Generation of a Genetically Engineered MCA13 Nonreactive Variant of the T36 Decline Isolate of *Citrus tristeza virus*

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ABSTRACT. A full-length infectious clone of the T36 isolate of *Citrus tristeza virus* (CTV) was modified to inactivate the epitope detected by the selective monoclonal antibody MCA13. The MCA13 reactive site, which was mapped to a single amino acid residue has been correlated with the presence of severe isolates of CTV in Florida. An MCA13 nonreactive full-length cDNA clone (pCTV9R-MCA13NR) was engineered from an MCA13 reactive full-length infectious cDNA clone (pCTV9R) by a single nucleotide change, UUU (Phe) to UAU (Tyr), at amino acid position 124 in the CP gene. Progeny virions from transcript-inoculated *Nicotiana benthamiana* protoplasts were further amplified and used to infect young citrus trees. Although the virions were not reactive to MCA13, the initial results suggest that CTV9R-MCA13NR produced symptoms on alemow, Mexican lime, and sour orange plants similar to those of the cloned wild type virus. These data suggest that pathogenicity of CTV isolates is not specific to the MCA13 reactive epitope. Since the MCA13-reactive and -nonreactive variants of CTV isolate T36 differ by only a single nucleotide in the CP gene, but can be readily distinguished serologically, they may be useful for testing cross protection mechanisms.

Citrus tristeza virus (CTV) is a member of the genus *Closterovirus* of the *Closteroviridae*, a family with members having monopartite genomes (*Closterovirus*, *Ampelovirus*) and bipartite or tripartite genomes (*Crinivirus*) (10, 11). CTV has long, flexuous, filamentous (2000 × 10-12 nm) bipolar particles with two coat proteins: a major coat protein (CP) covering >95% of the genome, and a minor coat protein (CPm) encapsidating the 5' 630 nucleotides (nt) of the genome (3, 23). The 19.3 kilobase single-stranded positive-sense RNA genome is organized into 12 open reading frames (ORFs) (Fig. 1A) (9, 15). The 5' ORFs 1a and 1b are translated directly from the genomic RNA and expressed through polyprotein processing and +1 ribosomal frameshifting. The ten internal ORFs are expressed from 3' coterminal subgenomic (sg) RNAs (7). A full-length infectious cDNA clone was developed for CTV T36, an MCA13 reactive Florida decline-inducing isolate, and the *in vitro*-produced RNA transcripts from the cDNA clone initiated replication in *Nicotiana benthamiana* protoplasts (19). Progeny virions derived from the infectious clone were serially passaged through *N. benthamiana* protoplasts and used to infect citrus plants, thus fulfilling Koch's postulates for this genetically complex plant virus (21).

CTV is one of the most economically important virus diseases of citrus because of the severity of damage it causes and the value of individual citrus trees, whose productive life can span up to 100 years (2). CTV induces severe stem pitting and decline diseases of citrus trees depending on the virus isolate, citrus cultivar and/or the scion/root stock combination. However, some CTV isolates or populations cause mild symptoms in Mexican lime indicator hosts and induce no decline or stem pitting symptoms. Bioindexing on specific indicator hosts (4) is the most reliable method to examine the type and severity of symptoms caused by CTV isolates. But this is a time consuming and laborious method.

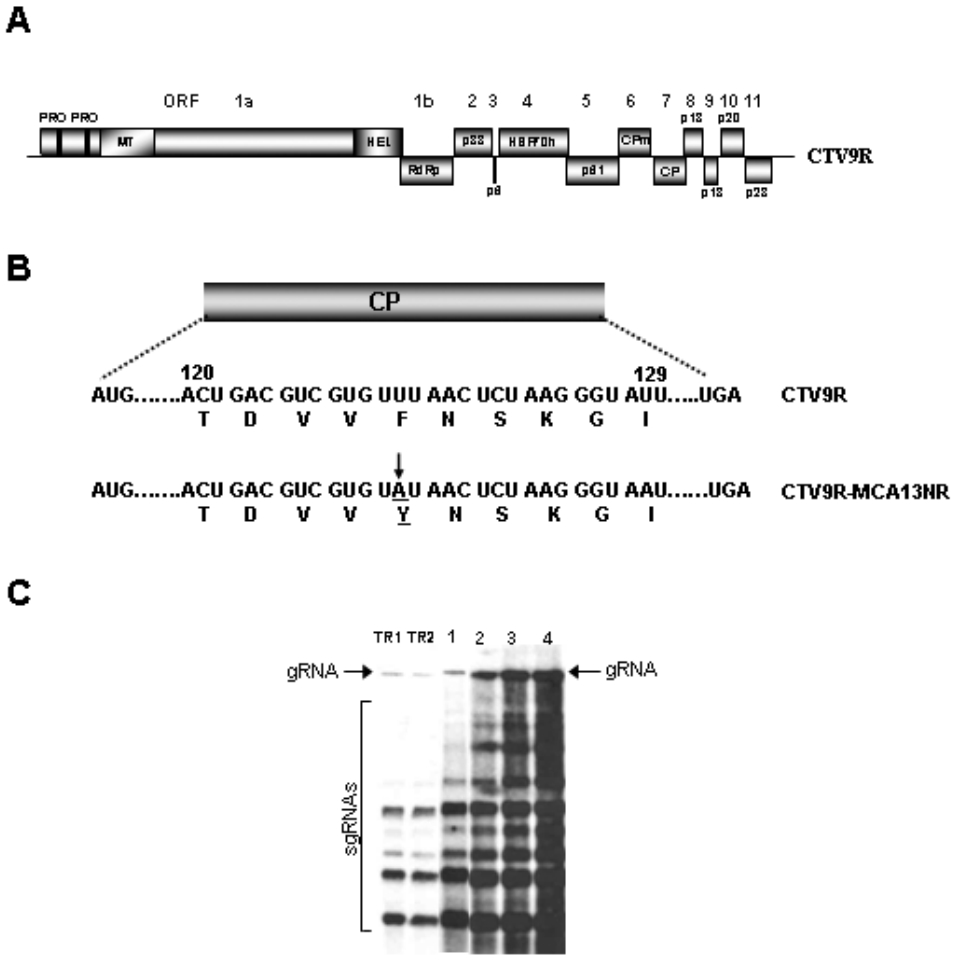

Fig. 1. (A). Genome organization of wild type CTV (CTV9R). Open boxes represent ORFs and their translation products. PRO, papain-like protease; MT, methyltransferase-like; HEL, helicase-like; RdRp, RNA dependent RNA polymerase-like domains; HSP70h, HSP70 homolog; CP, major coat protein; CPm, minor coat protein. (B). Enlarged view of CP ORF, showing codons encoding amino acids 120 to 129 of MCA13 reactive virus (CTV9R) and MCA13 non-reactive virus (CTV9R-MCA13NR). Single nucleotide change (UUU→UAU) that results in change from phenylalanine (F) to tyrosine (Y) at amino acid 124 is indicated with an arrow and underlined. (C). Hybridization analysis of amplification of CTV9R-MCA13NR in *N. benthamiana* protoplasts. Samples of total RNA from protoplasts inoculated with *in vitro* transcripts of CTV9R (lane TR1) and CTV9R-MCA13NR (lane TR2) harvested at 4 days post-inoculation. Total RNA from protoplasts inoculated with progeny virions of CTV9R-MCA13NR from the first (1), second (2), third (3), and fourth (4) rounds of protoplast passages. Northern hybridization blots were carried out using a 3' positive-stranded RNA specific riboprobe. Positions of the genomic and 3' coterminal sgRNAs are indicated by an arrow and a bracket, respectively.

The monoclonal antibody MCA13, which was developed against CTV T36, reacts with decline isolates in Florida and a majority of decline and stem pitting isolates from various citrus growing regions (16). In general, MCA13 fails to react with most mild isolates and is used as a presumptive rapid indicator for isolates capable of inflicting economic damage. MCA13 is used to screen plant material in the Florida citrus

budwood registration program to preclude propagation of budwood containing potentially damaging isolates, while allowing propagation of budwood carrying mild isolates already endemic in the state (25).

The MCA13 reactive site was mapped to a single amino acid in the CP by *in vitro* studies in *Escherichia coli* (14). Mutation of a single nucleotide resulting in a phenylalanine to tyrosine change at amino acid position 124 of the CP abolished the MCA13 reactivity of a severe isolate, whereas the CP of a mild isolate with a position 124 change from tyrosine to phenylalanine reacted positively with the MCA13 antibody (14). Since most severe isolates are MCA13 reactive and most mild CTV isolates are MCA13 nonreactive, there is a general perception that MCA13 reactivity is associated with severe isolates of CTV. However, evidence for direct involvement of the MCA13 reactive epitope with symptom induction has not been demonstrated. The infection of citrus plants with the cloned T36 virus (19, 21) allowed us to investigate the *in vivo* involvement of the MCA13 epitope in symptom development or symptom severity.

Here we report engineering an MCA13 non-reactive variant (pCTV9R-MCA13NR) from an MCA13 reactive infectious cDNA clone of CTV T36 (pCTV9R), the infection of citrus plants, and a preliminary evaluation of the symptoms induced on indicator hosts. Future applications of serologically distinct, nearly identical variants of CTV isolate T36 are also discussed.

MATERIALS AND METHODS

Virus isolates and indicator plants. A full-length cloned virus (CTV9R) of decline isolate CTV T36, reactive to MCA13 and inducing a mild to moderate seedling yellows reaction in sour orange (21); and wild type CTV T30, an MCA13 nonreactive Florida mild isolate (1), were maintained in Alemow and Mexican lime plants in a growth room at Citrus Research and Education Center (CREC), Lake Alfred, FL. Healthy Alemow, Mexican lime, and sour orange seedlings were propagated in a greenhouse.

Bioindexing on indicator hosts. Biocharacterization of the MCA13 nonreactive virus was done by graft-inoculating four to six seedlings of 6-9 mo old Alemow, Mexican lime, and sour orange. Inoculations with the parental CTV9R and wild type CTV T30 were included as controls. The graft-inoculated plants were incubated in a growth room or air conditioned greenhouse for monitoring symptom development. Bark from twigs of Alemow and Mexican lime plants was removed for assessment of stem pitting symptoms 4-6 mo post-inoculation. Bioindexing experiments were repeated at least twice.

Construction of MCA13 nonreactive full-length cDNA clone. An MCA13 nonreactive cDNA clone was engineered from a full-length cDNA clone (pCTV9R) of the MCA13 reactive Florida CTV isolate T36 (19, 22) by a single nucleotide change, UUU (Phe) to UAU (Tyr) at nucleotide position 16522, using mutagenic oligonucleotides for PCR amplification (Fig. 1B) followed by overlap extension PCR (8). For cloning convenience, PCR products were first ligated into pCTV9R-Δp33 (20) and later transferred into pCTV9R to obtain pCTV9-MCA13NR.

A positive primer C-579 [*5'-GCAAT*CTCGAGACTAGTTAGTGC TGTCTCTCCGTAT ATC-3' with an *Xho* I restriction site (underlined) and nonviral sequence (italics)] corresponding to nt positions 11658-11685 and a negative primer C-1158 (5'-AATACCCTTAGAGTTA**T**ACAC-GACGTCAGTCCA-3' mutated nt in bold and underlined) corresponding to positions 16538-16506 were used to amplify a fragment between nts 11658 and 16538. A second fragment was amplified between nts 16506 and 17298 using a plus sense primer C-1157 (5'-TGGACTGACGTCGTGT-

ATAACTCTAAGGGTATT-3', mutated nt in bold and underlined) corresponding to nt 16506-16538 and a minus sense primer C-538 (5'-CGTGTCTAAGTCACGCTAAACAA AGTGAC-3') corresponding to nt positions 17298-17269. Both PCR products were amplified with *Pfu* DNA polymerase (Stratagene) using pCTV9R as a template. The gel purified PCR products were used as templates for overlap extension PCR (8) with C-579 and C-538 primers using Vent DNA polymerase (New England Biolabs, Inc., Beverly, MA). The amplified overlap PCR product was digested with *Xho* I (present in C-579 primer) and *Pst* I (at nt 17205) and ligated into a similarly digested pCTVR-Δp33 (20) to obtain pCTVR-Δp33 (MCA13NR).

The DNA fragment between *Pme*I and *Not*I restriction sites (nts 11869-19293) from pCTVR-Δp33(MCA13NR) was ligated into *Pme*I and *Not*I digested pCTV9R to obtain pCTV9R-MCA13NR.

The ligation products were transformed into *Escherichia coli* strain JM109 and plated on LB agar-amp plates. The LB agar plates were incubated overnight at 37°C, followed by 2 days at room temperature (22). The colonies were screened by mini-prep analysis (18), and the presence of the desired point mutation in the CP gene was confirmed by DNA sequencing with an automatic sequencer (Applied Biosystems, model 373) at the Interdisciplinary Center for Biotechnology Research DNA sequencing core facility of the University of Florida, Gainesville, FL.

Amplification of CTV9R-MCA13NR in *N. benthamiana* protoplasts. Isolation and inoculation of *N. benthamiana* mesophyll protoplasts with *in vitro*-produced RNA transcripts from *Not*I-linearized pCTV9R-MCA13NR were as described previously (12, 19). Protoplasts were harvested at 4 days post-inoculation (dpi) and divided into two portions. One half was used to isolate total nucleic acids to examine the levels of replication; the other half was stored at -70°C for subsequent extraction of progeny virions to inoculate the next batch of protoplasts as described in Satyanarayana et al. (21). Virions in crude sap were amplified by eight successive passages in protoplasts. The final passage of virions was inoculated to a larger set of protoplasts ($10\text{-}12 \times 10^6$) to create virus inoculum for infecting citrus plants. Virions were extracted from frozen protoplasts by suspending in 40 mM sodium phosphate buffer, pH 8.2, and further concentrated by centrifugation on a 70% sucrose cushion as described (17).

ELISA and Western blot analyses. Double antibody sandwich indirect ELISA (DAS-I ELISA) was performed as described previously (5). Purified IgG from rabbit polyclonal antibody CTV-908 (1 µg/ml) was used as coating antibody. MCA13 and a broadly reactive CTV monoclonal antibody ECTV172 were used as detecting antibodies.

Virions from wild type CTV T30-, CTV9R-, and CTV9R-MCA13NR-infected plants were partially purified by centrifugation (38,000 rpm) through a 20% sucrose cushion in a SW41 rotor for 75 min at 4°C. The virus pellet was suspended in 1 × Laemmli buffer [50 mM Tris-Cl (pH 6.8), 2.5% β-mercaptoethanol, 2% sodium dodecyl sulfate, 0.1% bromophenol blue, and 10% glycerol] for SDS-PAGE, followed by immunoblot analysis. The blots were developed using the ECL Western blotting system using MCA13 and broad spectrum CTV-908 polyclonal antibodies against CP as per the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ).

RESULTS AND DISCUSSION

Development of the MCA13 nonreactive full-length cDNA clone. A full-length cDNA clone

pCTV9R (19, 22) of CTV T36, an MCA13 reactive Florida decline isolate, was modified into an MCA13 non-reactive virus (CTV9R-MCA13NR) by a single nucleotide change—UUU (Phe) to UAU (Tyr) at nucleotide position 16522—using mutagenic oligonucleotides for PCR amplification, followed by overlap extension PCR (see Materials and Methods section, Fig. 1B).

Amplification of CTV9R-MCA13NR in protoplasts. Previously, we developed a method to infect citrus plants with virions amplified by serial passages in *N. benthamiana* protoplasts (21). *In vitro*-generated RNA transcripts were used to inoculate *N. benthamiana* protoplasts by PEG-mediated transfection. Total nucleic acids were extracted from 4 dpi protoplasts and analyzed by Northern blot hybridization using a 3' end plus-strand RNA specific riboprobe. The *in vitro* transcripts from pCTV9R-MCA13NR initiated replication and produced genomic and sgRNAs approximately similar to that of the wild type CTV9R (Fig. 1C, compare TR1 and TR2). As expected, a single nucleotide change in the CP gene did not affect the replication of the MCA13 non-reactive virus, since it was reported previously that none of the ten 3' terminal genes of CTV is required for replication of the virus in protoplasts (19).

We extracted the progeny virions from the other half of transcript inoculated protoplasts and inoculated subsequent batches of protoplasts to amplify the progeny virions (Fig. 1C). By the seventh sequential passage the titer of progeny virions had been amplified to the maximum potential level. A set of 12 independent inoculations, each with ~1×10^6 protoplasts, was done with virions of the seventh passage.

The progeny virions from the final batch of protoplasts were examined for MCA13 reactivity by DAS-I ELISA. They failed to react with MCA13 antibody, but reacted strongly with broad spectrum CTV monoclonal antibody ECTV172 (data not shown). This result suggests that the virus retained the single nucleotide change in the CP gene, and that the mutation was not reverting during passage in protoplasts.

Infection of citrus plants with CTV9R-MCA13NR. Virions were concentrated and partially purified from extracts of the final set of protoplasts by centrifugation on a 70% sucrose cushion. Virus-containing fractions from the sucrose cushion were used to bark-flap inoculate four seedlings of Alemow (17). The test plants were pre-indexed by ELISA to eliminate the possibility of unintentional infection during their propagation in the screen house. Inoculated plants were pruned 2 weeks after inoculation to encourage production of fresh shoots. After 6-8 weeks, testing by DAS-I ELISA indicated all four inoculated plants were infected (Table 1).

ELISA and Western blot analyses of virions from CTV9R-MCA13NR-infected plants. The CTV9R-MCA13NR-infected plants failed to react with MCA13 antibody in DAS-I ELISA (Table 1). However, these plants reacted positively with ECTV172 antibodies, with virus concentrations comparable to that of wild type CTV9R (Table 1). As expected, CTV T30 (mild isolate) failed to react with the MCA13 antibody but reacted with ECTV172. Wild type CTV9R reacted positively with MCA13 as well as ECTV172 antibodies (Table 1).

We also examined partially purified virions from CTV9R-MCA13NR-infected plants nine months after infection by Western immunoblot analysis using MCA13 and CTV-908 IgG antibodies. Virions from CTV9R- and CTV T30-infected plants were used as controls. Virions from CTV9R-MCA13NR- and CTV T30-infected plants failed to react with MCA13 antibody, but reacted strongly with CTV-908 IgG antibod-

TABLE 1
REACTIVITY OF VIRIONS FROM CTV9R-MCA13NR INFECTED PLANTS TO MCA13 AND ECTV172 ANTIBODIES IN DAS-I ELISA

Virus	MCA13[a]	ECTV172[b]
CTV9R-MCA13NR	0.11 ± 0.007[c]	3.38 ± 0.023
CTV9R	1.21 ± 0.019	3.36 ± 0.016
CTV T30	0.11 ± 0.002	3.05 ± 0.025
Healthy	0.10 ± 0.004	0.09 ± 0.001

[a]MCA13 antibody used at 1:20,000 dilution.
[b]ECT172 antibody used at 1:200,000 dilution.
[c]ELISA values (A_{405}) are an average of three wells ± standard deviation.

ies (Fig. 2). Virions from CTV9R-infected plants reacted positively with MCA13 and CTV-908 IgG. These data suggest that the phenylalanine at position 124 in the CP gene is critical in determining the MCA13 reactivity of CTV isolates *in vivo* and support the *in vitro* findings of Pappu et al. (14). It also showed that the point mutation introduced into the CP gene was stable in citrus plants nine months after infection.

Biology of the CTV9R-MCA13NR on indicator hosts. Initial tests were made to compare the biology of CTV9R-MCA13NR to that of CTV9R (21) and CTV T30 (1) on several indicator hosts. CTV9R-MCA13NR produced characteristic vein clearing and leaf cupping symptoms on Alemow and Mexican lime plants 2-3 mo after inoculation (Fig. 3A and data not shown) and induced severe stem pits on twigs 4-6 mo after inoculation (Fig. 3C, c), similar to that of CTV9R (Fig. 3C, b). The mild isolate CTV T30 induced only mild symptoms on twigs (Fig. 3C, d) and leaves (data not shown) of Alemow and Mexican lime plants. Sour orange plants infected with CTV9R-MCA13NR and CTV9R exhibited a moderate "seedling yellows" reaction 8-12 weeks after inoculation, and were equally stunted, with smaller internodes, 3-4 mo after inoculation when compared to healthy sour orange plants (Fig. 3B).

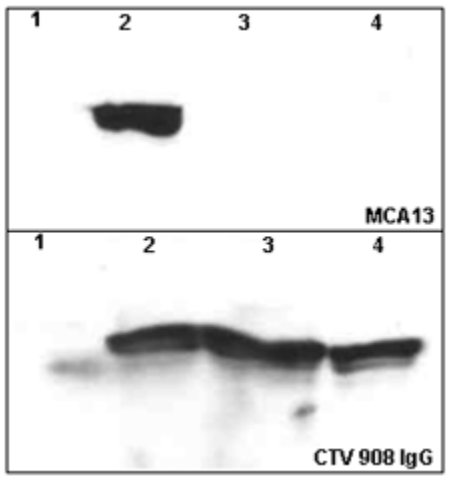

Fig. 2. Western immunoblot analysis of partially purified virions from (1) healthy, (2) wild type CTV9R, (3) CTV9R-MCA13NR, and (4) CTV T30 infected citrus plants. The blot was probed with the monoclonal antibody MCA13 or the broadly reactive CTV-specific polyclonal antibody 908 IgG.

The failure to reduce symptoms by genetically engineering T36 to an MCA13 non-reactive form suggests that pathogenicity of CTV isolates is not specifically linked to the MCA13 epitope even though there has been a general apparent correlation of expression of decline and stem pitting symptoms with reaction to MCA13. These results support observations with certain naturally occurring isolates in the international CTV collection in Beltsville, MD and isolates from Florida, in which a correlation between MCA13 reactivity and isolate severity was not always observed (6, 26). More-

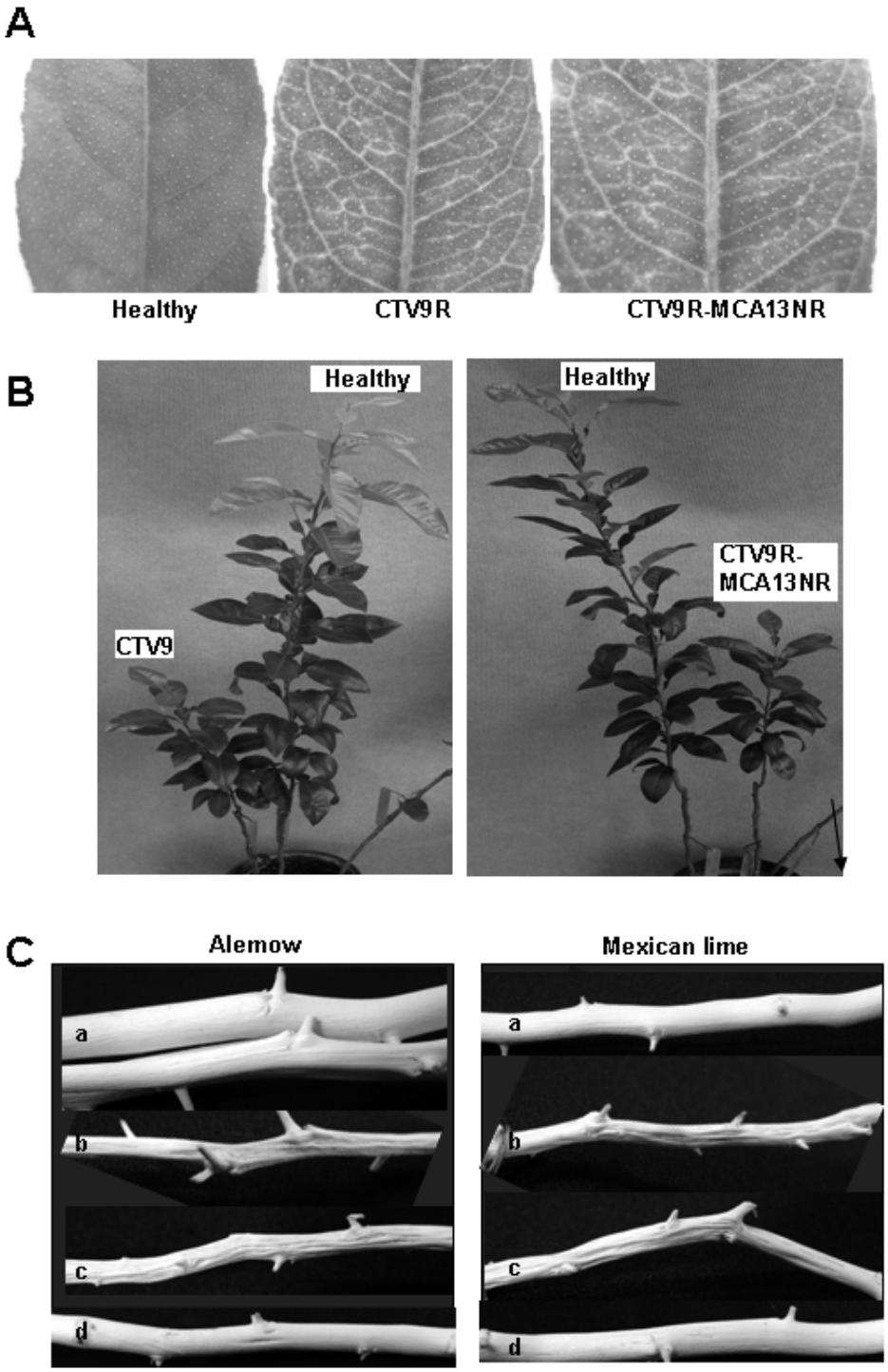

Fig. 3. (A) Healthy Alemow leaf and leaves showing vein clearing symptoms 8 weeks after inoculation with CTV9R and CTV9R-MCA13NR. (B) Sour orange plants infected with CTV9R and CTV9R-MCA13NR 3 mo after inoculation. Note the stunting of plants when compared to healthy plants. (C) Stem pitting symptoms induced by CTV9R (b), CTV9R-MCA13NR (c), and CTV T30 (d) on Alemow and Mexican lime plants 6 mo after inoculation. Stems from uninoculated plants (a).

over, recently we found that an infectious cDNA clone of T36, in which the p23 ORF and the 3' non-translated region had been exchanged with the analogous region from T30, failed to induce the seedling yellows reaction on sour orange (M. R. Albiach-Martí, unpublished). We also observed that deletion of the p33 and p18, or p33, p18 and p13 ORFs from isolate T36 reduced foliar symptoms on Alemow and Mexican lime plants (24), which further support the concept that the MCA13 reactive epitope is not coupled with pathogenicity. Our data suggest that conservation of the MCA13 reactive epitope in a majority of severe isolates and its association with severe symptoms is coincidental and not causal.

Although the MCA13-reactive and -nonreactive variants of CTV T36 differ by only a single nucleotide in the CP gene, they can be readily distinguished serologically using MCA13. These variants provide a protectant-challenge system with 99.99% sequence homology between components, yet are easily discriminated to examine the mechanisms of cross protection, virus-strain interactions, and movement within host plants. Similar studies on the mechanisms of cross protection can be done using MCA13 reactive deletion mutants as protectant viruses and the wild type MCA13 reactive CTV9R as challenge virus. However, although the deletion mutants induce milder symptoms, they are not serologically distinguishable from the wild type CTV9R, and monitoring the replication of the challenge virus required RT-PCR analysis (27) or hybridization using oligonucleotide probes (13). The availability of serologically distinct MCA13-non-reactive virus greatly simplified monitoring the replication of the challenge isolate using DAS-I ELISA. Similarly, this technique could also be used to engineer a 'marker', such as a restriction site(s), into a wild type CTV9R to examine the epidemiology of CTV, using the marker virus under natural conditions of virus spread.

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