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Short communication

Use of degenerate primers in a RT-PCR assay for the identification and analysis of some filamentous viruses, with special reference to clostero- and vitiviruses of the grapevine

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Abstract

RT-PCR with degenerate primers was used for the screening of the genome of some members of the *Closterovirus*, *Vitivirus* and *Trichovirus* genera. Two sets of primers, targeted to conserved sequences of the heat shock protein 70 homologue of closteroviruses or to the RNA dependent RNA polymerase genes of tricho- and vitiviruses, amplified the expected fragments from total RNA extracts or double-stranded RNAs of infected plants. Amplified cDNAs were cloned, sequenced and phylogenetically analyzed. Results support the allocation of grapevine viruses A, B, D and heracleum latent virus (HLV) in the genus *Vitivirus*, whereas, the detection of a HSP70 homologue in grapevine leafroll-associated viruses agrees with their assignment in the genus *Closterovirus*. The use of degenerate primers for the identification of grapevine viruses belonging to *Vitivirus* and *Closterovirus* genera is envisaged.

Leafroll and rugose wood are highly detrimental diseases of the grapevine (Martelli, 1993), adversely affecting the plant vigour and longevity, as well as the quality and quantity of the yield (Walter and Martelli, 1997). Both diseases have a viral origin. Closteroviruses are involved in the aetiology of leafroll (Martelli et al., 1997a) and members of the newly established genus *Vitivirus* (Martelli et al., 1997b) in the aetiology of two of the syndromes of the rugose wood complex (Boscia et al., 1997). To date, seven serologically distinct closteroviruses, denoted grapevine leafroll-associated viruses 1 to 7 (GLRaV-1 to GLRaV-7), were found to be associated with grapevine leafroll disease (Boscia et al., 1995; Choueiri et al., 1996). Four vitiviruses, i.e. grapevine viruses A to D (GVA, GVB, GVC, and GVD), have been isolated by sap transmission from vines with rugose wood symptoms (Martelli et al., 1997a; Boscia et al., 1997). These viruses are either serologically distinct (closteroviruses) (Martelli et al., 1997a) or very distantly related (vitiviruses) (Goszsczynski et al., 1996; Choueiri et al., 1997).

Molecular information is available for vitiviruses limitedly to GVA (Minafra et al., 1997; Rubinson et al., 1997), GVB (Saldarelli et al., 1996) and GVD (Abou Ghanem et al., 1997). As to grapevine closteroviruses, genomes of GLRaV-2 and GLRaV-3 were almost completely sequenced (Abou Ghanem et al., 1998; Zhu et al., 1998; Ling et al., 1998) thus extending previous molecular information pertaining to short genome regions (Habili and Rezaian, 1995, Habili et al., 1995, 1997; Fazeli et al., 1998). The HSP-70 homologue gene, a hallmark of the family *Closteroviridae* was identified and sequenced only for GLRaV-2 and GLRaV-3. The acquisition of molecular information on other grapevine closteroviruses

appeared therefore desirable not only for investigating taxonomic relationships among them, but also in view of the development of RT-PCR methods, with virus specific primers for their sensitive and reliable identification.

Recent reports describe the use of degenerate oligonucleotides targeted to conserved amino acidic stretches of the replicase (RdRp) of carmoviruses (Morozov et al., 1995) and the HSP-70 homologue of closteroviruses and criniviruses (Karasev et al., 1994; Tian et al., 1996) for detecting related viruses for evolutive and taxonomic purposes. Similarly, we designed degenerate primers on conserved domains of the replicase (RdRp) of viti- and trichoviruses, and used previously described degenerate primers targeted to HSP-70-related sequences of closteroviruses (Tian et al., 1996), for the identification of filamentous grapevine viruses.

Sources for total RNA (T-RNA) (Mackenzie et al., 1997) or double stranded RNA (dsRNA) (Saldarelli et al., 1994) extracts from geographically different isolates of the viruses under study were as listed in Table 1. Highly degenerate oligonucleotides for the detection of vitiviruses and trichoviruses were designed on conserved motifs II and V (Koonin, 1991) of the RdRp domains of GVA, GVB and apple chlorotic leaf spot virus ACLSV (Table 2), using previously published sequences (Minafra et al., 1997; Saldarelli et al., 1996; German et al., 1990). Primers dPR1 (sense) and dPR2 (complementary), amplifying a DNA region of about 363 nucleotides that expands between Koonin's motifs II and V (Koonin, 1991), were chosen for their maximum residue conservation, minimum codon degeneracy and possibility of amplifying a small region of the genome. Degenerate primers HSP-P-1 and HSP-P-2 for the detection of GLRaVs were those designed by Tian et al. (1996) on the conserved phosphate 1 and 2 motifs of the HSP-70 homologues of several clostero- and criniviruses. Reverse transcription and PCR amplifications of T-RNA or dsRNA preparations were as described by Tian et al. (1996) with the only modification of priming cDNA synthesis with random hexanucleotide mixture (Boehringer, Mannheim).

Dideoxy chain termination sequencing was performed on recombinant plasmids containing cloned RT-PCR products (Sambrook et al., 1989) in the *Sma I*

Table 1. Sources of viruses

Isolate	Virus	Geographical origin	Host
TO1	GVA	Italy	*N. benthamiana*
PA3	GVA	Italy	*N. benthamiana*
Is151*	GVA	Italy	*N. benthamiana*
BR V6	GVA	Italy	*N. benthamiana*
FR1	GVA	France	*N. benthamiana*
SA36	GVA	Canada	*N. benthamiana*
SA646	GVA	Canada	*N. benthamiana*
CY1	GVA	Cyprus	*N. benthamiana*
Y20	GVA	Yemen	*N. benthamiana*
BA11	GVB	Italy	*N. occidentalis*
Se*	GVB	USA	*N. occidentalis*
Primus	GVD*	Italy	*N. occidentalis*
Ch*	ACLSV	China	*C. quinoa*
HLV-M	HLV*	Scotland	*C. quinoa*
PVT-Sal	PVT*	Peru	*N. occidentalis*
FR6*	GLRaV-1 + GVA	France	grapevine
GW913	GLRaV-1	France	grapevine
BA*	GLRaV-2	USA	*N. benthamiana*
MT38*	GLRaV-3 + GVA + GFkV	Italy	grapevine
RaV106*	GLRaV-4	USA	grapevine
Y252	GLRaV-4	Israel	grapevine
DD85	GLRaV-4 + GVA	Turkey	grapevine
RaV100*	GLRaV-5	USA	grapevine
Y220	GLRaV-5	Syria	grapevine
AA42*	GLRaV-7 + GVA	Albania	grapevine

*: isolates used for cloning.

Table 2. Degenerate primers for amplifications of trichoviruses and vitiviruses

dPR1	
Nucleotide sequence	5′GCD AAR GCN GGN CAR ACH HTV GCB TGY TT3′
Amino acids sequence	AKAGQTI/LACF
dPR2	
Nucleotide sequence	5′RAA YTC NCC NSW RAA NCK CAT3′
Amino acids sequence	MRFS/TGEF

(N = A + C + G + T; R = A + G; Y = C + T; K = T+ G; S = C + G;
W = A + T; H = A + T + C; B = T + C + G; D = A + T + G; V = A + G + C).

site of pGEM4z (Promega). The sequences obtained were assembled with the DNA Strider software (Marck, 1988) and compared for similarity against the non redundant sequence database of the National Centre for Biotechnology Information, Bethesda, USA, using the BLAST alghoritm (Altschul et al., 1990). Protein sequences were aligned with Clustal V (Higgins and Sharp, 1988) and further refined manually. Phylogenetic and bootstrap analysis were performed by the parsimony method using the Phylip package software (Felsenstein, 1989).

Primers dPR1 and dPR2 effectively amplified tricho- and vitiviruses by RT-PCR. The expected DNA fragment of 363 nucleotides was specifically amplified from reverse transcribed cDNA synthesized on T-RNA extracts from herbaceous hosts infected by GVD, ACLSV, potato virus T (PVT), HLV and all the tested GVA and GVB isolates (Figure 1). Two RT-PCR products were observed sometimes with some GVA isolates, but these were not further studied. Specificity of primers dPR1 and dPR2 was also confirmed by the lack of amplification of reverse transcribed GLRaV-2 dsRNA, and non reverse transcribed T-RNA from healthy *N. benthamiana* and grapevine.

When used for the amplification by RT-PCR of several grapevine leafroll associated closteroviruses, degenerate primers HSP-P-1 and HSP-P-2 gave the expected fragments (ca. 600 bp) both from total or double stranded RNAs from infected grapevines (GLRaV-1, -3, -4,-5, -7) or *N. benthamiana* (GLRaV-2) (Figure 2). All GLRaVs isolates listed in Table 1 were successfully amplified and gave cDNAs of similar size. As observed by Langeveld et al. (1991), substitution of complementary primer with random hexanucleotides for cDNA synthesis gave very specific DNA products.

ACLSV, PVT, HLV, GVD, GLRaV-1, GLRaV-3 and GLRaV-7 sequences were submitted to the EMBL database under the accession numbers Y15889, Y15988, Y15893, Y15892, Y15890, Y15891 and Y15987, GLRaV-4 and GLRaV-5 to the GenBank database under the accession numbers AF039553 and AF039552, respectively, while only the cDNAs of GVA-Is151 and GVB-Se were cloned and sequenced as representative of all GVA and GVB isolates. The latter sequences were found strictly conserved to the previously published sequences (Minafra et al., 1997; Saldarelli et al., 1996). Sequences of RT-PCR products from tricho- and vitivirus revealed a compact 363 nt organisation in both known (GVA, GVB, ACLSV and PVT) and unknown (HLV, GVD) genomes. No gaps were found when these sequences were aligned. All products had a deduced amino acid composition of 121 residues which, when aligned, showed a high level of conservation especially among vitiviruses (not shown). Identity of amino acids in the genus *Vitivirus* spanned

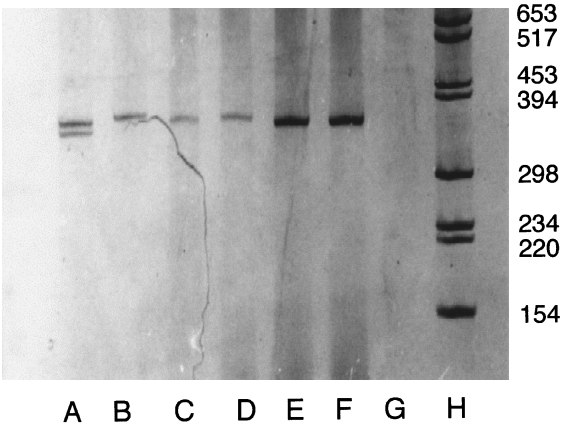

Figure 1. 5% polyacrylamide gel electrophoresis of RT-PCR amplified DNA fragments from tricho- and vitivirus infected tissues. Samples are: GVA-Is151 (lane A), GVB-Se (lane B), GVD-Primus (lane C), ACLSV-Ch (lane D), HLV-M (lane E), PVT-Sal (lane F), healthy *N. benthamiana* (lane G) and DNA molecular weight marker (lane H).

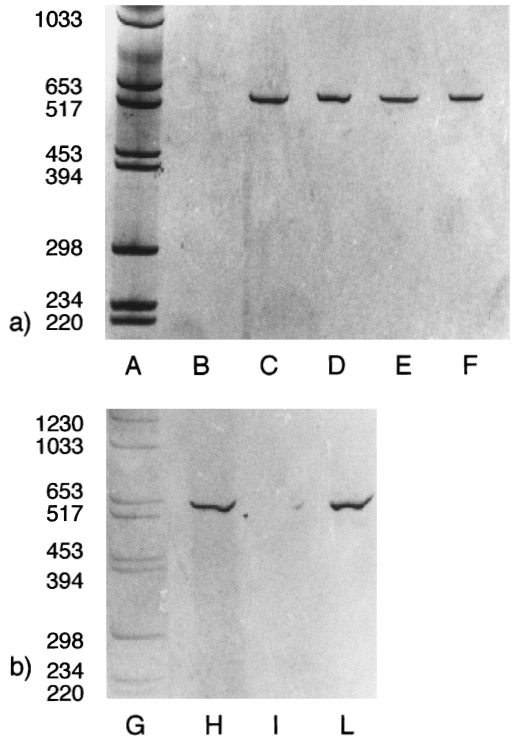

Figure 2. RT-PCR analysis of different GLRaV isolates using degenerate HSP-70 primers. Gel (a): DNA molecular weight marker (A), healthy grapevine (B), GLRaV-1 FR6 (C), GLRaV-2 BA (D), GLRaV-3 MT38 (E), GLRaV-7 AA42 (F). Gel (b): DNA molecular weight marker (G), GLRaV-4 RaV106 (H), healthy grapevine (I) and GLRaV-5 RaV100 (L).

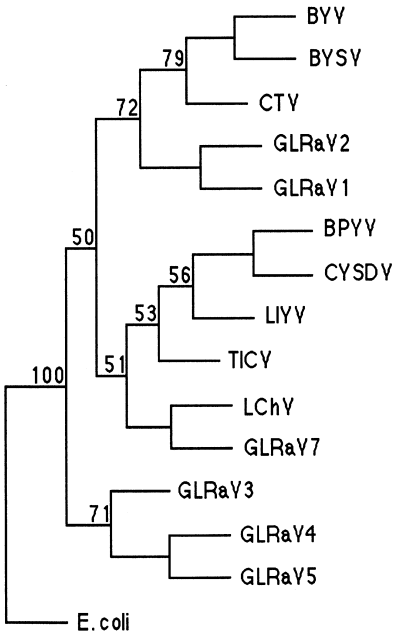

Figure 3. Phylogenetic tree of members of the family *Closteroviridae* using the HSP-70 sequences comprised between phosphate 1 and 2 motifs. Viruses and respective accession numbers are: BYV = beet yellows virus (P37092); CTV = citrus tristeza virus (A49804); BYSV = beet yellow stunt virus (1388133); BPYV = beet pseudo yellows virus (1778162); CYSDV= cucurbit yellow stunting disorder virus (1778164); LIYV = lettuce infectious yellows virus (641985); TICV= tomato infectious chlorosis virus (1778166); LChV = little cherry virus (Y10237). The tree was produced with the PROTPARS and DRAWGRAM programs of the Phylip package. Numbers indicate bootstrapping values. Outgroup is the related domain of *Escherichia coli* (accession P04475).

from 76 to 81%, while pairwise comparisons between tricho- and vitiviruses gave values around 50%.

Alignment of the deduced amino acids of HSP-70 regions amplified from GLRaVs confirmed the heterogeneity of these genes. When superimposed to the previously published alignment of whitefly- and aphid-transmitted members of the family *Closteroviridae* (Tian et al., 1996) it was not possible to identify a consensus for grapevine closteroviruses, although some GLRaVs showed a high level of homology. This was the case of GLRaV-1 and GLRaV-2 and of GLRaV-4 and GLRaV-5, which showed 91% identical amino acid residues. The possibility that such a high homology could originate from a mixed infection was ruled out because: (i) GLRaV-2 dsRNAs were isolated from mechanically inoculated *N. benthamiana* plants that contained no other closteroviruses; (ii) when the GLRaV-1 grapevine source FR6 was RT-PCR tested with GLRaV-2 specific primers (Abou-Ghanem et al., 1998) no amplification products were obtained. Comparable results, confirming the specificity of our GLRaV-4 and GLRaV-5 sequences have recently been reported (Routh et al., 1998).

In phylogenetic tree generated using the regions comprised between RdRp motifs II and V, tricho- and vitiviruses grouped consistently with their current taxonomic classification lending further support to the recent establishment of the genus *Vitivirus* as distinct from *Trichovirus* (Martelli et al., 1997b) (not shown). By converse, although our data provide molecular evidence that GLRaV-1, -4, -5 and -7 can be regarded as *bona fide* members of the family *Closteroviridae* possessing the HSP70 gene which is unique to this family, an incompletely coherent picture emerged from the analysis of closterovirus HSP-70 domain comprised between phosphate 1 and 2 motifs (Figure 3).

As already reported by Tian et al. (1996), two major lineages constituted by aphid- (CTV, BYV and BYSV), or whitefly- (BPYV, CYSDV, LIYV, and TICV) and mealybug- (LChV) transmitted viruses could be distinguished. GLRaVs were distributed among these lineages (GLRaV-1, -2 and -7) or gave origin to a third grouping (GLRaV-3, -4 and -5). After this work was finished, three papers were published describing the genome organizations of GLRaV-2 BA (Abou Ghanem et al., 1998) a further GLRaV-2 isolate (Zhu et al., 1998) and GLRaV-3 (Ling et al., 1998). Altough our GLRaV-3 HSP-70 gene sequence differs from that reported by Ling et al. (1998), they grouped togheter in phylogenetic analysis (not shown). Phylogenetic relationships reported by these authors considering different genes, supports our results.

The original purposes underlying the present investigation were: (i) to evaluate RT-PCR with degenerate primers for the study of three taxa (*Vitivirus, Trichovirus and Closteroviridae*), that have undergone taxonomic re-arrangement in the last few years and that comprise several species affecting grapevines; (ii) to develop reagents useful for acquiring new sequence data of still unknown viruses associated with grapevine leafroll and rugose wood. Both goals were achieved. The successful amplification of molecularly known (GVA, GVB) and unknown vitiviruses (GVD, HLV), trichoviruses (PVT), a different strain of ACLSV, and closteroviruses (GLRaV-1, -4, -5, -7) proved these tools to be efficient for collecting sequence data useful for both evolutive and diagnostic studies. Particularly for filamentous grapevine viruses, the acquisition of new sequence information constitutes the starting point for the molecular study of unknown regions of the genomes of these viruses and for the development of more sensitive and simpler diagnostic tools.

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