Vitis 36 (1), 35–38 (1997)

Identification and typing of grapevine phytoplasma amplified by graft transmission to periwinkle

by

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S u m m a r y : Yellows of grapevine are spreading in many parts of the world and have been attributed to mycoplasma-like organisms (MLOs; phytoplasma). A classification and basic understanding of grapevine phytoplasma requires samples of sufficient size which are not easy to prepare. Various phytoplasma isolates have been graft-transmitted from grapevine to periwinkle. Using PCR we show that the various phytoplasma types found in grafted periwinkle faithfully match the type found in the donor grapevine. PCR analysis of phytoplasma in periwinkle was more pronounced than the respective analysis in the donor grapevine. Therefore transmission to periwinkle may facilitate the study of grapevine phytoplasma.

K e y w o r d s : phytoplasma, grapevine yellows, periwinkle, PCR, restricted PCR.

Introduction

Grapevine yellows have been found in various parts of the world including North America, France, Germany, Italy, Israel and Australia (MAGAREY *et al.* 1988; CAUDWELL 1990, 1993; GRANATA and GRIMALDI 1991; BOVEY and MARTELLI 1992). The causative agents of many yellows in plants were determined by electron microscopy to be mycoplasma-like organisms (MLOs; CAUDWELL *et al.* 1971; MEIGNOZ *et al.* 1992). The name 'Phytoplasma' was later assigned to the group of plant MLOs which belong to the class Mollicutes, characterized by their small genome size (680 to 1,600 kbp) and lack of a cell wall (NAMBA *et al.* 1993 a; GUNDERSEN *et al.* 1994).

Phytoplasma can be detected in plants by fluorescence staining (DALE 1988), serology (CHEN *et al.* 1993), and molecular hybridization (KIRKPATRICK *et al.* 1987; SEARS *et al.* 1989; DAIRE *et al.* 1992; NEIMARK and KIRKPATRICK 1993). In recent years, phytoplasma have mostly been diagnosed by polymerase chain reaction (PCR) with "universal" primers carrying phytoplasma-conserved sequences of the 16S ribosomal DNA (AHRENS and SEEMÜLLER 1992; LEE *et al.* 1993; NAMBA *et al.* 1993 b; RASIN 1994; MAIXNER *et al.* 1995). These universal primers distinguish between phytoplasma and other microorganisms, but not among the various phytoplasma species. In order to further classify the phytoplasma type, the PCR products can be used as templates for nested PCR with type-specific primers (LEE *et al.* 1993, 1994), or subjected to restriction analysis (DAIRE *et al.* 1993; DAVIS *et al.* 1993; LEE *et al.* 1993, 1994).

Several types of phytoplasma have been found in plants belonging, among others, to the following subgroups: Aster Yellows (AY), Western-X Disease (WX) and Elm Yellows, with each subgroup being further subdivided (GUNDERSEN *et al.* 1994; SEEMÜLLER *et al.* 1994). PCR, nested PCR and restriction analysis of PCR products have indicated the occurrence of a variety of phytoplasma types in grapevine (LEE *et al.* 1994).

Phytoplasma are often transmitted by leafhoppers. However, in grapevine, only a few cases have hitherto been reported to be leafhopper-transmissible. In France, the causative agent of the grapevine yellows disease flavescence dorée is transmitted by *Scaphoideus titanus* (CAUDWELL *et al.* 1971; MEIGNOZ *et al.* 1992) and in Germany the Vergilbungskrankheit is transmitted by *Hyalesthes obsoletus* (MAIXNER *et al.* 1995). Many plants are affected by phytoplasma and many types infect periwinkle (*Catharanthus roseus*). Various phytoplasma have been transmitted to periwinkle by dodders or insects (see for example, LEE *et al.* 1994). The agents of grapevine diseases Vergilbungskrankheit and flavescence dorée have been transmitted from grapevine to periwinkle *via* dodder (DAVIS *et al.* 1993; MAIXNER *et al.* 1994).

The graft-transmission of a wider range of phytoplasma from grapevine to periwinkle is reported here. We also show that the infected periwinkle faithfully represents the type of phytoplasma in the donor grapevine. Consequently, a better source for further studies on grapevine phytoplasma has become available.

Materials and methods

P l a n t m a t e r i a l : Grapevines exhibiting yellows symptoms served as source material for grafting and DNA extraction. Samples were collected from Chardonnay, Gewürztraminer, Merlot, French Colombard, Cabernet franc, Carignane and Cabernet Sauvignon vines grown in various parts of Israel: the Golan Heights (north), Zora'a (center) and the Arad valley (south). A healthy grapevine (cv. Mission) which has served as a disease-free indicator for years, was used as negative control.

G r a f t t r a n s m i s s i o n : Shoot tips of infected grapevines were wedge-grafted onto the stem of healthy periwinkle plants. The graft site was covered with plastic to keep it moist. Symptoms usually developed within 2-3 months.

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Extraction of phytoplasma-enriched DNA from grapevine: 1 g of the main veins of grapevine leaves was macerated with a chilled mortar in 13 ml of freshly prepared extraction buffer: 0.1 M $KHPO_4$, 0.03 M KH_2PO_4, 0.3 M sucrose, 0.15 % bovine serum albumin, 2 % PVP-10 and 0.3 M ascorbic acid. The extract was clarified by a brief centrifugation at 2,000 *g*, followed by a 30 min centrifugation at 30,000 *g* and 4 °C. The pellet was resuspended in 3 ml of buffer (5 mM Tris-HCl, pH 7.0, 10 mM EDTA, 0.1 % 2-mercaptoethanol) and brought to 1 % with SDS. After incubation for 10 min at room temperature, 5 M potassium acetate was added at 1/3 of the volume and the sample was chilled on ice for 30 min. Following centrifugation, the supernatant fluid was mixed with 1 vol of chloroform, shaken and centrifuged. The aqueous phase was transferred to another tube, and DNA was precipitated by adding 0.6 vol of isopropanol and incubating at -70 °C for 15 min. The precipitated DNA was pelleted by centrifugation, washed with 70 % cold ethanol and resuspended in 100 µl H_2O. Phytoplasma-enriched DNA was extracted from periwinkle according to MAIXNER *et al.* (1995).

Fig. 1: Transmission of yellows from grapevine to periwinkle. A scion from a diseased grapevine (cv. Chardonnay from the Golan Heights) was grafted onto periwinkle. Symptoms on the grafted periwinkle (3 months after grafting) are shown on the left. A control periwinkle (grafted with a healthy grapevine shoot tip) is shown on the right.

PCR analysis: Universal primers (detecting any plant phytoplasma) and group-specific primers were used. Primers were synthesized according to published sequences (LEE *et al.* 1994) and information provided by Dr. R. E. DAVIS (USDA, Beltsville, MD, USA). The following primer pairs were used:

1. Universal primers: ACGACTGCTGCTAAGACTGG and TGACGGGCGGTGTGTACAAACCCC
2. AY-specific primers: TAAAAGACCTAGCAATAGG and CAATCCGAACTGAGACTGT
3. WX-specific primers: AAGAGTGGAAAAACTCCC and TCCGAACTGAGATTC

DNA (20 ng) was subjected to PCR with Appligene's Taq polymerase and Appligene's buffer at a Mg^{++} concentration of 2.5 mM. The reaction mixture (50 µl) was brought to 94 °C (5 min), and cooled to 55 °C. The Taq polymerase was added at this point and the mixture was incubated at 72 °C for 2 min. The reaction mixture was then subjected to 40 cycles of 92 °C (30 s), 55 °C (30 s) and 72 °C (30 s). In the last cycle, the elongation step (72 °C) was extended to 7 min. The PCR products were analyzed by electrophoresis on 1 % agarose gels. Restriction-pattern analysis (LEE *et al.* 1993) was carried out on 15 µl of PCR product. Cleavage with *Mse* I, *Alu* I, *Hpa* II , or *Kpn* I was performed in the buffer specific to the corresponding enzyme in a total volume of 30 µl, at 37 °C for 20 h. Fragments were separated by electrophoresis on 5 % polyacrylamide gels.

PCR products, amplified from phytoplasma-infected grapevine with group-specific primers were also obtained

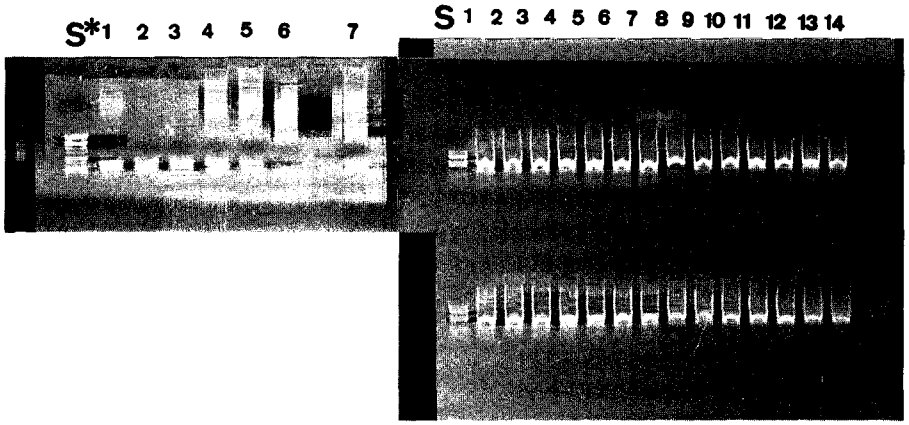

Fig. 2: PCR analysis of infected grapevine and periwinkle plants. PCR was conducted with universal primers. The left frame represents the negative controls. Lane S*: Size markers. Lane 1: PCR without any DNA template. Lanes 2 and 3: PCR with DNA from healthy periwinkle. Lanes 4 and 5: PCR with a DNA template from a healthy Mission grapevine. Lane 6: PCR with an irrelevant DNA template. Lane 7: Positive control; PCR product of cloned AY DNA. Right frame: PCR products from grapevine and periwinkle. Samples in each lane represent the donor grapevine (upper row) and its respective recipient periwinkle plant (lower row). Lane S: Size markers. The origin of infection in the various lanes are the following diseased grapevines: Lane 1: Gewürztraminer. Lanes 2, 3, 7, 9, 11, 12: Chardonnay. Lane 4: Merlot. Lane 5: French Colombard. Lanes 6, 10: Cabernet franc.Lane 13: Carignane. Lane 14: positive control of cloned DNA.

from Dr. R. E.Davis. The DNA was cloned into the plasmid pGEM-T (Promega) and amplified products of the respective plasmids served as internal positive controls in every PCR assay.

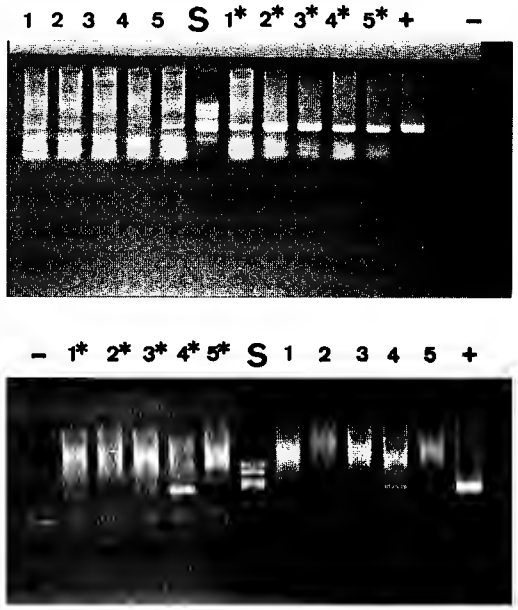

Fig. 3 (top): Nested PCR analysis of infected grapevine and periwinkle plants. The first PCR was conducted with universal primers and the second with primers specific for AY. PCR was carried out with DNA from the donor grapevine plants and the respective recipient periwinkle plants. Donor and respective recipient plants are designated by the same number (asterisk denotes periwinkle). The origin of infection is from the following diseased grapevines: Lanes 1 and 1*: Merlot. Lanes 2 and 2*: French Colombard. Lanes 3 and 3*: Cabernet Sauvignon. Lanes 4, 5, 4*, and 5*: Carignane. Lane S: Size markers. Lane +: Positive control. PCR was performed with a cloned AY DNA. Lane -: negative control (no template).

Fig. 4 (bottom): Nested PCR analysis of infected grapevine and periwinkle plants. PCR was conducted with primers specific for WX. Lanes and sample sources see Fig. 3.

Results

Disease transmission experiments were carried out by grafting scions from yellows exhibiting grapevines onto periwinkle. The grafted periwinkle developed disease symptoms within 3 months (Fig. 1) in 42 % (36/85) of the cases. Grafting scions from non-infected grapevines did not produce symptoms in periwinkle.

Each symptomatic grapevine donor was checked by PCR with universal primers to determine whether they carry any phytoplasma at all. The same test was performed with periwinkle DNA. All 45 symptom-showing grafted periwinkle plants were thus found to carry phytoplasma (Fig. 2).

Typing experiments were carried out to verify that the transmitted phytoplasma faithfully represented the exact form of pathogen present in the donor grapevine. In general, two forms of typing were performed: subgroup typing by nested PCR with group-specific primers, and individual typing by restriction analysis of the various group-specific nested PCR products. The major subgroups of grapevine phytoplasma found in Israel were AY and WX (Figs. 3 and 4). Figs. 3 and 4 also demonstrate that the phytoplasma transmitted to periwinkle were of the same subgroup as in the donor grapevine. Restriction analysis of the various nested PCR products with a variety of enzymes (Figs. 5 and 6) also indicated that the amplified phytoplasma-related DNAs from grapevine and the respective periwinkle were identical.

Discussion

Periwinkle is a common host for quite a number of different phytoplasma types. In grapevine the causative agents of Vergilbungskrankheit and flavescence dorée were transmitted to periwinkle by dodder. The procedure reported here can be used with any phytoplasma-infected grapevine. The transmission of phytoplasma by grafting is far easier than by dodder and can be performed on a larger scale.

The grafting of grapevine shoot tips onto periwinkle is relatively easy, and transmission of the respective phytoplasma to the grafted periwinkle is efficient. Symptom development and PCR analysis with universal primers confirmed the presence of phytoplasma sequences in the diseased periwinkle. Group-specific nested PCR analysis and restriction analysis of the PCR products indicated a perfect match between the type of phytoplasma in the donor grapevine and that in the recipient periwinkle. Phytoplasma titers in periwinkle were higher than in grape-

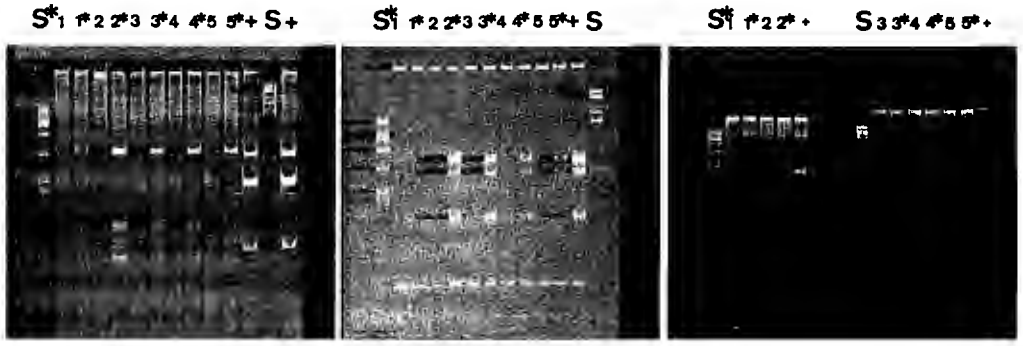

Fig. 5: Restriction analysis of PCR products from infected grapevine and periwinkle plants. The PCR product of the AY-specific nested PCR was digested with *Mse* I (left frame), *Alu* I (middle frame) or *Hpa* II (right frame). The origin of infection is from the following diseased grapevines: Lanes 1 and 1*: Merlot. Lanes 2 and 2*: French Colombard. Lanes 3 and 3*: Cabernet franc. Lanes 4, 4*, 5 and 5*: Carignane. Lane +: positive control. PCR was performed with cloned AY DNA and then submitted to restriction analysis. Lanes S and S*: size markers.

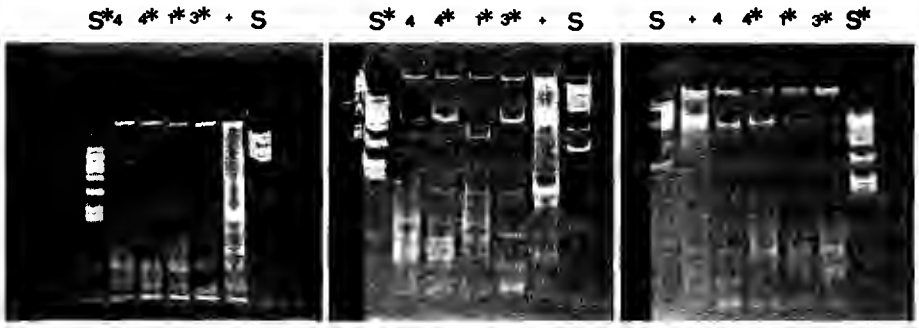

Fig. 6: Restriction analysis of PCR products from infected grapevine and periwinkle plants. The PCR product of the WX-specific nested PCR was submitted to digestion with *Mse* I (left frame), *Alu* I (middle frame) and *Hpa* II (right frame). Lanes 1*, 3* and 4*: DNA was extracted from periwinkle (origin of infection Merlot, Cabernet franc and Carignane). Lane 4: DNA was extracted from a Carignane grapevine. Lane +: positive control of restricted cloned WX DNA. Lanes S and S*: size markers.

vine making their isolation as well as the extraction of their nucleic acid easier in the former than in the latter. Since the phytoplasma composition in the periwinkle faithfully represents the situation in the respective donor grapevine, an easier in-depth study of grapevine-associated phytoplasma can now be conducted.

Acknowledgement

This project was supported by a grant from the Israel-U.S. Binational Agricultural Research and Development Foundation, BARD (#US 2335/95).

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Received October 2, 1996