

## Evidence for a Latent Viruslike Agent in Cassava

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## ABSTRACT

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Several species of double-stranded RNA (dsRNA) were found to occur in the cassava (*Manihot esculenta*) clone Secundina, which was thought to be virus-free. A large dsRNA molecule ( $6.6 \times 10^6 M_r$  = relative molecular mass) (L-dsRNA) was shown to be graft, but not mechanically transmitted and occurred primarily in a nuclear-rich fraction isolated from dsRNA containing Secundina plants. Viruslike particles were not detected in plants containing the L-dsRNA using a variety of purification procedures.

L-dsRNA was also found in two non-Secundina *M. esculenta* introductions. Smaller dsRNAs (S-dsRNAs), ranging mainly between 0.61 and  $0.41 \times 10^6 M_r$ , were also detected in some Secundina plants containing the L-dsRNA. The S-dsRNAs were not transmissible by mechanical inoculation or grafting. All dsRNAs could be eliminated by meristem-tip culture of infected plants. DsRNA analysis is recommended for indexing cassava for this latent viruslike agent.

Cassava (*Manihot esculenta* Crantz) is a vegetatively propagated crop commonly grown in the tropics. Many viruslike diseases of cassava have been reported to occur in Latin America but the causal agents for most of these diseases remain unknown (13). Cassava common mosaic virus (CCMV) (3) (a potyvirus) is the only Latin American cassava virus that has been characterized. As a result, virus specific antisera are not available so virus indexing programs are often based on mechanical and graft inoculations to indicator plants. Double-stranded RNA (dsRNA) analysis could be used to increase the speed and accuracy of cassava virus detection if unique dsRNA species could be associated with diseased plants. DsRNA analysis has been used to suggest the viral etiology of several viruslike diseases without the detection of virus particles (7,9,15). This allows for possible disease diagnosis without bioassay or serology.

To index cassava viruslike diseases using dsRNA analysis, we first needed to determine if dsRNA species occurred in plants that were thought to be virus-free. In this report, we describe a latent viruslike agent that was present in the cassava virus indicator clone Secundina (13). Detection of the agent was rapid and reliable using established dsRNA procedures.

## MATERIALS AND METHODS

Woody stem cuttings of Secundina were provided by the Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia. Cuttings of *Manihot websterae* Rogers & Appan were obtained from the Centro Internacional de Mejoramiento de Maiz y Trigo (CIMMYT), Juarez, Mexico. CCMV-infected cuttings of *M. esculenta* were obtained from Ingreso Agricultura Druetta, Formosa, Argentina. CCMV-infected Secundina clones were produced by grafting CCMV-infected buds onto CCMV-free Secundina. Cuttings were rooted under mist and transplanted into 4-in. pots. Cassava meristem-tips were cultured as described by Kartha et al (11). Lettuce necrotic yellows virus (LNYV) and sonchus yellow net virus (SYNV) were obtained from H.-T. Hsu, American Type Culture Collection. All cassava plants were grown

under quarantine conditions at the U. S. Plant Introduction Station at Glenn Dale, MD.

**DsRNA purification and gel electrophoresis.** DsRNA was extracted from 0.5–1.0 g of leaf tissue using the method of Jordan et al (10). DsRNA was fractionated by gel electrophoresis using 5% polyacrylamide (acrylamide:bis-acrylamide ratio was 40:1) slab gels ( $16 \times 18 \times 0.15$  cm, 20 mA for 18 hr) or horizontal, submerged, 1% agarose gels ( $11 \times 14 \times 1.5$  cm, 150 mA for 4 hr) in 40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.8. Gels were stained with 100 ng/ml of ethidium bromide for 30 min and destained in distilled water for 15 min.

Enzymatic digestions were done as in Gildow et al (6) for DNase I and RNase A (Sigma Chemical Co., St. Louis, MO) and according to Maniatis et al (14) for S1 nuclease (Sigma). Cucumber mosaic virus (CMV) single-stranded genomic RNA, lambda DNA, and tobacco mosaic virus (TMV) replicative form (RF) dsRNA were used as nucleic acid controls for enzyme digestion. The procedure of Diaz-Ruiz and Kaper (4) was followed for the differential precipitation of nucleic acids.

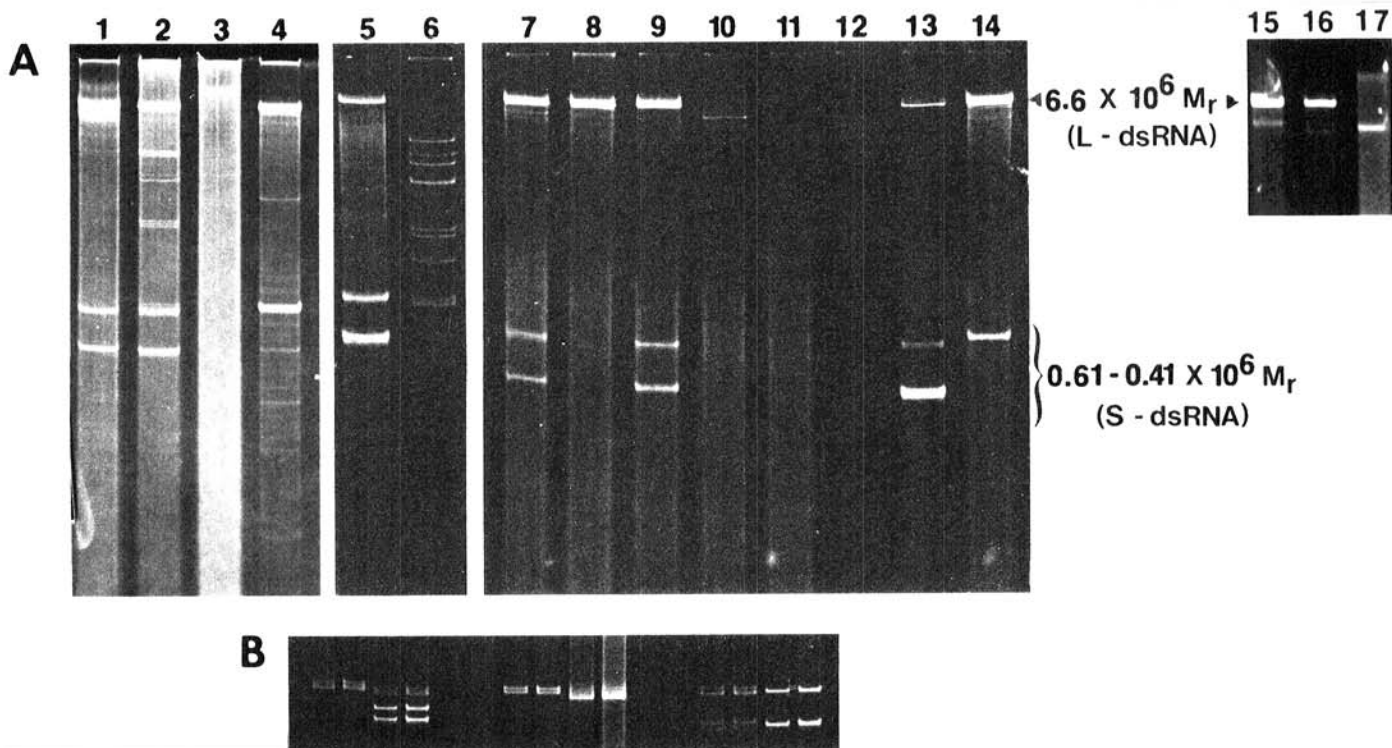
Molecular weight standards for size estimations of cassava dsRNA were citrus tristeza virus (CTV) RF dsRNA ( $13.3 \times 10^6 M_r$  = relative molecular mass), *Phaseolus vulgaris* 'Black Turtle Soup' (BTS) dsRNA ( $9.67 \times 10^6 M_r$ ) (21), TMV RF dsRNA ( $4.1 \times 10^6 M_r$ ), wound tumor virus (WTV) genomic dsRNAs (2.9, 2.4, 2.2, 1.8, 1.15, 1.1, 0.8, 0.6, and  $0.33 \times 10^6 M_r$ ) (18). CTV, BTS, and WTV dsRNAs were provided by R. Jordan, ARS, Beltsville, MD. Molecular weights were determined on 1% agarose gels for the high  $M_r$  dsRNA and on 5% polyacrylamide gels for the low  $M_r$  dsRNA by the method of Bozarth and Harley (2).

**Intracellular location of dsRNA.** The procedure of Powell et al (17) was used for the subcellular fractionation of cassava leaf extracts with the following initial modification: Ten grams of cassava leaves were homogenized with a razor blade in a buffer consisting of 50 mM Tris-Cl, 1 mM EDTA, and 3 mM 2-mercaptoethanol, pH 7.4. Samples from each of the fractions were stained with acetocarmine and examined with a light microscope (1,000 $\times$ ).

**DsRNA infectivity studies.** Graft inoculations were made by "chip budding" pieces of Secundina stem that contained the dsRNA molecules onto dsRNA-free *M. websterae* plants. Grafted plants were grown in a growth chamber at 28 C with 14-hr days. Leaves were tested at weekly intervals for the appearance of dsRNA. DsRNA-free *M. websterae* were also mechanically inoculated with dsRNA-containing Secundina sap, Secundina total nucleic acids (2  $\mu$ g), undenatured Secundina dsRNA (2  $\mu$ g), and heat denatured Secundina dsRNA (2  $\mu$ g). One gram of

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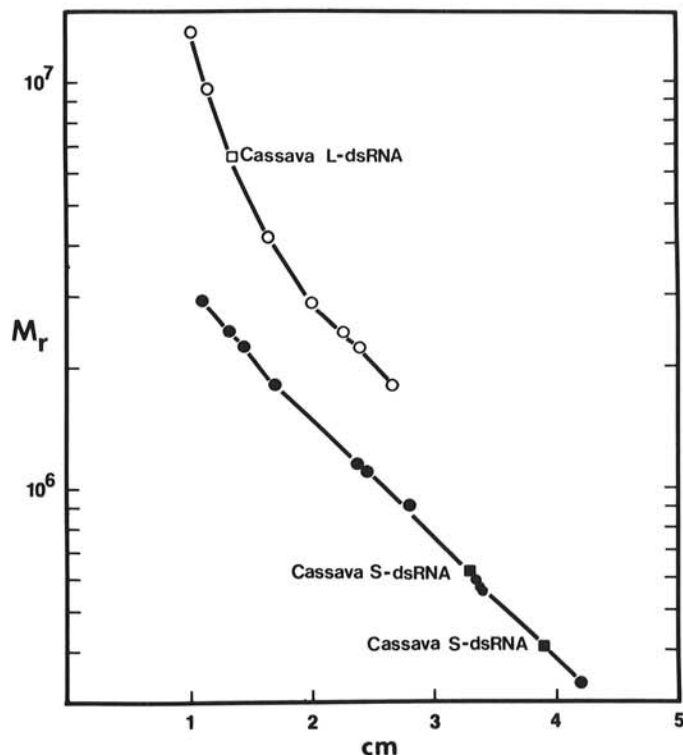
**Fig. 1.** Variety of double-stranded RNAs (dsRNA) isolated from cassava plants. DsRNAs were extracted from 0.5–1.0 g of cassava leaves as described. Electrophoresis was in 5% polyacrylamide gels in 40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.8, for 16 hr at 20 mA. Gels were stained with 100 ng/ml ethidium bromide. **A,** Lane 6 wound tumor virus dsRNA; lanes 1, 2, 4, 5, 7, 8, 9, 13, and 14 are extracts from the cassava clone Secundina; lanes 1, 7, 8 (Secundina), and 10 (non-Secundina *Manihot esculenta* clone) are extracts from cassava common mosaic virus (CCMV) infected plants; lanes 3, 11, and 12 are extracts from different *M. websterae* plants. Lanes 15–17 are from a 1% agarose gel which compared the large cassava dsRNA with those of TMV (lane 17) and CCMV (lanes 15–16). **B,** A portion of a 5% polyacrylamide gel which shows the variability of the dsRNAs that occur between  $0.61\text{--}0.41 \times 10^6 M_r$  ( $M_r$  = relative molecular mass) (S-dsRNA) in the clone Secundina.

undenatured or heat denatured TMV genomic RNA was inoculated onto *Chenopodium quinoa* Willd. as an infectivity control. Samples were heat denatured by boiling for 2 min followed by quick chilling on ice (19).

## RESULTS

**Variety of dsRNAs isolated from cassava plants.** DsRNA extractions of Secundina plants thought to be virus-free and those infected with CCMV revealed numerous dsRNA species (Fig. 1). Reproducible bands occurred at  $6.6$ ,  $0.61$ , and  $0.41 \times 10^6 M_r$  (Fig. 2). The  $6.6 \times 10^6 M_r$  band is referred to as L-dsRNA and the  $0.61\text{--}0.41 \times 10^6$  bands are collectively referred to as S-dsRNAs. The occurrence of other bands varied from one extraction to another. Lanes 1, 7, 8, and 10 (Fig. 1A) are dsRNAs extracted from different plants infected with CCMV. DsRNA gel patterns of CCMV-infected cassava always produced one, and usually two, bands occurring at or slightly above the TMV RF ( $4.1 \times 10^6 M_r$ ) band (Fig. 1A, lanes 15–17). This size range is where a full length potexvirus RF (about  $4.2 \times 10^6 M_r$ ) would occur. Lanes 1, 2, 4, 5, 7, 8, 9, 13, and 14 (Fig. 1A) are dsRNAs obtained from different apparently healthy (2,4,9,13,14) and CCMV-infected (1,7,8) plants of the virus indicator clone Secundina. All of the Secundina plants at the U. S. Plant Introduction Station contained the L-dsRNA band and usually the S-dsRNA bands. Out of 30 Latin American cassava introductions (non-Secundina clones) screened, only two had the L-dsRNA band, and only plants containing the L-dsRNA band contained the S-dsRNAs. However, not all plants containing the L-dsRNA band contained the S-dsRNAs (Fig. 1A, lane 8). Variability among the S-dsRNAs can be seen (Fig. 1B). Individual plants (0.5 g of tissue) of the clone Secundina were screened for dsRNA and resulted in a variety of S-dsRNA banding patterns.

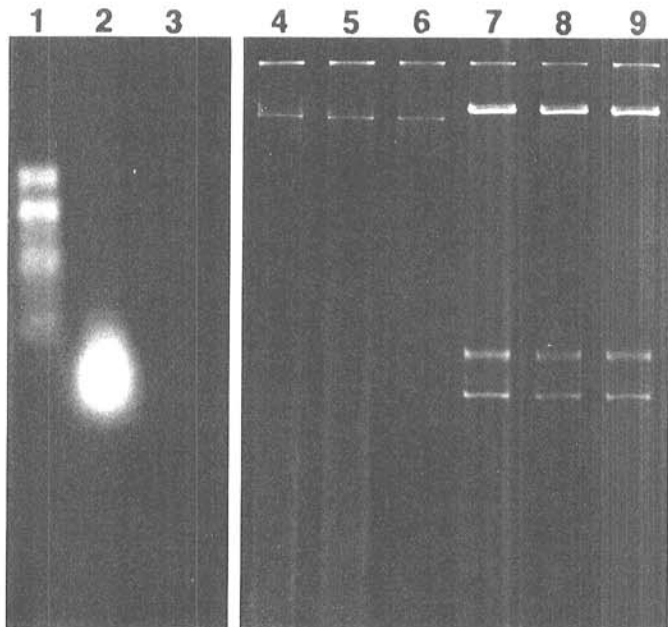
Cassava dsRNAs bound dsRNA specific antibodies (5), precipitated in 4.0 M LiCl, were resistant to DNase I, and were resistant to RNase A digestion in high salt (0.3 M NaCl, 30 mM



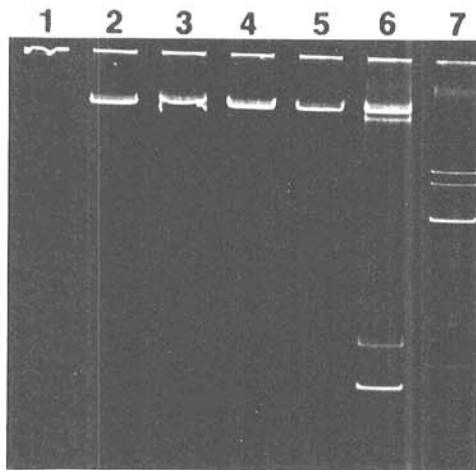
**Fig. 2.** Molecular weight determination of major cassava dsRNAs. o-o: The relative molecular mass of the large dsRNA (L-dsRNA) ( $\square$ ) from cassava was estimated by comparing it with the dsRNAs of CTV, BTS, TMV, and WTV dsRNAs 1–4. DsRNAs were fractionated on a 1% agarose gel. ●-●: The  $M_r$  of the small dsRNAs (S-dsRNAs) ( $\blacksquare$ ) from cassava were estimated by comparing them to wound tumor virus dsRNAs that were fractionated on a 5% polyacrylamide gel.

trisodium citrate) but degraded in low salt (15 mM NaCl, 0.15 mM trisodium citrate) conditions (data not shown). Cassava dsRNAs (Fig. 3, lanes 7–9) were resistant to S1 nuclease (single-strand specific) digestion in both low and high salt conditions compared with cucumber mosaic virus single stranded RNAs (susceptible to digestion) (Fig. 3, lanes 1–3) and TMV RF (resistant to digestion) (Fig. 3, lanes 4–6).

**Biological characterization of the *Secundina* dsRNAs.** None of the dsRNAs were mechanically transmitted by sap to *M.*



**Fig. 3.** Nuclease S1 digestion of cassava dsRNAs. DNase I treated nucleic acids were incubated with 800 units per milliliter of S1 nuclease for 30 min at 22 C under either high salt (300 mM NaCl, 30mM trisodium citrate) or low salt (15 mM NaCl, 0.15 mM trisodium citrate) conditions. Lanes 1–3 are cucumber mosaic virus RNA (10 µg) (fractionated on a 1% agarose gel) incubated with no enzyme, incubated with S1 and high salt, and incubated with S1 and low salt, respectively. Lanes 4–6 are tobacco mosaic virus replicative form dsRNA extracted from 0.5 g of infected tobacco tissue incubated with no enzyme, incubated with S1 and high salt, and incubated with S1 and low salt, respectively. Lanes 7–9 are cassava dsRNAs extracted from 1 g of *Secundina* tissue incubated with no enzyme, incubated with high salt, and incubated with low salt, respectively. Lanes 4–9 are from a 5% polyacrylamide gel.



**Fig. 4.** Graft transmission of cassava dsRNA. DsRNAs were extracted from 1 g of tissue and fractionated on a 5% polyacrylamide gel. Lane 1, mock inoculated *Manihot websterae*; lanes 2–5, DsRNA extracts from different *M. websterae* plants graft inoculated with *Secundina*; lane 6, DsRNAs extracted from *Secundina* inoculum source; lane 7, cucumber mosaic virus replicative form dsRNAs.

*websterae*, *Pisum sativum* L. 8221, *C. quinoa*, *Nicotiana tobacum* L. 'Xanthi' and 'Samsun,' chinese cabbage (*Brassica chinensis*), or radish (*Raphanus sativus*). Cassava L- and S-dsRNAs were not transmitted to *M. websterae* by mechanical inoculation using total nucleic acids from *Secundina*, or purified undenatured or heat denatured dsRNA as inoculum. Inoculations with TMV genomic RNA, either undenatured or heat denatured, resulted in numerous lesions on *C. quinoa* (data not shown). Graft inoculations, made by chip budding infected pieces of *Secundina* stem onto dsRNA-free *M. websterae* plants, showed that the L-dsRNA could be detected after 6 wk in all of the plants grafted with *Secundina* but in none of the plants inoculated with dsRNA-free buds from *M. websterae* (Fig. 4). The S-dsRNAs were not detected in the graft inoculated plants after 6 mo of observation. Meristem-tip culture of infected *Secundina* resulted in plants free of all dsRNA.

All attempts to purify virions from infected cassava tissue by using standard differential centrifugation procedures, as well as protocols for the purification of tomato spotted wilt virus (20), carrot mottle virus (16), and SYNIV (8) failed as determined by analytical sucrose density gradient centrifugation and electron microscopy. There were no cytopathological differences detected by electron microscopy of thin sections that compared dsRNA containing *Secundina* with dsRNA-free *Secundina*. CCMV inclusions were seen in dsRNA containing *Secundina* coinfecting with CCMV (data not shown).

**Intracellular location of dsRNA.** Subcellular fractionation of cassava leaf tissue resulted in the following four fractions: 1, 1,075 g supernatant; 2, top of the glycerol gradient; 3, chloroplast-rich fraction; and 4, nuclei-rich fraction. The chloroplast fraction contained some nuclei, and the nuclear fraction contained some chloroplasts as determined by light microscopy. DsRNA extraction of these fractions showed that the majority of the L-dsRNA band was located in the nuclear fraction (Fig. 5, lane 5). A small amount of L-dsRNA band was seen in the chloroplast fraction (Fig. 5, lane 4). No dsRNA was found at the top of the glycerol gradient (nonchloroplast, nonnuclear fraction, pelleted at 1,075 g



**Fig. 5.** Subcellular location of cassava dsRNAs. Ten grams of *Secundina* tissue were homogenized with a razor blade and filtered through Miracloth. The filtrate was centrifuged at 1,075 g and the pellet resuspended in 2 ml of buffer, layered on a discontinuous glycerol gradient (50, 75, and 90% w/w), then centrifuged at 20,000 rpm for 2 hr in a Beckman SW 40 rotor. Lane 1, dsRNA extracted directly from *Secundina* tissue; lane 2, dsRNA extracted from the 1,075 g supernatant; lane 3, dsRNA extracted from the top of the gradient; lane 4, dsRNA extracted from the chloroplast fraction; and lane 5, dsRNA extracted from the nuclear fraction.



in 15 min) (Fig. 5, lane 3). L and S-dsRNAs were detected in the 1,075 g supernatant (Fig. 5, lane 2) and in Secundina tissue that was extracted directly for dsRNAs (Fig. 5, lane 1). However, S-dsRNAs were not detected in the glycerol gradient (Fig. 5, lanes 3-5).

The presence of the  $6.6 \times 10^6 M_r$  L-dsRNA in nuclear fractions, plus a report of rhabdovirus-like particles in nuclei of cassava plants (12), prompted us to compare dsRNAs extracted from Secundina with dsRNAs extracted from *Nicotiana clevelandii* Gray plants infected with either LNYV or SYNIV (plant rhabdoviruses). DsRNAs were not detected in extracts from 1-10 g of rhabdovirus infected tissue using 1% agarose gels (data not shown).

## DISCUSSION

The size of the large dsRNA ( $6.6 \times 10^6 M_r$ ) suggests that it might be an RF resulting from a potyvirus infection (single-stranded RNA of  $3.0-3.5 \times 10^6 M_r$ ). However, potyvirus dsRNA is difficult to detect from 0.5 g of leaf tissue, whereas the cassava dsRNA is readily detected in this amount of tissue. Potyviruses are also mechanically transmissible, whereas the cassava agent is not. In addition, no potyvirus inclusions were seen in thin sections of L-dsRNA containing leaf tissue. Therefore, it is doubtful that the  $6.6 \times 10^6 M_r$  L-dsRNA is a product of a classic potyvirus infection.

Kitajima and Costa (12) detected rhabdovirus-like particles in the perinuclear space of symptomless cassava plants. They were not able to detect the particles in leaf dip preparations and they were not able to transmit the particles mechanically. The extreme scarcity of the viruslike particles (possibly allowing them to go undetected in our thin sections), the lack of symptoms in infected plants, and their association with the nucleus make the particles observed by Kitajima and Costa (12) candidates for the virus responsible for the production of the dsRNAs seen in this study. However, if the particles observed by Kitajima and Costa (12) are related to the Secundina dsRNAs, they must be functionally different from LNYV and SYNIV, because these rhabdoviruses produce no detectable dsRNA.

The relationship, if any, between the L-dsRNA and the S-dsRNAs remains unclear. There are some similarities between the S-dsRNAs and the dsRNA genomes of cryptic viruses (1). Cryptic viruses are latent, contain small segmented dsRNA genomes ( $1.2-0.85 \times 10^6 M_r$ ), are not transmitted mechanically or by grafting, and are seed transmitted. However, the small cassava dsRNAs do not appear to be encapsidated and have not yet been shown to be seed transmitted. It appears that the S-dsRNAs are dependent on the L-dsRNA for replication because they were only detected in plants containing the L-dsRNA.

Because the agent described in this paper is latent, even in the virus indicator clone Secundina, dsRNA analysis is the only method currently available to index for this agent. The dsRNA is detectable in small quantities (0.5 g) of cassava tissue using standard dsRNA extraction procedures. DsRNA analysis may also allow for the detection of CCMV (Fig. 1A, lane 10).

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