COST Action: FA0807 "Integrated Management of Phytoplasma Epidemics in Different Crop Systems"
Short-term Scientific Mission (STSM) Report

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STSM topic: Investigations on presence and expression of collagene-like genes in stolbur phytoplasma isolates

COST STSM Reference number: ECOST-STSM-FA0807- 10359

Grant Letter Reference Code: COST-STSM-ECOST-STSM-FA0807-130512-016521

STSM dates: from 13-05-2012 to 26-05-2012

Purpose of the visit

A gene encoding a collagen-like secreted protein was identified upon a stolbur phytoplasma genome survey. In order to check collagen-like protein expression in stolbur phytoplasma, collagen-like gene was cloned in pBAD expression vector for *E. coli*. The expressed protein was purified and injected to mice in order to produce anti-collagen like monospecific polyclonal antibodies. The aim of the STSM is to assess collagen-like expression in various stolbur isolates by western blot analysis of stolbur infected periwinkles and to detect the presence of collagene-like genes in a collection of stolbur phytoplasma isolates.

Description of the work

Protein, DNA and RNA extraction

In order to assess collagen-like expression in various stolbur isolates by western blot analysis, extraction of proteins was performed from the following samples of stolbur infected tomato and periwinkle from the collection of stolbur phytoplasma isolates of the host laboratory: T-PO, PO, GGY, P7, MOL, Char 1, Char 2, STOL-C and LG. Approximately 1 g of midveins from infected plants as well as from healthy tomato and periwinkle were crushed in 1.5 mL of Laemmli buffer (50 mM Tris-HCl pH 6.8, 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 0.1 (w/v) bromophenol blue, 10% (v/v) glycerol). Extracts were transferred to clean 1.5 mL tubes and incubated for 15 min at 100 ˚C and centrifuged for 1 min at 10 000 g. Supernatant containing proteins was transferred to a clean tube. Proteins, DNA and RNA were previously extracted from individual insects of stolbur phytoplasma vector species *Reptalus panzeri* and *Hyalesthes obsoletus* by using TRI Reagent®.

PCR amplification

Following primers were used to detect the presence of collagen-like gene (*coll-like*) as well as *stamp* gene and hyper variable *vmp*3 gene encoding a membrane protein with collagen-like repeated motifs at C-terminal part:

- coll F1/R1 (Foissac et al., unpublished) – *coll-like* gene
- vmp3 F4/R3 followed by vmp3 F5/R4, and vmp3 F1/R1 (Salar and Foissac, unpublished) – *vmp3* gene
- stamp F/R0 folllowed by stamp F1/R1 (Fabre at al., 2011) – *stamp* gene.

PCR products were subjected to the electrophoresis in 1% agarose gels in 0.5X TBE buffer for 1 h at 100 V.

Western blot and immuno-dot blot analyses

All conditions for Western blot analysis to assess collagen-like expression in various stolbur isolates from plant and insect samples had to be optimized in order to test the anti-*coll*-like antibody and to achieve a desirable sensitivity of the reaction. Proteins extracted from infected and healthy plants and insects, together with positive and negative control (proteins extracted from *E. coli* expressing *coll*-like protein and calmoduline, respectively) were separated by electrophoresis on 12.5% SDS-PAGE at 50 V for approximately 16 h (overnight). After the electrophoresis, proteins were transferred to the nitrocellulose or PVDF membrane by semi-dry transfer system. Transfer conditions were varied (150 or 200 mA) together with the time of the transfer (30 min or 1h). After the transfer, the membrane was saturated in blocking solution (TBS buffer with 5% non-fat dry milk) for 1 h followed by incubation with primary antibody for 1-2h. Membrane was washed 3 times in TBS-Tween 20 (0.2%) and 2 times in TBS buffer and incubated with anti-mouse secondary antibody conjugated to peroxidise. After the washes, western blot was revealed by using WestPico Luminol chemilluminescent substrate.
For dot-blot, 2-5 µl of protein extracts were applied directly on the membrane, followed by the immunodetection as described for western blot analysis.

Description of the main results

PCR amplification

- *coll-like* gene

  Amplicons of collagen-like gene variable in size were obtained from 4 *Reptalus panzeri* samples and from the positive control PO stolbur strain, while from *Hyalesthes obsoletus* samples as well from healthy periwinkle, no amplification was detected (Fig 1).

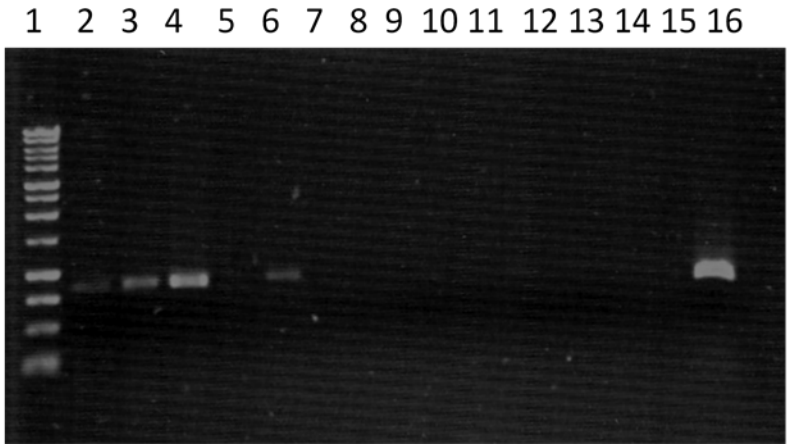

Fig 1. Amplification of stolbur collagen-like gene. Lane 1 – DNA ladder; lanes 2-8 – *Reptalus panzeri* samples; Lanes 9-13 – *Hyalesthes obsoletus* samples; lane 14 – water control; lane 15 – healthy periwinkle; lane 16 – PO strain from periwinkle

- *vmp*3 gene

Since *vmp*3 is a variable membrane protein gene with a C-terminal part containing collagen-like (GXY) repeats the number of which varies from one strain to another, the amplicons obtained from different *R. panzeri* and *H. obsoletus* samples were of different size (Fig 2.).
When N-terminal part of the gene was amplified by vmp3 F1/R1 primers, amplicons obtained were not that variable in size. However, the protocol needs to be optimized further (Fig 3.).

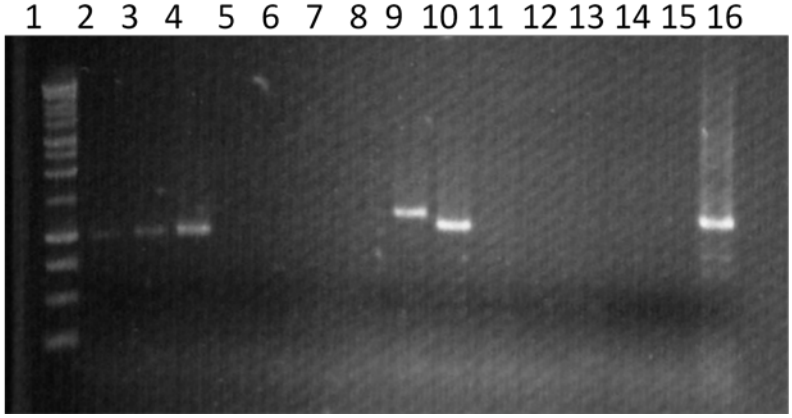

Fig 2. Amplification of C-terminus of stolbur *vmp*3 gene in a direct PCR by using vmp3 F4/R3 primers. Order of samples same as on Fig 1.

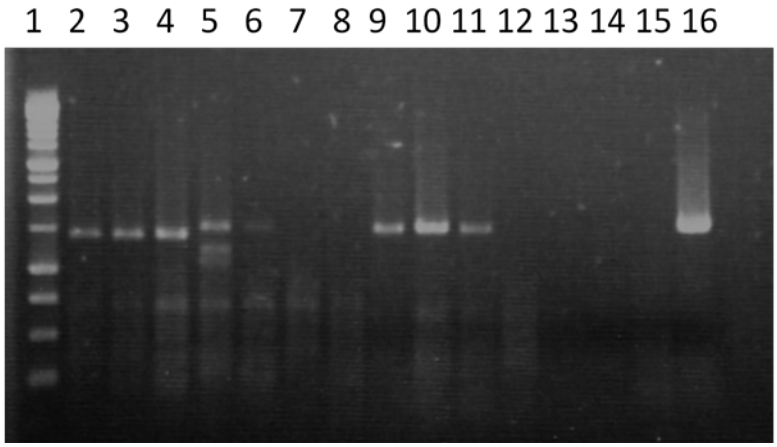

Fig 3. Amplification of N-terminus of stolbur *vmp*3 gene in a direct PCR by using vmp3 F1/R1 primers. Order of samples same as on Fig 1.

- *stamp* gene

In order to verify the stolbur phytoplasma presence in insect samples, stamp gene was additionally amplified (Fig 4.) and amplicons obtained in nested PCR by using stamp F1/R1 were sent for sequencing.

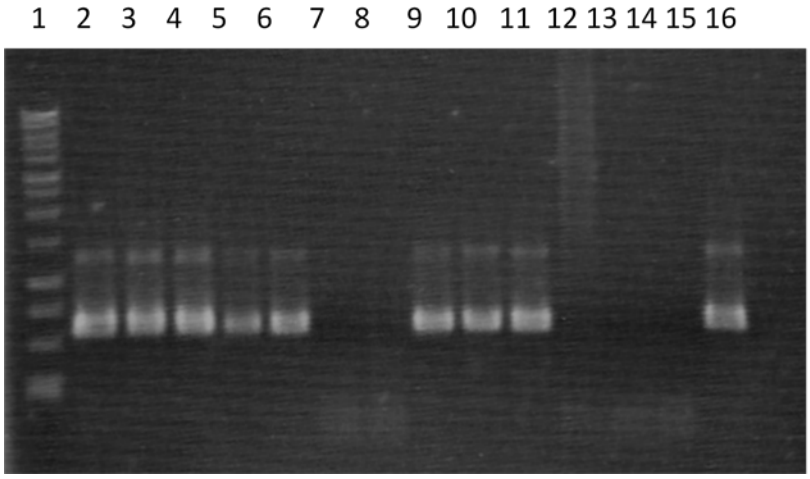

Fig 4. Amplification of *stamp* gene by nested PCR. Order of samples same as on Fig 1.

Western blot and immuno-dot blot analyses

Optimization of conditions for the western blot analysis of stolbur phytoplasma *coll*-like protein has shown that the best results were obtained when PVDF membrane was used with 30 minutes transfer at 150 mA (Fig 5). When nitrocellulose membrane was used with a longer transfer and when higher current was applied, no positive reaction was detected, probably due to unsuccessful transfer and leakage of proteins through the membrane. Since the optimization process is time-consuming and sample-consuming, once when the optimal conditions were reached most of the newly isolated samples were consumed and *coll*-like expression was detected only in the positive control sample of total proteins from *E.coli* expressing *coll*-like protein (Fig 5.). However, in dot blot analysis with crude protein extract, *coll*-like expression was detected in stolbur-infected tomato T-PO (Fig 6.).

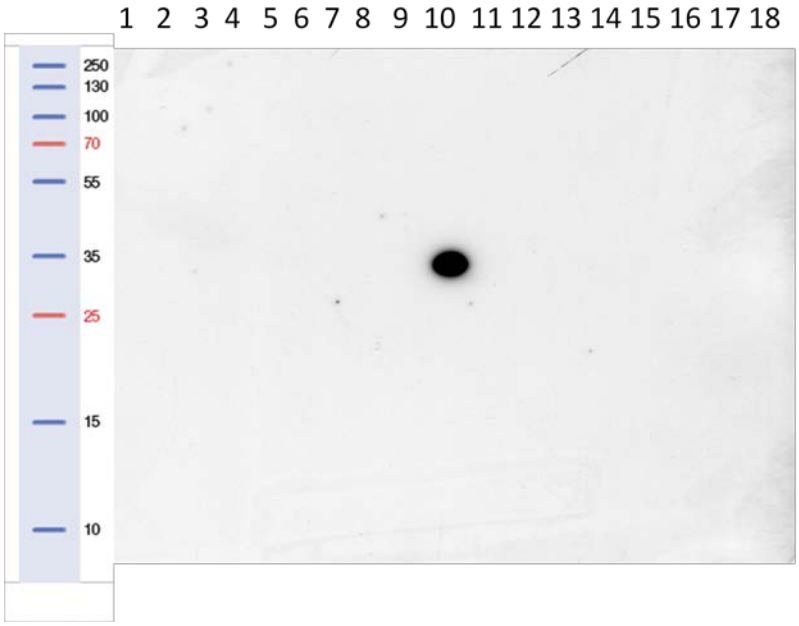

Fig 5. *Coll*-like expression detected in Western blot with anti-*coll*-like antibody. Lanes 1 to 8 – total proteins of healthy and infected plants; lane 9 – total proteins of *E.coli* expressing calmoduline; lane 10 – total proteins of *E.coli* expressing *coll*-like protein; lanes 11 to 18 - total proteins of healthy and infected insects.

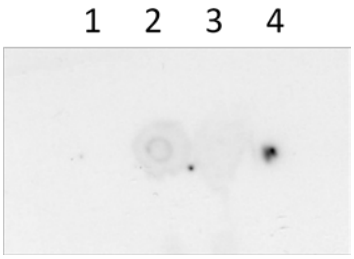

Fig 6. Immuno-Dot blot analysis detecting *coll*-like expression in stolbur-infected tomato T-PO (lane 2) and *E.coli* expressing *coll*-like protein (lane 4). In healthy tomato (lane 1) and *E.coli* expressing calmoduline no expression was detected.

Summary

The presence of stolbur phytoplasma *coll*-like gene was detected in *R. panzeri* samples with amplicons of different size, showing the presence of variable number of repeats. C-terminal part of *vmp*3 gene containing collagen-like repeats was also successfully amplified from both *R. panzeri* and *H. obsoletus* samples, revealing considerable difference in number of repeats. Dot blot analysis has detected the expression of *coll*-like protein in tomato infected with PO strain and *E.coli* expressing *coll*-like protein. Conditions for the assessment of the *coll*-like expression in various stolbur isolates from infected plants and insects by Western blot analysis were optimized. However, the sensitivity of the reaction was not completely satisfactory. Also, the optimization process was time- and sample-consuming, and once the optimal conditions were reached, it was not possible to repeat the experiment due to the complete consumption of samples.

Future collaboration with host institution

During this STSM, a protocol for the assessment of the expression of stolbur *coll*-like protein was successfully optimized. Since the sensitivity of the reaction was not satisfactory and the optimization was time- and sample-consuming, the main goal could not be completely achieved. Collaboration with the host institution in the near future should enable us to complete it by having the new protein extracts from plants with high phytoplasma titer and pooled insect samples instead of one insect per sample.