European Journal of Plant Pathology **107:** 411–420, 2001.

Characterization and detection of several filamentous viruses of cherry: Adaptation of an alternative cloning method (DOP-PCR), and modification of an RNA extraction protocol

M.E. Rott and W. Jelkmann
Federal Biological Research Center for Agriculture and Forestry, Institute for Plant Protection in Fruit Crops, Schwabenheimer Straße 101, D-69221 Dossenheim, Germany (Phone: +6221 86805 21; Fax: +6221 86805 15; E-mail: mike.rott@urz.uni-heidelberg.de)

Accepted 5 Febraury 2001

Key words: necrotic, rusty, mottle, foveavirus, silica

Abstract

For the identification and analysis of new RNA plant viruses infecting fruit trees, an initial step often involves the laborious procedure of isolation and cDNA synthesis and cloning from purified viral dsRNA. For subsequent RT-PCR detection of these and other viruses from tissue with high phenolic and polysaccharide concentrations, a simple and efficient extraction protocol for viral nucleic acid is also important. A method for rapid cDNA cloning from small amounts of purified dsRNA using a modification of degenerate oligo primed polymerase chain reaction (DOP-PCR), and a modification of a protocol for effective extraction of viral RNA for use in RT-PCR are presented. Both methods were used to analyze a number of mottling diseases described in cherry. The causal agents for two of these diseases have been previously described, Cherry green ring mottle virus, a tentative member of the foveaviruses, and *Cherry mottle leaf virus*, a member of the trichoviruses. For the diseases cherry rusty mottle and cherry necrotic rusty mottle, data are presented identifying viruses associated with each disease. Viruses associated with cherry rusty mottle, cherry necrotic rusty mottle and European isolates of cherry mottle leaf diseases, are closely related to Cherry green ring mottle virus and can be tentatively included in the foveavirus genus. An additional virus, related to cherry green ring mottle virus, was discovered by RT-PCR cloning and appears to be a common latent virus of cherry. Finally, isolates of cherry necrotic mottle disease could be assayed positive by RT-PCR for a virus

Abbreviations: ASPV – *Apple stem pitting virus*; CGRMV – Cherry green ring mottle virus ; CMLV – *Cherry mottle leaf virus*; CNM – cherry necrotic mottling; CNRM – cherry necrotic rusty mottle; CNRMV – Cherry necrotic rusty mottle virus; CRM – cherry rusty mottle; DOP-PCR – degenerate oligo primed-polymerase chain reaction; GRSPaV – *Grapevine rupestris stem pitting associated virus*; LChV – *Little cherry virus*.

Introduction

Cherry is host to a number of spherical and filamentous viral agents, which can cause a variety of disease symptoms. Furthermore, there are diseases of cherry which have been described, and thought to be induced by uncharacterized viral agents, but for which no known disease agent has been found (Németh, 1986). Over the last couple of years the RNA genomes of Cherry green ring mottle virus (CGRMV) (tentative foveavirus) (Zhang et al., 1998a) and *Cherry mottle leaf virus* (CMLV) (James et al., 2000) (trichovirus) have been sequenced and characterized. CGRMV has a wide geographical distribution and has been found in parts of North America, Europe, Africa and New Zealand (Németh, 1986). Characteristic symptoms include a yellow and green mottle pattern in mature leaves 4 to 6 weeks after petals fall. Fruit can have necrotic pits

with rings and have a bitter off-flavor taste. CMLV was first discovered in Oregon in the 1920s and has since been found in other parts of North America, Europe and South Africa (Németh, 1986). Symptoms include an irregular chlorotic mottle and distortion of terminal leaves. Fruit can be small and tasteless. Diseases of unknown etiology suspected to be of viral origin include cherry necrotic rusty mottle (CNRM), cherry rusty mottle (CRM) and cherry necrotic mottle leaf (CNM) (Desvignes, 1999; Németh, 1986; Wadley and Nyland, 1976). CNRM, also known as cherry bark blister, has been reported in North America, Chile, New Zealand and parts of Europe (Németh, 1986). Important disease characteristics are brown angular necrotic spots, rusty chlorotic areas and shot holes of the leaves, blisters, gum pockets and general necrosis of the bark. In North America, two agents of CRM have been described, a mild and a severe form (Németh, 1986). In Europe, the term CRM covers a number of different diseases with common characteristics and symptoms (Desvignes, 1999), but it is unclear whether one or more infectious agents are responsible. The European disease is characterized by symptoms first appearing on mature leaves in July consisting of yellowing of tertiary and quaternary veins with leaves gradually developing a pale green color. At a later stage, rusty mottling develops on the yellowish-green leaf surface (Desvignes, 1999). Cherry necrotic mottle leaf (CNM) is similar to CNRM, displaying yellowing leaves spotted with small rings or black lines along the veins later in the fall. The bark becomes necrotic with reduced growth (Desvignes, 1999).

With the increasing international exchange of germplasm, which generally cannot be visually assessed for viral infections and may contain latent virus infections, the development of reliable detection methods is of critical importance. For this reason, and to identify the agents of these diseases, trees infected with CGRMV and CMLV, and samples from trees showing disease symptoms characteristic of CNRM, CRM and CNM were tested with several PCR primers to identify and partially characterize putative viral agents.

To initially characterize CNRM, dsRNA was extracted from infected plant material for cloning by a modified degenerate oligonucleotide primed PCR (DOP-PCR) technique. This proved a rapid and convenient method to obtain preliminary sequence information of unknown plant RNA viruses. To detect virus in woody tissue, a modified silica capture method for RNA isolation from infected cherry trees was developed. This method represents an inexpensive and simple means for obtaining sufficient nucleic acid from tree leaves of a quality appropriate for RT-PCR.

Materials and methods

Virus isolates

The following disease isolates were collected, indexed and maintained in the field at the Dossenheim research station near Heidelberg Germany; CRM1097, CRM6988, CRM2386, CRM12186, CNRM12086, CNRM1685, LChV697, LChV6498 and LChV6598. Isolates CRM106, CML5498 and CGRMV5398 were kindly provided by T. Hasler (Wädenswihl, CH). Isolates CNRM3, CNRM4, CNRM7, CRMV9, CML13, CNM10, CNM11 and CNM12 were kindly provided by J-C. Desvignes (CTIFL, F) (Desvignes, 1999). D. James (B.C., CAN) provided the North American isolate of CMLV. Isolates CRM797 and CRM979 were kindly provided by A.N. Adams (East Malling, UK).

DOP-PCR

dsRNA from a tree displaying symptoms of necrotic rusty mottle (isolate 12086) was extracted and reverse transcribed essentially as described in (Keim-Konrad and Jelkmann, 1996). Reverse transcription was primed with one of the following primers, DOP1 (5′ CCGACTCGAGNNNNNNATGTGG 3′), DOP2 (5′ CCGACTCGAGATGNNNNNNTG 3′), DOP3 (5′ CCGACTCGAGNNNNNNAAATGC 3′), DOP4 (5′ CCGACTCGAGNNNNNNTTTACG 3′), DOP5 (5′ CCGACTCGAGNNNNNNTTCAGG 3′), DOP6 (5′ CCGACTCGAGNNNNNNAAGTCC 3′), DOP7 (5′ CCGACTCGAGANNNNNNTCTGG 3′) where N is any nucleotide. DOP-PCR was then performed using the cDNA as template together with the corresponding primer (Joos et al., 1995). Each sample volume of 50 µl contained, 10 mM Tris-HCl pH 8.4, 2 mM $MgCl_2$, 50 mM KCl, 100 µM gelatin, 200 µM each dNTP, 2 µM primer, 1.5 units Taq polymerase (PE Applied Biosystems) and 1 µl cDNA. Two sets of amplification cycles were done following an initial denaturation at 94 °C for 5 min in a Perkin Elmer 480 (PE Applied Biosystems). The first set consisted of five cycles of 94 °C 1 min, 30 °C 1.5 min, temperature ramping from 30 °C to 72 °C for 3 min. The second set consisted of 35 cycles of 94 °C for 1 min, 62 °C for 1 min and 72 °C for 3 min + 1 extra second/cycle.

A final elongation step was done at 72 °C for 10 min. Amplified fragments were electrophoresed on a 1.2% agarose gel, purified using Qiax II (Qiagen) and ligated into a prepared T-overhang vector (Hadjeb and Berkowitz, 1996).

RT-PCR

Reverse transcription reactions were performed as per conditions supplied with MMLV-RT (BRL) using a mixture of both random hexamers and oligo (dT) as primers and 5 µl of RNA isolated using the modified silica method. For some RT-PCR cloning experiments, the primer BoeoligodT (5′ GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV 3′) , where V is C, G or A) was used in the RT reaction and the primer Boeanchor (5′ GACCACGCGTATCGATGTCGAC 3′) used in the subsequent PCR. PCR conditions used were 75 mM Tris–HCl pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% (w/v) Tween 20, 2.5 mM $MgCl_2$, 0.2 µM each of dATP, dTTP, dCTP and dGTP, 0.2 µM each primer, and approximately 5 units Taq polymerase (Pluthero, 1993) and 1 µl cDNA in a total volume of 50 µl. PCR primer pairs were NRM48L (5′ GAATTGACTCCTCGGTGGGTTTA 3′) and NRM48U (5′ TTAATGATCTTCGTGGCTTGTTG 3′); GRM8316 (5′ CCTATAGCCAGTCTTCATATTATG 3′) and GRM7950 (5′ GCAGCCTTTGACTTTTTTGAG 3′); NEG1U (5′ AGTTCGCAGCYTTTGAYTTYTTTG 3′) and NEG1L (5′ GAKGGRWTTGCGRGGTTTATCA 3′); ERMUP (5′ GCGCTTTTGATTTCTTTGAG 3′) and ERMLO (5′ GGACAGGCCCACTTATTTACT 3′); and the primer Uni1 (5′ GCAGCYTTTGAYTTYTTTGARGC 3′) and BoeoligodT. A thermocycle program consisting of 35 cycles of 94 °C for 35 s, 52 or 55 °C, respectively, for 40 s, and 72 °C for 40 s was performed in a Robocycler (Stratagene).

Plant nucleic acid extraction

Leaf sample of 300 mg were ground in 3 to 4 ml grinding buffer (4.0 M guanidine thiocyanate, 0.2 M NaOAc pH 5.2, 25 mM EDTA, 1.0 M KOAc and 2.5% w/v PVP-40). This solution could be stored at 4 °C for several days. 500 µl of the ground plant material was then transferred to a 1.5 ml eppendorf tube to which was added 100 µl 10% *N*-lauryl sarkosyl and 5 µl 2-mercaptoethanol. The mixture was incubated at 70 °C with intermittent shaking for 10 min, placed on ice for 5 min, and then centrifuged at 13,000 rpm for 10 min. 300 µl was then transferred to a new tube to which was added 150 µl EtOH, 300 µl 6 M NaI and 25 µl resuspended silica, incubated at room temp for 10 min with intermittent shaking and then centrifuged at 6,000 rpm for 1 min. The pellet was resuspended in 500 µl wash buffer (10.0 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 50.0 mM NaCl, 50% ethanol) and centrifuged at 6,000 rpm for 1 min. The wash step was repeated once, and the pellet allowed to dry for several minutes at room temperature before resuspending in 150 µl H_2O. The mixture was incubated at 70 °C for 4 min, centrifuged at 13,000 rpm for 3 min, and the supernatant transferred to a new tube and stored at −20 °C. The NaI solution was prepared by first dissolving 0.75 g Na_2SO_3 in 40 ml water then 36 g NaI. Solution was stored in dark bottle at 4 °C. Silica was prepared by adding 60 g silica particles (Sigma S5631) to 500 ml distilled H_2O. The silica was well mixed and allowed to settle for 24 h. The upper 470 ml of supernatant was discarded, 500 ml of distilled H_2O added the suspension mixed well and allowed to settle for another 5 h. The upper 440 ml of solution was then discarded and the remaining 60 ml slurry was adjusted to pH 2.0 with HCl, autoclaved and stored in a dark bottle at room temperature or aliquoted into 1.5 ml eppendorf tubes for storage at 4 °C for several months.

Sequence analysis

Cloned PCR fragments were sequenced using an Applied Biosystems Inc automatic sequencer. Sequences were assembled and analyzed using the HUSAR program (German Cancer Research Center, Heidelberg, Germany). CLUSTALX (Higgins and Sharp, 1989), was used to generate the multiple alignments. Pairwise percent similarities were calculated as (100 × sum of the matches)/(length − gap residues (sequence 1) − gaps residues (sequence 2)).

Results

DOP-PCR

For this study, dsRNA was isolated from a cherry tree with CNRM disease. A clear dsRNA band of between 8 and 9 kilobases (kb) in size was obtained suggesting presence of a virus infected this tree (Figure 1a). Since only a small amount of dsRNA could be obtained, an alternative strategy for cloning from this dsRNA

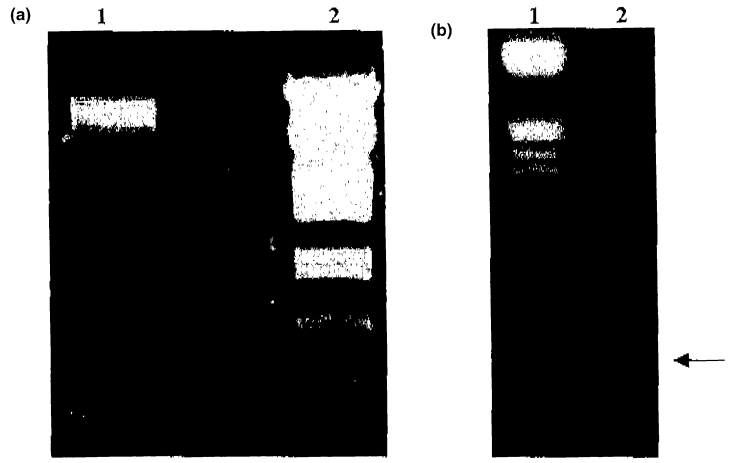


Figure 1. (a) dsRNA extracted from cherry necrotic rusty mottle isolate 12086. Lane 1, dsRNA. Lane2, λ DNA marker digested with *Eco*R1/*Hind*III. (b) DOP-PCR results using DOP4 primer. Lane1, λ DNA marker digested with *Eco*R1/*Hind*III. Lane2, amplified DNA fragments from cDNA synthesized to dsRNA from 1a. Viral specific band is indicated with an arrow.

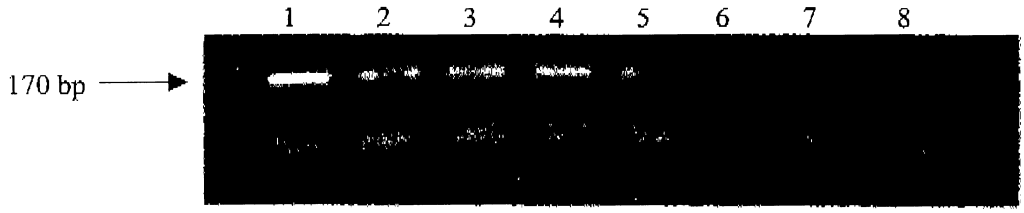


Figure 2. Presence of a 170 bp fragment amplified with primers NRM48U/NRM48L from trees displaying cherry necrotic rusty mottle symptoms, but not from CGRMV infected trees. Lanes 1 CNRM12086, 2 CNRM1885, 3 CNRM3, 4 CNRM4, 5 CNRM7, 6 CGRM105, 7 CGRM9 and 10 negative control.

was employed using a modification of the degenerate oligonucleotide primed-PCR (DOP-PCR) protocol (Joos et al., 1995). A small fragment was obtained using the DOP4 primer (Figure 1b) and cloned. Several fragments were amplified with the other primers, but none were viral specific as determined by cloning and sequencing. The DOP2 primer gave a smear. Clone NRM48 obtained from DOP4 was sequenced and shown to contain a viral specific insert of 262 nucleotides coding for part of an RNA-dependent RNA polymerase. At both nucleotide and predicted amino acid sequence levels, significant homology was found between this sequence and that of cherry green ring mottle virus (CGRMV) (65.8% and 71.3%, respectively) suggesting that the cloned virus was distinct from, but related to, CGRMV (data not shown). Based on the sequence of this cloned fragment, the PCR primers NRM48U and NRM48L were synthesized to amplify a product of 170 base pairs. The NRM48U/L primer pair was able to amplify the expected sized product from isolates of CNRM, but not CGRMV infected or uninfected tissue (Figure 2) indicating that this fragment probably represented a virus sequence associated with CNRM disease.

Analysis with CGRMV and CMLV specific PCR primers

Primers GRM7950 and GRM8316, located within the coat protein gene and the 3′ nontranslated region of the CGRMV genomic sequence, (nucleotide positions 7950 and 8316) were used in RT-PCR detection assays with nucleic acid isolated through the modified silica capture method. The expected viral specific fragment was amplified from trees infected with CGRMV, but not from trees infected with CNRM (Table 1). These primers were also able to amplify the expected

Table 1. Summary of PCR results using the 4 different primer pairs, GRM (GRM8361/GRM7950), NEG (NEG1U/NEG1L), ERM (ERMUP/ERMLO), and CML (CML13A/CML4A)

Isolate	RT-PCR Primers			
	GRM7950/8316	NEG1U/1L	ERMUP/LO	CML13A/4A
CRM1097	+	+	+	−
CRM6988	−	−	+	−
CRM2386	+	+	+	−
CRM12186	+	+	+	−
CRM106	+	−	+	−
CRM797	+	−	+	−
CRM997	+	−	−	−
CNRM12086	−	+	−	−
CNRM1685	−	+	−	−
CNRM3	−	+	+	−
CNRM4	−	+	−	−
CNRM7	−	+	+	−
CGRMV9	+	+	+	−
CGRMV5398	+	+	−	−
CML13	+	+/−	+	−
CML5498	+	+	−	−
CMLV	−	−	−	+
CNM10	+/−	−	+	−
CNM11	+/−	+	−	−
CNM12	+	+	−	−
LChV697	−	−	+	−
LChV6498	−	−	+	−
LChV6598	−	−	−	−
Control1	−	−	+	−
Control2	−	−	+	−
Control3	−	−	+	−
Control4	−	−	−	−
Control5	−	−	+	−
Control6	−	−	+	−

CRM = cherry rusty mottle diseased trees. CNRM = cherry necrotic rusty mottle diseased trees. CGRMV = trees infected with cherry green ring mottle virus. CMLV = cherry mottle leaf virus infected trees (North American isolate). CML = cherry mottle diseased trees not infected with CMLV. CNM = cherry necrotic mottle leaf disease infected trees of European origin. LChV = trees infected with little cherry virus. Control trees, are healthly looking trees. + = strong band, − = no band, and +/− = weak band.

fragment from two trees infected with European isolates of CML, but not a North American isolate of CMLV (see Table 1). In addition, the primer pair GRM7950/8316 amplified the expected fragment from isolates of CNM, and all but one isolate of CRM. A PCR product was not obtained with the GRM7950/83216 primers from leaf tissue from visually healthy trees and *Little cherry virus* (LChV) infected tree tissue.

To further clarify the relationship between the European and North American isolates of CMLV with CGRMV, the published primers for the detection of CMLV (CML13A and CML4A) (James et al., 1999) were used to test the various isolates by RT-PCR. The CML13A/4A primers, amplified the expected sized fragment from the North American isolate of CMLV only (Table 1). No amplified products could be obtained with these primers in RT-PCR assays from the two European isolates of CMLV. Likewise, isolates of CNRM, CRM, CNM, control trees infected with LChV, and healthy control trees, tested negative with the CML13A/4A primers (see Table 1).

3′ end sequence comparisons

To further investigate relationships between CGRMV and viruses associated with CNRM and CRM, 3′ end sequences were compared. Sequences compared were from the published sequences of CGRMV, CNRM (CNRM12086) (Rott and Jelkmann, 2001). In addition, the amplification products from another isolate of CNRM (CNRM1685), two isolates of CRM (CRM12186 and CRM2386) and an additional isolate of CGRMV (CGRMV105), obtained from either the primer combination Uni1/GRM8361 or Uni1/Boeanchor using cDNA primed with BoeoligodT, were cloned and sequenced. Figure 3a shows an alignment of these sequences. From isolate CRM12186 two very different but related viral sequences were obtained; one which closely matched the sequence obtained from isolate CRM2386, and the other only distantly (CRM3T3), indicating that this tree was infected with at least two viruses. Figure 3b shows percent similarity between these different viral sequences. Part of the cloned sequences contains the C-terminal region of the coat protein, the translation products of which are shown in Figure 4 along with the percent similarities. The nucleotide sequence from CGRMV isolate CGRMV105 has a similarity match of 91.4% with the published sequence of CGRMV, and the two were identical at the amino acid level. The two CRM isolates, CRM12186 and CRM2386 show very high similarity with each other and with the two CGRMV sequences (greater than 97%) at both nucleotide and amino acid sequence levels. Between the two CNRM isolates CNRM1685 and CNRM12086, nucleotide sequences were 98.1% similar, and identical at the amino acid sequence level. The CNRM nucleotide sequences only shared between 75.3% and 76.9% similarity with both CGRMV and CRM sequences. Higher similarities were found at the amino acid sequence level (90.7–92.0%). The sequence obtained from CRM3T3 was the most divergent, sharing between 47.1–50.1% similarity at the nucleotide sequence level and 53.9–57.3% similarity at the amino acid sequence level to the other viral sequences.

Based on the Figure 3a alignment, two sets of primers were synthesized, NEG1U and NEG1L which should detect CGRMV, CGRMV105, CNRM12086, CNRM1685, CRMV12186 and CRM2386; and ERMUP and ERMLO, which should detect only CRM3T3 in RT-PCR assays. Results are shown in Table 1. NEG1U/1L amplify all isolates of CGRMV and CNRM as expected. Not surprisingly, they also detected the two European isolates of CMLV, but only 3 of the 7 isolates of CRM. Two of the 3 CNM isolates could also be detected with the NEG1U/1L primers. *Little cherry virus* (LChV) infected and healthy control trees were negative.

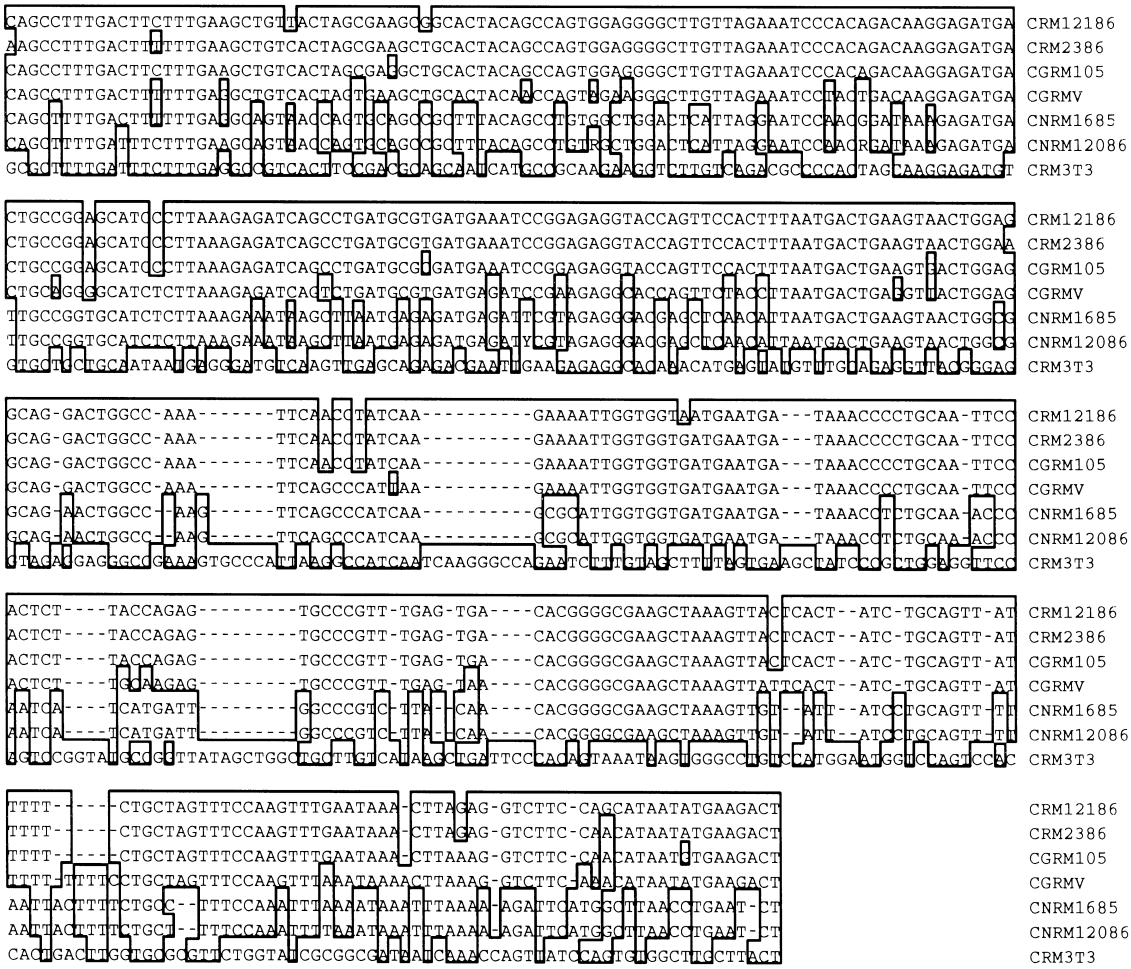
Primers ERMUP/LO amplified the expected viral specific fragment from a number of different samples including isolates of CRM, CNRM, CGRMV, CMLV, LChV, CNM and healthy leaf material used as negative controls in RT-PCR assays. Furthermore, multiple trees grafted from the same source of CRM tested either positive or negative with the ERMUP/LO primers (data not shown) suggesting that the virus may already have been in saplings before grafting. In addition, visually healthy looking control trees which tested positive with the ERMUP/LO primers by RT-PCR did not appear different than trees which tested negative. Taken together, these results identify a new virus that could not be associated with a disease. As a result, we tentatively propose naming this new, apparently latent virus of cherry, Cherry virus B (CVB). *Cherry virus A* (CVA) is the designation for a latent capillovirus of cherry.

Discussion

DOP-PCR

Degenerate oligonucleotide primed-PCR (DOP-PCR) is a very sensitive method originally developed for random amplification from small quantities of chromosomal DNA. We have adapted this method for the amplification of cDNA synthesized from purified viral dsRNA and report the first partial cloning of a plant virus using this method. In DOP-PCR a single primer, consisting of a 5′ and 3′ defined sequence flanking a random sequence of 6 nucleotides, was used for amplification. During the first 5 PCR cycles, a low stringency annealing temperature was used followed by a controlled ramp in temperature to the elongation temperature to facilitate priming of the oligonucleotide. A further 35 cycles using a high stringency annealing temperature preferentially amplified the products generated during the first 5 cycles. In the original DOP-PCR procedure, the primer DOP1 was used to randomly amplify from chromosomal DNA (Joos et al., 1995). To increase the chance of obtaining an amplified product from viral dsRNA as starting material, several other primer sequences were developed and analyzed (DOP2 to DOP7) which varied in sequence

(a)



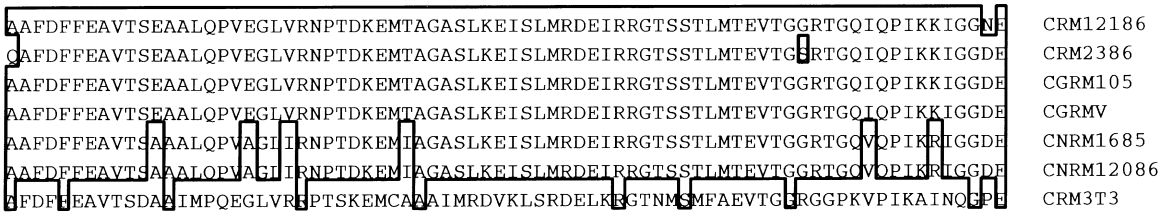
(b)

	CRM12186	CRM2386	CGRMV105	CGRMV	CNRM1685	CNRM12086	CRM3T3
CRM12186	100	98.4	97.9	90.9	75.8	76.0	49.2
CRM2386		100	98.1	91.6	75.5	75.3	48.7
CGRMV105			100	91.4	75.5	75.8	49.4
CGRMV				100	77.6	76.9	50.1
CNRM1685					100	98.1	46.9
CNRM12086						100	47.1
CRM3T3							100

Figure 3. (a) 3′ genomic nucleotide sequence alignment generated using CLUSTALX of 7 virus isolates obtained from trees showing symptoms of cherry rusty mottle (CRM), cherry green ring mottle (CGRM) and cherry necrotic rusty mottle (CNRM). Conserved nucleotides are boxed. The first nucleotide depicted for CGRMV corresponds to nucleotide 7952 of the genomic sequence. (b) Table showing percent nucleotide sequence similarity between the 7 viral sequences. Pairwise percent similarities were calculated as (100 × sum of the matches)/(length − gap residues (sequence 1) − gap residues (sequence 2)).

and number of 3′ defined nucleotides. The DOP2 primer has only 3 defined-3′ end nucleotides and is probably too unspecific as it gave a smear instead of distinct bands on an agarose. DOP7 is similar to DOP1, with the exception that it has 5 compared to 6 defined nucleotides at the 3′ end. It was hoped that due to the lower specificity, DOP7 would amplify more fragments then DOP1, but both primers did not amplify

(a)



(b)

	CRM12186	CRM2386	CGRMV105	CGRMV	CNRM1685	CNRM12086	CRM3T3
CRM12186	100	97.3	98.7	98.7	90.7	90.7	57.3
CRM2386		100	98.7	98.7	90.7	90.7	56.0
CGRMV105			100	100	92.0	92.0	57.3
CGRMV				100	92.0	90.8	56.6
CNRM1685					100	100	54.7
CNRM12086						100	53.9
CRM3T3							100

Figure 4. (a) Amino acid sequence alignments of C-terminal regions of the coat proteins of 7 viruses isolated from trees showing symptoms of cherry rusty mottle (CRM), cherry green ring mottle (CGRM) and cherry necrotic rusty mottle (CNRM) as generated using CLUSTALX. Conserved amino acids are boxed. The first amino acid depicted for CGRMV corresponds to amino acid 194 of the CGRMV coat protein. (b) Table showing percent amino acid sequence similarity between the 7 viral sequences. Pairwise percent similarities were calculated as (100 × sum of the matches)/(length − gap residues (sequence 1) − gap residues (sequence 2)).

any fragments. The only distinct viral specific fragment was obtained from DOP4. In the original DOP-PCR protocol with genomic DNA as a template, one defined primer may be sufficient due to the large size of the target. When applying this method to the relatively small genomes of plant RNA viruses, it is important to test several different DOP primers with the assumption that not all will give amplified products. A disadvantage of DOP-PCR cloning from viral dsRNA is that generally only a few small cDNAs are obtained. However, the obtained information is often sufficient for an initial analysis and is offset by the rapidity, ease and simplicity with which this information is obtained. In addition to using DOP-PCR to clone CNRMV, we have also used this method to obtain viral specific clones to Grapevine leafroll associated virus-1 (Turturo et al., 2000), Grapevine leafroll associated virus-7 (Turturo et al., 2000), as well as, *Grapevine leafroll associated virus-3* and Little cherry virus-2 (Rott and Jelkmann, unpublished results).

Nucleic acid extraction using modified silica capture

One of the difficulties with the routine use of RT-PCR for the detection of plant RNA viruses is obtaining sufficient and pure RNA starting material, cost effectively and efficiently (Korschineck et al., 1991; Minafra et al., 1992). Tree tissues or other plant tissues which contain high levels of phenolic or polysaccharide compounds are particularly difficult from which to purify (Demeke and Adams, 1992; Henson and French, 1993). Purification by immunocapture of virus particles using specific antibodies for subsequent use in RT-PCR has also been described (Wetzel et al., 1992). The method of Mackenzie et al. (1997), which uses the commercially available RNeasy extraction columns from Qiagen, fulfils many of the above criteria, its only shortcoming being the relative expense of the method. For this reason, alternative methods were explored. The modified silica capture method used here has all of the advantages of the MacKenzie method with a significant cost saving, making it a valuable additional tool for RT-PCR detection from virus infected plants.

Relationships and detection of a latent virus of cherry and viruses causing mottling disease in cherry

Viruses have been identified in association with the mottling diseases, CNRM, CRM, CNM and CML, in addition to a previously undescribed latent virus for which we tentatively propose the name CVB. All these viruses are related CGRMV, a tentative member of the newly formed foveavirus plant virus genus (Martelli and Jelkmann, 1998). The type member of the foveavirus genus is *Apple stem pitting virus* (ASPV) and the only other definitive member is *(Grapevine) Rupestris*

stem pitting associated virus-1 (GRSPaV-1) (Meng et al., 1998; Zhang et al., 1998a). Based on RT-PCR and limited sequence analysis, viruses associated with CRM and CML can be tentatively classified as strains of CGRMV, whereas the virus associated with CNRM and the latent virus CVB can be classified as distinct. Further data are required in order to classify the virus associated with CNM as either a strain of CGRMV or a distinct virus.

In North America, the causal agent of CML, *Cherry mottle leaf virus* (CMLV) was recently described and classified as a trichovirus (James et al., 2000). This virus was detected in our North American isolate of CML but not in 2 European isolates, whereas in the 2 European isolates a strain of CGRMV was found that was not present in the North American isolate. This would suggest that there are two distinct viruses associated with CML disease.

CRM describes a group of diseases with similar symptomatoloy (Desvignes, 1999) reflected in the varying ability of primers GRM and NEG to amplify from the different isolates in RT-PCR assays. Previous work with CGRMV showed that primers designed to 4 different isolates of CGRMV did not necessarily cross amplify (Zhang et al., 1998b), also demonstrating high heterogeneity between isolates. For the foveaviruses ASPV (Jelkmann, 1994) and GRSPaV-1 (Meng et al., 1998; Zhang et al., 1998a), significant sequence variants have also been observed (Meng et al., 1999a; Schwarz and Jelkmann, 1998; Zhang et al., 1998b). ASPV is also the causal agent of pear vein yellows, as well as being related to pear stony pit and leaf and fruit disorders of quince. In RT-PCR tests, the same set of primers could amplify from 24 of 33 isolates of ASPV and 14 of 14 isolates of pear displaying symptoms of pear vein yellows, stony pit, red mottle, quince sooty ringspot and quince fruit deformation. Partial sequences from some of these isolates revealed nucleic acid homologies of between 81.7% and 99.3%. Studies with GRSPaV-1 showed that only 85% of grapevines indexed positive for GRSPaV-1, could be tested positive in RT-PCR assays (Meng et al., 1999b). Partial sequences from 3 isolates of GRSPaV-1 showed sequence homologies of between 75.7% and 99%, while partial sequences from 5 isolates of GRSPaV-1 shared between 82% and 99% sequence identity (Meng et al., 1999a; Zhang et al., 1998b). CGRMV, as a tentative member of the foveaviruses, also can be characterized as consisting of a family of closely related sequence variants, which would appear to be a common characteristic of the genus foveavirus.

Acknowledgements

This work was funded from the European Community Contract Number FAIR-CT97-3889.

References

Demeke T and Adams RP (1992) The effect of plant polysaccharides and buffer additives on PCR. Biotechniques 12: 332–334

Desvignes JC (1999) Virus disease of fruit trees. Ctifl, Paris

Hadjeb N and Berkowitz GA (1996) Preparation of T-overhang vectors with high PCR product cloning efficiency. Biotechniques 20: 20–22

Henson JM and French R (1993) The polymerase chain reaction and plant disease diagnosis. Annual Review of Plant Pathology 31: 81–109

Higgins DG and Sharp PM (1989) Fast and sensitive multiple sequence alignments on a microcomputer. CABIOS 5: 151–153

James D, Jelkmann W and Upton C (1999) Specific detection of cherry mottle leaf virus using digoxigenin-labeled cDNA probes and RT-PCR. Plant Disease 83: 235–239

James D, Jelkmann W and Upton C (2000) Nucleotide sequence and genome organization of cherry mottle leaf virus and its relationship to members of the Trichovirus genus. Archives of Virology 145: 995–1007

Jelkmann W (1994) Nucleotide sequences of apple stem pitting virus and of the coat protein gene of a similar virus from pear associated with vein yellows disease and their relationship with potex- and carlaviruses. Journal of General Virology 75: 1535–1542

Joos S, Schütz B, Benze M and Licher P (1995) Detection of Chromosomal Imbalances Using DOP-PCR and Comparative Genomic Hybridization (CGH) In: Boehringer Mannheim – PCR Applications Manual. (pp 142–152) Boehringer Mannheim GmbH, Biochemica

Keim-Konrad R and Jelkmann W (1996) Genome analysis of the 3′-terminal part of the little cherry disease associated dsRNA reveals a monopartite clostero-like virus. Archives of Virology 141: 1437–1451

Korschineck I, Himmler G, Sagl R, Steinkellner H and Katinger HWD (1991) A PCR membrane spot assay for the detection of plum pox virus RNA in bark of infected trees. Journal of Virological Methods 31: 139–146

MacKenzie DJ, McLean MA, Mukerji S and Green M (1997) Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction. Plant Disease 81: 222–226

Martelli GP and Jelkmann W (1998) Foveavirus, a new plant virus genus. Archives of Virology 143: 1245–1249

Meng B, Zhu H-Y and Gonsalves D (1999a) Rupestris stem pitting associated virus-1 consists of a family of sequence variants. Archives of Virology 144: 2071–2085

Meng BZ, Pang SZ, Forsline PL, McFerson JR and Gonsalves D (1998) Nucleotide sequence and genome structure of grapevine rupestris stem pitting associated virus-1 reveal similarities to apple stem pitting virus. Journal of General Virology 79: 2059–2069

Meng BZ, Johnson R, Peressini S, Forsline PL and Gonsalves D (1999b) Rupestris stem pitting associated virus-1 is consistently detected in grapevines that are infected with rupestris stem pitting. European Journal of Plant Pathology 105: 191–199

Minafra A, Hadidi A and Martelli GP (1992) Detection of grapevine closterovirus A in infected grapevine tissue by reverse transcription-polymerase chain reaction. Vitus 31: 221–227

Németh M (1986) Virus, mycoplasma and rickettsia diseases of fruit trees. Martinus Nijhoff/ Dordrecht/Boston/Lancaster

Pluthero FG (1993) Rapid purification of high-activity Taq DNA polymerase. Nucleic Acids Research 21: 4850–4851

Rott ME and Jelkmann W (2001) Complete nucleotide sequence of cherry necrotic rusty mottle virus. Archives of Virology 146: 395–401

Schwarz K and Jelkmann W (1998) Detection and characterization of European apple stem pitting virus sources from apple and pear by PCR and partial sequence analysis. Acta Horticulturae 1: 75–85

Turturo C, Rott ME, Minafra A, Saldarelli P, Jelkmann W and Martelli GP (2000) Partial molecular characterization and RT-PCR detection of grapevine leafroll associated virus 7 ICVG 13th meeting Adelaide, South Australia

Turturo C, Rott ME, Minafra A, Saldarelli P, Martelli GP and Jelkmann W (2000) Grapevine leafroll associated virus 1: Partial cloning and RT-PCR detection. ICVG 13th meeting Adelaide, South Australia

Wadley BN and Nyland G (1976) Rusty mottle group In: Gilmer RM, Moore JD, Nyland G, Welsh M F and Pine TS (eds) Virus Diseases and Noninfectious Disorders of Stone Fruits in North America (pp 242–249) Agriculture Research Service United State Department of Agriculture, Washington, DC, USA

Wetzel T, Candresse T, Macquaire G, Ravelonandro M and Dunez J (1992) A highly sensitive immunocapture polymerase chain reaction method for plum pox potyvirus detection. Journal of Virological Methods 39: 27–37

Zhang YP, Kirkpatrick BC, Smart CD and Uyemoto JK (1998a) cDNA cloning and molecular characterization of cherry green ring mottle virus. Journal of General Virology 79: 2275–2281

Zhang YP, Uyemoto JK, Golino DA and Rowhani A (1998b) Nucleotide sequence and RT-PCR detection of a virus associated with grapevine rupestris stem-pitting disease. Phytopathology 88: 1231–1237

Zhang YP, Uyemoto JK and Kirkpatrick BC (1998c) Analysis of Double-Stranded RNAs from Cherry Trees with Stem Pitting in California. Plant Disease 82: 871–874