanus Ball (Hemiptera Cicadellidae) is a phloem-feeder leafhopper vector of '*Candidatus* Phytoplasma vitis', the phytoplasma responsible of "flavescence dorée" (FD), a grape yellows afflicting several wine production areas in Europe. Natural symbionts of the leafhopper for symbiotic control of FD transmission could be used. Previous work showed the presence of a CFB bacterium, '*Candidatus* Cardinium sp.' (named ST1-C) in several body districts of *S. titanus*. Our work was aimed to investigate the life cycle of ST1-C endosymbiont inside and outside of insect body. We fed *S. titanus* individuals on artificial media and tested insects and diets after the treatment for the presence of the ST1-C endosymbiont by qualitative and quantitative PCR, showing the release of the bacterium during feeding. We also performed fluorescent in situ hybridization on the sugar solutions after insect feeding, detecting ST1-C cells that confirmed its release into the medium. We tested by ST1-C-specific PCR DNA extracted from small areas of grape leaves exposed to *S. titanus,* identifying the bacterium in the majority of pierced leaves. Our experiments suggest that the ST1-C endosymbiont can undergo a horizontal transmission and have a complex life cycle possibly involving more than one host.

Key words: "flavescence dorée", *Scaphoideus titanus*, *Cardinium*, symbiotic control, horizontal transmission.

Introduction

"Flavescence dorée" (FD) is one of grape yellows that afflicts several wine production areas in Europe, from Portugal to Hungary (Angelini *et al.*, 2001; Bianco *et al*., 2001). FD is caused by a bacterium, '*Candidatus* Phytoplasma vitis', transmitted by the leafhopper *Scaphoideus titanus* Ball (Hemiptera Cicadellidae), which inoculates the phytoplasma into the phloem of healthy plants during feeding on the sap. FD transmission control is therefore a high priority in European wine producing areas. An innovative strategy is the 'symbiotic control' approach (Bextine *et al.*, 2004), in which microorganisms living in symbiosis with the insect host could exploit mechanisms for reducing vector competence (Beard *et al*., 1998) or interfering with the pathogen itself. Previous works (Marzorati *et al.*, 2006; Bigliardi *et al.*, 2006), demonstrated the presence in *S. titanus* of a CFB bacterium, '*Candidatus* Cardinium sp.' named ST1-C, in several organs and tissues of the vector of FD. Furthermore, it has been highlighted that ST1-C and '*Ca.* Phytoplasma vitis' have a complex life cycle in the body of this leafhopper and are co-localized in different organs and tissues. In this way, the endosymbiont population could be a useful tool for insect and/or phytoplasma biocontrol. The aim of this work is to investigate over the life cycle of ST1-C endosymbiont inside and outside of insect body.

Materials and methods

A total of 120 *S. titanus* individuals were collected across summer seasons 2005 and 2006 in vineyards without symptoms of FD, and in greenhouse at Di.Va.P.R.A. campus. 40 insects to be studied by transmission electron microscopy and by in situ hybridization were dissected. 80 insects were used for feeding trials on artificial medium and on small areas grapevine leaves. DNA extraction was performed according to a method previously described by Doyle and Doyle (1990) or with DNeasy Plant mini kit (QIAGEN GmbH, Germany). A suitable method of artificial feeding-cage was set up, using 2 ml tubes as insect chambers. The tube caps were filled with 250 µl of 5% sucrose in TE and sealed with parafilm. Antibiotic treatment consisted in the same artificial insect diet + rifampicin (300 µg/ml). A cage system on grapevine leaves was set up. Small plastic insect chambers were placed on the surface of grape leaves to force *S. titanus* to feed on a small leaf area. Direct and Nested specific PCR with primers Endo F1, Endo F2 and Endo R3 (Marzorati *et al.*, 2006), were set up to detect this endosymbiont in the body of individuals of *S. titanus*, in their artificial diet, and in small areas of grape leaves after a 48 hours feeding period. Specific quantitative real-time PCR assays with primers Endo F1 and Endo R3 were set up to quantify ST1-C endosymbiont in both *S. titanus* (with and without antibiotic treatment) and in his diet after 48 hours of feeding period. In situ hybridization (ISH), and fluorescent in situ hybridization were set up to identify ST1-C endosymbiont respectively in the body of *S. titanus* and in his artificial diets after feeding period; optical and fluorescence microscopy were used to find out where the probe bounded in the analyzed samples.

Results and discussion

We initially examined wild *S. titanus* individuals for the presence of ST1-C in the salivary glands. We consistently observed ST1-C cells in all the salivary glands examined by TEM. The identification of the bacterium was confirmed by ISH analysis. Small spots of cells were clearly visible in the salivary glands within transversal sections of the insect head. To verify if *S. titanus* can release ST1-C in the feeding medium we prepared two caging groups of adult individuals: the first one fed on the artificial diet and the second one fed on the same diet supplemented with antibiotic. The second group worked as a control group for confirming the release of the bacterium. We first checked the capability of *S. titanus* to live on the artificial medium. Some individuals were able to survive on the artificial diet for periods up to 30 days. We sampled both the artificial diets and the insect individuals at different time of feeding, to search for the presence of ST1-C by a standard qualitative and quantitative PCR. By using a ST1-C specific quantitative PCR assay, we found that the range of 16S rRNA gene copy number of the bacterium was 1.66×10^1 – 1.92×10^8 in the insect body. This number decreased of two orders of magnitude in the insects treated with the antibiotic. According to quantitative PCR the bacterium was released in the sugar solution since we were able to detect 1.66×10^1 – 5.33×10^7 16S rRNA gene copy per each sugar solution. The release of the bacterium during feeding was confirmed by analyzing the sugar solution of the control cage treated with antibiotic. Only one of fifteen separate feeding solutions analyzed by quantitative PCR showed a PCR signal. The experiments indicate that when the bacterium survival in the insect body is impaired by the antibiotic, the bacterium cannot be released in the diet, differently from the individuals not exposed to the antibiotic. To confirm the release of the bacterium during feeding we probed the sugar solutions after the feeding of the insect with a 16S rRNA ST1-C - specific fluorescent DNA probe. We were able to detect single or pairs of bacterial cells that positively responded to the specific probe, confirming that the bacterium was unambiguously released during feeding. We also fed individuals of *S. titanus* captured in the field, on leaves of grapevine plant never exposed to the leafhopper. We forced the insect to bite the leaves on the nervations by keeping it within small cages put on the leaf surface. In this way the insect could only bite a 5 cm^2 area of the leaf. Hence we extracted the DNA from the bitten area and from another equal leaf area placed at 2 cm from the one where the insect was feeding and that have never been in contact with the insect. We identified ST1-C by PCR in the 68% of the bitten leaves, while no one of the control leaves positively responded to the PCR test.

The overall data indicate that the life cycle and the transmission pattern of ST1-C is much more complex than previously shown. ST1-C endosymbiont of *S. titanus*, resulted to be released during the insect feeding process. We can hence suppose that this endosymbiont can undergo, besides vertical transovarial transmission (Marzorati *et al*., 2006), a horizontal pathway of transmission. This hypothesis suggests a complex life cycle of the bacterium, involving more than one host.

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