Epitope Diversity of Citrus Tristeza Virus Isolates in Spain

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ABSTRACT. Fifty-nine Spanish citrus tristeza virus (CTV) isolates from the IVIA collection were tested against nine CTV-specific monoclonal antibodies (MCAs) by double antibody sandwich indirect ELISA (ELISA-DASI). Six different epitopes were detected, and seven distinct serogroups were defined by their reaction pattern with the different MCAs. Five of the MCAs recognized all Spanish CTV sources tested. Changes in epitope composition, observed in some isolates after aphid transmission or after propagation in certain hosts and graft transmission, indicated that some field isolates are mixtures of strains. The analysis of 1,613 CTV-infected trees collected from areas with variable CTV incidence showed that up to 18% of the samples from trees in heavily infested areas (>80% incidence) reacted with all MCAs, whereas all samples from areas with less than 2% CTV incidence failed to react with some MCAs. This suggests that mixtures of strains are more abundant in areas where CTV has been established for a long time. The analysis of 3,231 CTV-infected trees by ELISA-DAS with MCAs 3DF1, 3CA5, and MC13 showed that only 8.7% reacted with MC13, whereas 99.6 % reacted with 3DF1 and 100% with 3CA5. A mixture of 3DF1 and 3CA5 detected all isolates. Under Spanish conditions, the reaction with MC13 was not correlated with the decline of trees on sour orange in the field. However, all known severe strains reacted with MC13 and 10E3 antibodies.

Index words. CTV isolates, strain mixtures, serogroups, monoclonal antibodies, epitopes, isolate segregation.

The existence of numerous strains of citrus tristeza virus (CTV) worldwide is well known and has also been studied in Spain (1,2,3,8,15,16,20,22). Common CTV strains in Spain are relatively mild (1) and do not cause damage in trees grafted on tolerant rootstocks. However, severe strains with destructive potential have been detected occasionally in budwood introduced illegally (2,16). An eradication plan (Ministry of Agriculture Order, July 30, 1986) based on ELISA-DAS was conducted to eliminate these isolates (3). Spain is one of the countries in the Mediterranean area where CTV is actively spreading (6,7,11,23), especially in areas where *Aphis gossypii* has become predominant in recent years (17). Techniques for quick and reliable identification of severe CTV strains are needed to control the potential spread of these strains into new areas.

Production of different CTV-specific monoclonal antibodies (MCAs) in Spain (27,28), California (14), Florida (24), Taiwan (26), Morocco (31), and Cuba (Batista, 1992, personal communication), and the development of various ELISA procedures based on monoclonal and polyclonal antibody combinations (4,5,9) have improved general CTV diagnosis and provided a means to differentiate CTV isolates by epitopic variations in the coat protein (10,24,26,29).

Information on the epitopic characteristics of CTV isolates present in a given area can be used to generate MCAs able either to react against all CTV isolates or to discriminate isolates based on virulence. MCAs are useful to detect mixed infections in a plant, to follow translocation of particular isolates in cross-protection studies, to detect CTV in plants transformed with CTV coat protein genes, and to monitor infection in challenged transgenic plants.

In this paper we report information on epitope diversity among Spanish CTV isolates developed in tests of an isolate collection and from field surveys.

MATERIALS AND METHODS

Antibodies. Polyclonal antibodies were purified from M1-CTV rabbit antiserum prepared to untreated CTV

particles purified from fruit albedo (unpublished data). Purified MCAs 3DF1, 3CA5, and 3BH6 to a Spanish CTV isolate (28) (INGENASA, Madrid); MC13 and MC14 (24) to a Florida isolate; supernatant fluid to MCAs IIIAD5 and IIA1 from California (14) and; MCAs 10E3 and 4H6H from Taiwan (26); were used.

ELISA procedure and serogrouping. Double antibody sandwich indirect (DASI) ELISA (9) was used to study epitope variability and to define serogroups based on reaction patterns of each MCA against a panel of CTV isolates. Maxisorp immunoplates (NUNC) were coated with 1 μg/ml M1 antibodies. After washing, 200 μl of plant extract (1/20 w/v) prepared in phosphate buffered saline (PBS), pH 7.2-7.4 with 0.2% DIECA, was added per well and the plates incubated overnight at 4C. CTV-specific MCAs were used as intermediate antibodies at the following concentrations (dilutions made in PBS which contained 1% crude rabbit serum): 3DF1 (0.05 μg/ml), 3CA5 (0.05 μg/ml), 3BH6 (0.15 μg/ml), MC13 (0.125 μg/ml), MC14 (0.20 μg/ml), IIIAD5 (1/80), IIA1 (1/50), 10E3 (1/25), and 4H6H (1/40), and incubated for 2 hr at 37C. After washing, goat anti-mouse immunoglobulins conjugated to alkaline phosphatase (Boehringer Mannheim) were added at a 1/5,000 dilution in PBS. Other details were as described previously (4). There were four well repetitions per CTV isolate, and each plate had eight wells with healthy control extract. The plate reader (Flow Titertek Multiskan MKII Plus) was zeroed with an empty plate. A reaction was considered positive when the mean optical density (405 nm) of the extract was at least double the mean ELISA value of the healthy control extract. CTV isolates were placed in serogroups according to their reaction pattern with the panel of MCAs.

Virus isolates. Fifty-nine Spanish CTV isolates from the collection of the Instituto Valenciano de Investigaciones Agrarias (IVIA) were used in this study. These isolates were maintained in a screenhouse, and most had been evaluated previously by host range symptomatology (1) and double-stranded RNA (dsRNA) analysis (20). Some had also been characterized by peptide map analysis (26) and by reaction with some selected MCAs (3,28,29). The original field isolates were propagated on sweet orange seedlings and then transmitted to Mexican lime by *A. gossypii* (15). In many cases, the original and aphid-transmitted subisolates were in the collection. This collection also included different subisolates segregated by aphid or graft transmission from the mild CTV isolate T.385. These differed from the parent source by symptoms and/or dsRNA patterns (20,21). Other isolates were T.343, obtained by filtration through specific hosts (12); T.302, which was symptomless in Mexican lime (unpublished data); and T.388, a very severe isolate (2,3) introduced with foreign budwood and later eradicated.

In addition to isolates in the collection, many field isolates were analyzed in three experiments. Three budsticks were collected from each tree (7) to prepare extracts. In the first experiment, 1,613 CTV-infected trees were assayed by ELISA-DASI with nine MCAs. Based on previous surveys (6), 750 samples were collected from areas with over 80% CTV incidence, 540 samples were from areas with 10 to 20% CTV incidence, and the remaining 323 samples were from areas with less than 2% infected trees.

In the second experiment, an ELISA-DAS biotin/streptavidin system (4) was used to analyze 3,231 additional CTV-infected trees with the MCAs 3DF1, 3CA5, and MC13. These samples were collected from different areas in the Valencian community.

In the third experiment, 92 trees grafted on sour orange were analyzed with 3DF1, 3CA5, and MC13 in ELISA-DAS. Thirty-five trees were without apparent symptoms of tristeza (except for honeycombing in the sour orange bark), 27 trees showed early decline symptoms, and 30 trees showed severe decline. The trees were selected near Moncada, Valencia.

To confirm CTV infection, all samples were previously analyzed by ELISA-DAS biotin/streptavidin with a mixture of MCAs 3DF1 and 3CA5 (INGENASA) that reacts with all CTV isolates tested (5).

RESULTS

Seven groups were defined in the IVIA collection of CTV isolates according to the reaction pattern with the nine MCAs (Table 1). The most frequent reaction was in group 4 (47.5% of the isolates). These isolates reacted to all MCAs except MC13 and 10E3. Group 3 was next with 23.7% of the isolates. These reacted to all MCAs except MC13. The least frequent reaction patterns were those of groups 5 and 6, which included only 1.7% of the isolates in the IVIA collection.

All isolates assayed reacted with at least five MCAs, and 17% of the isolates reacted with all the MCAs assayed (group 1).

At least six epitopes were detected, five of them are represented, respectively, by MCAs MC13, 10E3, IIIA5, 4H6H, and 3DF1. The last epitope is defined by the four MCAs that reacted with all CTV isolates tested (IIA1, MC14, 3BH6, and 3CA5). The epitope represented by MC13 is the least frequent and was present only in groups 1 and 3. The epitope represented by 3DF1 is present in all groups except 7, and the epitope(s) represented by the MCAs IIA1, MC14, 3BH6, and 3CA5 is (are) present in all CTV isolates tested.

Changes in epitope composition of specific isolates of the IVIA collection were observed after aphid transmission, propagation in different hosts, and after graft transmission. The parent source of isolate T.397 reacted with all MCAs; but a subculture, T.397-P, obtained by transmission with *A. gossypii* failed to react with MCAs MC13, 10E3, IIIA5, and 3DF1. Similarly, isolate CA.343, when maintained in Mexican lime, failed only to react with MCAs MC13 and 10E3; whereas, when maintained in Lisbon lemon, it also failed to react with the MCAs IIIA5 and 3H6H. Isolate T.385 reacted negatively only with MC13; but a graft-transmitted sub-isolate, T.385-37, failed also to react with MCAs 10E3 and IIIA5.

Analysis of samples in the first field survey showed that up to 18% of the infected trees from areas with more than 80% of CTV incidence reacted with all MCAs assayed, whereas only 1.1% of the samples from areas with CTV incidence between 10% and 20% reacted to all MCAs, and none of the

TABLE 1
SEROGROUPS OF 59 SPANISH CITRUS TRISTEZA VIRUS (CTV) ISOLATES FROM THE IVIA COLLECTION DEFINED BY REACTIONS WITH NINE DIFFERENT MONOCLONAL ANTIBODIES

MCA	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
MC13	+		+				
10E3	+	+	+				
IIIA5	+	+	+	+			
4H6H	+	+		+	+		+
3DF1	+	+	+	+	+	+	
IIA1	+	+	+	+	+	+	+
MC14	+	+	+	+	+	+	+
3BH6	+	+	+	+	+	+	+
3CA5	+	+	+	+	+	+	+
Representative isolate	T.388	T.300	T.373	T.302	T.385-37	Ca.343-2	T.397-p
Proportion in collection	17%	23.7%	3.4%	47.5%	1.7%	1.7%	5%

samples from areas with less than 2% incidence of CTV reacted with all MCAs.

In the second experiment, analysis of 3,231 field samples by the ELISA-DAS biotin/streptavidin system showed that 100% of the samples reacted with MCA 3CA5, 99.63% of them were recognized by MCA 3DF1, and only 8.70% reacted against MC13.

In the last experiment with 92 trees grafted on sour orange, the 35 trees without decline symptoms reacted with 3DF1 and 3CA5 but not with MC13, while all 27 trees with early decline symptoms reacted with 3DF1 and 3CA5, and only one of 27 reacted with MC13. Of the 30 trees with severe tristeza decline symptoms, only three reacted positively with MC13, whereas all reacted with 3DF1 and 3CA5.

DISCUSSION AND CONCLUSIONS

At least six different epitopes and seven distinct serological reaction patterns were found among the Spanish CTV isolates kept in the IVIA collection. In a complementary study of an international collection of CTV under quarantine at USDA facilities in Beltsville, Maryland, at least 16 different serogroups could be defined (Garnsey et al., unpublished results). These results show that it is possible to detect serological variability in the coat protein among CTV isolates and that diversity increases as number of geographical areas tested increases. The serological diversity of Spanish CTV isolates is less than that found worldwide. Analysis of a group of Brazilian CTV isolates (kindly provided by Dr. G. Müller) with the same panel of MCAs in 1992 revealed only one serogroup (every isolate reacting with all MCAs). This serological homogeneity probably reflects the presence of complex strain mixtures in field trees where the isolates were obtained and supports our observations in Spain, that the percentage of isolates reacting to all MCAs is highest where CTV is most common.

In Table 1 we show an isolate type representing each serogroup. The isolates reacting with all MCAs (group 1) are generally severe and some of them induce seedling yellows and/or stem pitting on sweet orange and grapefruit. These isolates were generally detected in illegal budwood introductions or were derived from these original sources by aphid transmission. They are maintained in the IVIA collection, but they do not represent isolates common in Spain and, perhaps, no longer exist in field trees.

The most common isolates belong to groups 2 and 4 and are represented by isolates T.300 and T.302, respectively. These isolates are highly transmissible by aphids and are symptomless or induce mild or moderate symptoms in Mexican lime. Many isolates of these groups cause decline of trees on sour orange.

Changes in epitope composition involving the loss of up to four epitopes were observed after aphid transmission of some isolates. Similar changes were also observed after propagation in different hosts or in common propagation of isolates by graft transmission. Changes in biological activity and DNA pattern after filtration through different hosts or after graft multiplication have been reported in Spanish isolates (20,22). Our results confirm these changes and indicate that, in some cases, they may affect the CTV coat protein. Variations in the characteristics of CTV isolates after aphid transmission or passage through certain hosts have been observed by several authors using biological indexing (16, 22, 25,30), dsRNA analysis (18, 20, 22), or serological reactivity (19). These results, and those reported here, are an indication that mixtures of CTV strains occur in field trees.

Mixtures of isolates are more likely to occur in areas with high CTV incidence. This is probably the reason for the presence in these areas of a high number of trees (18%) reacting with all MCAs, compared with the low ratio of trees showing this reaction pattern (0.53% of 863 samples) in areas with less CTV incidence (20% to less than 2% CTV infected trees).

The reaction with MC13 was not necessarily correlated with the decline of trees on sour orange under Spanish conditions. Only 10% of the trees which were showing severe decline in the Moncada area of Valencia reacted against MC13. Nevertheless, known severe isolates from the IVIA collection reacted with MC13 as previously reported (24). MCA 10E3 (26) was similar to MC13 for recognizing severe isolates. No direct comparison of the relative severity of Spanish decline isolates, which do not react to MC13, has been made with Florida decline isolates, which do react to MC13. Decline isolates from Florida frequently cause stunting and chlorosis in glasshouse-grown sweet orange trees grafted on sour orange. This reaction is uncommon with Spanish isolates.

ELISA-DASI is a good technique to study epitope diversity among CTV isolates since it provides high sensitivity (4) and can be used on a large scale. It is also a convenient technique to analyze small plants since minimal amounts of tissue are required. The MCAs 3DF1 and 3CA5 were the most efficient for detecting Spanish isolates by ELISA-DAS, especially using the biotin/streptavidin system. Currently, a mixture of both monoclonals is being used for detection purposes (5).

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