GARLIC VIRAL COMPLEX: IDENTIFICATION OF *POTYVIRUSES* AND *CARLAVIRUS* IN CENTRAL BRAZIL*

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ABSTRACT

Garlic viruses often occur in complex infections in nature. In this study, a garlic virus complex, collected in fields in Brazil, was purified. RT-PCR was performed using specific primers designed from the consensus regions of the coat protein genes of *Onion yellow dwarf virus*, a garlic strain (OYDV-G) and *Leek yellow stripe virus* (LYSV). cDNA of *Garlic common latent virus* (GCLV) was synthesized using oligo-dT and random primers. By these procedures individual garlic virus genomes were isolated and sequenced. The nucleotide sequence analysis associated with serological data reveals the presence of two Potyvirus OYDV-G and LYSV, and GCLV, a *Carlavirus*, simultaneously infecting garlic plants. Deduced amino acid sequences of the Brazilian isolates were compared with related viruses reported in different geographical regions of the world. The analysis showed closed relations considering the Brazilian isolates of OYDV-G and GCLV, and large divergence considering LYSV isolate. The detection of these virus species was confirmed by specific reactions observed when coat protein genes of the Brazilian isolates were used as probes in dot-blot and Southern blot hybridization assays. In field natural viral re-infection of virus-free garlic was evaluated.

Key words: OYDV-G, LYSV, GCLV, detection, coat protein, PCR, probes, epidemiology.

RESUMO

Complexo viral do alho: Identificação de *Potyvirus* e *Carlavirus* na região central do Brasil

Infecções virais em alho são normalmente causadas por um complexo viral. Neste estudo, um complexo viral de alho, coletado em campo, foi purificado. Procedeu-se à amplificação por RT-PCR usando oligonucleotídeos desenhados para regiões-consenso dos genes das proteínas capsidiais de *Onion yellow dwarf virus*, estirpe do alho e de *Leek yellow stripe virus*. cDNA de *Garlic common latent virus* foi sintetizado usando oligo-dT e oligonucleotídeos aleatórios. Por estes procedimentos clones de diferentes espécies virais foram isolados e sequenciados. A análise das sequências nucleotídicas e os resultados sorológicos revelaram a presença dos *Potyvirus* OYDV-G e LYSV e do *Carlavirus* GCLV, simultaneamente infetando plantas de alho. As seqüências de aminoácidos deduzidos dos isolados brasileiros foram comparadas com aquelas de vírus relacionados, relatados em diferentes regiões do mundo. A análise mostrou pequena variabilidade em relação aos isolados brasileiros de OYDV-G e GCLV, e maior divergência em relação ao isolado de LYSV. A detecção destas espécies virais também foi obtida por reações específicas observadas quando o gene da proteína capsídica dos isolados brasileiros foi usado como sonda em ensaios de hibridização do tipo dot-blot e Southern blot. Em campo, a re-infecção natural de alho livre de vírus foi avaliada.

INTRODUCTION

Commercial garlic (*Allium sativum* L.) is vegetatively propagated by bulbs. This system often leads to the accumulation of viruses in plant materials, permitting their dissemination and inducing yield losses during successive cultivation (Davis, 1995). A disease known as the "garlic viral complex" is usually induced by

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Gene sequences corresponding to OYDV-G, LYSV and GCLV coat proteins are accessible in GenBank under numbers AF228414, AF228415 and AF228416, respectively.

simultaneous infections of several viruses belonging to different taxonomic groups (Van Dijk, 1993a; Van Dijk, 1993b). It is difficult to separate single virus species from the viral complex in garlic, as similar characteristics exist among them, in particular their overlapping, narrow experimental host ranges and similar symptoms on naturally infected hosts. These biological features are limiting factors in obtaining homogenous virus isolates, consequently the production of specific antibodies to the individual virus species in the garlic virus complex has been hindered.

A variable number of Potyviruses and Carlaviruses have been reported infecting garlic plants, but their identity remains to be elucidated and they probably represent mixtures of well known viruses or their strains (Kobayashi *et al*. 1996; Tsuneyoshi *et al.*, 1998; Van der Vlugt *et al.*, 1999). The occurrence of three distinct groups of Potyviruses infecting *Allium* spp. has recently been proposed (Tsuneyoshi *et al.*, 1998), based on their sequence homology. Two distinct virus species have been denoted *Onion yellow dwarf virus* (OYDV), família *Potyviridae* gênero Potyvirus, garlic (G) and onion (O) strains, and *Leek yellow stripe virus* (LYSV), família *Potyviridae* gênero Potyvirus, based on high levels of coat protein (CP) amino acid sequence identities. A third group was named *Wakegi yellow dwarf virus* (WYDV), which is closely related to Potyviruses isolated from shallot (*Allium cepa* var. *ascalonicum* Backer), rakkyo (*A. chinese* G. Don), Welsh onion (*A. fistulosum* L.) and Wakegi onion. Recently, Van der Vlugt *et al.* (1999) suggested that WYDV can be classified as one isolate of *Shallot yellow stripe virus* (SYSV), família *Potyviridae* gênero Potyvirus, based on biological, serological and molecular data. Limited data is available in the literature for *Carlaviruses* (Van Dijk, 1993a), and as a consequence, the identity and classification of Carlavirus species infecting garlic remains uncertain. Carlavirus infecting garlic plants in the world include *Garlic common latent virus* (GCLV) and *Shallot latent virus* (SLV), as *Garlic latent virus* (GLV), the Japanese designation of garlic isolates of SLV.

In addition to the Potyvirus and Carlavirus, garlic plants have often been infected with viruses from *Rymovirus*, a mite-borne filamentous viruses, which are now all *Garlic virus A-D* (Helguera *et al.*, 1997) considered as members of the new genus *Allexivirus*, recently ratified.

In Brazil, garlic is largely cultivated in different geographical areas of the country. Typical virus symptoms have been found in all commercial fields of garlic. The agents detected in different areas of the country were reported as most likely being a garlic viral complex, comprised of Potyviruses and Carlaviruses (Assis *et al.* 1995; Daniels, 1999; Dusi, 1995; Fajardo *et al.*, 2000). However, the precise identity of these pathogens and their relationship with other garlic viruses described elsewhere has not yet been determined.

In this report we present results on serology, the use of molecular probes, and coat protein (CP) gene sequences of the garlic viral complex found in Central Brazil, confirming the presence of three virus species. They represent new isolates of the OYDV-G, LYSV and GCLV and were denoted OYDV-G_{BR}, $LYSV_{BR}$ and $GCLV_{BR}$.

MATERIAL AND METHODS

Isolation of the garlic virus complex

In a previous survey conducted in Brazil, serologically related garlic viruses were identified in a virus complex (Dusi, 1995; Assis *et al.*, 1995; Daniels, 1999). The original garlic viral complex collected in the field in Brasília-DF was maintained in a greenhouse through mechanical inoculation of virus-free garlic plants and of *Chenopodium quinoa* Willd. using a 0.05 M phosphate buffer, at pH 7.2. Symptoms were monitored weekly and plants were checked for the virus infection by ISEM (immunosorbent electron microscopy).

Decoration tests were performed using polyclonal antiserum directed to the Potyviruses OYDV-G, LYSV, Garlic yellow stripe virus (there is evidence that this name does not refer to a particular virus, but a virus complex), and to *Shallot latent virus* (SLV) and *Carnation latent virus* (CLV) from the genus Carlavirus. The CLV antiserum cross reacted to GCLV.

Isolation of purified virus, viral RNA and total RNA

The garlic virus complex was purified from infected garlic leaves as described by Carvalho & Shepherd (1983). Total RNA of infected garlic plants was extracted according to Chomczynsky & Sacchi (1987). Viral RNA was also extracted from purified virus preparations using the following procedure. Pellets obtained after centrifugation over a 20% sucrose cushion were resuspended in 400 µl RNA extraction buffer (0.2 M NaAc, 10 mM EDTA, pH 5.0) and RNAs were extracted after adding SDS to a final concentration of 1% (w/v), followed by phenol extractions. RNA was precipitated using ethanol dissolved in DEPC-treated water and analyzed by electrophoresis on a 1.2% agarose gel.

Onion plants infected with a strain of OYDV (OYDV-O) were maintained for comparative purposes and submitted to the same procedures as the garlic viruses. Total RNA of these plants was extracted using the same procedure described above and served as a control.

cDNA synthesis

In order to obtain GCLV cDNA, reverse transcription (RT) was conducted using approximately 1 µg of viral RNA and 0.2 µg of oligonucleotide oligo $(dT)_{12\text{-}18}$ using a "Time Saver cDNA Synthesis Kit" (Pharmacia Biotech). *Eco RI*/*Not I* adaptors were added after first strand synthesis according to the manufacturer's instructions to facilitate cloning.

RT and PCR amplification

For PCR, primers were designed from consensus sequences of OYDV-G and LYSV strains available in the literature or based on data bank sequences (Kobayashi *et al.*,

1996). The primers denoted 1OYDV-G (5' TTA CAT TCT AAT ACC AAG CA 3'), 2OYDV-G (5' GCA GGA GAT GGG GAG GAC GC 3'), 1LYSV (5' TCA CTG CAT ATG CGC ACC AT 3') and 2LYSV (5' GCA CCA TAC AGT GAA TTG AG 3') were employed for cDNA synthesis and PCR amplification of OYDV-G and LYSV, respectively. Expected sizes of the OYDV-G and LYSV amplified PCR products were 774 bp and approximately 1000 bp, respectively.

First strand cDNA synthesis was performed using AMV-reverse transcriptase and 100 ng of the primers 1OYDV-G and 1LYSV. Reaction conditions were in accordance with the manufacturer's instructions.

For PCR, 10 µl of the RT mix was added to a 50 µl polymerase reaction mixture containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each dNTPs, 2.5 U of Taq DNA polymerase and 100 ng of each of the primers 1OYDV-G and 2OYDV-G or 1LYSV and 2LYSV. PCR steps were 94 °C for 5 min, followed by 35 cycles (94 °C / 1 min, 50 °C / 2 min and 72 °C / 2 min) and a final amplification at 72 °C for 7 min RNA extracted from OYDV-O virions was submitted to the same cycles of amplification using the same set of primers described for OYDV-G.

Cloning and sequencing of the cDNA and of the amplified PCR fragment clones

Amplified PCR and cDNA fragments were cloned in the pGEM-T vector (Promega) or *Eco RI* site of the pBS/KS$^+$ (Stratagene), respectively. Recombinant clones from at least three independent RT-PCR reactions, were sequenced by the chain termination method, in an automatic ABI PRISM 377 DNA sequencer (Applied Biosystems). Sequences were compiled and analyzed using algorithms of the GCG package from the University of Wisconsin. GenBank searches were performed using BLAST.

Hybridization Assays

PCR fragments of 335 bp from OYDV-G and LYSV, obtained using Langeveld *et al.* (1991) primers, and GCLV cDNA clones, obtained as previously described, were used to prepare radioactive probes according to manufacturer's instructions of the "Ready to Go Labelling Kit" (Pharmacia Biotech). Total RNA from infected plants, and purified virus RNA extracted from a field garlic virus complex were employed in the hybridization assays. Southern and Dot blot hybridizations were performed according to Sambrook *et al.* (1989). Total RNA from healthy plants and viral RNA extracted from onion plants infected with OYDV-O were used as controls.

Serological detection of garlic viruses in infected field material

Serological assays using polyclonal antibodies against the coat proteins of OYDV-G, LYSV, GCLV and against a garlic virus complex (mainly consisting of the genera *Potyvirus* and *Carlavirus*), were performed on garlic plants cultivated during three successive seasons in two experimental fields (Embrapa Hortaliças and Água Fria) starting from virus-free bulbs stocks. Leaf samples were taken from infected plants in the field, diluted 1:10 (w:v) and analyzed by DAS-Elisa according to standard methods.

RESULTS

Isolation of garlic virus complex

Neither in inoculated garlic plants nor in *C. quinoa* local lesion extracts there was evidence that a single virus species was occuring. By ISEM in the same sample extract it was often observed that virus particles were decorated by two or more antisera.

PCR, cloning, sequence determination and analysis of garlic virus complex

DNA fragments comprising the complete CP gene were successfully amplified for OYDV-G and LYSV species by RT-PCR. To LYSV, a part of the polymerase (NIb) gene was included in the amplified PCR fragment. Using the primers 1OYDV-G and 2OYDV-G, DNA fragments of expected sizes (774 bp) were successfully amplified. With the primers 1LYSV and 2LYSV, single specific DNA bands of approximately 1000 bp were amplified by RT-PCR. The specificity of these amplified bands to each viral RNA was confirmed by the expected size of the amplified PCR product displayed. The amplified fragments were purified, cloned into pGEM-T vector and sequenced.

The open reading frame (ORF) representing the CP gene of OYDV-G Brazilian isolate contained 774 nucleotides (Figure 1), thereby potentially coding for a protein of 257 amino acids residues with a predicted M_r of about 28 kDa. The ORF in the $LYSV_{BR}$ CP gene contained 870 nucleotides and encoded a deduced protein of 289 amino acids and a predicted M_r of 31 kDa (Figure 2). For both OYDV-G_{BR} and $LYSV_{BR}$ isolates, the predicted gene product approximately corresponded in size to the CP proteins as determined by SDS polyacrylamide gels in other studies. No amplification was observed for OYDV-O using the same primer combinations as used for OYDV-G.

The nucleotide sequence of a large viral clone obtained by cDNA synthesis showed an open reading frame of 960 nucleotides, potentially coding for a protein of 319 amino acids residues with a predicted M_r of about 36 kDa. This ORF had high homology to GCLV (Figure 3). A search in the EMBL protein database revealed that the deduced amino acid sequences of OYDV-G_{BR}, $LYSV_{BR}$ and $GCLV_{BR}$ clones had high homology with the CP proteins of each reported virus species found elsewhere.

Coat protein amino acid sequence identity of the OYDV-G_{BR} to those of other OYDV-G isolates reported in garlic varied between 95.0-99.2%, whereas for $LYSV_{BR}$ CP protein ranged between 85.9-88.6% (Table 1A and 1B). For the $GCLV_{BR}$ isolate the amino acid sequence identity ranged from 94.0 to 94.5 with other GCLV isolates and 53.2-53.7% identity with GLV isolates, the related Carlavirus sequences

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  1 GCAGGAGATGGGGAGGACGCAGCTGCACAATCAAGCACATCAAAACAAGTTTCGAAGCAG
    A  G  D  G  E  D  A  A  A  Q  S  S  T  S  K  Q  V  S  K  Q

 61 AAGGATAAAGACGTTGATGCAGGCACAACCGGAAAATTCACAGTGCCAAGGATTAAAGCA
    K  D  K  D  V  D  A  G  T  T  G  K  F  T  V  P  R  I  K  A

121 TTGTCTGACAAAATGCGCTTTCCGAAAGTTGGTAAAAGCGTAGTTCTCAATGCGGAGCAC
    L  S  D  K  M  R  F  P  K  V  G  K  S  V  V  L  N  A  E  H

181 TTGTTGGCATACAAACCAGATCAAATTGAATTATACAACACACGAGCAACACAGCAACAA
    L  L  A  Y  K  P  D  Q  I  E  L  Y  N  T  R  A  T  Q  Q  Q

241 TTTGAAAATTGGTTTGGTGCGATCAAAAAGGAATATGACGTGAATGACGAACAGATGAAG
    F  E  N  W  F  G  A  I  K  K  E  Y  D  V  N  D  E  Q  M  K

301 ATAATACTGAACGGGTTGATGGTTTGGTGTATTGAGAACGGCACGTCTCCAAATTTATCA
    I  I  L  N  G  L  M  V  W  C  I  E  N  G  T  S  P  N  L  S

361 GGCAATTGGACTATGATGGACGGTGACGAGCAGGTTGAGTATCCCTTGGCACCGATTCTG
    G  N  W  T  M  M  D  G  D  E  Q  V  E  Y  P  L  A  P  I  L

421 GACAACGCAAAACCGACGTTCAGACAAATAATGGCACATTTCAGTGACGCAGCTGAAGCG
    D  N  A  K  P  T  F  R  Q  I  M  A  H  F  S  D  A  A  E  A

481 TATATTGAGTATAGAAATGCCACTGAAAAATACATGCCCCGGTATGGACTTCAGCGAAAC
    Y  I  E  Y  R  N  A  T  E  K  Y  M  P  R  Y  G  L  Q  R  N

541 CTAACAGAATTAAGTTTAGCACGTTACGCATTCGACTTTTATGAGATGACTTCAAAAACT
    L  T  E  L  S  L  A  R  Y  A  F  D  F  Y  E  M  T  S  K  T

601 CCCAAGCGAGCTAAGGAAGCACACATGCAAATGAAGGCGGCAGCGGTTAGAGGGCAACT
    P  K  R  A  K  E  A  H  M  Q  M  K  A  A  A  V  R  G  A  T

661 AACCGTTTGTTTGGCCTGGATGGTAATGTAAACACGACAGAAGAGGACACGGAAAGACAC
    N  R  L  F  G  L  D  G  N  V  N  T  T  E  E  D  T  E  R  H

721 ACAGCAGCAGATGTAAACAAGAACCAACACACGTTGCTTGGTATTAGAATGTAA 774
    T  A  A  D  V  N  K  N  Q  H  T  L  L  G  I  R  M  *
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FIG. 1 - Complete nucleotide sequence (above) and amino acid sequence (below) of *Onion yellow dwarf virus* Brazilian isolate coat protein. The nucleotides are numbered from the 5' terminus of the viral RNA. The asterisk indicates the stop codon. The DAG motif related to aphid transmission is underlined.

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  1 GCCGGCGACGAACTAGATGCAGGGACACAAGCAAGCAAGAATCAAAGGAATAACGCAGAC
    A  G  D  E  L  D  A  G  T  Q  A  S  K  N  Q  R  N  N  A  D

 61 AAATCTATTGAGCAACGAAGCCCACTAGTGTCACAAACAAATTTAAACGAGGCGAAGGGC
    K  S  I  E  Q  R  S  P  L  V  S  Q  T  N  L  N  E  A  K  G

121 AGTGGGAGTAGTTCGGGTCAAAACGTGAACAGAGACAGAGATGTAAATGTCGGCACCACA
    S  G  S  S  S  G  Q  N  V  N  R  D  R  D  V  N  V  G  T  T

181 GGAACTTTTAGTGTACCACGGATAAAACAAATCCCACAAAAAGGCATAGTAATTCCAATG
    G  T  F  S  V  P  R  I  K  Q  I  P  Q  K  G  I  V  I  P  M

241 GACGGAGGGAAATCAATACTCAACTTAGACCATCTACTACAATACAAGCCAAGTCAATTA
    D  G  G  K  S  I  L  N  L  D  H  L  L  Q  Y  K  P  S  Q  L

301 TGCATATCAAACACTAGAGCCACGAAGGCACAATTTATGACTTGGAAGGCGAGGCTGCAA
    C  I  S  N  T  R  A  T  K  A  Q  F  M  T  W  K  A  R  L  Q

361 GAGGAATATGGCGTCACTGAGAGTGAGATGAGCATCATTCTAAATGGCTTAATGGTGTGG
    E  E  Y  G  V  T  E  S  E  M  S  I  I  L  N  G  L  M  V  W

421 TGCATTGAGAACGGGACTTCACCCAATATAAACGGCGTTTGGACAATGATGGATGGCGAG
    C  I  E  N  G  T  S  P  N  I  N  G  V  W  T  M  M  D  G  E

481 GAGCAAGTCGAATTTCCTTTACGCCCTGTTGTTGAGCACGCACAACCAACGCTACGTCAG
    E  Q  V  E  F  P  L  R  P  V  V  E  H  A  Q  P  T  L  R  Q

541 ATAATGGCGCACTTCTCAGCATTAGCAGAAGCCTACATTGAGATGAGGAACTCAGAGCAG
    I  M  A  H  F  S  A  L  A  E  A  Y  I  E  M  R  N  S  E  Q

601 GCTTACATGCCGCGATATGGATTACAAAGAAATCTTACAGATATGGGTCTCGCACGGTAT
    A  Y  M  P  R  Y  G  L  Q  R  N  L  T  D  M  G  L  A  R  Y

661 TCATTTGACTTCTATGAAATCACATCAAGAACACCAGTTAGAGCGCGCGAGGCTCATGCA
    S  F  D  F  Y  E  I  T  S  R  T  P  V  R  A  R  E  A  H  A

721 CAAATGAAAGCAGCTGCCTTACGTAATTCAAGGCCAAAGCTGTTTGGATTAGACGGCAAC
    Q  M  K  A  A  A  L  R  N  S  R  P  K  L  F  G  L  D  G  N

781 GTCACAACCACGGATGAGGACACGGAGAGGCACACGGCACATGACGTGAATGCACGGATG
    V  T  T  T  D  E  D  T  E  R  H  T  A  H  D  V  N  A  R  M

841 CACCATCTTGATGGTGCGCATATGCAGTGA 870
    H  H  L  D  G  A  H  M  Q  *
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FIG. 2 - Complete nucleotide sequence (above) and amino acid sequence (below) of *Leek yellow stripe virus* Brazilian isolate coat protein. The nucleotides are numbered from the 5' terminus of the viral RNA. The asterisk indicates the stop codon. The DAG motif related to aphid transmission is underlined.

available in literature (Table 1C). Remarkably for LYSV, the homology of Brazilian LYSV significantly differs from those previously described from other countries (Table 1B).

Comparing the nucleotide sequences of the OYDV-G_{BR}, $LYSV_{BR}$ and $GCLV_{BR}$ coat protein gene with published sequences, 95.1%, 81.6% and 83.3% identities were found with other OYDV-G, LYSV and GCLV isolates, respectively (Table 1A, 1B and 1C).

Hybridization based identification of garlic viruses in field samples

A dot-hybridization was performed using radioactive probes obtained from specific cDNA clones of $GCLV_{BR}$. The results showed that probes were able to readily recognize the homologous virus in total RNA extracted from garlic tissues infected with the virus complex (Figure 4). Specific reactions were observed in similar dot-hybridization assays using OYDV-G_{BR} and $LYSV_{BR}$ radioactive probes (data not shown).

A Southern blot using a specific OYDV-G_{BR} PCR probe demonstrated that under high stringency it is possible to distinguish between OYDV-G and LYSV (Figure 5A and 5B), the two major Potyviruses infecting garlic under field conditions, since the heterologous reactions were absentees.

Virus re-infection under field conditions

Virus-free garlic plants cultivated during three successive growing seasons in two distinct geographical areas were analyzed by ELISA using a polyclonal antiserum against a virus complex and against three specific garlic viruses. In agreement with the sequencing data, the results of the serological tests showed that two Potyviruses, OYDV-G and LYSV, and a Carlavirus, GCLV, re-infected the garlic plants under field conditions. The results indicate that these viruses are widely spread in the garlic growing areas in Brazil, often occurring in mixed infections, and causing significant degeneration of the crop after a few multiplications under field conditions.

DISCUSSION

Determination of the identity of the viruses that make up the garlic virus complex is important not only from the taxonomical point of view, but it constitutes an essential requirement for the production of virus free-garlic stocks and the development of detection methods. The virus identity is also important to the study of virus epidemiology and to support breeding programs for the development of garlic cultivars resistant to virus infection.

The identification of virus infections in garlic plants has been based on serology, host range and sequence data of

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  1 ATGTCAGTGAGTGAAACAGAGGAACAGAGATCGCGAAGACTGGCTTCAGAGCGAGGCGAT
    M  S  V  S  E  T  E  E  Q  R  S  R  R  L  A  S  E  R  G  D

 61 GCTGAACGACGGAAAAATGATGCAGCAGTGAGAGCTAGGCAGGATGCTGCAATCGATTCT
    A  E  R  R  K  N  D  A  A  V  R  A  R  Q  D  A  A  I  D  S

121 GAGGAACCTGCTGACTTGCAAGAAACGAGCGTTAATGATGTTGATCTGCGTCAAATGGAA
    E  E  P  A  D  L  Q  E  T  S  V  N  D  V  D  L  R  Q  M  E

181 AATAGGGTCCAGGAAGCCAAGCGGTTTTTGGAGCGCTTTAACAAGCTTAAGAAGTTCCAG
    N  R  V  Q  E  A  K  R  F  L  E  R  F  N  K  L  K  K  F  Q

241 GCGGACAATATGACGGCAGGTGAGATCAAGAATGGAGGGTTTGAAACTGGGAGGCCAAAA
    A  D  N  M  T  A  G  E  I  K  N  G  G  F  E  T  G  R  P  K

301 CTGAACATTGCGGCCAATTTGCGTGGCGACACTGCTAATGTATTTACTAGGCCCAGCATG
    L  N  I  A  A  N  L  R  G  D  T  A  N  V  F  T  R  P  S  M

361 GATGCTTTAATAGCATTGGACTTCAAAGCTGAATCCTTGGCTGTCGCGACTGCTGAAGAC
    D  A  L  I  A  L  D  F  K  A  E  S  L  A  V  A  T  A  E  D

421 CTAGCTGCTATCACTGCTAAATTTGAACAGCTTGGGGTGCCAACTGAGAGATTAGCTCCA
    L  A  A  I  T  A  K  F  E  Q  L  G  V  P  T  E  R  L  A  P

481 CTTTGTTGGTCGATTGCAAGGTACTGTGCAGATACAAGTTCTTCATATGTGGCTGATCCG
    L  C  W  S  I  A  R  Y  C  A  D  T  S  S  S  Y  V  A  D  P

541 AAAGGAACTTTTGAATACCCAGGGGGTGCTATAACAAGGGACGCTGTTTATGCCGTCATC
    K  G  T  F  E  Y  P  G  G  A  I  T  R  D  A  V  Y  A  V  I

601 AAAGAAGTTACGACTCTGAGGGCTTTCTGCAGAGCTTTTGCACCAGTGGTTTGGAATGAA
    K  E  V  T  T  L  R  A  F  C  R  A  F  A  P  V  V  W  N  E

661 ATGTTAATCGCTAAAAGACCTCCTGCTGGCTGGCAAACCAAAGGTTACACTGCTAGTACA
    M  L  I  A  K  R  P  P  A  G  W  Q  T  K  G  Y  T  A  S  T

721 AAGTATGCTGCCTTTGATACTTTCGATTACGTACTTAATTCTGCTTGCGTCCAGCCACTT
    K  Y  A  A  F  D  T  F  D  Y  V  L  N  S  A  C  V  Q  P  L

781 GAGGGGATTATACGGGTTCCAACTGATGAGGAGACCATAGCTCACATGACCAACAAACGG
    E  G  I  I  R  V  P  T  D  E  E  T  I  A  H  M  T  N  K  R

841 ATTGCTATTGATAGGAATAGGCGCAATGGCCGGTTTTCAAGCACAAACAGTTTAGTTACT
    I  A  I  D  R  N  R  R  N  G  R  F  S  S  T  N  S  L  V  T

901 GGGGGCATGTTCGGTAAGGATATCAAGCTAAATTTCAATGGATCCAACAATGCAGACTAG 960
    G  G  M  F  G  K  D  I  K  L  N  F  N  G  S  N  N  A  D  *
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FIG. 3 - Complete nucleotide sequence (above) and amino acid sequence (below) of *Garlic common latent virus* Brazilian isolate coat protein. The nucleotides are numbered from the 5' terminus of the viral RNA. The asterisk indicates the stop codon.

the coat protein gene (Kobayashi *et al.*, 1996; Tsuneyoshi *et al.*, 1998; Van der Vlugt *et al.*, 1999). In Brazil, however, the identity of garlic viruses causing severe losses in bulb production has not yet been established. In this study, three virus species were found simultaneously infecting garlic plants in Brazil. Based on amino acid homology and serological reactions these viruses were identified as two isolates of the *Potyvirus* genus OYDV-G_{BR} and $LYSV_{BR}$, and an isolate of the *Carlavirus* genus, $GCLV_{BR}$. These characterized virus isolates had nucleotide sequence identities of 95.1%, 81.6% and 83.3% and amino acid sequence identities of 99.2%, 88.6% and 94.5% with homologous OYDV-G, LYSV and GCLV characterized elsewhere, respectively. Within the same species, virus strains also showed high specificity. The non amplification of OYDV-O, included for comparison, with the primers specific to OYDV-G corroborate the biological and molecular differences found between these two distinct strains. OYDV-G very rarely can infect onion plants and vice versa (Conci *et al.*, 1992) and comparison of the deduced aminoacid sequences showed a similarity of 88% between the viral coat proteins of onion and garlic strains of OYDV (Kobayashi *et al.*, 1996).

These three virus species seem to be widely spread in the garlic growing areas in Brazil and in many other countries around the world causing significant degeneration of the crop

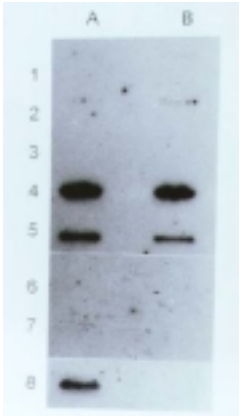

FIG. 4 - Dot blot hybridization with a P^{32} cDNA probe from $GCLV_{BR}$. Samples represented: (1A and 1B) Total RNA extracted from healthy onion (*Allium cepa*) plants; (2A and 2B) Total RNA extracted from healthy garlic (*Allium sativum*) plants; (3A and 3B) Total RNA extracted from onion plants infected with OYDV-O; (4A and 4B) Total RNA from field infected garlic plants; (5A and 5B) Purified cDNA clone from $GCLV_{BR}$; (6A and 6B) Purified PCR fragment of OYDV-O; (7A and 7B) Purified PCR fragment from the garlic *Potyvirus* complex; (8A) Purified RNA of garlic virus complex; (8B) Purified RNA of OYDV-O.

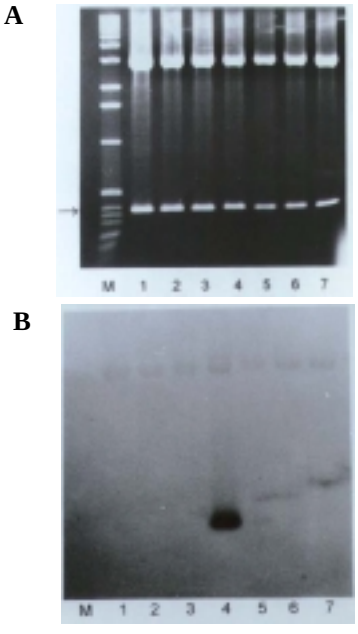

FIG. 5 - Southern blot hybridization of $LYSV_{BR}$ (lanes 1, 2, 3, 5, 6 and 7) and OYDV-G_{BR} (lane 4) fragments of the coat protein (335 bp, arrow). (A) agarose gel analysis of cloned fragments digested with *Pst I*/*Nco I* restriction enzymes; (B) hybridization with OYDV-G_{BR} specific P^{32} DNA clone. (M) molecular marker 1 kb ladder DNA.

TABLE 1 - Pairwise percent identities of coat protein amino acid sequences (below diagonal) and coat protein nucleotide sequences (above diagonal) among related (A) *Onion yellow dwarf virus* (OYDV-G), (B) *Leek yellow stripe virus* (LYSV) and (C) *Garlic common latent virus* and *Garlic latent Carlavirus* isolates from distinct geographical regions.

(A)

cDNA clone[1]	OYDV-G_{BR}	OYDV-G_{AR1}	OYDV-G_{IND}	OYDV-G_{JP}	OYDV-G_{AR2}	OYDV-G_{UAE}
OYDV-G_{BR}	-	95.1	84.5	83.7	83.8	84.2
OYDV-G_{AR1}	99.2	-	84.5	84.4	84.4	84.1
OYDV-G_{IND}	96.5	95.7	-	88.9	87.9	99.4
OYDV-G_{JP}	96.1	95.3	97.7	-	86.6	88.2
OYDV-G_{AR2}	96.1	95.3	96.5	96.5	-	87.2
OYDV-G_{UAE}	95.0	94.2	98.5	96.1	95.0	-

(B)

cDNA clone[1]	$LYSV_{BR}$	$LYSV_{UAE}$	$LYSV_{CHI}$	$LYSV_{IND}$	$LYSV_{L}$	$LYSV_{JP}$
$LYSV_{BR}$	-	81.6	80.6	80.0	81.1	80.2
$LYSV_{UAE}$	88.6	-	82.0	79.7	82.0	80.0
$LYSV_{CHI}$	88.6	88.6	-	79.0	81.0	77.4
$LYSV_{IND}$	86.2	85.9	86.9	-	90.6	84.0
$LYSV_{L}$	85.9	84.8	85.8	95.5	-	84.0
$LYSV_{JP}$	85.9	84.8	86.2	90.3	89.3	-

(C)

cDNA clone[1]	$GCLV_{BR}$	$GCLV_{1}$	$GCLV_{2}$	GLV_{1}	GLV_{2}	GLV_{3}
$GCLV_{BR}$	-	83.3	81.4	56.7	56.5	56.5
$GCLV_{1}$	94.5	-	99.3	46.6	45.7	46.9
$GCLV_{2}$	94.0	100.0	-	49.4	46.3	47.6
GLV_{1}	53.7	54.3	54.3	-	77.0	78.2
GLV_{2}	53.2	52.5	52.5	91.9	-	97.0
GLV_{3}	53.2	52.5	52.5	91.9	99.3	-

[1]Accession numbers: OYDV-G_{BR}: AF228414 (garlic-Brazil), OYDV-G_{AR1}: X89402 (garlic-Argentina 1), OYDV-G_{IND}: AB000841 (garlic-Indonesia), OYDV-G_{UAE}: AB000839 (garlic-UAE), OYDV-G_{AR2}: AB000837 (garlic-Argentina 2), OYDV-G_{JP}: AB000838 (garlic-Japan). $LYSV_{BR}$: AF228415 (garlic-Brazil), $LYSV_{UAE}$: AB005611 (garlic-UAE), $LYSV_{L}$: X89711 (leek), $LYSV_{CHI}$: AB005610 (garlic-China), $LYSV_{JP}$: D11118 (garlic-Japan), $LYSV_{IND}$: AB005612 (leek-Indonesia). $GCLV_{BR}$: AF228416 (garlic-Brazil), $GCLV_{1}$: X81139 (garlic-type 2), $GCLV_{2}$: X81138 (garlic-type 1), GLV_{1}: D73379, GLV_{2}: D11161, GLV_{3}: D28591.

after only a few multiplications under field conditions (Fajardo *et al.*, 2000; Takaichi *et al*., 2001). ELISA results show that virus-free garlic plants cultivated during three successive growing seasons were re-infected by OYDV-G and LYSV, and by GCLV under field conditions.

In contrast to other virus genera, serology is not a very good parameter for virus differentiation among viruses of the genus *Potyvirus*, as serological cross reactions often cause misinterpretation of results (Conci *et al.*, 1999). Although serology can be used for Potyvirus detection, it is not suitable for Potyvirus taxonomy (Shukla & Ward, 1988). These observations support the application of molecular techniques for characterization of the garlic virus complex, as demonstrated by others (Lot *et al.* 1998; Nagakubo *et al.*, 1994; Kobayashi *et al.*, 1996; Tsuneyoshi *et al.*, 1998; Van der Vlugt *et al.*, 1999). The sequence of the coat protein gene has been used as an efficient tool in defining the Potyvirus species (Shukla & Ward, 1988).

Although sequence alignment showed high homology values, allowing a precise determination of the garlic virus species, the variation observed can be explained by natural strain variation among virus isolates. No correlation between geographical location and sequence homology in the coat protein genes of distinct isolates of OYDV has been observed (Tsuneyoshi *et al.*, 1998; Van der Vlugt *et al.*, 1999). The hypothesis of independent evolution of OYDV isolates adapting to garlic plants as hosts (Tsuneyoshi *et al.*, 1998) is partially confirmed in this research, since the OYDV-G_{BR} displayed a high homology with other isolates characterized elsewhere, and the highest homology was observed with an Argentinean strain. For the $LYSV_{BR}$ isolate infecting garlic plants in Brazil, this seems not to be the case, as it showed a significantly lower amino acid homology with homologous isolates from distinct geographical areas. Other selection pressures, such as environmental conditions, alternative hosts, vector efficiency, may therefore be involved in the generation of these genetic variations, implying an adaptation of this virus to distinct ecological niches.

Considering the virus from the *Carlavirus* genus studied, the $GCLV_{BR}$ showed a similar variation to that observed in OYDV-G isolates. The significance of the close relationship (over 94% amino acid sequence identity) between

$GCLV_{BR}$ and the two isolates remains to be investigated. Most likely, this garlic virus may have been introduced in Brazil with bulbs from Asian countries, notably from China.

In addition to the RT-PCR (Langeveld *et al.*, 1991; Takaichi *et al.*, 1998; Tsuneyoshi & Sumi, 1996), the analysis of clones obtained from the virus genomes can also be used to determine the variability of distinct viruses in the garlic virus complex (Tsuneyoshi *et al.*, 1998). RT-PCR using specific primers and hybridization with molecular probes can be helpful in the detection of new garlic viruses not yet characterized (Sumi *et al.*, 1993). In this work, PCR fragments from OYDV-G and LYSV were labeled and used as probes. Under high stringency conditions, no cross-reaction was observed between coat protein sequences of these two Potyviruses, showing that the virus isolates characterized belonged to distinct *Potyvirus* species and specific probes can be used for virus differentiation, providing an accurate method for detection of mixed infections of garlic viruses. In addition, garlic viruses can be detected from total RNA extracts, as demonstrated for $GCLV_{BR}$.

The present results indicate that these distinct detection and virus differentiation methods can be successfully used to monitor a high quality program of virus-free garlic production associated with an efficient program of virus eradication from garlic by meristem-tip culture and thermotherapy (Torres *et al.*, 2000).

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