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# Transgenic melon and squash expressing coat protein genes of aphid-borne viruses do not assist the spread of an aphid non-transmissible strain of cucumber mosaic virus in the field

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Transgenic melon and squash containing the coat protein (CP) gene of the aphid transmissible strain WL of cucumber mosaic cucumovirus (CMV) were grown under field conditions to determine if they would assist the spread of the aphid non-transmissible strain C of CMV, possibly through heterologous encapsidation and recombination. Transgenic melon were susceptible to CMV strain C whereas transgenic squash were resistant although the latter occasionally developed chlorotic blotches on lower leaves. Transgenic squash line ZW-20, one of the parents of commercialized cultivar Freedom II, which expresses the CP genes of the aphid transmissible strains FL of zucchini yellow mosaic (ZYMV) and watermelon mosaic virus 2 (WMV 2) potyviruses was also tested. Line ZW-20 is resistant to ZYMV and WMV 2 but is susceptible to CMV. Field experiments conducted over two consecutive years showed that aphid-vectored spread of CMV strain C did not occur from any of the CMV strain C-challenge inoculated transgenic plants to any of the uninoculated CMV-susceptible non-transgenic plants. Although CMV was detected in 3% (22/764) of the uninoculated plants, several assays including ELISA, RT-PCR-RFLP, identification of CP amino acid at position 168, and aphid transmission tests demonstrated that these CMV isolates were distinct from strain C. Instead, they were non-targeted CMV isolates that came from outside the field plots. This is the first report on field experiments designed to determine the potential of transgenic plants expressing CP genes for triggering changes in virus-vector specificity. Our results indicate that transgenic plants expressing CP genes of aphid transmissible strains of CMV, ZYMV, and WMV 2 are unlikely to mediate the spread of aphid non-transmissible strains of CMV. This finding is of practical relevance because transgenic crops expressing the three CP genes are targeted for commercial release, and because CMV is economically important, has a wide host range, and is widespread worldwide.

*Keywords*: Pathogen-derived resistance; cucumber mosaic virus; environmental safety issues; virus-vector specificity; heterologous encapsidation; recombination; aphid-mediated virus spread; field experiments; risk assessment

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## Introduction

The concept of pathogen-derived resistance (Sanford and Johnston, 1985) has opened new avenues for the development of virus-resistant plants. Since the first report that transgenic tobacco plants expressing the coat protein (CP) gene of tobacco mosaic tobamovirus (TMV) were protected from infections by TMV (Powell-Abel *et al*., 1986), pathogen-derived resistance has been applied to numerous

viruses and a range of host plants, including agronomically important crops (Fuchs and Gonsalves, 1997). Transgenic potato (Kaniewski *et al*., 1990; Malnoe *et al*., 1994; Thomas *et al*., 1997), cucumber (Gonsalves *et al*., 1992), squash (Tricoli *et al*., 1995), tomato (Fuchs *et al*., 1996), cantaloupe (Fuchs *et al*., 1997), and papaya (Lius *et al*., 1997) plants containing CP genes have been effective for controlling viral diseases under field conditions.

'Freedom II', a yellow crookneck squash F1 hybrid cultivar, was the first virus-resistant transgenic crop plant commercially released in the United States. It is highly resistant to single and mixed infections by zucchini yellow mosaic potyvirus (ZYMV) and/or watermelon mosaic virus 2 potyvirus (WMV 2) (Fuchs and Gonsalves, 1995; Tricoli *et al*., 1995). Since the release of Freedom II, transgenic squash line CZW-3 has been deregulated, while transgenic papaya line 55-1 has been commercialized in the United States. Squash line CZW-3 is resistant to ZYMV, WMV 2, and cucumber mosaic cucumovirus (CMV) (Tricoli *et al*., 1995). Papaya line 55-1 is resistant to papaya ringspot type p potyvirus (PRSV) (Gonsalves, 1998; Lius *et al*., 1997). Undoubtedly, more virus-resistant transgenic crops will be commercialized because of their benefits to agriculture.

Environmental safety issues have been expressed with the commercialization of virus-resistant transgenic plants, in particular those expressing viral CP genes. These issues include the development of modified viruses, the creation of novel viruses, and the escape of virus-derived transgenes into free-living crop relatives (DeZoeten, 1991; Hancock *et al*., 1996; Hull, 1989; Miller *et al*., 1997; Palukaitis, 1991; Rissler and Mellon, 1996; Tepfer, 1993; Tolin, 1991). Modified and novel viruses can develop through heterologous encapsidation and recombination. In heterologous encapsidation, genomes of challenge viruses can be partially or completely encapsidated by CP subunits produced by transgenic plants expressing viral CP genes (DeZoeten, 1991; Hull, 1989; Miller *et al*., 1997; Palukaitis, 1991; Rissler and Mellon, 1996; Tepfer, 1993; Tolin, 1991). This can have significant consequences given the functions of the CP. For example, in the case of insect-borne viruses, the CP is critical for the specificity of transmission (Atreya *et al*., 1990; Chen and Francki, 1990; Harrison and Murant, 1984). Thus, transgenic plants expressing the CP of aphid-borne viruses have the potential to mediate the vector transmissibility of aphid non-transmissible viruses. Also, recombination between transgene mRNAs and RNA of challenge viruses can drive the development of chimeric genomes that contain segments of two distinct RNA species (Allison *et al*., 1996). Chimeric RNA genomes can acquire new properties compared to the original challenge viral RNA. As a consequence of heterologous encapsidation and recombination, transgenic plants expressing CP genes might modify existing viruses and create new virus species with possible altered vector specificity and expanded host range (DeZoeten, 1991; Hull, 1989; Miller *et al*., 1997; Palukaitis, 1991; Rissler and Mellon, 1996; Tepfer, 1993; Tolin, 1991).

Indeed, some reports have shown that CPs expressed by transgenic plants can partially or completely encapsidate the genome of challenge RNA viruses (Candelier-Harvey and Hull, 1993; Farinelli *et al*., 1992; Hammond and Dienelt, 1997; Holt and Beachy, 1991; Lecoq *et al*., 1993; Osbourn *et al*., 1990). Further, Lecoq *et al*. (1993) showed that the CP of plum pox potyvirus in *Nicotiana benthamiana* mediated the vector transmissibility of an aphid non-transmissible strain of ZYMV. Also, recombination between viral transgene transcripts and the genome of challenge viruses has been shown to trigger systemic infections of virus mutants that were not capable of translocating from the initial infection sites (Gal *et al*., 1992; Greene and Allison, 1994; Greene and Allison, 1996; Schoelz and Wintermantel, 1993; Wintermantel and Schoelz, 1996). Most of these experiments were done in the laboratory, and under conditions of high to moderate selection pressure to facilitate the recovery of viruses with new properties. Will heterologous encapsidation and recombination occur in the field with transgenic crops grown under commercial conditions? Field experiments are critical to assess the environmental impact of virus-resistant transgenic plants.

We are examining environmental issues related to virus spread using transgenic plants that contain CP genes derived from aphid-borne viruses. This study primarily focused on field experiments with melon and squash expressing the CP gene of the aphid transmissible CMV strain WL. Our main objective was to determine if such transgenic plants enabled the spread of the aphid non-transmissible CMV strain C.

## Materials and methods

### *Plant material*

Transgenic melon (*Cucumis melo* L.) and squash (*Cucurbita pepo* L.) plants expressing the CP gene of the aphid transmissible CMV strain WL (Gonsalves *et al*., 1982) were obtained by *Agrobacterium tumefaciens*-mediated transformation using the binary vector pGA482GG-cpCMV-WL-5-14 (Namba *et al*., 1991). This vector contained within its T-DNA borders the CP gene of the aphid transmissible CMV strain WL. The CMV CP gene construct comprised a cDNA copy of the full length CP gene and the first 90 nucleotides of the untranslated 3′ end region located downstream of the CP translation stop codon (Namba *et al*., 1991; Slightom, 1991). Expression of the CP gene was regulated by the cauliflower mosaic caulimovirus (CaMV) 35S transcript promoter, the inter-

genic region of RNA3 of CMV strain C, and the CaMV 35S terminator sequence (Slightom, 1991). Several lines of transgenic melon and squash were used. These lines were selected based on their relative level of CP expression and degree of resistance to infections by CMV. The development of some of the melon lines was reported previously (Gonsalves *et al*., 1994).

We also used transgenic squash line ZW-20 which is the virus-resistant parent of commercial cultivar Freedom II (Tricoli *et al*., 1995). Transgenic ZW-20 plants were obtained by *A. tumefaciens*-mediated transformation using the binary vector pPRBN-ZYMV72/WMBN22 (Tricoli *et al*., 1995). This vector contained within its T-DNA borders the CP genes of the aphid transmissible strains FL of ZYMV and WMV 2 (Tricoli *et al*., 1995). The two CP gene constructs consisted of full length CP genes starting at the putative proteolytic cleavage site Q/S (Quemada *et al*., 1990), and ca. 40 nucleotides of the 3′ end untranslated region located downstream of the translation stop codon (Quemada *et al*., 1990; Slightom, 1991). Expression of the ZYMV and WMV 2 CP genes was driven by the CaMV 35S promoter, the intergenic region of RNA3 of CMV strain C, and the CaMV 35S terminator sequences (Slightom, 1991). Transgenic line ZW-20 is highly resistant to ZYMV and WMV 2 (Fuchs and Gonsalves, 1995; Tricoli *et al*., 1995), but is susceptible to CMV (data not shown).

Constitutive expression of CPs in transgenic melon and squash was detected by enzyme-linked immunosorbent assay (ELISA) using appropriate γ-globulins developed in our laboratory.

Non-transgenic plants of the melon cvs TopMark and Hales Best Jumbo, and non-transgenic plants of the squash cvs Pavo and Seneca were used as recipients for aphid inoculations and negative controls for ELISA.

### *CMV strains and mechanical inoculations*

CMV strains WL (Gonsalves *et al*., 1982), C (Provvidenti, 1976), Cat (Rodriguez, 1993), and Fny (Banik and Zitter, 1990) were used as inocula. Plants were mechanically-inoculated by rubbing Corundum-dusted cotyledons and the two first expanded leaves with a 1:20 dilution of leaf extracts of summer squash cv President or melon cv Hales Best Jumbo infected by one of the four CMV strains. Inoculations were done in the greenhouse one week prior to transplanting to the field sites. Most of the transgenic plants were destined to field experiments but some were kept in the greenhouse for aphid transmissions.

### *Open field experiments*

Open field experiments were carried out under permits issued by APHIS-USDA. Melon plants were tested on two farms (1–2) that were 5 km apart. Plots A and B were 100 m apart on farm 1, and plot E was 500 m away from the former two. Plots C and D were 100 m apart on farm 2. In 1994, squash and melon plants were tested on seven plots (F–L) that were located on three new farms (3–5). Farms 3 and 4 were 1 km apart and farm 5 was 3 km away from the former two. Plots F, G, and I were 100 m apart from each other on farm 3. Plots H and L were 100 m apart on farm 5, and plots J and K were 100 m apart on farm 4.

Squash and melon seedlings were transplanted on bare soil 1 and 2 m apart, and 2 and 4 m between rows, respectively. The size of squash and melon plots, each composed of 100 to 300 plants, was approximately 30 m × 40 m and 20 m × 30 m, respectively. A complete block design was used for each plot, except plot C, with random allocations of the two treatments (uninoculated and mechanically-inoculated plants). A near equal or 1:2 ratio of inoculated versus uninoculated plants was used to create conditions of high disease pressure.

Since previous field experiments with transgenic melon and squash showed that aphid-vectored virus transmission is efficient on the different farms that were selected (Fuchs and Gonsalves, 1995; Fuchs *et al*., 1997), indigenous aphid populations were not monitored.

### *Screenhouse experiments*

Screenhouse experiments were carried out under permits issed by APHIS-USDA. Screenhouses measured 12 × 6 × 3 m or 24 × 6 × 3 m. They covered three rows of plants which were 1.5 m apart. Beds were prepared with black polyethylene plastic mulch and a single drip irrigation line at the center. Melon plants were tested in 1993, 1994, and 1995, and squash plants were analyzed during the last two years. Melon and squash were tested in different screenhouses, as well as control and transgenic plants. Also, CMV strain WL- and CMV strain C-inoculated control plants were set in different screenhouses. Each row was planted by alternating uninoculated and inoculated plants. Conditions of high disease pressure were achieved with a 1:1 ratio of inoculated over uninoculated plants.

### *Data collection*

CMV spread was measured on all uninoculated plants by regularly assessing symptom development. Uninoculated plants that became symptomatic were further characterized by ELISA to identify the virus(es) causing symptoms. Leaf samples were collected for ELISA as soon as symptoms were seen. ELISA was run and interpreted as described previously (Fuchs and Gonsalves, 1995). If ELISA results were ambiguous, bioassays were carried out on squash cv. President, a systemic host, or *Chenopodium quinoa*, a local lesion host. Symptomatic plants were kept in the field throughout the 1993 season while they were rogued in 1994 to minimize the incidence of undesired viruses. Plants that reacted positively for CMV in ELISA were further analyzed to identify the infecting isolates.

*Establishment of CMV cultures*

Leaf samples that were collected on CMV-infected plants in the field were used as inoculum to establish CMV cultures on *N. benthamiana* after local lesion cloning on *C. quinoa*.

*RT-PCR-RFLP analysis of CMV isolates*

RT-PCR-RFLP analysis was used to unambiguously classify the CMV isolates into one of the two subgroups. Total RNA (5 µg) from CMV-infected *N. benthamiana* (Pinck *et al*., 1988), an oligonucleotide complementary to nt 778-797 of the CMV CP gene (downstream primer A: 5′ CGGCATTCGACCTACCTGT 3′) and reverse transcriptase from avian myeloblastosis virus were used to obtain the first cDNA strand (Sambrook *et al*., 1989). The second cDNA strand was synthesized and amplified by PCR using an oligonucleotide corresponding to nt 295-318 of the CMV CP gene (upstream primer B: 5′ TATGATAAGAAGCTTGTTTCGCGC 3′), primer A and Taq DNA polymerase (Sambrook *et al*., 1989). Primers A and B were designed in conserved regions of the CMV CP gene (Palukaitis *et al*., 1992; Rizos *et al*., 1992; Singh *et al*., 1995). The amplified DNA product (486 bp for CMV subgroup I isolates and 501 bp for CMV subgroup II isolates) was purified on 1.5% low melting point agarose gels and digested with Msp I or Eco R I.

*Identification of CMV CP residue 168*

If RT-PCR-RFLP analysis indicated that the CMV isolates from the field belonging to subgroup I, another RT-PCR assay combined with sequencing of the amplified CP gene fragment was performed to deduce the amino acid sequence of a 39 residue polypeptide (CP amino acids 142–181) including residue 168. Oligonucleotides corresponding to nt 544–563 (downstream primer C: 5′ TACACGAGGACGGCGTACTT 3′) and nt 409–428 (upstream primer D: 5′ GCCGCCATCTCTGCTATGTT 3′) of the CMV CP gene were used. Primers C and D were designed in conserved regions (Palukaitis *et al*., 1992). The 155 bp amplified DNA fragment obtained from a single RT-PCR was purified on 1.5% low melting agarose gels and directly sequenced. Sequencing was performed using either the fmol™ DNA system (Promega, Madison, WI) and $^{32}$P-labeled primers C or D, the dideoxynucleotide chain termination method and Sequenase® DNA polymerase (US Biochemicals, Cleveland, OH), or the Prism™ Ready Reaction DyeDeoxy™ Teminator Cycle system (Applied Biosystems, Foster City, CA) with an ABI 273A DNA sequencer.

*Aphid transmission experiments*

Nonviruliferous *M. persicae* were reared on healthy cabbage in a growth chamber where temperature (18 °C ±2) and photoperiod (14 hours of light) were controlled. After a 2–4 hour starvation period, aphids were deposited on symptomatic transgenic plants previously inoculated with CMV strain C. Following a 2–5 min acquisition period on symptomatic leaves, groups of 5 or 10 aphids found in probing positions were transferred individually to healthy summer squash cv President. Each transmission experiment consisted of five plants that received five aphids and five plants that received ten aphids. In addition, aphids feeding on squash plants infected by CMV strains WL or C were used as controls for each transmission experiment. Also, CMV strain C was mechanically transferred from inoculated transgenic plants to summer squash cv President to assess the infectivity of the inoculum. Aphids were allowed to feed on test plants for 16–20 hours before being killed with an insecticide. Recipient plants were monitored for symptom development and tested for CMV infection by ELISA three weeks after exposure to the aphids.

## Results

*Properties of transgenic melon and squash plants*

The four transgenic melon lines used in this study were selected for their difference in CMV strain WL CP expression level. Melon lines H-3 and H-7 expressed relatively high levels of CP while lines T-22 and T-64 had almost no detectable levels of CP, as shown by ELISA. The OD 405 nm readings were 0.045–0.106 for lines H-3 and H-7, 0.026–0.032 for lines T-22 and T-64, and 0.025 for healthy non-transgenic melons, after 30 min substrate hydrolysis. Regardless of the CP expression level, the four transgenic lines were susceptible to infections by CMV in a 1993 field trial with aphid-vectored inoculations of the aphid transmissible CMV subgroup I strain Fny (Fig. 1). Lines T-22 and T-64 were as susceptible as controls with systemic symptoms consisting of mosaic, chlorosis, mottling, prominent downward curling of young leaves, reduced leaf laminae and stunted growth. Lines H-3 and H-7 showed a slight delay (2–3 weeks) in the onset of disease but developed systemic symptoms similar to those observed on controls. Similar to aphid-vectored inoculations, the four transgenic melon lines were equally susceptible to mechanical inoculations of the aphid non-transmissible CMV subgroup I strain C in the greenhouse (data not shown).

The two transgenic squash lines S-3 and S-4 expressing the CP gene of CMV strain WL were selected for their relatively high expression levels of CP, as shown by ELISA. OD 405 nm readings were 0.073–0.165 for both lines and 0.020 for healthy non-transgenic squash controls after 30 min substrate hydrolysis. Contrary to the four transgenic melon lines, squash lines S-3 and S-4 were highly resistant to infections by CMV (Fig. 1). A 1993 field trial with vector-mediated inoculations of the

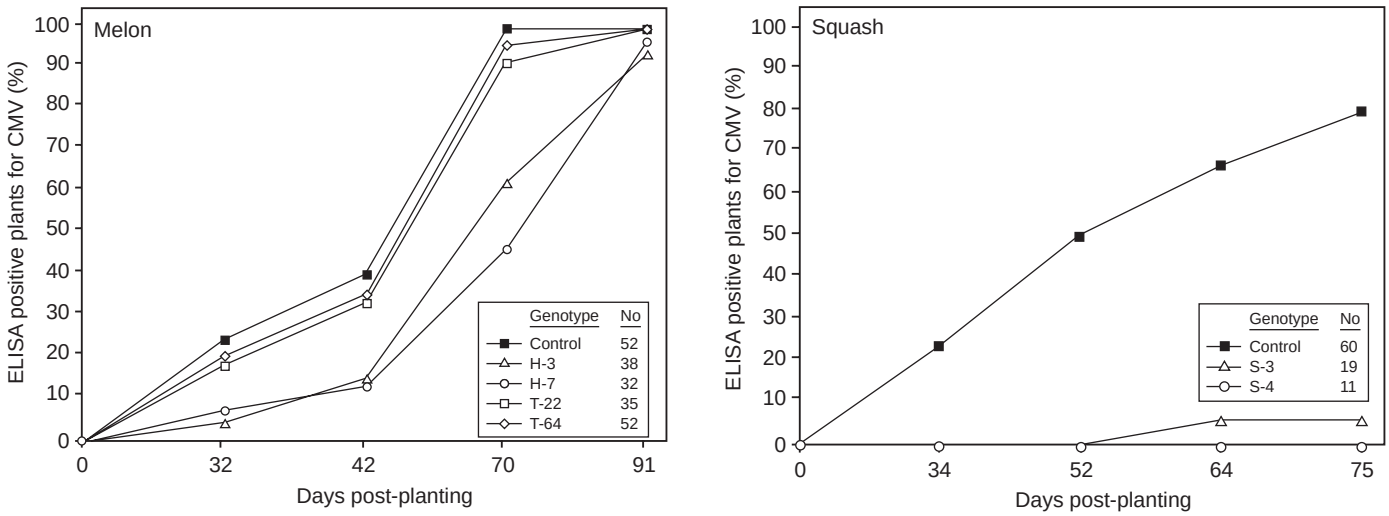


**Fig. 1.** Resistance of transgenic melon (top) and squash (bottom) expressing the CP gene of CMV strain WL to aphid-vectored inoculations of CMV subgroup I strain Fny under field conditions in 1993. Data represent cumulative percent of ELISA positive plants for CMV. Melon and squash were transplanted to two separate fields that were 5 km apart on June 30 and July 22, 1993, respectively. Mechanically-inoculated control plants served as virus source for aphid transmissions. Transgenic melon (line T-64, open diamond; line T-22, open squares; line H-3, open triangle; and line H-7, open circle) showed systemic infection like controls (closed square). Transgenic squash (line S-3, open triangle; and line S-4, open circle) showed no systemic symptoms but eventually chlorotic blotches on older leaves. The number of plants tested is indicated for each genotype. Resistance to CMV was evaluated by ELISA because WMV 2 and ZYMV were also detected in 65 and 55% of the test plants, respectively, although these two potyviruses were not deliberately introduced in the fields. CMV-infected plants had OD 405 nm readings at least 30 times higher (1.2–0.8 vs 0.025) than healthy controls, and at least 5 times higher than uninoculated transgenic plants (1.2–0.8 vs 0.165), after 30 min substrate hydrolysis.

aphid transmissible CMV subgroup I strain Fny showed that S-3 and S-4 plants did not develop systemic symptoms, but occasionally exhibited chlorotic blotches on lower leaves. CMV was detected in leaf tissue of a few plants that exhibited chlorotic blotches (6%) but not in symptomless surrounding area. Lines S-3 and S-4 showed a similar localized reaction upon mechanical inoculation with aphid non-transmissible CMV subgroup I strain C in the greenhouse (data not shown).

Transgenic squash line ZW-20 (Tricoli *et al*., 1995), which is the virus-resistant parent of commercial cultivar Freedom II, was also used in this study. ZW-20 is highly resistant to ZYMV and WMV 2 (Fuchs and Gonsalves, 1995; Tricoli *et al*., 1995), but is susceptible to CMV (data not shown).

### *Properties of CMV strains WL and C*

Characterizing distinct features of CMV strain WL, which supplied the CP gene expressed by the majority of transgenic plants, and CMV strain C, which was used as inoculum to challenge transgenic plants, was essential to our study. The differences in vector transmissibility, serological relatedness, nucleotide homologies of the CP genes, and amino acid at position 168 of the CP between these two CMV strains are illustrated below.

*Aphid transmissibility.* CMV strain WL was highly transmissible by *Myzus persicae* while strain C was not (Table 1), confirming previous findings (Quemada *et al*., 1991; Rodriguez, 1993). Interestingly, strain WL showed a higher transmission rate when melon was used as recipient for aphids rather than squash.

*Serological relationship.* Previously it was proposed that strain WL belongs to CMV subgroup II while strain C belongs to CMV subgroup I (Edwards and Gonsalves, 1983). Our ELISA data confirmed this classification. After 30 min substrate hydrolysis, strain WL had OD 405 nm readings of 0.28 and 0.71 with γ-globulins specific to subgroup I and II, respectively. Strain C had

**Table 1.** Aphid transmissibility of CMV strains C and WL

| *Virus source*[a] | *CMV strain* | *CMV infection Infected/Tested* | % |
|---|---|---|---|
| Melon | C | 0/50 | 0 |
| | WL | 40/50 | 80 |
| Squash | C | 0/50 | 0 |
| | WL | 28/50 | 56 |

[a]Nonviruliferous *Myzus persicae* were allowed to probe on infected plants for 2–5 min. Aphids found in probing position were transferred individually to healthy squash plants. Each transmission experiment consisted of 5 plants that received 5 aphids and 5 plants that received 10 aphids. Aphids were maintained on test plants for 16–20 h and then were treated with an insecticide. Aphid-recipient plants were analyzed for CMV infection by symptom and ELISA. Infected samples had OD 405 nm readings at least 20 times higher (1.2–0.6 vs 0.03) than healthy controls after 30 min substrate hydrolysis.

OD 405 nm values of 1.26 and 0.34 with γ-globulins specific to subgroup I and II, respectively. Healthy control plants had OD 405 nm readings of 0.09 with both γ-globulins.

*Nucleotide homologies of CP genes.* The CP genes of CMV strains WL and C have 76% homologies at the nucleotide level (Quemada *et al*., 1989). Differences in nucleotide composition of the two CP genes were revealed by a reverse transcription-polymerase chain reaction-restriction fragment length polymorphism (RT-PCR-RFLP) assay. Strain WL, like other CMV subgroup II strains, showed 3 fragments (197, 124, and 82 bp) while strain C, like other CMV subgroup I strains, showed 2 fragments (335 and 151 bp) upon Msp I digestion of the 486 bp amplified DNA product (Fig. 2).

*Differences between CMV strain C and other subgroup I strains.* It was critical to unambiguously distinguish CMV strain C from other subgroup I strains because of possible contamination of our field plots by non-targeted aphid transmissible CMV subgroup I strains. The uniqueness of the amino acid at position 168 of the CP of CMV strain C allowed us to make this discrimination. Strain C carries a phenylalanine 168 (Phe, codon TTT) (Quemada *et al*., 1989) while all the other CMV subgroup I and II strains sequenced so far, except strains M (Owens *et al*., 1990) and Hawaii (Hu *et al*., 1995), contain a tyrosine 168 (Tyr, codon TAT) (Palukaitis *et al*., 1992). The difference in codon usage for amino acid 168 between strain C and the aphid transmissible subgroup I strain Cat (Rodriguez, 1993) is illustrated in Fig. 3.

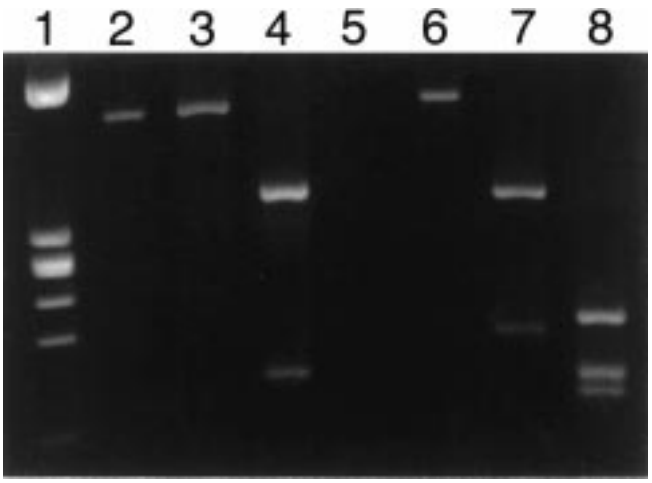


**Fig. 2.** Characterization of CMV subgroup I strain C and subgroup II strain WL by RT-PCR-RFLP analysis. DNA fragments were analyzed by electrophoresis on ethidium bromide-containing 3% agarose gels. Sources of DNA were as follows: lane 1, marker øX174 RF DNA digested with Hae III (603, 310, 281, 271, 234, 194, and 118 bp fragments); lane 2, undigested strain C (486 bp fragment); lane 3, EcoR I digest of strain C (486 bp fragment); lane 4, Msp I digest of strain C (335 and 151 bp fragments); lane 5, negative control, healthy plant; lane 6, undigested strain WL (501 bp fragment); lane 7, EcoR I digest of strain WL (332 and 169 bp fragments); lane 8, Msp I digest of strain WL (197, 124, 82 bp fragments and a 28 bp fragment that is not visible on the gel but is deduced from nucleotide sequence data).

*Rationale of the field trials and data collection*

The basic questions that we wanted to answer were: (1) Will CPs from aphid transmissible viruses in transgenic plants mediate the spread of the aphid non-transmissible CMV strain C in the field? and (2) Will the level of CP expression and the degree of resistance to CMV infection in transgenic plants influence the spread of CMV strain C? The experimental approach was to mechanically inoculate transgenic plants with the aphid non-transmissible CMV strain C and observe for subsequent transmission of this strain to uninoculated CMV-susceptible non-

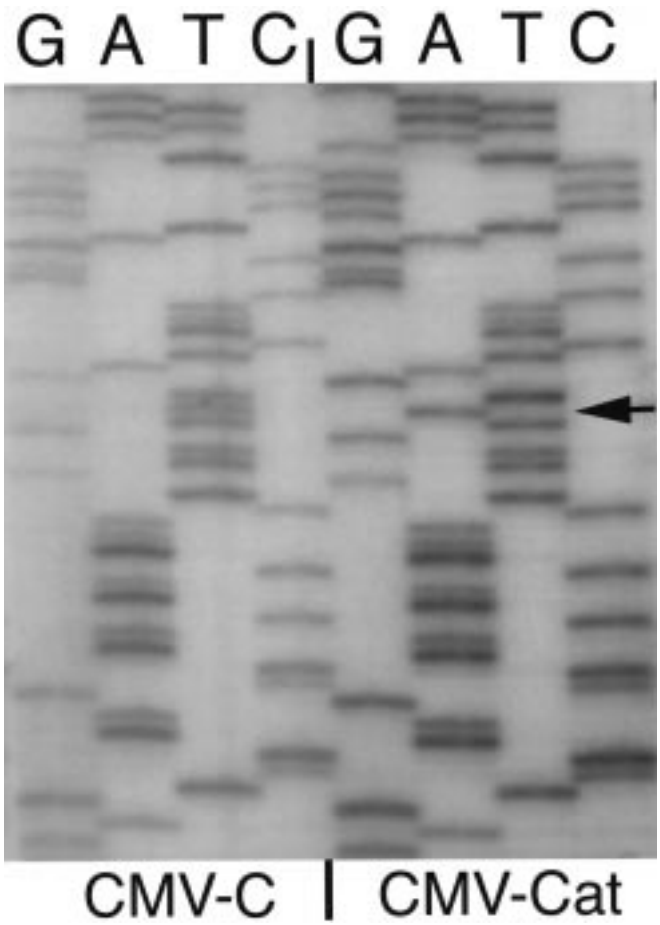


**Fig. 3.** Sequencing of a 155 bp CP gene fragment obtained from CMV subgroup I strains C and Cat. Amino acid at position 168 (codon TAT 502–504) is a phelyalanine (codon TTT) for strain C and a tyrosine (codon TAT) for strain Cat. The position of T 503 for strain C (left) and A 503 for strain Cat (right) is shown by a single arrow. $^{32}$P-labelled reactions obtained with the fmol™ DNA sequencing system were loaded on 8% denaturing polyacrylamide gels, electrophoresed for 2 h at 60 W, and autoradiographed.

transgenic plants in the field. The component and characteristics of the field plots used in two consecutive years are summarized in Table 2. Control plots were also set to confirm the lack of spread of CMV strain C, determine the relative influx of CMV from the outside into our field plots, evaluate the effect of natural CMV infection in mediating the spread of CMV strain C, and analyze the rate of transmission of the aphid transmissible strain Cat.

Spread of CMV was achieved in all field plots by indigenous aphid populations. Plants that became infected by CMV were identifed by visual monitoring of symptom development and ELISA. A number of assays were subsequently run to discriminate between the aphid non-transmissible strain C and aphid transmissible CMV strains, and to monitor the influx of non-targeted viruses into our field plots. CMV isolates recovered in the field were classified into subgroup I and II by ELISA with γ-globulins specific to each subgroup, and RT-PCR-RFLP analysis. CMV isolates that belonged to subgroup I were further characterized to distinguish strain C from other strains. Identification of CMV strain C was carried out by RT-PCR and sequencing of the 155 bp amplified DNA product to determine the CP amino acid at position 168. Finally, aphid transmission experiments were done to characterize the vector transmissibility of CMV subgroup I isolates.

### *Melon plants expressing the CP of the aphid transmissible CMV strain WL do not mediate the spread of the aphid non-transmissible CMV strain C*

CMV-susceptible transgenic melon lines with high (H-3 and H-7) and low (T-22 and T-64) levels of CP expression were used to assess whether the CP expression level would affect the spread of the aphid non-transmissible CMV strain C.

The incidence of natural CMV infections is summarized in Table 3. In plots with CMV strain C-inoculated transgenic plants (A, B, F, and G), a few uninoculated plants reacted positively for CMV in ELISA (4–5%) at the end of the growing seasons, 61 and 68 days post-planting (dpp) in 1993 and 1994, respectively (Table 3). Most of these CMV-infected plants developed typical CMV symptoms consisting of mosaic, chlorosis, downward curling of apical leaves and plants stunting starting at 53 and 42 dpp in 1993 and 1994, respectively.

**Table 2.** Summary of field plots and rationale

| *Year* | *Farm* | *Field plot* | *Crop* | *Inoculated plants* | *CMV Challenge* | *Objectives* |
|---|---|---|---|---|---|---|
| 1993 | 1 | A, B | melon | H-3, T-22 | C | Assess whether transgenic melon plants that express relative high (H-3) or low (T-22) levels of CMV strain WL CP and are susceptible to CMV strain C might assist the spread of the aphid non-transmissible strain C |
| | 2 | C | melon | control | none | Evaluate the base line of natural CMV infection |
| | 2 | D | melon | control | C | Confirm the lack of vector transmissibility of the aphid non-transmissible CMV strain C |
| | 1 | E | melon | control | Cat | Examine the rate of transmission of the aphid transmissible CMV strain Cat by indigenous aphid populations |
| 1994 | 3 | F, G | melon | H-7, T-64 | C | Assess whether transgenic melon plants that express relative high (H-7) or low (T-64) levels of CMV strain WL CP and are susceptible to CMV strain C might assist the spread of the aphid non-transmissible strain C |
| | 5 | H | melon | control | C | Evaluate simultaneously the base line of natural CMV infection and the lack of vector transmissibility of the aphid non-transmissible strain C in a melon field |
| | 3 | I | squash | S-3, S-4 | C | Assess whether transgenic squash plants that express relative high levels of CMV strain WL CP and are resistant to CMV strain C might assist the spread of the aphid non-transmissible strain C |
| | 4 | J | squash | ZW-20 | C | Assess whether transgenic squash plants that express relative low levels of the CP genes of ZYMV and WMV 2 and are susceptible to CMV strain C might assist the spread of the aphid non-transmissible strain C |
| | 4, 5 | K, L | squash | control | C | Examine simultaneously the base line of natural CMV infection and the lack of vector transmissibility of the aphid non transmissible strain C in squash fields |

**Table 3.** Incidence of aphid-vectored CMV in the field

| *Field plot* | *Crop* | *Line* | *Challenge strain* | *No. mechanically-inoculated plants* | *CMV infection Infected/Tested*[a] | % |
|---|---|---|---|---|---|---|
| A, F | melon | H-3, H-7 | C | 128 | 9/170 | 5.3 |
| B, G | | T-22, T-64 | C | 78 | 7/170 | 4.1 |
| C | | control | none | 0 | 7/100 | 7.0 |
| D, H | | control | C | 200 | 7/200 | 3.5 |
| E | | control | Cat | 70 | 43/50 | 86.0 |
| I | squash | S-3 | C | 50 | 2/110 | 1.8 |
| J | | ZW-20 | C | 52 | 4/164 | 2.4 |
| K, L | | control | C | 216 | 9/216 | 4.2 |

[a]Cumulative number of uninoculated plants that became infected by CMV at 61 and 68 dpp in 1993 and 1994, respectively, over the number of uninoculated plants analyzed. CMV-infected plants were identified by ELISA. Infected samples had OD 405 nm readings at least 10 times higher than healthy controls (1.25–0.35 vs 0.03) after 30 min substrate hydrolysis

Interestingly, CMV-infected plants were located at the edges of the field plots, suggesting that infections came from outside the field plots. In plots where the aphid non-transmissible CMV strain C or no virus were introduced (C, D, and H), CMV infection rates were low (3.5–7%). In contrast, CMV infection rates were high (86%) in plot E, where the aphid transmissible subgroup I strain Cat was introduced (Table 3).

Leaf samples of CMV-infected plants from all field plots, except C and E, were used to establish 23 independent CMV cultures in *N. benthamiana*. All 23 cultures gave higher ELISA reactions with subgroup I than sugroup II γ-globulins (0.67 vs 0.23, and 0.05 for uninoculated controls after 30 min substrate hydrolysis). Furthermore, RT-PCR-RFLP analysis showed that the 486 bp amplified DNA product from all the isolates recovered in the field was not digested by EcoR I (Fig. 2, lane 3) but yielded 2 smaller fragments (335 and 151 bp) upon digestion with Msp I (Fig. 2, lane 4). These results indicated that the 23 CMV isolates from the field belonged to subgroup I, like strain C (Table 4).

Identification of CP residue at position 168 showed that none of the 23 CMV isolates recovered in the field had a Phe like CMV strain C. Instead, some (12 of 23) had a Tyr 168 and others (11 of 23) carried a His 168 (Table 4). Besides residue 168, the other 38 amino acids deduced from the nucleotide sequence of the 155 bp amplified DNA fragments were identical to those of strain C. As control, sequencing of the 155 bp fragment amplified from the inoculum recovered from a few CMV strain C-inoculated transgenic plants revealed Phe 168, as expected. These data demonstrated that the 23 CMV isolates from the field were different from strain C that was deliberately inoculated to transgenic plants.

Analysis of the vector transmissibility of the 23 CMV isolates recovered in the field showed that *Myzus persicae* transmitted each of them at an average rate of 42% (Table 4). As expected, control experiments con-

**Table 4.** Characterization of CMV isolates from the field

| *Field plot* | *No*[a] | *RT-PCR-RFLP*[b] *subgr. I* | *subgr. II* | *CP aa reside 168*[c] *Phe* | *Tyr* | *His* | *Aphid transmissibility Infection rate*[d] | % |
|---|---|---|---|---|---|---|---|---|
| A, F | 9 | 9/9 | 0/9 | 0/9 | 4/9 | 5/9 | 40/90 | 44 |
| B, G | 7 | 7/7 | 0/7 | 0/7 | 6/7 | 1/7 | 28/70 | 40 |
| D, H | 7 | 7/7 | 0/7 | 0/7 | 2/7 | 5/7 | 26/70 | 37 |
| I | 2 | 2/2 | 0/2 | 0/2 | 2/2 | 0/2 | 6/20 | 30 |
| J | 4 | 4/4 | 0/4 | 0/4 | 3/4 | 1/4 | 19/40 | 48 |
| K, L | 9 | 9/9 | 0/9 | 0/9 | 6/9 | 3/9 | 48/90 | 53 |

[a]Number of CMV-infected plants recovered in each field plot.
[b]The 486 bp amplified DNA fragment was not digested by Eco R I and yielded two bands of 335 and 151 bp upon digestion with Msp I.
[c]Identification of amino acid at position 168 of the CP was done as described in Experimental Protocol.
[d]Number of aphid-inoculated squash plants that became infected with CMV over the total number of squash plants inoculated by aphids.
CMV-infected plants were identified by monitoring of symptom development and ELISA.

firmed that strain WL was efficiently transmitted (28 of 68 plants infected) while strain C was not (none of the 82 plants infected). These data substantiated our previous tests by demonstrating that the 23 CMV isolates from the field were not CMV strain C.

Visual assessment of cross contamination indicated that symptoms caused by viruses other than CMV developed in all field plots regardless whether plants were mechanically-inoculated with CMV. Symptoms consisted of chlorosis, mosaic, deformations, reduced leaf laminae, shoestringing, necrosis and stunted growth. In 1993, symptoms were observed on a few plants (2%) early in the season (18 dpp), increasing to 77% at 48 dpp and 100% at 61 dpp. In 1994, symptoms occurred on a few plants (8%) at 30 dpp, and on 23% at 45 dpp. ELISA indicated the presence of WMV 2, ZYMV, tobacco ringspot nepovirus (TRSV), and tomato ringspot nepovirus (ToRSV). WMV 2 was predominant, infecting 80% of test plants in 1993 and 6% in 1994. ZYMV was detected in 23 and 1% of the test plants in 1993 and 1994, respectively, while the incidence of TRSV and ToRSV was very low in 1994 with only 11 and 1% infected plants, respectively. The latter two viruses were not detected in 1993.

*Squash plants expressing CPs of aphid transmissible viruses do not mediate the spread of the aphid non-transmissible CMV strain C*

Squash lines S-3 and S-4 were used to determine if transgenic plants expressing high levels of CP and displaying local but no systemic infection to CMV would assist the spread of the aphid non-transmissible CMV strain C. Besides lines S-3 and S-4, transgenic squash line ZW-20 was tested to detemine if heterologous CPs would assist the spread of the aphid non-transmissible strain CMv strain C.

Similar to the melon trials, CMV infections were low in uninoculated non-transgenic plants (Table 3). The incidence of CMV infection was similarly low in test plots with transgenic plants (I, J) and in control plots with non-transgenic plants (K, L) (2 vs 4%). The majority of plants (75%) that were ELISA positive showed typical CMV symptoms consisting of mosaic, chlorosis, downward curling, and stunted growth. Also, most of CMV-infected plants were identified late in growing season (42 dpp) and were located at the edges of the field plots, suggesting that infections came from external sources.

Leaf samples from squash plants that reacted positively for CMV in ELISA were used to establish 15 independent CMV cultures on *N. benthamiana*. Most of these cultures had higher ELISA values with γ-globulins specific to CMV subgroup I than subgroup II (0.42 vs 0.28, and 0.04 for healthy control after 30 min substrate hydrolysis), suggesting that they belonged to CMV subgroup I. RT-PCR-RFLP analysis confirmed this classification, with Msp I digestion patterns of the 486 bp amplified DNA products showing 2 fragments of 335 and 151 bp (Fig. 2, lane 4). These data demonstrated that the 15 CMV isolates from the field belonged to subgroup I, like strain C (Table 4).

Identification of the CP residue at position 168 showed that, unlike CMV strain C, none of the 15 isolates from the field had a Phe. Eleven of the 15 isolates contained a Tyr 168 and four had a His 168 (Fig. 3). Besides amino acid 168, the other 38 residues deduced from the nucleotide sequence of the 155 bp amplified fragments were identical to those of CMV strain C. As expected, sequencing of the 155 bp DNA product amplified from the inoculum recovered from a few CMV strain C-inoculated plants revealed the presence of Phe 168. Furthermore, *M. persicae* transmitted each of the 15 isolates from the field at an average rate of 39%, in contrast to strain C which was not transmissible (Table 4). These data demonstrated that the 15 CMV isolates from the field were different from strain C.

In addition to typical CMV symptoms, symptoms of chlorosis, mosaic, deformations, reduced leaf laminae, and stunted growth were also observed. These symptoms, which indicated the presence of viruses other than CMV, were observed on a few uninoculated plants in plots I (6%) and K (7%) but on the majority of uninoculated plants in plots L (76%) and J (100%) at 45 dpp. ELISA revealed the presence of WMV 2, TRSV, and ToRSV.

*Screenhouse experiments*

Some of the transgenic melon (H-3, H-7, T-22, and T-64) and squash (S-3 and S-4) lines which were tested in open fields were also analyzed in screenhouses that were established in the field. Screenhouse provided optimal conditions to carry out virus transmission studies in the field with limited interference from non-targeted viruses.

Ninety five transgenic melon and thirty one transgenic squash were inoculated with the aphid non-transmissible CMV strain C (Table 5). *M. persicae* were deposited once a week on symptomatic leaves starting at the time symptoms were visible. Spread of CMV strain C to uninoculated non-transgenic plants was monitored by symptom development and ELISA. Results showed that CMV strain C did not spread to any of the 119 uninoculated melon and any of the 31 uninoculated squash plants (Table 5). In similar experiments with non-transgenic plants, strain WL was efficiently transmitted (28 infected melons of 33, and 22 infected squash of 45) but not strain C (none of 7 melon infected, and none of 8 squash infected) (Table 5). Similar to the aphid transmission experiments performed in the laboratory, strain WL had a higher transmission rate from melon than squash (85 vs 49%). To verify the infectivity of the inoculum, CMV strain C was sucessfully transferred by

**Table 5.** Incidence of aphid-vectored CMV infection in screenhouses established in the field

| *Crop* | *Line* | *Challenge strain* | *No inoculated plants* | *CMV infection Infected/Tested*[a] | % |
|---|---|---|---|---|---|
| Melon | H-3, H-7 | C | 50 | 0/60 | 0 |
| | T-22, T-64 | C | 45 | 0/59 | 0 |
| | control | C | 11 | 0/7 | 0 |
| | control | WL | 33 | 28/33 | 85 |
| Squash | S-3, S-4, | C | 31 | 0/31 | 0 |
| | control | C | 10 | 0/8 | 0 |
| | control | WL | 45 | 22/45 | 49 |

[a]Number of uninoculated plants that became infected with CMV over the total number of plants analyzed. CMV infections were determined by monitoring of symptom development and ELISA using γ-globulins specific to CMV subgroup I and II strains. Infected samples had OD 405 nm readings at least 12 times higher than healthy controls (1.2–0.45 vs 0.035) after 30 min substrate hydrolysis.

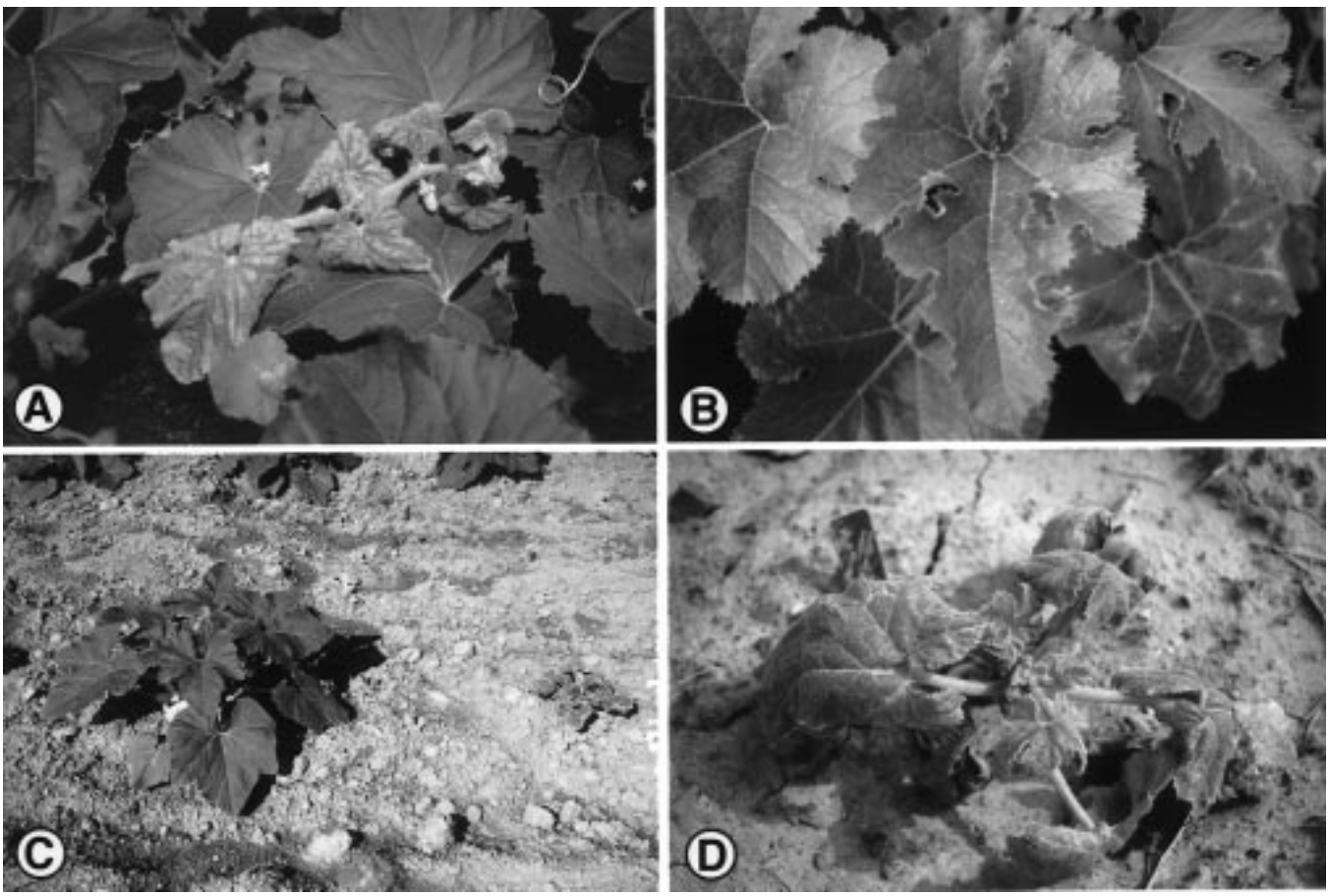


**Fig. 4.** Symptoms of CMV strain C on mechanically-inoculated transgenic melon and squash plants. Symptoms were on: (A) Melon line H-3 showing chlorosis and downward curling of the leaves on top of a healthy non-transgenic plant in a screenhouse, (B) Squash line S-3 showing chlorotic blotches along main veins of inoculated leaves and asymptomatic uninoculated upper leaves in a screenhouse, (C) Squash line ZW-20 showing chlorosis and stunted growth (right) while an uninoculated non-transgenic plant shows normal growth (left) in the field, and (D) close-up of squash line ZW-20 showing foliar chlorosis and downward curling in the field.

hand rubbing from a few infected transgenic plants grown in screenhouses to healthy squash plants in the greenhouse.

## Discussion

This is the first report on risk assessment of potential changes in virus-vector specificity mediated by viral CP genes in transgenic plants that were tested under field conditions. We examined how transgenic melon and squash expressing CP genes of the aphid transmissible strains WL of CMV or FL of ZYMV and WMV 2 might affect the spread of the aphid non-transmissible CMV strain C. Our field experiments, which were conducted under conditions of high disease pressure but low selection pressure, showed that transgenic plants expressing these CP genes did not mediate the spread of the aphid non-transmissible CMV strain C.

Spread of the aphid non-transmissible CMV strain C did not occur despite optimal conditions of interaction between CPs and CP mRNAs in transgenic plants, and CMV strain C. Optimal conditions of interaction included the use of CP genes from highly aphid transmissible virus strains (Quemada *et al*., 1990; Quemada *et al*., 1991; Rodriguez, 1993; Tricoli *et al*., 1995) in transgenic plants, the very close proximity of CMV strain C-infected transgenic plants and uninoculated non-transgenic aphid-recipient plants, and the establishment of field plots when populations of alate aphid are abundant (Nawrocka *et al*., 1975). Our findings have practical significance because (1) CMV has a wide host range, (2) CMV is economically important worldwide, (3) trangenic plants expressing the CMV CP gene have been produced (Fuchs *et al*., 1997; Fuchs *et al*., 1996; Gonsalves *et al*., 1992; Tricoli *et al*., 1995), and (4) transgenic plants expressing the CMV CP gene are likely to be commercialized.

Heterologous encapsidation is one mechanism by which CPs of aphid transmissible virus strains in transgenic plants could have assisted the spread of the aphid non-transmissible CMV strain C. Although spread of CMV strain C was not observed, there is no reason to believe that heterologous encapsidation could not occur when CMV strain C infects transgenic plants expressing the CP gene of CMV strain WL. In fact, Candelier and Hull (1993) showed that the RNAs of CMV can be encapsidated by the CP of alfalfa mosaic alfamovirus in transgenic plants. Furthermore, Gera *et al*. (1979) provided evidence that CMV virions assembled with the RNAs of a poorly aphid transmissible isolate and the CP subunits of a highly aphid transmissible isolate were efficiently transmitted by aphids after membrane acquisition. Also, heterologous encapsidation has been suspected for CMV in mixed infected non-transgenic plants (Perry and Francki, 1992). Interestingly, heterologous encapsidation in transgenic plants has been demonstrated for virus species with rod-shaped (tobamoviruses) (Holt and Beachy, 1991; Osbourn *et al*., 1990), flexuous (potyviruses) (Farinelli *et al*., 1992; Hammond and Dienelt, 1997; Lecoq *et al*., 1993), and bacilliform (Candelier-Harvey and Hull, 1993), but not spherical particles (nepoviruses) (Cooper *et al*., 1994), like CMV.

Recombination is another mechanism by which the aphid non-transmissible CMV strain C could have been rendered aphid transmissible. Unlike heterologous encapsidation, however, several factors act against recombination as a possible mechanism inducing changes in virus-vector specificity. It is known that the 3′ untranslated region of viral RNAs is involved in RNA recombination with viral transgene mRNAs (Greene and Allison, 1994; 1996). In our case, the CMV, WMV 2 and ZYMV CP gene constructs contain only a short segment of their 3′ untranslated sequences, ca. 90 nt for CMV (Namba *et al*., 1991; Slightom, 1991) and ca. 40 nt for WMV 2 and ZYMV (Namba *et al*., 1991; Slightom, 1991; Tricoli *et al*., 1995). These short segments correspond to 35 and 15% of the 3′ untranslated region of CMV RNA 3 (Quemada *et al*., 1989) and the potyviral genomic RNA (Quemada *et al*., 1990), respectively. Importantly, the three CP genes constructs lack the extreme 3′ terminus of the 3′ untranslated region. In fact, for an aphid transmissible variant of CMV strain C to emerge through recombination, it would need to incorporate part or the entire region of the CP mRNA of CMV strain WL that governs aphid transmissibility. The attributes of the three CP gene constructs considerably lessen the chances of such recombination events to occur and then result in aphid-vectored spread of the recombinant isolate under field conditions. In other words, the probabilities that recombination will make CMV strain C aphid transmissible are remote.

Although low levels of CMV infection were observed in our field experiments, characterization of the CMV isolates recovered in the field showed that they were non-targeted isolates that were introduced via aphids from outside our fields. None of the 38 CMV isolates from the field contained a Phe at position 168 of the CP which is specific to strain C. Additionally, all CMV isolates from the field were aphid transmissible. If CMV strain C was rendered aphid transmissible through heterologous encapsidation, the virions subsequently recovered in uninoculated plants would have been aphid non-transmissible. If CMV strain C was rendered aphid transmissible through recombination, aphid transmissible CMV variants that belong to subgroup II, like strain WL, would have been recovered in uninoculated plants. Although all CMV isolates from the field were aphid transmissible, they all belonged to subgroup I. Lastly, our screenhouse experiments, which excluded non-targeted viruses, substantiated our data from open field experiments by showing that CMV strain C was not transmitted from inoculated transgenic plants.

Identification of CP residue 168 revealed a His (codon CAT) in 39% (15/38) of the CMV isolates from the field. Residue His 168, which resulted from a single nucleotide substitution (C instead of T) at position 502 of the CP gene relative to the commonly found Tyr (codon TAT) 168, was not expected since it has not been reported (Palukaitis *et al*., 1992). Thus, His 168 suggests higher rates of natural variation in the CMV CP than previously reported. Limited information is available on the genetic variability of natural populations of CMV. Fraile *et al*. (1997) analyzed numerous CMV isolates from the field by RNase protection assay using probes complementary to different regions of the genomic RNAs. While this approach is powerful to detect mixed infections, reassortants and recombinants, it does not provide insights on the variations at the nucleotide and amino acid levels. Our approach advances another possibility to investigate variations in the CP of CMV.

So far, most studies on risk assessment of transgenic plants expressing viral CPS have been conducted in the laboratory and greenhouse. While such experiments are valuable to address potential impacts and their frequency of occurrence, they give limited insights in environmental risk assessment. The present work extends the research on risk assessment into the field with transgenic crops that are targeted for commercial release, thus providing more relevant information to actual agricultural practices.

Our data show that field scale use of transgenic plants expressing the CMV CP gene did not affect the spread or development of modified viruses and the creation of novel virus species. Can our results on transgenic cucurbits and CMV be extrapolated to other transgenic crops engineered for virus resistance and other CP gene constructs? At this point, it is preliminary to attempt such generalization since more field work is needed. For example, field studies are needed with potyviruses for which heterologous encapsidation is well documented (Farinelli *et al*., 1992; Hammond and Dienelt, 1997; Lecoq *et al*., 1993). Also more work is needed with transgenic perennial crops that could serve as reservoirs of CPs and CP gene mRNAs for longer periods of time than annual crops, therefore increasing the chances of heterologous encapsidation and recombination. Nevertheless, our results support the predictions of APHIS-USDA and others (Falk and Bruening, 1994) that transgenic plants expressing virus CP genes will likely have little to no detectable environmental impact beyond those of natural background events.

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