In Situ Immunofluorescence for the Detection of Citrus Tristeza Virus Inclusion Bodies

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ABSTRACT

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Transverse and longitudinal cryostat-cut sections, 30–40 μm thick, were prepared from bark and petioles of five citrus species that were healthy or infected with various isolates of citrus tristeza virus (CTV). Sections were immersed in tetramethylrhodamine isothiocyanate (TRITC) labeled or fluorescein isothiocyanate (FITC) labeled immunoglobulin (IgG) for 1 hr at room temperature, rinsed in phosphate-buffered saline, mounted, and viewed with a fluorescence microscope. Orange-red fluorescing bodies were readily observed in phloem fibers, sieve tubes, and parenchyma cells of CTV-infected tissue sections incubated in TRITC-labeled IgG specific to CTV. Yellow-green fluorescence of the same cells was observed after incubation in FITC-labeled IgG. Autofluorescence was more evident with FITC-labeled IgG than with TRITC-labeled IgG. No fluorescing bodies were observed in sections incubated in normal IgG or in fluorescent labeled IgG specific for either tobacco etch virus or Pierce's disease bacterium or in sections of healthy tissue incubated in TRITC-labeled CTV IgG. The same fluorescing bodies in CTV-infected tissue also were stained by Azure A, indicating that the nucleoprotein-containing inclusion bodies of CTV were the fluorescing structures.

Citrus tristeza virus (CTV) is one of the most serious virus problems of citrus worldwide. The virus is a member of the closterovirus group with long, flexuous rod particles approximately 2,000 nm long and 10 nm wide. Cytological (17,21,22), transmission, and translocation studies (18) have demonstrated that CTV is phloem-associated.

Schneider (22) reported the presence of chromatic cells in the phloem of CTV-infected tissue. The chromatic cells were usually parenchyma or parenchymalike cells adjacent to sieve tubes that contained a thick, lightly stained cytoplasm with dark-staining masses of strands or needlelike objects. Central vacuoles, if present, were small and often divided. He suggested that these cells were the primary cytological symptom from CTV infection and that they were involved in the development of wood pitting, vein clearing, and seedling yellows symptoms. Christie and Edwardson (4) and Garnsey et al (5) found that Azure A stained inclusion bodies in CTV-infected tissue.

Using electron microscopy (1,11,12, 17,23,24,26), viral aggregates of CTV in infected citrus tissues have been observed. Vesicular structures (1,12,14,23), as well as parallel tubes thought to be tubular P-protein (1), also have been reported in such infected tissues. Vacuolated, reddish-staining cells along with banded and fibrous inclusions were observed by Christie and Edwardson (4) in fresh CTV-infected tissue stained with Azure A.

Fluorescent antibody microscopy has previously been used to detect the presence of plant viruses and inclusion bodies. Nagaraj and Black (16) used fluorescent antibodies to detect and localize wound tumor virus antigen in plant tumors. Tobacco mosaic virus antigen was detected in tobacco mesophyll protoplasts using fluorescent-labeled antibodies (25). Rao et al (19) used fluorescein isothiocyanate (FITC) labeled antibodies to detect inclusion bodies of clover yellow mosaic virus (CYMV) in epidermal strips of CYMV-infected cowpea. Tsuchizaki et al (27) also detected CTV antigens in phloem cells of infected fruits using FITC-labeled, CTV-specific immunoglobulin G (IgG). Using the same technique, Sasaki et al (20) reported that they were able to differentiate between mild and severe isolates of CTV by the number of fluorescing cells observed. One problem observed with the use of FITC labels for citrus tissue is the presence of autofluorescing materials in sections (3).

The purpose of this study was to determine 1) if tetramethylrhodamine isothiocyanate (TRITC) labeled IgG specific for CTV could be used to detect CTV antigens in infected plant tissues and if it had any advantage over the FITC method, 2) if the observed fluorescing structures were the same inclusion bodies as those revealed by Azure A staining, and 3) if freezing, desiccation, and fixation of the tissue before treatment affected the detection of CTV antigens by fluorescent antibody or Azure A staining procedures.

MATERIALS AND METHODS

Virus isolates and plant material. Five isolates of CTV, each in five host plants, were used in this study. The T-4 isolates cause strong veinclearing, stunting, and stem pitting in Mexican lime (*Citrus aurantifolia* (Christm.) Swingle), no visible decline on sweet orange (*C. sinensis* (L.) Osbeck) on sour orange rootstocks (*C. aurantium* L.), and no seedling yellows symptoms. Isolate T-3, originally described by Grant and Higgins (10), causes severe symptoms on Mexican lime, severe decline of sweet orange on sour orange rootstock, and seedling yellows symptom on Eureka lemon (*C. limon* (L.) Burm. f.) and sour orange seedlings. The T-36 isolate was originally collected from a sweet orange tree on sour orange rootstock suffering from quick decline (7) and produces severe symptoms on Mexican lime, and a mild seedling yellows reaction. Isolates T-30 and T-26 produce mild symptoms on Mexican lime but no decline or seedling yellows reaction.

Sweet orange, Eureka lemon, Etrog citron (*C. medica* L.), sour orange, and Mexican lime plants were graft-inoculated with each isolate. Samples of petioles and bark were later obtained from the infected plants and healthy controls. The presence of systemic infection was verified by ELISA.

Antiserum. The antiserum used in this study was that prepared to whole, unfixed CTV isolate T-4, as previously described (8,9). IgG was separated from the whole serum using the protein A-Sepharose affinity chromatography procedure of Miller and Stone (15). The concentration of IgG was estimated spectrophotometrically using $E^{0.1\%}_{1\,cm} = 1.4$ (at 280 nm). The anti-CTV IgG and normal serum IgG were conjugated to either fluorescein isothiocyanate (FITC) (Miles Laboratories, Elkhart, IN 46515) or to tetramethylrhodamine isothiocyanate (TRITC) (Research Organics,

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Inc., Cleveland, OH) using the methods of Blakeslee and Baines (2) and Brlansky et al (3), respectively.

Tissue preparation. Sections of young bark and petioles of virus-infected and healthy citrus were cut using a Harris WRC cryostat. Transverse and longitudinal sections 30–40 μm thick were immersed in labeled IgG to CTV, labeled normal (nonimmunized) IgG, or in labeled control IgGs specific for tobacco etch virus or Pierce's disease bacterium. Labeled IgG was diluted 1/20 with phosphate-buffered saline (PBS) (pH 7.0) before use. Sections were incubated at room temperature for 1 hr or at 37 C for 30 min. Tissue sections were washed in PBS for 10–20 min, mounted on microscope slides in Aqua Mount (Lerner Laboratories, New Haven, CT 06513), and observed with a Leitz Dialux fluorescence microscope (E. Leitz, Inc., Rockleigh, NJ 07647) in the 560–590 nm wavelength range for TRITC (Leitz filter block N2), and in the 380–420 nm range (Leitz filter block H2) for FITC. Fluorescing structures were photographed using Kodak Technical Pan 2415 black and white film.

Orientation of the sections and fluorescing structures was noted. Then sections were removed from the slides, washed for 5 min in PBS to remove the mounting medium, and stained for 5 min in 0.05% Azure A in ethylene glycol monomethyl ether (2-methoxyethanol) that was buffered with 0.2 M Na_2HPO_4 just before use. After staining, the sections were washed sequentially for 5 min in 95% ethanol and ethylene glycol monomethyl ether acetate (2-methoxyethyl acetate) and then mounted on slides in Euparal (G.B.I. Ltd., Manchester, England). Sections were viewed on the Leitz Dialux microscope using transmitted light. Photographs were made of the magenta staining inclusions using Kodak Panotomic X black and white film.

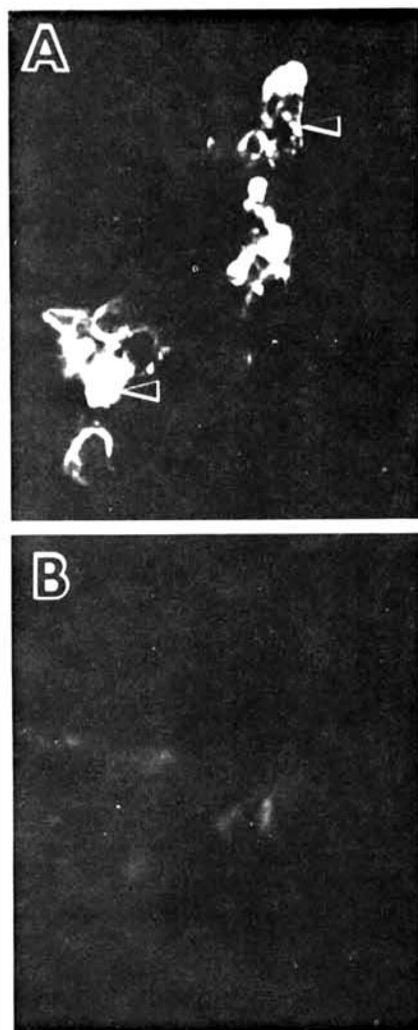

Fig. 1. Immunofluorescence of structures in the phloem of plants using tetramethylrhodamine isothiocyanate (TRITC) labeled antisera. **(A)** Fluorescing structures in the phloem cells of petioles from Mexican lime plants (*Citrus aurantifolia*) infected with citrus tristeza virus isolate T-36 × 690. **(B)** Tissue section of the same petiole incubated in TRITC-labeled normal serum with no fluorescing structures in the phloem × 690.

Fig. 2. In situ immunofluorescence and Azure A staining of petiole tissue sections from citrus tristeza virus (CTV), T-36 isolate, infected Mexican lime (*Citrus aurantifolia*) plants. **(A)** Fluorescing structures in phloem of petiole from CTV-infected Mexican lime plant × 276. **(B)** Same tissue section as shown in A, but stained with Azure A showing that the same fluorescing structures are CTV inclusion bodies × 276.

Tissue treatments. Petioles and bark tissue samples (1 cm long) of virus-infected and healthy citrus were excised from greenhouse-grown plants and either dried over calcium sulfate or silicagel, frozen at −70 C, or fixed in 3% glutaraldehyde for 8–12 hr, then rinsed and stored in 0.06 M phosphate buffer (pH 6.8) at 4 C. Samples were removed at various intervals and in situ immunofluorescence (ISIF) and Azure A staining were done to determine the effect of treatment and storage time on staining of the inclusion bodies.

Fresh tissue sections of petioles and bark were tested to determine if pretreatment with such agents as acetone, Driselase, or Triton X-100 would affect the penetration of Azure A or the fluorescent-labeled CTV IgG. Cryostat sections were immersed in 100% acetone, 0.1 and 1% solutions of Driselase in 0.1 M sodium citrate buffer (pH 8.0), or in 5% Triton X-100 in PBS. Samples from these treatments were then processed as described above for Azure A and ISIF staining.

RESULTS

Orange-red fluorescing structures were observed inside phloem, phloem fiber, and parenchyma cells in all CTV-infected plant tissues when sections were incubated in TRITC-labeled IgG specific to CTV (Fig. 1A), but not in sections incubated in TRITC-labeled normal serum IgG (Fig. 1B) or in labeled IgG specific for tobacco etch virus or Pierce's disease bacterium. Fluorescing structures were not present in any tissue sections from healthy plants. When the same sections were stained with Azure A and observed with the light microscope, most of the same structures were stained magenta as previously reported for CTV inclusion bodies (6). The fluorescing bodies and the magenta inclusion bodies were similar in structure and in form (Fig. 2). Sections of CTV-infected tissue not incubated in TRITC-labeled IgG also had structures in the phloem that stained magenta with the Azure A procedure. Tissue sections of healthy citrus tissue did not contain any magenta-staining bodies in the phloem and associated tissues when stained with Azure A.

Inclusions in CTV-infected tissue incubated in CTV-specific IgG labeled with FITC fluoresced a yellow-green color. However, autofluorescence of the xylem and oil glands occurred in all sections (healthy, infected, incubated, or not incubated in FITC-labeled IgG) when viewed at 380–420 nm wavelength range. Some autofluorescence also occurred in the 560–590 nm range for TRITC, but only in thicker sections (50–60 μm).

Storage of CTV-infected petioles and bark tissue before ISIF or Azure A treatment was easily accomplished by desiccation over calcium sulfate or silica gel or freezing the tissue at −70 C. Inclusion bodies of all five CTV isolates were detected in citron and sweet orange using ISIF or Azure A in tissue pieces subjected to these storage conditions for up to 6 wk. Small pieces of virus-infected or healthy tissue that were fixed in 3% glutaraldehyde and stored in phosphate buffer at 4 C before ISIF or Azure A treatment often exhibited nonspecific fluorescence or magenta cellular staining.

Pretreatment of tissue sections with acetone, Driselase, or Triton X-100 showed no apparent effect on either the fluorescent CTV IgG or Azure A stain.

DISCUSSION

Citrus tristeza virus antigen was readily detected in citrus phloem using CTV-specific IgG labeled with TRITC. This simple and easy fluorescence procedure proved useful in the diagnosis of CTV infections. The use of TRITC was preferred over FITC as a fluorescent label since less autofluorescence of the xylem and other plant tissues occurred in the 560–590 nm range. The fluorescing bodies observed in CTV-infected tissues also stained magenta with the Azure A procedure. They were the inclusion bodies described previously (4,5). The desiccation of CTV-infected tissue over silica gel or calcium sulfate proved to be a useful procedure, especially when large numbers of samples were harvested before processing. Inclusion bodies formed as a result of infection by CTV are mainly aggregates of the long, flexuous, threadlike particles of the virus (13,18) and they probably represent the contents of the chromatic cells described by Schneider (22,23). The formation of these inclusion bodies and their significance in virus-infected plant tissues is unknown. However, their presence by the detection methods described is useful in the detection of CTV.

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