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Purification of phytoplasma DNA for sequencing the "flavescence dorée" and stolbur phytoplasma genomes

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Abstract

In order to compare genomes of the "flavescence dorée" and stolbur phytoplasmas, we produced fractions from infected plants enriched in phytoplasma DNA. DNA of phytoplasma strains FD92 and stolbur PO were respectively prepared from broad beans and tomato plants by repeated bis-benzymide-CsCl buoyant density gradient centrifugations. Sequence of cloned DNAs were homologous to known phytoplasma sequences in 27 % to 54 % of the cases. According to these data, the sequencing of the genome of these two organisms can be undertaken. A draft of the FD92 phytoplasma genome using the pyrosequencing technology will be first envisaged.

Key words: *Mollicutes*, "bois noir", CsCl gradient, genome sequencing.

Introduction

Vineyards of southern Europe are affected by the "flavescence dorée" phytoplasma (FD), inducing an epidemic and quarantine disease of the grapevine. FD is transmitted by the ampelophagic leafhopper of nearctic origin *Scaphoideus titanus* Ball, and has an important negative economic impact on grapevine production (Boudon-Padieu, 2002). Another grapevine yellows causing serious damage in Euro-mediterranean vineyards is the "bois noir" (BN) disease. BN is associated with the endemic stolbur phytoplasma which is mainly transmitted by a polyphagous planthopper, *Hyalesthes obsoletus* Signoret (Fos *et al.,* 1992). Stolbur phytoplasmas (Stolp) are reported across Europe, parts of Asia and Africa. They affect a wide range of crops including tomato, potato pepper, tobacco, lavender and strawberry.

Whereas important progress has been made in phytoplasma classification and ecology, little is known about mechanisms of phytopathogenicity and transmission by insects; these research areas should benefit from the knowledge and comparative analysis of phytoplasma genomes.

Recently, the complete genome sequences of 2 lines of '*Candidatus* Phytoplasma asteris', OY-M (860 kbp) and AY-WB (706 kbp) have been published. Genome characterization is underway for '*Ca.* P. pruni', '*Ca.* P. mali' and '*Ca.* P. australiense'. A modified suppression subtractive hybridization (SSH) method allowed to isolate some nearly pure Stolp DNA (line PO) from infected *Catharanthus roseus* periwinkle plants, and to characterize 15% of stolbur phytoplasma genome (Cimerman *et al.*, 2006). However this subtractive method did not provide gene libraries representative enough for sequencing the Stolp genome. For FDp no SSH gene libraries could be prepared. As a consequence, the classical CsCl density gradient method was preferred for the preparation of enriched sources of phytoplasma DNA.

Materials and methods

FD92 phytoplasma was transmitted to *Vicia faba* var. Aqua Dulce using leafhoppers collected in FD-affected vineyards in South-west of France in 1992, and maintained since that time by continuous serial transmissions from broad bean to broad bean using the alternative leafhopper *Euscelidius variegatus* (Kirschbaum). Stolbur phytoplasma (PO) was transmitted by grafting *Solanum lycopersicum* tomato plants var. Elsa Craig.

Phytoplasma DNA was enriched from host plant DNA, by 4 repeated bis-benzymide Cesium chloride density gradients (Kollar and Seemüller, 1989). The bis-benzymide Hoechst 33258 binds preferentially to adenine + thymine (A+T)-rich sequences thereby reducing their buoyant density. So, the phytoplasma DNA band was characterized by a lower buoyant density than that of the host DNA. Total nucleic acids were extracted from stems and leaf midribs of infected broad beans, periwinkles or tomatoes by the cetyltrimethylammonium bromide (CTAB) procedure (Murray and Thompson, 1980). Purified nucleic acid pellets were resuspended in 1X TE buffer. One mg of total DNA was used for each CsCl gradient. The same procedure was performed with healthy plants as a control.

DNA was mechanically sheared or cut by *Hind*III. Nebulization of DNA was performed using nebulizers (Invitrogen life technologies) according to the manufacturer's instructions to achieve a 1-10 kbp size range. After reparation, DNA was ligated in dephosphorylated pBS vector, previouly linearized by *Hinc*II and *Sma*I or by *Hind*III. Plasmids were cloned in *E.coli* DH10B cells, and purified using SV Miniprep Promega Kit. Inserts of recombinant plasmids were sequenced on ABI Prism sequencer.

Results

After the first centrifugation, the expected enriched phytoplasma DNA was visible as a faint band above the main band of the host DNA (figure 1, gradient 1) by

comparison to the healthy plants tubes (HBB). The upper band was removed from tubes and pooled to initiate a second CsCl centrifugation. The second centrifugation of four pooled fractions (gradient 2) resulted in a strongly reduced lower band of host DNA, and a brighter upper band expected to contain the phytoplasma DNA. This upper band was collected and introduced in a third gradient (gradient 3) from which, the visible band was again submitted to a fourth gradient. The 4th gradient clearly provided a low density DNA fraction for FD and stolbur phytoplasmas. Hosts plants DNA bands were no longer visible (gradient 4). After final centrifugation, the phytoplasma was destained by isopropyl alcohol partitioning and cleaned of CsCl by dialysis in 1X TE buffer.

Purified DNA fractions have been obtained from FD infected broad beans (3 to 7 µg per fraction), and stolbur PO infected tomatoes (4 to 52 µg per fraction). To evaluate the quantity of phytoplasma DNA in enriched fractions, plasmidic libraries after nebulization which generates randomly sheared DNA fragments were produced. A representative number of clones was sequenced in order to define the proportion of clones containing phytoplasma DNA.

Sequence similarities with '*Ca.* P. asteris' and stolbur phytoplasma or plant sequences revealed that, in the case of FD92, the upper band contained 27% of FD DNA, 12% of plant DNA, and 61% of DNA without any homology with recorded sequences. In the same way, the upper band purified from stolbur PO infected tomato plants contained 54% of Stolp DNA, 7% of tomato plant DNA and 39% of orphan sequences.

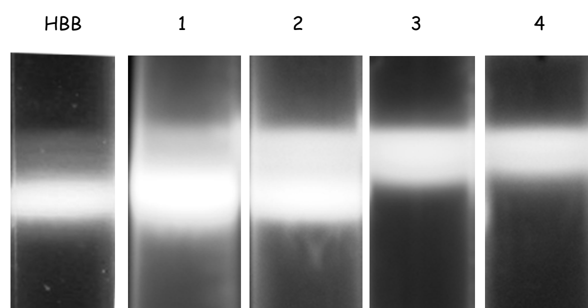

Figure 1. FD92 phytoplasma DNA purification from midribs and stems of infected broad beans in bis-benzymide cesium chloride density gradients. HBB: 1 mg healthy broad bean DNA. Gradient 1: 1 mg total DNA/tube, 2: pool of the upper band from 8 gradients 1, 3: upper band from gradient 2, 4: upper band from gradient 3.

Discussion

As previously demonstrated, purification of phytoplasma DNA from broad beans, and tomatoes respectively infected by 2 different phytoplasmas, clearly demonstrates the validity of the repeated bis-benzymide-CsCl buoyant density gradients which results in highly enriched phytoplasma band. By this way, DNA sources is available for the complete genome sequencing of these 2 organisms. The FD92 phytoplasma genome sequence is planned by the use of the pyrosequencing technology (Margulies *et al.*, 2005) which avoids the fastidious setting up of genomic libraries with a high number of clones.

First results will be presented for FD and commented regarding to the technologic improvements as well as to the comparative genomics point of view.

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References

BOUDON-PADIEU E., 2002.- Flavescence dorée of the grapevine: knowledge and new developments in epidemiology, etiology and diagnosis.- *Atti Giornate Fitopatologiche,* 1: 15-34.

CIMERMAN A., ARNAUD G., FOISSAC X., 2006.- Stolbur phytoplasma survey achieved using a suppressive subtractive hybridization approach with high specifity.- *Applied and Environmental Microbiology,* 72: 3274-3283.

FOS A., DANET J. L., ZREIK L., GARNIER M., BOVE J. M., 1992.- Use of a monoclonal antibody to detect the stolbur mycoplasma-like organism in plants and insects and to identify a vector in France.- *Plant Disease,* 76: 1092-1096.

KOLLAR A., SEEMÜLLER E., 1989.- Bases composition of the dna of mycoplasma-like organisms associated with various plant diseases.- *Journal of Phytopathology,* 127: 177-186.

MARGULIES M., EGHOLM M., ALTMAN W. E., ATTIYA S., BADER J. S., BEMBEN L. A., BERKA J., BRAVERMAN M. S., CHEN Y. J., CHEN Z. T., DEWELL S. B., DU L., FIERRO J. M., GOMES X. V., GODWIN B. C., HE W., HELGESEN S., HO C. H., IRZYK G. P., JANDO S. C., ALENQUER M. L. I., JARVIE T. P., JIRAGE K. B., KIM J. B., KNIGHT J. R., LANZA J. R., LEAMON J. H., LEFKOWITZ S. M., LEI M., LI J., LOHMAN K. L., LU H., MAKHIJANI V. B., MCDADE K. E., MCKENNA M. P., MYERS E. W., NICKERSON E., NOBILE J. R., PLANT R., PUC B. P., RONAN M. T., ROTH G. T., SARKIS G. J., SIMONS J. F., SIMPSON J. W., SRINIVASAN M., TARTARO K. R., TOMASZ A., VOGT K. A., VOLKMER G. A., WANG S. H., WANG Y., WEINER M. P., YU P. G., BEGLEY R. F., ROTHBERG J. M., 2005.- Genome sequencing in microfabricated high-density picolitre. Reactors.- *Nature,* 437: 376-380.

MURRAY M. G., THOMPSON W. F., 1980.- Rapid isolation of high molecular plant DNA.- *Nucleic Acids Reseach,* 8: 4321-4326.

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