experimental methods can be used in economics for basic, applied, and policy research. Such a demonstration presents a real challenge to the commonly held belief that economics is not a laboratory science as a matter of principle.

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Research Articles

Delay of Disease Development in Transgenic Plants That Express the Tobacco Mosaic Virus Coat Protein Gene

PATRICIA POWELL ABEL, RICHARD S. NELSON, BARUN DE, NANCY HOFFMANN, STEPHEN G. ROGERS, ROBERT T. FRALEY, ROGER N. BEACHY

A chimeric gene containing a cloned cDNA of the coat protein (CP) gene of tobacco mosaic virus (TMV) was introduced into tobacco cells on a Ti plasmid of *Agrobacterium tumefaciens* from which tumor inducing genes had been removed. Plants regenerated from transformed cells expressed TMV mRNA and CP as a nuclear trait. Seedlings from self-fertilized transgenic plants were inoculated with TMV and observed for development of disease symptoms. The seedlings that expressed the CP gene were delayed in symptom development and 10 to 60 percent of the transgenic plants failed to develop symptoms for the duration of the experiments. Increasing the concentration of TMV in the inoculum shortened the delay in appearance of symptoms. The results of these experiments indicate that plants can be genetically transformed for resistance to virus disease development.

FOR A NUMBER OF YEARS, AGRICULTURALISTS HAVE INOCULATED plants with mild strains of viruses or viroids to prevent more virulent strains from infecting the plant and causing severe disease symptoms. This practice, referred to as cross-protection, has been used to reduce yield losses in crops such as tomatoes, potatoes, and citrus, due to tomato mosaic virus (TMV), potato spindle tuber viroid, and citrus tristeza virus, respectively (*1*). In addition to cross-protection, there are several other types of plant resistance responses to pathogens. These responses are often nonspecific and have been categorized as induced resistance. Extensive discussions on the nature and utility of cross-protection and induced resistance have been presented (*2–4*). Successful cross-protection is most often judged by the ability of the first virus to suppress or delay disease symptoms caused by the superinfecting (challenge) virus. In some cases replication of the superinfecting virus is suppressed (*5*). In most cases symptoms are suppressed or delayed for a period of time, after which the severe strain overcomes the protection, and symptoms develop (*6*).

A number of models have been proposed to explain cross-protection. Gibbs (*7*) suggested that replication of the inducing virus depletes the host cell of a component needed for replication of the challenger. Another hypothesis proposes that capsid protein

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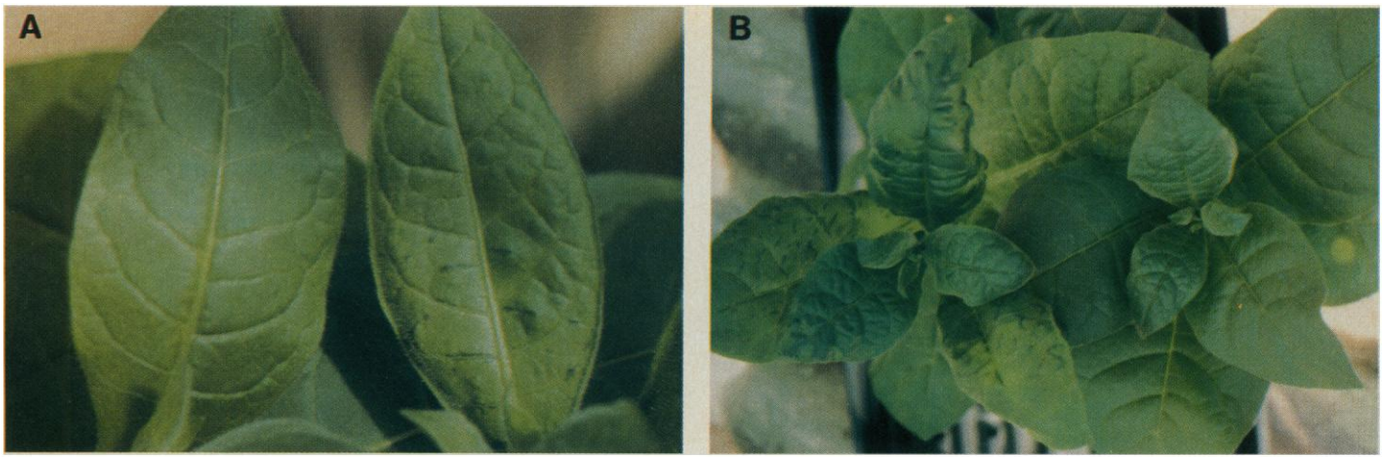

Fig. 1. Suppression of symptoms, due to viral infection, by genetically engineered plants. (A) Leaves from plants inoculated with TMV 10 days previously. Leaf at left is part of a transgenic plant containing and expressing the coat protein (CP) gene from TMV. Leaf at right is part of a transgenic plant not containing the CP gene. The leaf from the plant not containing the CP gene shows characteristic symptoms of a systemic infection by TMV (that is, light and dark green areas and leaf margin deformity). (B) Normal and transgenic plants 14 days after inoculation with TMV. Nontransformed *N. tabacum* cv. Xanthi plants (left) develop typical systemic symptoms of TMV infection during this period of time. The transgenic plant containing and expressing the TMV coat protein gene (right) has not developed symptoms.

produced by the first infection encapsidates the RNA of the challenging strain, thereby preventing its replication (*8*). Experiments with viroids (*9*) and with defective strains of tobacco mosaic virus (*10*), however, argue against the involvement of virus capsid protein in cross-protection. It has also been suggested that uncoating of the challenge virus is blocked by the inducing virus (*11*). Palukaitis and Zaitlin (*4*) proposed that sense or antisense RNA of the inducing virus anneals with RNA of the challenger virus thereby blocking replication of the challenge virus. From these examples it is evident that although cross-protection has been used to reduce economic losses caused by virus infection, the mechanism responsible for cross-protection has not been elucidated.

There are potential disadvantages to the widespread use of cross-protection in field agriculture. First, the mild strain (protecting strain) might undergo mutation to a highly virulent form which could be selected during replication, thereby leading to extensive crop losses rather than protection (*4*). Second, the protecting strain might act in synergism with a nonrelated virus resulting in a disease condition that is more severe than that caused by either virus alone. Such a condition occurs when TMV and cucumber mosaic virus concurrently infect tomato plants (*12*). Third, a protecting virus in one plant might be a severe pathogen in another, thereby making it unwise to spread the virus extensively in field situations. Fourth, the protecting strain may itself cause a small but significant loss in yield (*3*). Most or all of these objections could be overcome if cross-protection was engendered in plants as the result of expression of a single viral gene, rather than as a result of infection with an intact virus.

To further understand the mechanisms of cross-protection, and to produce plants that carry a heritable genetic trait for viral resistance, we began a series of experiments designed to express gene sequences of TMV as chimeric nuclear genes in transgenic tobacco plants. These plants would then be tested for the capacity to suppress symptom development upon infection by a severe strain of TMV. We now report the results of experiments showing that plants can be genetically engineered to suppress symptoms due to viral infection (Fig. 1).

Construction of the chimeric gene encoding the TMV–coat protein. The complete nucleotide sequence of the U_1 (common) strain of TMV has been determined by sequencing cloned complementary DNA's (cDNA's) derived from viral RNA (*13*). Nucleotides 70 to 4919 encode two polypeptides that are postulated to be subunits of the TMV replicase (*14*). The 130-kD polypeptide terminates at an amber codon which is infrequently suppressed to produce a 180-kD polypeptide which terminates at nucleotide 4919 (*15*). Both the 130-kD and 180-kD polypeptides have been identified in virus-infected cells (*16*). Nucleotides 4903 to 5709 encode a 30-kD polypeptide which has been implicated in facilitating the movement of virus from cell to cell (*17*). The coat or capsid protein (CP) is encoded within the nucleotides 5712 to 6190. The 3′ untranslated region of the RNA extends to nucleotide 6395. A double-stranded (ds) cDNA of the CP cistron of TMV-RNA was generated and cloned into the plasmid pUC9 (Fig. 2). The cDNA containing the viral coat protein (CP) coding sequence was excised from the pUC plasmid by restriction with Aha III [nucleotide 5707] (*13*) and with Bam HI. To confirm that the cloned cDNA contained an open reading frame encoding the TMV-CP, we cloned the ds-cDNA into a plasmid for in vitro transcription, and the transcript was translated in vitro in a wheat germ system. The results of these experiments (Fig. 3) demonstrate that the protein product of these reactions comigrates with TMV-CP during electrophoresis and is immunoreactive with antiserum to CP produced in rabbits.

The coat protein coding sequence was ultimately inserted into the expression cassette vector pMON316 (*18*) to create a chimeric gene containing the 35*S* promoter from cauliflower mosaic virus (CaMV) and a polyadenylation signal from the nopaline synthase gene (NOS) (Fig. 2). Leaf disks of *Nicotiana tabacum* cv. Xanthi were inoculated with *Agrobacterium tumefaciens* cells containing the pTM319::pTiB6S3-SE cointegrate plasmid, and transformed cells were selected for kanamycin resistance and regenerated into plants as described (*19*). A total of eight different transgenic plants were regenerated and analyzed for expression of the chimeric gene.

Quantitation of gene copy number and expression of the chimeric gene in transgenic plants. The number of chimeric TMV-CP genes present in transgenic plants was determined (Fig. 4A). The expected size of the Bam HI fragment containing the CP gene is approximately 2.3 kb. The copy number of the chimeric TMV-CP gene was one to five per diploid genome for six of the transgenic plants, and more than ten copies for two others (Fig. 4A). Plants having high copy numbers of the CP gene were shown to have multiple sites of insertion (*20*). Since there are greater numbers of copies of the TMV-CP gene in some plants than in others, but not

equivalent differences in the amounts of RNA or protein (Fig. 4, B and C), it is probable that some copies of the gene are silent in plants with high numbers of genes.

RNA was isolated from transgenic plants and analyzed for TMV-related messenger RNA (mRNA) (Fig. 4B). As controls in these experiments, RNA isolated from nontransformed plants was included on each blot. The size of the chimeric mRNA is expected to equal the sum of the cDNA (697 nucleotides), the 5′ untranslated nucleotides (42 nucleotides), the approximately 160 nucleotides contributed by the NOS 3′ end, plus polyadenylate residues—altogether about 950 to 1000 nucleotides in length. This RNA would, therefore, be at least 200 nucleotides longer than the low molecular size component (LMC) RNA, the subgenomic RNA encoding the CP in TMV-infected cells (*21*). The RNA blot analyses confirmed that the transcript, found only in transgenic plants carrying pTM319, was larger than the LMC RNA (Fig. 4B). This transcript binds to oligo(dT) cellulose, confirming that it is polyadenylated (*20*). In addition to the CP mRNA of about 0.95 kb, a second RNA of about 2 kb containing sequences of TMV RNA and binding to oligo(dT) cellulose, was found. This RNA presumably results either from multiple sites of transcription initiation, or multiple sites of polyadenylation of the transcript. The reasons for the variability in the relative amounts of these two RNA's is unknown. There was no more than a two- to fivefold variability in mRNA levels between different transgenic plants.

The amount of TMV-CP that accumulated in transgenic plants

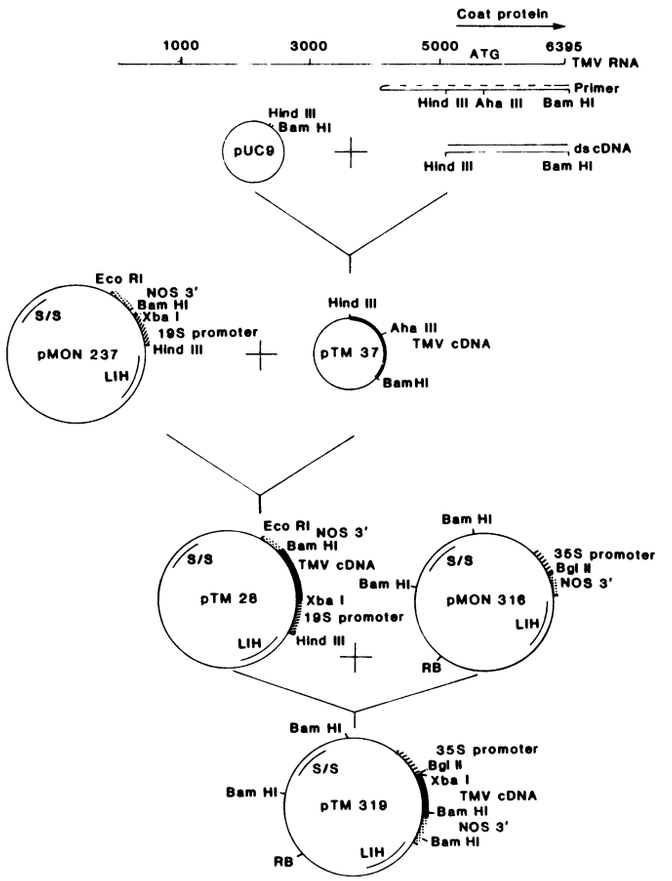

Fig. 2. Construction of the TMV-CP cDNA clone and the intermediate plasmids. A cDNA clone of the 3′ region of TMV RNA (containing nucleotides 5707 to 6395 and including the coat protein cistron) was generated (*29*) by reverse transcriptase with a 35-base oligonucleotide as primer. The primer was complementary to 26 nucleotides at the 3′ end of the viral RNA; the remaining nucleotides generated a Bam HI site. Second strand synthesis was done with the Klenow fragment of DNA polymerase, and the Hind III–Bam HI fragment of TMV cDNA was cloned into the plasmid pUC9 (*30*). The resultant plasmid, pTM37, was digested with Aha III (nucleotide 5707) and Bam HI to excise the CP sequences. This fragment was ligated into the vector pMON 237 (*31*) at the Xba I site which was made blunt, and the Bam HI site to produce pTM 28. The CP coding sequence was excised from pTM 28 by digestion with Xba I and Bam HI and transferred to another plasmid to acquire a Bgl II site at the 5′ end of the coding sequence and an Eco RI site at the 3′ end. The Bgl II–Eco RI fragment was inserted into the expression cassette vector pMON 316 between the CaMV 35*S* promoter (*18*) and the NOS 3′ untranslated region. The resultant plasmid pTM 319 was mated into *A. tumefaciens* strain GV 3111 carrying the disarmed pTi B6S3-SE plasmid (*32*). Recombination between the plasmid LIH region and the LIH region of the transferred DNA (T-DNA) was selected by screening for *A. tumefaciens* colonies resistant to spectinomycin and streptomycin (S/S on the plasmid). These colonies were subsequently used to transform *N. tabacum* cv. Xanthi leaf disks which were then regenerated into whole plants (*19*).

Table 1. Segregation of the TMV-CP gene in transgenic plants and chi-square (χ^2) analysis for a 3:1 expression ratio. Seeds were germinated in a greenhouse under natural light supplemented with artificial light (150 μE m^{-2} sec^{-1}) to produce 14-hour days and 10-hour nights. Seedlings were transplanted 20 days after planting. Leaf disks from the two youngest expanding leaves were sampled, frozen in liquid nitrogen, and ground in extraction buffer (0.035*M* potassium phosphate buffer, *p*H 7.5, 0.4*M* NaCl, 10 m*M* 2-mercaptoethanol). Tissue was ground in extraction buffer at a ratio of 1 ml of buffer to 1 gram of fresh leaf. After centrifugation of the crude extracts, supernatants were diluted 1:1 with Laemmli buffer mix (*37*) and boiled. Samples were analyzed for the presence of CP, after SDS-PAGE and by immunological reactions (*38*). *P*, probability of such a χ^2 value or larger for 3:1 ratio. CP, coat protein; T_2, second generation from transformation event.

Transformant	Number of T_2 seedlings		χ_1^2 (3:1)	*P*
	Expressing CP	Not expressing CP		
3646	42	17	0.36	0.5–0.7
3773	32	8	0.17	0.5–0.7
3404	41	17	0.58	0.3–0.5
3648	39	1	10.8	<0.005

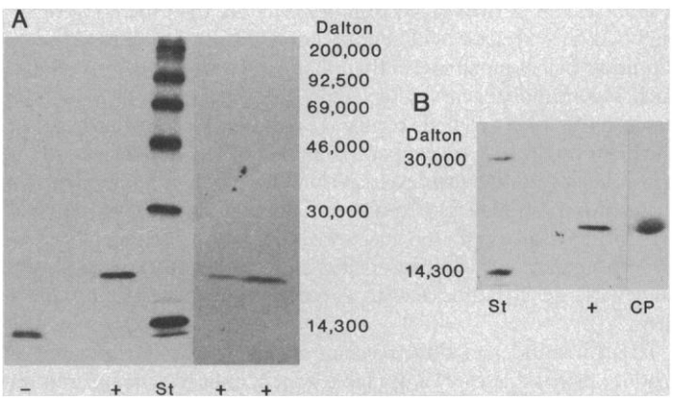

Fig. 3. In vitro translation of a transcript made in vitro. TMV-CP cDNA containing nucleotides 5707 to 6395 of TMV-RNA was ligated into the plasmids SP65 and SP64 (Promega Biotec), restricted with Bam HI or Eco RI, respectively, and used as the template for SP6 polymerase (*33*). ^{3}H-labeled uridine triphosphate (UTP) was used to label the RNA used as the template in a wheat germ extract in an in vitro translation reaction. Products of the translation reaction were labeled with [^{3}H]leucine. (A) Translation product when RNA in the positive sense (+) was used as template and when antisense (−) RNA was used. The center lane contains molecular size standards. The lanes at the right contain the translation products that were immunoprecipated with antiserum to TMV. (B) Electrophoretic mobility of the translation product (+) and authentic coat protein (CP). Authentic CP was stained with Coomassie brilliant blue.

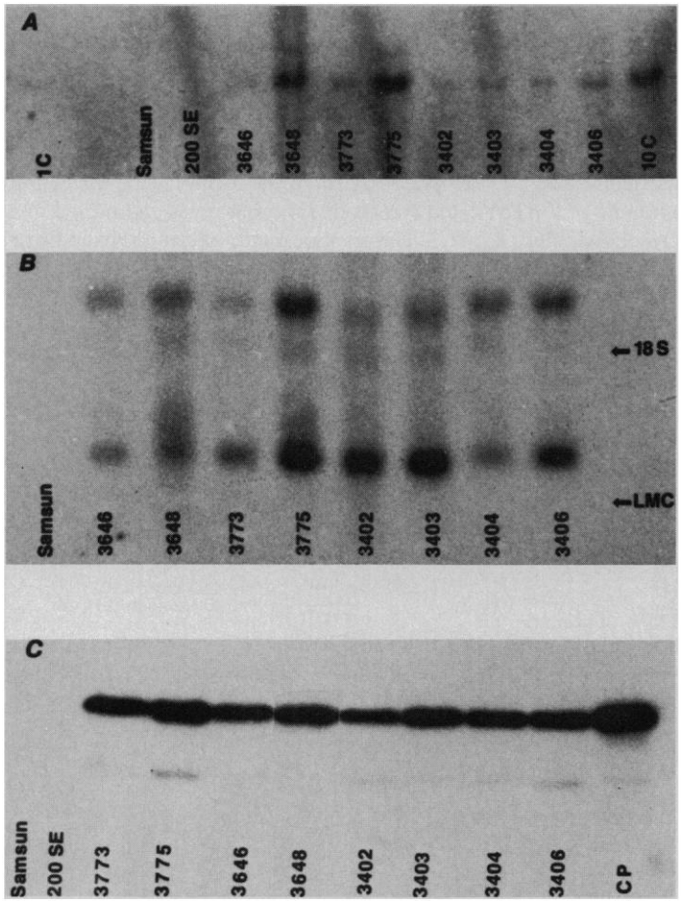

Fig. 4. Integration and expression of the chimeric CP gene in transgenic plants. (A) Quantitation of TMV-CP DNA sequences in transgenic plants. Genomic DNA was isolated from nontransformed tobacco plants and transgenic plants containing the vector alone, or the vector plus the CP gene. DNA (10 μg) was digested with Bam HI, separated on 1 percent agarose gels, transferred to nitrocellulose (*34*), and hybridized with ^{32}P-labeled transcripts of the cDNA (as in Fig. 4B). Reconstructions representing one copy (1C) and ten copies (10C) per genome equivalent of pTM319 are indicated. Lanes containing DNA from normal tobacco leaves (Samsun) are as indicated. Sample 200 SE shows DNA from transgenic plants containing only the vector. The remaining lanes show DNA from leaves of individual transgenic plants containing pTM 319 (Fig. 2). (B) Detection of the TMV coat protein related transcripts in tissue from transgenic plants. RNA was isolated from leaves by the method of Haffner *et al.* (*35*) with minor modifications. Total RNA was separated on a 1.0 percent agarose gel containing formaldehyde and transferred to nitrocellulose (*29*). The blot was hybridized with a ^{32}P-labeled in vitro transcription product complementary to CP mRNA. The transcript probe was prepared as described in Fig. 3 except that [^{32}P]GTP was used in place of [^{3}H]UTP. The first lane (Samsun) contains 6 μg of RNA from leaves of a normal tobacco plant (nontransformed). Successive lanes contain 6 μg of total RNA from leaves of eight individual transgenic plants. The positions of 18*S* ribosomal RNA (rRNA) and LMC, the CP mRNA present in TMV-infected plants (*21*), are indicated. The slight variability in the amount of RNA placed in different lanes is reflected in the amount of probe bound to the 18*S* rRNA. (C) Detection of coat protein in leaves of transgenic plants. Protein was isolated by powdering leaf samples in liquid nitrogen followed by grinding in 0.03*M* potassium phosphate buffer, *p*H 7.5, 0.4*M* NaCl, and 10 m*M* 2-mercaptoethanol. The protein concentration of clarified supernatants was determined by the method of Bradford (*36*). The supernatant was combined with Laemmli sample buffer (*37*) in a 1:1 ratio and boiled for 3 minutes. Total protein (50 μg) from eight individual transformants was separated on a 12.5 percent sodium dodecyl sulfate (SDS) polyacrylamide gel, transferred to nitrocellulose (*38*), and reacted first with rabbit antibody to TMV, then with ^{125}I-labeled donkey antiserum to rabbit serum (Amersham). Proteins extracted from normal Samsun tobacco leaves and transgenic plants containing the vector alone (200 SE) are indicated. The right lane contained 100 ng of purified TMV-CP. The identity of the protein migrating ahead of the CP is unknown.

was determined as described in Fig. 4C. The amount of antigen in each sample was determined by blotting 100 ng of purified TMV-CP on each filter. The amount of coat protein in transgenic plants was between 20 and 50 ng per 50 μg of leaf protein (Fig. 4C), or about 0.1 percent of the extracted leaf protein. Furthermore the amount of TMV-CP, as a percentage of cell protein, was approximately equivalent in leaves at the top, middle, and bottom of the transgenic plants (*22*).

Molecules of TMV-CP assemble to multimeric forms depending upon the concentration of the CP, *p*H, ionic strength, and temperature. Interaction of the monomeric and aggregated states of the CP with viral RNA in vitro has been extensively studied (*23*). To determine whether or not the CP produced in transgenic plants is assembled into multimeric forms, protein was extracted from leaves of these plants under conditions that favor maintenance of the 20*S* disk (*24*). Leaf protein was subjected to velocity sedimentation in sucrose gradients, with hemoglobin (4*S*) and catalase (11*S*) as marker proteins in accompanying parallel gradients. As shown in Fig. 5, TMV-CP was located only in fractions 1 to 5, indicating that the coat protein which accumulates in transgenic plants consists of monomers and multimers with sedimentation coefficients of about 4*S*. The lack of more complex multimers, such as the 20*S* disks, probably reflects the relatively low concentration (estimated to be no more than 0.1 percent of cell protein) of the monomers in leaf tissues.

Infection of transgenic plants with TMV. Experiments were initiated to determine whether transgenic plants could suppress symptoms caused by TMV infection, as in viral cross-protection. In our initial experiments rooted cuttings of transgenic plant 3646, and control plants, consisting of either plants that contained the vector without the CP gene, or nontransformed plants, were infected. Two or three leaves of the rooted cuttings were inoculated by rubbing them with a suspension containing 0.5 μg of TMV per milliliter of phosphate buffer and symptom development was monitored daily in the greenhouse. In experiments with 17 rooted cuttings, each of the 10 plants that did not contain the chimeric CP gene developed classical symptoms of TMV infection. Four of the plants that expressed the CP gene and developed symptoms did so from 2 to 14 days later than any of the controls. Three of the seven transgenic plants expressing the CP gene did not develop symptoms over a 20-day period of observation (*25*).

The limiting factor in experiments with rooted cuttings is in obtaining a sufficient number of uniform plants to carry out experiments that are statistically significant. Therefore all other experiments were done with seedling progeny of self-fertilized transgenic plants. If transgenic plants express the CP gene at a single locus, three of every four seedlings should contain TMV-CP. Seedlings were classified as "expressors" (E) or "nonexpressors" (NE) of TMV-CP on the basis of immunoblot analysis. Expressor and nonexpressor plants were indistinguishable from each other as judged by growth rates, physical appearance, and fertility. An example of the analysis of the seedling progeny of transgenic plant 3773 (Fig. 6) demonstrates that 32 of the 40 seedlings tested expressed the TMV-CP gene. Seedling progeny of transgenic plants 3646, 3773, and 3404 each segregate with a ratio consistent with expression of the TMV-CP gene at a single locus (Table 1). The segregation ratio of seedlings from plant 3648 did not fit the 3:1 expression model, suggesting that the CP gene was expressed from genes inserted at multiple loci. Plant 3648 contained multiple copies of the CP gene (Fig. 4A).

To determine whether transgenic seedlings could suppress symptoms caused by infection, plants were inoculated with TMV. Progeny that did not contain the CP gene and seedlings of nontransformed plants served as controls. In one experiment a total

of 37 to 40 seedlings in the three- to four-leaf stage from each of four transgenic plant lines were inoculated with a suspension of TMV (0.25 μg/ml). These plants were held in a growth chamber under conditions that favored rapid appearance of symptoms. Under these conditions typical symptoms of TMV infection appeared in 3 to 4 days on all control seedlings. In contrast, seedlings of transgenic plants that expressed the TMV-CP were significantly delayed in symptom development compared with the controls (Fig. 7). The results of these experiments indicated that delay in symptom appearance is correlated with expression of the TMV-CP cDNA. In every experiment there were some inoculated transgenic seedlings that did not develop symptoms during the observation period. For example, in one experiment 47 percent of seedlings derived from transgenic plant 3404 that expressed TMV-CP did not exhibit symptoms 30 days after inoculation (*25*). In other examples, 4 to 28 percent of the CP-expressing seedlings did not develop symptoms within 12 days after inoculation (the duration of the experiment).

One of the features of cross-protection against TMV is that increasing the concentration of virus in the challenge infection reduces the length of time between the challenge infection and symptom development (*6*). Such an experiment was carried out with transgenic plants that expressed the TMV-CP gene. Seedlings from transgenic lines 3773 and 3404 were inoculated with increasing amounts of U_1-TMV and symptom appearance was monitored in a greenhouse (Fig. 8). The delay in appearance of symptoms in those plants expressing the CP gene was again apparent when compared with segregants that were not expressing CP. In addition, as the concentration of TMV in the inoculum was decreased from 2.0 μg/ml to 0.4 μg/ml, the delay in symptom appearance in the expressing plants increased from about 1 day to about 10 days as judged by comparison with nonexpressing controls. These results are similar to the cross-protection experiments reported for TMV in tomato (*6*). In our experiment, which was carried out in a greenhouse, a large percentage of the transgenic plants (up to 60 percent of plants

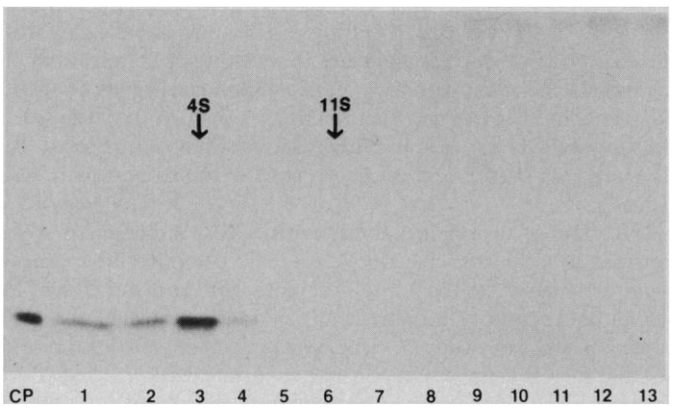

Fig. 5. Determination of the sedimentation coefficient of the coat protein in transformed plants. Protein was isolated from leaves of transgenic plants by grinding in 0.03*M* sodium phosphate, *p*H 7.0, at 20°C, conditions which favor maintenance of CP in higher states of aggregation (*24*). The protein was then placed on a linear sucrose gradient (5 to 30 percent) and subjected to centrifugation in a Beckman SW40 rotor at 35,000 rev/min for 16 hours. Fractions (0.8 ml) were collected, concentrated, boiled with Laemmli sample buffer, and subjected to immunoblot analysis as in Fig. 4. Catalase (11*S*) and hemoglobin (4*S*) were placed on a parallel gradient as standards to determine sedimentation coefficients.

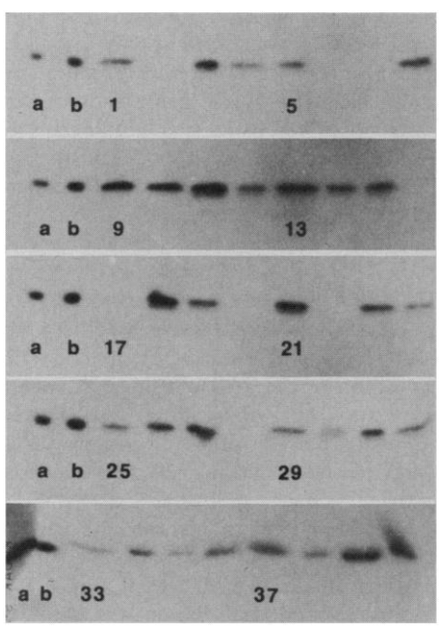

Fig. 6. Segregation of expression of the CP gene in seedlings. Forty micrograms of protein extracted from leaves of seedlings grown from self-fertilized transgenic plants (line 3773) were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis as in Fig. 4C. Of the 40 seedlings assayed, 8 did not express the coat protein gene, and 32 did. Lanes a and b contain 10 and 20 ng of TMV-CP, respectively.

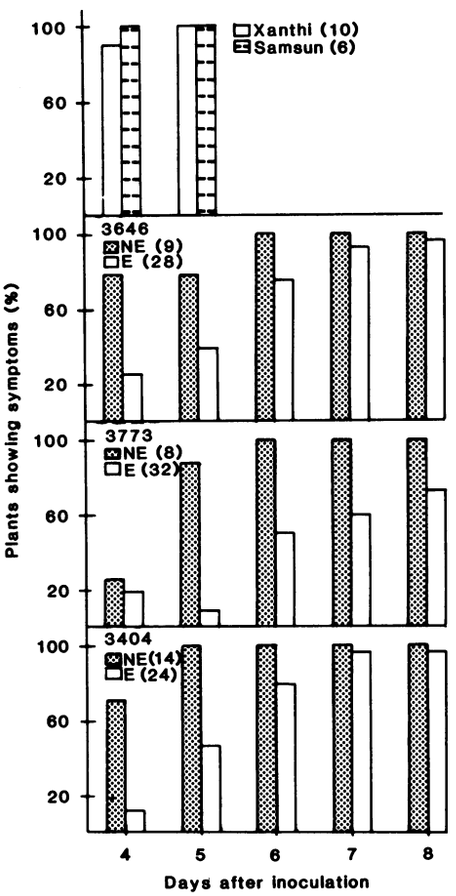

Fig. 7. The percentage of plants showing TMV infection on their leaves at successive days after inoculation with purified TMV. Seeds of each plant type, *N. tabacum* cv. Xanthi and cv. Samsun and transgenic lines 3646, 3773, 3404, and 3648, were germinated and assayed for CP (Table 1). Four days later the two youngest expanding leaves were inoculated with a suspension of purified TMV (0.25 μg/ml); carborundum (330 grit, Fisher Scientific) was used as an abrasive. The seedlings were then placed in growth chambers in a light-dark cycle of 14 hours of light and 10 hours of darkness; the temperature was 26°C for the light period and 19°C for the dark period. The relative humidity was approximately 50 percent and light flux density (PAR), supplied by fluorescent and incandescent lamps, was 340 μE m^{-2} sec^{-1}. Plants were scored as having positive symptoms when the first and second leaves above the inoculated leaves showed obvious vein clearing. CP analyses were not completed until all visual observations were recorded in order to avoid observation bias. NE, segregated seedlings from transgenic plants which were not expressing coat protein; E, segregated progeny from transgenic plants which were expressing coat protein. Numbers in parentheses indicate sample size for each plant type. Plants expressing CP were delayed in disease expression compared with respective nonexpressing plants for each plant line as determined by the Mann-Whitney *U* test ($\alpha = 0.005$). The test was conservative in that (i) no corrections were made for ties in scoring, and (ii) in the case of line 3773 (E progeny at 4 and 5 days after inoculation) once a plant was scored positive for symptom development it was never released from that category during calculations. A fourth transgenic plant line, 3648, had only one NE plant. The 39 E plants in line 3648 showed symptom development patterns similar to transgenic plant line 3773 (*28*).

inoculated with 0.4 μg of TMV per milliliter) did not develop symptoms during the 19-day period of the experiment (Fig. 8).

Toward an understanding for cross-protection. A double-stranded cDNA encoding the coat protein (CP) of TMV was ligated to the CaMV 35*S* promoter and NOS 3′ sequences, and introduced into tobacco cells on a Ti plasmid. Eight different transgenic plants regenerated from transformed cells produce CP to the level of about 0.1 percent of the extractable cellular protein. Bevan *et al.* (*26*) recently reported the results of similar experiments and provided data to indicate the TMV-CP accumulated to a level of about 0.002 percent of the cell protein (*27*). The reasons for the apparent differences in levels of expression of a similar gene in transgenic plants generated in different laboratories remains to be determined. It may reflect differences in the 35*S* promoter fragment, or the host plant genotype used in the different laboratories.

On inoculation with TMV, symptom development in transgenic plants expressing the CP gene was altered compared to control plants. First, not all transgenic plants developed disease symptoms. The results of virus replication experiments have indicated that plants that do not develop symptoms are not infected (*28*). As was expected, increasing the concentration of TMV in the inoculum increases the number of plants that become infected and develop symptoms (Fig. 8). Second, plants that developed symptoms did so 1 to 10 days later than the control plants, depending on the concentration of TMV in the inoculum (Figs. 7 and 8). Both of these observations are consistent with the hypothesis that the number of sites susceptible to infection are reduced in leaves of transgenic plants that express the CP gene compared with leaves of control plants.

Although Bevan *et al.* (*26*) reported the accumulation of TMV-CP in transgenic plants, they did not discuss virus infection of the plants. If the level of expression in these plants is indeed 50 to 100 times lower than in the plants described above, it may be possible to use the plants with lower CP levels to establish a threshold level of gene expression that is needed to give protection.

Delay in symptom development such as that demonstrated in Figs. 1, 7, and 8 is a measure of the degree of cross-protection in these plants (*3*). Whether or not the delay in symptom development in the transgenic plants is the result of a mechanism similar to that of classical cross-protection remains to be determined. However, identification of the mechanism (or mechanisms) of classical cross-protection will be technically difficult. Identifying the mechanism that gives rise to resistance in transgenic plants will be somewhat easier since it involves the expression of a single virus-related gene, rather than on replication of closely related viruses. Regardless of the precise mechanism involved in the virus disease resistance in these transgenic plants, the methods described here provide a way of producing virus-resistant plants that should complement those used in classical plant breeding.

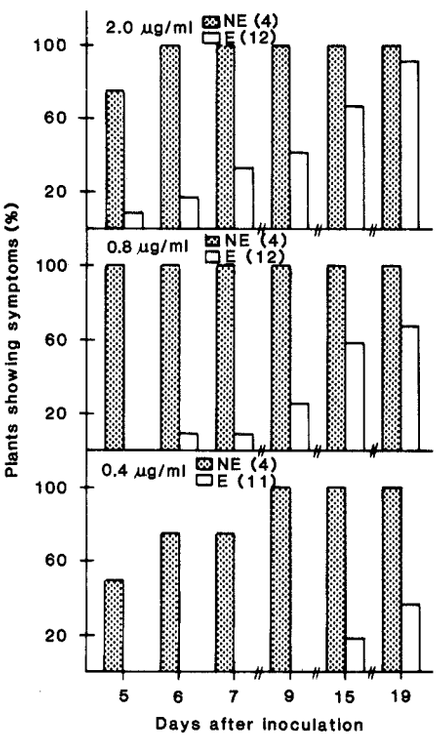

Fig. 8. The percentage of plants showing symptoms of TMV infection at succeeding days after inoculation with various concentrations of purified U_1-TMV. Seeds of transgenic lines 3773 and 3404 were germinated and assayed for CP (Table 1), or for the presence of nopaline (*39*). Approximately 4 days later, the two youngest expanding leaves of each plant were inoculated with suspensions containing purified TMV at 0.4, 0.8, or 2.0 μg/ml. The seedlings were kept in a greenhouse under conditions described in Table 1. Plants were scored as having positive symptoms when leaves above the inoculated leaves showed vein clearing that subsequently led to severe chlorosis. Equal numbers of plants from lines 3773 and 3404 were used except for the inoculum at 0.4 μg/ml, where one less 3773 plant was used. NE, segregated progeny from transgenic plants that did not express coat protein. E, segregated progeny from transgenic plants that expressed the coat protein gene. Numbers in parentheses indicate the sample size for each plant type.

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