

## Expression of Coat Protein Gene from Cucumber Mosaic Virus Strain C in Tobacco: Protection Against Infections by CMV Strains Transmitted Mechanically or by Aphids

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### ABSTRACT

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The coat protein (CP) gene from cucumber mosaic virus (CMV) strain C was engineered for expression in plants and transferred into the genome of tobacco (*Nicotiana tabacum* 'Xanthi'). Transfer of the CP gene was confirmed, and plants containing it produced the expected 1,400-nucleotide mRNA and 24-kDa protein products, which were detected by northern and western blots, respectively. Transgenic tobacco plant lines were infected with CMV strains C and Chi of subgroup I and strain WL of

subgroup II, transmitted mechanically or by aphids. The effectiveness of the protection varied in different transgenic plant lines, ranging from almost complete protection to none, depending upon the challenge strain. These experiments demonstrate that within a group of transgenic plants expressing the CP gene of CMV strain C (subgroup I), plant lines can be found that show a significant degree of protection when challenged with CMV strains of either subgroup.

*Additional keywords:* engineered protection, protein analysis, range of protection.

Several studies have shown that virus infection can be reduced by the expression of a viral coat protein (CP) gene in transgenic plants (7,11,13,16,18,23,28,29,31,38-41). This CP-mediated protection has many characteristics in common with the well-known phenomenon of viral cross-protection. For example, in viral cross-protection, plants inoculated with a mild viral strain are usually protected against infections by more severe strains of the same virus, but not against inoculation with the viral RNA (8,34), although exceptions have been reported (45). This difference in susceptibility to virions and viral RNA is also observed in most cases of CP-mediated protection (23,29,40,41), although there have been exceptions reported as well (11).

One additional characteristic of viral cross-protection is that the protecting strain is not effective against all other strains of the same virus or against other viral types (34,42,45). This difference in effectiveness has also been found in CP-mediated protection. Transgenic plants expressing the CP gene from tobacco mosaic virus (TMV) strain UI are protected more effectively against that strain than against TMV strain PV230 (28,29). Plants expressing the CP gene from alfalfa mosaic virus (AIMV) strain 425 are protected more effectively against that strain than against the distantly related tobacco streak virus (TSV). Reciprocal results were observed with transgenic plants expressing the CP gene from TSV (41). The CP gene from tobacco rattle virus (TRV) strain TCM provides protection against pea early browning virus (which has a CP nearly identical to that of strain TCM), but not against TRV strain PLB, whose CP shares an amino acid identity of only 39% with that of strain TCM (39). In studies testing distinct viruses, plants expressing the TMV CP gene had delayed symptom development when challenged with potato virus X (PVX), cucumber mosaic virus (CMV), potato virus Y (PVY), and AIMV; plants expressing the AIMV CP gene also had delayed symptom development when challenged with PVX and CMV, but not when challenged with TMV strain UI (4). Furthermore, Stark and Beachy (36) found that the soybean mosaic virus CP gene provides

protection against symptom expression in infections by other potyviruses. In addition, the TMV CP gene provides protection against other tobamoviruses (27).

CMV is one of the viruses for which CP-mediated protection has been demonstrated (7). It is one of the most economically important plant viruses, because of its wide host range and the large number of different strains isolated (17,33). Over 39 strains of CMV have been identified, and they appear to fall into two biologically and biochemically distinguishable groups. Subgroup I includes strains C, D, Fny, and Y; subgroup II includes strains Q and WL. Within the subgroups, CP amino acid sequences share more than 95% sequence identity (7,32). However, the degree of identity between the subgroups is only approximately 80% (32).

Because CMV has numerous strains, it is important to determine the extent of protection that the CP of one strain provides against others. It has been shown that plants expressing the CP gene from a strain in CMV subgroup I (strain D) are protected against infection by another strain from the same subgroup (strain C) (7). The extent of protection against more distantly related CMV strains (i.e., those from the other subgroup) needs to be determined. The results of such experiments have practical significance, because commercial transgenic plants are likely to be grown in a wide range of geographic locations and are therefore likely to encounter strains from both subgroups.

In addition to strain variability, the method used to infect plants must also be considered. Most tests with plants expressing viral CP genes have used mechanical means to transfer the challenge viruses. More normal infection conditions must be used to determine the actual level of resistance of transgenic plants prior to testing in the field. This is especially pertinent for plants engineered for resistance to aphid-transmissible viruses, such as CMV. In mechanical inoculation, plants are briefly exposed to the challenge viruses (albeit at a high dose), whereas plants in the field are continually exposed to various levels of challenge by aphids.

We report here experiments with transgenic tobacco plants expressing the CP gene from CMV strain C (CMV-C) and inoculated (mechanically or by aphids) with CMV strains WL (CMV-WL) and Chi (CMV-Chi). CMV-WL is a member of subgroup II,

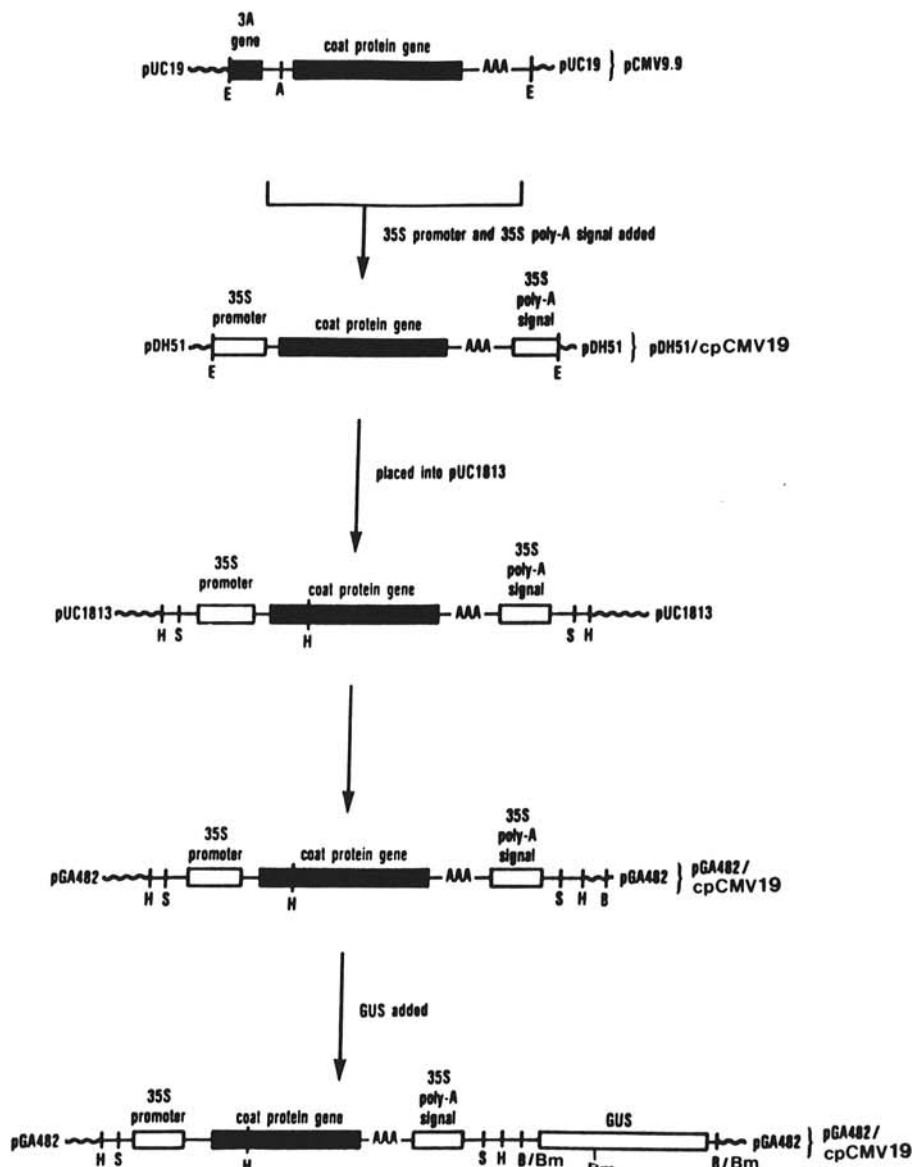
and its CP differs from that of CMV-C by approximately 30% at the nucleotide sequence level and 20% at the amino acid sequence level (32). CMV-Chi has been found to be serologically related to CMV-C (20), and therefore its CP amino acid sequence should differ from that of CMV-C by only a few percentage points (32). CMV-Chi was selected for these studies because it is a highly virulent strain with especially stable virus particles (20).

## MATERIALS AND METHODS

**Plasmids.** The plasmid pDH51 contains a polylinker site flanked by the cauliflower mosaic virus (CaMV) 35S transcript promoter and 35S polyadenylation signal (30). The plasmid pUC1813 contains a polylinker that provides two symmetrically placed sites for several restriction enzymes (19). The *Agrobacterium tumefaciens* Ti plasmid derivative pGA482 contains a kanamycin

resistance marker, neomycin phosphotransferase II (NPT II), driven by the nopaline synthase (Nos) promoter (expressible in both bacteria and plants), as well as a polylinker, both of which are located within the transferred (T-DNA) region (2). The gene for  $\beta$ -glucuronidase (GUS) was removed from the plasmid pBI221 (Clontech Laboratories, Inc., Palo Alto, CA). This gene is driven by the CaMV 35S promoter and is terminated by the Nos polyadenylation signal (15).

**Engineering of the CMV-C CP gene for expression in plants.** The CMV-C CP gene was excised from the plasmid pCMV9.9 (32) as an *AccI-EcoRI* fragment (Fig. 1). This fragment contains all of the sequences corresponding to RNA 4 of CMV-C, except for the 5'-terminal eight nucleotides. In addition, 85 adenine residues were added to this fragment at its 3' end, by polyadenylation of the CMV-C RNA prior to first-strand cDNA synthesis (32). The *AccI-EcoRI* fragment was treated with the Klenow fragment



**Fig. 1.** Engineering the coat protein (CP) gene of cucumber mosaic virus strain C (CMV-C) for expression in plants. The gene was removed from the plasmid pCMV9.9 (32) by means of the restriction enzymes *AccI* and *EcoRI*; the 1.1-kb fragment contains most of the RNA 4 of CMV-C. This DNA fragment was treated with the Klenow fragment of *Escherichia coli* DNA polymerase I (25) and cloned into the *SmaI* site of the cauliflower mosaic virus 35S gene vector pDH51 (30), to obtain the clone pDH51/cpCMV19. The CMV-C CP gene expression cassette was removed from pDH51/cpCMV19 by means of *EcoRI* and cloned into the *EcoRI* site of pUC1813 (19) to add flanking *HindIII* sites. The larger *HindIII* fragment (1.9 kb) was isolated by a partial *HindIII* digest and cloned into the *Agrobacterium* binary vector pGA482 (2), to obtain the clone pGA482/cpCMV19. To aid in the detection of transgenic plants containing the engineered CMV-C CP gene, the  $\beta$ -glucuronidase (GUS) gene (15) was cloned into the *BglII* site of pGA482/cpCMV19. This binary vector was complete, except for the addition of a selectable marker gene (Fig. 2) to aid its transfer into various *Agrobacterium* strains. The restriction enzyme sites are A, *AccI*; B, *BglII*; Bm, *BamHI*; B/Bm, indigestible fusion of *BglII* and *BamHI* sites; E, *EcoRI*; H, *HindIII*; and S, *SmaI*.

of DNA polymerase I (25) in order to generate blunt ends and was then ligated into the *Sma*I site of pDH51. The resulting clones were analyzed by restriction enzyme digestion to determine the orientation of the insert with respect to the CaMV 35S transcript promoter and polyadenylation signals. One clone that mapped correctly, pDH51/cpCMV19 (Fig. 1), was sequenced to confirm its structure.

This CMV-C CP gene expression cassette was removed from pDH51/cpCMV19 by complete digestion with *Eco*RI and was ligated into the *Eco*RI site of pUC1813. This fragment was then removed by a partial *Hind*III digestion and ligated into the *Hind*III site of pGA482. The structure of the resulting plasmid, pGA482/cpCMV19 (Figs. 1 and 2), was verified by restriction enzyme site mapping.

The GUS gene was added to pGA482/cpCMV19 as follows. The plasmid pBI221 was digested with *Eco*RI and *Hind*III, and the 3-kb GUS expression cassette (15) was isolated, treated with the Klenow fragment of *Escherichia coli* DNA polymerase I to generate blunt ends, and then ligated into the *Sma*I site of pUC1813, resulting in the clone pUC1813GUS. The GUS-containing fragment was obtained by a partial *Bam*HI digestion of pUC1813GUS and was ligated into the *Bgl*II site of pGA482/cpCMV19, to generate the plasmid pGA482GG/cpCMV19 (Figs. 1 and 2).

To facilitate the transfer of this binary construction into various *Agrobacterium* host strains, a bacterial gentamicin gene (1) was introduced into the *Sal*I site adjacent to the left T-DNA border of pGA482GG/cpCMV19, resulting in the plasmid pGA482GG/cpCMV19 (Fig. 2).

**Transformation of tobacco tissues.** The pGA482GG/cpCMV19 construct was transformed into the *A. tumefaciens* disarmed strain LBA4404 (12) by the method described by An (3). The leaf disk transformation system (14) was then used to introduce the T-DNA region of pGA482GG/cpCMV19 into cells of tobacco, *Nicotiana tabacum* 'Xanthi-nc.' Transformed cells were selected by their ability to grow shoots and roots in the presence of kanamycin at 500 µg/ml. After transfer to soil, kanamycin-resistant plantlets were assayed for the expression of GUS according to the procedure of Jefferson et al (15).

**RNA and protein analyses of CMV-C CP.** For northern blot analysis, the RNA isolation procedure of Logemann et al (24) was followed, and gel electrophoresis and RNA transfer onto nylon filters were done as described by Maniatis et al (25). The filter was hybridized against a nick-translated <sup>32</sup>P-labeled CMV-C CP gene fragment isolated from pCMV9.9 (32). The hybridization and washing conditions were as described by Maniatis et al (25).

The size and stability of the CMV-C CP was checked by western blot analysis. Total leaf proteins were isolated from leaf tissue by the method of Monroy and Schwartzbach (26), fractionated by electrophoresis on a 12% polyacrylamide gel, and transferred to nitrocellulose as described by Towbin et al (37). Antibodies to CMV-C CP were prepared by Southern Biotech (St. Petersburg, FL), using protein that had been purified by disruption of whole virions in Laemmli gel buffer, subsequent electrophoresis under denaturing conditions (21), and excision of the CP band after visualization with cold 0.2 M KCl. CMV-C protein bands were detected by the CMV-C CP antibody and alkaline phosphatase-conjugated goat anti-rabbit antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

For enzyme-linked immunosorbent assay (ELISA), anti-CMV-C antibody raised against whole virions was used. Known amounts of purified CP in nontransformed tobacco extract were used as standards for calculating the levels of CP in transformed plants, by the procedure of Clark and Adams (6). Calculations were based on OD<sub>405</sub> readings of 0.1–0.3 for extracts of transformed plants, versus readings of 0.03 or less for extracts of controls (healthy nontransformed plants). Total soluble protein concentrations were determined by the method of Bradford (5), and the level of CP produced by plants was expressed as a percentage of the total soluble protein. For detection of viral infection, the antibodies described above or CMV-iv antibodies

(obtained from Agdia, Inc., Elkhart, IN) were used. These assays were used to confirm the visual evaluation of symptoms.

**Mechanical inoculation tests.** R<sub>0</sub> plants were challenged with a non-aphid-transmissible isolate of CMV-C (R. Rodriguez and D. Gonsalves, unpublished data). Tobacco leaves infected with CMV-C were ground in PEN buffer (9), and the extract was rubbed on leaves dusted with Carborundum abrasive (two leaves per plant, the first two nonembryonic leaves). In the R<sub>0</sub> plant infections, the controls were regenerated from leaf pieces by the same tissue culture procedures as those for obtaining transgenic plants, except that the controls were not cocultivated with *Agrobacterium* and were not subjected to kanamycin selection. Plants were examined daily for symptoms. At 6 wk postinoculation, samples were taken from all plants for ELISA, to quantitate the level of virus.

R<sub>1</sub> plants were germinated under sterile conditions in the presence of kanamycin (500 µg/ml) and assayed for GUS activity and the presence of CMV-CP. Inoculations were then carried out as described above. Control plants (line 042288.3A) contained the Nos-NPT II gene but had no detectable GUS activity and no CMV-C CP. For inoculations, CMV strains C, WL (10), and Chi (20) were propagated by infecting tobacco leaves, and the inocula were prepared by grinding leaves in PEN buffer. The infectivity of these dilutions was measured by inoculating *Chenopodium quinoa* with the extracts used for infecting tobacco and then counting the number of local lesions per leaf on this host.

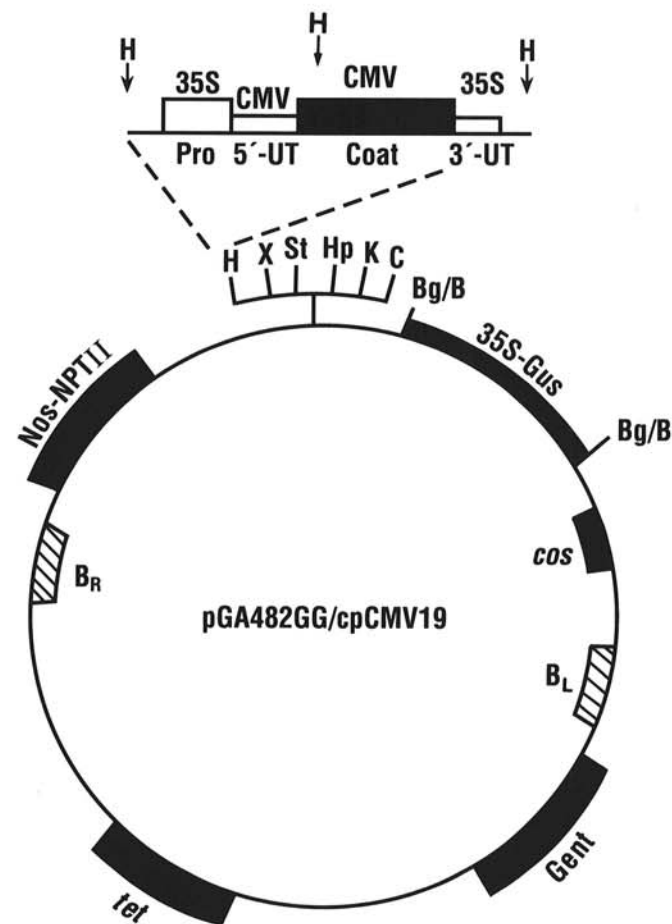


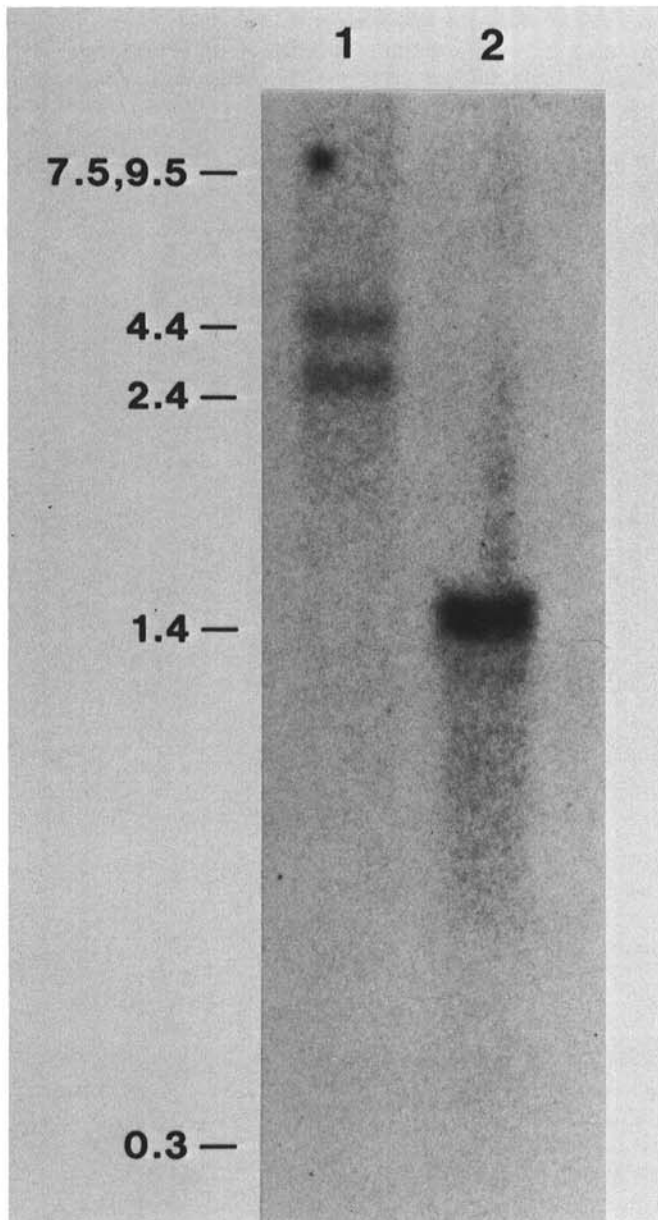
Fig. 2. Organization of the binary vector pGA482GG/cpCMV19. This plasmid vector contains the T-DNA border fragments (B<sub>R</sub> and B<sub>L</sub>) of pTi37, the *cos* site from bacterial phage λ, a restriction enzyme polylinker, and the fusion gene for nopaline synthase and neomycin phosphotransferase II (Nos-NPT II). The bacterial gentamicin (*Gent*) gene (1) was added to aid the identification of plasmid transfer into *Agrobacterium*, and *tet* refers to the tetracycline gene present in the original pGA482 vector (2). The restriction enzyme sites are Bg/B, indigestible fusion of *Bgl*II and *Bam*HI sites; C, *Clal*; H, *Hind*III; Hp, *Hpa*I; K, *Kpn*I; St, *Stu*I; and X, *Xba*I.



Therefore, all measures of virus dose reported in this paper are in terms of local lesions per leaf of *C. quinoa*, a standard that we believe provides an accurate measurement of the biological activity of a particular virus inoculum. In order to confirm the visual measurements of infections, ELISA was performed as described above.

**Aphid transmission.** Three strains of CMV were used in aphid transmission trials: 1) an aphid-transmissible isolate of CMV-C (CMV-Cat), 2) CMV-WL, and 3) CMV-Chi. The aphid transmissibility of these isolates was previously determined (R. Rodriguez and D. Gonsalves, unpublished data). Aphids (*Myzus persicae*) were reared on pepper plants (*Capsicum annuum*). True leaves of zucchini plants (cv. President), which had been infected

with one of the CMV isolates 7–10 days prior to virus acquisition access feeding, served as virus sources. The test plants were inoculated at the three- to four-leaf stage. Groups of about 30 aphids that had been starved for about 2 h were placed on freshly excised symptomatic zucchini leaves and allowed to feed for 5 min. Five aphids were transferred to each test plant and left to feed overnight; then the plants were sprayed with nicotine to kill the aphids. In order to have uniform inoculation conditions after acquisition access feeding, aphids (in groups of five) were alternately placed on test plants of different treatments. The test plants were kept in a glasshouse throughout the experiment. The plants were observed for symptoms at 2- to 3-day intervals and tested by ELISA 20 days after aphid inoculation. The experiment was concluded 25 days postinoculation. To confirm the visual assessment of infection, ELISA was performed with antisera to CMV-C and CMV-WL as described by Edwards and Gonsalves (9).



**Fig. 3.** Check of the mRNA transcription product of cucumber mosaic virus strain C (CMV-C) resulting from the CMV-C coat protein (CP) gene expression cassette. Total RNA was isolated from transformed tobacco plant 021088.3 as described in Materials and Methods, electrophoresed in a formaldehyde agarose gel, and transferred to a nylon filter. This filter was probed with the CMV-C CP gene isolated from clone pCMV9.9 (32), which was  $^{32}\text{P}$ -labeled by nick translation. Lane 1, RNA size standard (Bethesda Research Laboratories, Gaithersburg, MD); lane 2, 10  $\mu\text{g}$  of total mRNA isolated from transgenic plant 021088.3. The CMV-C CP mRNA band is about 1,400 nucleotides.

## RESULTS

**$R_0$  plants.** *Kanamycin-resistant plantlets and GUS expression.* After 4 wk on kanamycin-containing shooting medium, tobacco leaf pieces cocultivated with *A. tumefaciens* strain LBA4404, which contained the engineered CMV CP gene (Fig. 1) located between the T-DNA borders of the binary plasmid pGA482GG/cpCMV19 (Fig. 2), developed numerous shoot-producing calluses. The calluses (one per leaf piece, on average) produced shoots continuously over a period of 3–4 wk after the appearance of the first shoot. To sample as many independent transformation events as possible, only one shoot per callus was excised and transferred to rooting medium. A total of 128 independent shoots were rooted.

After 2–3 wk on rooting medium in the presence of kanamycin (500  $\mu\text{g}/\text{ml}$ ), plantlets were transplanted to potting soil. Leaf samples (each about 0.1 g) were collected and assayed for GUS activity (15). Positive GUS assays were recorded for 50% of the plantlets (data not shown), confirming their transformed status. ELISA showed that some of the GUS-negative plants nevertheless contained and expressed the CMV-C CP gene (data not shown).

*RNA and protein analyses of CMV-C CP.* Three putatively transformed plantlets were analyzed for the expression of CMV-C mRNA by hybridizing the nick-translated insert of pCMV9.9 (32) against slot-blotted total plant RNAs. Hybridization signals were observed only in RNAs isolated from the putative transformed plants; no hybridization signals were observed in RNAs isolated from nontransformed plants (data not shown). The size of this CMV-C mRNA was checked by extracting total RNA from the  $R_0$  transgenic plant 021088.3 and using it for northern blot analysis. The autoradiograph detected a single mRNA band of about 1,400 nucleotides (Fig. 3). In vivo translation of CMV-C CP mRNA was checked by western blots using antibodies raised against CMV-C CP. In an analysis of protein extracted from the leaves of the  $R_0$  transgenic plant 021088.1, this technique detected a CMV-C CP-specific band having the same mobility (24 kDa) as purified CMV CP (Fig. 4).

*Mechanical inoculation with CMV-C.* Fifteen kanamycin-resistant  $R_0$  tobacco plants were assayed for GUS activity and were subsequently tested by ELISA for the presence of CMV-C CP (Table 1). These plants were then challenged with a viral inoculum prepared by grinding a CMV-C-infected leaf in PEN buffer (1:10, w/v), and the number of days until symptoms were observed was recorded. In addition, four nontransgenic control plants, derived by tissue culture, were challenged with the same viral inoculum as the transgenic plants. Table 1 presents the development of symptoms in the transgenic plants relative to the nontransgenic control plants. Five of the 15 transgenic plants tested were asymptomatic 6 wk postinoculation (31 days after symptoms had developed on the controls). One other plant (042288.6) had barely detectable symptoms. Reduced symptoms were observed only in plants that expressed the CP gene. There were, however, plants that expressed the CP gene but showed symptoms; for example, plants 042288.1 and 042288.2 expressed high CP levels but developed symptoms at nearly the same time

as the control plants (Table 1).

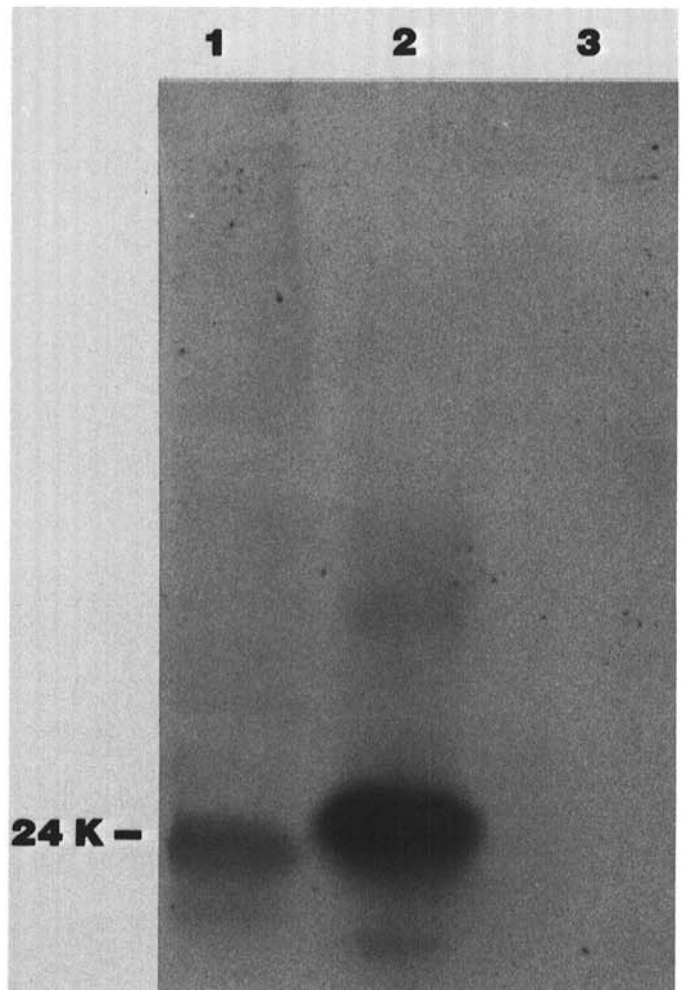
ELISA was performed 6 wk postinoculation, to measure the CP levels in plants (Table 1). Presumably, significant increases in CP levels reflected virus replication. As expected, most symptomatic plants (except 042288.12) had high levels of viral antigens. Asymptomatic plants, however, showed varying levels of viral antigens. For example, three asymptomatic plants (041988.1, 041988.2, and 042288.13) had ELISA values that were less than 5% of that of the infected control, indicating virus inhibition. In contrast, one asymptomatic plant (042288.5) had high levels of viral antigen, thus suggesting that viral replication could be high even in a plant with the asymptomatic phenotype. In this case, protection appears to be manifested by a suppression of symptoms rather than inhibition of viral accumulation. These two types of protected plants suggest that CP-mediated protection may operate through more than one mechanism.

**R<sub>1</sub> plants.** *Mechanical inoculation with CMV-C, CMV-WL, and CMV-Chi.* Several R<sub>0</sub> lines were advanced to the R<sub>1</sub> generation by self-fertilization. The offspring from two of the plants (041988.2 and 042288.3) used in the experiments described above, as well as offspring from two other R<sub>0</sub> plants (040388.28 and 040388.32), were chosen for further study. (The other R<sub>0</sub> plant lines that showed excellent protection [Table 1] were sterile and thus could not be advanced.) R<sub>1</sub> line 041988.2A was derived from 041988.2, which had been found to accumulate the highest level of CMV-C CP as determined by ELISA (0.16% of total soluble protein) and remained asymptomatic following inoculation with CMV-C (Table 1). R<sub>1</sub> lines 040388.28A and 040388.32A were moderately expressing lines (0.07% of total soluble protein) from R<sub>0</sub> plants that had not been challenge-inoculated with CMV-C. All three lines expressed the GUS gene in addition to the CMV-C CP gene. R<sub>1</sub> line 042288.3A (derived from 042288.3) was selected as a control transgenic line, because it was kanamycin-resistant but did not contain detectable levels of GUS or CMV-C CP (Table 1). This plant line also showed symptoms similar to those of the nontransgenic control plants.

Seeds of the CP-gene-expressing lines were germinated in a medium containing kanamycin (500 µg/ml) and then challenged with viruses. Plant lines 040388.28A and 040388.32A showed

marginal levels of protection against CMV-WL and essentially no protection against CMV-C (Fig. 5). In contrast, plant line 041988.2A showed excellent protection against CMV-C and CMV-Chi (subgroup I) and moderate protection against CMV-WL (subgroup II) (Fig. 6). It should be noted that only 47% of the control plants inoculated with CMV-C became infected during this test (Fig. 6). However, none of the corresponding 041988.2A plants showed infection. Typically, most of the transgenic plants that became infected developed symptoms about 10 days after the control plants, and their symptoms were as severe as those of the control plants.

*Aphid transmission.* Mechanical inoculation is an effective and the most common method for inoculating plants with many plant viruses, including CMV. However, CMV is primarily transmitted in nature by aphids in a nonpersistent manner. Thus, R<sub>1</sub> transgenic tobacco plants of lines 042288.3A (not expressing the CP gene) and 041988.2A (expressing the CP gene) were used to test the effect of CP-mediated protection against CMV inoculation by aphids. Unlike those in the previous experiment, these R<sub>1</sub> plants were not preselected by germinating in a medium containing kanamycin; instead the seeds were germinated in potting soil. Segregation of the transgenic phenotype was determined during the experiment by GUS assays. As expected, none of the nontrans-



**Fig. 4.** Check of the coat protein (CP) translation product obtained from plants expressing the CP gene from cucumber mosaic virus strain C (CMV-C). Total protein was extracted from the leaves of transgenic tobacco plant 021088.1 as described in Materials and Methods. About 50 µg of the protein extract, treated with sodium dodecyl sulfate, was subjected to electrophoresis through a 12% polyacrylamide gel and blotted on a nylon filter. CMV-C CP was detected as described in Materials and Methods. Lane 1, total soluble protein from plant 021088.1; lane 2, purified CMV-C CP added to total soluble protein from a nontransformed tobacco plant; lane 3, total soluble protein from a nontransformed tobacco plant.

**TABLE 1.** Symptom expression and enzyme-linked immunosorbent assay (ELISA) of R<sub>0</sub> transgenic tobacco plants inoculated with cucumber mosaic virus strain C<sup>a</sup>

Plant	Days to symptom expression	Relative symptom <sup>b</sup>	ELISA (OD <sub>405</sub> )	
			0 days	44 days
Control	13	Same	0.00	2.04
041988.1	...	NS <sup>c</sup>	0.14	ND <sup>d</sup>
041988.2	...	NS	0.44	0.09
042288.1	13	Same	0.36	2.21
042288.2	13	Same	0.13	2.20
042288.3	13	Same	0.00	1.76
042288.4	...	NS	0.15	0.29
042288.5	...	NS	0.40	2.17
042288.6	13	Mild	0.17	2.22
042288.7	13	Same	0.08	2.23
042288.8	13	Same	0.15	2.15
042288.9	16	Same	0.00	2.00
042288.10	17	Same	0.06	2.15
042288.11	13	Same	0.12	2.11
042288.12	17	Same	0.38	0.69
042288.13	...	NS	0.38	0.06

<sup>a</sup> Negative control plants showed symptoms by 13 days postinoculation; only one was sampled for ELISA. ELISA was performed on the days indicated, blanking on a sample that contained substrate buffer plus substrate. The extracts at 0 days were 10 times more concentrated than those at 44 days. This allowed for the detection of coat protein gene expression in transgenic plants at 0 days but not at 44 days.

<sup>b</sup> Same = symptoms similar to those of the control; mild = milder mosaic than in the control.

<sup>c</sup> No symptoms.

<sup>d</sup> Not detectable (below the level of detection, because of the dilution factor described in footnote a).

genic control plants (cv. Havana 423) and none of the 042288.3A plants were GUS-positive. On the other hand, 55 of 75 (73%) of the transformed R<sub>1</sub> plants from plant line 041988.2A were GUS-positive, which is expected for the segregation of a single dominant gene.

Aphid transmission tests with the three CMV strains are summarized in Table 2. Considerable differences were observed in the aphid transmission efficiency for the different strains. CMV-Chi had the highest rates of transmission (60–62%) to nontransformed plants (Havana 423) and the control transgenic plants (line 042288.3A). The transmission rate of CMV-Cat and CMV-WL ranged from 10 to 24% for the corresponding plants. Only one of the 75 aphid-inoculated 041988.2A plants (the total number inoculated with CMV-C, CMV-WL, and CMV-Chi) became symptomatic (Table 2). This symptomatic plant had no GUS activity, indicating that it was a segregant that did not contain the CMV-C CP gene. ELISA, performed on all plants, verified our visual evaluations, as symptomatic plants had much higher OD<sub>405</sub> values than asymptomatic plants (data not shown).

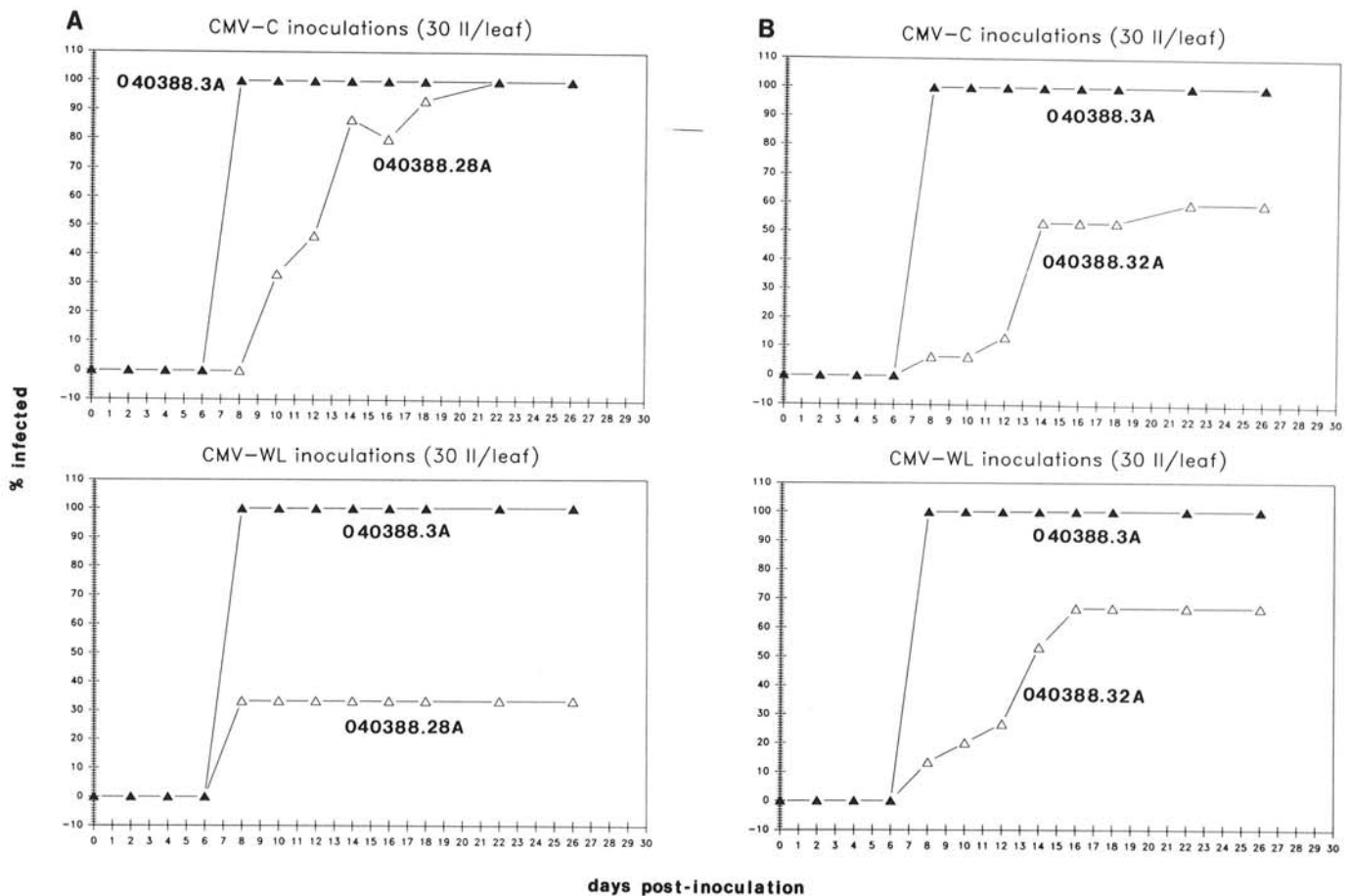
## DISCUSSION

Tobacco tissue cocultivated with *A. tumefaciens* strain LBA4404 containing the binary vector pGA482GG/cpCMV19 yielded transformed R<sub>0</sub> tobacco plants resistant to kanamycin at 500 µg/ml, of which 50% showed expression of the GUS reporter gene. However, some of the GUS-negative plants accumulated detectable levels of CMV-C CP, a result that is consistent with the construction of the vector (Figs. 1 and 2) and the direc-

tional transfer (from the right border to the left border) of the T-DNA regions (43). A check of the mRNA transcript from the engineered CMV-C CP gene showed the presence of a 1,400-nucleotide band (Fig. 3), which is consistent with the expected length if the RNA contains 10 nucleotides from the CaMV 35S promoter region, 1,100 nucleotides of the CMV sequence (32), 200 nucleotides from the CaMV 35S 3'-untranslated region, and an assumed addition of a polyadenylate tract of approximately 100 nucleotides. A check for the protein product, presumably translated from this mRNA species, revealed the presence of a full-length CMV-C CP polypeptide (Fig. 4), and this western blot indicates that this CMV-C CP is not extensively degraded. The CMV-C mRNA and protein checks both indicate that the engineered CMV-C CP expression cassette is functioning as expected in transgenic tobacco plants.

We have shown that transgenic plants (i.e., line 041988.2A; Fig. 6) expressing the CP gene of one subgroup of CMV can provide protection against strains from the other subgroup and against a biologically distinct strain (i.e., the more severe CMV-Chi). Furthermore, our data show that protection is effective against aphid inoculations. This extends to cucumoviruses the observations made by Lawson et al (22) for the potyvirus PVY, that CP-mediated protection is effective against inoculations by aphids.

With the assumed high degree of shared amino acid identity, transgenic plants expressing the CMV-C CP gene would be expected to be better protected against infection by CMV-Chi (a member of the same subgroup) than against infection by CMV-WL (a member of a different subgroup). Indeed, this trend was



**Fig. 5.** Infection of transgenic R<sub>1</sub> plant lines 040388.28A, 040388.32A, and 040388.3A with cucumber mosaic virus strains C (CMV-C) and WL (CMV-WL). R<sub>1</sub> plants from these lines (at least 10 plants from each line) were inoculated with CMV-C and CMV-WL, with a viral dose of 30 local lesions (II) per leaf. The strengths of the inocula were determined by inoculating the local lesion host *Chenopodium quinoa* (see Materials and Methods). **A**, Infection results for plant line 040388.28A (open triangles); **B**, results for similar infections of plant line 040388.32A (open triangles). Plant line 040388.3A (solid triangles) was used as a control, and in both experiments this control line reached 100% infection, indicating that the inoculation procedure (see Materials and Methods) was extremely effective.



observed in transgenic plant line 041988.2A. However, different results were obtained for plant lines 040388.28A and 040388.32A; these lines showed essentially no protection against infection by the homologous virus strain but showed moderate protection against the heterologous strain (Fig. 5).

Thus, in this study, the degree of amino acid sequence identity between expressed and challenge viruses did not show a direct correlation with the observed level of protection. In fact, it is surprising that in two transgenic plant lines the protection was greater against the heterologous strain than against the homologous strain. This result differs from those reported by others (4,28,29,36,39,41; see also the introduction) for plants expressing the CP genes of other viruses. However, in these previous studies, the degree of CP amino acid sequence identity between the expressed and the challenge viruses was much less than that between the CMV subgroups. It appears that at the level of CP

amino acid divergence involved in the present study (about 20%) the predictability of CP protection is low. Clearly, a larger number of transgenic lines and CMV strains need to be tested in order to determine if the degree of shared amino acid identity can be used as a predictor of protection against the many different strains in both CMV subgroups. Such data would help in predicting the performance of transgenic plants under field conditions. Because the CMV subgroups are widely distributed, protection against both subgroups is required. If protection is generally effective only against closely related strains, the usefulness of transgenic plants expressing one CP gene may be somewhat restricted, and their use will require careful selection to fit the needs of a particular plant species in a particular geographic location. For example, surveys in New York State found that strains in subgroup II (e.g., CMV-WL) were dominant in peppers, and those in subgroup I (e.g., CMV-C) were dominant in other crops, such as tomato and cucurbits (20). However, 16% of the tested samples had strains of both subgroups; thus, as a practical strategy, it might be prudent to have transgenic plants that express the CP genes of both subgroups. Efforts to accomplish this goal are in progress.

Our data on the  $R_0$  plants suggest that the CP levels in transgenic plants are not absolute predictors of how well a plant will be protected from viral infections. Similar observations have been reported by others (22,36). One difficulty in determining the significance of the CP levels measured in this study and others is the accuracy of the measurements. We have found measurements of "absolute" CP levels to be quite variable, depending upon the completeness of the tissue disruption and the physiological state of the plant from which the tissue was obtained. On the other hand, relative levels of expression between plant lines are generally constant. Efforts were made in this study to compare CP levels of plants of similar age and physiological state, but it is not clear that the absolute measured levels are especially relevant to the observed protection, since the means by which CP-mediated protection acts (and thus the amounts of CP and the timing required for its effect) are unknown. The possible complexity of the CP-mediated protection mechanism is indicated by recent work, which shows that protection may result from the action of CP on more than one portion of the viral infection cycle (44).

It is conceivable that factors other than the accumulation of CP may influence the protection observed in particular plant lines. These factors may include the level of CP accumulated in specific tissues of transformed plants. Furthermore, somaclonal variation produced by tissue culture may have resulted in plants with altered metabolism. This alteration may affect tissue-specific expression and may also result in some protection against viral infection, independent of the expressed CP gene. These effects will have to be accounted for in attempting to determine the effectiveness of CP-mediated protection.

The aphid transmission experiment indicates that CP-mediated protection does extend to CMV transmitted by its natural vectors. Therefore, aphids do not appear to be delivering viruses in a manner that overcomes the CP-mediated protection mechanism,

TABLE 2. Transgenic  $R_1$  and control tobacco plants infected after aphid inoculation with cucumber mosaic virus (CMV) strains C, Chi, and WL

Plant line <sup>c</sup>	No. of infected plants/No. of test plants <sup>b</sup>		
	CMV-Cat <sup>c</sup>	CMV-Chi	CMV-WL
Havana 423	3/25	16/25	6/25
042288.3A (CP <sup>-</sup> )	4/25	15/25	4/25
041988.2A (CP <sup>+</sup> )	0/25	1/25	0/25

<sup>a</sup> Havana 423, nontransformed; 042288.3A, transformed but not expressing the coat protein gene; 042288.2A, transformed and expressing the coat protein gene.

<sup>b</sup> Five aphids from a population feeding on infected zucchini were used to inoculate each plant. The experiment was concluded 25 days after inoculation.

<sup>c</sup> An aphid-transmissible isolate of strain C.

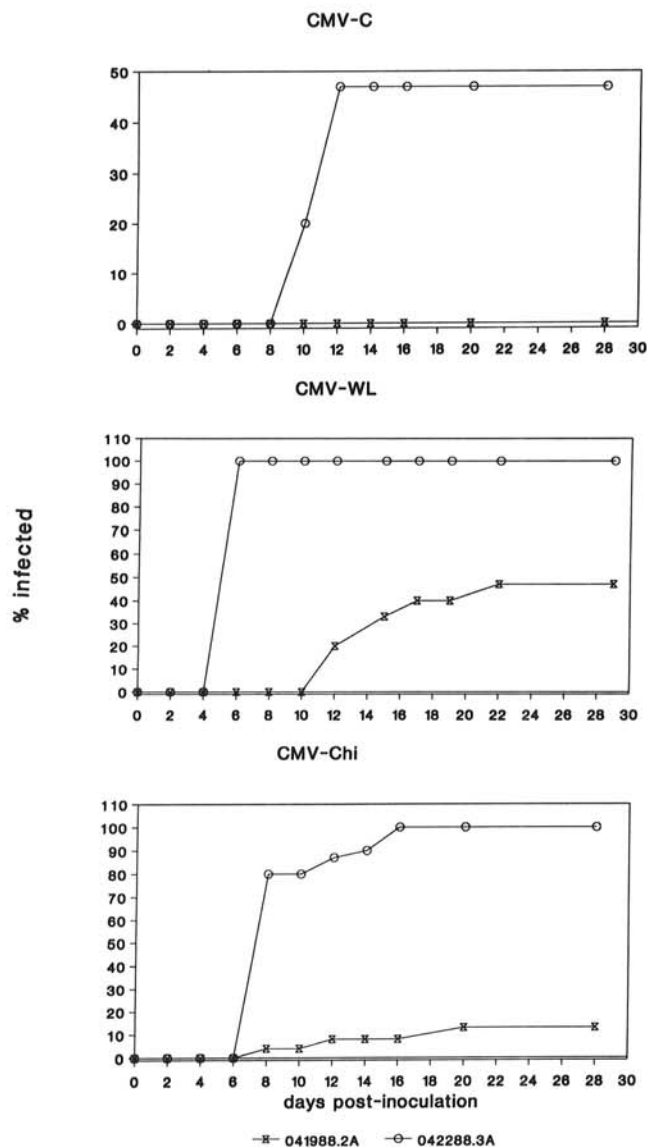


Fig. 6. Infection of the transgenic  $R_1$  plant line 041988.2A, which accumulates detectable levels (0.16%) of coat protein from cucumber mosaic virus (CMV) strain C, and plant line 041988.3A, which does not accumulate detectable levels of coat protein.  $R_1$  plants from these lines were inoculated with CMV strains C (CMV-C), WL (CMV-WL), and Chi (CMV-Chi). The symbol used to designate each plant line is shown at the bottom of the lower frame. Results were obtained from the infection of at least 10  $R_1$  plants with each strain, and the course of infection was followed by enzyme-linked immunosorbent assays for 28 days after infection. For each infection, the amount of virus used yielded 30–90 local lesions on the host *Chenopodium quinoa* (see Materials and Methods).

at least at the level of aphid feeding in this experiment. In the field, however, plants experience repeated inoculations over the course of a growing season; therefore, they have the potential to be exposed to much higher virus levels and numerous infections. The long-term effectiveness of cucumber plants that express CMV-C CP, as engineered in this report, is being tested under natural field conditions. Initial (35) and extensive field tests (D. Gonsalves, P. P. Chee, and J. L. Slightom, *unpublished data*) indicate that these transgenic cucumber plants have high levels of protection against CMV-C infections transmitted by aphids.

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## Molecular Plant Pathology

### A Pathogenicity Locus from *Xanthomonas citri* Evaluable Status from Several Pathovars of *X. campestris* Elicit Characteristic Lesions in Citrus

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## SUMMARY

Wang-Guang Robert DeGroot, Ronald W. Schubert, and Deep W. Chahal. 1991. A pathogenicity locus from *Xanthomonas citri* elicits characteristic lesions in citrus. *Phytopathology* 81:327-331.

*Xanthomonas citri* pathovars appear to have different pathogenicity profiles. However, high sequence homology between the DNAs of a subset of these pathovars from citrus and *Xanthomonas* such as those from *X. campestris* from the citrus production pathogen *Xanthomonas* strains suggest a common ancestry with the pathovars. A pathogenicity locus from *X. campestris* was transferred into citrus and graft-inoculated citrus. Citrus canker-like lesions were induced and citrus canker-like lesions were induced in citrus. The pathovars were evaluated as potential citrus canker pathogens. The citrus canker-like lesions induced in citrus were similar to those of citrus canker-like lesions induced in citrus by citrus canker-like lesions induced in citrus. The pathovars were evaluated as potential citrus canker pathogens. The citrus canker-like lesions induced in citrus were similar to those of citrus canker-like lesions induced in citrus by citrus canker-like lesions induced in citrus.

**KEY WORDS:** Citrus canker, *Xanthomonas citri*, citrus canker, citrus canker.

*Xanthomonas citri* pathovars are highly specific citrus canker pathogens. However, high sequence homology between the DNAs of a subset of these pathovars from citrus and *Xanthomonas* such as those from *X. campestris* from the citrus production pathogen *Xanthomonas* strains suggest a common ancestry with the pathovars. A pathogenicity locus from *X. campestris* was transferred into citrus and graft-inoculated citrus. Citrus canker-like lesions were induced and citrus canker-like lesions were induced in citrus. The pathovars were evaluated as potential citrus canker pathogens. The citrus canker-like lesions induced in citrus were similar to those of citrus canker-like lesions induced in citrus by citrus canker-like lesions induced in citrus.

*Xanthomonas citri* (Hesse) Dickey is the causal agent of a citrus canker disease. The host range of the pathogen includes citrus, guava, and mango, as well as ornamentals such as colea, hibiscus, and geranium. Citrus canker is a major citrus production disease in Florida and other citrus producing areas. Citrus canker is caused by a bacterium which produces a necrotic lesion on citrus leaves, stems, and fruit (1). The pathogen is a Gram-negative bacterium which produces a necrotic lesion on citrus leaves, stems, and fruit (1). The pathogen is a Gram-negative bacterium which produces a necrotic lesion on citrus leaves, stems, and fruit (1).

Several pathovars of citrus canker have been developed in Florida. These pathovars produce only mild to moderate citrus canker lesions on citrus leaves, stems, and fruit (1).

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