Characterization of Monoclonal Antibodies for Identification of the Severe Strains of 'Capão Bonito' *Citrus tristeza virus*

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ABSTRACT. The severe 'Capão Bonito' (CB) isolates of *Citrus tristeza virus* (CTV) have limited distribution in Brazil, but affect both sweet orange and rootstocks varieties, especially Pera sweet orange grafted on Rangpur lime. The CB complex has at least two different CTV types expressing different coat proteins, whose genes were cloned and expressed in *Escherichia coli*. These proteins, CB-104 and CB-22, were used as antigens, allowing us to obtain three groups of monoclonal antibodies (MAb). All CTV isolates from CB reacted strongly in DASI-ELISA with MAb 30 which seems to react with a conserved epitope. Whereas MAbs 30 and IC.04 react with conformational epitopes, MAbs 37 and 39 recognize linear epitopes on the viral coat protein. It is possible that MAbs 37, 39 and IC.04 can be used diagnostically, and may discriminate severe CB isolates.

Citrus tristeza virus (CTV) is endemic in Brazil due to its introduction many years ago, the presence of the highly efficient vector, the brown citrus aphid, and the use of CTV-tolerant rootstock species that maintain the virus as permanent reservoirs. For the more susceptible varieties such as Pera sweet orange, Mexican lime, and grapefruit, control has been achieved by cross protection using mild protective isolates of the virus. Amongst all Brazilian CTV isolates, the complex known as 'Capão Bonito' (CB) has been considered the most severe due the economic damage caused especially in sweet orange varieties grafted on Rangpur lime (6). Both scion and rootstock varieties infected with the CB complex develop severe stem pitting, and all other tristeza symptoms, except decline (7).

Biological diagnosis of CB isolates has been conducted using Mexican lime and Pera sweet orange as indicators, but there is no significant difference in response compared with other severe CTV complexes. Polyclonal antisera and monoclonal antibodies such as MCA 13 (11), which react with Florida severe isolates, or 3DF1 and 3CA5 (15) that react to a wide range of CTV isolates with differing symptoms, were unable to discriminate CB isolates (4). The occurrence of mixtures in the CB isolates, with at least two CTV types both with similar CP gene expression rate (16) allowed us to use both recombinant coat proteins, called CB-22 and CB-104, as antigens for producing monoclonal antibodies (MAbs) (13). MAb IC.04 has specificity for the protein CB-22, which has high homology with the isolates that induce weak CTV symptoms in Mexican lime. MAb 39 has specificity to the protein CB-104, which has high homology with severe isolates. The third group of monoclonal antibodies, MAbs 30 and 37, recognize both proteins. In this paper, we report the characterization of these MAbs that can probably be used for the identification and differentiation of severe strains of CTV present in the CB complex.

MATERIALS AND METHODS

Polyclonal antiserum 1006 was prepared in rabbits against whole purified virus particles from Mexican lime (1). Monoclonal antibodies against recombinant coat proteins CB-22 and CB-104 were obtained by Stach-Machado et al. (13). Samples were collected from trees with severe

TABLE 1
ABSORBANCE AT 405 NM (OD_{405}) OBTAINED BY DASI-ELISA USING *CITRUS TRISTEZA VIRUS* POLYCLONAL ANTIBODY 1006 ANTISERUM (2 µG/ML) AS COATING ANTIBODY, AND THE MONOCLONAL ANTIBODIES (2 µG/ML) AS DETECTION ANTIBODIES (30.G. 02, 37.G. 11, 39.08 AND IC04-08).

Sample	30.G.02	37.G.11	39.08	IC04-08
Pera I	++++	+	+	—
Pera II	+++	+	+	—
Valencia I	+++	+	—	—
Valencia II	+++	+	+	—
Calderon I	+++	+	—	—
Calderon II	+++	+	+	—
CB-22	++++	+++	—	++
CB-104	+++	++	++	—
Negative control	—	—	—	—

The reactions were scored as +++++ for measurements greater than 2.0; ++++ for measurements of 1.5 to 2.0; +++ for measurements of 1.0 to 1.5; ++ for measurements of 0.5 to 1.0; + for measurements of 0.5 to 0.1; — for measurements less than 0.1.

symptoms of CB tristeza at the Núcleo de Agronomia do Sudeste, Capão Bonito. Different varieties of sweet orange, including nucellar lines of Pera, were also collected from the mother plant blocks at the Centro APTA Citros Sylvio Moreira, Cordeirópolis. These plants did not show tristeza symptoms, but all of them were infected with the mild and protective isolates in the cross protection program. *In vitro* shoot-tip grafted Pera-IAC sweet orange was used as negative control. Samples of 1 mg of lyophilized bark tissue were powdered in liquid nitrogen, and homogenized in extraction buffer (PBS, 0.05% Tween 20-PBS, 2% PVP) at a 1:10 (w/v) dilution. All ELISA tests were performed using double antibody sandwich indirect (DASI)-ELISA, according Garnsey and Cambra (3). Western blots were carried out under denaturing conditions (SDS-PAGE in 12.5% acrylamide gels). The proteins were electrophoretically transferred onto Hybond-C membrane (Amersham) using semi-dry equipment (Biorad), as described by Towbin et al. (17).

TABLE 2
ABSORBANCE AT 405 NM OBTAINED BY DASI-ELISA USING DIFFERENT *CITRUS TRISTEZA VIRUS* MONOCLONAL ANTIBODIES (2 µG/ML) FOR COATING AND POLYCLONAL ANTIBODIES FROM ANTISERUM 1006 (2 µG/ML) AS INTERMEDIATE ANTIBODIES.

Sample	30.G.02	37.G.11	39.08	IC04-08
Pera I	+++++	++	++	++
Pera II	++++	—	—	—
Valencia I	+++++	++	++	++
Valencia II	+++++	++	++	++
Calderon I	+++++	—	+	+
Calderon II	+++++	—	—	—
CB-22	+++++	+++++	—	+++++
CB-104	+++++	+++++	+++++	—
Negative control	—	—	—	—

The reactions were scored as +++++ for measurements greater than 2.0; ++++ for measurements of 1.5 to 2.0; +++ for measurements of 1.0 to 1.5; ++ for measurements of 0.5 to 1.0; + for measurements of 0.5 to 0.1; — for measurements less than 0.1.

The dot-immunobinding assay (DIBA) was performed according Rocha-Peña et al. (12).

RESULTS AND DISCUSSION

Several monoclonal antibodies have been developed against CTV that display varying degrees of reactivity to various CTV isolates (11, 15), but none of them is able to discriminate the CB strains of CTV. The coat protein gene sequences of several geographically and biologically distinct isolates have been cloned (5, 10, 14) and recombinant proteins expressed in *Escherichia coli* have been used as antigens for production of polyclonal antisera (2, 8, 9).

Recombinant coat proteins, named CB-22 and CB-104, were used as antigens for producing MAbs, and four of them from three different fusions were selected for immunore-

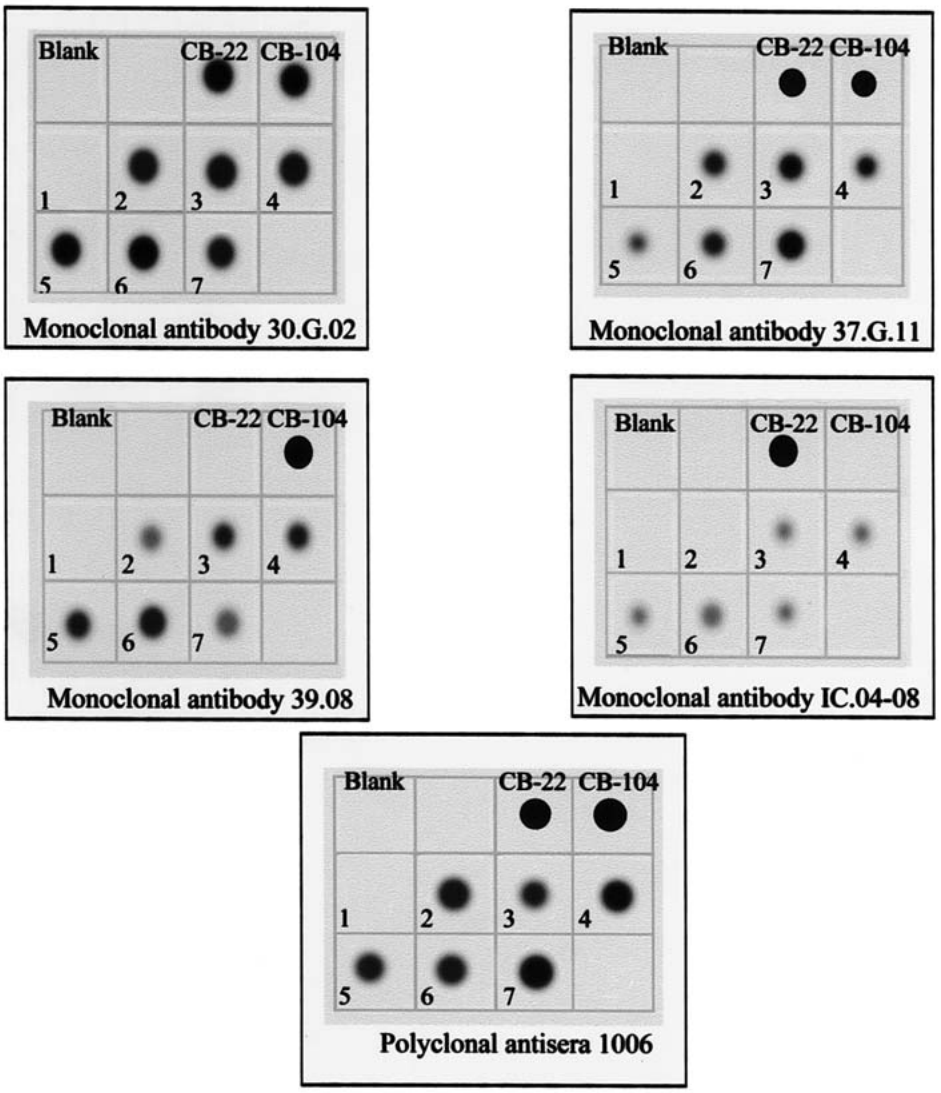

Fig. 1. DIBA carried out with samples of sweet orange varieties infected with *Citrus tristeza virus* complex CB: 1. Negative control; 2. Calderon I; 3. Calderon II; 4. Valencia I; 5. Valencia II; 6. Pera I; 7. Pera II.

activity by DASI-ELISA with recombinant CB-CTV proteins (13). Sweet orange trees with severe symptoms of CB tristeza maintained under field conditions for over 20 yr were used to determine the range of reactivity of the MAbs. The results obtained in DASI-ELISA using polyclonal anti-CTV as coating antibody and the MAbs as detection antibodies are presented in Table 1. All samples of sweet orange showed positive reaction against MAb 30.G.02. Weakly positive reactions were obtained with MAb 37.G.11 and 39.08, whereas IC.04-08 failed to react. When the monoclonal antibodies were used as coating antibodies (Table 2), MAb 30.G.02 still reacted positively with all extracts, but the reaction pattern of the other MAbs changed significantly.

The MAbs were tested using Western blot analysis (Fig. 2). MAb 30.G.02 reacted with *E. coli*-expressed CP protein CB-22 and CB-104, but failed to recognize the protein in the extract of CTV-infected citrus bark tissue. Whereas MAb 37.G.11 which reacted with *E. coli*-expressed CP protein CB-22 and CB-104, was able to detect two bands with molecular weights of approximately 27 and 26 kDa, corresponding to CP1 and CP2, respectively. The explanation for this is probably that the epitope recognized by Mab 30.G.02 is susceptible to denatur-

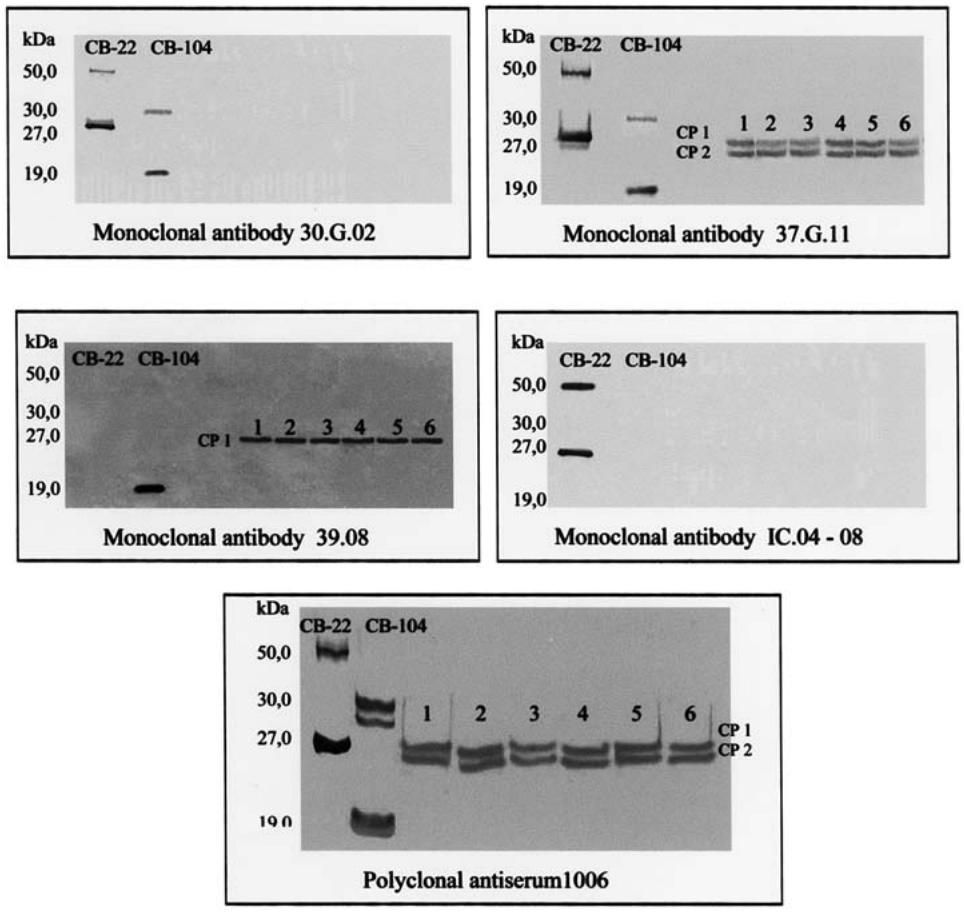

Fig. 2. Immunoblotting of total proteins from the sweet orange bark tissue after electrophoretic analysis in 12% SDS-PAGE. (1) Calderon; (2) Calderon II; (3) Valência I; (4) Valência II; (5) Pêra I e; (6) Pêra II.

ation. MAbs 39.08 and IC.04-08 reacted strongly with the *E. coli*-expressed protein CB-104 or CB-22 respectively, only MAb 39.08 reacted with one protein band of 27 kDa.

The dot-immunobinding assay (DIBA) was used to compare the results obtained by DASI-ELISA and Western blot. The results (Fig. 1) showed that all monoclonal antibodies and polyclonal antiserum react positively with all samples of plants, although the intensity of the reaction varies among the antibodies tested. The intensity of reaction between MAb 30.G.02 and polyclonal antiserum 1006 was similar, and the reactivity of MAbs 39.08 and 37.G.11 were comparable each other. MAb IC.04-08 showed the lowest reaction when compared with the other antibodies.

The pattern of reactivity of monoclonal antibody was compared using samples of Pera sweet orange (old and new clones), infected with CTV, but symptomless. All 16 samples (Table 3) showed strong positive reaction with MAbs 30.G.02, 37.G.11 and 39.08, and only six samples give weak positive reactions with MAb IC.04-08 using polyclonal antiserum 1006 as coated antibody. In contrast, when the monoclonal antibodies were used as coating antibodies (Table 4), only MAb 30.G.02 reacted positively with all plant extracts, and the other monoclonal antibodies were negative.

These results indicate that these monoclonal antibodies obtained against recombinant proteins CB-104 and CB-22 react differentially to the CTV tested. Although direct compe-

TABLE 3
ABSORBANCE AT 405 NM (OD_{405}) OBTAINED BY DASI-ELISA USING THE POLYCLONAL 1006 ANTI-*CITRUS TRISTEZA VIRUS* ANTISERUM (2 MG/ML) AS COATING ANTIBODY, AND THE MONOCLONAL ANTIBODIES (2 MG/ML) AS DETECTION ANTIBODIES.

Sample	30.G.02	37.G.11	39.08	IC .04-08
Sweet oranges				
Pera IAC	+++++	+++	++++	—
Pera Olímpia	+++++	+++	+++	—
Pera EEL	+++++	++	+++	—
Pera Bianchi	+++++	+++	+++	—
Hamlin	+++++	++	++++	++
Rubi	+++++	+++	+++	++
Natal	+++++	++	+++	—
Valencia	++++	++	+++	—
Navel Cabula	++	++	+++	+
Navel Baianinha	++++	+++	+++	+
Mandarins				
do Rio	+++++	+++	++++	—
Ponkan	++++	++	++	—
Cravo	+++++	+++	+++	—
Tangor Murcott	++++	++	++	—
Eureka lemon	+++	++	++	—
Taiti lime	+++++	+++	++++	—
Control				
CB-104	+++++	+++	+++	—
CB-22	+++++	+++	—	+++
Negative	—	—	—	—

The reactions were scored as +++++ for measurements greater than 2.0; ++++ for measurements of 1.5 to 2.0; +++ for measurements of 1.0 to 1.5; ++ for measurements of 0.5 to 1.0; + for measurements of 0.5 to 0.1; — for measurements less than 0.1.

TABLE 4
ABSORBANCE AT 405 NM OBTAINED BY DASI-ELISA USING DIFFERENT *CITRUS TRISTEZA VIRUS* MONOCLONAL ANTIBODIES (2 MG/ML) FOR COATING AND POLYCLONAL ANTIBODIES FROM ANTISERUM 1006 (2 MG/ML) AS INTERMEDIATE ANTIBODIES

Sample	30.G.02	37.G.11	39.08	IC.04-08
Sweet oranges				
Pera IAC	++++	+	—	+
Pera Olímpia	+++	—	—	+
Pera EEL	+++	+	—	+
Pera Bianchi	+++	—	—	—
Hamlin	+++	+	—	—
Rubi	+++	—	—	—
Natal	+++	—	—	—
Valencia	+++	—	—	—
Navel Cabula	++	—	—	—
Navel Baianinha	++	—	—	+
Mandarins				
do Rio	+++	—	—	—
Ponkan	+++	—	—	—
Cravo	+++	—	—	—
Tangor Murcott	+++	—	—	—
Eureka lemon	+++	—	—	—
Taiti lime	+++	—	—	—
Control				
CB-104	+++	++	+++	
CB-22	+++	++	—	+++
Negative	—	—	—	—

The reactions were scored as +++++ for measurements greater than 2.0; ++++ for measurements of 1.5 to 2.0; +++ for measurements of 1.0 to 1.5; ++ for measurements of 0.5 to 1.0; + for measurements of 0.5 to 0.1; — for measurements less than 0.1.

tition assays between these four MAbs were not performed, the differential reactions of each MAbs observed in DASI-ELISA, Western blot and DIBA suggested that they recognize different epitopes. MAb 30.G.02 can be used as universal antibody against the Brazilian isolates in large scale screening like DASI-ELISA.

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