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Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure

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Abstract

A polymerase chain reaction procedure was developed which enables specific amplification of a ribosomal sequence from the mycoplasmalike organism (MLO) associated with German grapevine yellows (Vergilbungskrankheit, VK) and stolbur-related diseases of solanaceous plants. Successful amplification from all samples prepared from various cultivars collected in different viticultural areas indicates that the causal agent is a relatively homogeneous organism. Amplification was also achieved with template DNA prepared from naturally infected weeds in vineyards such as *Convolvolus arvensis* and *Solanum nigrum*, and from the planthopper *Hyalesthes obsoletus* that was collected in the vineyards. Feeding of insects of this species on grapevine seedlings resulted in the development of typical yellows symptoms by the grapes. *H. obsoletus* could therefore be identified as a vector of Vergilbungskrankheit.

Abbreviations: FD = Flavescence dorée; GY = Grapevine yellows; MLO = Mycoplasmalike organism; PCR = Polymerase chain reaction; RFLP = restriction fragment length polymorphism; VK = Vergilbungskrankheit (German grapevine yellows).

Introduction

Grapevine yellows (GY) diseases, a complex of serious disorders of *Vitis vinifera* L., occur in viticultural areas world wide. A GY disease called 'Vergilbungskrankheit' (VK) occurs in several viticultural areas of Germany. Mycoplasmalike organisms (MLO) have been found associated with flavescence dorée (FD), a yellows disease in southern France [Caudwell *et al.*, 1971a], which is transmitted by the leafhopper *Scaphoideus titanus* Ball [Schvester *et al.*, 1961]. Recently, MLOs were detected in *V. vinifera* affected by other European and American grapevine yellows diseases [Bianco *et al.*, 1993; Chen *et al.*, 1993; Daire *et al.*, 1993a; Daire *et al.*, 1993b; Davis *et al.*, 1993; Maixner and Ahrens, 1993; Prince *et al.*, 1993] by employing the polymerase chain reaction or DNA hybridization techniques. Attempts to detect MLOs in symptomatic vines by other methods frequently failed, apparently due to their low titer in grapevine.

Although the various GY induce similar symptoms, the MLOs detected in vines affected by FD and other GY are not identical. The FD-MLO is related to the pathogen associated with elm yellows [Daire *et al.*, 1992; 1993a] while the pathogens associated with 'Vergilbungskrankheit' in Germany, bois noir in northern France and different GY diseases of the Mediterranean exhibit a close relationship to the pathogen associated with stolbur of solanaceous plants [Daire *et al.*,

1993b; Maixner *et al.*, 1994]. These GYs occur in areas where the vector of FD is not present, or they proved to be not transmissible by *S. titanus* [Caudwell *et al.*, 1971b]. However, analysis of the spatial distribution of GY in Germany [Maixner, 1993] and Italy [Credi and Callegari, 1988] revealed aggregations of infected vines in the vineyards, which indicate a spread of these diseases in the field. Field observations by Caudwell *et al.* [1972] led to the assumption, that bois noir is presumably spread by vectors that are not specialized grapevine feeders. Numerous leafhopper and planthopper species including known MLO vectors have been found in vineyards [Vidano *et al.*, 1987; Maixner, unpublished data], but no vector of a GY beside FD was yet identified. A preliminary report on transmission of VK by *H. obsoletus* has been published [Maixner, 1994].

The VK-MLO and a pathogen associated with stolbur of solanaceous plants (STOL-MLO) are closely related. Seemüller *et al.* [1994] determined the level of homology of their 16S ribosomal DNA sequences as more than 99%. According to these authors, VK- and STOL-MLOs form a subgroup of the aster yellows strain cluster. They both exhibit approximately 97% homology with typical aster yellows strains. Schneider *et al.* [1993] classified the stolbur-type MLO strains as a subgroup of the aster yellows group on the basis of restriction fragment length polymorphism (RFLP) analysis.

In the present work, we report the development of oligonucleotide primers for the polymerase chain reaction (PCR) which proved to be specific for stolbur-type MLOs. We applied this technique to detect MLOs associated with Vergilbungskrankheit. Furthermore, the method was employed in epidemiological studies that led to the detection of alternative host plants of the pathogen in vineyards and succeeded in the identification of a planthopper vector of VK.

Materials and methods

Plant samples

Samples were collected from symptomatic grapevines of eight different cultivars grown in five viticultural areas of Germany (Table 1). Entire leaves were taken from symptomatic shoots between July and September 1993. Leaves of grapevine seedlings grown in a greenhouse served as healthy controls.

Healthy periwinkle (*Catharanthus roseus*) and the following MLO strains maintained in periwinkle were used for comparison: AP, apple proliferation from Italy (obtained from L. Carraro, Universita degli Studi, Udine, Italy); AT, Apple proliferation, Germany; VAC, Witches broom of *Vaccinium myrtillus*, Germany (both obtained from R. Marwitz, Biologische Bundesanstalt, Berlin, Germany); ACLR, Aster yellows-related strain from an apricot tree affected by apricot chlorotic leaf roll, Spain (obtained from G. Llacer, IVIA, Moncada-Valencia, Spain, via F. Dosba, INRA, Bordeaux, France); EY, Elm yellows, New York and ASHY, Ash yellows, New York (both obtained from W. Sinclair, Cornell University, Ithaca NY, USA); MOL, Molières disease of cherry, France (obtained from F. Dosba, INRA, Bordeaux, France); PYLR, Peach yellow leaf roll, California, a strain of Western X-disease (obtained from M.F. Clark, Horticultural Research International, East Malling, UK); SAY, American western aster yellows (obtained from B.C. Kirkpatrick, University of California, Davis CA, USA); STOL, Stolbur of *Capsicum annuum*, Serbia (obtained from D. Sutic, University of Beograd, Serbia, Via R. Marwitz), STOLF, Stolbur of *Lycopersicon esculentum*, France (obtained from M.-T. Cousin, INRA, Versailles, France). Beside periwinkle isolates, samples were collected in the field from *Prunus armeniaca* (apricot) with yellowing and decline symptoms.

Weeds grown in VK affected vineyards which exhibited symptoms of a MLO infection were included in this study. We sampled bindweed, *Convolvolus arvensis*, with stunting symptoms, and black nightshade, *Solanum nigrum*, which exhibited virescence. Samples were further prepared from leaf midribs of *Vicia faba* and grapevine seedlings which served as host plants for planthoppers in transmission experiments.

Insect samples

The Cixiids *Oliarus panzeri* and *Hyalesthes obsoletus*, the Delphacid *Asiracea clavicornis*, and

Table 1. Origin of grapevines with symptoms of Vergilbungskrankheit used as sources for DNA-extraction

Viticultural area	Location	Cultivar	Symptoms	PCR (fStol/rStol)
Mosel-Saar-Ruwer	Kues	Riesling	+	+
	Bernkastel	Riesling	+	+
	Bernkastel	Müller-Thurgau	+	+
	Bernkastel	Scheurebe	+	+
	Platten	Riesling	+	+
	Lieser	Riesling	+	+
	Enkirch	Riesling	+	+
	Kröv	Riesling	+	+
	Wolf	Riesling	+	+
	Graach	Riesling	+	+
	Wehlen	Riesling	+	+
	Senheim	Riesling	+	+
Mittelrhein	Boppard	Optima	+	+
	Boppard	Dunkelfelder	+	+
	Boppard	Spätburgunder	+	+
	Boppard	Kerner	+	+
	Boppard	Müller-Thurgau	+	+
	Boppard	Riesling	+	+
	Boppard	Ruländer	+	+
	Boppard	Scheurebe	+	+
Rheinhessen	Nierstein	Scheurebe	+	+
Nahe	Monzingen	Riesling[1]	+	+
Rheinpfalz	Herrenletten	Riesling[2]	+	+
Greenhouse	Healthy control	Seedling	–	–

[1] Collected by H.P. Lipps, SLVA Bad Kreuznach, Germany.
[2] Collected by M. Rüdel, SLFA Neustadt/Weinstr., Germany.

the Deltocephalid *Neoaliturus fenestratus* were collected individually from weeds and grapevine in vineyards. Laboratory-reared *Fiberiella florii* served as healthy controls (provided by G. Krczal, Landesanstalt für Pflanzenschutz Mainz, Germany). Insects were fed in pot-cages on *Vicia faba* for one week and on grapevine seedlings for another week. They were then removed from the plants and stored at –20 °C.

Isolation of DNA

For extraction of DNA from grapevine samples, leaf midribs (60 mg) or whole insects were ground in 2-ml microcentrifuge tubes with 750 µl of buffer (2% cetyltrimethylammoniumbromide (CTAB), 1.4 M NaCl, 0.2% mercaptoethanole, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0), 250 µl of buffer were added and the samples incubated for 10 min at 60 °C. The supernatant was transferred to a 1.5-ml cup after centrifugation for 1 min at 3200 g, and one volume of chloroform/isoamylalcohol (24:1, v/v) was added. The mixture was centrifuged for 5 min at 3200 g and one volume of ice-cold isopropanole was added to the supernatant. The preparation was stored at –20 °C for 20 min and centrifuged at 14900 g for 6 min. The pellet was washed with 70% ethanol, dried, and resuspended in 50 µl of sterile water. DNA from *C. roseus* and *P. armeniaca* was extracted according to Ahrens and Seemüller [1992]. Individual insects were crushed in 400 µl buffer (2% CTAB, 1.4 M NaCl, 10 mM EDTA, 100 mM Tris-HCl, pH 8.0) [X. DAIRE, pers. comm.] and prepared as described for grapevine.

Sequencing and primer selection

To obtain primers specific for VK and stolbur, variable regions of the 16S rRNA gene and the

spacer region separating the 16S and 23S rRNA genes of VK- and STOL-MLO were examined for specific sequences. The 16S rDNA sequences of these MLOs [Seemüller *et al.*, 1994] (AC numbers X76428 (VK) and X76427 (STOL)) were compared to sequences available in the database of the European Molecular Biology Laboratory, Heidelberg: AT-MLO (AC-Number: X68375), AAY-MLO, American aster yellows (X-68373), SAY-MLO (M86340), WX-MLO, Western X-disease (L04682); ACLR-MLO (X68338), OAY-MLO, Virescence of *Oenothera hookeri* (M30790), PPER-MLO, European stone fruit yellows of peach (X68374), VAC-MLO (X76430), SCWL-MLO, Sugarcane white leaf (X76432), ULW-MLO, elm witches' broom (X68376), and ASHY-MLO (X68339).

Prior to sequencing of the spacer region of VK MLO, the region was amplified by a PCR using primers P1 from the 5′ region of the 16S rRNA gene [Deng and Hiruki, 1991], and P7 from the 5′ region of the 23S rRNA gene(obtained from B.C. Kirkpatrick, Davis, USA). These primers allow the amplification of a DNA fragment consisting of the complete 16S rRNA gene together with the 16S-23S spacer region and the 5′ terminal region of the 23S rRNA gene, which was used as template for the following sequencing procedure. Amplification was achieved with 35 cycles for 1 min at 94 °C for denaturation, 1 min at 52 °C of annealing, and 30 s at 72 °C of elongation.

The PCR products obtained were electrophoresed in a 1.5% horizontal agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0; 5 V cm^{-1}), and the target DNA fragments were eluted from the gel using the Quiaex kit according to the instructions of the manufacturer (Quiagen). Sequencing of the 16S–23S rDNA spacer region was performed using the dsDNA Cycle Sequencing System following the manufacturer's recommendation (BRL-Life Technologies). Primer rP7 was end-labeled with [^{32}]gamma dATP and used in an asymmetric PCR reaction consisting of 20 cycles of 45 s at 95 °C, 5 s at 50 °C, and 30 s at 72 °C. Amplification products were electrophoresed in 6% Long Ranger (AT Biochem) buffer gradient gel, which was vacuum-dried and exposed to X-ray film at room temperature.

The 16S–23S rDNA spacer sequence of VK-MLO was compared to analogous sequences of SAY-MLO [Kuske and Kirkpatrick, 1992] WX-MLO [B.C. Kirkpatrick, pers. comm.], OAY-MLO [Lim and Sears, 1989], ASHY-, ULW- and SCWL-MLO [R. Mäurer, pers. comm.], and AT-, AP-, ACLR-, KV-, P.Per, P.arm.- MLOs [Ahrens, 1994].

MLO-detection by PCR

PCR amplifications were carried out with primers U1/U4 [Ahrens and Seemüller, 1992] and fStol/rStol developed in this study, utilizing the thermal cyclers PE 480 (Perkin Elmer Cetus) and OmniGene TR3 (Hybaid Ltd.). With primers U1/U4, 40 cycles were carried out with 60 s at 94 °C of denaturation, 60 s at 55 °C of annealing and 30 s at 72 °C of elongation. With primers fStol/rStol, we performed 35 cycles of 94 °C for 60 s, 58 °C for 60 s, and 72 °C for 30 s. Reaction mixtures of 25 µl contained 50–100 ng of template DNA, 250 nM of each primer, 100 µM of each of four dNTPs (Pharmacia), 0.75 Units of Replitherm DNA polymerase and polymerase buffer (Biozym Diagnostik) and were overlaid by 25 µl of mineral oil. Water instead of template DNA was added to the reaction mixture for controls.

PCR products obtained with primers U1/U4 were analyzed by polyacrylamide gel electrophoresis (10 % polyacrylamide, 10 V cm^{-1}, TBE buffer: 45 mM Tris-borate, 1 mM EDTA, pH 8.0) of restriction fragments, obtained by *Alu*I digestion of amplified fragments according to Ahrens and Seemüller [1992].

Products obtained with primers fStol/rStol were analyzed by electrophoresis of 5 µl of each reaction mixture in a 1.5% horizontal agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0; 5 V cm^{-1}). DNA was stained with ethidium bromide and visualized in UV-light. Molecular weight standards (1 kb ladder, BRL life Technologies) were used on each gel.

Results

Selection and specificity of primers fStol and rStol

The primers fStol and rStol which were designed as a result of multiple comparisons of the 16S rRNA gene and 16S–23S spacer sequences of STOL and VK MLOs with sequences of various

other MLOs have the sequence 5′-GCCATCAT-TAAGTTGGGGA-3′ and 5′-AGATGTGACC-TATTTTGGTGG-3′, respectively. Primer fStol exhibited a difference of at least four bases to the corresponding sequences of the other MLOs, while less than 50% homology was observed between the primer rStol and the sequences of the other MLOs. PCR using these primers resulted in the amplification of fragments of approximately 570 bp from template DNA isolated from symptomatic grapevine or from periwinkle plants infected by the stolbur and Molières disease MLOs (Fig. 1). No amplification was achieved with DNA prepared from healthy grapevine and periwinkle.

The results of PCR amplification with primers fStol/rStol and template DNA prepared from healthy plants and various MLO isolates are shown in Fig. 2. With the parameters applied, amplification of a fragment of approximately 570 bp was only achieved with DNA of the stolbur isolate. All other samples tested in this study, which previously proved to be MLO infected, led to negative results.

Detection of VK MLO in grapevine, weeds, and insects

DNA fragments of identical size were amplified from all samples collected from symptomatic grapevine of various cultivars in different viticultural areas of Germany (Table 1; Fig. 3).

Bindweed, *C. arvensis*, and black nightshade, *S. nigrum*, that showed stunting and virescence symptoms, respectively, were frequently found in vineyards. MLOs were detected by the DAPI

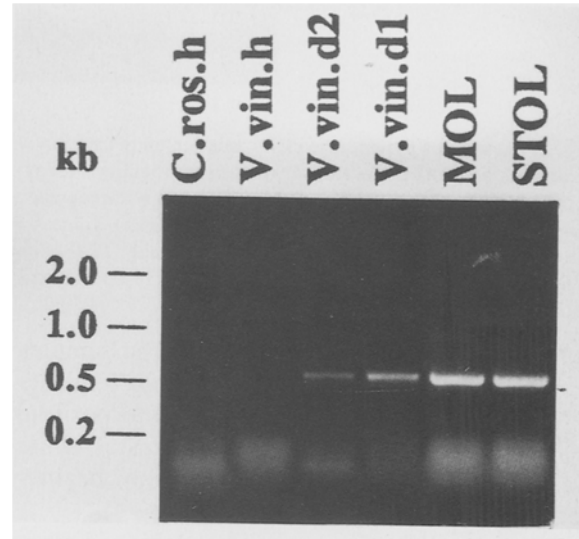

Fig. 1. Results of DNA amplification with primers fStol/rStol and template DNA prepared from periwinkle and grapevine. C.ros.h = Healthy periwinkle; V.vin.h = Healthy grapevine; V.vin.d2, V.vin.d1 = Field grown grapevines showing symptoms of Vergilbungskrankheit; MOL = Periwinkle isolate of Molières disease; STOL = Periwinkle isolate of Stolbur.

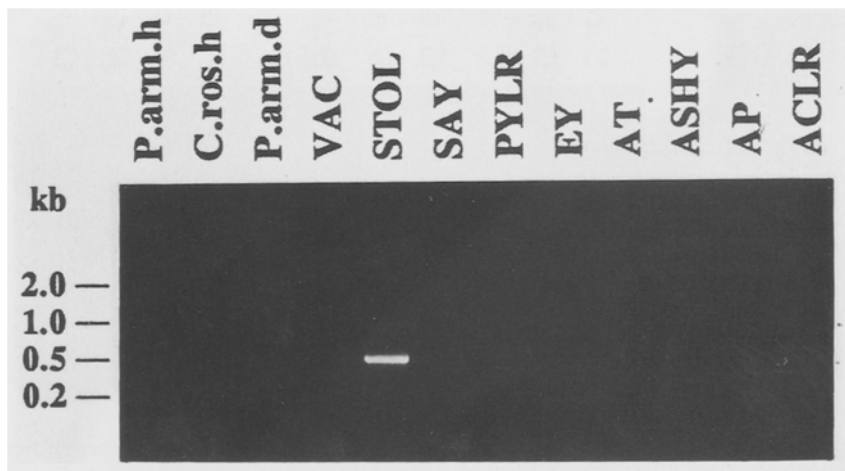

Fig. 2. Results of DNA amplification with primers fStol/rStol and template DNA prepared from periwinkle and *Prunus armeniaca*. The MLO isolates represent major MLO groups and subgroups proposed by Seemüller *et al.* (1994). P.arm.h = *Prunus armeniaca* healthy; C.ros.h = Periwinkle healthy; P.arm.d = *P. armeniaca* with yellowing and decline symptoms; VAC = Witches broom of *Vaccinium myrtillus*; STOL, Stolbur of *Capsicum annuum*; SAY, American western aster yellows; PYLR = Peach yellow leaf roll, a strain of Western X-disease; EY = Elm yellows; AP, AT = Apple proliferation; ASHY = Ash yellows; ACLR = Aster yellows-related strain from an apricot tree affected by apricot chlorotic leaf roll.

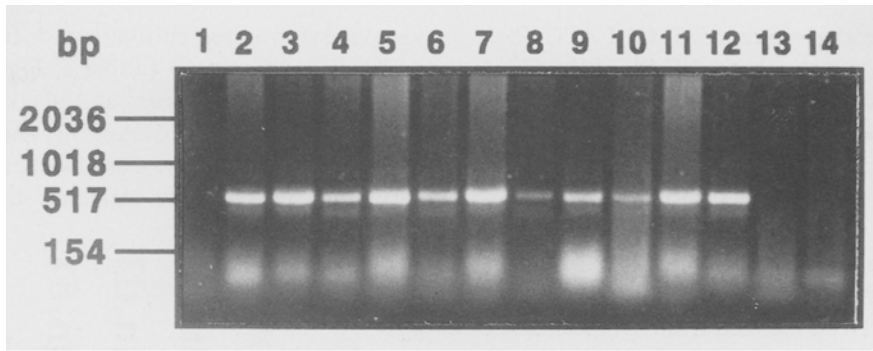

Fig. 3. Results of polymerase chain reaction amplification with primers fStol/rStol and template DNA prepared from *V. vinifera* of various cultivars collected from different viticultural areas of Germany: 1 = Healthy grapevine seedling; 2–12: Vines exhibiting symptoms of VK; 2 = 'Riesling', Mosel-area; 3 = 'Scheurebe', Mosel; 4 = 'Müller-Thurgau', Mosel; 5 = 'Riesling', Middel-Rhine; 6 = 'Kerner', Middel-Rhine; 7 = 'Ruländer', Middel-Rhine; 8 = 'Spätburgunder', Middel-Rhine; 9 = 'Dunkelfelder', Middel-Rhine; 10 = 'Riesling', Palatine; 11 = 'Riesling', Nahe; 12 = 'Scheurebe', Rheinhessen; 13 = Symptomless 'Riesling', Mosel; 14 = Water control. A fragment of approximately 570 bp was amplified from all samples collected from symptomatic vines.

staining technique [Seemüller, 1976] in samples from plants exhibiting such symptoms. Template DNA obtained from these plants led to positive results in PCR with the primers fStol/rStol, while no amplification was observed with healthy seedlings of the respective weeds (Fig. 4).

DNA-amplification was also achieved with template DNA prepared from the Cixiid *H. obsoletus* collected on bindweed in vineyards. Seventeen of 45 planthoppers (38 %) of this species that were examined tested positively. No amplification was observed with other species of Auchenorrhyncha collected in vineyards, the Cixiid *O. panzeri* (n = 21), the Delphacid *A. clav-*

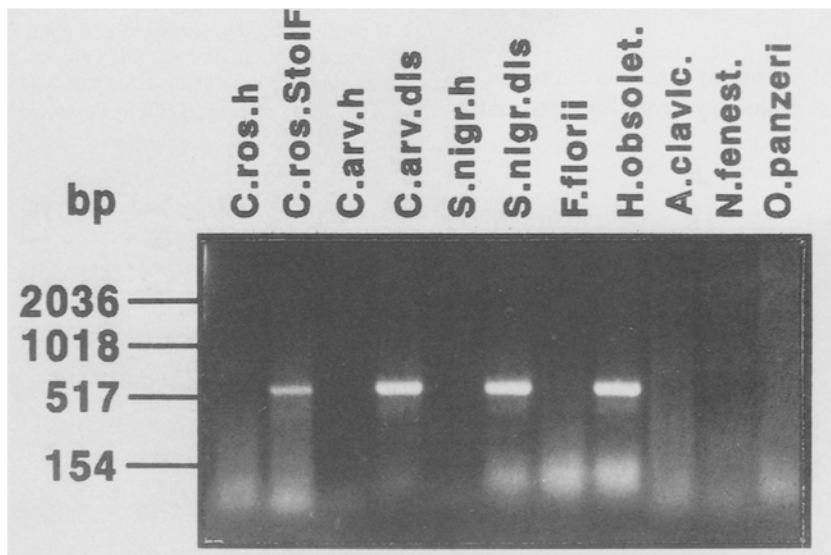

Fig. 4. Polymerase chain reaction amplification with primers fStol/rStol using template DNA prepared from plants and insects. C.ros.h = Healthy periwinkle; C.ros.StolF = Strain StolF of stolbur MLO maintained in periwinkle; C.arv.h = Healthy seedling of bindweed *Convolvolus arvensis*; C.arv.dis. = *C. arvensis* diseased; S.nigr.h = Healthy seedling of black nightshade *Solanum nigrum*; S.nigr.dis = *S.nigrum* diseased; F. florii = Laboratory-bread healthy *Fiberiella floii*; Auchenorrhyncha collected in a vineyard affected by VK: H. obsolet. = *Hyalesthes obsoletus*; A. clavic. = *Asiraca clavicornis*; N.fenest. = *Neoaliturus fenestratus*; O.panzeri = *Oliarus panzeri*. A DNA fragment corresponding in size with the fragments amplified from symptomatic grapevines was amplified from the StolF isolate, from symptomatic bindweed and nightshade, and also from the Cixiid *H. obsoletus*.

icornis (n = 14), the Deltocephalid *N. fenestratus* (n = 36) and the laboratory bred *F. florii.*

Transmission of VK MLO

Disease symptoms developed in *V. faba* and grapevine seedlings upon feeding of *H. obsoletus* collected on bindweed. Symptomatic *V. faba* exhibited small, cup-shaped leaves and a restricted growth. VK-like symptoms such as lack of lignification of the wood and pustules along the shoots developed in infected grapevine, while typical leaf symptoms did not develop under greenhouse conditions.

From bindweed on which *H. obsoletus* was collected, as well as from planthoppers surviving the transmission experiments and from healthy *F. florii*, DNA was extracted. Additional sources for DNA extraction were symptomatic *V. faba* and grapevine seedlings, a symptomatic vine from the field, and non-inoculated bean and grapevine controls. Samples from *C. arvensis*, *H. obsoletus*, *V. faba* and grapevines used for feeding of *H. obsoletus*, as well as the field grown grapevine tested positively in PCR with primers fStol/rStol (Fig. 5). PCR with the primers U1/U4 and subsequent restriction digest of amplification products revealed the typical group-II restriction fragment profile according to Ahrens and Seemüller [1992] for all fStol/rStol positive samples (data not shown). This profile was previously described for VK-affected grapevine [Maixner *et al.*, 1994].

Three of eight tested *V. faba* (38%) and 4 of 10 tested grapevines (40%) which were used for transmission experiments with *H. obsoletus*, tested positively, although only 113 of 172 (66%) planthoppers survived one week on broad bean, and not more than 3 of 122 (2.5%) survived on grapevine.

Discussion

The detection of MLOs associated with yellows diseases of grapevine by PCR using mollicutes-specific primers [Ahrens and Seemüller, 1992] is a reliable technique [Maixner *et al.*, 1994; Daire *et al.*, 1993a]. However, the procedure is not suitable for routine testing, since restriction site analysis of the amplification products is required to identify MLO DNA. The aim of this study was to develop specific primers for the detection of the MLO associated with Vergilbungskrankheit so that

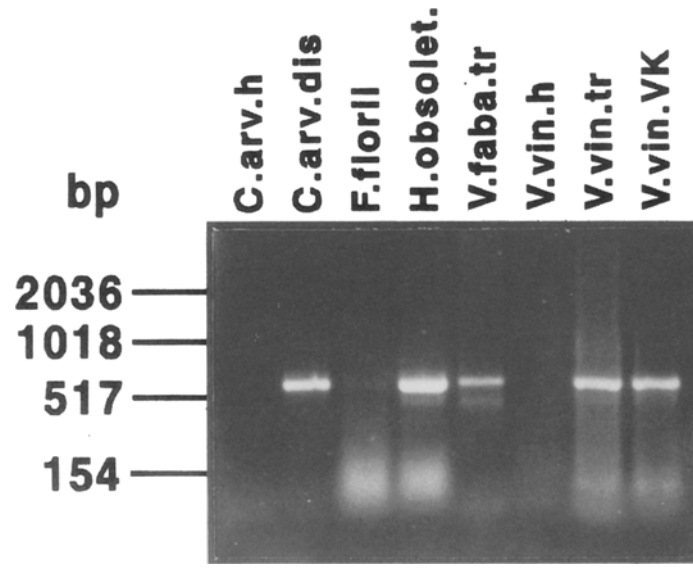

Fig. 5. Amplification products obtained by polymerase chain reaction amplification using primers fStol/rStol. C.arv.h = Healthy bindweed (*Convolvolus arvensis*) seedling; C.arv.dis. = *C. arvensis* with stunting symptoms collected in a vineyard affected by VK; F. florii = Laboratory bread healthy *Fiberiella florii*; H.obsolet. = *Hyalesthes obsoletus* collected from C.arv.dis in a vineyard affected by VK; V.faba.tr = *Vicia faba* seedling on which H.obsolet had been fed; Seedling = Healthy grapevine seedling; V.vin.tr = Grapevine seedling on which H.obsolet had been fed; V.vin.VK = Grapevine with symptoms of VK growing in the vineyard where C.arv.dis and H.obsolet had been collected.

post-amplification detection procedures are not necessary. This would make both diagnosis and epidemiological studies more convenient.

The primers fStol/rStol specifically amplified the target sequence from the MLOs associated with VK, stolbur and Molières disease. These pathogens form a distinct subgroup together with Tomato Big Bud MLO (TBB) in the classification schemes proposed by Schneider *et al.* [1993] and Seemüller *et al.* [1994]. The MLO-isolates used for comparison represent six of seven major MLO-groups proposed by Schneider *et al.* [1993], as well as four of five strain clusters and six of eight subgroups established by Seemüller *et al.* [1994] on the basis of sequence comparisons of 16S ribosomal DNA. Amplification was only achieved with the stolbur-type MLOs. Based on these results, the primers fStol/rStol were found suitable for the specific detection of pathogens of the subgroup of stolbur-related MLOs, including the pathogen associated with Vergilbungskrankheit.

All symptomatic field grown grapevines tested positively using the fStol/rStol primers. Fragments of identical size were amplified although the samples originated from various cultivars collected in different viticultural areas of Germany. This implies that the German grapevine yellows known as Vergilbungskrankheit is caused by a relatively homogeneous organism. Daire *et al.* [1993a, 1993b] detected MLOs of the same subgroup than the pathogen of VK in GY-diseased vines of different European and Mediterranean countries. However, other MLOs could be detected in vines exhibiting GY symptoms in France, Italy, and North America [Bianco *et al.*, 1993; Chen *et al.*, 1993; Daire *et al.*, 1993a; Davis *et al.*, 1993; Prince *et al.*, 1993].

The epidemiology of most grapevine yellows diseases is not well understood. Alternative hosts of the pathogens and vectors are still largely unknown. However, MLOs could be detected in wild *Vitis riparia* Michx. adjacent to vineyards affected by grapevine yellows in New York [Chen *et al.*, 1993]. The results presented in this work demonstrate, that stolbur-type pathogens are widespread in common weeds such as bindweed and nightshade in German vineyards. Fos *et al.* [1992] identified *C. arvensis* and *Solanum dulcamara* as wild hosts of the pathogen associated with stolbur of tomato in southern France. The significance of these weeds for the epidemiology of VK needs to be further investigated. Occurrence in the same weed species, transmission by one planthopper vector and the high level of sequence homology [Seemüller *et al.*, 1994] indicate that VK- and STOL-MLO may be strains of the same organism.

The vector of FD, the deltocephalid leafhopper *Scaphoideus titanus* [Schvester *et al.*, 1961] was the only natural vector of a grapevine yellows MLO identified as yet. This species is not present in Germany and, on the other hand, is not able to transmit bois noir [Caudwell *et al.*, 1971b], a yellows disease occurring in France that is similar or identical to VK [Caudwell, 1989].

Attempts to identify vectors of GY other than FD have been made in the United States [Maixner *et al.*, 1993], Australia [Osmelak *et al.*, 1989], and Italy [Vidano *et al.*, 1987; Di Terlizzi *et al.*, 1993]. Alma *et al.* [1993] transmitted MLOs to clover and periwinkle with leafhoppers collected in GY-affected vineyards, but only feeding of *S. titanus* resulted in the development of yellows symptoms in grapevine.

We started our study with a few promising species that were selected out of more than 40 species of Auchenorrhyncha collected in vineyards of the Mosel-valley [Maixner, unpublished data]. Of these, *H. obsoletus* had already been identified as a vector of Stolbur [Fos *et al.*, 1992], and the three other species *O. panzeri*, *N. fenestratus*, and *A. clavicornis* were frequently collected in affected vineyards. We were able to detect MLOs by PCR with primers fStol/rStol in *H. obsoletus*, a species that obviously preferred bindweed in the vineyards. Nymphs of this planthopper were frequently found feeding on the roots of MLO-infected bindweed. Nevertheless, detection of a particular MLO in an insect is not sufficient to identify this species as a vector, since MLOs can be acquired by and detected in nonvector species [Lefol *et al.*, 1993; Vega *et al.*, 1993]. It was therefore mandatory to confirm by transmission trials that *H. obsoletus* is a vector of VK. The induction of VK symptoms in grapevine seedlings after feeding by the planthopper, along with the detection of MLOs in those plants confirmed this hypothesis. For the first time a vector of a grapevine yellows diseases other than flavescence dorée has been identified.

The slow spread of VK in the field, as

compared to FD, is possibly due to the fact that *H. obsoletus* is only an erratic feeder on grapevine [Vidano *et al.*, 1987]. Although the planthopper was numerous on bindweed, it was rarely found feeding on grapevines. The high proportion of positive-testing planthoppers of 38% does not represent the contamination of the field populations, since the insects where collected preferably from symptomatic bindweed. FOS *et al.* [1992], on the other hand, found 25% of a *H. obsoletus* population contaminated by stolbur but only a low incidence of the disease in tomato fields. These authors too, assign this phenomenon to the preference of the planthopper for other plants.

More investigations are required to investigate the vector efficiency of *H. obsoletus* in the field and to understand the importance of this planthopper for the spread of VK. Furthermore, it should be considered, that other species of Auchenorrhyncha may be involved in the epidemiology of VK. *N. fenestratus*, for example, was frequently found in the vineyards. This leafhopper, the vector of safflower phyllody, belongs to a genus, which is known or suspected to transmit various mollicutes [Klein, 1992]. The methods described in this work will be useful tools for further studies on these epidemiological problems.

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