Two Components Associated with Citrus Ringspot Virus

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ABSTRACT. Infectious preparations of citrus ringspot virus (CRSV) were prepared by differential centrifugation, but individual fractions following sucrose density gradient centrifugation were not infectious. Mixtures of fractions approximately 3.5 cm deep and 5.5 cm deep in the gradients were highly infectious. The two components appeared to depend on each other for infectivity. Efforts to infect citrus or herbaceous plants with single components were not successful.
Index words. infectivity assays, virus purification.

Citrus ringspot was first described by Wallace and Drake (2) in 1968. The cause of the disease has not been determined, but it is presumed to be a virus and is referred to as citrus ringspot virus (CRSV). CRSV is readily transmitted to many herbaceous plants by sap inoculation and produces local lesions on *Chenopodium quinoa*, but the infectivity in extracts is unstable, and there are no reports on the physical and chemical properties of the virus. Several individuals (unpublished observations of S. M. Garnsey, D. Gonsalves, R. F. Lee, and L. W. Timmer) have observed repeatedly that the infectivity associated with CRSV appears to be lost following fractionation by sucrose density gradient centrifugation (SDGC).

This paper shows that the infectivity associated with CRSV is resolved into two widely separated components by SDGC.

MATERIALS AND METHODS

Virus source. The CRSV isolate used in this study was CRSV-4 that had been through several successive single lesion transfers on *C. quinoa* followed by transfer to *Gomphrena globosa* and subsequently to Duncan grapefruit (1).

Partial purification. Infectivity assays, partial purification trials and sucrose density gradients were done using 0.05 M Tris, 0.1% ascorbic acid, 0.1% L-cysteine, and 0.5% 2-mercaptoethanol (2-ME), adjusted to pH 8.0 with HCl (TACM). Young leaves of Duncan grapefruit with symptoms were used in purification trials. Leaves were pulverized in the presence of liquid nitrogen. The resulting frozen powder was transferred to a mortar and pestle at room temperature and ground with 2 or 7 ml of TACM per g of leaf tissue. All subsequent purification steps were done at 4 C. The extracts were filtered through cheesecloth and, in some experiments, stirred with Freon for one min followed by clarification by centrifugation for 10 min at 12,000 *g*. Linear gradients of 10-40% sucrose in TACM were prepared in Beckman SW 41 rotor tubes. Clarified extracts prepared in 2 ml of buffer per g of leaf tissue were applied to gradients without concentration. Extracts in 7 mls per g of leaf tissue were subjected to one or more cycles of differential centrifugation (12,000 *g* for 10 min followed by 300,000 *g* for 1 hr) and the resulting pellets were suspended in TACM (1.0 ml/6 g of starting tissue). One ml preparations were layered on the sucrose gradients and centrifuged for 2.5 hr at 38,000 rpm at 4 C. The gradients were fractionated into 0.6-ml fractions using an ISCO gradient fractionator.

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Infectivity assay. Extracts and fractions from gradients were assayed for infectivity on *C. quinoa*. Leaves were dusted with 600 mesh carborundum, and 50 μl of extracts, individual gradient fractions, or equal volume mixtures of two gradient fractions were spotted and rubbed onto leaves. The plants were maintained in a greenhouse and lesions were counted 6 to 8 days after inoculation.

Assay for possible mixed infection. Individual top and bottom gradient fractions that were known to be infectious when mixed were used to inoculate *C. quinoa*, *G. globosa* and Duncan grapefruit. At various times after inoculation, extracts from these plants were mixed with the appropriate top or bottom fractions from SDGC and used to inoculate *C. quinoa*.

RESULTS AND DISCUSSION

Infectivity associated with CRSV has been shown to be stabilized to a degree by making extracts in Tris buffer, pH 8.0 containing 0.5% 2-ME (1). Unpublished studies at Lake Alfred have shown the addition of L-cysteine and ascorbic acid to the Tris buffer further stabilizes the infectivity. Thus, TACM was used for all extractions, partial purifications and infectivity trails reported here. Using Tris buffer below pH 8.0 or omitting 2-ME always resulted in severe loss of infectivity.

Infectivity assays on *C. quinoa* of extracts of young Duncan grapefruit leaves with symptoms of CRSV (prepared using 1 g of tissue per 10 ml of TACM) produced 80 to confluent lesions per leaf. As previously reported (1), most of this infectivity was lost if the preparation was kept on ice for 24 hr before assay. The infectivity in extracts could be recovered in high-speed pellets following differential centrifugation, but these preparations also lost most of their infectivity after 24 hr on ice. Individual fractions from SDGC of clarified extracts or preparations concentrated by differential centrifugation were not infectious, but some infectivity could be recovered from a mixture of all fractions from the gradient. This observation led to the finding that at least two components that are widely separated by SDGC are required for infectivity of CRSV. In various experiments, maximum infectivity was always associated with mixtures of fractions that were approximately 3.5 cm and 5.5 cm deep in the gradients. To readily demonstrate the presence of infectious components following SDGC, it was necessary to perform the partial purification and assay fractions for infectivity on the same day. Partial purification by SDGC did not stabilize the infectivity. Very little infectivity could be detected when the fractions were kept on ice overnight. This indicates that infectivity assays cannot be used to monitor CRSV purification schemes that require more than 1 day.

The observation that two fractions that are readily separated by SDGC are required for infectivity suggested citrus ringspot may be caused by a mixed infection of two viruses. Symptoms were not observed on *C. quinoa*, *G. globosa*, or Duncan grapefruit plants that had been inoculated with either top or bottom fractions. Extracts from these plants which had been inoculated with single fractions failed to produce local lesions on *C. quinoa* when mixed with a freshly prepared top or bottom fraction from SDGC. These observations suggest CRSV is not a mixed infection of two competent viruses and indicate that the top and bottom fractions contain portions of a divided viral genome that depend on each other for infectivity.

The location of the infectious components of CRSV on sucrose gradients should prove useful in further studies to characterize the virus and develop diagnostic tests for indexing.

LITERATURE CITED

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