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D-RNA Molecules Associated with Subisolates of the VT Strain of Citrus Tristeza Virus which Induce Different Seedling-Yellows Reactions

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Abstract. Citrus tristeza virus (CTV) strains were previously catalogued as seedling-yellows (SY) and non-SY (nSY) types, according to their yellowing and stunting effects on indicator seedlings. Among subisolates of the VT strain, which were selected from chronically infected Alemow plants, there was a correlation between the presence of 2.4-, 2.7- and 4.5-kb D-RNAs, and SY and nSY reactions, respectively. Similarly, plants infected with Mor-T subisolates, which cause SY, contained D-RNAs of 2.6 to 2.8 kb, while nSY subisolates from recovered sour orange tissue contained a major D-RNA of 5.1 kb. Plants harboring the 2.7-kb D-RNA were protected against challenge inoculation with a subisolate harboring the 4.5-kb D-RNA. This study suggests that the nSY reaction results either from the absence of SY gene(s) in the genomes of certain CTV strains or through the suppression of the effects of SY gene(s) by D-RNAs with 5′ parts larger than 4000 nt.

Key words: closteroviridae, interference, plant-virus-interaction

Introduction

Citrus tristeza virus (CTV) (1,2), a member of the closterovirus group and the *Closteroviridae* family (3–7) is an important pathogen, causing considerable economic losses to citrus industries worldwide. Citrus trees infected with CTV display two main types of disease: (i) quick decline of sweet oranges (SwO) (*Citrus sinensis* L.) and of some other species grafted on the sour orange (*C. aurantium*) rootstock (8); and (ii) stem pitting of grapefruit (*C. paradisi*) and pummelo (*C. grandis*) (9). Other manifestations of infection with CTV include the seedling-yellows (SY) reaction (9–12) which is primarily a disease of experimentally inoculated plants but which might also be encountered in the field in top-grafted plants. Seedlings of sour orange, lemon (*C. limon*) and grapefruit become chlorotic and stunted when inoculated with CTV-SY isolates, but no symptoms are elicited when SwO or mandarin (*C. reticulata*) is inoculated (1,13). The CTV-SY phenomenon is one of the long-standing enigmas in citrus virology. The early studies of McClean & van der Planck (9), Fraser (10) and Wallace (11) all suggested a complex aetiology of the CTV-SY disease. There have been reports of spontaneous recovery from SY infection by sour orange plants which initially showed SY symptoms, and of the elimination of the SY causal agent by the passage of SY-inducing CTV subisolates through SY-sensitive citrus hosts such as grapefruit and sour orange (12), which has led to the emergence of non-SY (nSY) isolates. These phenomena have given rise to the hypothesis that the CTV-SY reaction is caused by two separate components: the CTV agent, capable of autonomous replication and responsible for the quick decline and the lime reaction; and a second component, responsible for the SY reaction and able to replicate only in plants harboring the CTV component.

The CTV particles contain a single-component positive-stranded genomic RNA of 19296 nt for the Florida isolate, T36 (14) and of 19226 nt for the VT strain from Israel (15). The genomes of these CTV strains showed considerable sequence deviation

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within the 5′ half, but were found to have similar organization and to encompass 12 ORFs which potentially code for at least 17 protein products. In addition to the large replicative form (RF) RNA molecule, the infected plants contain a nested set of at least nine smaller species of 3′-co-terminal single-and double-stranded subgenomic RNAs (sgRNAs). These sgRNAs correspond to the 3′-terminal ORFs (16,17). Cloning of the VT strain of CTV revealed the presence of several defective (D) RNAs of various sizes, composed of the 5′ and 3′ termini of the genomic RNA with extensive internal deletions, along with the full-length virus. The sizes of the termini varied among species, with minimal lengths of 442 nt and 858 nt from the 3′ and the 5′ termini, respectively, resulting in different sizes of D-RNAs with different junction sites (18,19).

Inoculation of VT on the sour orange indicator resulted in SY symptoms (20). Later infections of sour orange seedlings by grafting with CTV-VT infected Alemow budwood resulted in inconsistent SY reactions; and not all plants showed the SY symptoms. Recently, we selected subisolates of two CTV strains, VT and Mor-T (21), which differed in their SY reactions on sour orange seedlings. The present paper reports the association of D-RNAs with 5′ termini larger then 4000 nt, with VT and Mor-T subisolates which do not elicit the SY reaction. D-RNAs may be involved in the long-standing enigma of the complex etiology of the SY-CTV reaction.

Materials and Methods

Virus Sources and Propagation

The VT strain was originally isolated in 1970 from a SwO cv. Valencia tree grafted on sour orange. The tree showed advanced quick-decline symptoms. Inoculation of sour orange plants with the VT inoculum maintained in sour lime caused typical SY symptoms (20). Later passages of the VT strain from sour lime and Alemow plants to sour orange often resulted in inconsistent SY reactions: not all sour orange seedlings showed the SY symptoms, even when inoculum from a single Alemow plant was used to infect groups of plants from a single seed source (Bar-Joseph, unpublished). Subisolates of CTV-VT (Table 2) were randomly selected in 1994 from chronically infected Alemow plants which had been graft inoculated several years earlier (1988 to 1994) with different passages of this strain. The VT subisolates were maintained in a propagation glass-house with temperatures ranging between 15 and 35°C. The SY reaction was assayed by grafting chip buds from infected Alemow stems onto sour orange seedlings grown in a temperature-controlled glass-house facility with incandescent illumination to complete 20 h of light, and two temperature regimes (TR) of 26/18°C or 29/21°C for the normal and the semi-warm TR, respectively. In both TRs the high and low temperatures were maintained for 8 and 12 h, respectively, and the adjustment from the high daytime to the low night time level and *vice versa* took 2 h. The SY reactions were recorded 8 and 16 weeks after inoculation, for the normal and semi-warm TR, respectively. The Mor-T isolate originated from a declining Minneola tangelo tree (21). The virus was propagated in Alemow and was used to inoculate a group of sour orange seedlings, some of which were inarched with the CTV-tolerant rootstock Go-Tou. Sour orange twigs and leaves showing SY and SY recovery, respectively, were used to infect sour orange and Alemow seedlings.

Northern Blotting

Double-stranded (ds) RNAs were isolated from 5–7 g of Alemow or sour orange tissues, according to Dodds and Bar-Joseph (22). The RNAs were separated by electrophoresis in formamide-formaldehyde denaturating, 1.1% agarose gels, prepared in MOPS buffer, transferred to Hybond N + membranes. The hybridization probes consisted of a 611-bp and a 762-bp cDNA fragment from the 3′ and 5′ ends of CTV-VT genome, respectively (15). The DNA probes were either non-radioactively labeled using the Gene Images Random Prime Labeling Module Kit from Amersham or radioactively labeled with ^{32}P according to Mawassi et al. (17). RNA probes labeled with ^{32}32P-UTP were synthesized, with the Riboprobe System-T7 kit (Promega) according to the manufacturer's instructions, from cDNA fragments of 611 bp and 762 bp of the CTV-VT 3′ and 5′ ends, respectively, cloned in pGEM (Promega).

Antibodies to CTV, ELISA and Western Blotting

Antibodies for ELISA capture were prepared in sheep primed with recombinant CTV coat protein (rCTV-

Table 1. Primers used for preparing cDNA fragments from two CTV-VT subisolates

Primer Code	Sequence (5′ to 3′)	Polarity	Binding Site in CTV-VT Genome
P1	CGGTTGGCAGCAGAAGAC	−	906–917
P2	GATGGACCTATGTTGGCCCCCCATAG	−	19203–19227
P3	CAAATTCACCCGTACCCTCCGGAAATC	+	8–34
P4	AGCGAAGGATATCATCCA	−	692–709
P5	TGGCGCATATGTTAATGC	+	18611–18628
P6	ATGGACCATTGTTGGCCCC	−	19206–19227
P7	GAGGACGCCGACGTGTC	−	2470–2486
P8	CTTCAGTGCTAGCTGTGTTG	−	18377–18397
P9	GCTACGTTCGTCACGTATAC	+	1301–1321
P10	ATGCATATGAGCATTCGACGTCT	+	17260–17276
P11	GTCCGACTTCATAGAGTGTAC	−	17837–17857
P12	GATGGGCACCGGAATGGC	−	738–755
dT14V	ATGACCAATCAGATGGCAC$(T)_{14}$V*		
AD	ATGACCAATCAGATGGCAC		

*V represents either A, C, or G (31).

CP) antigen and boosted with a partially purified CTV preparation. The second antibodies were obtained from egg yolks of chickens immunized with rCTV-CP. The ELISA procedure for CTV viral antigen quantification in different tissues, which were soaked overnight in the antibody-coated ELISA wells, was according to Bar-Joseph et al. (23).

RT-PCR Amplification Restriction and Sequencing Analyses of cDNA Fragments from the VT5 and VT12 Subisolates

The cDNAs were prepared from dsRNA templates of VT5 and VT12, with primers P1 and P2 for the first-strand synthesis, and primers P3–P4 and P5–P6 for nested and direct PCR amplification (Table 1). The cDNA fragments were separated by electrophoresis on 1% agarose gel. The bands were excised from the gel and tested with the restriction enzymes, Sac I and Nsi I (Promega). For sequence analysis we used primers P7 and P9; P10 and P11; P10 and P8 to obtain three cDNA fragments located at ORF1 (1300–2486), ORFs 9 + 10(17260–17857) and ORFs 9 + 10 + 11(17260–18397), respectively. The cDNA fragments were cloned into the pUC 57/T (Fermentas) and sequenced from both sides by using Sequenase Version 2 from USB. Sequences of at least 150 bases were read from the 5′ and 3′ termini of each of the cDNA fragments. The dsRNAs from Alemow plants infected with two Mor-T subisolates, designated #a and #b for SY-recovered and SY-reacting plants, respectively, were poly-A tailed and used for first-strand cDNA synthesis with primer dT14V (Table 1) and for second-strand synthesis with primers P9 and P8, for nested PCR amplification of the viral 3′ and with primers P12 and AD for the viral 5′. The cDNA fragments were separated by electrophoresis on 1% agarose gel, cloned into pUC 57/T (Fermentas). Sequencing from both sides of the 3′ fragments, was performed by using Sequenase Version 2 from USB and the 5′ sequence was determined with the aid of an automatic sequencing machine.

Interference Experiments Between Two VT Subisolates Harboring Different D-RNAs

Two groups of 9 month old Alemow seedlings were graft inoculated at heights of 25–30 and 30 cm, with two chip buds from Alemow plants infected with VT5 or VT12, respectively. Two weeks post-infection (wpi), the plants were pruned and allowed to develop two side branches. Tests for the presence of the specific D-RNAs were conducted after 10 wpi. The plants where challenged, 20 wpi by top grafting with stems infected with the reciprocal subisolates. Two lateral buds were allowed to sprout from each of the protected plants and leaf and stem bark tissue were tested for the presence of D-RNAs by Northern blotting.

Results

Biological Characterization of VT and Mor-T Subisolates

Table 2 summarizes the differing SY reactions of sour orange seedlings graft-inoculated with eleven CTV-VT subisolates. Plants infected with VT subisolates 1, 5–10 and 13 showed typical SY symptoms, including short internodes and thorns and small leaves (not shown). Plants infected with VT subisolates 3, 4 and 12 developed similarly to non-infected plants or, occasionally, showed a few smaller leaves and were defined as nSY. The root systems of the SY-reacting sour orange plants were especially damaged and reduced in weight, compared with the control non-infected or the nSY plants (not shown). Among the plants with SY symptoms which were maintained under the high TRs, a previously unrecorded symptom, midday wilting, was noticed 5–6 weeks after infection (not shown).

The virus titers estimated by ELISA, of sour orange plants maintained under two TRs after infection with various VT subisolates are presented in Table 2. The virus titers under both TRs were significantly higher for the nSY than for the SY subisolates (Table 2). The virus titers of the apical parts (Table 2) and of the root and rootlets (not shown) of plants infected with VT subisolates 1, 7, 8 and 10, which showed wilting and SY reactions under the higher TR, were either similar to or only slightly above those of the healthy controls. Moreover, grafting of apical sour orange leaves with severe SY symptoms on Alemow plants resulted in 4/18 of virus-free plants. Contrary to these profound differences among the sour orange plants, the ELISA titers of Alemow plants infected with VT5 or VT12 were similar (not shown).

Infection of the Mor-T#o isolate on sour orange plants caused a severe SY reaction in all ($n = 6$) infected plants. These symptoms persisted for at least 18 mo, except for the plants which were inarched with the Go Tou rootstock. In the inarched plants, apparently recovered twigs with larger leaves were observed after 1–2 years. ELISA assays showed higher titers for the SY-recovered (SYr) parts compared with the SY leaves (not shown). The sour orange plants ($n = 4$) infected with stem pieces from the SY part showed typical SY symptoms. The sour orange plants infected with SYr leaves showed different symptoms, with 2, 2 and 1 plants showing nSY, SY and intermediate reactions respectively. Virus titers estimated by ELISA, in sour orange petioles were > 1.0 or < 0.2 OD/405 nm for plants with Mor-T nSY and SY subisolates, respectively.

Variation in D-RNA Composition of Plants Infected with VT and Mor-T Subisolates

Hybridization with an approximately 0.7-kb cDNA probe or riboprobe from the 5′ end of the VT genome with dsRNA extracts from Alemow plants, revealed the presence of the large RF and the low-molecular-weight tristeza 5′-corresponding RNA molecules (LMT) (18) and D-RNAs. VT-subisolates 6–8 and 13, and 1, 5, 9, 10, with apparently similar SY reactions, showed the presence of two types of D-RNAs, of 2.4 kb and 2.7 kb, respectively. The three nSY subisolates (3,4 and 12) showed the presence of a 4.5-kb D-RNA (Fig. 1A and Table 2). The hybridization patterns of dsRNAs extracted from sour orange seedlings infected with VT subisolates VT12 (nSY)

Table 2. A catalog of the seedling yellows (SY) reactions, the associated D-RNAs and the ELISA reaction of sour orange plants infected with different subisolates of the VT strain of citrus tristeza virus, under two temperature regimes

VT Subisolate	1	3	4	5	6	7	8	9	10	12	13	Control
Reaction	SY	nSY	nSY	SY	SY	SY	SY	SY	SY	nSY	SY	nSY
DI RNAs (kb)	2.7	4.5	4.5	2.7	2.4	2.4	2.7	2.7	2.7	4.5	2.4	/
ELISA (OD 405 nm)												
TR 26/18°C	ND	2.0*/1.5	2.1	0.44/0.48	0.7/0/0.32	0.47/0	0.04	0/0.66/1.04/0	0.49/0.14/0.32	1.45/2.05	ND	0/0.05
TR 29/21°C	0	2.0**	2.02	0.70	0.66	0.06	0.11	0.30	0	2.0	0.83	0

* Each figure represents a reading of upper stem tissue from a different plant.

** Average of two sour orange plants. ND - not determined, TR - temperature regime.

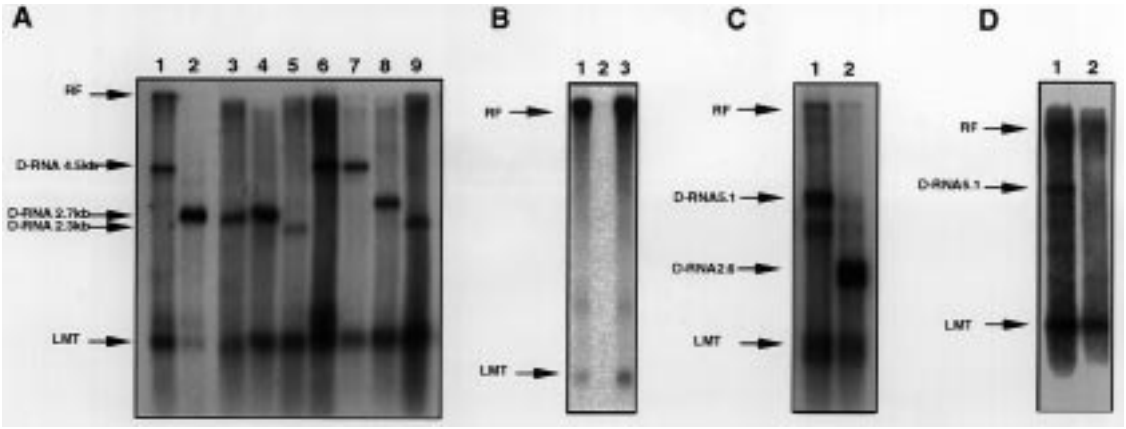

Fig. 1. Northern blot hybridization of dsRNA extracts from: A. Alemow plants infected with different subisolates of the VT strain of CTV, using a non-radioactive cDNA probe from the distal 5′ end of the CTV genome (Lanes 1 to 9 represent subisolates 12, 5, 9, 10, 6, 4, 3, 1 and 13, respectively. B. Sour orange plants infected with VT subisolates 12 and 5 (lanes 1 and 2, respectively) and with inoculum from an Alemow plant which was included in the interference experiments, showed a dominant 4.5-kb and a minor 2.7-kb D-RNAs (lane 3). C. Alemow plants inoculated with Mor-T subisolates #a1 (nSY) and #b1 (SY) (lanes 1 and 2, respectively) and with subisolates #e1 (nSY) and #c1 (SY) (D. Lanes 1 and 2, respectively).

and VT5 (SY) are shown in Fig. 2B. Only weak or no hybridization signals of genomic and/or defective RNA could be located in bark and leaves from the sour orange plant which showed severe SY compared with those from the nSY plant.

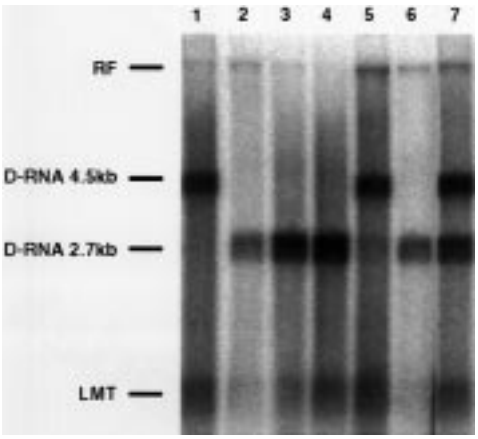

Fig. 2. Northern blot analyses of interference between two CTV-VT subisolates containing different D-RNAs. Lanes 1 and 2 show hybridization with dsRNA extracts from Alemow plants infected with VT subisolates 12 and 5. Lanes 3, 4 and 6 represent plants protected with VT5 and challenge inoculated with VT12, at 41 weeks post challenge inoculation. Lanes 5 and 7 represent plants protected with VT12 and challenged with VT5. Note the absence of the D-RNA harbored by the challenging subisolate in plants protected with the smaller (2.7 kb) D-RNA (lanes 3, 4 and 6) and the considerably larger amounts of the challenging (2.7 kb) D-RNA in lane 7 than in lane 5.

Hybridization of dsRNAs from Alemow plants inoculated with Mor-T subisolates #a1 (nSY) and #b1 (SY), showed the presence of major large (ca. 5.1 kb) and small (ca. 2.6 kb) D-RNAs respectively (Fig. 1C). One of the SY Mor-T subisolates #c1 showed only weak bands of D-RNA molecules compared with the nSY subisolate #e1, which showed the major D-RNA of ca. 5.1 kb (Fig. 1D, lane 1). Sequence analyses revealed that SY subisolate #b1 contained two D-RNAs of 2634 and 2815 nt with junctions of their 5′ termini located at positions 1772 and 1521, whereas the nSY subisolate #a1, contained a major D-RNA of 5125 nt, with the junction of the 5′ terminus located at position 4376 (Fig. 3B).

RT-PCR and Sequencing Analyses of VT Subisolates

The hybridization with the VT 5′ probe with different VT and Mor-T subisolates suggested a close relationship between their genomic RNAs. In order to examine the genomic composition of the VT5 (SY) and the VT12 (nSY) subisolates, we compared the sequences of termini of their genomes by means of nested RT-PCR and sequencing analyses. Primers P1 and P2 were used for first-strand cDNA synthesis and primers P3, P4, P5 and P6 (Table 1) for PCR

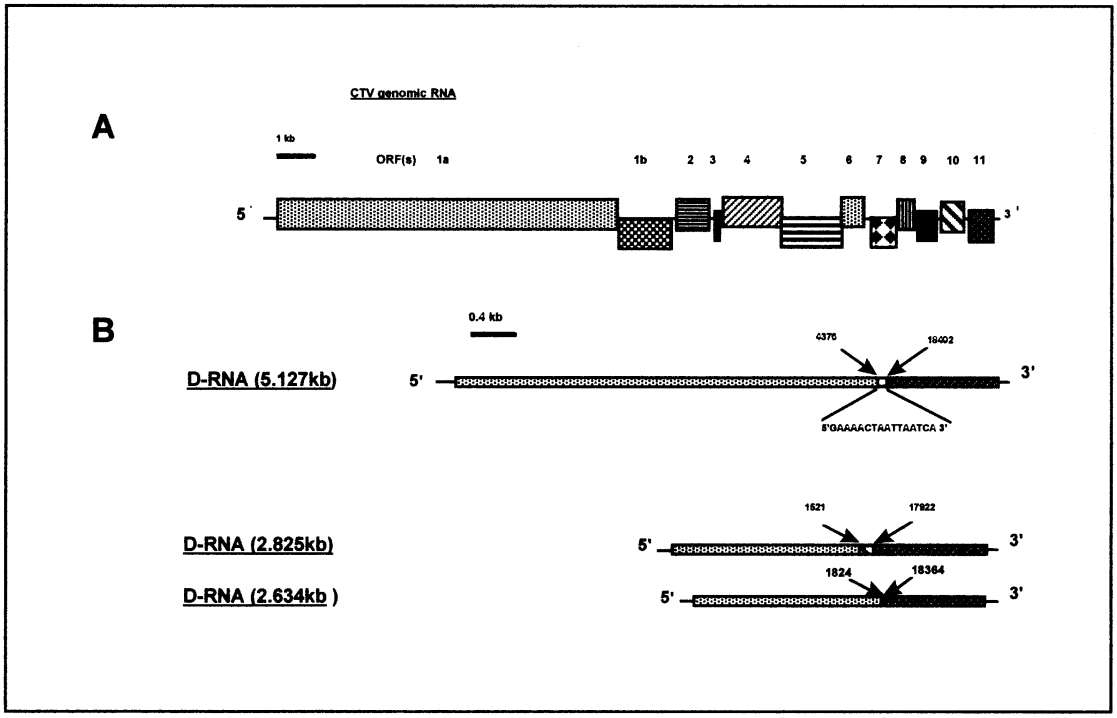

Fig. 3. A. Diagram of the genomic organization of CTV. B.
The structure of the three CTV-Mor-T-D-RNAs compared with the CTV genomic RNA. Note the presence of a 16-nt sequence apparently of non-viral origin at the junction site of the 5.1-kb D-RNA.

amplification. The resulting cDNA fragments for both subisolates gave the expected lengths for the 5′ (8-709) and 3′ (18611–19227) ends of their genome. Restriction analysis of these products with SacI and NsiI gave restriction fragments of identical size (not shown). Sequence analyses of internal regions, at least 150 nt in length, of three cDNA fragments positioned at different regions of the VT genome (positions 1300–2486, 17260–17857 and 17260–18397) did not reveal any sequence deviation between the products obtained from the dsRNAs of the VT5 (SY) and the VT12 (nSY) subisolates (not shown).

Interference Between Small and Large D-RNAs

The possibility of interference between two VT subisolates, VT5 and VT12, harboring the 2.7- and the 4.5-kb D-RNAs, respectively, was tested in Alemow plants. The dsRNAs from plants which had first received a protective inoculation with either the VT5 or the VT12 subisolate and were later challenged by top grafting with the reciprocal subisolate, were hybridized with the 5′-specific probe. At 18 weeks post challenge inoculation (wpci), the basal parts of each combination had predominantly the D-RNAs of the protective isolate (not shown). Later tests at 41 wpci showed only the 2.7-kb D-RNA in the basal parts of plants protected with VT5 (Fig. 2, lanes 3, 4 and 6). Plants protected with VT12 showed the presence of either a conspicuous or a weak band of the challenging 2.7-kb D-RNA in addition to the 4.5-kb D-RNA (Fig. 2, lanes 5 and 7, respectively).

Sour orange seedlings infected with Alemow tissues from the interference experiments, which harbored both the 2.7- and the 4.5-kb D-RNAs, showed a range of SY reactions. Plants inoculated with tissue predominantly harboring the 2.7-kb D-RNA showed typical SY reactions, gave only low ELISA titers and contained negligible amounts of viral dsRNAs (not shown), whereas plants infected with a dominant population of the 4.5-kb D-RNA gave an intermediate (nSY/SY) reaction and showed

stronger ELISA titers and higher dsRNAs concentrations (Fig. 2B, lane 3).

Discussion

Selection and Characterization of VT and Recovered Mor-T Subisolates

Biological and molecular characterization of 11 VT subisolates, which were randomly selected from chronically infected Alemow plants, revealed the presence of eight SY and three nSY subisolates. The VT subisolates caused similar symptoms and comparable ELISA reactions in Alemow plants (not shown). The virus titers were considerably higher in sour orange plants infected with nSY than in those infected with SY subisolates. These differences were consistent among plants which were maintained under different TRs (Table 2). Low virus titers or the absence of virus (indicated by negative reactions on indicator plants) in sour orange leaves and roots showing severe SY symptoms, suggest the possibility that the SY isolates emit a long-distance signal for a hypersensitive reaction. A similar situation has been previously observed in mature trees infected with CTV-Mor-T, where the collapse of the sweet orange/sour orange combination often preceded the spread and redistribution of the virus towards the upper parts of the infected trees (21).

The profound differences among the sour orange reactions to the various VT-subisolates were associated with the presence of different major D-RNAs. The nSY subisolates, 3, 4 and 12, showed the presence of a major band of 4.5-kb D-RNA, whereas the eight SY subisolates, 1, 5–10 and 13, showed the presence of two smaller D-RNAs of 2.4 and 2.7 kb, with no apparent difference in the intensity of the SY reaction to subisolates which contained either of the smaller D-RNAs. Infection of sour orange with tissues from Alemow plants concomitantly infected with mixtures of VT5 and VT12 resulted in reactions ranging from SY to nSY, with virus titers depending on the relative concentrations of the 2.7- and 4.5-D-RNAs in the inoculum source.

Previously, we showed variations in the presence of the 2.4-, 2.7- and 4.5-kb D-RNAs in Alemow plants infected with budwood from a single VT-infected source plant (18). Differences in D-RNA populations might have accounted for the previously noticed inconsistencies in the SY reaction of sour orange plants infected with VT strain (Bar-Joseph, unpublished). The selection of VT subisolates which show a more consistent SY reaction was correlated with the presence of a major type of D-RNA (Table 2). One probable reason for obtaining apparently stable subisolates was their selection from chronically infected plants ($>$ 2–3 years after inoculation) at a time when a single type of D-RNA had become dominant.

Interference Between VT Subisolates Harboring Different D-RNAs

After 18 wpci the basal parts of the Alemow plants which had been infected with either of the VT subisolates showed only the D-RNAs of their corresponding protective subisolates. Later tests conducted at 41 wpci showed continued protection, with the 2.7-kb D-RNA, and mixed populations of the 2.7- and the 4.5-kb D-RNAs in the basal parts of plants protected with the 2.7- and 4.5-kb D-RNA, respectively (Fig. 3). These results are probably indicative of a competitive situation whereas the smaller D-RNA were more effectively transcribed than the larger one. It has been generally thought that smaller D-RNAs can replicate more efficiently than larger D-RNAs (24) and co-transfection experiments with different sizes of D-RNAs have demonstrated that the replication of the larger D-RNA was strongly inhibited by replication of the smaller genomic coronavirus D-RNA (25).

D-RNAs and the Enigma of CTV-SY

D-RNAs of 4.5 and 5.1 kb, which share at least 4043 nt of the 5′ end of the CTV genome were correlated with the amelioration of the SY symptoms by certain VT and Mor-T subisolates. The SY reaction was observed with a Mor-T subisolate which contained low concentrations of D-RNAs (Fig. 1D, lane 2) and with several VT and Mor-T subisolates which harbored D-RNAs of 2.4 and 2.8 kb. Sequence analyses of VT-associated D-RNAs showed considerable differences among their genomic compositions. The 2.4- to 2.8-kb D-RNAs showed 5′ genomic termini of up to 1926 nt and not less than 940 nt for the 3′ part (for details see Fig. 2 in (5)) while the 4.5-kb D-RNA showed a short 3′ end of 440 nt and a long (4043 nt) 5′ end. Similarly, the two D-RNAs, from the

Mor-T- SY subisolate had 5′ end which extended to 1772 nt and a 3′ end of at least 862 nt, whereas the D-RNA of the nSY subisolate was composed of a large 5′ part of 4376 nt and a 3′ end of 736 nt (Fig. 3). A 16-nt sequence, 5′-GAAAACTAATTTATCA, with no homology to other regions of the CTV genome was found at the junction site (Fig. 3). A different short sequence, probably of host origin had previously been observed at the junction site of the 2.4-kb D-RNA (19).

The CTV-SY phenomenon is one of the long-standing enigmas in citrus virology. The finding that both the CTV and the CTV-SY diseases could be transferred by mechanical inoculation of preparations of CTV particles (26,27) raised the question (28) of the dual-component theory of the causal agent of the CTV-SY disease (12). Dodds et al. (29) noted an association between two dsRNAs of about 0.8 and 2.7 kb and SwO trees infected with SY subisolates. Molecular characterization associated the 0.8-kbp dsRNA with the replicative subgenomic RNA coding for ORF11 (940 nt) (16,17,30,31) and hybridization with a 3′-specific probe did not reveal quantitative differences in the amounts of the 0.8-kbp dsRNAs from SY and nSY plants (not shown). Moreover, low-molecular-weight D-RNAs of 2.4 kb were located in Alemow infected with nSY isolates Mik-T and Ach-T (32) (not shown).

CTV isolates were previously classified by a variety of criteria into subisolates which differed in host reactions, vector transmissibility and dsRNAs patterns (29,33–37). The variability among subisolates was considered as an indication of the high frequency of mixed CTV infections.

D-RNAs were previously implicated in the variability between the dsRNA patterns of parental isolates and their subisolates (5,35) and the present findings indicate a correlation between certain D-RNAs and host reactions, and support a working hypothesis that the nSY reaction results either from the absence of SY gene(s) or through the suppression of their effects by D-RNAs with 5′ parts larger than 4000 nt.

The genomic and D-RNA fragments of the two differentially reacting VT subisolates were found to show a complete sequence identity. Nevertheless, the possibility that a minimal sequence deviation between other parts of their genomes is involved in these biological differences cannot at the present be completely ruled out. Moreover, the question of the mechanism that causes SY symptoms in sour orange tissues, which contain only low concentrations of viruses or D-RNA remains to be answered.

D-RNAs have been isolated from a broad spectrum of animal viruses and, more recently, also from a large number of plant viruses (for recent reviews, see (38)). Different D-RNAs have previously been reported to have different effects on disease expression: while D-RNAs of tombusviruses had attenuating effects on infection (39,40), the D-RNAs associated with the turnip crinkle virus tended to increase the severity of symptoms (41) and the D-RNAs associated with broad bean mottle virus had no effect on some host plants but intensified the severity of symptoms in others (42). The correlation between the SY reactions of sour orange seedlings and the genomic composition of the D-RNAs in the Alemow inoculum, support the notion that the host type is a major determinant of the biological effects of D-RNAs (43).

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