Partial characterization of the lettuce infectious yellows virus genomic RNAs, identification of the coat protein gene and comparison of its amino acid sequence with those of other filamentous RNA plant viruses

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Purified virions of lettuce infectious yellows virus (LIYV), a tentative member of the closterovirus group, contained two RNAs of approximately 8500 and 7300 nucleotides (RNAs 1 and 2 respectively) and a single coat protein species with Mr of approximately 28000. LIYV-infected plants contained multiple dsRNAs. The two largest were the correct size for the replicative forms of LIYV virion RNAs 1 and 2. To assess the relationships between LIYV RNAs 1 and 2, cDNAs corresponding to the virion RNAs were cloned. Northern blot hybridization analysis showed no detectable sequence homology between these RNAs. A partial amino acid sequence obtained from purified LIYV coat protein was found to align in the most upstream of four complete open reading frames (ORFs) identified in a LIYV RNA 2 cDNA clone. The identity of this ORF was confirmed as the LIYV coat protein gene by immunological analysis of the gene product expressed in vitro and in Escherichia coli. Computer analysis of the LIYV coat protein amino acid sequence indicated that it belongs to a large family of proteins forming filamentous capsids of RNA plant viruses. The LIYV coat protein appears to be most closely related to the coat proteins of two closteroviruses, beet yellows virus and citrus tristeza virus.

Introduction

Lettuce infectious yellows virus (LIYV) was first identified by Duffus et al. (1982) when it occurred in epidemic proportions in the agricultural areas of the southwestern U.S.A. Large losses occurred in lettuce, sugarbeet, melon and squash crops because of stunting, leaf rolling, yellowing and brittleness in infected plants. Extracts from infected plants contained flexuous, filamentous virus-like particles which measured 1800 to 2000 nm in length (Duffus et al., 1986). Examination of infected tissues indicated that infected plants contained intracellular inclusions, including vesicles similar to those associated with infection by beet yellows closterovirus (BYV; Duffus et al., 1986). These closterovirus-like characteristics led to the tentative inclusion of LIYV in the closterovirus group (Francki et al., 1991).

The closterovirus group is composed of structurally similar, flexuous, filamentous plant viruses which cause interveinal yellowing symptoms in infected plants, but which are variable in other characteristics such as particle length (Bar-Joseph & Murant, 1982). There are currently over 30 closterovirus-like viruses, including LIYV, which have been tentatively included in this diverse group (for a review see Coffin & Coutts, 1993). However, classification of these viruses is evolving as information about their molecular biology emerges. For example, differences in genome size and organization between BYV, type member of the subgroup B closteroviruses, and apple chlorotic leafspot virus (ACLSV), a proposed subgroup A closterovirus, have been identified which suggest that these two viruses might be best placed in separate taxonomic groups (German et al., 1990; Agranovsky et al., 1991a). The relationships among the closteroviruses have been further elucidated by using comparative sequence analysis to establish a phylogeny of filamentous plant virus coat proteins (Dolja et al., 1991; Boyko et al., 1991).

Even though LIYV is similar to the closteroviruses in particle morphology, cytopathology and disease symptoms, it differs from other viruses within the group in several respects. Firstly, LIYV is transmitted semi-persistently by the sweet potato whitefly, Bemisia tabaci...
(Gennadius); aphid transmission is more typical of the clusteroviruses. Secondly, we have shown preliminary evidence for two high M, RNAs in LIYV-infected plants and purified virions (Klaassen et al., 1992). The clusteroviruses characterized so far contain only one ssRNA.

We initiated further studies of the LIYV genome with two primary objectives. First, nucleotide sequence data of the two LIYV RNAs present in infected plants and purified virions would help to determine their relatedness to each other. Second, information about the LIYV gene products and genome organization would be useful in clarifying the relationship between LIYV and other clusteroviruses. In this paper, we report partial cDNA cloning and hybridization analysis of the RNAs found in LIYV-infected plants and purified virions. We have also identified and sequenced the LIYV coat protein gene, and will discuss the tentative phylogenetic relationship of the LIYV coat protein with those of other filamentous plant viruses.

Methods

Virion purification and RNA isolation. LIYV was propagated in Chenopodium murale and Nicotiana clevelandii, using the whitefly vector B. tabaci as described by Duffus et al. (1986). Three LIYV isolates were used during these studies. Isolates 87 and 90 were kindly supplied by R. Creamer (University of California at Riverside, U.S.A.) and isolate 92 was kindly supplied by J. Duffus (U.S. Department of Agriculture, Salinas, Calif., U.S.A.). These were originally obtained from LIYV-infected Lactuca sativa L. in the Imperial Valley of California, U.S.A.

LIYV virions were purified using a protocol obtained by modifying and compiling steps from previously described protocols for the isolation of filamentous plant viruses (Duffus et al., 1986; Lockhart et al., 1992). LIYV-infected tissue (100 g fresh weight) was ground in 5 ml TE. Triton X-100 was added to the suspension at a final concentration of 2% (v/v) and again stirred for 1 to 2 h at 4°C, centrifuged in a Beckman 70Ti rotor for 1 h at 93000 g. The pellets were resuspended overnight at 4°C in 5 ml TE. Triton X-100 was added to the suspension at a final concentration of 2% (v/v) and again stirred for 1 to 2 h at 4°C, followed by centrifugation in a Beckman 70Ti rotor for 1 h at 93000 g. The supernatant was layered over 4 ml of 20% (w/v) sucrose in TE (0.01 M-Tris–HCl pH 7.9, 0.5 mM-sodium acetate 0.5 mM-EDTA). Specific dsRNA extracts were treated with RNase A (10 μg) in 0.2 mM NaCl or RNase alone (Morris & Dodds, 1979).

cDNA cloning and Northern blot hybridization analysis. Virion RNA (1 μg) was polyadenylated as described by Huiet et al. (1992) and used for cDNA synthesis. cDNA synthesis and subsequent cloning were performed using the Superscript Plasmid System cDNA synthesis and cloning kit (BRL). To determine the size of cDNA inserts, recombinant plasmids (approximately 200) were digested with MluI, followed by electrophoresis in 0.8% agarose gels in TAE.

cDNA clones were further analysed using Northern blot hybridization. LIYV virion RNAs and total ssRNAs from healthy and LIYV-infected plants were denatured, separated by gel electrophoresis and transferred to Hybond-N+ (Amersham) in 50 mM-NaOH for 4 h, according to the manufacturer's protocol. Double-stranded RNAs from LIYV-infected plants were denatured by soaking agarose gels in 50 mM-NaOH for 15 min at room temperature. Gels were then equilibrated in 0.5 x TBE (89 mM-Tris–HCl pH 8.0, 89 mM-boric acid, 2 mM-EDTA) for 1 h at 4°C and the denatured dsRNAs were electrophoretically transferred to Hybond-N+ according to the manufacturer's protocol. All filters were rinsed in 2 x SSC and hybridized to 32P-labelled LIYV cDNA clones according to the procedure of Amasino (1986). The cDNAs were labelled using the Sequenase Random-Primed DNA Labeling Kit (USB).

Nucleotide sequence analysis. DNA sequencing reactions were performed on both strands of selected cDNA clones by the dideoxy-nucleotide chain termination method of Sanger et al. (1977) using the Sequenase kit (USB). The sequences of internal regions of the cDNA inserts were obtained from deletion clones generated by exonuclease III and nuclease S1 digestion (Henikoff, 1984), or by using synthetic oligonucleotides complementary to internal regions of the cDNA to prime sequencing reactions. The nucleotide sequence of the LIYV coat protein open reading frame (ORF) was obtained from two independent cDNA clones, pSP105 and pSP122. Nucleotide sequences were analysed using Genetics Computer Group sequence analysis software from the University of Wisconsin (Devereux et al., 1984).

Coat protein isolation and analysis. Purified LIYV virions were analysed by SDS-PAGE as previously described (Klaassen & Falk, 1989). Purified LIYV coat protein and a proteolysis product were used for amino acid sequence analysis. Proteolytic fragments were generated by incubation of gel-purified LIYV coat protein in 10 volumes of a solution containing 10 mg/ml cyanogen bromide and 70% formic acid for 16 h at room temperature. The resulting peptides were separated by SDS-PAGE and transferred to ProBlot membrane (Applied Biosystems). After staining, the major cleavage product was excised and subjected to automated Edman degradation (at the Protein Structure Laboratory, University of California at Davis, Calif., U.S.A.).

Expression of the LIYV coat protein ORF. An 800 bp PCR (Saiki et al., 1988) fragment containing the entire putative LIYV coat protein ORF was generated using primers P2-206 (5’TGAATCCCATGGA-TACAG 3’), identical to nucleotides 891 to 900 of cDNA clone pSP105.
and including an *EcoRI* site and P2-307 (5'-GGATCCCCCCCCATGCCC-TGGAGGTAG3'); complementary to nucleotides 1674 to 1664 of cDNA clone pSP105 and including *BamHI* and *NcoI* sites. The *EcoRI-BamHI* PCR fragment was subcloned into the expression vector pGEMEX-1 (Promega), in the multiple cloning site of the cDNA clone pSP105 and including *EcoRI-BamHI* sites). Two clones, pG41CP and pG45CP, with inserts of the expected size were selected and partially sequenced to confirm the orientation and identity of the PCR fragment and to ensure that the LIYV coat protein ORF was in frame with the T7 gene 10.

In vitro transcription and translation analyses were used to confirm the identity of the LIYV coat protein ORF. Transcripts from clone pG41CP were generated in *vitro* using T3 RNA polymerase and translated in wheat germ extract in the presence of [35S]methionine as described by Huiet *et al.* (1992). Labelled proteins were analysed by electrophoresis on 12% SDS-polyacrylamide gels followed by autoradiography. Protein products were further analysed by immunoprecipitation using polyclonal antibodies to the purified virions of LIYV (kindly supplied by J. Duffus) brome mosaic bromovirus and radiography. Protein products were further analysed by immunoprecipitation using polyclonal antibodies to the purified virions of LIYV (kindly supplied by J. Duffus) brome mosaic bromovirus and radiography. Protein products were further analysed by immunoprecipitation using polyclonal antibodies to the purified virions of LIYV (kindly supplied by J. Duffus) brome mosaic bromovirus and radiography. Protein products were further analysed by immunoprecipitation using polyclonal antibodies to the purified virions of LIYV (kindly supplied by J. Duffus) brome mosaic bromovirus and radiography. Protein products were further analysed by immunoprecipitation using polyclonal antibodies to the purified virions of LIYV (kindly supplied by J. Duffus) brome mosaic bromovirus and radiography.

The LIYV coat protein ORF, contained in clone pG41CP, was also expressed as a fusion protein in *E. coli* according to the manufacturer’s protocol. Proteins were analysed by SDS-PAGE and either visualized by staining with Coomassie brilliant blue G-250, or transferred to nitrocellulose for Western blot analysis (Burnette, 1981). Western blots were probed with antisera to the purified virions of LIYV and LMV and to the T7 gene 10 leader peptide. Serological reactions were detected using goat anti-rabbit IgG conjugated with alkaline phosphatase, and nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. 

Computer-assisted analysis of amino acid sequences. The coat protein amino acid sequences of filamentous RNA plant viruses representing six groups were inferred from the corresponding nucleotide sequences (Dolja *et al.*, 1991; Ochi *et al.*, 1992; Sekiya *et al.*, 1991; Yoshikawa *et al.*, 1992). Local and global alignments of these coat protein sequences were created as previously described (Dolja *et al.*, 1991). A cluster dendrogram was generated using the KITCH program in the PHYLIP package, which implements the Fitch-Margoliash distance matrix algorithm under the molecular clock assumption (Felsenstein, 1989). The alignment used for cluster analysis was produced by fitting the coat protein amino acid sequences of LIYV, BYV, citrus tristeza closterovirus (CTV), apple stem grooving capillovirus (ASGV) and potato capillovirus T (PVT) into previously published global alignments (Dolja *et al.*, 1991) of the coat proteins of filamentous RNA plant viruses. Positions in the alignment including gaps were omitted from the distance matrix calculations.

**Results**

**Virion purification and RNA isolation**

LIYV virions were purified from infected plants in order to identify the LIYV coat protein and the number and sizes of the genomic RNAs. Attempts to purify LIYV virions from infected plants by using established procedures resulted in low yields of virions which were only partially pure. As a result, RNA extracted from these preparations also contained varying amounts of contaminating low Mr nucleic acids and virion RNA was often degraded by contaminating RNases. To obtain the more highly purified LIYV coat protein and virion RNA needed for molecular analysis, several protocols for the isolation of filamentous plant viruses were modified and tested. The method described here consistently yielded a single opaque band in Cs2SO4–sucrose gradients, approximately 8.2 cm below the meniscus. This band contained a major protein species of approximately 28K and lesser amounts of high Mr proteins (around 55K) as determined by SDS–PAGE (data not shown). Western blot analysis showed that the LIYV antiserum reacted strongly with the 28K protein, indicating that it was the LIYV coat protein (data not shown).

When RNA was extracted from purified LIYV virions and analysed by denaturing agarose gel electrophoresis, two high Mr RNAs of approximately equimolar amounts were resolved (Fig. 1a). These RNAs, referred to as RNAs 1 and 2, were estimated to be 8500 and 7300 nucleotides respectively. When dsRNAs were extracted from LIYV-infected plants and analysed by non-denaturing agarose gel electrophoresis, two high Mr dsRNAs were identified (Fig. 2a). These corresponded in

![Fig. 1. (a) An ethidium bromide-stained 1% agarose gel showing RNAs after denaturation and subsequent electrophoresis. All RNAs except those in lane 1 were isolated from purified virions. Lane 1, RNA ladder (BRL); lane 2, tobacco mosaic tobamovirus RNA; lane 3, BYV RNA; lane 4, LIYV RNAs. Numbers to the left of lane 1 are sizes of the RNA markers in kb. The upper left arrow (14.5 kb) points to BYV RNA. The approximate sizes of LIYV RNAs 1 and 2 are given on the right. (b) Northern blot hybridization analysis of LIYV virion RNAs. Duplicate Northern blots of equivalent samples of LIYV denatured virion RNAs to those in lane 4, hybridized to the cDNA clones pSP182 (lane 5) and pSP130 (lane 6).](image-url)
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Analysis of LIYV cDNA clones
cDNA was made to LIYV virion RNAs and cloned in order to obtain initial nucleotide sequence data on LIYV RNAs 1 and 2 and to identify the LIYV coat protein ORF. Oligo(dT) primers did not prime LIYV virion RNAs for reverse transcription, suggesting that the RNAs were not polyadenylated. Therefore, LIYV virion RNAs were polyadenylated in vitro to facilitate priming cDNA synthesis and cloning the 3'-proximal regions of the RNAs.

Two-hundred recombinant plasmids were screened and found to contain cDNA inserts smaller than 4000 bp. Twenty recombinant plasmids containing the largest cDNA inserts (2000 to 4000 bp) were labelled and used as probes in Northern blot hybridization experiments. Each of the clones hybridized specifically with either LIYV RNA 1 or 2, but not both (Fig. 1b). Eight clones (including pSP130) hybridized with RNA 1 and 12 (including pSP130) hybridized with RNA 2. LIYV RNAs 1 and 2 were also identified by hybridization analysis using total ssRNAs from LIYV-infected plants (data not shown). No hybridization was detected with total ssRNAs from healthy plants (data not shown).

Northern blot hybridization analysis of dsRNAs from LIYV-infected plants gave results which corresponded to those for LIYV virion RNAs and total ssRNAs. Each clone hybridized specifically with one of the high $M_r$ dsRNAs corresponding to the replicative forms of LIYV RNAs 1 or 2 (Fig. 2b). In addition, the clones could be differentiated by their hybridization to different subsets of lower $M_r$ dsRNAs (Fig. 2b). The results from the hybridization experiments using LIYV virion RNAs, total ssRNAs and dsRNAs indicated that the cDNA clones belong to two groups, each group representing one of two non-homologous LIYV RNAs.

Identification of the LIYV coat protein gene
We wanted to identify the LIYV coat protein gene to be able to compare its amino acid sequence with those of other filamentous plant viruses (Dolja et al., 1991). The nucleotide sequence of the largest LIYV cDNA clone, pSP105 (corresponding to LIYV RNA 2), was determined in an attempt to identify any putative ORFs which might encode the LIYV coat protein. pSP105 was found to contain 3892 bp of cDNA. Computer analysis of the nucleotide sequence revealed that the most upstream ORF in the positive strand (virion polarity) could encode a protein with an $M_r$ of 27752. No other ORFs were identified within pSP105 that were the correct size to encode the LIYV coat protein. The nucleotide sequence of this 28K ORF (Fig. 3) was also determined from an independent cDNA clone, pSP122. No nucleotide sequence variations in this ORF were found between the two clones.

As a next step towards identifying the LIYV coat protein gene, a partial amino acid sequence was obtained directly from gel-purified LIYV coat protein. Initial sequencing efforts indicated that the N terminus of the LIYV coat protein was blocked. Therefore, a proteolytic fragment was isolated and an amino acid sequence of nine residues was determined (Ile-Asn-Asn-Ile-Arg-Ser-Ala-Gly-Ile; Fig. 3). This amino acid sequence was found to align perfectly within the predicted amino acid sequence of the 28K ORF.

The 28K ORF was expressed in vitro to determine whether it encoded the LIYV coat protein. Nucleotide sequence analysis of the 5'-terminal region of clone pG41CP verified that the 28K ORF had been cloned into pGEMEX-1 in the correct orientation to express the ORF. When clone pG41CP was transcribed and translated in vitro, a product of approximately 28K was seen...
LIYV RNA 2 contains the coat protein gene

884 TTAAGATATGGGATACAGATGGAATGATGTGGATCGGAAACGATACAGGAA
   M D T D G D N D V F G S G N D T R N
943

944 TAATGATGATAAGAAGAAGGAATGAAACCAAAACATTCTGACAATTTCTCAATACTCAT
   N D D K K K E E M K Q N I S D N S Q I I
1003

1004 ATCAACCGAGGATCATGAGCTGACATCATGGGAAGTATATGCAAGAGGATTTGCTCAA
   S T R D H E A D I I G S I S K E D L S K
1063

1064 AATCGTTGTCGCGTCGACGGACGAGTCTGTAGTGAATGTTCAAAGATTAG
   I V V R V D R H D A L S A N D V Q S F R
1123

1124 AGAAGGCTATGATAAACATTCTGACGAGGAAATACGTTATCTGAGCTCAAACATATTGACAA
   E A M I N F M R D K P N R N Q P S D K
1183

1184 ATGTATTATTGTCTAGCTAGGAAAGTGTGTTTTATAATGCTGATAATTCTGAGCTGACTTCGCG
   L I I A M E V G V Y Q M V I N L G T S A
1243

1244 TAAATTGGGTAAAGCTCAACATCTGATGAGAGAGAAAGGATTTGTCCAA
   K L G N A N N L E F T I A Y D Q E T R T
1303

1304 ATATAAGGCTGCGAGATTTTGGTGAAATTATATGCATGACGTGAGAGGCATCAGCAGAATGT
   Y K V A D F V N M Q S R M R N S P N V
1363

1364 TGTTAGGCAATATGCAAGAGCAGCAGGAAAGGACAAATTTAAACATAAAGGAGTGCTGGAAT
   V R Q Y A R A M E K T I N N I R S A G I
1423

1424 CATATAACAGCCAATGGAATTTTTGCGACGGAAACATGGGAGGCTGTCATCTTACAGAAACTC
   I N S N G V L A A K H G V L A S Y R N S
1483

1484 TTACAGCGACTTTGCTGTTTGTGGTAAAGCAGACACCCTGATGCTCAACTCTACTTCTGCT
   Y S D F A V G F G N D T T D A Q L T S L
1543

1544 AATGTTAGCTAGAAAACAGCAGTATTTGCAAGGAGGTTGGCTAGCTGAGCATTACAA
   M L A R K Q A L C G G S V E H Y N
1603

1604 TACTATGCGTGTAGCTAACCTTAACATCCATGTGGAGGGCGGAAATTGATGAAGTGGAG
   T M Q L A N L K H P C *
1663

1664 CTAACCTCCAG 1674

GATTGAGGTC

Fig. 3. Sequence of nucleotides 884 to 1674 of cDNA clone pSP105 (shown as the LIYV RNA 2 positive-polarity strand). The predicted amino acid sequence of the 28K ORF is shown below the corresponding codons. An asterisk indicates the stop codon at the end of the ORF. The nucleotide sequence of primers P2-306 and P2-307 (the complementary sequence) are underlined (see Methods). The nine amino acid residues sequenced from a proteolytic fragment of purified LIYV coat protein are shown in bold letters.

by SDS-PAGE (data not shown). When the in vitro product was further analysed by immunoprecipitation followed by SDS-PAGE, the 28K product was specifically precipitated by LIYV antiserum, although slight amounts of the product were also precipitated using LMV antiserum (data not shown).

To test whether the reaction with LMV antiserum indicated a weak serological relationship or was non-specific precipitation, the 28K ORF product was analysed using Western blots. First, pG41CP was expressed as a fusion protein in E. coli. This yielded a protein with a M₀ of 56000, the size expected for a fusion protein encoded by the S'-terminal region of T7 gene 10 and the 28K ORF. Western blot analysis showed that the LIYV antiserum reacted specifically with a 56K protein and with the LIYV coat protein (Fig. 4). Antiserum to the T7
gene 10 leader peptide reacted with a 56K protein and
the leader peptide encoded by the 5'-terminal region
of T7 gene 10, but not with the LIYV coat protein (Fig. 4).
LMV antiserum did not react with a 56K protein or with
the LIYV coat protein (Fig. 4). These results indicate
that the 28K ORF is the LIYV coat protein gene.

Analysis of coat protein amino acid sequences

We compared the deduced amino acid sequence of the
28K ORF with the coat protein sequences of other
filamentous plant viruses to identify any similarities and
possible relationships. Preliminary local sequence simi-
larity analysis suggested that the amino acid sequence of
the LIYV coat protein was most similar to the coat
protein sequences of two closteroviruses, BYV and CTV
data not shown). Stepwise multiple alignments of the
three sequences over nearly their entire length gave an
adjusted alignment score of 7.6 S.D. above the random
expectation for each step. This can be considered an
indication of genuine relatedness. An evolutionary
distance of 179.1 was obtained, further indicating their
relatedness. Analysis of the alignment revealed 18
conserved positions, 21 similar positions, and 16 posi-
tions occupied by hydrophobic amino acid residues (Fig.
5). The LIYV coat protein possesses the longest N-
terminal variable region of the three aligned proteins (54
residues as compared to 38 residues in CTV and nine
residues in BYV).

As previously demonstrated, all filamentous plant coat
proteins share three conserved amino acid residues:
serine, arginine and aspartic acid (Dolja et al., 1991).
The corresponding residues were also conserved in the LIYV
coop protein amino acid sequence (residues Ser-117, Arg-
160 and Asp-202; Fig. 5).

The most conserved central part of the alignment
shown in Fig. 5 was incorporated into a global alignment
of the coat proteins of representatives of six groups of
filamentous plant viruses. This alignment was used to
generate a cluster dendrogram illustrating the sequence
relationships, and tentative phylogenetic relationships,
inside this large family of virion coat proteins (Fig. 6).
The resulting dendrogram shows four main groups of
closely related coat proteins. The first group is composed
of the coat proteins of ASGV and PVT together with
ACLSV, the second of LIYV and the two closteroviruses
BYV and CTV, the third of potyviruses and bymoviruses
and the fourth of potexviruses and carlaviruses. This
analysis supports clustering the LIYV coat protein with
the coat proteins of the closteroviruses, although it
shows that the coat proteins of BYV and CTV are more
V. Klaassen, M. Boeshore, V. Dolja & B. Falk, unpublished results). These results suggest the possibility of a tested to date (87, 90 and 92 as well as five field isolates; 2 do not share significant sequence homology. Two RNAs, RNAs 1 and 2, in purified LIYV virions and molecular analysis of the LIYV genome in order to define LIYV characteristics further and to determine more accurately its relationship to the closteroviruses.

Our data indicate that LIYV is distinguished from the monopartite closteroviruses. The relationship between LIYV, BYV and CTV that is suggested by the coat protein sequence analysis. It has been shown that the coat protein genes of positive-strand RNA viruses display extreme evolutionary mobility, crossing the borders of virus families and even superfamilies, possibly as a result of RNA recombination (Lai, 1992; Dolja & Carrington, 1992). Therefore, we are not able to predict whether other ORFs in the LIYV, BYV and CTV genomes will show a similar level of relatedness. Two genes of particular interest are those coding for the heat shock protein 70-related protein and the diverged homologue of the coat protein.

The taxonomic significance of this potential difference in genome organization between LIYV and the closteroviruses was investigated by comparing the coat protein genes and products. A partial amino acid sequence obtained from purified LIYV coat protein indicated that the 28K ORF of cDNA clone pSP105 (corresponding to RNA 2) encodes the coat protein. The identity of this ORF was confirmed by immunological analysis of its product expressed both in vitro and in E. coli. The upstream location of the LIYV coat protein gene (V. Klaassen, M. Boeshore, V. Dolja & B. Falk, unpublished results) is similar to the location of the BYV (and possibly the CTV) coat protein gene (Agranovsky et al., 1991a) and stands in contrast to most other filamentous plant viruses whose coat proteins are encoded by the 3'-terminal ORF (Dolja et al., 1991).

Phylogenetic analysis based on the amino acid sequence similarity of the LIYV coat protein to those of other filamentous RNA plant viruses suggests that the LIYV, BYV and CTV coat protein genes are closely related. Although these data indicate that the coat protein genes of the two closteroviruses, BYV and CTV, are more closely related to each other than to the LIYV coat protein gene, they support earlier suggestions that LIYV is related to the closteroviruses (Duffus et al., 1986).

It remains to be determined whether comparisons of other ORFs will support the close evolutionary relationship between LIYV, BYV and CTV that is suggested by the coat protein sequence analysis. It has been shown that the coat protein genes of positive-strand RNA viruses display extreme evolutionary mobility, crossing the borders of virus families and even superfamilies, possibly as a result of RNA recombination (Lai, 1992; Dolja & Carrington, 1992). Therefore, we are not able to predict whether other ORFs in the LIYV, BYV and CTV genomes will show a similar level of relatedness. Two genes of particular interest are those coding for the heat shock protein 70-related protein and the diverged homologue of the coat protein. Both are present in BYV (Agranovsky et al., 1991b; Boyko et al., 1992) and CTV (Pappu et al., 1993; Boyko et al., 1992). This is consistent with the close relationship indicated by the coat protein sequence analysis. The conservation of these two genes in the LIYV genome would provide additional evidence for a close relationship between LIYV and the two closteroviruses.

Regardless of the degree of evolutionary relatedness indicated by future comparison of LIYV, BYV and CTV ORFs, the presence of two RNAs represents a potential difference in genome organization from that of the monopartite closteroviruses. The relationship between the LIYV and the closteroviruses may be similar to that between BaYMV, the type member of the bymoviruses.
and the potyviruses. Despite a close genetic relationship between BaYMV RNA 1 and the 3'-terminal region of the monopartite potyvirus genome, there are major differences in the organization of BaYMV RNA 2 and the 5'-terminal region of the potyvirus genome (Kashiwazaki et al., 1991). The BaYMV bipartite genome is not merely a split analogue to the monopartite genome of potyviruses; these data support previous suggestions that the bymoviruses and potyviruses be classified into separate taxonomic groups (Kashiwazaki et al., 1989).

By analogy, it may be appropriate ultimately to classify LIYV and the closteroviruses into separate taxonomic groups. Further sequence analysis of LIYV RNAs 1 and 2 is required to define more clearly the relatedness and genomic organization of these RNAs and to assess more definitively their relationship to the closteroviruses.

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