Mutagenic Analysis and Localization of a Highly Conserved Epitope Near the Amino Terminal End of the Citrus Tristeza Closterovirus Capsid Protein

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ABSTRACT

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The monoclonal antibody (MAb) 3DF1 is the first commercially available citrus tristeza closterovirus (CTV)-specific MAb. It detects a broad spectrum of CTV isolates from various parts of the world. To precisely map the antigenic determinant recognized by 3DF1, the capsid protein (CP) genes of four 3DF1-nonreactive isolates were cloned as complementary DNA and their nucleotide sequences determined. Comparison of the deduced CP sequences of the four nonreactive isolates with those of previously sequenced 3DF1-reactive isolates revealed differences at three positions near their amino terminal ends. The amino acids Asp-2, Lys-13, and Phe-28 were conserved in all the 3DF1-reactive isolates, but they were replaced by Gly, Thr/Asp, and Tyr, respectively, in the CPs of the nonreactive isolates. Site-specific mutations were introduced into the cloned CP genes of the 3DF1-nonreactive isolate B215 and the 3DF1-reactive isolate T36. The serological reactivities of the wild-type and mutant CPs of B215 and T36 expressed as recombinant fusion proteins in *Escherichia coli* were evaluated by Western blot analysis. A point mutation (A→G) resulting in an Asp→Gly change at amino acid position 2 of the CP of isolate T36 abolished the reactivity with the MAb, whereas a reverse mutation resulting in a Gly→Asp change at the same position conferred reactivity on the CP of the nonreactive B215 isolate. The implications of the observed antigenic diversity on virus detection are discussed.

Citrus tristeza closterovirus (CTV) is the causal agent of one of the most economically important diseases of citrus worldwide (2). The virus consists of a single-stranded, positive-sense RNA genome of approximately 20,000 nucleotides packaged in flexuous particles 2,000-nm long (1). The capsid protein (CP) is encoded by a 669-nucleotide (nt) open reading frame with a calculated M_r of 25,000 (25 kDa) (38), which is located near the 3′ end of the CTV genome (26). The CP genes of several biologically and geographically distinct CTV isolates have been cloned and sequenced, and sequence comparisons reveal that there is over 80% sequence identity among various CTV strains (19,30). Phylogenetic analysis of the CP sequences reveal that the sequence information can potentially be correlated with the biological properties of the virus isolates (mild versus severe) (22).

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There is a great degree of diversity in the symptoms caused by CTV. The most common and economically important symptom patterns are the decline of sweet orange trees grafted on sour orange rootstock or pitting of stems and/or branches of trees irrespective of the rootstock and scion combination. Isolates that induce varying degrees of such severe symptoms and those that cause only mild symptoms in Mexican lime without causing decline or stem pitting have been described (6). A standardized host reaction test has been described to better characterize the biological differences of various CTV isolates (7).

Successful management of tristeza involves citrus sanitation and certification programs to prevent the introduction of CTV (21) and the eradication of infected trees when the CTV incidence is low (16). Both approaches require early and efficient virus detection (35), and several CTV-specific monoclonal antibodies (MAb) were developed in recent years for this purpose (34). Among them, MAb 3DF1, developed in Spain, is the first MAb that became commercially available (42). It can detect most of the

CTV isolates from different parts of the world irrespective of their biological properties (S. M. Garnsey, *unpublished data*) and more than 3.5 million tests have been made with it in Spain and other citrus producing countries (M. Cambra, *unpublished data*).

Electrophoretic analyses of the CTV preparations show, in addition to the intact 25 kDa of CP, the presence of at least two smaller hydrolysis products, CP1 and CP2 of M_r 24 kDa and 21 kDa, respectively (17,39). The 21 kDa of CP2 is more pronounced than the 24 kDa of CP1 on both the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and Western blots. The 24 kDa of CP1 may be absent in some preparations depending on the citrus host and the tissue extraction method used (38; H. R. Pappu, *unpublished data*). While the majority of the MAbs tested detect all three CP forms (intact CP, CP1, and CP2), MAb 3DF1 reacts only with the intact CP and not with CP1 and CP2 (10,39). Previous studies suggest that CP1 and CP2 originate as specific hydrolysis products of the intact CP. Direct protein sequencing of all three CP polypeptides showed that CP1 may result from cleavage of the intact CP anywhere between amino acid positions 11 and 15 from the amino terminus, whereas CP2 may result from cleavage between amino acid positions 25 and 26 of the amino terminus of the intact CP (38). CP1 and CP2, therefore, lack the amino terminal region of the intact CP. Since 3DF1 does not recognize CP1 and CP2, it was proposed that the epitope it recognizes was localized within the amino terminal 26 amino acids of the intact CP (38).

MAb 3DF1 detects over 95% of the approximately 220 biologically diverse CTV isolates described from over 26 countries (S. M. Garnsey, *unpublished data*). Because of its broad spectrum reactivity to various CTV isolates, it is the most widely used CTV-specific MAb in the world (15; M. Cambra and S. M. Garnsey, *unpublished data*). Its broad specificity suggests that the sequence of the amino terminal region of the CTV CP is highly conserved among various geographically and biologically diverse isolates of CTV. Precise mapping of the sequences that contribute to the epitope recognized by 3DF1 would help in understanding the basis for the serological diversity of the virus and devising more reliable detection methods. The sequences of the CP genes of various CTV isolates were used as the basis to test the effect of amino acid substitutions on the specificity of 3DF1 and subsequently to map its highly conserved epitope on the CTV CP. A preliminary report of these results has been published (28).

MATERIALS AND METHODS

Virus isolates. CTV isolates used in this study are listed in Table 1. The origin and biological and serological properties of the CTV isolates M16A and M27A are described by Kano et al. (12). These isolates, redesignated and hereafter referred to as B188 and B215, respectively, are not recognized by 3DF1. Two additional 3DF1-nonreactive isolates, Sat/JC-DW-5 (41) redesignated as B213 and T397P from Spain (3), were used. A 3DF1-reactive isolate, T36 described previously from Florida (36), also was used. All the isolates were maintained under quarantine at the Exotic Citrus Pathogen Collection Facility at the Beltsville Agricultural Research Center of the United States Department of Agriculture, Beltsville, MD (9).

TABLE 1. List of citrus tristeza closterovirus (CTV) isolates used in the study

Isolate	Origin	Symptom severity[a]	Serological reactivity[b] MCA-13	3DF1	1053	Reference
B188[c]	Japan	mild	–	–	+	12
B213[c]	Korea	mild	–	–	+	41
B215[c]	Japan	mild	–	–	+	12
T397P	Spain	mild	–	–	+	3
T36	Florida	severe	+	+	+	36
T30	Florida	mild	–	+	+	36

[a] Symptom severity is expressed as a cumulative score obtained by inoculating a selected group of indicator host species (7).
[b] Serological reactivity of the isolates determined by ELISA and Western blotting with selected CTV-specific antibodies. Monoclonal antibody MCA-13 reacted primarily with severe isolates that induce decline and/or stem pitting on infected citrus (32). Monoclonal antibody 3DF1 reacted with the majority of the isolates irrespective of their biological activity. Polyclonal antisera 1053 prepared to purified CTV preparation reacted with all of the isolates used in this study.
[c] The isolates designated in the B series are named according to Garnsey et al. (9). B188 was originally described as M16a (12), B213 as Sat/JC-DW-5 (41), and B215 as M27a (12).

Cloning, sequencing, and in vitro mutagenesis of the CP genes. Using total nucleic acid extracts of CTV-infected citrus leaf tissue (25), the CP genes of B188, B213, B215, and T397P were amplified using a coupled, one-tube, reverse transcription polymerase chain reaction (RT-PCR) procedure adapted from Pappu et al. (31). The oligonucleotide 5′ CCGAGCTTCTTAACCTACGCG 3′ was used as the genome-sense primer. It anneals to the CTV sequence 133 nt upstream of the CP gene (26,38). The oligonucleotide 5′ CACTACCGTCCGCTATAATCG 3′, which anneals 158 nt downstream of the CP gene, served as the genome-antisense primer (26,38). The resulting polymerase chain reaction product, which included the CP gene and the flanking sequences, was cloned into *Sma*I-digested pUC118 and the double-stranded DNA templates were sequenced using Sequenase 2.0 kit (U.S. Biochemical, Cleveland). All DNA manipulations were done as described by Sambrook et al. (37). Sequences were analyzed using the software package from the University of Wisconsin's Genetics Computer Group (5), Clustal V (11), or Seqaid II (33). Site-directed in vitro mutagenesis was performed according to Kunkel et al. (13). M13 forward- and reverse-sequencing primers and the oligonucleotides used for in vitro mutagenesis were purchased from the DNA Synthesis Core Facility of the Interdisciplinary Center for Biotechnology Research, University of Florida.

Expression of recombinant CP in *Escherichia coli*. For the expression of the wild-type and mutant CPs of B215 and T36 as fusion proteins, the respective CP genes were subcloned into a pETH3a vector (20) using primers specific to the 5′ and 3′ ends of the CP gene. Oligonucleotides 5′ TATGGGCGACGAAACAAAG 3′ and 5′ TATGGACGACGAAACAAAG 3′ served as the genome-sense primers for B215 and T36 CP genes, respectively. Oligonucleotide 5′ GAATTCGCGGCCGCTCAACGTGTGTTAAATTTCC 3′ served as the genome-antisense primer for both CP genes. Following RT-PCR, the resulting 700-bp polymerase chain reaction products were initially cloned into *Sma*I-digested pUC118 and

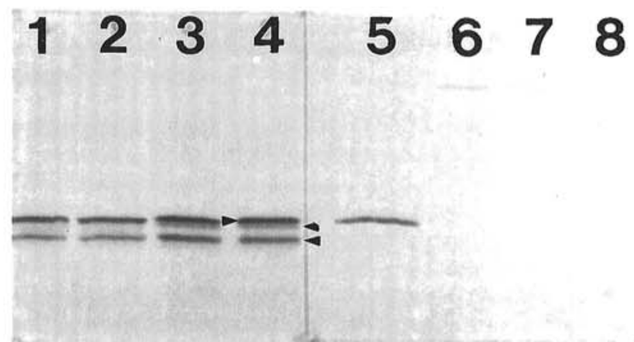

Fig. 1. Western blot analysis of citrus tissue extracts from plants infected with 3DF1-reactive and -nonreactive isolates of citrus tristeza closterovirus (CTV). Lanes 1 to 4 were treated with CTV-specific polyclonal antibodies. Lanes 5 to 8 were treated with monoclonal antibody 3DF1. Lanes 1 and 6, CTV isolate B188; lanes 2 and 7, CTV isolate B213; Lanes 3 and 8, CTV isolate B215; lanes 4 and 5, CTV isolate T36. In addition to the intact capsid protein (CP), the smaller hydrolysis products, CP1 and CP2 of the intact CP, also were detected by polyclonal antibodies (lanes 1 to 4), whereas 3DF1 reacted only with the intact CP (lane 5). CP1 is more pronounced in lanes 3 and 4 than in lanes 1 and 2. The three CP polypeptides (intact CP, CP1, and CP2, from top to bottom, respectively) are indicated by arrowheads in lane 4.

then subcloned as *Xba*I/*Eco*RI fragments into *Nhe*I/*Eco*RI-digested pETH3a. Cloning into a pETH vector using the above restriction sites resulted in six additional codons at the 5′ end of the CP genes of T36 and B215. Induction and purification of the fusion proteins from *E. coli* strain BL21(DE3)pLysS were performed as described previously (18,40).

Western blotting. Western blot analysis of SDS-PAGE-separated proteins (14) was performed using CTV-specific polyclonal antibodies (PAb) (1053) produced against purified preparations of isolate T26, or MAbs MCA-13 (32) or 3DF1 (42).

RESULTS

Serological specificity of 3DF1. On Western blots of tissue extracts from plants infected with a 3DF1-reactive CTV isolate, the smaller hydrolysis products of the intact CP were not detected by 3DF1 (Fig. 1, lane 5), whereas MAbs and PAbs developed to CTV generally react with all three CTV-specific CP forms (the intact CP, CP1, and CP2). Examples of this are shown in Fig. 1, lanes 3 and 4. CP2 was more pronounced than CP1 (Fig. 1, lanes 1 and 2). Isolates B188, B213, and B215 did not react with 3DF1 (Fig. 1, lanes 6, 7, and 8, respectively). The serological reactivities of the isolates used are given in Table 1.

Cloning and sequencing of the CP gene. cDNA cloning and sequencing of the CP genes of CTV isolates B188, B213, B215, and T397P showed an open reading frame of 669 nt, coding for a protein of 223 amino acids. The CPs shared more than 90% amino acid sequence similarity with those of previously sequenced isolates (19,29,30). Most of the nucleotide differences were silent and did not result in amino acid sequence differences (Fig. 2). Compared to the CPs of 3DF1-reactive isolates, the CPs of B188, B213, B215, and T397P differed at three amino acid positions all near their amino terminal ends. While all the 3DF1-reactive isolates had Asp (D), Lys (K), and Phe (F) at positions 2, 13, and 28, respectively (30); these amino acids were replaced by Gly (G), Thr (T), and Tyr (Y) in the CPs of 3DF1-nonreactive isolates B188, B215, and T397P; and G, D, and Y in B213 (Fig. 2).

Expression of the CP genes in *E. coli*. The CP genes of 3DF1-reactive and -nonreactive isolates were expressed in *E. coli* as fusion proteins. Each recombinant CP was expressed to a high level and was easily detected by Coomassie blue staining of the SDS-PAGE used to separate the total proteins from the bacterial extracts (data not shown).

Effect of site-directed mutagenesis on 3DF1 reactivity. To evaluate the role of the three variant amino acids in conferring the 3DF1 reactivity, the codon for each of the variant amino acids in the wild-type B215 CP was individually changed to that of the 3DF1-reactive isolate (G→D, T→K, and Y→F). The resulting mutant CP genes were sequenced to confirm the introduction of the desired mutation. The wild-type and mutant CPs were expressed as fusion proteins in *E. coli* and their serological reactivity with 3DF1 was analyzed by Western blotting. Of the three individual mutations, a G→D mutation in the amino acid position 2 of the CP conferred reactivity on the previously 3DF1-nonreac-

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      3DF1
   reactivity
                          I          II
                                                          50
T36     +   MDDETKKLKNKNKETKEGDDVVAAESSFSSVNLHIDPTLITMNDVRQLST
B188    -   -G----------T--------------Y----------------------
B213    -   -G----------D--------------Y----------------------
B215    -   -G----------T--------------Y----------------------
T397P   -   -G----------T--------------Y----------------------

                                                         100
T36         QQNAALNRDLFLTLKGKHPNLPDKDKDFHIAMMLYRLAVKSSSLQSDDDA
B188        --------------------------------------------------
B213        --------------------------------------------------
B215        --------------------------------------------------
T397P       --------------------------------------------------

                                   ↓                     150
T36         TGITYTREGVEVDLSDKLWTDVVFNSKGIGNRTNALRVWGRTNDALYLAF
B188        -----------------------Y--------------------------
B213        -----------------------Y--------------------------
B215        -----------------------Y--------------------------
T397P       -----------------------Y--------------------------

                                                         200
T36         CRQNRNLSYGGRPLDAGIPAGYHYLCADFLTGAGLTDLECAVYIQAKEQL
B188        --------------------------------------------------
B213        --------------------------------------------------
B215        --------------------------------------------------
T397P       --------------------------------------------------

                                223
T36         LKKRGADDVVVTNVRQLGKFNTR
B188        -----------------------
B213        -----------------------
B215        -----------------------
T397P       -----------------------
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Fig. 2. Multiple alignment of the deduced amino acid sequences (single letter code) of the capsid protein (CP) genes of citrus tristeza closterovirus isolates T36, B188, B213, B215, and T397P. Identical residues are indicated by dashed lines. The revised amino acid sequence of the T36 CP gene is from Pappu et al. (26). The 3DF1 reactivity of each isolate is indicated by + for positive and – for negative. Isolates B188, B213, and B215 were not recognized by the monoclonal antibody MCA-13, and the phenylalanine residue (F) at position 124, found critical for the binding of MCA-13 (29), is indicated by the arrow. The putative proteolytic cleavage sites (38) in the amino terminus of the intact CP resulting in CP1 and CP2 are indicated as I and II, respectively.

tive isolate B215 (Fig. 3, lane 2), while the mutations at amino acid positions 13 and 28 did not have any apparent effect on the CPs' reactivity (data not shown). The *E. coli*-expressed protein preparations showed some nonspecific reaction with 3DF1 (Fig. 3, lanes 1, 2, and 3).

To further confirm the role of the amino acid at position 2 in determining the specificity of the MAb, reverse mutations (D→G, K→T, and F→Y) were introduced into the CP of the 3DF1-reactive isolate T36. Western blot analysis showed that a single amino acid substitution (D→G) abolished the reactivity of 3DF1 with T36 (Fig. 3, lane 3). All the wild-type and mutant CPs of 3DF1-reactive and -nonreactive isolates were recognized by CTV-specific PAbs, thus confirming the antigenic identity of the recombinant fusion proteins expressed in *E. coli* (data not shown).

DISCUSSION

The CTV-specific MAb 3DF1 detects most CTV isolates from different parts of the world. A peculiarity of this MAb is that it does not react with the smaller, apparent hydrolysis products, CP1 and CP2 of the intact CP. By mutagenic analysis of the CTV CP, we precisely mapped the sequences critical for the epitope recognized by 3DF1. Data presented here provide further evidence for the critical role of a single amino acid residue in antibody binding (29). Previous studies led to the speculation that the epitope for MAb 3DF1 is localized near the amino terminus of the CP (38). Computer-based analysis of the CPs of 3DF1-reactive isolates failed to precisely identify the region involved in the antigenic determinant, except that the N terminal 15-amino acid stretch was one of the three regions that showed a relatively higher antigenic index (data not shown). The observation that MAb 3DF1 failed to react with certain CTV isolates was exploited by cloning and sequencing the CP genes of these nonreactive isolates for comparison with the CP genes of reactive isolates. Sequence comparisons identified the amino acids that could potentially contribute to the antigen-antibody specificity (Fig. 2). Subsequent mutational analyses showed that the amino acid at position 2 in the CP was critical if the epitope was to be recognized by MAb 3DF1. A hydrophilic, negatively charged residue (Asp) conferred reactivity on an otherwise nonreactive CP, and its replacement by another hydrophilic but uncharged residue (Gly) abolished the serological reactivity with 3DF1. Further mutations

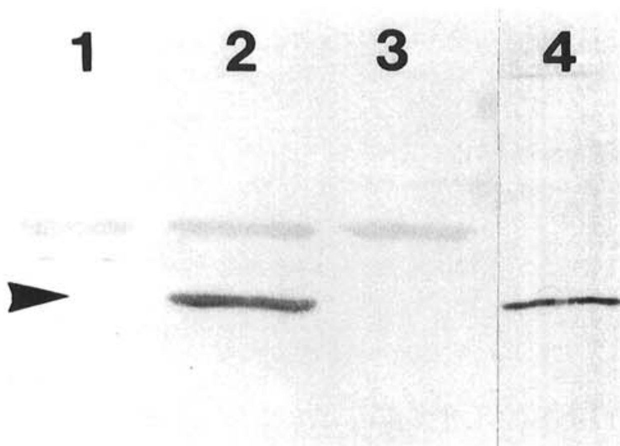

Fig. 3. The effect of site-directed mutations introduced into the citrus tristeza closterovirus (CTV) capsid protein (CP) on the reactivity of CTV-specific monoclonal antibody 3DF1. The mutant CPs were expressed as recombinant fusion proteins in *E. coli* and were analyzed by Western blot. Lane 1, proteins induced by a pETH vector without the CP gene; lane 2, *E. coli*-expressed mutant CP (G→D) of B215; lane 3, *E. coli*-expressed mutant CP (D→G) of T36; lane 4, extract of T36-infected tissue. The position of the CTV CP is indicated by an arrowhead.

aimed at substituting chemically different amino acid residues at this site, as well as mutations in the flanking sequence, should reveal more information on the specificity of the binding of 3DF1.

The broad specificity of MAb 3DF1 can be explained by the fact that the amino terminal sequence of the CP is highly conserved among geographically and biologically distinct isolates of CTV whose CP genes have been sequenced to date (18,19,29,30). However, the sequence divergence seen at the amino terminal regions of the four 3DF1-nonreactive isolates from geographically distinct areas including Spain, Japan, and Korea led us to speculate that variants of CTV may arise as a consequence of independent mutational events. Alternatively, the various 3DF1-nonreactive isolates might have originated from a single source and been spread by the movement of infected plant material.

The finding that 3DF1 reacted with the CP of CTV in Western blots suggested that the epitope for the MAb was largely dependent on primary structure rather than on conformation of the CP. Its localization near the amino terminal region of the CP suggested that it was probably surface exposed. This is supported by the fact that virus particles attached to 3DF1-coated electron microscope grids (42). Moreover, the antigen used for the production of the MAb was prepared under nondenaturing conditions (42) that are expected to preserve the native antigenic structure of the virion. Serological studies of various CTV-specific MAbs suggest that the CP contains several epitopes (3,8,43). In previous work, the critical amino acid residue in the epitope for the CTV-specific MAb MCA-13 is identified by mutational analysis of the CP gene (29), which also may be an indicator of the symptom severity (24). The 3DF1-nonreactive isolates used in this study are not known to cause decline and/or stem pitting and were not reactive with MCA-13 (Table 1). Sequence analysis revealed that the phenylalanine residue (Phe-124), which is critical for the binding of MCA-13 (29), was absent in these isolates (Fig. 2). Mapping the epitopes identified by other CTV MAbs would provide a better understanding of the nature and distribution of the antigenic determinants, as well as the molecular basis for the antigenic diversity of the CTV CP. Such information would be useful in formulating decisions on the choice of various monoclonals for field surveys in a given geographic region. Using deletion constructs of the CTV CP expressed in *E. coli*, regions of the CP that are potentially involved in the specificity of selected CTV-specific MAbs are being delineated (23). By using this approach, the epitope for CTV-specific MAb 3E10 from Taiwan (41), which appears to be common to most CTV isolates, was localized near the carboxy terminal region of the CP (O. Nikolaeva and R. F. Lee, *unpublished data*).

We have demonstrated that the antigenic determinant for MAb 3DF1 is localized near the amino terminal end of the intact CP of CTV. This conclusion was supported by molecular analyses and the observation that it was susceptible to hydrolysis by plant proteases, with the result that the smaller CP1 and CP2 were not detected by 3DF1. The removal of amino acids near the amino terminus reduced the amount of intact CP molecules available in tissue extracts for detection of CTV by 3DF1. The data presented here and previously (29) demonstrate that a point mutation in the CTV CP gene, resulting in a single amino acid substitution, could drastically alter the serological reactivity of a MAb. This might also explain the basis for the existence and/or emergence of new serotypes of CTV. In view of the antigenic diversity extant among CTV isolates, new variants may exist or arise that may not be detected by a given MAb (27). Detection can be further complicated if the antigenic determinant is subject to removal by host proteolytic enzymes as in the case of MAb 3DF1. To preclude such false negatives, it is advisable to use a combination of MAbs (4) or PAbs that recognize different epitopes on the CTV CP to improve the sensitivity of CTV detection and the effectiveness of certification programs.

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