Protection of Transgenic Plants Expressing the Coat Protein Gene of Watermelon Mosaic Virus II or Zucchini Yellow Mosaic Virus Against Six Potyviruses

Shigetou Namba, Kaishu Ling, Carol Gonsalves, Jerry L. Slightom, and Dennis Gonsalves

First, second, third, and fifth authors: Department of Plant Pathology, Cornell University, Geneva, NY 14456; fourth author: Molecular Biology Unit 7242, The Upjohn Company, Kalamazoo, MI 49007.

Permanent address of first author: Laboratory of Plant Pathology, Faculty of Agriculture, University of Tokyo, Bunkyo-Ku, Tokyo, Japan.

We thank E. D. Williams and D. K. Hummer for their technical assistance; C. Moore, M. Namba, and N. Lu for help in regeneration of transgenic plants; and S. E. Ecker-Day for maintenance of these plants in the greenhouse. This work was supported by a grant from Asgrow Seed Company and The Upjohn Company.

Accepted for publication 22 May 1992.

ABSTRACT

Namba, S., Ling, K., Gonsalves, C., Slightom, J. L., and Gonsalves, D. 1992. Protection of transgenic plants expressing the coat protein gene of watermelon mosaic virus II or zucchini yellow mosaic virus against six potyviruses. Phytopathology 82:940-946.

The coat protein (CP) genes of watermelon mosaic virus II (WMV II) and zucchini yellow mosaic virus (ZYMV) were engineered for expression in plant tissues and subsequently transferred into *Nicotiana benthamiana* by *Agrobacterium tumefaciens*. Transgenic *N. benthamiana* plants expressing the CP gene of WMV II or ZYMV showed protection against symptom development when inoculated with WMV II and six other potyviruses: bean yellow mosaic (BYMV), potato Y (PVY), pea mosaic (PeaMV), clover yellow vein (CYVV), pepper mottle (PeMV), and tobacco etch (TEV). The level of protection depended on the challenge virus and the inoculum strength, with the best protection being against the lower inoculum dose. Overall, transgenic plants that expressed WMV II or ZYMV CP genes showed highest resistance to WMV II, followed by CYVV; then the group of BYMV, TEV and PeMV; and then PVY and PeaMV. Transgenic plants expressing the WMV II CP gene generally showed better protection against these potyviruses than those expressing the ZYMV CP gene. Given this and other reports, it appears that transgenic plants that express a potyvirus CP gene will show at least a noticeable level of protection against symptom development when challenged by other potyviruses.

Recent approaches utilizing gene transfer technologies have shown promise for obtaining plants that are resistant or tolerant to virus infection (3). The most widely used approach, coat protein–mediated protection (CPMP), was first shown in tobacco plants that expressed the tobacco mosaic virus (TMV) coat protein (CP) gene (29). This technology has since been successfully used with CP genes of many other viruses, including cucumber mosaic virus (CMV) (7,28,31), potato virus Y (PVY) (19,22), potato virus X (PVX) (12,15,19,22), potato virus S (27), tobacco streak virus (TSV) (45), alfalfa mosaic virus (AlMV) (14,25,41,42), soybean mosaic virus (SMV) (40), tobacco rattle virus (TRV) (43,44), papaya ringspot virus (PRV) (24), potato leafroll virus (PLRV) (20), and tobacco etch virus (TEV) (23). Generally, CPMP is effective only against the virus, or related strains, for which the CP gene is expressed (3).

However, two reports (24,40) showed that transgenic plants expressing the CP genes of potyviruses (9,16) are resistant to other distinct potyviruses. Stark and Beachy (40) reported that tobacco plants expressing the SMV CP gene are resistant to TEV and PVY, two potyviruses that are not closely related to SMV (33,36). Interestingly, this resistance is conferred despite the fact that tobacco is not a host of SMV. Elsewhere (24) we also showed that tobacco plants expressing the CP gene of PRV (another potyvirus that does not infect tobacco) were highly tolerant of infection by TEV, PVY, and pepper mottle virus (PeMV). These results have obvious implications, since it becomes theoretically possible to use one or a limited number of potyvirus CP genes to protect plants against infections by other potyviruses.

In the two previous reports (24,40), however, the relative effectiveness of protection afforded by CPMP could not be compared between homologous and heterologous virus infections, because tobacco is not a host of SMV or PRV. This critical comparison is desirable because the relative degree of protection against heterologous potyviruses may not be sufficient to economically protect plants in the field. In addition, a limited amount of data has been collected on homologous CPMP for potyviruses, with the only reported CPMP cases being PVY in potato (19,22) and TEV in tobacco (23).

Thus, we wanted to gather additional data on homologous CPMP for a potyvirus and to expand on the data base for heterologous CPMP by potyviruses. In our experiments, we used CP genes of two important potyviruses that infect cucurbits: water-

melon mosaic virus II (WMV II) and zucchini yellow mosaic (ZYMV). WMV II and ZYMV viruses are distantly related, since their CP genes share about 70% identity at the nucleotide and amino acid sequence levels (32). The CP genes were transferred into *Nicotiana benthamiana* Domin., a host (5,10,30) to WMV II and the other potyviruses used to test heterologous protection.

In this report we show that *N. benthamiana* plants that express the CP gene of WMV II or ZYMV provide a range of protection levels against challenge by WMV II and six other potyviruses. The level of protection against the latter heterologous potyviruses varied considerably and was dependent on the challenge potyvirus used and its inoculum dose.

MATERIALS AND METHODS

Materials. Restriction endonucleases *Eco*RI, *Hin*dIII, *Nco*I, *Xba*I, and *Xho*I were obtained from New England Biolabs (Beverly, MA), Promega (Madison, WI), or Boehringer-Mannheim (Indianapolis, IN). *Taq* polymerase and polymerase chain reaction (PCR) kits were obtained from Perkin-Elmer Cetus Instruments (Norwalk, CT). [α-^{32}P]dATP was obtained from either Amersham (Arlington Heights, IL) or Du Pont (Wilmington, DE). T7 DNA polymerase (Sequenase version 2) was obtained from U.S. Biochemical (Cleveland, OH), and T4 ligase was obtained from either Collaborative Research (Lexington, MA) or Promega. Oligonucleotide synthesis was done with an Applied Biosystems instrument (model 380A, Foster City, CA).

Vectors and plant virus coat protein gene clones. The construction of the expression vector, pUC18CPexp, which has the CaMV 35S promoter fused to the CMV CP gene 5′-untranslated region, has been described elsewhere (37,38). The *Agrobacterium tumefaciens* Ti plasmid-derived binary vector pGA482 has been described by An (1). This vector was modified by the addition of the GUS gene fusion system (18) and the bacterial gentamicin resistance genes to obtain pGA482GG (31). Template DNAs for PCR amplification of the two plant virus CP genes were obtained from the following clones: WMV II from clone pWMVII-3.2 and ZYMV from clone pZYMV-15 (33).

PCR amplification. Each oligonucleotide primer used for PCR amplification contained 20 bp of shared sequence identity flanking the respective CP gene region to be modified (by the addition of flanking *Nco*I sites) and amplified. The 5′-*Nco*I sites were added so that the translation start codon (ATG) was fused to the first CP gene codon after the expected protease cleavage site (32,33) that started with a G-nucleotide. For the engineering of WMV II and ZYMV CP genes, the codon selected was one amino acid codon 3′ of the protease cleavage site. These types of fusions were necessary to preserve the consensus translation initiation site (AAXXATGG) and the *Nco*I restriction enzyme recognition site (CCATGG) (37,38).

PCR amplification reactions consisted of using about 50 ng of each primer and 100 ng of template plasmid DNA in a total reaction volume of 100 μl, using the protocols from the manufacturer (Perkin-Elmer Cetus).

Capsid protein expression cassettes and their transfer to a binary plasmid. The PCR amplified CP gene regions were cloned into pUC18CPexp, and the resulting clones were restriction enzyme–mapped and sequenced to ensure accuracy of the PCR amplification and ligation steps (37,38). The following clones were obtained: pUC18cpWMVII5 containing WMV II CP gene and pUC18cpZYMV6 containing ZYMV CP gene. The nucleotide sequence analyses of these cloned inserts (35) showed a ligation error in clone pUC18cpZYMV-6, where a C nucleotide was deleted from the expected 5′-*Nco*I recognition site, which resulted in the loss of this restriction enzyme recognition site (Fig. 1). Each CP gene expression cassette was removed from the pUC18 plasmid by *Hin*dIII digestion, and the gel-purified DNA fragments were cloned into the *Agrobacterium*-derived binary vector pGA482GG (31) to obtain the following clones: pGA482GG/cpWMVII5-2 and pGA482GG/cpZYMV6-7. The CP$^-$ binary vector pGA482GGCPexp3-1 was also constructed for obtaining CP$^-$ transgenic plants that contained the exact T-DNA region (Nos-NPTII and GUS genes, and the CPexp cassette), except that no CP gene was included. These binary vectors were transferred into avirulent *Agrobacterium* strain C58Z707 (13) using the method described by An (2). Transformed *Agrobacterium* was selected on plates supplemented with 60 μg/ml of gentamicin and 50 μg/ml of kanamycin.

Transformation of *N. benthamiana*. The leaf disk transformation system (17) was used for the introduction of the T-DNA regions of pGA482GG/cpWMVII5-2, pGA482GG/cpZYMV6-7, and the control CP$^-$ vector pGA482GG/CPexp3-1 into *N. benthamiana*. Transformed cells were selected by their ability to grow and regenerate shoots and roots in the presence of 300 μg/ml of kanamycin. Transgenic tissues were regenerated as described by Sanchez-Serrano et al (34), and R_0 plants were selfed to get R_1 progenies.

Analysis of transgenic plants. R_1 plants resistant to kanamycin were analyzed for expression of GUS and virus CP genes. GUS gene expression was assayed by the method of Jefferson (18), and the presence of the CP gene was checked by DNA blot hybridization (39). Detection of CP in transgenic plants was done by a modified double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) described by Namba et al (28). The basic modification from the DAS-ELISA method of Clark and Adams (6) involved mixing the antigens and conjugated antibodies in the same well instead of the usual addition of antigens, incubation, washing, and then the addition of conjugated antibodies. Coating immunoglobulins were at 2 μg/ml, and the conjugated antibodies were used at a dilution of 1/500. Tissue extracts were

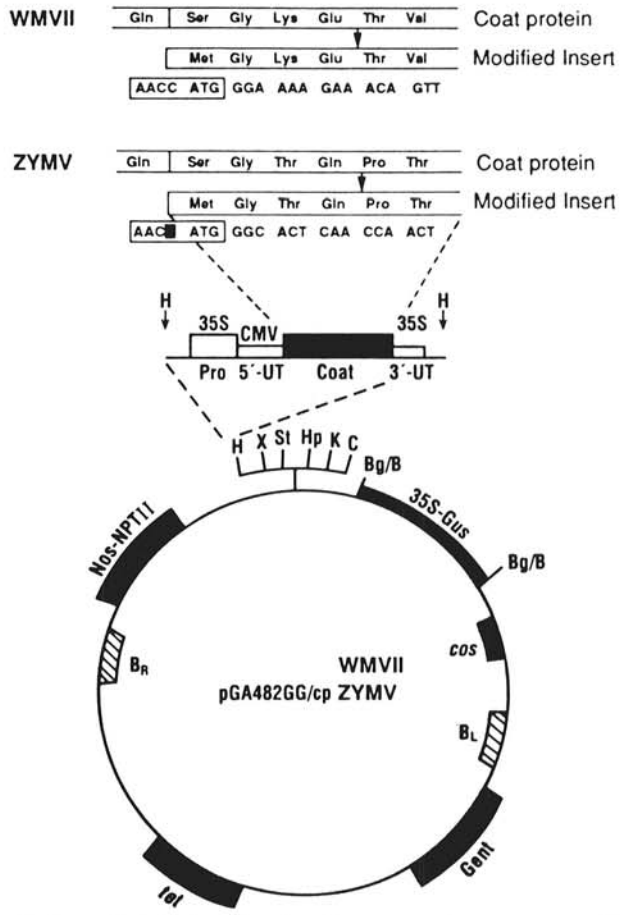

Fig. 1. N-terminal fusions for the plant expression vectors used for expression of WMV II or ZYMV coat protein (CP) genes. Each expression cassette was constructed using the custom polymerase chain reaction engineering technique described by Slightom (37,38), and each potyvirus CP gene was engineered by obtaining a translational fusion that upon expression yielded a CP nearly identical to the native CP. Restriction enzyme sites are: Bg/B, nondigestible fusion between a *Bgl*II and *Bam*HI sites; C, *Cla*I; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; St, *Stu*I, and X, *Xba*I.

diluted at 1/20. Antibodies to ZYMV-FL (46) were prepared in our laboratory, and antiserum to WMV II was kindly provided by D. Purcifull, University of Florida. The amount of CP in leaf tissues was determined with the procedures described by Ling et al (24), which involved comparing ELISA readings of extracts of transgenic plants with readings of a standard curve obtained by diluting known amounts of purifed CP (24) with leaf extracts from healthy *N. benthamiana*. The amount of total soluble protein in transgenic leaf tissue was determined by a Bio-Rad protein assay kit.

Inoculation of transgenic plants with potyviruses. Seven different potyviruses were used in infectivity studies. Bean yellow mosaic virus (BYMV), clover yellow vein virus (CYVV), and WMV II (isolate 76-2) were all maintained on *N. benthamiana*. Pea mosaic virus (PeaMV), potato virus Y (PVY; isolate MKC), pepper mottle virus (PeMV; isolate PV2), and TEV were maintained on tobacco (*N. tabacum* L. cv. Havana 423). The potyviruses were provided by R. Provvidenti of Cornell University, Geneva, New York. Inocula were applied to the lower two or three leaves of *N. benthamiana* test plants that were at the six- to eight-leaf stage. Relative infectivity of each extract was determined by local lesion assay on *Chenopodium quinoa*, except for PeMV, which was applied at dilutions of 1/100 and 1/500. Plants were observed daily for systemic symptom expression for 28 days following inoculation. Plants were scored as symptomatic when any leaf above the inoculated leaves showed veinclearing, mosaic, or necrotic symptoms.

RESULTS

Construction of CP gene expression cassettes and transfer into *N. benthamiana*. A significant feature of each expression cassette is that the translation start codon is located within a single amino acid of the proposed (32,33) N-terminus of each CP (Fig. 1). Additionally, the nucleotides surrounding each translation start codon conformed to the consensus sequence, AAXXATGG, described by Kozak (21) and Lutchke et al (26), which was expected to ensure efficient translation. These features were significantly different from the expression cassette, pUC18cpPRV-4, which we tested earlier (24).

Fifty and 25 independently transformed plants with the WMV II and ZYMV CP gene, respectively, were regenerated from each *A. tumefaciens* strain used to infect *N. benthamiana*. All transformed plants that were selected for further analysis gave positive GUS expression. Additionally, genomic DNAs were isolated from two of the GUS-positive transgenic plants, and these DNAs were checked for the presence of the engineered CP gene by blot hybridization (39) against the respective radioactive-labeled WMV II or ZYMV CP gene. These plants showed the presence of the expected 1.5 kb *Hin*dIII fragment that corresponds to the size of the full-length expression cassettes, which included about 600 bp from the CaMV-CMV promoter region (36), and about 900 bp from each of the respective CP genes (data not shown).

Expression of CP genes in transgenic *N. benthamiana* tissues. The levels of WMV II CP gene expression in *N. benthamiana* plants varied from less than 0.1% to 0.7% of the total soluble protein, with the largest number of these plants containing WMV II CP levels between less than 0.1 and 0.3% (Fig. 2). In contrast, none of the transgenic plants with the ZYMV CP gene had CP levels above 0.1%.(Fig. 2).

Testing CPMP of R_1 transgenic *N. benthamiana* plants that express the WMV II or ZYMV CP gene against WMV II infection. Under our greenhouse conditions, WMV II produced mild veinclearing on the younger leaves of *N. benthamiana* within a week after inoculation, followed by mild mosaic symptoms, leaf rolling, and stunted growth. ZYMV-FL did not systemically infect *N. benthamiana*, but the virus is detectable in inoculated leaves (46).

R_0 plants of *N. benthamiana* expressing the CP gene of ZYMV or WMV II were initially screened by inoculation with WMV II. Twenty WMV II-CP, eight ZYMV-CP, and 10 CP^- transgenic *N. benthamiana* plants were challenge-inoculated with WMV II. Only one CP gene–expressing plant, which expressed the ZYMV-CP gene, showed symptoms (data not shown). Thirteen days after inoculation each plant was bioassayed by mechanical inoculation of its leaf extract onto *C. quinoa*. WMV II was recovered from 12 of 20 WMV II-CP, six of eight ZYMV-CP, and all 10 CP^- R_0 plants. Subsequently, three R_0 test plants from each plant group with the WMV II or ZYMV CP gene were selected and advanced to R_1 plants for further testing. (These plants are listed in Table 1.)

R_1 progeny plants derived from the selected self-fertilized transgenic *N. benthamiana* plants were tested for expression of GUS and the respective CP genes. To determine the relation between GUS and CP gene expression, 10 GUS-positive seedlings from each R_1 plant line were tested in ELISA for the presence of CP. All them tested ELISA-positive for the respective CP, with the exception of line 2214. Previous tests (Table 1) had also failed to detect ZYMV CP in line 2214, even though the CP gene could be detected by Southern blot hybridization (data not shown). These data suggested that GUS and CP genes were closely linked, as expected. The segregation ratios (Table 1) indicated that most of the selected plant lines contained multiple CP genes; only plant lines 2902-25 (WMV II CP) and 2503-25 (CP^-) showed the 3:1 ratio expected from the transfer and integration of the T-DNA region at a single locus.

R_1 seedlings (10 or 12 seedlings per line) from each of those treatments that tested positive for GUS expression were also challenge-inoculated with WMV II (Table 1). Typical symptoms of WMV II infection appeared about 6 days after inoculation of transformed CP^- control plants. The three lines expressing WMV II CP showed some resistance or tolerance, in that 50–90% remained symptomless after 25 days. However, relatively high levels of infectious WMV II were detected in asymptomatic plants of lines 2902 and 2914, but not at all in line 2901. CP^+ plants expressed milder symptoms than CP^- plants. Virus was recovered in all but three of the symptomatic plants expressing WMV II CP gene, and the titers of infectious virus were similar to that of those recovered from infected control plants. Plant line 2901 showed the best level of resistance even though it accumulated a lower level of CP (0.075%) compared to line 2902 (0.360%). The lack of correlation between the amount of CP accumulated and the level of CPMP has been reported for other potyviruses (3,19,24,40).

Although the three selected transgenic ZYMV CP gene–expressing lines accumulated relatively low levels of ZYMV CP (not detectable by ELISA to 0.023%), they showed reasonable levels of protection against symptoms, in that 70–100% of the inoculated plants in each line were asymptomatic (Table 1). Infectious virus, however, was recovered from 18 of the 27 symptomless plants. The symptoms on most of the plants were milder than the control plants (CP^-). Plant line 2217 showed the best overall

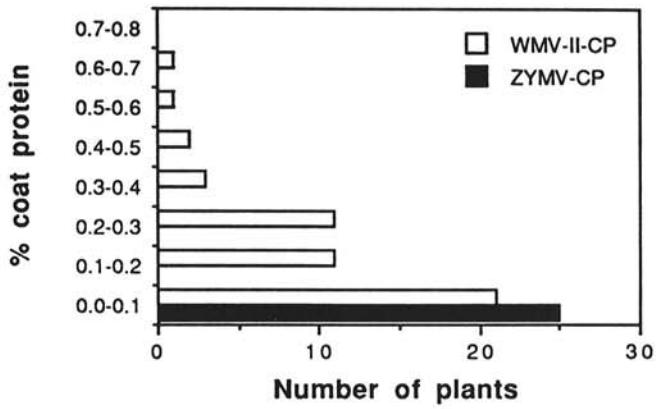

Fig. 2. Coat protein (CP) levels found in R_0 transgenic plants that express the CP gene of watermelon mosaic virus II (WMV II) or zucchini yellow mosaic virus (ZYMV). CP levels were detected by enzyme-linked immunosorbent assay (ELISA) of leaf extracts and compared to ELISA reactions of known amounts of purified WMV II or ZYMV CP. The amount of total leaf soluble protein isolated from the leaves of plants was determined by a Bio-Rad protein assay kit.

resistance, with 50% of the plants being asymptomatic and showing no infection by WMV II.

Heterologous CPMP of transgenic *N. benthamiana* R_1 plants expressing WMV II or ZYMV CP gene. R_1 progeny transgenic plants from a control CP^- line and from lines 2901-10 (WMV II-CP) and 2217 (ZYMV-CP) were challenge-inoculated with TEV, PVY, PeMV, BYMV, CYVV, and PeaMV in addition to WMV II. Both lines had shown the highest levels of resistance to WMV II in the previous tests (Table 1). The data resulting from challenge inoculations with these potyviruses are summarized in Table 2.

Transgenic plants expressing the WMV II CP gene showed delays in symptom expression with all viruses, but the degree of delay and the number of transgenic plants that became infected differed considerably (Table 2). For example, PeaMV-inoculated plants showed the earliest and most severe symptoms, and in contrast, only 33% of the plants challenged with CYVV showed symptoms (Table 2). WMV II CP gene-expressing plants did not develop symptoms following inoculations with TEV (Table 2). Resistance to the potyviruses was also found to be dependent on the inoculum strength. This was especially evident for BYMV and PeMV. In the BYMV test, plants showed essentially no protection at the higher inoculum dose (120 local lesions per leaf), whereas none of the inoculated plants developed symptoms at the lower inoculum dose (15 local lesions per leaf) (Table 2). Generally, CP^+ plants that became infected showed milder (but yet definite) symptoms than those on comparably infected CP^- control transgenic plants. The two exceptions were CP^+ plants infected with PeaMV and PeMV. These plants showed severe symptoms when inoculated at high inocula doses.

In general, a higher percentage of inoculated ZYMV CP^+ plants showed symptoms than similarly inoculated WMV II CP^+ plants at high inocula doses, but results at lower inocula doses were comparable (Table 2). At high inocula doses, 90% or more of the inoculated ZYMV CP^+ plants showed symptoms, with one exception. The lone exception was plants inoculated with WMV II, of which only 20% showed symptoms. It was noted that ZYMV CP^+ plants showed no resistance to TEV infection, which is in contrast to WMV II CP^+ plants. Delay in symptom development and severity of symptoms on ZYMV CP^+ plants were similar to those observed on WMV II CP^+ plants. That is, symptoms were generally milder and slower to develop than those observed on comparable CP^- plants.

DISCUSSION

We have shown that transgenic *N. benthamiana* plants with the CP genes of WMV II or ZYMV show noticeable levels of protection against symptom development when challenged with four other potyviruses—BYMV, CYVV, PeMV, and TEV—and lesser protection against PVY and PeaMV (Table 2). Stark and Beachy (40) first showed that transgenic tobacco plants expressing SMV CP gene were protected against unrelated potyviruses (TEV and PVY), and thus proposed that CPMP derived from the expression of one particular potyvirus CP gene could possess a form of broad-spectrum protection against infections by heterologous potyviruses. This observation has been recently confirmed by Ling et al (24), who obtained similar results when transgenic plants that express the PRV CP gene were challenge-inoculated with TEV, PVY, and PeMV. Given the results of these combined experiments, we conclude that a degree of broad-spectrum CPMP may generally result from the expression of most any potyvirus CP gene.

In general, we found here that the level of heterologous CPMP in *N. benthamiana* is less than the level of homologous CPMP (thus far tested only for WMV II); however, transgenic plants that express a potyvirus CP gene have, in this and other reports (24,40), shown a detectable level of heterologous CPMP against the potyviruses used for inoculation. The most impressive level of heterologous CPMP that we observed involves transgenic *N. benthamiana* plants that express the WMV II CP gene, since they showed high levels of heterologous CPMP against two (TEV and CYVV) of the six tested potyviruses at high inocula strengths (Table 2). Except for these two cases, the type of heterologous CPMP that we observed in *N. benthamiana* plants that express the CP gene of either WMV II or ZYMV was highly dependent on inoculum strength (Table 2). For two potyviruses (BYMV and PeMV) the level of CPMP was very high when inoculum strengths were low, but this protection was almost nonexistent when the inoculum strengths were raised by about 10-fold (Table 2). This is in contrast with the observation made by Stark and Beachy (40) that a tobacco line, homozygous for expressing the SMV CP gene, was effectively protected against a 10-fold range of TEV and PVY inoculum strength. However, it should be noted that the plant lines used in our experiments are R_1 plants, none of which are homozygous for the CP gene. Thus, CPMP in our plants may not be optimum, and one could expect that selections from subsequent generations could yield plants that possess a higher level of CPMP. Also, a survey of a larger population of independent transformants could reveal a plant line that possesses a higher level of CPMP. In fact, Stark and Beachy (40) showed that one particular transgenic tobacco line did possess a much higher level of CPMP than any other tested transformants.

An important goal of this work was to determine the feasibility of expressing the CP genes of a limited number of potyviruses to obtain CPMP against a larger number of potyviruses. The

TABLE 1. Inoculation of watermelon mosaic virus II (WMV II) to transgenic *Nicotiana benthamiana* R_1 plants expressing coat protein (CP) genes of WMV II or zucchini yellow mosaic virus (ZYMV)

CP source Plant line	R_1 plants[a] (GUS^+:GUS^-)	CP (%)[b]	Inoculated plants[c] With symptoms (%) Virus[d]	 No virus	 Without symptoms (%) Virus	 No virus
WMV II						
2901-10	12.6:1	0.075	0	10	0	90
2902-25	3:1	0.360	30 (100)	0	70 (64)	0
2914-25	all +	0.045	30 (360)	20	50 (220)	0
ZYMV						
2214	36:1	0.000	0	0	100 (196)	0
2217	all +	0.023	20 (268)	0	30 (276)	50
2223	all +	0.015	25 (237)	0	42 (146)	33
Control[e]						
2503-25	3.1:1	0.000	100 (204)	0	0	0

[a] Seedlings from seeds of R_0 plants (37–112 plants per line) were tested for GUS expression. The data are presented as segregation ratios.

[b] Percentage of CP in total soluble leaf proteins of transgenic plants.

[c] Ten or 12 plants were inoculated, and symptoms were read 25 days after inoculation. Recovery tests were made for all plants. The numbers outside the parentheses represent the percentage of plants with or without symptoms, and the numbers in parentheses represent the average number of lesions that inocula from test plants produced on *Chenopodium quinoa*.

[d] Virus = virus recovered by inoculation to *C. quinoa* (see c, above); no virus = virus not detected by inoculation to *C. quinoa*.

[e] Transgenic plants transformed with CPexp plant expression vector (contains NPTII and GUS genes but no CP genes).

variability that we observed in the level of CPMP (Table 2) suggests that the expression of no one potyvirus CP gene will provide sufficient CPMP against all potyviruses, at least from the expressed potyvirus CP genes that we have tested thus far. Nevertheless, the degree of heterologous CPMP could be of significant economical value in some crops, provided that there is a knowledge of which potyviruses infect a particular crop in a particular field environment. It would be interesting to test such control strategies with cucurbits, since they are infected by PRV, WMV II, and ZYMV. Fortunately, all the elements needed to test this strategy with cucurbit plants are available. First, all three potyvirus CP genes have been engineered and shown to express in plant tissues; and second, procedures for cucurbit regeneration and transformation have been developed, e.g., for melon (8,11) and cucumbers (4).

It is interesting to note that after the analysis of about 25–50 independent transgenic plants for each engineered CP gene, many more plants were found to accumulate high levels of the WMV II CP than the ZYMV CP (Fig. 2). For the ZYMV expression cassette, none of the 50 independent transformants accumulated over 0.2% ZYMV CP in the total soluble protein. The fact that more plants accumulate high levels of WMV II CP is surprising, and this result does not appear to be directly related to the number of gene copies present (Table 1; and unpublished), since most of the transformed plants studied here apparently contain multiple copies of the respective CP gene. Other reasons for this disparity

TABLE 2. Resistance of transgenic R_1 *Nicotiana benthamiana* plants expressing the CP genes of watermelon mosaic virus II (WMV II) and zucchini yellow mosaic virus (ZYMV) to infection by seven potyviruses

CP gene expressed[a]	Virus[b] Inocula[c]	Plants inoculated (no.)	CP^+ with symptoms (%) Days after inoculation 2	3	4	6	8	10	12	14	16	Symptom severity[d] Control	Transgenic
WMV II													
	BYMV												
	120	12	0	[0][e]	100	100	100	100	100	100	100	+++	++
	15	10	0	0	0	0	0	0	0	[0]	0	+++	−
	PVY												
	120	10	0	0	20	[20]	60	60	60	60	60	+++	++
	16	8	0	0	0	[38]	50	50	50	50	50	+++	++
	PeaMV												
	100	10	0	50	[80]	90	90	100	100	100	100	+++	+++
	15	11	0	[0]	0	73	91	91	91	91	91	+++	++
	CYVV												
	120	12	0	0	[0]	0	33	33	33	33	33	++	+
	8	10	0	0	0	0	0	0	0	[0]	0	+	−
	PeMV												
	1/10	12	0	0	[25]	50	75	100	100	100	100	+++	+++
	1/5,000	10	0	0	0	0	0	0	[0]	0	0	+++	−
	TEV												
	174	12	0	[0]	0	0	0	0	0	0	0	++	−
	WMV II												
	66	10	0	0	0	0	0	[0]	10	10	10	++	+
ZYMV													
	BYMV												
	120	10	0	[0]	90	90	90	90	90	90	90	+++	++
	15	11	0	0	0	0	[0]	0	9	9	9	+++	++
	PVY												
	120	10	0	0	0	[20]	20	60	90	90	90	+++	++
	16	10	0	0	0	[0]	0	0	10	10	10	+++	++
	PeaMV												
	100	11	0	[0]	0	55	55	64	91	91	91	+++	+++
	15	10	0	0	0	[20]	30	30	50	50	50	+++	++
	CYVV												
	120	10	0	0	[10]	10	80	80	80	100	100	+++	+++
	8	10	0	0	0	0	0	0	[0]	0	0	+++	++
	PeMV												
	1/10	10	0	0	[40]	70	100	100	100	100	100	+++	+++
	1/500	10	0	0	0	0	20	20	20	[20]	20	+++	+
	TEV												
	174	11	0	[18]	36	36	36	45	91	91	91	++	+
	WMV II												
	66	10	0	0	0	0	0	[0]	20	20	20	++	+

[a] By R_1 transgenic plants of line 2901-10 (WMV II) and of line 2217 (ZYMV).

[b] Used for inoculation: BYMV = bean yellow mosaic virus, PVY = potato virus Y, PeaMV = pea mosaic virus, CYVV = clover yellow vein virus, PeMV = pepper mottle virus, TEV = tobacco etch virus, WMV II = watermelon mosaic virus II.

[c] Average numbers of local lesions per inoculated *Chenopodium quinoa*. Inocula of PeMV was measured by relative dilutions (g/ml).

[d] Symptoms on transgenic plants were compared to the symptoms produced on inoculated control plants. The controls were R_1 plants of line 2503-25, which contain NPTII and GUS genes but no CP genes. +++ = Severe symptoms with stunting, severe chlorosis, and sometimes death of plants; ++ = distinct chlorosis or mosaic patterns, moderate stunting; + = mild symptoms consisting primarily of mild chlorosis or mosaic patterns; − = no symptoms.

[e] Squares indicate the days after inoculation at which 80-100% of the control (CP^-) plants showed symptoms. For example, 100% of the control plants inoculated with BYMV (120 lesions per leaf) showed symptoms at 3 days postinoculation. Ten control plants were inoculated per treatment.

cannot be readily determined; one possibility could be due to the use of different translational fusion codons for each construction (Fig. 1), which may have altered translation or CP stability, or to the fact that the different CPs may inherently possess different stabilities in a plant environment. In this latter case ZYMV CP may be subjected to higher rates of turnover than WMV II CP. Comparisons of the expression levels derived from the ZYMV expression cassette is also complicated by the use of a nonconsensus translation initiation sequence (CAACATGG instead of AACCATGG) (26), which was lost during the engineering process (38). The resolution of all of these possible effects will require additional gene engineering and more detailed analyses of how these CPs are accumulated or turned over in plant tissues.

Very recently, Lindbo and Dougherty (23) showed that transgenic tobacco expressing altered forms of TEV CP, as well as transgenic plants that produce plus- or minus-sense transcripts but no TEV CP, are resistant to infection by TEV. It will be very interesting to determine if such plants are resistant to other potyviruses.

In summary, we have shown that CP genes of plant viruses can be efficiently engineered by the CPCR method (37), and that transgenic plants expressing the CP gene of WMV II or ZYMV do provide a level of broad-spectrum protection against other potyviruses. The degree of protection varies in an unpredictable way, which may be influenced by the viral CP (artifacts dependent on gene engineering, stability of the CP in plant tissues, and so on), number of integrated gene copies, and the host species. Lastly, the stage is now set to test in the field the practicality of using broad-spectrum resistance in crops of economic value.

LITERATURE CITED

1. An, G. 1986. Development of plant promoter expression vectors and their use for analysis of differential activity of nopaline synthase promoter in transformed tobacco cells. Plant Physiol. 81:86-91.
2. An, G. 1987. Binary Ti vectors for plant transformation and promoter analysis. Methods Enzymol. 153:292-305.
3. Beachy, R. N., Loesch-Fries, S., and Tumer, N. E. 1990. Coat protein-mediated resistance against virus infection. Annu. Rev. Phytopathol. 28:451-74.
4. Chee, P. P. 1990. Transformation of *Cucumis sativus* tissue by *Agrobacterium tumefaciens* and the regeneration of transformed plants. Plant Cell Rep. 9:245-248.
5. Christie, S. R., and Crawford, W. E. 1978. Plant range of *Nicotiana benthamiana.* Plant Dis. Rep. 62:20-22.
6. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method for enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
7. Cuozzo, M., O'Connell, K. M., Kaniewski, W., Fang, R.-X., Chua, N.-H., and Tumer, N. E. 1988. Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat protein or its antisense RNA. Bio/Technology 6:549-557.
8. Dong, J-Z., Yang, M-Z., Jia, S-R., and Chua, N-H. 1991. Transformation of melon (*Cucumis melo* L.) and expression from the cauliflower mosaic virus 35S promoter in transgenic melon plants. Bio/Technology 9:858-863.
9. Dougherty, W. G., and Carrington, J. C. 1988. Expression and function of potyviral gene products. Annu. Rev. Phytopathol. 26:123-143.
10. Edwardson, J. R. 1974. Host Ranges of Viruses in the PVY-Group. Monogr. 5. Florida Agricultural Experiment Station, University of Florida, Gainesville. 225 pp.
11. Fang, G., and Grumet, R. 1990. *Agrobacterium tumefaciens* mediated transformation and regeneration of muskmelon plants. Plant Cell Rep. 9:160-164.
12. Hemenway, C., Fang, R.-X., Kaniewski, W. K., Chua, N.-H., and Tumer, N. E. 1988. Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. EMBO J. 7:1273-1280.
13. Hepburn, A. G., White, J., Pearson, L., Maunders, M. J., Clarke, L. E., Prescott, A. G., and Blundy, K. S. 1985. The use of pNJ5000 as an intermediate vector for the genetic manipulation of Agrobacterium Ti-plasmids. J. Gen. Microbiol. 131:2961-2969.
14. Hill, K. K., Jarvis-Egan, N., Halk, E. L., Krahn, K. J., Liao, L. W., Mathewson, R. S., Merlo, D. J., Nelson, S. E., Rashka, K. E., and Loesch-Fries, L. 1991. The development of virus-resistant alfalfa, *Medicago sativa* L. Bio/Technology 9:373-377.
15. Hoekema, A., Huisman, M. J., Molendijk, L., Van den Elzen, P. J. M., and Cornelissen, B. J. C. 1989. The genetic engineering of two commercial potato cultivars for resistance to potato virus X. Bio/Technology 7:273-278.
16. Hollings, M., and Brunt, A. A. 1981. Potyviruses. Pages 732-777 in: Handbook of Plant Virus Infections and Comparative Diagnosis. E. Kurstak, ed. Elsevier/North-Holland Biomedical Press, New York. 943 pp.
17. Horsch, R. B., Fry, J. E., Hoffman, N. L., Eichholtz, D., Rogers, S. G., and Fraley, R. T. 1985. A simple and general method for transferring genes into plants. Science 227:1229-1231.
18. Jefferson, R. A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. 5:387-405.
19. Kaniewski, W. K., Lawson, C., Sammons, B., Haley, L., Hart, J., Delannay, X., and Tumer, N. E. 1990. Field resistance of transgenic Russet Burbank potato to effects of infection by potato virus X and potato virus Y. Bio/Technology 8:750-754.
20. Kawchuk, L. M., Martin, R. R., and McPherson, J. 1990. Resistance in transgenic potato expressing the potato leafroll virus coat protein gene. Mol. Plant-Microbe Interact. 3:301-307.
21. Kozak, M. 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44:283-292.
22. Lawson, C., Kaniewski, W., Haley, L., Rozman, R., Newell, C., Sanders, P., and Tumer, N. E. 1990. Engineering resistance to mixed virus infection in a commercial potato cultivar: Resistance to potato virus X and potato virus Y in transgenic russet burbank. Bio/Technology 8:127-134.
23. Lindbo, J. A., and Dougherty, W. G. 1992. Pathogen-derived resistance to a potyvirus: Immune and resistant phenotypes in transgenic tobacco expressing altered forms of a potyvirus coat protein nucleotide sequence. Mol. Plant-Microbe Interact. 5:144-153.
24. Ling, K., Namba, S., Gonsalves, C., Slightom, J. L., and Gonsalves, D. 1991. Protection against detrimental effects of potyvirus infections in transgenic tobacco plants expressing the papaya ringspot virus coat protein gene. Bio/Technology 9:752-758.
25. Loesch-Fries, L. S., Merlo, D., Zinnen, T., Burhop, L., Hill, K., Krahn, K., Jarvis, N., Nelson, S., and Halk, E. 1987. Expression of alfalfa mosaic virus RNA 4 in transgenic plants confers virus resistance. EMBO J. 6:1845-1851.
26. Lutchke, H. A., Chow, K. C., Mickel, F. S., Moss, K. A., Kern H. F., and Scheele, G. A. 1987. Selection of AUG initiation codons differs in plants and animals. EMBO J. 6:43-48.
27. Mackenzie, D. J., Tremaine, J. H., and McPherson, J. 1991. Genetically engineered resistance to potato virus S in potato cultivar Russet Burbank. Mol. Plant-Microbe Interact. 4:95-102.
28. Namba, S., Ling, K., Gonsalves, C., Gonsalves, D., and Slightom, J. L. 1991. Expression of the gene encoding the coat protein of cucumber mosaic virus strain (CMV)-WL appears to provide protection to tobacco plants against infection by several different CMV strains. Gene 107:181-188.
29. Powell-Abel, P., Nelson, R. S., De, B., Hoffmann, N., Rogers, S. G., Fraley, R. T., and Beachy, R. N. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738-743.
30. Quacquarelli, A., and Avgelis, A. 1975. *Nicotiana benthamiana* Domin. as host for plant viruses. Phytopathol. Mediterr. 14:36-39.
31. Quemada, H. D., Gonsalves, D., and Slightom, J. L. 1991. Expression of coat protein gene from cucumber mosaic virus strain C in tobacco: Protection against infections by CMV strains transmitted mechanically or by aphids. Phytopathology 81:794-802.
32. Quemada, H. D., L'Hostis, B., Gonsalves, D., Heinrikson, R., Reardon, I., Hiebert, E. L., Sieu, L. C., and Slightom, J. L. 1990. The nucleotide sequences of cDNA clones of the 3′ coding and untranslated regions of papaya ringspot virus strains W and P. J. Gen. Virol. 71:203-210.
33. Quemada, H. D., Sieu, L. C., Siemieniak, D. R., Gonsalves, D., and Slightom, J. L. 1990. Watermelon mosaic virus II and zucchini yellow mosaic virus: Cloning of 3′-terminal regions, nucleotide sequences, and phylogenic comparisons. J. Gen. Virol. 71:1451-1460.
34. Sanchez-Serrano, J. J., Leil, M., O'Connor, A., Schell, J., and Willmitzer, L. 1987. Wound-induced expression of a potato proteinase inhibitor II gene in transgenic tobacco plants. EMBO J. 6:303-306.
35. Siemieniak, D. R., Sieu, L. C., and Slightom, J. L. 1991. Strategy and methods for directly sequencing cosmid clones. Anal. Biochem. 192:441-448.
36. Shukla, D. D., and Ward, C. W. 1989. Identification and classification of potyviruses on the basis of coat protein sequence data and serology. Arch. Virol. 106:171-200.

37. Slightom, J. L. 1991. Custom PCR engineering of a plant expression vector. Gene 100:251-255.
38. Slightom, J. L., Drong, R. F., Sieu, L., and Chee, P. P. 1991. Custom polymerase chain reaction engineering plant expression vector and genes for plant expression. B16. Pages 1-55 in: Plant Molecular Biology Manual. S. B. Gelvin and R. A. Schilperoort, eds. Kluwer, Dordrecht, Netherlands.
39. Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.
40. Stark, D. M., and Beachy, R. N. 1989. Protection against potyvirus infection in transgenic plants: Evidence for broad spectrum resistance. Bio/Technology 7:1257-1262.
41. Tumer, N. E., Kaniewski, W., Haley, L., Gehrke, L., Lodge, J. K., and Sanders, P. 1991. The second amino acid of alfalfa mosaic virus coat protein is critical for coat protein-mediated protection. Proc. Natl. Acad. Sci. USA. 88:2331-2335.
42. Tumer, N. E., O'Connell, K. M., Nelson, R. S., Sanders, P. R., Beachy, R. N., Fraley, R. T., and Shah, D. M. 1987. Expression of alfalfa mosaic virus coat protein gene confers cross-protection in transgenic tobacco and tomato plants. EMBO J. 6:1181-1188.
43. Van Dun, C. M. P., and Bol, J. F. 1988. Transgenic tobacco plants accumulating tobacco rattle virus coat protein resist infection with tobacco rattle virus and pea early browning virus. Virology 167:649-652.
44. Van Dun, C. M. P., Bol, J. F., and Van Vloten-Doting, L. 1987. Expression of alfalfa mosaic virus and tobacco rattle virus coat protein genes in transgenic tobacco plants. Virology 159:299-305.
45. Van Dun, C. M. P., Overduin, B., Van Vloten-Doting, L., and Bol, J. F. 1988. Transgenic tobacco expressing tobacco streak virus or mutated alfalfa mosaic virus coat protein does not cross-protect against alfalfa mosaic virus infection. Virology 164:383-389.
46. Wang, H. L., Gonsalves, D., Provvidenti, R., and Zitter, T. A. 1991. Comparative biological and serological properties of four strains for zucchini yellow mosaic virus. Plant Dis. 76:530-535.