A strong-stop DNA in rice plants infected with rice tungro bacilliform virus

Yiming Bao and Roger Hull*

Department of Virus Research, John Innes Institute, John Innes Centre for Plant Science Research, Colney Lane, Norwich NR4 7UH, U.K.

A virus-specific small nucleic acid (strong-stop DNA) was identified in rice plants infected with rice tungro bacilliform virus, but not in the virus particles. This nucleic acid was shown to consist of about 595 deoxyribonucleotides with about 70 ribonucleotides covalently linked at the 5' end. Hybridization with sequence-specific oligonucleotides showed that the ribonucleotides were from the plant cytoplasmic tRNA\textsuperscript{\textasciitilde et} sequence. PCR analysis detected hairpin structures at the 3' end of the DNA.

Introduction

The pararetroviruses (Hull & Will, 1989; Temin, 1989) involve reverse transcription in their replication. There are two such groups among plant viruses, the caulimoviruses (Harrison et al., 1971) which have isometric particles of which most are transmitted by aphids, and the badnaviruses (Lockhart, 1990) which have bacilliform particles of which most are transmitted by mealybugs. Rice tungro bacilliform virus (RTBV) is a proposed member of the latter group (Jones et al., 1991). Members of both groups have several common features in their genome structures which indicate similarities in the mechanism of genome replication. All of them have circular double-stranded genomes with discontinuities at specific sites on each strand. The genomes are transcribed asymmetrically and encode, or putatively encode, reverse transcriptase. The replication mechanism is well understood for the type member of the caulimovirus group, cauliflower mosaic virus (CaMV) but there is little published information for badnaviruses.

In the replication model proposed for CaMV (Hull & Covey, 1983a, b; Pleiffer & Hohn, 1983), the viral DNA with discontinuities enters the nucleus where it becomes a circular, covalently closed DNA and forms a minichromosome. This is the template for transcription producing the 35S RNA (Menissier et al., 1983; Olszewski et al., 1982) which has a terminal direct repeat of 180 nucleotides (nt) (Covey et al., 1981; Guilley et al., 1983). In the cytoplasm, the 3' end of the host cell tRNA\textsuperscript{\textasciitilde et} anneals to 15 complementary nucleotides located approximately 600 nt from the 5' end of the 35S RNA, and serves as a primer for reverse transcription towards the 5' end until the template is exhausted. At this stage, a minus-strand DNA molecule of about 600 nt covalently linked to the tRNA primer would be produced (Condit & Meagher, 1983; Covey et al., 1983; Guilley et al., 1983; Turner & Covey, 1984). This DNA molecule is similar to the strong-stop DNA of retroviruses (Varmus & Swanstrom, 1982). As the 35S RNA is copied into strong-stop DNA, RNase H degrades the template enabling a template switch to the 3' end of the 35S RNA, due to the terminal repeat.

The double-stranded circular genome of RTBV has two discontinuities which are located at specific sites, one on each strand (Jones et al., 1991). The full-length genomic clones of 8 kbp from three Philippine isolates, one of which was shown to be infectious (Dasgupta et al., 1991), have been sequenced (Hay et al., 1991; Qu et al., 1991; Kano et al., 1992). The two discontinuities in RTBV DNA were mapped (Bao & Hull, 1992), and a potential primer binding site for tRNA\textsuperscript{\textasciitilde et} similar to that of CaMV was found near the minus-strand discontinuity. This suggests that tRNA\textsuperscript{\textasciitilde et} serves as the primer for minus-strand DNA synthesis but does not prove it. Qu et al. (1991) identified a more-than-genome length RNA transcript for RTBV, mapping the 5' end to nt 7354 and the 3' terminus to nt 7620.

Turner & Covey (1984) characterized the strong-stop DNA (which they termed sa-DNA) from CaMV-infected turnip tissues. They showed that it was of the expected size for strong-stop DNA and had RNA with properties consistent with being tRNA\textsuperscript{\textasciitilde et} covalently linked to its 5' end. Virus-specific DNA molecules with hairpin structures were also found in CaMV-infected turnip plants (Covey & Turner, 1986; Turner & Covey, 1988). Here, we report on the identification and characterization of a strong-stop DNA for RTBV and compare it with that of...
CaMV. We show that it has tRNA<sub>ret</sub> covalently linked to its 5' end and that at least some of the molecules have a hairpin structure at the 3' end.

**Methods**

**Nucleic acid preparation.** Two grams of RTBV-agroinfected rice leaves (Dasgupta et al., 1991) or CaMV (Cabb B-Jl)-infected turnip leaves were cut into pieces and ground in a mortar after freezing with liquid N<sub>2</sub>. The leaf powder was then added to 15 ml of extraction buffer consisting of 100 mm-Tris–HCl pH 8.0, 50 mm-EDTA pH 8.0, 500 mm-NaCl and 1:25% SDS preincubated at 65 °C, and incubated at 65 °C for 15 min. Five ml of 5-m-potassium acetate was added and the mixture was shaken vigorously for 2 min and then placed on ice for 20 min. The mixture was centrifuged at 3000 r.p.m. at 4 °C in a Sorvall RT6000 centrifuge for 20 min, the supernatant was transferred to another tube and extracted with chloroform–isoamyl alcohol (24:1, v/v). Two-thirds volume of isopropanol was added to precipitate the nucleic acid. The pellet obtained after centrifugation was dried under vacuum and resuspended in 1 ml of distilled water.

**Nuclease treatment.** RNase A was added to the total nucleic acid to a final concentration of 20 µg/ml and the mixture was incubated at 37 °C for 1 h. The nucleic acid was used directly for non-denaturing gel electrophoresis.

**m<sup>2</sup>G cleavage by aniline hydrochloride.** Thirty-seven µl of total nucleic acid was mixed with 1 µl of 2 M-NaOH and 2 µl of 2 M-EDTA (pH 8.0) and incubated at 20 °C for 15 min. Two µl of 2 M-acetic acid was added to adjust the pH of the solution to 5. Forty µl of 0.3 M-aniline hydrochloride, dissolved in 0.2 M-sodium acetate (pH 4.5), was added, the mixture was incubated at 37 °C for 4 h and the nucleic acid was then precipitated by the addition of 2.5 volumes of ethanol. The pellet was dried under vacuum and resuspended in 30 µl of distilled water.

**Alkali treatment.** Total nucleic acid was treated in 0.2 M-NaOH at 65 °C for 1 h. The solution was neutralized by adding a one-tenth volume of 3 M-sodium acetate (pH 4.8) and nucleic acid was precipitated and resuspended as described above.

**Gel electrophoresis.** Non-denaturing gels were 0.7% agarose in 1 x Tris–borate–EDTA buffer (Sambrook et al., 1989). A gel slice containing nucleic acid of the size in question was cut and nucleic acid was extracted by the liquid nitrogen freeze–squeeze method (Gaastra & Jorgensen, 1984). For denaturing gels, 3 µl of total nucleic acid was mixed with 2 µl of 0.1 M-sodium phosphate buffer pH 7.0, 3:3 µl of glyoxal (deionized) and 11:7 µl of formamide (deionized) and incubated at 55 °C for 20 min. The samples were loaded in a 25% low melting temperature agarose/0.5% agarose gel in 1 x Tris-acetate-EDTA buffer (Sambrook et al., 1989) and the DNA was blotted onto nylon filters (Hybond-N, Amersham) with 20 x SSC directly after electrophoresis and fixed to the filter in a u.v. cross-linker (Stratagene). The filter was soaked in 20 x Tris–HCL pH 8.0 at 65 °C for 10 min to remove the glyoxal (Sambrook et al., 1989). For non-denaturing gels, DNA in gel was depurinated, denatured, neutralized and transferred to nylon filters as described by Sambrook et al. (1989). The filters were prehybridized in 6 x SSC, 5 x Denhardt’s reagent, 0.5% SDS and 0.2 mg/ml calf thymus DNA (Sigma) for 2 h at 65 °C. Random primer-labelled plasmids and DNA fragments or 5’ end-labelled oligonucleotides were added to the prehybridization solution, and hybridization was carried out for 8 to 16 h. The filters were washed twice with 6 x SSC for 30 min each. The hybridization and washing temperatures were 42 °C for V647, 37 °C and 50 °C for V688, 39 °C for V750, and 65 °C for pJH112, probes A, B and C. To reprobe the filter, the probe was stripped by soaking the filter in 0.1% SDS heated to 100 °C and incubating at room temperature for 30 min.

**PCR of strong-stop DNA and sequencing.** Primers were used to amplify strong-stop DNA were V406 (7457-AAGCTCCTCTCTGTCCTCT-7439, complementary to the plus-strand of the RTBV genome) and V418 (7457-AACCTGGCTCTGATACCA-1, complementary to the plus-strand of the RTBV genome). PCR amplification was performed in 1 x Taq polymerase buffer, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 5 units of Taq DNA polymerase with DNA template (nucleic acid obtained from 0.5 to 1.0 kb region of total nucleic acid in agarose gel) and primers in a total volume of 100 µl. The PCR program was 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C for 35 cycles. One µl of T4 DNA polymerase (10 units, BRL) was added to the reaction after PCR and the mixture was incubated at 37 °C for 1 h. The PCR product was electrophoresed in a 1.2% agarose gel in 1 x TAE buffer and stained with ethidium bromide. A band of about 580 bp was cut from the gel, the DNA was recovered by the liquid nitrogen freeze–squeeze method (Gaasta & Jorgensen, 1984) and then ligated into Smal-digested plBluescript (Stratagene). Recombinant plasmid mini-preparations were made by the boiling method (Sambrook et al., 1989) and the DNA was used for dsDNA sequencing (Hsiao, 1991).

**Results and Discussion**

Southern blots of native total nucleic acid from RTBV-infected rice plants were probed with labelled DNA from different regions of the RTBV genome (Fig. 1). The probe of the whole RTBV genome revealed four major discrete bands and a diffuse band of about 600 nt (Fig. 1, lane 1). These bands were not found in nucleic acid from healthy plants (Fig. 1, lane 3). The diffuse band was detected with probe C (Fig. 1, lane 13) but not with probes A or B (Fig. 1, lanes 5 and 9). Treatment of the total nucleic acid with RNase A did not affect the diffuse band (Fig. 1, lanes 2 and 14). The electrophoresis conditions used here are not capable of resolving differences in migration shifts in this size range. More precise results were obtained under appropriate conditions as shown in Fig. 3.) The diffuse band was not detected after DNase I treatment (data not shown). This
indicated that it was all or mostly DNA. The position on the genome to which this DNA mapped and its size suggested that this DNA was similar to the CaMV sa-DNA or strong-stop DNA. This DNA is termed strong-stop DNA of RTBV.

Turner & Covey (1984) reported that CaMV sa-DNA was virion-associated, but in RTBV, the strong-stop DNA was not detected in nucleic acid preparations from partially purified virus (Fig. 1, lanes 4 and 16). This may indicate that the replicative complexes undergo encapsidation at a step after the switch from the 5' end to the 3' end of the template.

To characterize the RTBV strong-stop DNA further, denaturing agarose gel electrophoresis was carried out (see Methods) with nucleic acid from CaMV-infected turnip plants being used as control (Fig. 3, lanes 1 and 4). The sizes of the denatured nucleic acids were 665 and 650 bases for RTBV and CaMV respectively.

Aniline hydrochloride treatment cleaves nucleic acids at m'G residues (Simsek et al., 1973); such a residue was found at nt 46 in plant cytoplasmic tRNA^Met (Sprinzl et al., 1989). Alkali treatment removes ribonucleotides but not deoxyribonucleotides. After treatment with aniline hydrochloride the virus-specific nucleic acids were estimated to be 625 and 610 bases for RTBV and CaMV respectively and after alkali treatment 595 and 580 bases respectively.

Based on the sequence of the bean cytoplasmic tRNA^Met (Canaday et al., 1980), three oligonucleotides were designed to be complementary to the three-loop region (Fig. 2). These oligonucleotides were 5'-labelled and were used to probe the same filter depicted in Fig. 3. The result is shown in Fig. 4. V750 hybridized to both the untreated and aniline hydrochloride-treated strong-stop DNA (Fig. 4b, lanes 1, 2, 4 and 5) whereas V688 hybridized only to the untreated DNA (Fig. 4a, lanes 1 and 4). This is in accord with the aniline hydrochloride cutting the tRNA at the m'G, releasing the 5' portion including the loop complementary to V688. It also indicates that the RNA was attached to the 5' end of the DNA. From Fig. 4, it can be seen that the probes hybridized to the CaMV samples (Fig. 4b, lanes 4 and 5) more than to the RTBV samples (Fig. 4b, lanes 1 and 2). This is probably because there was more CaMV than RTBV DNA on the blot (see Fig. 3 which was probed with the same amount of radioactivity in probes of the same specific activity). Oligonucleotide V647 failed to
hybridize to either strong-stop DNA (data not shown), most likely owing to the large differences reported for hybridization efficiencies to different regions of the tRNAs (Kumazawa et al., 1992).

From these results, we conclude that the strong-stop DNA of RTBV consists of 595 nt of DNA with about 70 ribonucleotides covalently linked to it. The initiation site of the major RTBV transcript was mapped to nt 7354 (Qu et al., 1991) and thus the strong-stop DNA would be expected to be 648 nt which is about 50 nt larger than we determined. In the control CaMV, our estimate of 580 nt of DNA is close to the 589 nt indicated from the data of Guilley et al. (1982). To determine the cause of this discrepancy between the measurement and estimate of the size of RTBV strong-stop DNA, the 5' end of the transcript of RTBV which we used was mapped by the RNase protection method (Y. Bao & R. Hull, unpublished). It was mapped to nt 7404 and 7405, which would give a strong-stop DNA of 597 or 598 nt in length which is very close to the value of 595 nt we obtained. In both RTBV and CaMV, about 70 ribonucleotides were linked to strong stop-DNA which almost matches the expected length of tRNA_Met. For both strong-stop DNAs, the position of m^7G was about 30 nt from the 3' end of tRNA_Met, which is consistent with the published sequences of plant cytoplasmic tRNA_Met's (Sprinzl et al., 1989).

In CaMV, the strong-stop DNA has been reported to form a hairpin structure at its 3' end (Marco & Howell, 1984). In an experiment to investigate the 3' end of RTBV strong-stop DNA, terminal transferase was used to add poly(G) to the 3' end. PCR was then performed using oligo(dC) and another oligonucleotide, V418. However nothing could be amplified. To obtain sequences in the hairpin region, oligonucleotides V406 and V418 were used to amplify the strong-stop DNA. Nucleic acid obtained from the 0.5 to 1.0 kbp region of total nucleic acid in agarose gel, which contains the strong-stop DNA, was used as the template. A fragment of about 580 bp was obtained after PCR and was cloned into pBluescript. Several clones were obtained and two of them were partially sequenced from the V406 end.

Fig. 3. Southern blot of 2.5% low melting agarose-0.5% agarose gel electrophoresis of strong-stop DNA in 1 x TAE buffer. All the samples were treated with glyoxal and formamide. Lane M, size markers with sizes (bp) shown on the left-hand side; lanes 1 to 3, total DNA from RTBV-agroinfected rice; lanes 4 to 6, total DNA from CaMV-infected turnip. Lanes 2 and 5, total DNAs were treated with aniline hydrochloride; lanes 3 and 6, total DNAs were treated with NaOH. The probes were 32P-labelled pJlIS2 and a full-length clone of CaMV.

Fig. 4. Southern blot hybridization using 32P-end-labelled oligonucleotides V688 (a) and V750 (b). The filter is the same as in Fig. 3.
Two slightly different sequences were found (Fig. 5, bottom line in b and c). By comparison with the RTBV genomic sequence, it was found that V406 uses the plus-strand complementary to the strong-stop DNA as template for a certain distance and then strand-switches to the minus-strand of the strong-stop DNA as template (Fig. 5a, thick line). The ability of V406 to use the plus-strand complementary to the strong-stop DNA as the template is strongly indicative of the existence of a hairpin structure at the 3' end of the strong-stop DNA. There are at least two possible models for strand-switching. In the first, the PCR product might reflect strand-switching during reverse transcription forming the strong-stop DNA. In the second, extensions from V406 could strand-switch because, once V406 annealed to the plus-strand of the strong-stop DNA, stable secondary structures were formed in the rest of the 3' end region of the strong-stop DNA during the cooling-down step of PCR. Two inverted repeat sequences were found near the strand-switch region (shaded lines in Fig. 5a) which can form the stem structures shown in Fig. 5(b, c). These, together with the other stable secondary structures, could force V406 to strand-switch to the minus-strand of the strong-stop DNA. In the second model, one would expect the hairpin molecules of the strong-stop DNA to be longer than normal strong-stop DNA and therefore move slower than those non-hairpin molecules in the denaturing gel. They were not readily detected in our experiments. This suggests either that the first model is correct or that only a small amount of the full-length strong-stop DNA has the hairpin structure and would be detected only by PCR.

Priming by tRNA in reverse transcription is considered to be a common feature in pararetroviruses except for hepadnaviruses (Mason et al., 1987). However in many cases, definitive proof that a tRNA is involved is lacking, and the evidence that is cited is the complementarity of the sequence to that of the 3' end of specific tRNAs. In this paper we have shown that the RTBV strong-stop DNA has an RNA attached to the 5' end with many properties consistent with it being a tRNA.

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References


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