Breakdown of Cross Protection Between Strains of Tobacco Mosaic Virus Due to Susceptibility of Dark Green Areas to Superinfection

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ABSTRACT

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Reciprocal cross protection between the common strain of tobacco mosaic virus (TMV-C) and TMV-P (a necrotizing strain in *Nicotiana sylvestris*) was investigated in plants of *N. tabacum* 'Samsun' and 'Xanthi'. When a concentration of challenge inoculum of 1 μg/ml or higher of either TMV-P or TMV-C was used, there was superinfection of plants infected with the heterologous strain. The susceptibility to superinfection was associated with dark green areas of mosaic leaves, which were more susceptible to superinfection than the neighboring light green areas. Challenge inoculation with TMV-P RNA overcame the protection afforded by light green areas in TMV-C-infected Samsun and Xanthi. Systemic superinfection by the challenge strain occurred for all plants in which superinfection was detected in either dark or light green areas. Thus, once infection was initiated there was not extensive protection against the subsequent spread of the challenge virus. Protoplasts from dark and light green areas from *N. sylvestris* systemically infected with TMV-C were receptive to the attachment and/or uptake of the ^{32}P-labeled TMV-P. Superinfection with virions or RNA from TMV-P was detected in protoplasts from both dark and light green areas, but virus accumulation was delayed and lessened in protoplasts from light green areas. The susceptibility of plants and protoplasts to superinfection may be a result of the uneven distribution of the protecting strain of TMV in infected plants.

Cross protection, the activity of a virus in a plant preventing the expression of a subsequent challenge virus (11), was first described in the late 1920s (23,44), but diseases caused by citrus tristeza virus and papaya ringspot virus appear to be the only extant examples in which cross protection is used commercially (24,35,47). Anxiety about potential problems could explain the paucity of examples of cross protection being used for biological control of diseases in the field (43). These include the protection being overcome (superinfection) by a severe strain, the possibility of spreading the mild protecting virus to other hosts in which its effects might be severe, the possible synergistic reaction of the protecting virus with an unrelated virus, and the change of the mild protecting strain to a more severe form. Although cross protection has been the subject of much research, speculation, and review (17,18,37,43,48), the mechanism(s) is not fully understood.

The occurrence of superinfection that leads to a breakdown of protection in plants infected by a virus has been demonstrated for several host-virus combinations (3,5,16,20,29). Also, the use of either naturally selected or mutant mild strains of virus as immunizing agents in commercial crops commonly provides only incomplete or partial protection (7,8,14,47). Fulton (16) suggested that superinfection in these cases may result because 1) the protecting mild strain may not completely invade the plant; 2) the protecting strain may not reach a concentration sufficient to occupy all infection sites or all multiplication sites; or 3) the process involved in replication of one strain may be different from that of another strain so they do not interfere. None of these mechanisms has been fully demonstrated.

Superinfection of dark green areas, which frequently contain much less virus than the neighboring light green areas, has been studied with several viruses (2,15,21,33). Fulton (15) and Sherwood and Fulton (38) reported that dark green areas of *Nicotiana sylvestris* Speg. infected with tobacco mosaic virus (TMV) were more susceptible to superinfection by necrotizing strains than light green areas, but both the dark and light green areas were resistant to superinfection by the same mosaic-causing strain of TMV. Similarly, Reid and Matthews (33) reported that dark green areas in Chinese cabbage (*Brassica pekinensis* 'Wong Bok') infected with turnip yellow mosaic virus appeared resistant to superinfection by the same virus. In contrast, Loebenstein et al (21) found that dark green areas developed in leaves of *N. tabacum* L. 'Xanthi-nc' and 'White Burley' after inoculation with cucumber mosaic virus (CMV, Price No. 6) were resistant to reinfection by three additional strains of CMV, but not to infection by TMV.

The differential susceptibility of dark green areas to superinfection indicates their importance in the events in cross protection that occur between plant virus strains. In this study we investigated superinfection between strains of TMV in plants of *N. tabacum* cultivars Samsun and Xanthi and protoplasts isolated from *N. sylvestris*.

MATERIALS AND METHODS

Plants and growing conditions. Samsun and Xanthi seeds were sown in a commercial soil mix in 10-cm plastic pots. One month later the seedlings were transplanted individually into pots containing the same soil mix. The greenhouse was maintained at 25–28 C.

Viruses, purifications, and inoculations. Two strains of TMV and tobacco etch virus (TEV) were used. The common strain of TMV (TMV-C) was propagated in *N. sylvestris* and a strain originally isolated from petunia (TMV-P) (38) was maintained in Samsun. TEV was propagated in Samsun. Both strains of TMV were purified by differential centrifugation (36). TEV was purified following the procedure described by Purcifull and Hiebert (30). Inoculum for all viruses was prepared in 0.01 M phosphate buffer, pH 7.0. Plants were mechanically inoculated using Carborundum as an abrasive.

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RNA from both strains of TMV was isolated by phenol extraction using the method of Ralph and Berquist (31). RNA was stored frozen at −70 C in 1% KH_2PO_4, pH 7.0. RNA inoculum was prepared in the same buffer and plants were inoculated as before.

Labeling of TMV with ^{32}P. Germinated seeds of tomato (*Lycopersicon esculentum* Mill. 'Rutgers') were transplanted into pots containing steam-sterilized vermiculite. Seedlings were watered daily with Hoagland's mineral salt solution (19) deficient in phosphorous. Two seedlings with fully expanded opposite leaves were mechanically inoculated with 0.2 mg/ml of TMV-C or TMV-P. Developing true leaves were removed 4–5 days after inoculation. The petiole end of the leaves was immediately immersed in 250 μl of an aqueous solution containing 5 mCi of ^{32}P as orthophosphate, HCl free and carrier free (PBS.13A, Amersham, Arlington Heights, IL) in a 1.5-ml microfuge tube. Distilled water was added to the tube after the phosphorous solution was taken up by the leaves. Leaves were incubated for 72 h in a Percival growth chamber (Boone, IA) at 25 C in continuous light at approximately 6,000 lx. The leaves then were harvested and the virus was purified as described. Radioactivity of the purified virus was measured in a Quick-count Bioscan (QC 2000, Bioscan, Inc., Washington, DC). The radioactivity for purified virus was 3,077 cpm/μg for TMV-C, and 27,181 cpm/μg for TMV-P.

Production of cross absorbed IgG. Antisera against TMV-C or TMV-P were produced by injecting rabbits intramuscularly twice weekly for 5 wk with 1 mg of purified virus with Freund's complete adjuvant. Antiserum against TMV-C was cross absorbed with an equal volume of crude sap containing TMV-P diluted 1:10 in phosphate-buffered saline (PBS). Antiserum against TMV-P was similarly cross absorbed with TMV-C. The IgG fractions of each antiserum then were purified by ion-exchange chromatography using a DEAE-Trisacryl-M column washed with 35 mM NaCl and 25 mM Trizma base, pH 8.8. IgG was stored at −20 C. Anti-TMV-C IgG and anti-TMV-P IgG also were conjugated to alkaline phosphatase (Sigma type VII, P5521) using glutaraldehyde (10). Conjugated IgG was stored at 4 C.

Challenge inoculation on the entire leaf surface. Samsun and Xanthi plants were mechanically inoculated with 1 μg/ml of TMV-C or TMV-P at the two-leaf stage. Two to three weeks later plants systemically infected with TMV-C were challenge-inoculated with different concentrations of TMV-P (0.01, 0.1, 1, 5, 25, and 50 μg/ml), and vice versa. The challenge inoculum was applied to the two upper fully expanded leaves of the systemically infected plants. Leaves showed symptoms of systemic infection. Samples were collected from challenge-inoculated leaves and additional upper leaves of the plants 12 days after the challenge inoculation. Each sample consisted of two 7-mm leaf disks taken randomly from the leaves. All samples were individually ground in PBS containing 0.05% Tween (PBS-Tween) and 2% polyvinylpyrrolidone (PVP) and diluted 1:100. The presence of the challenge strain in each sample was determined by a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (10) and an infectivity test on *N. sylvestris* when TMV-P was used as the challenge strain. The upper leaves of the plants again were tested for the challenge strain 25 days after the challenge inoculation.

Challenge inoculation on dark and light green areas. Samsun and Xanthi plants were inoculated with TMV-C or TMV-P as before. Two to three weeks later plants systemically infected with TMV-C or TMV-P were separated into two sets. The first set consisted of plants showing well-defined dark green areas, and the second set consisted of plants exhibiting distinct light green areas. A third group consisted of healthy plants of the same age that were used as controls for the challenge inoculation. Six dark green areas and six light green areas on the two fully expanded upper leaves were marked with a circle about 1 cm in diameter (three areas per leaf). The same number of circles were marked on the two developed upper leaves of the healthy plants. Plants infected with TMV-C were mechanically challenge-inoculated within the marked circles with 1 μg/ml of TMV-P and vice versa. Challenge inoculations with TMV-P RNA (50 μg/ml of RNA) were carried out using the same experimental design. Protection to an unrelated virus also was tested by challenging dark and light green areas with 1 μg/ml of TEV. Samples were collected from inoculated areas and upper leaves of the plants 12 days after the challenge inoculation. Each sample consisted of two 7-mm leaf disks. A total of four samples were obtained from each test plant. The presence of the challenge strain (TMV-C or TMV-P) in the samples was tested as before, while TEV was detected by the protein-A sandwich ELISA (13). Data of systemic superinfection were statistically analyzed by the chi-square test and the values were compared by the two samples comparison for proportion test (40).

Preparation of protoplasts. Fully expanded leaves from 7- to 8-wk-old healthy and TMV-C infected *N. sylvestris* plants were used for isolation of mesophyll protoplasts. Dark and light green areas from mosaic leaves were separated with a razor blade. Leaves were surface-sterilized and the lower epidermis was gently scraped with a wire brush. Scraped leaves were incubated overnight in a solution of 13% mannitol containing 1% cellulase, 0.25% macerozyme, and 0.25% bovine serum albumin, pH 5.8. Protoplasts from healthy tissue and dark or light green area tissues were adjusted to a concentration of 3×10^5 protoplasts per milliliter. Viability of protoplasts was tested by staining with 1% Evans blue prepared in 13% mannitol. Only suspensions containing at least 85% viable protoplasts after isolation were used in further experiments.

Inoculation, culture, and sampling of inoculated protoplasts. Protoplasts were inoculated using a procedure slightly modified from that of Loesch-Fries and Hall (22). A pellet of 3×10^5 protoplasts was resuspended in 25 μl of 13% mannitol containing 5 μg of virus (TMV-P or TMV-C). The mixture was held for a few seconds and then transferred to a glass tube containing 200 μl of 3 mM $CaCl_2$ and 3 mM 2-[N-Morpholino]ethanesulfonic acid (MES) containing 40% (w/v) polyethylene glycol (PEG 1540, Polysciences, Inc., Warrington, PA), pH 5.8. The contents were mixed well, held for 10 s, and then diluted with 1 ml of 13% mannitol. After incubation for 20 min at room temperature, the protoplasts were sedimented and washed three times by centrifugation at 50 *g* and by adding 2–3 ml of 13% mannitol. The same procedure was used to inoculate protoplasts with ^{32}P-labeled virions.

Inoculation of protoplasts with viral RNA was conducted similarly as described for inoculation with virus, except that a pellet of 3×10^5 protoplasts was resuspended in 10 μl of 1% KH_2PO_4 containing 0.5 μg of RNA from TMV-C or TMV-P. Inoculated protoplasts were incubated on ice for 20 min and then washed once in 13% mannitol. Mock-inoculated protoplasts from healthy tissues and dark and light green area tissues were used as controls for all experiments.

After washing, protoplasts (3×10^5/ml) were resuspended in culture medium (1) containing 13% mannitol and 200 μg/ml of carbenicillin. Protoplasts were kept at 25 C under continuous light (2,000 lx).

Samples of 600 μl were taken from inoculated and mock-inoculated protoplasts at 0, 24, 48, and 72 h after inoculation. After sedimentation, protoplasts were resuspended in 600 μl of PBS-Tween containing 2% PVP. Samples were frozen at −20 C and superinfection was evaluated by double antibody sandwich ELISA and infectivity tests on *N. sylvestris*.

Fluorescent antibody staining of infected protoplasts. After incubation for 72 h in culture medium, protoplasts were prepared for immunofluorescent microscopy using a procedure slightly modified from that of Otsuki and Takebe (27). Glass slides were coated with Mayer's egg albumin. One hundred microliters of protoplast suspension was centrifuged at 400 *g* for 3 min. All but 10 μl of the supernatant was discarded. Protoplasts were resuspended in the remaining 10 μl of supernatant, placed on the slides, and quickly dried with warm air. Protoplasts were fixed in acetone for 30 min and then allowed to dry at room temperature. After the slides were washed in PBS for 15 min, 100 μl of the cross absorbed antiserum diluted 1:500 in PBS was added to the slides. Slides were incubated in a moist chamber for 2 h at 36 C and then washed in PBS for 15 min. One hundred microliters of rabbit IgG conjugated with fluorescein isothio-

cyanate (FITC) (Sigma, F-0382), diluted 1:100 in PBS, was added to the slides. Slides were incubated in a moist chamber for 2 h at 30 C and then were washed for 15 min in PBS. A few drops of glycerol in PBS (1:9, v/v) were placed on the slides, the cover slips were mounted, and protoplasts were observed with UV optics using an Olympus BH-2 microscope (Olympus Corp., Lake Success, NY).

Detection of association of virus with protoplasts. Protoplasts inoculated with ^{32}P-labeled TMV-P or TMV-C were washed as described to remove virions remaining in solution after inoculation. After the third washing, pellets of 3×10^5 protoplasts were resuspended in 1 ml of 13% mannitol. Radioactivity of inoculated and uninoculated protoplasts was measured in a Quickcount Bioscan (QC 2000) to determine attachment and/or uptake of virions in protoplasts.

ELISA. The double antibody sandwich ELISA procedure used was similar to that of Clark and Adams (10). ELISA plates were coated with 10 μg/ml anti-TMV-P IgG or 1 μg/ml anti-TMV-C IgG diluted in 0.05 M carbonate buffer, pH 9.6. After incubating and rinsing plates, samples of protoplasts were thawed at room temperature and then added to the plates (100 μl per well). When TMV-C was the challenge strain, samples of protoplasts were thawed, diluted 1:50 in PBS-Tween containing PVP, and then added to the plates. Known concentrations of the challenge strain being tested were added to other wells in the same ELISA plate. After incubation at 4 C overnight, plates were rinsed three times with PBS-Tween. Alkaline phosphatase-labeled anti-TMV-P IgG diluted 1:500, or alkaline phosphatase-labeled anti-TMV-C IgG diluted 1:800 in PBS-Tween containing 2% PVP and 0.2% ovalbumin, was added to the plates. After incubation and rinsing, *p*-nitrophenyl phosphate (Sigma, N-2765) dissolved in diethanolamine substrate buffer, pH 9.8, was added. Plates were read in a Bio-Tek EIA plate reader (Burlington, VT).

Quantification of the challenge strain produced in protoplasts. Yield of TMV-P in superinfected protoplasts was estimated by means of absorbance values from ELISA. Absorbance values obtained for inoculated protoplasts from healthy tissue and from dark and light green area tissues were subtracted from the absorbance values for their respective mock-inoculated samples. This procedure was used to eliminate absorbance due to nonspecific reaction between the anti-TMV-P IgG and the TMV-C strain already present in protoplasts from dark and light green areas. Absorbance values obtained for the standard concentrations of TMV-P (32, 64, 128, 256, 512, 1,024, and 2,048 ng/ml of virus) were analyzed by a multiple linear regression analysis (40). Subtracted absorbance values then were substituted into the regression equation in order to estimate the concentration of TMV-P in each type of protoplast at different intervals after inoculation.

Infectivity tests. Samples from mock-inoculated protoplasts and from protoplasts inoculated with TMV-P were diluted 1:3 in 0.2 M phosphate buffer, pH 6.8. Each sample then was mechanically inoculated on three half-leaves of *N. sylvestris* to test the infectivity of TMV-P produced in protoplasts. Lesions were counted 4–5 days after inoculation.

Quantification of TMV-C in tissues. Two-millimeter leaf disks were collected from dark and light green areas of *N. sylvestris* and Samsun and Xanthi infected with TMV-C. Samples were individually ground in PBS-Tween containing 2% PVP. To assure that the absorbance value from the sample in ELISA would be in the range of the standard curve, based on results from preliminary experiments, samples from dark green areas were diluted 1:6,000 to 1:8,000 and samples from light green areas were diluted 1:17,000 to 1:25,000. The concentration of TMV-C was estimated using a multiple linear regression equation obtained with the absorbance values of standard concentrations of TMV-C (16, 32, 64, 128, 256, 512, and 1,024 ng/ml of virus).

RESULTS

Superinfection of plants by challenge inoculation on the entire leaf surface. The threshold challenge inoculum concentration for superinfection was determined by inoculating cultivars Samsun and Xanthi systemically infected with TMV-C with different concentrations of TMV-P and vice versa. All Samsun and Xanthi plants systemically infected with TMV-C and challenge-inoculated with 1, 5, 25, or 50 μg/ml of TMV-P were susceptible to superinfection (data not shown). Results of ELISA and infectivity tests on *N. sylvestris* indicated that TMV-P was present in the inoculated leaves and in the upper leaves of all challenged plants. Only part of the challenge-inoculated plants were superinfected on the inoculated leaves as well as systemically when inoculated with TMV-P at 0.1 or 0.01 μg/ml. The susceptibility of Samsun and Xanthi systemically infected with TMV-P to superinfection with TMV-C were similar to those systemically infected with TMV-C and challenged with TMV-P (data not shown). Partial and complete protection was found when the concentration of TMV-C was 0.1 and 0.01 μg/ml, respectively. Healthy plants used as controls were systemically infected in all experiments regardless of the inoculum concentration.

Susceptibility of dark and light green areas to superinfection. Dark and light green areas of cultivars Samsun and Xanthi systemically infected with TMV-C showed different susceptibility to superinfection with TMV-P (Table 1). TMV-P superinfected 67 and 62% of the inoculated dark green areas of Samsun and Xanthi, respectively, based on ELISA and infectivity tests on *N. sylvestris*. Superinfection by TMV-P was not detected in inoculated light green areas of cultivar Samsun, but TMV-P was detected in 33% of the inoculated light green areas of cultivar Xanthi. The same difference in the susceptibility of dark and light green areas to superinfection occurred when Samsun and Xanthi plants systemically infected with TMV-P were challenged with TMV-C (Table 1). Superinfection by TMV-C occurred in

TABLE 1. Localized superinfection of dark green areas (DGA) and light green areas (LGA) of tobacco mosaic virus (TMV)-infected leaves of *Nicotiana tabacum* 'Samsun' and 'Xanthi' with the common strain of TMV (TMV-C), a strain of TMV that produces necrotic lesions on *N. sylvestris* (TMV-P), RNA of TMV-P, or tobacco etch virus (TEV)

Cultivar/ protective strain	Challenge inoculum	Number of areas superinfected[y,z]/number of challenge inoculated areas		
		Healthy (control)	DGA	LGA
Samsun/TMV-C	TMV-P	19/24	30/45	0/60
Xanthi/TMV-C	TMV-P	18/21	24/39	15/45
Samsun/TMV-P	TMV-C	4/12	19/24	3/33
Xanthi/TMV-P	TMV-C	9/18	22/33	4/36
Samsun/TMV-C	TMV-P RNA	33/33	51/51	32/54
Xanthi/TMV-C	TMV-P RNA	21/21	36/36	23/42
Samsun/TMV-C	TEV	10/12	24/27	27/27
Xanthi/TMV-C	TEV	10/12	27/30	20/27

[y] Plants were mechanically inoculated with 1 μg/ml of TMV-C or TMV-P at the two-leaf stage. Two to three weeks later plants systemically infected were challenge inoculated. Six dark green areas and six light green areas (1 cm diameter) on the two fully expanded upper leaves were marked (three areas per leaf). Healthy plants were similarly marked. Challenge inoculum of TMV-C or TMV-P was 1 μg/ml. The same experimental design was used for inoculations with TMV-P RNA (50 μg of RNA/ml) and TEV (1 μg/ml). Samples were collected from inoculated areas 12 days after the challenge inoculation.

[z] A double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was used to detect the challenge strain in each sample and, when TMV-P was used as the challenge inoculum, an infectivity test on *N. sylvestris* TMV-P also was used. Each sample contained leaf disks from two independently inoculated areas. ELISA plates were coated with 10 μg/ml anti-TMV-P IgG or 1 μg/ml anti-TMV-C IgG. Samples of 100 μl per well were added to the plates. After incubation at 4 C overnight, the bound virus was detected using alkaline phosphatase labeled anti-TMV-P IgG diluted 1:500 or alkaline phosphatase labeled anti-TMV-C IgG diluted 1:800 followed by *p*-nitrophenyl phosphate dissolved in diethanolamine substrate buffer. Plates were read in a Bio-Tek EIA plate reader. Samples were considered positive when the reading was higher than the average of the three negative control wells plus three standard deviations of the readings of those wells.

79 and 67% of inoculated dark green areas of Samsun and Xanthi, respectively, based on ELISA. On the other hand, TMV-C was detected in only 9% of inoculated light green areas of Samsun and 11% of inoculated light green areas of Xanthi.

Systemic superinfection of Samsun and Xanthi was directly related to superinfection of dark and light green areas. The number of Samsun and Xanthi plants superinfected systemically by TMV-P was greater when the challenge inoculum was applied to the dark green areas than when applied to the light green areas (Table 2). Systemic superinfection with TMV-C also was directly related to superinfection of dark or light green areas (Table 2).

TABLE 2. Systemic superinfection of *Nicotiana tabacum* 'Samsun' and 'Xanthi' systemically infected with tobacco mosaic virus (TMV), the common strain of TMV (TMV-C), a strain of TMV that produces necrotic lesions on *N. sylvestris* (TMV-P), RNA of TMV-P, or tobacco etch virus (TEV) inoculated on dark green areas (DGA) or light green areas (LGA)

Cultivar/ protective strain	Challenge inoculum	Number of plants superinfected systemically[y,z]/number of challenged plants		
		Healthy (control)	DGA	LGA
Samsun/TMV-C	TMV-P	7/8 a	13/15 a	0/20 b
Xanthi/TMV-C	TMV-P	7/7 a	10/13 b	2/15 c
Samsun/TMV-P	TMV-C	3/4 a	7/8 a	4/11 b
Xanthi/TMV-P	TMV-C	5/6 a	9/11 a	1/12 b
Samsun/TMV-C	TMV-P RNA	11/11 a	17/17 a	9/18 b
Xanthi/TMV-C	TMV-P RNA	7/7 a	12/12 a	10/14 b
Samsun/TMV-C	TEV	4/4 a	9/9 a	9/9 a
Xanthi/TMV-C	TEV	4/4 a	10/10 a	9/9 a

[y] Plants were mechanically inoculated wih 1 μg/ml of TMV-C or TMV-P at the two-leaf stage. Two to three weeks later plants systemically infected were challenge inoculated. Six dark green areas and six light green areas (1 cm diameter) on the two fully expanded upper leaves were marked (three areas per leaf). Healthy plants were similarly marked. Challenge inoculum of TMV-C or TMV-P was 1 μg/ml. The same experimental design was used for inoculations with TMV-P RNA (50 μg of RNA/ml) and TEV (1 μg/ml). Samples (four per plant) of two 7-mm leaf disks were collected from upper leaves 12 days after the challenge inoculation.

[z] A double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was used to detect the challenge strain in each sample and, when TMV-P was used as the challenge inoculum, an infectivity test on *N. sylvestris* TMV-P also was used. Each sample contained leaf disks from two independently inoculated areas. ELISA plates were coated with 10 μg/ml anti-TMV-P IgG or 1 μg/ml anti-TMV-C IgG. Samples of 100 μl per well were added to the plates. After incubation at 4 C overnight, the bound virus was detected using alkaline phosphatase labeled anti-TMV-P IgG diluted 1:500 or alkaline phosphatase labeled anti-TMV-C IgG diluted 1:800 followed by *p*-nitrophenyl phosphate dissolved in diethanolamine substrate buffer. Plates were read in a Bio-Tek EIA plate reader. Samples were considered positive when the reading was higher than the average of the three negative control wells plus three standard deviations of the readings of those wells. Means followed by the same letter in the row are not significantly different (two samples comparison for proportion, $P = 0.05$).

Dark and light green areas of cultivars Samsun and Xanthi systemically infected with TMV-C were more susceptible to superinfection with TMV-P RNA than with virus (Table 1). All dark green areas of Samsun and Xanthi challenge-inoculated with TMV-P RNA were superinfected 12 days after challenge inoculation and systemic infection also occurred (Table 2). Fifty-nine percent of light green areas of Samsun and 55% of light green areas of Xanthi were superinfected when inoculated with TMV-P RNA. When the TMV-P RNA challenge inoculum was applied to light green areas, 50 and 71% of Samsun and Xanthi plants became systemically infected, respectively (Table 2).

Dark and light green areas of cultivars Samsun and Xanthi infected with TMV-C and the leaves above these areas were equally and nearly 100% susceptible to superinfection with TEV (Tables 1 and 2).

Attachment of radiolabeled TMV to protoplasts. Protoplasts from dark and light green areas were isolated and challenge-inoculated to determine if the differential response of dark and light green areas to superinfection was due to an inability of the virus to become associated with the cells of these areas or an inability to replicate in the cells. Protoplasts from healthy (control) and dark and light green areas from *N. sylvestris* systemically infected with TMV-C were inoculated with ^{32}P-labeled TMV-P. The radioactivity counts indicated that protoplasts were as receptive to virus binding and/or uptake as compared to protoplasts from healthy tissues. The average cpm/3×10^5 protoplasts from three experiments was 1,928 $\pm$ 512, 1,574 $\pm$ 408, and 2,859 $\pm$ 672 for protoplasts from healthy tissue and dark and light green areas, respectively. Similar results were obtained when protoplasts from dark and light green areas from *N. sylvestris* systemically infected with TMV-C were inoculated with ^{32}P-labeled TMV-C. The average cpm/3×10^5 protoplasts from three experiments was 3,884 $\pm$ 818, 3,285 $\pm$ 522, and 2,494 $\pm$ 1,359 for protoplasts from healthy tissue and dark green and light green areas, respectively.

Infection and superinfection of protoplasts. Protoplasts from dark and light green areas from *N. sylvestris* systemically infected with TMV-C showed different levels of susceptibility to superinfection with TMV-P (Table 3). TMV-P antigen was first detected in protoplasts from dark green areas 24 h after challenge inoculation and showed a gradual accumulation through 72 h of incu-

TABLE 3. Superinfection of protoplasts from dark and light green areas from *Nicotiana sylvestris* infected with the common strain of tobacco mosaic virus (TMV-C), a necrotic lesion-causing strain of TMV (TMV-P), or TMV-P RNA

Origin of protoplast	Challenge inoculum[y]	Yield of TMV-P (μg)/3×10^5 protoplasts[z]			
		0 h	24 h	48 h	72 h
Healthy (control)	Virus	0.00	1.12 $\pm$ 0.46	2.01 $\pm$ 0.33	1.83 $\pm$ 0.11
	RNA	0.00	0.90 $\pm$ 0.54	1.43 $\pm$ 0.39	1.74 $\pm$ 0.31
Dark green areas	Virus	0.00	0.56 $\pm$ 0.33	0.73 $\pm$ 0.55	1.46 $\pm$ 0.22
	RNA	0.00	0.61 $\pm$ 0.80	0.51 $\pm$ 0.50	0.99 $\pm$ 0.61
Light green areas	Virus	0.00	0.00	0.23 $\pm$ 0.26	0.45 $\pm$ 0.40
	RNA	0.00	0.16 $\pm$ 0.25	0.63 $\pm$ 0.92	0.65 $\pm$ 0.69

[y] A pellet of 3×10^5 protoplasts was resuspended in 25 μl of 13% mannitol containing 5 μg of virus or 10 μl of 1% KH_2PO_4 containing 0.5 μg of RNA. Inoculated protoplasts were kept at 25 C under continuous light (2,000 lx).

[z] Yield of TMV-P in superinfected protoplasts was estimated by means of absorbance values from enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated with 10 μg/ml anti-TMV-P IgG or 1 μg/ml anti-TMV-C IgG. Samples of 100 μl per well were added to the plates. After incubation at 4 C overnight, the bound virus was detected using alkaline phosphatase labeled anti-TMV-P IgG diluted 1:500 followed by *p*-nitrophenyl phosphate dissolved in diethanolamine substrate buffer. Plates were read in a Bio-Tek EIA plate reader. Absorbance values obtained for inoculated protoplasts from healthy tissue and from dark and light green area tissues were subtracted from the absorbance values for their respective mock-inoculated samples to eliminate absorbance due to nonspecific reaction between the anti-TMV-P IgG and the TMV-C strain already present in protoplasts from dark and light green areas. Absorbance values obtained for the standard concentrations of TMV-P (32, 64, 128, 256, 512, 1,024, and 2,048 ng of virus per milliliter) were analyzed by a multiple linear regression analysis and used to obtain the concentration of TMV-P in the sample. Each value is the average $\pm$ standard deviation of four or three experiments when TMV-P or TMV-RNA was used as inoculum, respectively.

bation. The same was found with protoplasts from healthy tissue used as controls for the inoculation. Protoplasts from light green areas, on the other hand, showed partial protection to superinfection with TMV-P virions. Such partial protection was characterized by a delay of 24 h for detection of a measurable amount of TMV-P antigen in superinfected protoplasts and by a lower estimated yield of TMV-P compared with the yield of TMV-P in protoplasts from dark green areas and from healthy tissue. The susceptibility of protoplasts from light green areas to superinfection with TMV-P RNA was higher than that to TMV-P virions (Table 3). TMV-P antigen was detected in these protoplasts 24 h after inoculation, and a higher accumulation of TMV-P antigen was found after incubation for 72 h. The efficiency of inoculation of protoplasts, tested by the immunofluorescent assay, showed that when TMV-P virions were used as inoculum, an average of 80% of protoplasts from healthy tissue (control) were infected after 72 h of incubation. When TMV-P RNA was used as inoculum, an average of 50% of infected protoplasts was found after incubation for 72 h. The immunofluorescent assay also was used to try to estimate the percentage of protoplasts from dark and light green areas superinfected with TMV-P. However, because of cross-reaction between antiserum to TMV-P and antigen to TMV-C in the immunofluorescent assay, the test was ineffective. The problem of cross-reaction was not eliminated even when antiserum cross absorbed with the heterologous virus was used. Cross-reaction of the antisera was not a limiting factor for ELISA.

Infectivity tests on *N. sylvestris* indicated that protoplasts from dark and light green areas superinfected with either TMV-P virions or free viral RNA contained infectious TMV-P progeny based on the necrotic lesions produced on the inoculated leaves (Table 4). The lower number of local lesions produced by samples from superinfected protoplasts from dark and light green areas was a result of the interference due to the presence of TMV-C in the inoculum. Such interference between strains of a virus is a well-known phenomenon occurring when a mosaic-causing strain is inoculated in a mixture with a necrotic lesion-forming strain and decreases the number of lesions produced (34,39).

Results from attempts to superinfect protoplasts from dark and light green areas of TMV-C-infected *N. sylvestris* with the same mosaic-causing strain (TMV-C) were not conclusive. ELISA and infectivity assays on *N. tabacum* cv. Xanthi-nc were not sensitive enough to detect if additional replication of TMV-C occurred.

Concentration of TMV-C in tissues. Results from ELISA of 417 samples indicated that the concentrations of TMV-C in dark and light green areas from *N. sylvestris* and Samsun and Xanthi were not homogeneous (Fig. 1). The concentration of TMV-C for the majority of samples from dark green areas was smaller than the concentration of virus from light green areas. However, there was an overlapping range in which the concentrations of TMV-C in both types of tissue were similar.

DISCUSSION

TMV-C and TMV-P are two serologically related strains that reciprocally cross protect in Samsun and Xanthi. This protection, however, was found to depend on the concentration of the challenge strain inoculum. Inoculum equal to or higher than 1 μg/ml led to complete superinfection of the plants when the challenge inoculum was applied over the entire surface of two leaves. This is in opposition to findings with coat protein expressing transgenic plants where inocula of 1 or 2 μg/ml were still unable to fully overcome protection (26,28). On the other hand, in experiments where the challenge inoculum was applied to either dark or light green areas, the dark green areas were more susceptible to superinfection than the light green areas. This suggests that dark green areas are apparently responsible for the majority of breakdown in protection observed when the entire leaf surface was inoculated. Fulton (15) and Sherwood and Fulton (38) also showed that dark green areas of *N. sylvestris* infected with TMV were susceptible to superinfection with necrotizing strains of TMV, including TMV-P.

The *N. sylvestris*-TMV system used by Fulton (15) and Sherwood and Fulton (38) to study cross protection between strains of TMV had the disadvantage of not allowing further observation of protection against systemic invasion of the plants by the challenge strain. That is because all challenge strains of TMV used in their experiments were strains that caused localized necrotic lesions on *N. sylvestris*. This type of observation was possible in the present study because TMV-C and TMV-P systemically invade cultivars Samsun and Xanthi. Our results showed that systemic invasion by the challenge strain occurred for all plants of cultivars Samsun and Xanthi in which superinfection was detected in either dark or light green areas. These results suggest that protection in this system is apparently related to an early event in the infection process, rather than to prevent systemic movement of the challenge strain. Urban et al (42) reported that cross protection between TMV-C and TMV-P in *Arabidopsis thaliana* 'Columbia' was due to prevention of systemic movement of the challenge strain. They found that regardless of the virus strain inoculated first, the challenge strain multiplied in inoculated leaves to concentrations detectable by ELISA, but it did not move systemically in the plants. Because both studies used the same strains of TMV and similar experimental procedures to evaluate superinfection, it can be inferred that the host may play an important role in the systemic movement of the challenge strain.

Challenge inoculation with TMV-P RNA dramatically overcame the resistance of light green areas of Samsun and Xanthi to superinfection. This suggests that uncoating of the challenge strains may be involved in the resistance of light green areas to superinfection. Prevention of uncoating of the challenge strains was suggested by Sherwood and Fulton (38) as responsible for the resistance of light green areas of TMV-C infected *N. sylvestris*

TABLE 4. Infectivity of the progeny of the necrotic lesion-causing strain of tobacco mosaic virus (TMV-P) produced in protoplasts from healthy *Nicotiana sylvestris* and in protoplasts from dark and light green areas from *N. sylvestris* infected with the common strain of TMV (TMV-C) and superinfected with TMV-P or TMV-P RNA

Origin of protoplast	Challenge inoculum[y]	Number of local lesions on three half-leaves of *N. sylvestris*[z]			
		0 h	24 h	48 h	72 h
Healthy (control)	Virus	0.0	96 ± 39	231 ± 222	144 ± 64
	RNA	0.0	113 ± 96	189 ± 168	346 ± 322
Dark green areas	Virus	0.0	29 ± 15	84 ± 48	44 ± 62
	RNA	0.0	14 ± 13	27 ± 24	69 ± 66
Light green areas	Virus	0.0	0.0	3 ± 2	2 ± 3
	RNA	0.0	4 ± 7	15 ± 23	20 ± 33

[y] A pellet of 3×10^5 protoplasts was resuspended in 25 μl of 13% mannitol containing 5 μg of virus or 10 μl of 1% KH_2PO_4 containing 0.5 μg of RNA. Inoculated protoplasts were kept at 25 C under continuous light (2,000 lx).

[z] Samples from mock-inoculated protoplasts and from protoplasts inoculated with TMV-P were diluted 1:3 in 0.2 M phosphate buffer, pH 6.8. Each sample was inoculated on three half-leaves of *N. sylvestris* and lesions were counted 4–5 days after inoculation. Each value is the average ± standard deviation of four or three experiments when TMV-P or TMV-RNA was used as inoculum, respectively.

to superinfection with necrotic lesions causing strains of TMV, including TMV-P. Superinfection of plants after inoculation with the virus RNA also was reported by Dodds et al (12) for studies of cross protection between strains of CMV. Because not all plants of Samsun and Xanthi challenged on light green areas were superinfected with TMV-P RNA, it is suggested that other factor(s) may be in part responsible for their resistance to superinfection. Yamaya et al (46) found that transgenic tobacco plants expressing the entire genome of a mild strain of TMV were protected against challenge inoculation with TMV-RNA.

The experiments on superinfection of protoplasts from dark and light green areas from *N. sylvestris* indicated that the challenge strain can bind to protoplasts from both dark and light green areas. The use of radioactively labeled virions showed that virus attachment or uptake was not specific since TMV-P and TMV-C attached to protoplasts already infected with TMV-C. Whether the challenge strain only bound or entered the protoplasts 20 min after inoculation cannot be determined from these experiments. However, because protoplasts from dark and light green areas were superinfected with TMV-P, it is likely that at least part of the measured radioactivity could have come from virus particles within the protoplasts. Several washings of the protoplasts did not remove the labeled virions.

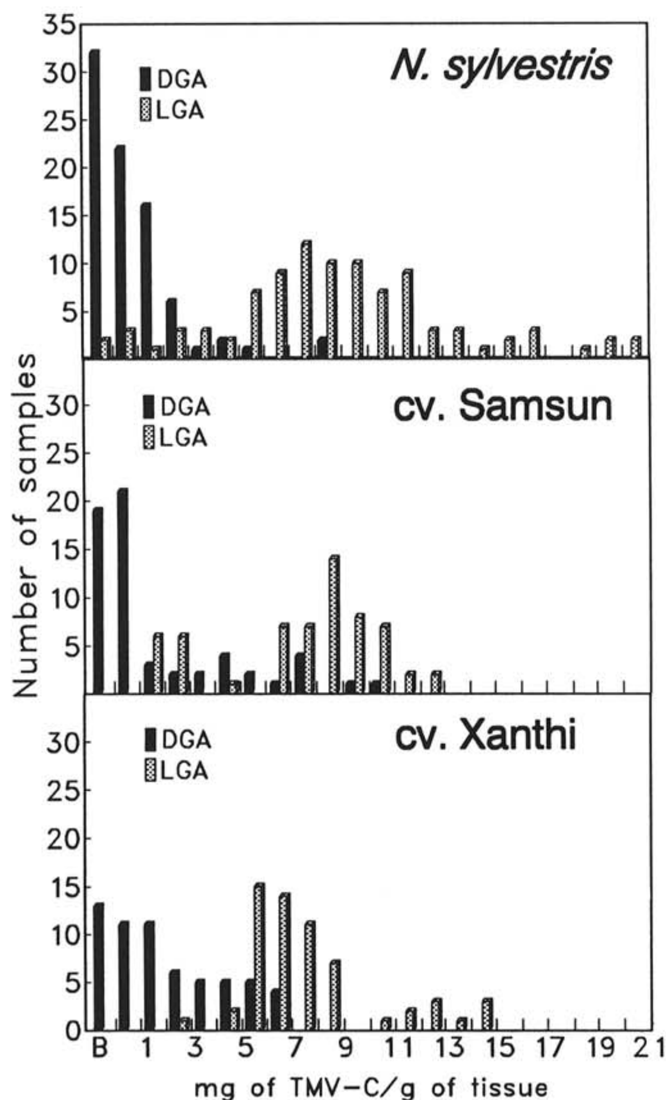

Fig. 1. The concentration of tobacco mosaic virus (TMV-C) in dark and light green areas of systemically infected plants was determined by double antibody sandwich enzyme-linked immunosorbent assay (ELISA). Two-millimeter leaf disks were collected from dark and light green areas of *Nicotiana sylvestris* and *N. tabacum* 'Samsun' and 'Xanthi' infected with TMV-C of a similar size and age of plants used for superinfection experiments. ELISA plates were coated with 1 μg/ml of anti-TMV-C IgG. Samples of 100 μl per well were added to the plates. After incubation at 4 C overnight, the bound virus was detected using alkaline phosphatase-labeled anti-TMV-C IgG diluted 1:800 followed by *p*-nitrophenyl phosphate dissolved in diethanolamine substrate buffer. Absorbance values obtained for the standard concentrations of TMV-C (16, 32, 64, 128, 256, 512, and 1,024 ng/ml of virus) were analyzed by a multiple linear regression analysis and used to obtain the concentration of TMV-C in the sample. B indicates samples with values below a detectable amount of virus by this analysis.

Our finding of only partial protection of light green areas of cultivars Samsun and Xanthi and of protoplasts from light green areas from *N. sylvestris* is not consistent with that found by Fulton (15) and Sherwood and Fulton (38) in mosaic leaves of *N. sylvestris*. They found that light green areas on whole leaves of *N. sylvestris* offered full protection against superinfection by TMV-P and other necrotic lesion-forming strains of TMV. Variation in protection at the level of whole plants and protoplasts also was found by Barker and Harrison (4) in studies of cross protection between strains S and E of raspberry ringspot virus (RRV). They found that recovered leaves of *N. benthamiana* systemically infected with RRV-S were protected against infection by RRV-E. However, when protoplasts from recovered leaves were inoculated with RRV-E, protection was partial, even though at least 98% of the protoplasts contained RRV-S antigen before challenge inoculation. In experiments with transgenic tobacco plants expressing coat protein, where protection is not complete, protection against TMV infection is similar to the protection expressed in protoplasts from transgenic plants (32). However, recently Wisniewski et al (45) have reported that protection in whole plants is due to both protection against initial infection and prevention of systemic spread of the challenge virus. This second protection cannot be easily accounted for in protoplasts studies.

An intriguing question from our experiments is what makes light green areas of *N. tabacum* cultivars Samsun and Xanthi and protoplasts from light green areas from *N. sylvestris* not fully protected against superinfection, while light green areas on whole leaves of *N. sylvestris* offered apparently full protection against superinfection. Sherwood and Fulton (38) suggested that the resistance of light green areas on whole leaves of *N. sylvestris* to superinfection was a result of the prevention of uncoating of the challenge strain. They concluded that the prevention of uncoating might be regulated by the kind and amount of viral coat protein already present in the cell. This was based on the knowledge that the average concentration of TMV in light green areas is higher than the concentration of virus in dark green areas (2,15,36). Work with transgenic tobacco plants that express TMV coat protein showed that the degree of resistance to TMV infection is directly related to the amount of coat protein accumulated in the plants in some cases (25). In the present study it also was observed that the average concentration of TMV-C in tissues and protoplasts from light green areas was higher than in tissues and protoplasts from dark green areas. In addition, it was found that even within dark and light green areas virus is not homogeneously distributed. The concentration of virus in some light green areas was similar to the concentration of virus in dark green areas that are susceptible to superinfection (15,38). Therefore, it is suggested that the uneven distribution of TMV-C in cells of light green areas may result in some cells with a virus concentration lower than the amount required to prevent superinfection. The absence of visible superinfection in light green areas on whole leaves of *N. sylvestris* may be attributed to subliminal infection, in which the challenge strain replicates in individual cells on inoculated leaves but remains restricted in its translocation to neighbor cells (45). Subliminal infections have been found to occur in other host-virus interactions (9,41,49). Experiments to determine whether light green areas of *N. sylvestris* are subliminally infected by the challenge strain might clarify this difference in protection. It has been proposed that a mechanism of coat protein-mediated protection and in some cases cross protection involves an inhibition of uncoating of the challenge virus (6,43). The use of transgenic plants that express the coat protein

gene of the virus evenly throughout the plant may offer greater protection than classical cross protection in cases where superinfection may be due to the uneven distribution of the protecting strain within the plant.

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