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A Heterogeneous Population of Defective RNAs Is Associated with *Lettuce infectious yellows virus*

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Preparations of dsRNAs and virion RNAs extracted from *Nicotiana clevelandii* plants infected with the bipartite *Lettuce infectious yellows virus* (LIYV) were found to contain multiple LIYV RNA species. In addition to the two LIYV genomic RNAs, three types of RNAs were observed: (a) 3′ coterminal subgenomic RNAs; (b) RNAs containing LIYV RNA 1 or RNA 2 5′ terminus but lacking the 3′ terminus; and (c) RNAs with both LIYV RNA 2 3′ and 5′ termini but each with a central extensive deletion, a structure typical of defective RNAs (D RNAs). No D RNA-like RNAs were detected for LIYV RNA 1. A reverse transcription followed by polymerase chain reaction (RT–PCR) strategy was used to clone from virion RNAs several LIYV RNA 2 D RNAs as cDNAs. Nucleotide sequence analysis of 43 cloned cDNAs showed in some D RNAs the presence of a stretch of 1–5 nt in the junction site that is repeated in the genomic RNA 2 in the two positions flanking the junction site or in close proximity. Some D RNAs contained in the junction site one or several extra nucleotides not present in the LIYV genomic RNA 2. Two of the cloned cDNAs were used to generate *in vitro* transcripts, and infectivity studies showed that both D RNAs were replication competent in protoplasts when coinoculated with LIYV RNAs 1 and 2 or with only LIYV RNA1. Neither D RNA showed obvious effects upon LIYV RNA 1 and RNA 2 accumulation in coinfected protoplasts. These data suggest that LIYV infections contain a heterogeneous population of LIYV RNA 2 D RNAs, and some are encapsidated into virions.

INTRODUCTION

Lettuce infectious yellows virus (LIYV) is the type member of the genus *Crinivirus* in the family *Closteroviridae* (Martelli *et al.,* 1999). All viruses of this family share several characteristic traits, such as phloem limitation within their plant hosts, transmission by specific Homopteran vectors in a semipersistent manner, filamentous and flexuous virions, and a large positive-sense single-stranded RNA genome (Dolja *et al.,* 1994; Agranovsky, 1996; Martelli *et al.,* 1999). So far, within the genus *Crinivirus* only the genome organization and nucleotide sequences of the two LIYV genomic RNAs have been determined (Klaassen *et al.,* 1995). LIYV RNA1 (8118 nt) codes for proteins associated with RNA replication. The open reading frame (ORF) 1A and 1B proteins contain the domains methyltransferase, helicase, and RNA-dependent polymerase, which are common to all Sindbis-like viruses (Dolja *et al.,* 1994). LIYV RNA 2 (7193 nt) contains the *Closteroviridae* hallmark gene array, encoding for p5, a small hydrophobic protein, a heat shock protein 70 homolog (HSP70), p59 (a protein of unknown function), the LIYV major capsid protein (CP), and the minor capsid protein (CPm) (Dolja *et al.,* 1994; Klaassen *et al.,* 1995). Recently Tian *et al.* (1999) showed the bipolar nature of the LIYV virion capsid, similar to that first discovered for viruses in the genus *Closterovirus, Beet yellows virus* (BYV; Agravnosky *et al.,* 1995) and *Citrus tristeza virus* (CTV; Febres *et al.,* 1996), and presented evidence for a possible role of the CPm in LIYV transmission by its natural vector, *Bemisia tabaci* Genn.

Defective RNAs (D RNAs) have been described within the family *Closteroviridae* for CTV (Mawasssi *et al.,* 1995; Yang *et al.,* 1997; Ayllón *et al.,* 1999) and for plant RNA viruses in other taxonomic groups (Graves *et al.,* 1996; White and Morris, 1999). D RNAs are deletion and/or rearrangement mutants of the viral genomic RNA and are unable to replicate in the absence of the wild-type or helper virus. In some cases, D RNAs can interfere with helper virus accumulation and affect the symptoms induced by the helper virus in host plants and are referred to as defective-interfering RNAs (DI RNAs). Within the genus *Crinivirus*, although smaller-than-genomic dsRNAs have been observed for LIYV, *Beet pseudo-yellows virus* (BPYV), and *Cucurbit yellow stunting disorder virus* (CYSDV; Klaassen *et al.,* 1995; Livieratos *et al.,* 1998), no D RNAs have been positively identified so far.

In this paper, we report the detection and characterization of a number of heterogeneous D RNAs associated with LIYV. Two D RNAs were cloned as cDNAs and transcripts generated *in vitro* were coinoculated to protoplasts with one or both LIYV genomic RNAs to study

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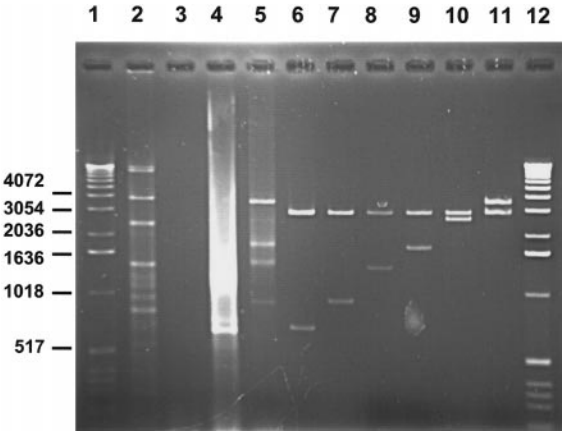

FIG. 1. Ethidium-bromide-stained 2% agarose gel showing double-stranded RNAs extracted from LIYV-infected *N. clevelandii* plants, reverse transcription and polymerase chain reaction (RT–PCR) products, and cloned cDNAs of LIYV-defective RNAs (D RNAs). Lanes 1 and 12 correspond to the 1-kb DNA ladder (GibcoBRL); lane 2, LIYV dsRNAs; lane 3, RT–PCR products from LIYV RNA 2 *in vitro* transcripts; lane 4, RT–PCR products from LIYV dsRNAs; lane 5, RT–PCR products from LIYV virion RNAs. The remaining lanes show D RNA cloned cDNAs obtained from LIYV virion RNA RT–PCR products, after restriction digestion with *Sac*I and *Sph*I. Lanes 6–11, clones M15, M39, M44, M27, M9, and M30, respectively.

their competence for replication and their ability to interfere with helper virus replication.

RESULTS

Detection and characterization of smaller-than-genomic LIYV RNAs

When LIYV dsRNAs were analyzed by agarose gel electrophoresis, several dsRNAs smaller than the full-length genomic dsRNAs were seen (Fig. 1, lane 2). To determine their origin, the dsRNAs were denatured and analyzed by Northern hybridization with probes corresponding to terminal and internal regions of the LIYV genomic RNAs 1 and 2 (see Fig. 2). For RNA 1, only three consistent and prominent dsRNAs were seen (Fig. 2). These included the dsRNA corresponding to the genomic RNA 1, which was detected by the 5′, middle, and 3′ probes; a second large dsRNA of ca. 7.0 kb, which hybridized only with the 5′ and middle probes (indicated as I in Fig. 2); and a dsRNA of ca. 1 kb, which hybridized only with the 3′ terminal probe (indicated as P32 in Fig. 2). The 1-kb dsRNA appears to be a dsRNA corresponding to the subgenomic RNA corresponding the LIYV RNA 1 ORF 2. Conversely, the ca. 7-kb dsRNA is not a conventional subgenomic RNA as it lacks 3′ homology with the genomic RNA 1, and the significance of this RNA is currently unknown. In contrast to the results for RNA 1, when probes corresponding to specific regions of LIYV RNA 2 were used, a more complex pattern was seen. dsRNAs corresponding to the subgenomic RNAs for the HSP70, p59, CP, and P26 ORFs were clearly identified (Fig. 2). These RNAs hybridized with probes corresponding to the respective ORF as well as to all downstream probes. In contrast, probes corresponding to regions of the genomic RNA but 5′ of the ORF did not hybridize with these subgenomic RNAs. Also, two dsRNAs were detected that hybridized with probes corresponding to the RNA 2 5′ end, the HSP70 homolog, and p59 genes but not with probes corresponding to the 3′ region of LIYV RNA 2 (indicated as II and III in Fig. 2). In addition, several dsRNAs that hybridized with probes corresponding to both 5′ and 3′ LIYV RNA 2 termini but not with probes from internal sequences were observed (e.g., RNAs A–D in Fig. 2). These data suggested that these RNAs contained the 5′ and 3′ termini but lacked various internal regions. Thus these were likely D RNAs associated with LIYV RNA 2.

The strongest hybridization signal with LIYV RNA 2 probes was obtained for a ca. 3.5-kb dsRNA (indicated by A in Fig. 2), similar in size to the largest nongenomic dsRNA observed by electrophoresis (Fig. 1, lane 2). Therefore, this 3.5-kb dsRNA was gel-purified and used as a template for RT–PCR with primers corresponding to the LIYV RNA 2 5′ and 3′ termini (primers Rmm501 and Rmm502T3 in Table 1). The resulting RT–PCR product was cloned, and the nucleotide sequences of three clones were partially or completely determined and analyzed. These analyses confirmed that this RNA had a

TABLE 1

Oligonucleotide Primers Used For RT-PCR Reactions

Primer	Position[a]	Sequence
Rmm501[b]	3′ terminus	5′ ATAAGAAT**GCGGCCGC**GGTCTAGTAT-ACGAGATACA 3′
Rmm502T3[b]	5′ terminus	5′ TGCA**GAGCTC***AATTAACCCTCACTAAA*-GGTAATCACAATTACCATTG 3′
CPf[c]	4270[a]	5′ GGAATAATGATGATAAGAAGAAAGA 3′
CPr	4850[a]	5′ CATCAGTGGGTGTCGTTACCAAAACC 3′
2L521f	521[a]	5′ ATGTGCAGGTATATTGTAAA 3′
2L1077r	1095[a]	5′ ATGAGCTGGCTAACAACCT 3′
2L4158f	4158[a]	5′ AACATTGACATTTACCTAGATTTCC 3′
2L963f	963[a]	5′ AAATACGTAGCAGAGTTGGTTGAAG 3′
2L5834r	5853[a]	5′ ATTAGACTCAACATCAATTG 3′
2L6234r	6253[a]	5′ CGTCGTCAA AGCTGTGGTCT 3′
2L6751r	6770[a]	5′ ATTTTTATTTTTAGAATATT 3′

[a] Position of first nucleotide in primers corresponding to the nucleotide sequence of LIYV RNA 2 (Klaassen *et al.,* 1995).

[b] Primer Rmm501 contains a *Not*I site (bold nucleotides) and is complementary to the 20 3′-terminal nucleotides of LIYV RNA 2 (nucleotides underlined). Primer Rmm502T3 includes an *Sst*I site (in bold), T3 promoter sequence (nucleotides in italics), and the 20 5′-terminal nucleotides of LIYV RNA 2 (nucleotides underlined; Klaassen *et al.,* 1996).

[c] "f" and "r" indicate primer orientation. "f" indicates that the primer is of the same polarity as LIYV RNA 2. "r" indicates the primer is complementary to LIYV RNA 2.

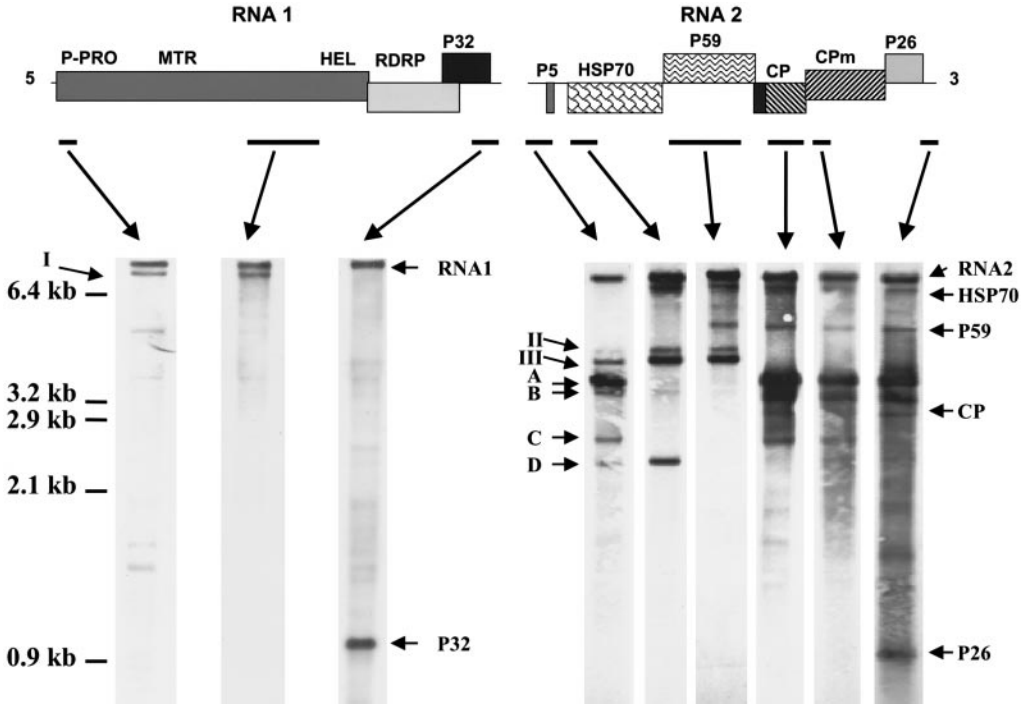

FIG. 2. Northern blot hybridization analysis of denatured dsRNAs from LIYV-infected *N. clevelandii* plants. A genomic map of the LIYV RNAs 1 and 2 is shown at the top (Klaassen *et al.,* 1995). Hybridizations were done using DIG–RNA probes corresponding to different regions of the LIYV genomic RNAs. Lines shown below the LIYV genomic map indicate probes. Arrows then show lanes corresponding to the indicated probes. At the right of the gels are indicated the dsRNAs corresponding to the 3′ coterminal subgenomic RNAs for the HSP70 homolog, CP, P59, P26 (RNA 2), and P32 (RNA 1) ORFs. At the left are indicated the ds RNAs of the 5′ coterminal subgenomic-like RNAs (I, II, and III) and of defective RNAs (A–D). Corresponding positions of marker RNAs are given at left.

typical D RNA structure. It contained the LIYV RNA 2 5′ and 3′ terminal sequences but had a single deletion between positions 627 and 4274 of LIYV RNA 2. Interestingly the junction site contained an extra G not present at position 628 or 4273 of the LIYV RNA 2 nucleotide sequence (Klaassen *et al.,* 1995). To determine whether this G residue was also present in the genomic RNA 2 of the infecting LIYV, RT–PCR was done using gel-purified full-length LIYV genomic dsRNA 2 and the primers 2L521f-2L1077r and 2L4158f-CPr (Table 1). The resulting products were cloned and their nucleotide sequences were determined. None contained an extra G, confirming that the G occurs at the junction site of the 3547 nt RNA but not in the coinfecting LIYV genomic RNA 2.

A large heterogeneous population of LIYV RNA 2 deletion mutants is present in LIYV infections

Our original Northern hybridization analyses suggested the possibility that several D RNAs were likely associated with LIYV RNA 2. To clearly identify these D RNAs, we performed RT–PCR using primers corresponding to both termini of LIYV RNA 2 (Rmm501 and Rmm501T3, Table 1) and LIYV-infected *Nicotiana clevelandii* dsRNAs as the template. To ensure that any D RNAs identified by this approach were not artifacts, we also performed RT–PCR using full-length RNA 2 transcripts as a control. No RT–PCR products were detected from full-length RNA 2 transcripts (Fig. 1 lane 3), indicating that under our conditions, RT–PCR did not produce artifacts nor were full-length cDNAs generated. As an additional control, the RNA 2 transcripts were used for RT–PCR with the internal primers CPf and CPr (Table 1). A product of 588 nt, the expected size, was obtained (not shown), indicating that the LIYV RNA 2 transcripts were good templates for RT–PCR. The LIYV dsRNA RT–PCR products appeared as a smear when analyzed by agarose gel electrophoresis, possibly as a result of amplification of a large population of different size RNAs having LIYV RNA 2 5′ and 3′ termini (Fig. 1, lane 4).

To determine whether or not D RNAs were present in LIYV virion preparations, we performed RT–PCR amplification from LIYV virion RNAs using the primers Rmm501 and Rmm501T3 (Table 1). RT–PCR yielded a number of discrete products ranging in size from ~1 to 3.5 kb (Fig. 1, lane 5). Because these RT–PCR products from LIYV virion RNAs showed an electrophoretic pattern less complex than that from dsRNAs, they were cloned and analyzed. Several recombinant plasmids were obtained with cDNA inserts ranging in size from 621 to 3548 nucleotides. Many plasmids had cDNA inserts that corre-

TABLE 2

Cloned cDNAs Corresponding to LIYV-Defective RNAs (D RNAs)

Type	D RNAs[a]	N[b]	Size	Junction[c]	Adjacent repeats[d]	Extra[e]
I	M2	1	910	624, 6907 **A**		
	M3	1	1364	618, 6447 **TGTCG**		
	M1	1	1738	251, 5706 **AG**		
	M11	1	1884	562, 5871 **TT**		
	M20	1	1931	589, 5851 **AAT**		
	M26	1	1985	346, 5554 **T**		
	M12	1	2259	313, 5247 **C**		
	M9	1	2506	621, 5308 **C**		
	M18[f]	1	3109	295, 4379 **AGAG**		
II	M15[f]	1	621	562, 7134 **T**	573, 7143 **ATAAA** 581, 7151 **TTT** 590, 7160 **ATTTA**	
	M44[f]	1	1347	615, 6461**ATT**	583, 6428 **TTCTTAAA** 596, 6442 **AAA** 622, 6468 **GAT**	
	M46[f]	1	1398	609, 6404**TTT**	616, 6412 **TTTG**	
	M16[f]	2	1405	616, 6404 **TTTG**	631, 6421 **TTCAT**	
	M4[f]	1	1419	620, 6394 **TC**	622, 6397 **GATA**	
	M38	1	1641	441, 5993 **TTT**	457, 6001 **AATTT**	
	M17	1	1687	347, 5853**TC**	351, 5857 **ATG**	
	M34[f]	5	1763	619, 6049 **GTCG**	623, 6054 **ATA**	
	M39[f]	1	904	691, 6984 **AAA**	694, 6987 **TGA**	**C**
III	M21[f]	1	1091	286, 6387	260, 6358 **TGCGA** 265, 6365 **AT** 270, 6371 **AAGAG** 277, 6379 **GAT**	
	M42[f]	1	1124	276, 6344	277, 6346 **GAT**	
	M23	1	1371	562, 6384	580, 6403 **TTTT**	**G**
	M28	1	1575	231, 5852	429, 5847 **GT**	**GTAT**
	M36	1	1600	189, 5782	186, 5781 **CAAAA**	**G**
	M14	1	1601	386, 5978	386, 5984 **AAAG**	**C**
	M5[f]	2	1897	619, 5915	608, 5903 **AT** 610, 5906 **TTT** 615, 5911 **ATTT** 619, 5916 **GT** 623, 5918 **ATATC** 631, 5926 **TTC**	**A**
	M10	1	1942	615, 5870	610, 5861 **TTT** 625, 5875 **ATCA**	**TGAAC**
	M19	1	2501	562, 5253	567, 5259 **GTT**	
	M30[f]	3	3548	628, 4273	621, 4262 **CGATA**	**G**
IV	M37[f]	2	1270	415, 6337		
	M13	1	1279	342, 6256		**G**
	M22	1	1688	470, 5973		
	M27	1	1690	328, 5830		
	M32	2	1925	462, 5729		

[a] Each LIYV D RNA is derived from LIYV RNA 2 but has a corresponding central deletion.

[b] Number of clones obtained for each D RNA.

[c] Type I and II D RNAs have in the junction site a stretch of a few nucleotides that is repeated on both sides of the junction in the genomic LIYV RNA 2. For these, the positions of the first nucleotides of both side repeats are indicated by two numbers followed by the repeated nucleotides. If no repeats are in the exact junction sites then the position of the first and last nucleotides of the deleted zones are indicated (Type III and IV D RNAs).

[d] If there are repeats nearby but not in the exact junction site, the positions and repeated nucleotides are indicated (Type II and III D RNAs). Some clones have more than one set of repeated nucleotides (i.e. M15 and M21).

[e] Nucleotide/s found in the D RNA junction site but not in the corresponding positions in the LIYV genomic RNA 2.

[f] Defective RNAs whose complete nucleotide sequences were determined are deposited in GenBank under Accession Nos. AF229370–AF229382.

sponded closely in size with the original LIYV virion RNA RT–PCR products (Fig. 1, lanes 6–11). The nucleotide sequences of 48 clones were determined and were analyzed. Five cDNA clones were found to result from a misannealing artifact in the RT–PCR reaction and are not presented. The remaining 43 clones all contained the 5′ and 3′ termini of the LIYV RNA 2 and an extensive single central deletion (Table 2 and Fig. 3). The sizes of the LIYV RNA 2 terminal sequences varied for different cDNA clones ranging from 188 to 693 nucleotides for the 5′ terminal sequences, while the RNA 2 3′ terminal sequences ranged from 58 to 2919 nucleotides (Table 2 and Fig. 3). None of the clones contained sequences corresponding to the LIYV RNA 2 HSP70 homolog and p59 ORFs. According to the nucleotide sequences in or adjacent to the junction site, we classified the clones into four types (Table 2): Type I clones had a stretch of a few nucleotides in the junction site that is repeated on both sides of the junction in the genomic LIYV RNA 2. Type II clones not only had nucleotide repeats in the exact junction site but also had repeats in other positions nearby to the junction site. Type III clones only had repeats nearby the junction site. Type IV clones lacked nucleotide repeats. In addition, ~30% of the clones (i.e.,

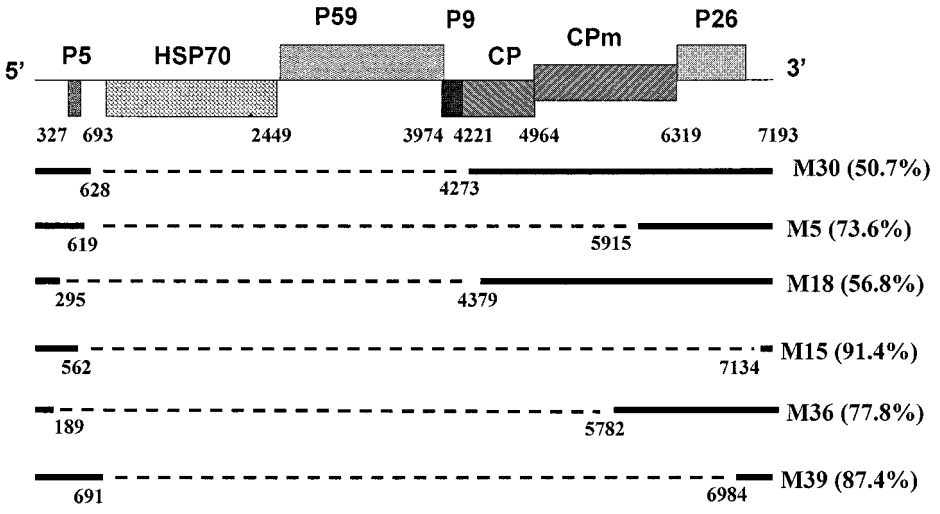

FIG. 3. Schematic representation of some LIYV D RNAs described in Table 2. The LIYV RNA 2 genomic map is indicated at the top. Numbers below map indicate the nucleotide position of corresponding ORF start codons. The position of the LIYV RNA 2 3′-most nucleotide is 7193. Continuous lines represent nucleotide sequences of D RNAs common to LIYV RNA 2. Discontinuous lines correspond to deleted regions not present in the corresponding D RNAs. Numbers given below lines indicate the LIYV RNA 2 D RNA junction positions (as given in Table 2). The deletion sizes (percentage respect the full-length genomic RNA) are indicated between parentheses.

M5 and M10 in Table 2) contained in the junction site extra nucleotide(s) not present in the genomic RNA sequence. Three of these clones (corresponding to D RNA M30; see Table 2 and Fig. 3) showed nucleotide sequences, including the extra G, identical to the clone obtained by RT–PCR from the gel-purified 3.5-kb dsRNA (see Fig.1, lane 2).

Replication of LIYV D RNAs in protoplasts

To determine whether the LIYV D RNAs were replication competent, we synthesized T3 transcripts from cDNA clones M5 and M18 (see Table 2 and Fig. 3) and inoculated them separately into protoplasts alone, with only LIYV RNA 1 transcripts, or with both LIYV RNA 1 and 2 transcripts. The original PCR primer, Rmm502T3, which was used for constructing D RNA cDNAs, was designed to contain a T3 promoter immediately adjacent to the LIYV RNA 2 5′-most nucleotide, thus resulting in transcripts that corresponded to D RNA sequences. Transcripts of M5 and M18 failed to accumulate when inoculated alone to protoplasts (not shown). However, D RNA accumulation was observed when each was coinoculated with LIYV RNA 1 or with LIYV RNAs 1 and 2 (Fig. 4). Both D RNAs accumulated rapidly and were detected at 48 h postinoculation. To determine whether these D RNAs interfered with the replication of LIYV genomic RNAs 1 or 2, protoplasts were coinoculated with LIYV RNA1, RNA 2, and D RNA M5 or M18 transcripts. Results varied slightly for six separate experiments; however, neither M5 nor M18 consistently appreciably affected LIYV RNA 1 or RNA 2 accumulation (Fig. 4).

DISCUSSION

Our results show that LIYV-infected plants contain, in addition to the two genomic RNAs, multiple smaller RNA species. These RNAs can be classified as three types according to their nature. The first was a set of typical 3′ coterminal subgenomic RNAs. Although our data here

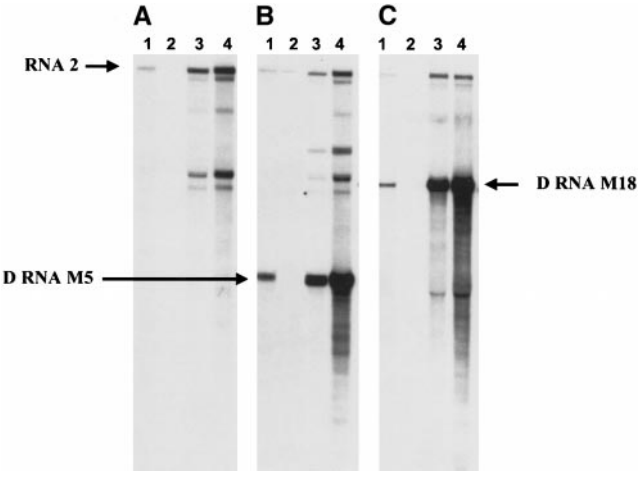

FIG. 4. Northern blot analysis of total RNAs extracted from *Nicotiana tabacum* protoplasts inoculated with transcripts of (A) LIYV genomic RNA 1 and RNA 2; (B) LIYV genomic RNA 1 and RNA 2 plus transcripts for the defective RNA M5 (see Table 2 and Fig. 3); and (C) LIYV genomic RNA 1 and RNA 2 plus defective RNA M18 (see Table 2 and Fig. 3). Defective RNAs and genomic LIYV RNA 1 and RNA 2 were inoculated in equimolar concentrations. Total RNAs were extracted from protoplasts 0, 24, 48, and 72 h after inoculation (lanes 1–4). Hybridizations were done using a DIG–RNA probe corresponding to LIYV RNA 2 3′ terminus.

show results for LIYV dsRNAs, similar results were obtained by us using total RNAs from LIYV-infected plants and protoplasts and by others for the *Closterovirus*, CTV (Hilf *et al.,* 1995). These LIYV subgenomic RNAs are the result of the expression of LIYV RNA 1 ORF 2 and RNA 2 ORFs 2–7, similar to that seen for other members of the *Closteroviridae* (Dolja *et al.,* 1994; Hilf *et al.,* 1995). We also observed RNAs that contained LIYV RNA 1 or RNA 2 5′ terminus but lacked the corresponding 3′ terminus. A similar RNA has been reported for CTV (Mawassi *et al.,* 1995), but its role in virus infection is as yet unknown. Similarly, the role of the LIYV 5′ subgenomic like RNAs is currently unknown. Finally, we detected RNAs that had LIYV RNA 2 5′ and 3′ termini but also had large central deletions. The structure of these RNAs is typical for D RNAs of plant viruses of several taxonomic groups including the family *Bromoviridae* and genera *Closterovirus* and *Potexvirus* (Graves *et al.,* 1996; Ayllón *et al.,* 1999; Damayanti *et al.,* 1999; Torrance *et al.,* 1999; White and Morris, 1999). We demonstrated that at least two of these RNAs were able to replicate in protoplasts if only LIYV genomic RNA 1 is present. The accumulation of these RNAs, while higher than that of the genomic RNA 2, did not interfere with the accumulation of LIYV genomic RNA1 or RNA 2. All of these characteristics fit the definition of D RNAs.

Curiously, no D RNAs derived from LIYV RNA 1 were observed here. If D RNAs are also associated with LIYV RNA 1, they did not accumulate to amounts as high as the LIYV RNA 2 D RNAs so as to be detected in our experiments. The preferential generation of D RNAs corresponding to only one genomic segment seems to be a general feature of multipartite viruses (Graves *et al.,* 1996). One possible explanation for the absence of detectable LIYV RNA 1 D RNAs could be the inability of RNA 1 to replicate *in trans* while RNA 2 and its derived D RNAs contain signals for *trans* replication. In an additional experiment, we constructed an artificial LIYV RNA 1 D RNA (deletion of nucleotides 448-5180). This D RNA was unable to replicate in protoplasts in presence of the helper virus, LIYV RNA 1, suggesting that *trans* replication of RNA-1-derived sequences may not be common (Yeh *et al.,* unpublished data). In contrast to results for RNA 1 the LIYV RNA 2 D RNAs formed a large heterogeneous population. As LIYV RNA 2 is replicated *in trans*, the RNA 2 D RNAs may also be suited to replication *in trans*. Comparison of all the LIYV D RNAs analyzed here showed that all contained at least the 5′-terminal 188 nt and the 3′-terminal 58 nt of LIYV RNA 2 (see Fig. 3). It seems likely that signals for RNA 2 replication *in trans* and possibly for encapsidation might be within one or both of these sequences.

The mechanisms by which plant virus D RNAs are generated have not been definitively demonstrated. The replicase-driven template switching model (Nagy and Simon, 1997) best fits most LIYV D RNAs. These D RNAs corresponded to LIYV genomic RNA 2 with a central extensive deletion and contained at their exact junction or in its vicinity a stretch of a few nucleotides that is repeated in or nearby the positions flanking the deleted segment. In the template switching model, the D RNAs are generated by a translocation event in which the polymerase, together with the nascent strand, falls off the template strand probably at regions of secondary structure. RNA synthesis then reinitiates at a different site with nucleotide sequence identical or similar to the jumping site. D RNAs with similar repeats also have been observed in some isolates of CTV (Ayllón *et al.,* 1999). However, the fact that other LIYV D RNAs contain extra nucleotides and/or no repeats seems to indicate that the mechanism for generating LIYV D RNAs could be more complex or more than one mechanism could be involved. Yang *et al.* (1997) proposed that recombination between a subgenomic RNA with distant parts from the 5′ end of CTV genomic RNA could explain an extra genomic cytosine found in the junction of several CTV D RNAs. Nagy and Bujarski (1997) also proposed a model in which A/U-rich sequences might promote recombination in *Brome mosaic virus* (BMV). In this model, the weak base pairing within the A/U-rich region can facilitate the release of the 3′ end of the incomplete nascent RNA. Also, the weak base pairing within the A/U-rich region can facilitate the reannealing in the acceptor strand by formation of a temporary bubble. The highly A/U-rich sequences could promote RNA polymerase slippage, resulting in the accidental mismatched repeats and/or the incorporation of nontemplated nucleotides. Approximately two-thirds of the LIYV genomic RNA 2 nucleotides are A and U (Klaassen *et al.,* 1995). Interestingly, the 5′ terminus of the all D RNA deleted sequences corresponded with a zone rich in A/U stretches that is located upstream the HSP70 homolog gene, which was lacking in all of the LIYV D RNAs seen here.

MATERIALS AND METHODS

LIYV maintenance and replication in protoplasts

LIYV was maintained by successive transmission to *Lactuca sativa* L., *Chenopodium murale* L., and *Nicotiana clevelandii* gray plants using the whitefly *Bemisia tabaci* (Klaassen *et al.,* 1994). Capped transcripts corresponding to LIYV RNAs were synthesized as previously described (Klaassen *et al.,* 1996). Transcripts were inoculated to protoplasts prepared from *Nicotiana tabacum* suspension cells (Passmore *et al.,* 1993) as described (Lindbo *et al.,* 1993), but here 1.2 million cells were used and the protoplast postinoculation incubation was at 26.5°C.

RNA analysis and extraction

Total RNAs from LIYV-inoculated *N. tabacum* protoplasts were isolated using TRI Reagent (MRO) according to the manufacturer's instructions. LIYV dsRNAs, virions, and virion RNAs were purified from LIYV-infected *N. clevelandii* plants and were analyzed as previously described (Klaassen *et al.,* 1994). LIYV dsRNAs were separated using agarose gel electrophoresis and gel-purified using the RNAid kit (BIO 101, La Jolla, CA.) according to the manufacturer's instructions. Denatured LIYV dsRNAs and protoplast total RNAs were analyzed by Northern hybridization using DIG-labeled RNA probes corresponding to specific genomic regions of LIYV RNA 1 and RNA 2 (see probes in Fig. 2) as previously described (Klaassen *et al.,* 1994–1996).

Reverse transcriptase and polymerase chain reaction (RT–PCR), cloning, and nucleotide sequence analysis

LIYV dsRNAs, virion ssRNAs, and *in vitro* transcripts were used as templates for synthesis of cDNAs using the oligonucleotide Rmm501, complementary to the 20 3′-terminal nucleotides of LIYV RNA 2 (Table 1) and AMV reverse transcriptase (RT; Promega Corp., MD) per the manufacturer's instructions. The cDNAs were PCR-amplified in a 20-μl reaction mixture containing PCR buffer, 1.5 mM $MgCl_2$, 1 mM of each of the four dNTPs, 2.5 U of *Taq* DNA polymerase (Promega), and 50 ng of each oligonucleotide: Rmm501 and Rmm502T3 (Table 1; Rmm502T3 contained a T3 promoter sequence followed by the 20 5′-terminal nucleotides of LIYV RNA 2). After an initial denaturing step at 94°C for 4 min, PCR was performed for 30 cycles, each at 94°C for 30 s, 50°C for 30 s, and 72°C for 8 min, followed by an extension step at 72°C for 10 min. RT–PCR products were separated by electrophoresis in 2% agarose gels and detected by ethidium bromide staining. RT–PCR products were cloned into pGEM-T (Promega) by incubating with DNA ligase (Promega) overnight at 4°C, followed by transformation into *Eschericia coli* DH5α (Sambrook *et al.,* 1989). Nucleotide sequences of the cloned cDNAs were determined in both directions using primers corresponding to the T7 and SP6 plasmid promoters and the ABI PRISM DNA sequencer 377 (Perkin–Elmer, Foster City, CA). Additionally a set of specific LIYV RNA 2 primers was used to determine internal nucleotide sequences (primers 2L521f, 2L4158f, 2L963f, 2L5834r, 2L6234r, and 2L6751r in Table 1). Nucleotide sequence alignment and identity were determined using the program GAP from the Wisconsin GCG software package (Devereux *et al.,* 1984).

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REFERENCES

Agranovsky, A. A. (1996). Principles of molecular organization, expression, and evolution of closteroviruses: Over the barriers. *Adv. Virus Res.* **47,** 119–158.

Agranovsky, A. A., Lesemann, D. E., Maiss, E., Hull, R., and Atabekov, J. G. (1995). Rattlesnake structure of filamentous plant RNA virus built of two capsid proteins. *Proc. Natl. Acad. Sci. USA* **92**, 470–473.

Ayllón, M. A., López, C., Navas-Castillo, J., Mawassi, M., Dawson, W. O., Guerri, J., Flores, R., and Moreno, P. (1999). New defective RNAs from citrus tristeza virus: Evidence for a replicase-driven template switching mechanism in their generation. *J. Gen. Virol.* **80,** 817–821.

Damayanti, T. A., Nagano, H., Mise, K., Furusawa I., and Okuno T. (1999). Brome mosaic virus defective RNAs generated during infection of barley plants. *J. Gen. Vir.* **80,** 2511–2518.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12,** 387–395.

Dolja, V. V., Karasev, A. V., and Koonin, E. V. (1994). Molecular biology and evolution of closterovirus: Sophisticated build-up of large RNA genomes. *Annu. Rev. Phytopathol.* **32,** 261–285.

Febres, V. J., Ashoulin, L., Mawasi, M., Frank, A., Bar-Joseph, M., Manjunath, K. L., Lee, R. F., and Niblett, C. L. (1996). The p27 protein is present at one end of citrus tristeza particles. *Phytopathology* **86,** 1331–1335.

Graves, M. V., Pogany, J., and Romero, J. (1996). Defective interfering RNAs and defective viruses associated with multipartite RNA viruses of plants. *Semin. Virol.* **7,** 399–408.

Hilf, M. E., Karasev, A. V., Pappu, H. R., Gumpf, D. J., Niblett, C. L, and Garnsey, S. M. (1995). Characterization of citrus tristeza virus subgenomic RNAs in infected tissue. *Virology* **208,** 576–582.

Klaassen, V. A., Boeshore, M., Dolja, V. V., and Falk, B. W. (1994). 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. *J. Gen. Virol.* **75,** 1525–1533.

Klaassen, V. A., Boeshore, M. L, Koonin, E. V., Tian, T., and Falk, B. W. (1995). Genome structure and phylogenetic analysis of lettuce infectious yellows virus, a whitefly-transmitted, bipartite closterovirus. *Virology* **208,** 99–110.

Klaassen, V. A., Mayhew, D., Fisher, D., and Falk, B. W. (1996). *In vitro* transcripts from cloned cDNAs of the lettuce infectious yellows closterovirus bipartite genomic RNAs are competent for replication in *Nicotiana benthamiana* protoplasts. *Virology* **222,** 169–175.

Lindbo, J. A., Silva-Rosales, L., Proebsting, W. M., and Dougherty, W. G. (1993). Induction of highly specific antiviral state in transgenic plants: Implications for regulation of gene expresion and virus resistance. *Plant Cell* **5,** 1749–1759.

Livieratos, I. C., Katis, N., and Coutts, R. H. A. (1998). Differentiation between cucurbit yellow stunting disorder virus and beet pseudo-yellows virus by a reverse transcription-polymerase chain reaction assay. *Plant pathol.* **47,** 362–369.

Martelli, G. P., Agranovsky, A. A., Bar-Joseph, M., Boscia, D., Candresse, T., Coutts, R. H. A., Dolja, V. V., Duffus, J. E., Falk, B. W., Gonsalves, D., Jelkmann, W., Karasev, A. V., Minafra, A., Murant, A., Namba, S., Niblett, C. L., Vetten, H. J., and Yoshikawa, N. (1999). Family *Closteroviridae*. *In*: "Seventh Report of the International Committee on Taxonomy of Viruses" (M. H. V. van Regenmortel *et al.,* Eds.)

Mawassi, M., E. Mietkiewska, M. E. Hilf, L. Ashoulin, A., Karasev, V., Gafny, R., Lee, R. F., Garnsey, S. M., Dawson, W. O., and Bar-Joseph, M. (1995). Multiple species of defective RNAs in plants infected with citrus tristeza virus. *Virology* **214,** 264–268.

Nagy, P. D., and Bujarski, J. J. (1997). Engineering of homologous

recombination hotspots with AU-rich sequences in brome mosaic virus. *J. Virol.* **71,** 3799–3810.

Nagy, P. D., and Simon, A. E. (1997). New insights into the mechanisms of RNA recombination. *Virology* **235,** 1–9.

Passmore, B. K., Sanger, M., Chin, L-S., Falk, B. W., and Bruening, G. (1993). A subviral, independently-replicating RNA stimulates the replication of beet western yellows luteovirus. *Proc. Natl. Acad. Sci. USA* **90,** 10168–10172.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning. A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Tian, T., Rubio, L., Yeh, H-H., Crawford, B., and Falk, B. W. (1999). Lettuce infectious yellows virus: *In vitro* acquisition analysis using partially purified virions and the whitefly *Bemisia tabaci*. *J. Gen. Virol.* **80,** 1111–1117.

Torrance L., Cowan G. H., Sokmen M. A., and Reavy B. (1999). A naturally occurring deleted form of RNA 2 of Potato mop-top virus. *J. Gen. Vir.* **80,** 2211–2215.

White K. A., and Morris, T. J. (1999). Defective and defective interfering RNAs of mopartite plus-strand RNA plant viruses. *In*: "Satellites and Defective Viral RNAs" (P. K. Vogt and A. O. Jackson, Eds.). Springer-Verlag, Berlin.

Yang G., Mawassi, M., Gofman, R., Gafny, R., and Bar-Joseph, M. (1997). Involvement of a subgenomic mRNA in the generation of a variable population of defective citrus tristeza virus molecules. *J. Virol.* **71,** 9800–9802.