SUPERINFECTION BETWEEN STRAINS OF TOBACCO MOSAIC VIRUS

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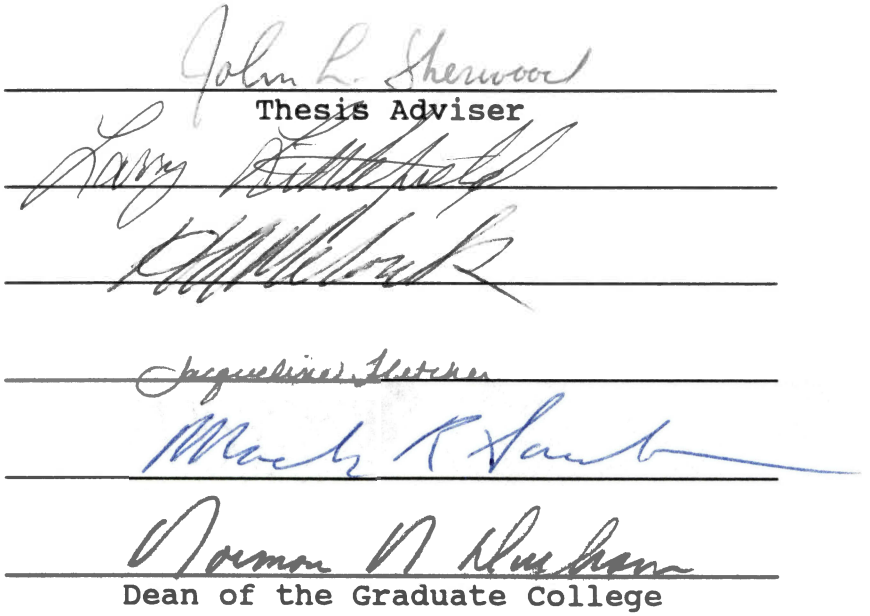

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CHAPTER I

INTRODUCTION

Cross protection, the activity of a virus in a plant preventing the expression of a subsequent challenge virus (Dodds, 1982), was first described in the late 1920s (Wingard, 1928; McKinney, 1929). Since then, studies on cross protection have focused on the mechanisms involved in this phenomenon and on its practical application for disease control.

The mechanism of cross protection is not fully understood, but it has been a subject of much research, speculation and review (Hamilton, 1980; Fulton, 1982; Zaitlin and Hull, 1987; Sherwood, 1987a; Urban et al., 1989). Many theories exist to explain cross protection, but experiments to test some of them are not yet possible. According to Fulton (1982), part of the difficulty in attempting to explain cross protection may be in looking for a single explanation for what may be a complex of reactions.

The use of cross protection for biological control of plant virus diseases was suggested over 50 years ago (Salaman, 1937; Johnson, 1937), 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 for control (Müller and Costa, 1977; Salibe, 1987; Yeh et al., 1988). Cross protection was also widely applied previously for control of tomato mosaic, caused by tobacco mosaic virus (TMV) (Rast, 1975; Fletcher and Rowe, 1975). Today growers rely primarily on resistant varieties to control the disease (Fulton, 1986). Anxiety about potential problems could explain the scarcity of examples of cross protection being used for biological control of diseases in the field (Urban et al., 1989). These include the protection being overcome by a severe isolate, 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.

Further research on cross protection is necessary to better understand the mechanisms involved in this phenomenon, and to more effectively use it to control virus diseases. The main purpose of this study was to investigate cross protection between two serologically related strains of TMV. The first goal was to study the susceptibility of protoplasts from dark and light green areas from Nicotiana sylvestris Spegaz & Comes infected with the common strain of TMV (TMV-C) to superinfection with the same strain and with a necrotic lesion causing strain. The necrotic lesion causing strain (designated TMV-P) was used earlier in cross protection experiments (Sherwood and Fulton, 1982). The

second objective was to study the susceptibility of dark and light green areas of N. tabacum L. cvs. Samsun and Xanthi, infected with TMV-C, to superinfection with TMV-P. The susceptibility of dark and light green areas produced by TMV-P on both cultivars was also tested using TMV-C as a challenger. Also, studies were made to determine if sap from N. sylvestris systemically infected with TMV-C contained an "antiviral factor" responsible for the resistance of dark green areas to viral infection.

CHAPTER II

LITERATURE REVIEW

Historical background of cross protection

The term cross protection, apparently introduced into the literature by Price (1940), has been widely used to describe the protection between related strains of the same virus. In addition to cross protection, this phenomenon has also been called cross immunization, mutual antagonism, acquired immunity, and preimmunization.

Cross protection was first reported by Wingard (1928). He observed that recovered[1] leaves of tobacco (Nicotiana tabacum L.) systemically infected with tobacco ringspot virus (TobRSV) were "immune" from further symptom development when reinoculated with the same virus.

McKinney (1929) noted that tobacco infected with a strain of tobacco mosaic virus (TMV) causing a light green mosaic did not develop additional symptoms when inoculated with a strain of TMV causing a yellow mosaic. Thung (1931) observed that when tobacco plants infected with a strain of TMV causing a white mosaic were inoculated with the common type strain, they did not develop symptoms of the common

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[1]Recovered leaves are defined as leaves that contain virus, but do not exhibit symptoms.

type. However, plants infected by the common type strain and challenged with the white type strain produced a mosaic suggestive of infection by both strains.

Salaman (1933) found that a mild strain of potato virus X (PVX) protected tobacco or Datura stramonium L. against infection by a severe strain. He also showed that the protection was specific. No protection was observed when PVX infected plants were challenged with potato virus Y (PVY) or TMV. Salaman (1937) later observed the same phenomenon between a mild strain and a severe strain of PVY.

Price (1932) showed that the new growth of tobacco systemically infected with TobRSV had mild or no symptoms and contained less virus than the previously inoculated leaves. Yet, the new leaves were protected from reinfection by most, but not all, strains of TobRSV (Price 1936a; 1936b).

Working with TMV and Nicotiana sylvestris Spegaz & Comes, Kunkel (1934) found that some strains of the virus caused mosaic symptoms, while others caused distinct localized necrotic lesions. Kunkel also found that leaves heavily inoculated with a strain causing mosaic became immune to infection with a local lesion strain, but were not protected against cucumber mosaic virus (CMV) or TobRSV. Attenuated strains of TMV also offered protection against the common or aucuba strains.

Costa and Carvalho (1961), in Brazil, reported that tobacco infected with a super-mild strain of tobacco streak

virus (TSV) did not become infected when reinoculated with severe strains of TSV and had a similar growth to those of healthy plants.

Theories on the mechanism of cross protection

Numerous theories have been proposed to explain the phenomenon of cross protection (Fulton, 1982; and Sherwood, 1987a). Urban et al. (1989) summarized four theories that have been suggested to explain cross protection: 1) utilization and depletion of host metabolites or structures; 2) specific sequestering of the nucleic acid of the challenge virus; 3) involvement of coat protein in inhibition of the infection of the challenge virus and 4) prevention of systemic spread of the challenge virus. They also pointed out that a single mechanism or any combination of each of them may contribute to the events leading to cross protection.

The utilization of a host metabolite or structure was suggested by Ross (1974). He pointed out that the initial virus would utilize the available ribosomes by a rapid increase in mRNA. More ribosomes than normal would bind to viral RNA and become unavailable for the introduced challenge nucleic acid, which would become susceptible to degradation before it could be expressed. According to Fulton (1982), it is difficult to accommodate the demonstrable evidence of specificity of cross protection by

this theory, since it could apply equally well to related or unrelated viruses. Another possible host constituent with a role in cross protection could be a protein that is required as part of the RNA-dependent RNA polymerase of an RNA virus (Ponz and Bruening, 1986). Depletion of this component by the first virus could prevent the challenge virus from replicating.

The specific sequestering of nucleic acid as a mechanism of cross protection was suggested by Palukaitis and Zaitlin (1984). They proposed that for positive-sense (+) RNA viruses, superinfection by the challenge strain would be reduced or prevented by the inhibition of synthesis of its (+) RNA. This could occur when nascent (-) RNA of the challenge strain became hybridized to the excess (+) RNA of the protecting strain. Huss et al. (1989) suggested that this model would explain their results on cross protection between arabis mosaic virus (ArMV) and grapevine fan leaf virus (GFLV) in Chenopodium quinoa Willd. ArMV and GFLV are members of the same sub-group of the nepoviruses, which are serologically unrelated, but have nucleotide sequences in common. If a mechanism is to account for the cross protection that occurs between strains of viroids as well as between strains of viruses it will most likely involve the regulation of replication of nucleic acid. This mechanism, however, would not be applied to explain cross protection between viruses with somewhat unrelated sequences such as sunn-hemp mosaic and tobacco mosaic viruses (Zinnen and

Fulton, 1986; Gibbs, 1986).

The involvement of the viral coat protein in cross protection in nontransgenic plants as well as in protection in transgenic plants has been supported by several experiments. De Zoeten and Fulton (1975) proposed a model to explain cross protection in which the viral RNA of the challenge strain would be encapsidated by the coat protein of the virus already present in the cell. The result of this encapsidation is that the challenger RNA is effectively prevented from replicating. Zaitlin (1976) tested their hypothesis using a coat protein mutant of TMV (PM1), which produces insoluble coat protein that does not encapsidate TMV RNA. When plants inoculated with the mutant were challenge inoculated with TMV (U1), a smaller amount of the U1 strain was recovered than in controls, indicating that protection was achieved. The results also suggested that encapsidation of the challenge RNA was not involved in protection in this system.

Horikoshi *et al*. (1987) suggested that the regulation of replication by the coat protein may be the basis of cross protection. This suggestion is based on experimental evidence that the coat protein of brome mosaic virus, *in vitro*, blocked the binding site of the replicase thereby interfering with RNA synthesis.

Sherwood and Fulton (1982) demonstrated that the specific basis of cross protection with TMV in *N. sylvestris* is the inability of the challenge virus to uncoat when

inoculated onto plants systemically infected with the protecting strain. Plants of N. sylvestris infected with the common strain of TMV develop a mosaic of dark and light green areas (Fulton 1951). When a mosaic leaf is inoculated with strains of TMV that produce necrotic local lesions, the lesions are restricted to the dark green areas of the mosaic (Fulton, 1951; Sherwood and Fulton, 1982). However, when the challenge inoculation is made with RNA of the necrotizing strains of TMV, necrotic lesions are produced in both dark and light green areas. This suggests that the necrotic lesion producing strains are unable to uncoat in the light green areas where the concentration of TMV is greatest. De Zoeten and Gaard (1984) reported that 2 to 7.5 times more TMV antigen was detectable in cell walls of light green areas than in those of dark green areas.

Dodds et al. (1985) found similar results in tomato with two strains of CMV. As with TMV in N. sylvestris, a breakdown of cross protection occurred when the tomato leaves infected with the mild strain (S) were challenged with viral RNA but not with the intact virion of the severe strain (P). However, the breakdown in cross protection, as measured by the presence of dsRNA or intact virion of the challenge strain, was only observed in the challenge inoculated leaves and not in the upper leaves.

Work with coat protein-free mutants of TMV has created certain controversy about the involvement of coat protein in cross protection. Sherwood (1987b) inoculated leaves of

N. sylvestris with the DT-1G coat protein-free mutant of TMV (Sarkar and Smitamana, 1981) or with the common strain of TMV (TMV-C). The leaves were then challenge inoculated with turnip mosaic virus (TuMV) to test non-specific protection, or with a necrotic lesion causing strain of TMV (TMV-N). The leaves inoculated with the coat protein-free strain had equal susceptibility to TuMV and TMV-N. When TMV-C was used as protectant, infection was less with TMV-N than with TuMV.

Gerber and Sarkar (1989), found that N. tabacum cv. Samsun inoculated with the coat protein-free mutant of TMV (DT-1G) showed up to 90% protection against the U1 strain of the same virus. They concluded that the presence of TMV coat protein was not essential for cross protection. Urban et al. (1989) suggested that these conflicting reports support the idea that the mechanism of cross protection may be distinct in different hosts.

Cross protection studies with sunn-hemp mosaic virus (SHMV) and TMV indicated that coat protein may be a factor in cross protection in some situations, but other factors may also be involved (Zinnen and Fulton, 1986). Cowpeas systemically infected with SHMV were completely protected against superinfection by either virion or RNA of a necrotic local lesion causing strain of SHMV, which had been produced by nitrous acid treatment. However, when cowpeas infected with SHMV were challenge inoculated with TMV-C RNA encapsidated in SHMV coat protein, or TMV-C intact virion, the plants challenged with the RNA encapsidated in SHMV coat

protein showed 5-27 time less infection than the TMV RNA encapsidated in TMV coat protein.

The involvement of coat protein in cross protection has also received support from experiments with transgenic plants. Powell Abel et al. (1986) demonstrated that transgenic tobacco plants that expressed the TMV coat protein gene delayed the development of systemic symptoms, when inoculated with TMV, as compared to non-transgenic plants. Loesch-Fries et al. (1987) reported similar results from studies of transgenic tobacco that expressed coat protein gene of alfalfa mosaic virus (AlMV). Plants that expressed the highest concentration of coat protein developed fewer primary infections following inoculation with AlMV and developed systemic infection slower than did plants that did not express coat protein. In both examples, transgenic plants were resistant to infection by virions but susceptible to infection by RNA. The resistance of transgenic plants that express the virus coat protein gene has also been shown in a number of other plant:virus combinations (Tumer et al., 1987; van Dun et al., 1987; Cuozzo et al., 1988; Hemenway et al., 1988; Lawson et al., 1989)

Prevention of systemic spread of the challenge strain as a mechanism of cross protection is supported by the work of Dodds (1982) and Dodds et al. (1985). They tested cross protection between two strains of CMV (S and P) in tomato and showed that the challenge strain was able to increase in

the inoculated leaves but did not move systemically. However, when the protecting strain was not systemically well established, an increase in the challenge strain also occurred in the upper leaves.

Urban et al. (1988) also demonstrated prevention of systemic spread of the challenge strain in tests of cross protection between two serologically distinct strains of TMV in Arabidopsis thaliana (L.) Heynh. They showed that the challenge strain multiplied in the inoculated leaves to concentrations detectable by ELISA, but it did not move systemically in the plant. They also suggested that the impairment of systemic spread of the challenge strain could be related to the interaction of the 30 kDa movement protein with a host component.

Blum et al. (1989) suggested a virus-induced, host specific inhibitor of viral transport. They hypothesized that the challenge virus cannot synthesize its movement protein because the protecting strain induces the host to synthesize an inhibitor of 30 kDa synthesis. They further suggested that if the challenge virus cannot use the movement system of the protecting strain or the protein has already dissipated, the challenge virus will be unable to spread. Additional support for the interaction of the 30 kDa protein with host protein comes from Moser et al. (1988). In N. tabacum cv. Samsun NN infected with TMV, the amount of movement protein in cell wall fractions decreased when necrosis became visible and the production of coat

protein ceased.

Cross protection and virus disease control

Although Salaman (1937) and Johnson (1937) suggested the use of cross protection for disease control over 50 years ago, there are few examples of the use of this technique for control of plant virus diseases. Research has focused on the mechanism of cross protection rather than its application to control virus diseases.

Cacao swollen shoot in Africa (Posnette and Todd, 1955) and passion fruit woodiness in Queensland, Australia (Simmonds, 1959) were the first virus diseases in which plants protected with mild strains showed good development and reduction of yield loss under field conditions. Programs for the control of these diseases by cross protection have not continued, and the diseases are still economically important.

Grant and Costa (1951) demonstrated the use of cross protection to control citrus tristeza virus (CTV) in Brazil. In 1961, Müller and Costa (1977) initiated a research program to utilize cross protection to control CTV in São Paulo State. After many years of field trials, they isolated 70 mild isolates of CTV from vigorous trees in severely infected groves. Of 70 mild isolates, 45 were field tested (Costa and Müller, 1980). Of these, 3 were satisfactory for orange, 2 for Galego lime, and 1 for Ruby

Red grapefruit. There was also a varied relationship between isolate severity and host. Isolates that were mild in Pêra sweet orange or grapefruit tended to be rather severe in Galego lime (Costa and Müller, 1980). In Florida, Cohen and Burnett (1961) also reported the CTV isolates from many declining sweet orange trees on sour orange rootstock induced less severe reaction in Key lime than did isolates from symptomless trees. Thus, isolates of CTV selected on the basis of mildness of symptoms in one citrus species were not necessarily mild in all citrus species.

The work in Brazil was significant because it demonstrated that naturally occurring isolates of CTV varied in their protecting capacity as well as in the symptoms caused. Protected citrus clones have satisfactorily been used in Brazil, Australia, India, Israel, Japan, South Africa and the USA (Hamilton, 1985). By 1987, a total of 50 million Pêra sweet orange trees protected with mild isolates of CTV had been planted in Brazil (Salibe, 1987).

Cross protection was also widely applied for control of tomato mosaic (Rast, 1975; Fletcher and Rowe, 1975; Broadbent, 1976; Channon et al., 1978). However, difficulties have developed in controlling tomato mosaic using cross protection. Mild strains generally protected less effectively at 25-30 C than at lower temperatures. The mild strain MII-16, produced by nitrous acid mutation of strain 1 (Rast, 1972), does not protect tomatoes against all other strains of the virus. In some areas where the MII-16

strain has been used, there has been an increase in the prevalence of strain 1. Presently, growers rely primarily on resistant varieties to control the disease (Fulton, 1986).

The use of cross protection to control papaya ringspot virus (PRV) was discussed by Costa et al. (1978), and has been investigated in Taiwan (Lin, 1980), Brazil (Rezende et al., 1981; Rezende,1985), and Hawaii (Yeh and Gonsalves, 1984; Yeh et al., 1988). A mild strain of PRV, produced by nitrous acid treatment, was used in field experiments in Taiwan. Papaya plants protected by the mild strain had 82% greater fruit yield than unprotected trees, resulting in a 111% increase in grower income. Protection was effective when protected plants were planted in solid blocks, and disease pressure within the test orchard was minimized by roguing severely infected plants once every 10 days up to flowering. However, protection waned under high disease pressure by other strains from areas near the protected trees. Due to the success of these experiments, more than one million papaya seedlings inoculated with the mild strain of PRV were planted in the field in 1986 (Yeh et al., 1988).

In Brazil, in spite of very satisfactory results of protection in greenhouse and field experiments, protected papaya plants in the field showed mild symptoms for only 6-8 months. Afterwards the symptoms increased in severity. Symptom intensification occurred in a synchronized manner in all plants. The change of symptoms was not considered a

breakdown of protection, but rather a change in the mild strains selected from the field due to mutation and selective competition (Rezende and Costa, 1987). The success of cross protection as a method of controlling papaya ringspot in Brazil depends upon finding more stable mild strains.

Although cross protection has been commercially applied to control only two diseases, it may be an effective option for control of many other diseases. Cross protection has been proposed for control of cauliflower mosaic virus in Brussels sprouts (Tomlinson and Shepherd, 1978), CMV in tomato (Dodds, 1982; Yoshida et al., 1985) and pepper (Tien and Chiang, 1983), TMV in sweet pepper (Goto et al., 1984), PVY in tobacco (Latorre and Flores, 1985), tomato aspermy virus in tomato (Kuti and Moline, 1986), and GFLV in grapevines (Huss et al., 1989). Fulton (1986) also suggested that avocado sun blotch, concave gum and psoriasis of citrus, and some stone and pome fruit virus diseases may be effectively controlled by cross protection. Urban et al. (1989) suggested that greater attention to the practical application of cross protection could result in control of more virus diseases. However, the decision to use this method to control virus diseases must be well thought out and precautions should be taken to prevent additional problems (Fulton, 1986).

CHAPTER III

INHIBITOR OF VIRUS INFECTION ASSOCIATED WITH HEALTHY AND TOBACCO MOSAIC VIRUS-INFECTED TISSUES OF *NICOTIANA SYLVESTRIS*

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ABSTRACT

A "dark green agent" has been suspected to be responsible for the low virus content and resistance to virus infection of dark green areas of the mosaic in tobacco mosaic (TMV) infected *Nicotiana* spp. Concentrated extracts from infected and healthy *N. sylvestris* caused inhibition of the infectivity of a TMV strain that produces necrotic localized lesions in *N. sylvestris*. The inhibitory effect of the substance present in healthy and TMV infected tissues was almost completely eliminated when the extract was diluted. Similar inhibition occurred when a concentrated extract of healthy *N. tabacum* cv. Xanthi-nc was used. Attempts to demonstrate the presence of an antiviral factor in tissues of *N. sylvestris* infected with the common strain

of TMV were not successful.

Nicotiana sylvestris Spegaz & Comes infected with the common strain of tobacco mosaic virus (TMV-C) shows a mosaic pattern of distinct dark green areas surrounded by light green areas. The dark green areas contain much less virus than the light green areas (Fulton, 1951; Sherwood, 1981). Dark green areas have also been shown to be cytologically normal and to maintain normal cytological connections with light green areas (Atkinson and Matthews, 1970). Virus-free plants have been regenerated from cells excised from dark green areas demonstrating that virus-free cells may exist in these areas (Murakishi and Carlson, 1976). However, what is responsible for the resistance of dark green areas to viral infection and/or replication as compared to neighboring light green areas is not known.

The existence of a diffusible "dark green agent" has been postulated as responsible for the reduced virus concentration of dark green tissues (Atkinson and Matthews, 1970; Murakishi and Carlson, 1976; Carlson and Murakishi, 1978). Sherwood (1981) tried to demonstrate the presence of a water soluble agent inhibiting viral multiplication in dark green tissue of N. sylvestris, but did not obtain conclusive results. Gera and Loebenstein (1988) found a substance inhibiting virus replication, which they called IGI, associated with the resistance of dark green areas developed in N. tabacum L. cv. Xanthi-nc infected with

cucumber mosaic virus (CMV) strain Price No. 6. The IGI was isolated from protoplasts obtained from dark green areas tissue and directly from green islands in leaf tissue.

The occurrence of antiviral compounds induced in plants as a result of systemic virus infection has been reported by many (Sela and Applebaum, 1962; Sela et al., 1964; Chadha and MacNeill, 1969; Antignus et al., 1971; Miczynski and MacNeill, 1976). Such compounds have been described mainly as factors causing inhibition of virus infectivity. However, in a few instances they have been indicated to be inhibitors of virus multiplication (Sela et al., 1965; Chadha and MacNeill, 1969).

The purpose of this study was to determine if the resistance of dark green areas of TMV-C infected N. sylvestris could be associated with the presence of an "antiviral factor".

MATERIALS AND METHODS

Virus strains and test-plants. Two strains of TMV, TMV-C which produces a systemic mosaic in N. sylvestris, and a petunia strain (TMV-P) which induces the hypersensitive reaction characterized by localized necrotic lesions in N. sylvestris, were used in this study. TMV-C was maintained in N. sylvestris, while TMV-P was propagated in N. tabacum L. cv. Samsun. Purification of the viruses was accomplished as described by Sherwood (1981). Test plants were grown in commercial soil mix in 10 cm plastic pots. They were watered once or twice daily and fertilized weekly with a

solution of commercial fertilizer (15-30-15).

Test for an "antiviral factor". The presence of an antiviral factor in N. sylvestris infected with TMV-C was assayed according to a procedure described by Chadha and MacNeill (1969). Plants of N. sylvestris were mechanically inoculated with a purified preparation of TMV-C (1 mg/ml) at the three leaf stage. Inoculated plants and healthy plants of the same age were kept in the greenhouse for 6-8 more wk. Fifteen grams of leaves from mosaic and healthy plants were harvested and frozen at -20 C overnight. Frozen tissues (1 g/ml) were ground in porcelain mortars in 0.2 M phosphate buffer, pH 6.8. The extracted juices were passed through cheesecloth and then clarified by centrifugation at 12,000 rpm for 20 min at 4 C in a Beckman rotor No. JA-17. Supernatants were then treated with four changes of equal volumes of hydrated calcium phosphate (HCP) prepared according to Fulton (1959). The extracts were then centrifuged for 20 min as before. Fifteen ml of phosphate buffer was similarly treated with HCP as a control. An aliquot of all three solutions was taken after each treatment with HCP and inoculated onto N. tabacum cv Xanthi-nc to test for any possible infectivity still present in the saps. The solutions obtained after the fourth treatment with HCP (approximately 250 ml each) were flash evaporated at 40 C in an Evapotec Rotary Evaporator (Büchi/ Brinkmann Instruments, NY), to a volume equals to 2 ml per 15 g of tissue. The control buffer was similarly treated.

Purified TMV-P was then added to a final concentration of 0.05 mg/ml of flash evaporated solution. The antiviral activity was assessed by inoculating half-leaves of N. sylvestris following a completely randomized design. A cotton swab was used to apply the inoculum and then inoculated plants were covered with a wet paper towel for 12-18 hr. Plants were kept in a greenhouse and localized necrotic lesions produced by TMV-P were counted 5-7 days after inoculation. Data were statistically analyzed and the means were separated by the Student-Newman-Keuls test (Steel and Torrie, 1981).

Test for an inhibitor of virus infectivity. Studies were carried out to assay for an inhibitor of virus infectivity in concentrated extracts from healthy N. sylvestris and N. tabacum cv. Xanthi-nc. Twelve grams of leaves from each species were frozen at -20 C overnight. The tissues were then ground in a mortar with 3 ml of 0.1 M phosphate buffer, pH 6.8. The sap was passed through cheesecloth and then centrifuged at 12,000 rpm for 20 min at 4 C in a Beckman rotor No. JA-17 to remove plant material. The supernatant was saved and portions were diluted to 2:1 and 1:1 in the same phosphate buffer. Purified TMV-P was added to each dilution to a final concentration of 0.02 mg/ml. Phosphate buffer containing the same amount of TMV-P was used as control. The presence of an inhibitor of virus infectivity was tested using the same experimental design for testing an antiviral factor. Counts of localized

lesions were statistically analyzed as described.

RESULTS AND DISCUSSION

Infectious TMV-C was removed from infected tissues of N. sylvestris by four treatments with HCP. This was confirmed by the absence of local lesions produced by extracts inoculated onto N. tabacum cv. Xanthi-nc. HCP binds to plant proteins of high molecular weight and to virus particles.

The production of an antiviral factor in different species of plants as a result of infection by TMV or potato virus Y has been reported by Sela and Applebaum (1962) and Chadha and MacNeill (1969). In the present study, three independent experiments were carried out to test for the presence of an antiviral factor in extracts from mosaic tissue of N. sylvestris. Extracts from TMV-C infected tissues and from healthy tissues of N. sylvestris caused a similar reduction in the average number of necrotic local lesions produced by TMV-P inoculated to N. sylvestris (Table 1). The reduction of infectivity of TMV-P caused by extract from healthy tissues indicates the presence of an inhibitor of virus infection in N. sylvestris.

Extracts from many healthy plants are known to contain substances which inhibit infection by viruses. For example, Phytolacca decandra L. (Allard, 1918) and Spinacia oleracea L. (Grant, 1934) contain substances which, when extracted and mixed with virus inoculum, inhibit infection of such plants as tobacco and N. glutinosa L. These inhibitors,

however, are frequently effective only when the virus is being inoculated to other species (Matthews, 1981). This was not the case for the inhibitor present in extracts from healthy N. sylvestris, since it inhibited infection of the same species of plant. Also, this inhibitor was only detected in a highly concentrated extract from that species (Table 2). As dilution of the extract increased to 2:1 or 1:1, the inhibitory effect of the crude sap was almost completely eliminated. The same inhibitory activity was found in a concentrated extract from healthy N. tabacum cv. Xanthi-nc (Table 2).

Antignus et al. (1971) pointed out that a major problem in assaying crude antiviral factor preparations for antiviral activity is the presence of inhibitory substances other than the antiviral factor present in the plant extract. They also showed that dilution, precipitation with 95% ethanol or ammonium sulfate, or filtration through a membrane were effective in removing inhibitors from TMV infected N. glutinosa and concentrating the antiviral factor. Dilution of the extracts after flash evaporation did not eliminate the effect of the natural inhibitor present in N. sylvestris, which could be masking the activity of an antiviral factor (Table 3). The dilution of the extracts eliminated the effect of the inhibitor as well as the activity of any possible antiviral factor that could have been left over in the extracts from infected tissues. These experiments were not continued due to the lack of

evidence for the occurrence of an antiviral factor in N. sylvestris infected with TMV-C.

TABLE 1. Inhibition of the infectivity of a necrotic lesion producing strain of tobacco mosaic virus (TMV-P) on Nicotiana sylvestris by extracts from leaves of healthy N. sylvestris and leaves of N. sylvestris infected with the common strain of TMV (TMV-C).

Treatment	Lesions per half-leaf[a,b]	Relative infectivity %
Control (buffer)	154.5 a	100.0
Healthy leaf extract	69.6 b	45.0
Infected leaf extract	55.4 b	35.8

[a] Average of 54 half-leaves from 3 independent experiments.

[b] Means followed by the same letter are not significantly different (Student-Newman-Keuls test, P=0.01).

TABLE 2. Reduction of the infectivity of a necrotic lesion causing strain of tobacco mosaic virus (TMV-P) on Nicotiana sylvestris by highly concentrated extracts from healthy leaves of N. sylvestris and N. tabacum cv. Xanthi-nc.

Treatment	Extract from N. sylvestris		Extract from N. tabacum cv. Xanthi-nc	
	Lesions/ half-leaf[a,b]	Relative infec- tivity %	Lesions/ half-leaf[c]	Relative infec- tivity %
Control (buffer)	261.3 a	100.0	221.9 a	100.0
Dilution 1:1 (v:v) (extract:buffer)	228.6 a	87.4	187.5 a	84.5
Dilution 2:1 (v:v) (extract:buffer)	166.5 ab	63.7	175.8 a	79.2
Dilution 4:1 (w:v) (tissue:buffer)	114.1 b	43.6	40.3 b	18.1

[a] Average of 24 half-leaves of N. sylvestris from 3 experiments.

[b] Means followed by the same letter are not significantly different (Student-Newman-Keuls test, P=0.01).

[c] Average of 18 half-leaves of N. sylvestris from 2 experiments.

TABLE 3. Reduction of the infectivity of a necrotic lesion causing strain of tobacco mosaic virus (TMV-P) on Nicotiana sylvestris by highly concentrated extracts from healthy leaves of N. sylvestris and leaves of N. sylvestris infected with the common strain of TMV (TMV-C).

Treatment	Local lesions/half-leaf[a,b]			
	Undiluted[c]	1:2 dilution	1:4 dilution	1:8 dilution
Control (buffer)	163.6 a	184.8 a	238.0 a	183.8 a
Healthy leaf extract	47.8 b	82.8 a	137.1 a	151.1 a
Infected leaf extract	38.5 b	116.8 a	166.0 a	130.0 a

[a] Average of 6 half-leaves N. sylvestris from one experiment.

[b] Means followed by the same letter within a column are not significantly different (Student-Newman-Keuls test, P=0.01).

[c] Extracts obtained after flash evaporation.

CHAPTER IV

SUPERINFECTION OF PROTOPLASTS FROM DARK AND LIGHT GREEN AREAS FROM NICOTIANA SYLVESTRIS INFECTED WITH TOBACCO MOSAIC VIRUS

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ABSTRACT

Dark and light green areas of whole leaves of N. sylvestris systemically infected with the common strain of tobacco mosaic virus (TMV-C) are susceptible and resistant, respectively, to superinfection by a necrotizing strain TMV-P. Protoplasts from dark and light green areas were receptive to the attachment and/or uptake of the ^{32}P labeled TMV-P. Protoplasts from dark and light green areas were also superinfected with virions or RNA from TMV-P as determined by ELISA and an infectivity test. Lower yield of TMV-P was estimated in superinfected protoplasts from light green areas as compared to that in superinfected protoplasts from dark green areas after incubation for 72 hr. Protoplasts from dark green areas could not be superinfected

with the same mosaic causing strain (TMV-C). The breakdown in protection in experiments with isolated protoplasts from light green areas may be a result of the uneven distribution of TMV-C in the leaves.

Strains of TMV have been used for many years to study cross protection in Nicotiana sylvestris Spegaz & Comes (Kunkel, 1934; Fulton, 1951; Sherwood and Fulton, 1982; Sherwood, 1987b; Rezende and Sherwood, 1990). Plants inoculated with the type or common strain of tobacco mosaic virus (TMV-C) develop a mosaic symptom characterized by dark green areas surrounded by a light green background. Other strains of TMV can be characterized by development of necrotic lesions on the inoculated leaves.

Fulton (1951) found that N. sylvestris systemically infected with strains of TMV causing mosaic were superinfected in the dark green areas, but not in the light green areas, by necrotizing strains of TMV. He also found that the inoculation of mosaic leaves with the strain of TMV that caused mosaic did not increase resistance of dark green areas to superinfection by necrotic type strains. From this he concluded that there were no virus free cells in the mosaic leaves and that superinfection occurred in cells already containing a mosaic type strain. The possible occurrence of virus-free cells in a mosaic leaf was demonstrated by Murakishi and Carlson (1976) by regeneration of virus-free plants from leaf pieces excised from dark

green areas of N. tabacum L. cv. White Burley infected with the Vulgare strain of TMV. Sherwood and Fulton (1982) showed that both dark and light green areas of mosaic leaves of N. sylvestris were susceptible to superinfection with the RNA from necrotizing strains of TMV. They concluded that protection in light green areas of N. sylvestris with mosaic could result from the prevention of uncoating of the challenge strains. This conclusion is based on the assumption that when the challenge strain is inoculated as virions it may enter cells of light green areas of the mosaic leaf but is unable to initiate the replicative cycle.

Rezende and Sherwood (1990) found that dark green areas of N. tabacum cvs. Samsun and Xanthi infected with TMV-C were more susceptible than light green areas to superinfection with a serologically related strain of TMV (TMV-P). Plants were similarly susceptible when TMV-P was the protecting strain and TMV-C was used as the challenge. Once the challenge strain replicated in the inoculated areas there was no prevention of systemic movement. These events led to a breakdown in protection. This indicates that protection in this system is related to an early event in the infection process rather than to prevention of systemic movement of the challenge strain.

Plant protoplasts have been used in the study of mechanisms of plant virus infection and replication, and protection between viruses. Although criticisms of the use of protoplasts have been raised (Coutts, 1980), classical

cross protection can be observed using protoplasts (Otsuki and Takebe, 1976; Barker and Harrison, 1978). Protoplasts from transgenic plants expressing the TMV coat protein gene have also been used to study the mechanisms of coat protein mediated protection (Register and Beachy, 1988).

Although cells from light and dark green areas of N. sylvestris systemically infected with TMV have been cultured, protoplasts from these areas have not been used to study the events of cross protection. In this study we investigated the uptake and superinfection of TMV-P in protoplasts from dark and light green areas from N. sylvestris infected with TMV-C. Also, studies were also done to test the susceptibility of protoplasts from dark green areas to superinfection with the same mosaic causing strain.

MATERIALS AND METHODS

Virus strains and purifications. The common strain of TMV (TMV-C) which produces systemic mosaic in N. sylvestris, and a strain originally isolated from petunia (TMV-P) which causes necrotic lesions in N. sylvestris were used in this study (Sherwood and Fulton, 1982). TMV-C was purified from N. sylvestris and TMV-P was purified from N. tabacum cv. Samsun by differential centrifugation as previously described (Sherwood, 1981). Viral nucleic acid (RNA) from both strains was isolated by phenol extraction using the method of Ralph and Berquist (1967). RNA was stored frozen at -70 C in 1% KH_2PO_4.

Plants and growing conditions. Seeds of N. sylvestris were sown in a commercial soil mix in 10 cm plastic pots. After 2-3 wk plants were individually transplanted to the same size pots containing the same commercial soil mix. Plants were grown in the greenhouse at 25-28 C, watered once or twice daily, and fertilized weekly with a commercial fertilizer (15-30-15) dissolved in water. Insects were controlled with Pydrin as needed.

Labeling of virions with ^{32}P. Seeds of tomato (Lycopersicum esculentum Mill. cv. Rutgers) were germinated on moist filter paper in a 10 cm Petri dish. Germinated seeds were transferred to pots of vermiculite that had previously been steamed for 20 min. Pots were placed in a growth chamber (Percival) at 25 C in continuous light at approximately 6000 lux. Seedlings were watered once every day with Hoagland's mineral salt solution (Hoagland, 1920) deficient in phosphorous. Two seedlings with fully expanded opposite leaves were mechanically inoculated with purified TMV-C or TMV-P diluted to 0.2 mg/ml in 0.01M phosphate buffer, pH 7.0. Developing true leaves were removed four to five 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 (Amersham, PBS.13A) in a plastic tube. Distilled water was added to the tube after leaves had taken up the phosphorous solution. Leaves were incubated for 72 hr in the same growth chamber as above. The leaves were

then harvested and the virus was purified. Radioactivity of purified virus was measured in a Quick-count Bioscan (QC 2000). For TMV-C it was 3,077 cpm/μg of virus, while for TMV-P it was 27,181 cpm/μg of virus.

Production of cross absorbed IgG. Antisera against TMV-C and TMV-P were produced by injecting rabbits intramuscularly twice weekly for 5 wk with 1 mg of purified virus with Freund's complete adjuvant. The IgG was isolated from antisera that had microprecipitin titer of 1024. 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. Cross absorbed antisera were individually precipitated with an equal volume of saturated ammonium sulfate, pH 7.2. The IgG fraction of each antiserum was then purified by ion-exchange chromatography using a DEAE-Trisacryl-M column washed with 35 mM NaCL, 25 mM Trisma-Base, pH 8.8. IgG was stored frozen at -20 C. Anti-TMV-C IgG and anti-TMV-P IgG were also conjugated to alkaline phosphatase (Sigma Type VII, P-5521) using glutaraldehyde (Clark and Adams, 1977). Conjugated IgG was stored at 4 C.

Preparation of protoplasts. Fully expanded leaves from 7-8 week-old healthy and TMV-C infected N. sylvestris 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 by immersing them

successively in 70% ethanol for 2 min, then 2 min in 10% sodium hypochlorite (Clorox). They were then rinsed with 3 changes of sterile distilled water. Subsequent steps were conducted in a laminar flow hood. The lower epidermis of the leaves was gently scraped with a wire brush. Scraped leaves were placed in Petri dishes with 25 ml of 13% mannitol for about 30 min. The mannitol solution was then replaced by 25 ml of 13% mannitol containing 1% cellulase (Calbiochem, 219466), 0.25% macerozyme (Calbiochem, 441201), and 0.25% bovine serum albumin (Sigma, A-4503), pH 5.8. Leaves were incubated overnight at room temperature in the dark. Suspensions of protoplasts were filtered through a nylon filter (70 μ mesh) to remove large debris. Filtrates were transferred to 50 ml screw-cap centrifuge tubes and protoplasts were sedimented at 500 rpm, for 4 min in a benchtop IEC HN-S centrifuge (Damon). The supernatant was discarded and protoplasts were resuspended in 4 ml of 13% mannitol. Protoplast suspensions were transferred to 13 X 100 mm sterile glass tubes. The suspensions were underlaid with 2 ml of 24.3% sucrose solution and then centrifuged for 10 min at 500 rpm. Protoplasts collected at the interface were transferred to beakers containing 5 ml of 13% mannitol solution. Protoplasts from healthy tissue, dark green and light green area tissues were adjusted to a concentration of 3×10^5 protoplasts/ml. 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 of protoplasts. Inoculation was done with slight modification of the procedure of Loesch-Fries and Hall (1980). Pellets of 3 X 10^5 protoplasts from healthy tissues (control), dark green and light green area tissues were resuspended in 25 μl of 13% mannitol containing 5 μg of virions (TMV-P or TMV-C). The mixtures were held for a few sec and transferred to glass tubes 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.), pH 5.8. The contents were mixed well and held for 10 sec. One ml of 13% mannitol was added and tubes were incubated for 20 min at room temperature. Protoplasts were sedimented at 500 rpm for 4 min and washed three times in 13% mannitol at room temperature. The same procedure was used to inoculate protoplasts with ^{32}P labeled virions.

Inoculation with viral nucleic acid (RNA) was done by resuspending 3 X 10^5 protoplasts in 10 μl of 1% KH_2PO_4 containing 0.5 μg of RNA from TMV-C or TMV-P. The mixtures were immediately transferred to glass tubes containing 100 μl of 40% PEG prepared as above. The mixtures were held for 10 sec, and then 1 ml of 13% mannitol was added. Protoplasts were incubated on ice for 20 min and then washed once in 13% mannitol. Mock inoculated protoplasts from healthy tissues, dark green and light green area tissues

were used as controls for all experiments.

Culture and sampling of inoculated protoplasts. After washing, protoplasts were resuspended in culture medium (Aoki and Takebe, 1969), containing 13% mannitol and 200 μg/ml of carbenicillin (3 X 10^5/ml). Four ml of protoplast suspension was transferred to a 25 cm^2 polystyrene tissue culture flask (Corning) and incubated for 72 hr at 25 C in continuous light at approximately 2000 lux. Every 12 hr flasks were gently swirled to prevent protoplasts from attaching to the bottom surface of the flasks.

Samples of 600 μl were taken from inoculated and mock inoculated protoplasts at zero, 24, 48, and 72 hr after inoculation. Protoplasts were sedimented in microcentrifuge tubes at 1000 rpm for 5 min in a microfuge (Savant). Protoplasts were resuspended in 600 μl of PBS containing 0.05% Tween (PBS-Tween) and 2% polyvinylpyrrolidone (PVP). Samples were frozen at -20 C for ELISA and infectivity tests.

Fluorescent-antibody staining of infected protoplasts. After incubation for 72 hr in culture medium, protoplasts were prepared for immunofluorescence microscopy. Glass slides were coated with Mayer's egg albumin. One hundred microliters of protoplast suspension was centrifuged at 1500 rpm 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 hr at 36 C, and then washed in PBS for 15 min. One hundred microliters of rabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma, F-0382), diluted 1:100 in PBS, was added to the slides. Slides were incubated in a moist chamber for 2 hr at 30 C and then were washed 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 an Olympus BH-2 microscope with UV.

Detection of virus attachment. 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 X 10^5 protoplasts were resuspended in 1 ml of 13% mannitol. Protoplasts inoculated with unlabeled virions were used as control. Radioactivity of inoculated and control samples was measured in a Quick-count Bioscan (QC 2000) to determine attachment and/or uptake of virions in protoplasts.

Enzyme linked immunosorbent assay (ELISA). The double sandwich ELISA procedure used was similar to that of Clark and Adams (1977). 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. Plates were incubated for two hr

at room temperature. Plates were rinsed 3 times with PBS-Tween. Samples of protoplasts were thawed at room temperature and then added to the plates (100 µl/well). When TMV-C was the challenge strain, samples of protoplasts were thawed, diluted to 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 3 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 for 5 hr at room temperature, plates were rinsed as above and p-nitrophenyl phosphate (Sigma, N-2765) dissolved in diethanolamine substrate buffer, pH 9.8 was added. After incubation for varied times, plates were read in a BIO-TEK-EIA plate reader (BIO-TEK Instrument, Inc, Burlington, VT).

Quantitation 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, 1024 and 2048 ng of virus/ml) were analyzed by a multiple linear regression analysis (Steel and Torrie, 1980). Subtracted absorbance values were then substituted into the regression equation in order to estimate the concentration of TMV-P in each type of protoplasts at different intervals after inoculation. The same procedure was applied to estimate the concentration of TMV-C when it was the challenge strain. The standard concentrations of TMV-C used were 16, 32, 64, 128, 256, 512 and 1024 ng of virus/ml.

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 was then mechanically inoculated on 3 half-leaves of N. sylvestris to test the infectivity of TMV-P produced in superinfeted protoplasts. Lesions were counted 4-5 days after inoculation. When TMV-C was the challenge strain, samples were diluted 1:50 in the same buffer and then inoculated on 3 half-leaves of N. tabacum cv. Xanthi-nc. Lesions were counted 4-5 days after inoculations. Lesions produced by samples from inoculated protoplasts were subtracted from lesions produced by samples from mock inoculated protoplasts in order to determine superinfection by TMV-C.

Quantitation of TMV-C in protoplasts and tissues. Samples of uninoculated protoplasts (3 x 10^5/ml) from dark and light green areas were diluted 1:100 in PBS Tween containing PVP and tested by ELISA. Concentration of TMV-C was estimated using a linear regression equation obtained by plotting the absorbance values from ELISA against standard concentrations of TMV-C (16, 32, 64, 128, 256, 512 and 1024 ng of virus/ml).

Leaf discs were collected from dark and light green areas with a 2 mm diameter cork borer. Discs were individually ground in PBS Tween containing PVP. Samples from dark green areas were diluted 1:6000 to 1:8000, while samples from light green areas were diluted 1:17000 to 1:25000. Samples were individually tested on ELISA and TMV-C concentration was estimated as before.

RESULTS

Attachment of radiolabeled TMV to protoplasts. When protoplasts from 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 (Table 4). The same receptivity was also found when protoplasts from both origins were inoculated with ^{32}P labeled TMV-C (Table 5).

Infection and superinfection of protoplasts. Protoplasts from dark and light green areas from N.

sylvestris systemically infected with TMV-C did not have complete protection against superinfection with TMV-P, but showed different levels of susceptibility to superinfection (Table 6). TMV-P antigen was first detected in protoplasts from dark green areas 24 hr after challenge inoculation and showed a gradual accumulation until 72 hr of incubation. 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 hr for detection of measurable amount of TMV-P antigen in superinfected protoplasts and by a lower estimated yield of TMV-P compared to 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 7). TMV-P antigen was detected in these protoplasts 24 hr after inoculation, and a higher accumulation of TMV-P antigen was found after incubation for 72 hr. 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 hr of incubation. When TMV-P RNA was used as inoculum, an average of 50% of infected protoplasts was found after incubation for 72 hr. The immunofluorescent assay was also used 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 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 was not a limiting factor for the ELISA tests.

Results of infectivity tests on N. sylvestris showed that protoplasts from dark and light green areas superinfected with either TMV-P virions or RNA contained infectious TMV-P progeny based on the necrotic lesions produced on the inoculated leaves (Tables 8 and 9). 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 (Sadasivan, 1940; Sherwood and Fulton, 1983).

Attempts to superinfect protoplasts from dark and light green areas of TMV-C infected N. sylvestris with the same mosaic causing strain (TMV-C) gave inconclusive results. ELISA tests and infectivity assays on N. tabacum cv. Xanthi-nc were not sensitive enough for detection of additional replication of TMV-C.

Concentration of TMV-C in protoplasts and tissues. Quantitative analysis of TMV-C antigen present in samples of protoplasts from dark and light green areas and from leaf pieces of dark and light green areas from N. sylvestris showed that the concentration of virus is not homogeneous (Table 10 and Fig. 1). The concentration of TMV-C in protoplasts from dark green areas varied from 1.017 to 51.870 μg/3 x 10^5 protoplasts, while in protoplasts from light green areas it varied from 6.381 to 155.57 μg/3 x 10^5 protoplasts (Table 10). This wide range in TMV-C concentration in both types of protoplasts results in some protoplasts from light green areas having a virus content similar to that present in protoplasts from dark green areas. The same variability in the concentration of TMV-C was found in tests with leaf discs obtained from dark and light green areas of mosaic leaves of N. sylvestris (Fig. 1). The majority of samples from dark green areas fell within the range of below detectable level (BDL) to 8-9 mg of TMV-C/g of tissue, while samples from light green areas showed concentration of virus varying from BDL to 21 mg/g of tissue. Overlapping concentrations of TMV-C in leaf discs from dark and light green areas were found within the range of BDL to 9 mg of virus/g of tissue. Concentration of TMV-C below detectable level in some leaf discs from dark and light green areas may be attributed to the dilution used for the samples.

DISCUSSION

The experiments on superinfection of protoplasts from dark and light green areas from N. sylvestris indicated that protoplasts from dark and light green areas were receptive to the challenge strain. The challenge virus could also replicate in the protoplasts from dark and light green areas.

The use of radioactively labeled virions to show attachment of virus particles to plant mesophyll protoplasts was reported by Zhuravlev et al. (1975) and Roenhorst et al. (1988). The results of this study suggest that TMV-C infected protoplasts from dark and light green areas were not protected from the challenge strain. 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, since 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. Also, several washings of the protoplasts did not remove the labeled virions.

Our finding of the protection in protoplasts from light green areas from TMV-C infected N. sylvestris is not consistent with that found by Fulton (1951) and Sherwood and Fulton (1982) in whole leaves. They found that light green

areas on whole leaves offered full protection against infection by TMV-P and other necrotic lesion forming strains of TMV. The same type of variation on protection was also found by Barker and Harrison (1978) in studies of cross protection between strains S and E of raspberry ringspot virus (RRV) at the level of whole plants and protoplasts. They found that recovered leaves of N. benthamiana Domin 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 (Register and Beachy, 1988).

An intriguing question from our experiments is what makes protoplasts from light green areas from TMV-C infected N. sylvestris partially protected against superinfection by TMV-P, while light green areas on whole leaves were fully protected against TMV-P. Sherwood and Fulton (1982) found that the resistance of light green areas on whole leaves of N. sylvestris to superinfection by TMV-P and other necrotizing strains of TMV was a result of the prevention of uncoating of the challenge strain. They suggested that the prevention of uncoating might be regulated by the kind and

amount of viral coat protein already present in the cell. This suggestion 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 (Fulton, 1951; Atkinson and Matthews, 1970; Sherwood, 1981). Work with transgenic tobacco plants that express the TMV coat protein gene showed that the degree of resistance to TMV infection was directly related to the amount of coat protein accumulated in the plants (Nejidat and Beachy, 1989). In the present study it was also observed that the average concentration of TMV-C in protoplasts from light green areas was higher than in protoplasts from dark green areas. In addition, it was also found that even within dark and light green areas TMV-C was not homogeneously distributed. Tests with leaf discs revealed that some samples from light green areas had concentration of TMV-C within the range of virus concentration in dark green areas, which are known as being susceptible to superinfection (Fulton, 1951; Sherwood and Fulton, 1982). 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. However, this breakdown in protection can be observed in experiments with isolated protoplasts from light green areas but may not be visibly expressed in light green areas on leaves inoculated by mechanical means. The absence of visible superinfection on 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. Subliminal infections have been found to occur in other host-virus interactions (Zaitlin and Keswani, 1964; Cheo, 1970; Sulzinski and Zaitlin, 1982).

The absence of the cell wall in experiments with protoplasts may be another factor that renders protoplasts more susceptible to superinfection. The cell wall is apparently the first barrier that a virus encounters during the inoculation process and cannot be dismissed in studies of cross protection. Development of techniques for inoculation and culture of isolated cells would open an opportunity to investigate the involvement of the cell wall in the process of adsorption of the challenge strain and subsequent superinfection of previously infected cells.

TABLE 4. Attachment or uptake of a necrotic lesion causing strain of tobacco mosaic virus (TMV-P) labeled with ^{32}P to protoplasts from healthy Nicotiana sylvestris and to protoplasts from dark and light green areas from N. sylvestris infected with the common strain of TMV (TMV-C).

Origin of protoplasts	Fraction	Radioactivity[a]
		cpm/3 x 10^5 protoplasts
Healthy (control)	Protoplasts after 20-min inoculation	1928 ± 512
	Solution from 3rd washing	14 ± 4
Dark green areas	Protoplasts after 20-min inoculation	1574 ± 408
	Solution from 3rd washing	15 ± 3
Light green	Protoplasts after 20-min inoculation	2859 ± 672
	Solution from 3rd washing	17 ± 4

[a] Average of three experiments.

TABLE 5. Attachment or uptake of the common strain of tobacco mosaic virus (TMV-C) labeled with ^{32}P to protoplasts from healthy Nicotiana sylvestris and to protoplasts from dark and light green areas from N. sylvestris infected with TMV-C.

Origin of protoplasts	Fractions	Radioactivity[a]
		cpm/3 x 10^5 protoplasts
Healthy (control)	Protoplasts after 20-min inoculation	3884 ± 818
	Solution from 3rd washing	68 ± 23
Dark green areas	Protoplasts after 20-min inoculation	3285 ± 522
	Solution from 3rd washing	43 ± 15
Light green areas	Protoplasts after 20-min inoculation	2494 ± 1359
	Solution from 3rd washing	43 ± 8

[a] Average of three experiments.

TABLE 6. Superinfection of protoplasts from dark and light green areas from Nicotiana sylvestris infected with the common strain of tobacco mosaic virus (TMV-C) with the necrotic lesion causing strain of TMV (TMV-P).

Origin of protoplasts	Exp.	Yield of TMV-P (μg)/3 X 10^5 protoplasts			
		0 h	24 h	48 h	72 h
Healthy (control)	I	0.000	1.365	1.773	1.824
	II	0.000	1.506	1.713	1.854
	III	0.000	0.465	2.142	1.686
	IV	0.000	1.155	2.412	1.956
	Ave.	0.000	1.122	2.010	1.830
	S.D.	0.000	0.461	0.328	0.111
Dark green areas	I	0.000	0.804	1.080	1.572
	II	0.000	0.882	1.230	1.713
	III	0.000	0.282	0.027	1.233
	IV	0.000	0.264	0.567	1.323
	Ave.	0.000	0.558	0.726	1.460
	S.D.	0.000	0.330	0.545	0.221
Light green areas	I	0.000	0.000	0.330	0.729
	II	0.000	0.000	0.552	0.843
	III	0.000	0.000	0.000	0.000
	IV	0.000	0.000	0.021	0.219
	Ave.	0.000	0.000	0.225	0.448
	S.D.	0.000	0.000	0.264	0.403

TABLE 7. Superinfection of protoplasts from dark and light green areas from Nicotiana sylvestris infected with the common strain of tobacco mosaic virus (TMV-C) with RNA from the necrotic lesion causing strain of TMV (TMV-P).

Origin of protoplasts	Exp.	Yield of TMV-P (μg)/3 X 10^5 protoplasts			
		0 h	24 h	48 h	72 h
Healthy (control)	I	0.000	0.852	1.446	1.692
	II	0.000	0.390	1.029	1.455
	III	0.000	1.467	1.815	2.079
	Ave.	0.000	0.903	1.430	1.742
	S.D.	0.000	0.540	0.393	0.314
Dark green areas	I	0.000	0.306	0.537	1.047
	II	0.000	0.000	0.000	0.351
	III	0.000	1.521	0.990	1.563
	Ave.	0.000	0.609	0.509	0.987
	S.D.	0.000	0.804	0.495	0.608
Light green areas	I	0.000	0.027	0.198	0.573
	II	0.000	0.000	0.000	0.000
	III	0.000	0.444	1.683	1.377
	Ave.	0.000	0.157	0.627	0.650
	S.D	0.000	0.248	0.919	0.691

TABLE 8. 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 virions.

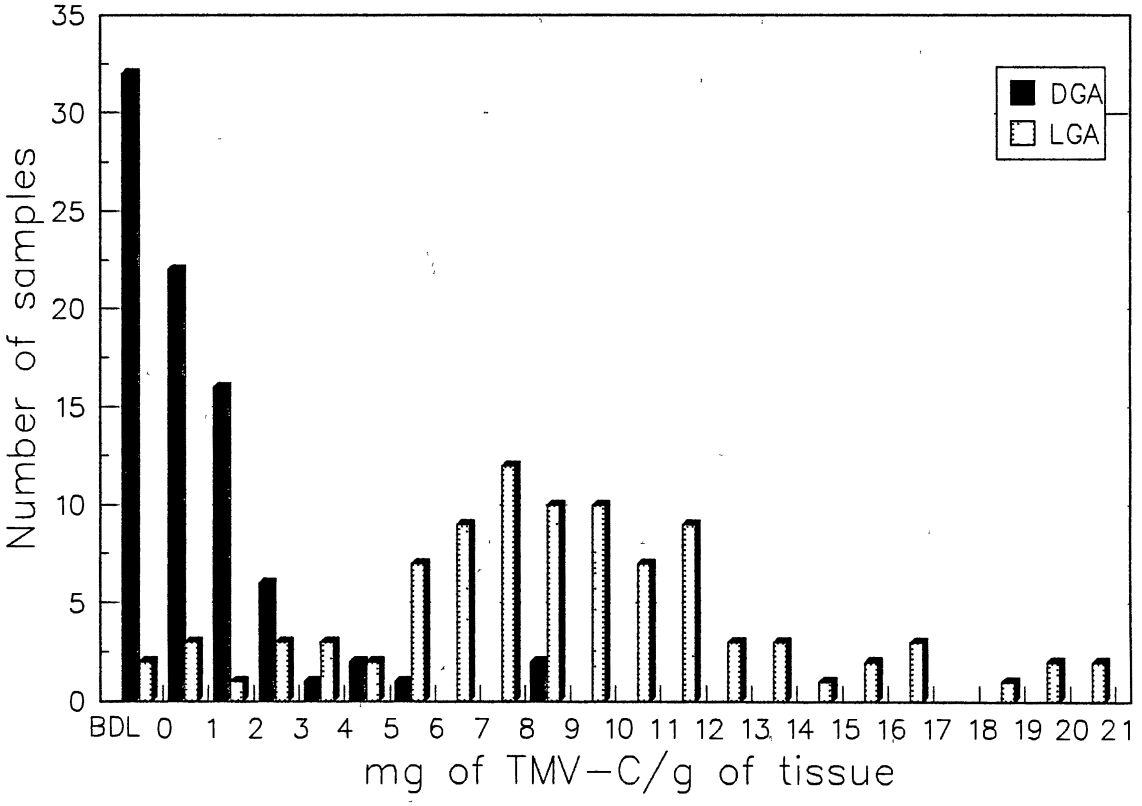
Origin of protoplasts	Exp.	No. of local lesions on 3 half-leaves of Nicotiana sylvestris			
		0 h	24 h	48 h	72 h
Healthy	I	0.0	88.0	431.0	214.0
(control)	II	0.0	85.0	414.0	152.0
	III	0.0	60.0	22.0	150.0
	IV	0.0	151.0	55.0	58.0
	Ave.	0.0	96.0	230.5	143.5
	S.D.	0.0	38.7	222.2	64.3
Dark	I	0.0	24.0	142.0	31.0
green	II	0.0	38.0	104.0	135.0
areas	III	0.0	9.0	35.0	1.0
	IV	0.0	43.0	56.0	9.0
	Ave.	0.0	28.5	84.2	44.0
	S.D.	0.0	15.2	48.1	61.9
Light	I	0.0	0.0	5.0	4.0
green	II	0.0	0.0	4.0	5.0
areas	III	0.0	0.0	0.0	0.0
	IV	0.0	0.0	2.0	0.0
	Ave.	0.0	0.0	2.8	2.3
	S.D.	0.0	0.0	2.2	2.6

TABLE 9. 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 the TMV-P RNA.

Origin of protoplasts	Exp.	No. of local lesions on 3 half-leaves of Nicotiana sylvestris			
		0 h	24 h	48 h	72 h
Healthy (control)	I	0.0	3.0	4.0	17.0
	II	0.0	180.0	233.0	360.0
	III	0.0	155.0	331.0	660.0
	Ave.	0.0	112.6	189.3	345.6
	S.D.	0.0	95.7	167.8	321.7
Dark green areas	I	0.0	0.0	1.0	4.0
	II	0.0	20.0	31.0	67.0
	III	0.0	23.0	48.0	136.0
	Ave.	0.0	14.3	26.6	69.0
	S.D.	0.0	12.5	23.8	66.0
Light green areas	I	0.0	0.0	0.0	1.0
	II	0.0	0.0	3.0	1.0
	III	0.0	12.0	41.0	58.0
	Ave.	0.0	4.0	14.6	20.0
	S.D.	0.0	6.9	22.8	32.9

TABLE 10. Concentration of the common strain of tobacco mosaic virus (TMV-C) in protoplasts from dark and light green areas from Nicotiana sylvestris systemically infected with TMV-C.

Plant group	Concentration in $\mu g/3 \times 10^5$ protoplasts	
	Dark green areas	Light green areas
I	1.017	14.240
II	7.290	34.820
III	51.870	155.570
IV	1.767	6.381



BDL = Below detectable level

FIGURE 1. Concentration of the common strain of tobacco mosaic virus (TMV-C) in leaf discs from dark green areas (DGA) and light green areas (LGA) of Nicotiana sylvestris.

CHAPTER V

SUSCEPTIBILITY OF DARK GREEN AREAS TO SUPERINFECTION LEADS TO BREAKDOWN OF CROSS PROTECTION WITH STRAINS OF TOBACCO MOSAIC VIRUS

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ABSTRACT

Reciprocal cross protection between the common strain of tobacco mosaic virus (TMV-C) and TMV-P (necrotizing strain in N. sylvestris) in plants of N. tabacum. cvs. Samsun and Xanthi was dependent on the concentration of the challenge inoculum. Concentrations of 1 μg/ml or higher of either TMV-P or TMV-C caused complete breakdown of protection in plants infected with the other virus. The susceptibility to superinfection of dark green areas on mosaic leaves of cvs. Samsun and Xanthi infected with TMV-C or TMV-P was apparently responsible for the majority of breakdown in protection. Dark green areas were much more susceptible to superinfection by virions than light green areas, and challenge inoculation with TMV-P RNA dramatically overcame the resistance to superinfection of light green

areas of TMV-C infected cvs. Samsun and Xanthi. Systemic superinfection by the challenge strain occurred in all plants in which superinfection was detected in either dark or light green areas. Dark and light green areas were equally susceptible to superinfection with tobacco etch virus. Uneven concentration of virus in dark and light green areas was suggested as an apparent cause for breakdown in protection between TMV-C and TMV-P.

Cross protection is a phenomenon in which plants infected with one strain of a virus are protected from the effects of subsequent infection by related strains (Wingard, 1928; McKinney, 1929). The mechanism(s) of cross protection has been the subject of much research, speculation and review (Fulton, 1982; Hamilton, 1980; Ponz and Bruening, 1986; Zaitlin and Hull, 1987; Sherwood, 1987a; Urban et al., 1989). Since the discovery of this phenomenon, cross protection has been used to establish relationships among viruses and for controlling diseases such as citrus tristeza (Müller and Costa, 1977; Costa and Müller, 1980) and papaya ringspot (Yeh et al., 1988). However, the phenomena that control the outcome of the interaction between virus strains in the host have not been fully explained.

Cross protection has also been used for control of tomato mosaic caused by tobacco mosaic virus (TMV) (Rast, 1975; Fletcher and Rowe, 1975). However, due to breakdown in protection growers now rely primarily on resistant

varieties to control the disease (Fulton, 1986). The occurrence of breakdown in protection has also been reported for other host-virus combinations. Holmes (1934) selected a masked strain of TMV for protection against the severe strain by culturing TMV infected tomato stem tissue at about 35 C. He found that when the masked strain was introduced about one wk before inoculation of the severe strain, the masked strain interfered with the movement of the severe strain, but a complete protection was not achieved.

Price (1936b) found that tobacco "ringspot no. 1" did not protect Nicotiana tabacum L. cv. Turkish and N. sylvestris Spegaz & Comes against yellow ringspot, although yellow ringspot did protect completely against "ringspot no. 1". Bald (1948) superinfected potatoes carrying a masked strain of PVX by inoculating a severe strain to the top leaves. Inoculation of the severe strain to the lower leaves rarely produced severe symptoms. Bawden and Kassanis (1951) described failures of cross protection by potato viruses thought to be closely serologically related.

Fulton (1951) found that mosaic leaves of N. sylvestris infected with TMV were susceptible to superinfection in dark green areas but not in light green areas when challenge inoculated with strains of TMV that cause localized necrotic lesions in that host. Since tissue of dark green areas contain much less virus than the light green areas, it was suggested that superinfection was related to the concentration of the mosaic causing strain in the leaves.

Fulton (1978) also reported superinfection between strains of tobacco streak virus (TSV) in recovered leaves of tobacco, but did not find evidence relating superinfection with low amount of the protecting virus in recovered leaves of the plants. Recovered leaves of TSV infected plants contained as much virus or more virus than symptomatic leaves of the same plants (Fulton, 1949).

The use of mild or mutant strains of virus as immunizing agents in commercial crops commonly provides only incomplete or partial protection (Fletcher and Rowe, 1975; Cassells and Herrick, 1977; Bar-Joseph, 1978; Burgyán and Gáborjányi, 1984; Yeh et al., 1988). Fulton (1978) suggested that superinfection in these cases may result from: 1) the protecting mild strain may not completely invade the plant; 2) it 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 demonstrated.

In this study we investigated the factor(s) that leads to breakdown of cross protection between strains of TMV in N. tabacum cvs. Samsun and Xanthi.

MATERIALS AND METHODS

Viruses and purifications. Two strains of TMV and tobacco etch virus (TEV) were used in this study. The common strain of TMV (TMV-C) was propagated in N. sylvestris

and a strain originally isolated from petunia (TMV-P) (Sherwood and Fulton, 1982) was maintained in N. tabacum cv. Samsun. TEV was propagated in N. tabacum cv. Samsun. Both strains of TMV were purified by differential centrifugation (Sherwood, 1981). TEV was purified following the procedure described by Purcifull and Hiebert (1982). One hundred grams of TEV infected tobacco leaves were homogenized in 150 ml of 20 mM [N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid] (HEPES), pH 7.5 containing 0.1% sodium sulfate and 21 ml of n-butanol. The suspension was filtered through cheesecloth and then centrifuged at 5000 rpm for 10 min at 4 C in a Beckman rotor JA-14. The virus was precipitated from the supernatant by adding Triton X-100 to 1% (v/v), polyethylene glycol (PEG, MW 8000) to 4% (w/v) and NaCl to 100 mM and stirring for 1 hr at 4 C. The mixture was centrifuged at 8000 rpm for 10 min at 4 C in the same rotor as before. The pellet was resuspended in 50 ml of 20 mM HEPES, pH 7.5 and then centrifuged at 8000 rpm for 10 min at 4 C. The virus was precipitated from the supernatant fluid by adding PEG to 8% and NaCl to 100 mM and stirring for 1 hr at 4 C. The solution was centrifuged at 8000 rpm for 15 min at 4 C in a Beckman rotor JA-17. The pellet was resuspended in 5 ml of 20 mM HEPES, pH 7.5. The concentration of TEV was determined based on absorbance at 260 nm (A_{260} = 2.4 = 1 mg/ml).

Viral nucleic acid (RNA) from both strains of TMV was isolated by phenol extraction using the method of Ralph and

Berquist (1967). RNA was stored frozen at -70 C in 1% KH_2PO_4, pH 7.0.

Test-plants and growing conditions. N. tabacum cvs. Samsun and Xanthi and N. sylvestris were used in these experiments. Seeds were sown in a commercial soil mix in 10 cm plastic pots. After 2-3 wk plants were individually transplanted to the same size pots with commercial soil mix. Plants were grown in the greenhouse at 25-28 C. Nutrients were supplied weekly with commercial fertilizer (15-30-15). Insects were controlled with Pydrin as required.

Production of cross absorbed IgG. Antisera against TMV-C and TMV-P were produced in rabbits by intramuscular injection of purified virus with Freund's complete adjuvant. Injections of 1 mg were given twice weekly for 5 wk. The IgG was isolated from antisera that had a microprecipitin titer of 1024. Antiserum to TMV-C was cross absorbed with an equal volume of sap containing TMV-P, diluted 1:10 in phosphate buffered saline (PBS). Antiserum to TMV-P was cross absorbed with TMV-C antigen as above. Cross absorbed antisera were individually precipitated with an equal volume of saturated ammonium sulfate, pH 7.2. The IgG fraction of each antiserum was then purified by ion-exchange chromatography using a DEAE-Trisacryl-M column washed with 35 mM NaCl, 25 mM Trisma-Base, pH 8.8. IgG was stored frozen at -20 C. Anti-TMV-C IgG and anti-TMV-P IgG were conjugated to alkaline phosphatase (Sigma Type VII, P-5521) using glutaraldehyde (Clark and Adams, 1977). Conjugated

IgG was stored at 4 C.

Antiserum against TEV was obtained from the American Type Culture Collection (PVAS 69).

Challenge inoculation on the entire leaf surface. Plants of N. tabacum cvs. Samsun and Xanthi were mechanically inoculated with 1 μg/ml of purified TMV-C or TMV-P at the two-leaf stage. The inoculum was prepared in 0.01 M phosphate buffer, pH 7.0. Two to three wk later plants systemically infected with TMV-C were challenge inoculated with TMV-P, and vice-versa. Different concentrations of the challenge strain (0.01, 0.1, 1, 5, 25 and 50 μg/ml) were used. The challenge inoculum was prepared in the same buffer and applied to the two upper fully expanded leaves of protected plants. For each test, two healthy plants of the same age were used as control for the challenge inoculation, while two plants inoculated with the protective strain were not challenged. Samples were collected from challenge inoculated leaves and upper leaves of the plants 12 days after the challenge inoculation. Each sample consisted of two 7 mm-leaf discs taken randomly from the leaves. These and other samples were obtained with an appropriate diameter cork borer. All samples were individually ground in PBS containing 0.05% Tween (PBS-Tween) and 2% polyvinylpyrrolidone (PVP), diluted 1:100. The presence of the challenge strain in each sample was tested by double antibody sandwich ELISA. An infectivity test on N. sylvestris was carried out for all experiments in

which TMV-P was used as the challenge strain. The upper leaves of the plants were retested for the challenge strain 25 days after the challenge inoculation.

Challenge inoculation on dark and light green areas. Plants of N. tabacum cvs. Samsun and Xanthi were inoculated with TMV-C or TMV-P as before. Two to three wk later plants systemically infected with TMV-C or TMV-P were separated into three 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. The third set 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 well developed upper leaves were marked with a circle about 1 cm in diameter (3 areas/leaf). The same number of circles were marked on the two developed upper leaves of the healthy plants. Plants infected with TMV-C were challenge inoculated with TMV-P and vice-versa. The challenge inoculum was prepared in 0.01 M phosphate buffer, pH 7.0 at the concentration of 1 μg/ml. The challenge strain was mechanically applied within the marked circles with a cotton swab. Plants were maintained in the greenhouse at 25-28 C. 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 discs. A total of 4 samples were obtained from each test plant. Samples were individually ground in PBS Tween containing PVP

and then diluted 1:100. The presence of the challenge strain in the samples was tested by the double antibody sandwich ELISA. Infectivity tests on N. sylvestris was carried out for all experiments in which TMV-P was the challenge strain. The presence of the challenge strain in the upper leaves of the test plants was retested 25 days after the challenge inoculation. 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 (Steel and Torrie, 1981).

Challenge inoculations with TMV-P RNA were carried out using the same experimental design. The inoculum (50 μg of RNA/ml) was prepared in 1% KH_2PO_4, pH 7.0. Sampling, evaluation of superinfection and statistical analysis were done as before.

Protection to an unrelated virus. Plants of N. tabacum cvs. Samsun and Xanthi were inoculated with 1 μg/ml of purified TMV-C. Two to three wk later plants were challenge inoculated on dark and light green areas with 1 μg/ml of TEV, using the same experimental design described before. TEV inoculum was prepared in 0.01 M phosphate buffer, pH 7.0. Samples were collected as before and leaf discs were ground in PBS-Tween, diluted 1:50. Superinfection with TEV was evaluated by protein-A sandwich ELISA. Data of systemic superinfection were statistically analyzed as before.

Enzyme linked immunosorbent assay (ELISA) procedures. The double antibody sandwich ELISA procedure used to test

superinfection with TMV-P or TMV-C was similar to that of Clark and Adams (1977). 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. Plates were incubated for two hr at room temperature and then rinsed 3 times with PBS-Tween. Samples diluted 1:100 in PBS-Tween containing 2% PVP were added to the plates (100 μl/well). Plates were incubated overnight at 4 C and then rinsed 3 times as before. 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 for 5 hr at room temperature, plates were rinsed as before and 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 (BIO-TEK Instrument, Inc, Burlington, VT).

The protein-A sandwich ELISA was used to test superinfection by TEV. ELISA plates were coated with 1 μg/ml protein A (Sigma P-6650) diluted in 0.05 M carbonate buffer, pH 9.6. Plates were incubated for 2 hr at room temperature. Plates were washed 3 times with PBS-Tween. TEV antiserum diluted 1:500 in PBS-Tween was added to the plates (100 μl/well). After incubation for 2 hr at room temperature, plates were washed as before. Samples diluted 1:100 in PBS-Tween were added to the plates. Plates were incubated at 4 C overnight and then washed 3 times with PBS-

Tween. TEV antiserum diluted 1:500 in PBS-Tween was added and plates were incubated at room temperature for 2 hr. Plates were washed with PBS-Tween. Alkaline phosphatase labeled protein-A (Sigma, P-9650) diluted 1:500 was added. After incubation at room temperature for 2 hr plates were washed 3 times with PBS-Tween and p-nitrophenyl phosphate dissolved in diethanolamine substrate buffer, pH 9.8 was added. Plates were read as before.

Infectivity tests. Infectivity tests on N. sylvestris were carried out for all cross protection experiments in which TMV-P was the challenge strain. An aliquot from all samples tested by ELISA was mechanically inoculated on 2 half-leaves of N. sylvestris. Lesions were counted 4-5 days after inoculation.

Quantitation of TMV-C. Two mm leaf discs were collected from dark and light green areas and individually ground in PBS-Tween containing 2% PVP. To assure the absorbance value from the sample in ELISA would be in the range of the standard curve, samples from dark green areas were diluted 1:6000 to 1:8000 and samples from light green areas were diluted 1:17000 to 1:25000. The concentration of TMV-C was estimated using a regression equation obtained with the absorbance values of standard concentrations of TMV-C (16, 32, 64, 128, 256, 512 and 1024 ng of virus/ml).

RESULTS

Superinfection of plants by challenge inoculation on the entire leaf surface. All plants of N. tabacum cvs. Samsun and Xanthi systemically infected with TMV-C and challenge inoculated with 1, 5, 25 or 50 μg of TMV-P/ml were susceptible to superinfection (Table 11). 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. When plants were inoculated with TMV-P at 0.1 or 0.01 μg/ml only part of the challenge inoculated plants were superinfected on the inoculated leaves as well as systemically. The susceptibility of cvs. Samsun and Xanthi systemically infected with TMV-P to superinfection with TMV-C were similar to those presented before (Table 12). ELISA tests showed that plants challenge inoculated with 1, 5, 25 or 50 μg of TMV-C/ml were not protected against local and systemic superinfection. On the other hand, partial or complete protection was found when the concentration of TMV-C was reduced to 0.1 or 0.01 μg/ml. 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 cvs. Samsun and Xanthi systemically infected with TMV-C showed different susceptibility to superinfection with TMV-P (Table 13). Results of ELISA and infectivity tests on N. sylvestris showed that TMV-P superinfected 67% and 62% of the

inoculated dark green areas of cvs. Samsun and Xanthi, respectively. Superinfection by TMV-P was not detected in inoculated light green areas of cv. Samsun, but it was found in 33% of the inoculated light green areas of cv. Xanthi. Systemic superinfection of cvs. Samsun and Xanthi was directly related to superinfection of dark and light green areas. The number of plants of cvs. Samsun and Xanthi superinfected systemically by TMV-P was greater when the challenge inoculum was applied to the dark green areas then when applied to the light green areas (Table 14). The same difference on the susceptibility of dark and light green areas to superinfection occurred when plants of cvs. Samsun and Xanthi systemically infected with TMV-P were challenged with TMV-C (Table 15). Results of ELISA tests showed that superinfection by TMV-C occurred in 79% and 67% of inoculated dark green areas of cvs. Samsun and Xanthi, respectively. On the other hand, TMV-C was detected in only 9% of inoculated light green areas of cv. Samsun and 11% of inoculated light green areas of cv. Xanthi. Systemic superinfection with TMV-C was also directly related to superinfection of dark or light green areas (Table 16). Delay in detection of the challenge strain in the upper leaves of the plants was observed in a few cases (Table 14 and 16).

Dark and light green areas of cvs. Samsun and Xanthi systemically infected with TMV-C were more susceptible to superinfection with the RNA from TMV-P than

with the intact virion (Table 17). All dark green areas of cvs. Samsun and Xanthi challenge inoculated with TMV-P RNA were superinfected 12 days after challenge inoculation. Fifty nine percent of light green areas of cv. Samsun and 55% of light green areas of cv. Xanthi were superinfected when inoculated with RNA from TMV-P. All plants of cvs. Samsun and Xanthi challenge inoculated on dark green areas with TMV-P RNA were systemically superinfected 12 days after inoculation (Table 18). When the TMV-P RNA challenge inoculum was applied to light green areas 50% of plants of cv. Samsun and 71% of plants of cv. Xanthi were systemically invaded by TMV-P.

Susceptibility of dark and light green areas to superinfection with TEV. Dark and light green areas of N. tabacum cvs. Samsun and Xanthi infected with TMV-C were equally susceptible to superinfection with an unrelated virus (Table 19). TEV was also found in the upper leaves of all plants challenge inoculated on dark and light green areas, 12 days after challenge inoculation (Table 20).

Concentration of TMV-C in tissues. ELISA data showed the concentration of TMV-C in leaf discs of dark and light green areas from N. tabacum cvs. Samsun and Xanthi was not homogeneous (Fig. 2 and 3). The concentration of TMV-C in dark green areas of cv. Samsun varied from below detectable level (BDL) to 10-11 mg of virus/g of tissue (Fig. 2). Thirty one percent of samples taken from dark green areas of cv. Samsun fell BDL while 35% fell within the range of

0-1 mg of TMV-C/g of tissue. The concentration of TMV-C in light green areas of cv. Samsun ranged from 1-13 mg of virus/g of tissue, with the majority of samples falling between 6-11 mg of virus/g of tissue. Dark green areas of cv. Xanthi showed concentrations of TMV-C varying from BDL to 6-7 mg of virus/g of tissue (Fig. 3). In this cultivar, 22% of the samples from dark green areas had TMV-C concentration below detectable level while 37% of the samples fell within the range of 0-2 mg of virus/g of tissue. The concentration of TMV-C in light green areas of cv. Xanthi varied from 2-15 mg of virus/g of tissue, with 78% of the samples showing concentration of virus between 5-9 mg/g of tissue.

DISCUSSION

TMV-C and TMV-P are two serologically related strains that showed cross protection in plants of N. sylvestris (Fulton, 1951; Sherwood and Fulton, 1982) and Arabidopsis thaliana (L.) Heynh. (Urban et al., 1988). Reciprocal cross protection tests between these strains showed that TMV-C and TMV-P also protected against each other in plants of N. tabacum cvs. Samsun and Xanthi. This protection, however, was found to be dependent on the concentration of the challenge strain inoculum. Complete or partial protection was observed when plants of cvs. Samsun and Xanthi systemically infected with one strain was challenge inoculated on to the entire surface of 2 leaves with 0.01 or 0.1 μg/ml of the other (challenge) strain. However, when

the concentration of the challenge strain was increased to 1 μg/ml or higher, breakdown in protection was found for all tested plants. This breakdown in protection was detected by means of serology and local lesion assay (when TMV-P was the challenge strain), since no change in the severity of the symptoms was noticed in doubly infected plants. Cassells and Herrick (1977) found that tomato plants systemically infected with a mild strain of TMV were superinfected by a severe strain, and that the development of severe symptoms was dependent on the concentration of the severe challenge inoculum. In plants challenged with low concentration of the challenge strain (2 μg/ml), severe strain antigen was detected in the upper leaves although severe strain symptoms did not develop over the period of observation. They suggested that for this host-virus system protection was apparently overcome as a consequence of the greater productivity and faster rate movement of the severe strain. Dodds et al. (1985) on the other hand found that a mild strain of cucumber mosaic virus (CMV-S) completely protected plants of tobacco, tomato, and squash from the effects of a more severe strain (CMV-P), and also prevented the accumulation of virions and ds RNAs of the challenge strain in the upper leaves of the plants.

The presence of dark green areas on mosaic leaves of cvs. Samsun and Xanthi infected with TMV-C or TMV-P was found in subsequent experiments to be apparently responsible for the majority of breakdown in protection observed before.

Experiments of challenge inoculation on dark or light green areas showed that dark green areas were much more susceptible to superinfection by the challenge strain than light green areas. Studies on cross protection between strains of TMV in N. sylvestris also showed that dark green areas were more susceptible than light green areas to superinfection by necrotizing strains, but resistant to superinfection by the same mosaic causing strain of TMV (Fulton, 1951; Sherwood and Fulton, 1982). On the other hand, resistance of dark green areas to superinfection by a virus has also been reported for other host-virus combinations. Reid and Matthews (1966) reported that dark green areas in Chinese cabbage (Brassica pekinensis Rupr. cv. Wong Bok) infected with turnip yellow mosaic virus (TYMV) were apparently resistant to reinfection by the same virus. Loebenstein et al. (1977) found that dark green areas developed in N. tabacum cvs. Xanthi-nc and White Burley following inoculation with cucumber mosaic virus (CMV) Price No. 6 were resistant to reinfection with three strains of CMV, but not to infection with TMV.

The N. sylvestris-TMV system used by Fulton (1951) and Sherwood and Fulton (1982) to study cross protection between strains of TMV had the disadvantage that it did not allow any further observation on the 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 since TMV-C and TMV-P systemically invade cvs. Samsun and Xanthi. Our results showed that systemic invasion by the challenge strain occurred for all plants of cvs. Samsun and Xanthi in which superinfection was detected in either dark or light green areas. These results suggest that protection in this system was apparently related to an early event in the infection process, rather than to prevention of systemic movement of the challenge strain. Urban et al. (1988), on the other hand, reported that cross protection between TMV-C and TMV-P in A. thaliana cv. 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. Urban et al. (1989) pointed out that the impairment of systemic movement of the challenge strain in A. thaliana may be due to an interaction of the 30 kDa movement protein with a host component. Since both studies used the same strains of TMV and similar experimental procedure to evaluate superinfection it can be inferred that the host played an important role in the systemic movement of the challenge strain.

The resistance of light green areas of cvs. Samsun and Xanthi to superinfection was specific for strains of TMV. Both dark and light green areas of cvs. Samsun and Xanthi

systemically infected with TMV-C were susceptible to superinfection with TEV. Also, infection with TMV-C did not affect the systemic movement of TEV in all challenged plants. Strain specificity is a characteristic of cross protection and it was reported in several other cases (Price, 1936b; Fulton, 1951; Zaitlin, 1976; Sherwood and Fulton, 1982).

Challenge inoculation with the nucleic acid (RNA) from TMV-P dramatically overcame the resistance of light green areas of cvs. Samsun and Xanthi to superinfection. Also, 100% of the inoculated dark green areas on both cultivars were superinfected when TMV-P RNA was used as inoculum. 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 found by Sherwood and Fulton (1982) as responsible for the resistance of light green areas of TMV-C infected N. sylvestris to superinfection with necrotic lesions causing strains of TMV, including TMV-P. Superinfection of plants following inoculation with the virus RNA was also reported by Dodds et al. (1985) for studies of cross protection between strains of CMV. Since not all plants of cvs. Samsun and Xanthi challenged on light green areas were superinfected with TMV-P RNA, it is suggested that other factor(s) may be responsible for their resistance to superinfection. All plants of cvs. Samsun and Xanthi superinfected in dark or light green areas following

inoculation with RNA were systemically invaded by the TMV-P.

The average virus concentration in dark green areas was much lower than in light green areas in TMV-C infected plants. Similar differences were also reported for N. sylvestris plants infected with TMV (Fulton, 1951; Atkinson and Matthews, 1970; Sherwood, 1981), Chinese cabbage infected with TYMV (Reid and Matthews, 1966) and N. tabacum cvs. Xanthi-nc and White Burley infected with CMV (Loebenstein et al., 1977). However, when samples of dark and light green from cvs. Samsun and Xanthi were individually analyzed it was observed that the concentration of TMV-C varied in both types of tissue. Variability of TMV-C concentration within dark and light green areas was also found in N. sylvestris plants infected with TMV-C (Rezende and Sherwood, this thesis, chap. IV). Since the highest susceptibility to superinfection was found in dark green areas, which had the greatest number of samples with very low virus concentration, it is proposed that superinfection may be related to the amount of virus present in the leaves. The lower number of light green areas superinfected by the challenge strain would be due to the smaller number of areas with a concentration of virus that allows superinfection to occur. This apparent relationship between virus concentration and superinfection suggests that there must be a limiting concentration of the protecting strain necessary in both dark and light green areas for complete protection to occur. Below that limit both types

of tissues are susceptible to superinfection. Work with isolated protoplasts from light green areas of N. sylvestris infected with TMV-C showed that they were partially susceptible to superinfection with TMV-P (Rezende and Sherwood, this thesis, chap. IV). Based on quantitative analysis of TMV-C antigen present in protoplasts and leaf discs from dark and light green areas of N. sylvestris Rezende and Sherwood suggested that susceptibility in this case might also be related to the uneven distribution of TMV-C in the leaves. Work with transgenic tobacco plants that express the TMV coat protein gene showed that the degree of resistance to TMV infection was directly related to the amount of coat protein accumulated in the plants (Nejidat and Beachy, 1989).

Development of techniques to increase the virus concentration in dark green areas would permit further investigation on the involvement of virus concentration on superinfection by the challenge strain. Also, challenge inoculation on dark and light green areas previously analyzed for virus concentration would allow studies to verify if there is a limiting amount of the protecting strain necessary for protection to occur.

TABLE 11. Susceptibility of Nicotiana tabacum cvs. Samsun and Xanthi systemically infected with the common strain of tobacco mosaic virus (TMV-C) to superinfection with different concentrations of a strain of TMV (TMV-P) that causes necrotic lesions on N. sylvestris.

Cultivar	Concentration of TMV-P (μg/ml)	No. of plants super-/No. of challenged plants infected[a]	
		Inoculated leaves	Upper leaves
Samsun	0.01	0/7	3/7
	0.1	5/9	7/9
	1.0	12/12	12/12
	5.0	3/3	3/3
	25.0	3/3	3/3
	50.0	6/6	6/6
Xanthi	0.01	1/7	3/7
	0.1	5/9	7/9
	1.0	9/9	9/9

[a] Based on ELISA and infectivity tests on N. sylvestris.

TABLE 12. Susceptibility of Nicotiana tabacum cvs. Samsun and Xanthi systemically infected with a strain of tobacco mosaic virus (TMV-P) that causes necrotic lesions on N. sylvestris to superinfection with different concentrations of the common strain of TMV (TMV-C).

Cultivar	Concentration of TMV-C (μg/ml)	No. of plants super-/No. of challenged plants infected[a]	
		Inoculated leaves	Upper leaves
Samsun	0.01	1/6	1/6
	0.1	4/6	4/6
	1.0	6/6	6/6
	5.0	3/3	3/3
	25.0	3/3	3/3
	50.0	6/6	6/6
Xanthi	0.01	0/6	0/6
	0.1	4/6	4/6
	1.0	3/3	3/3

[a] Based on ELISA tests.

TABLE 13. Susceptibility of dark and light green areas of Nicotiana tabacum cvs. Samsun and Xanthi systemically infected with the common strain of tobacco mosaic virus (TMV-C) to superinfection with a strain of TMV (TMV-P) that produces necrotic lesions on N. sylvestris.

Cultivar	No. of areas super-/No. of challenge inoculated areas[a]		
	Healthy (control)	Dark green areas	Light green areas
Samsun	19/24	30/45	0/60
Xanthi	18/21	24/39	15/45

[a] Each sample contained leaf discs from 2 independently inoculated areas.

TABLE 14. Susceptibility of Nicotiana tabacum cvs. Samsun and Xanthi systemically infected with the common strain of tobacco mosaic virus (TMV-C) to systemic superinfection with a strain of TMV (TMV-P) that produces necrotic lesions on N. sylvestris challenge inoculated on dark and light green areas separately.

Cultivar	Days after challenge inoculation	No. of plants super-/No. of challenged plants infected systemically[a]		
		Healthy (control)	Dark green areas	Light green areas
Samsun	12	7/8 a	13/15 a	0/20 b
	25	7/8 a	13/15 a	0/20 b
Xanthi	12	7/7 a	10/13 b	2/15 c
	25	7/7 a	11/13 b	5/15 c

[a] Means followed by the same letter in the row are not significantly different (Two samples comparison for proportion, P=0.05).

TABLE 15. Susceptibility of dark and light green areas of *Nicotiana tabacum* cvs. Samsun and Xanthi systemically infected with a strain of tobacco mosaic virus (TMV-P) that causes necrotic lesions on *N. sylvestris* to superinfection with the common strain of TMV (TMV-C).

Cultivar	No. of areas super-/No. of challenge inoculated areas[a]		
	Healthy (control)	Dark green areas	Light green areas
Samsun	4/12	19/24	3/33
Xanthi	9/18	22/33	4/36

[a] Each sample contained leaf discs from 2 independently inoculated areas.

TABLE 16. Susceptibility of Nicotiana tabacum cvs. Samsun and Xanthi systemically infected with a strain of tobacco mosaic virus (TMV-P) that produces necrotic lesions on N. sylvestris to systemic superinfection with the common strain of TMV (TMV-C) challenge inoculated on dark and light green areas separately.

Cultivar	Days after challenge inoculation	No. of plants super-/No. of challenged plants infected systemically[a]		
		Healthy (control)	Dark green areas	Light green areas
Samsun	12	3/4 a	7/8 a	3/11 b
	25	3/4 a	7/8 a	4/11 b
Xanthi	12	5/6 a	9/11 a	1/12 b
	25	6/6 a	9/11 a	4/12 b

[a] Means followed by the same letter in the row are not significantly different (Two samples comparison for proportion, P=0.05).

TABLE 17. Susceptibility of dark and light green areas of *Nicotiana tabacum* cvs. Samsun and Xanthi systemically infected with the common strain of tobacco mosaic virus (TMV-C) to superinfection with RNA from a strain of TMV (TMV-P) that causes necrotic lesions on *N. sylvestris*.

Cultivar	No. of areas super-/No. of challenge inoculated areas[a]		
	Healthy (control)	Dark green areas	Light green areas
Samsun	33/33	51/51	32/54
Xanthi	21/21	36/36	23/42

[a] Each sample contained leaf discs from 2 independently inoculated areas.

TABLE 18. Susceptibility of Nicotiana tabacum cvs. Samsun and Xanthi systemically infected the common strain of tobacco mosaic virus (TMV-C) to systemic superinfection after challenge inoculation on dark and light green areas with RNA from a strain of TMV (TMV-P) that causes necrotic lesions on N. sylvestris.

Cultivar	No. of plants superinfected systemically[a,b,c] / No. of challenged plants		
	Healthy (control)	Dark green areas	Light green areas
Samsun	11/11 a	17/17 a	9/18 b
Xanthi	7/7 a	12/12 a	10/14 b

[a] 12 days after challenge inoculation.

[b] Number of plants superinfected systemically remained the same 25 days after challenge inoculation.

[c] Means followed by the same letter in the row are not significantly different (Two samples comparison for proportion, P=0.05).

TABLE 19. Susceptibility of dark and light green areas of Nicotiana tabacum cvs. Samsun and Xanthi systemically infected with the common strain of tobacco mosaic virus (TMV-C) to superinfection with tobacco etch virus (TEV).

Cultivar	No. of areas super-/No. of challenge inoculated areas[a]		
	Healthy (control)	Dark green areas	Light green areas
Samsun	10/12	24/27	27/27
Xanthi	10/12	27/30	20/27

[a] Each sample contained leaf discs from 2 independently inoculated areas.

TABLE 20. Susceptibility of Nicotiana tabacum cvs. Samsun and Xanthi systemically infected with the common strain of tobacco mosaic virus (TMV-C) to systemic superinfection with tobacco etch virus (TEV).

Cultivar	No. of plants superinfected systemically[a,b] / No. of challenged plants		
	Healthy (control)	Dark green areas	Light green areas
Samsun	4/4 a	9/9 a	9/9 a
Xanthi	4/4 a	10/10 a	9/9 a

[a] 12 days after challenge inoculation.

[b] Means followed by the same letter in the row are not significantly different (Two samples comparison for proportion, P=0.05).

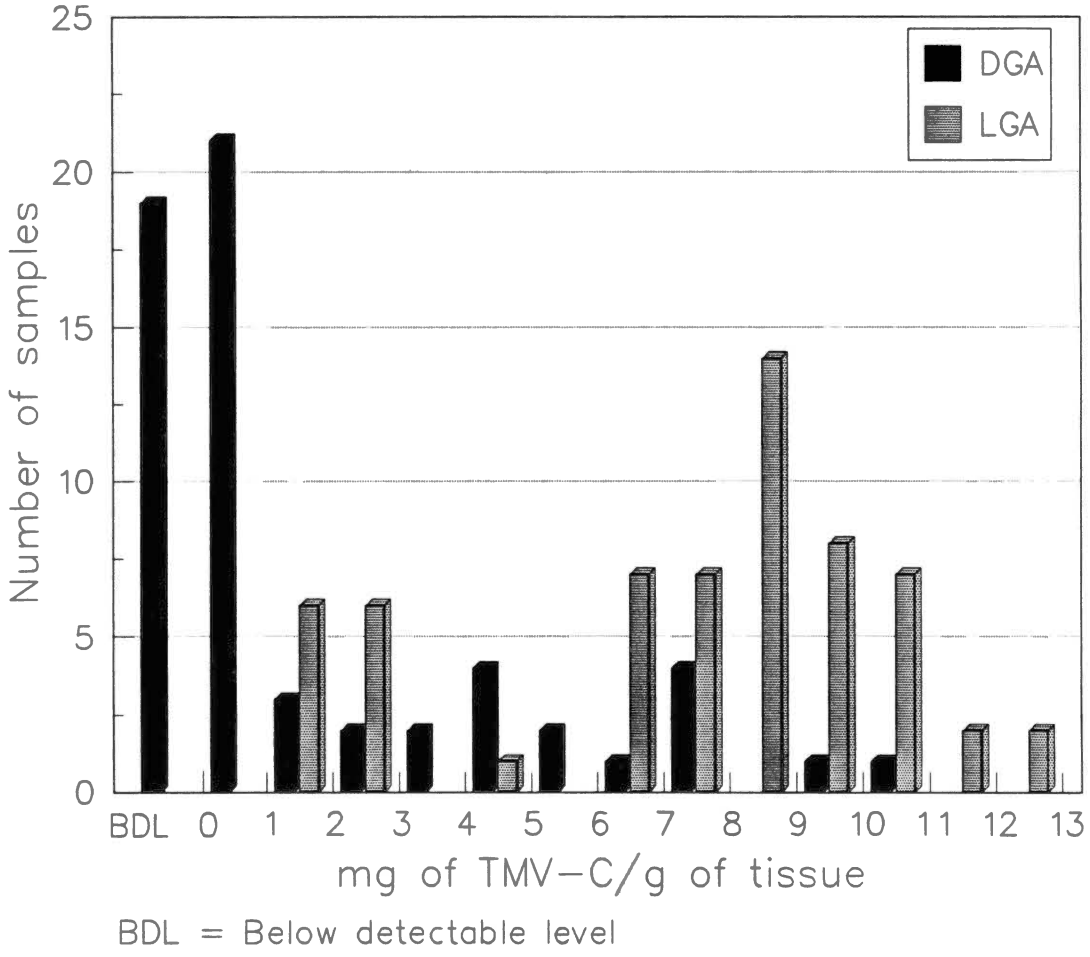

FIGURE 2. Concentration of the common strain of tobacco mosaic virus (TMV-C) in leaf discs from dark green areas (DGR) and light green areas (LGA) of Nicotiana tabacum cv. Samsun.

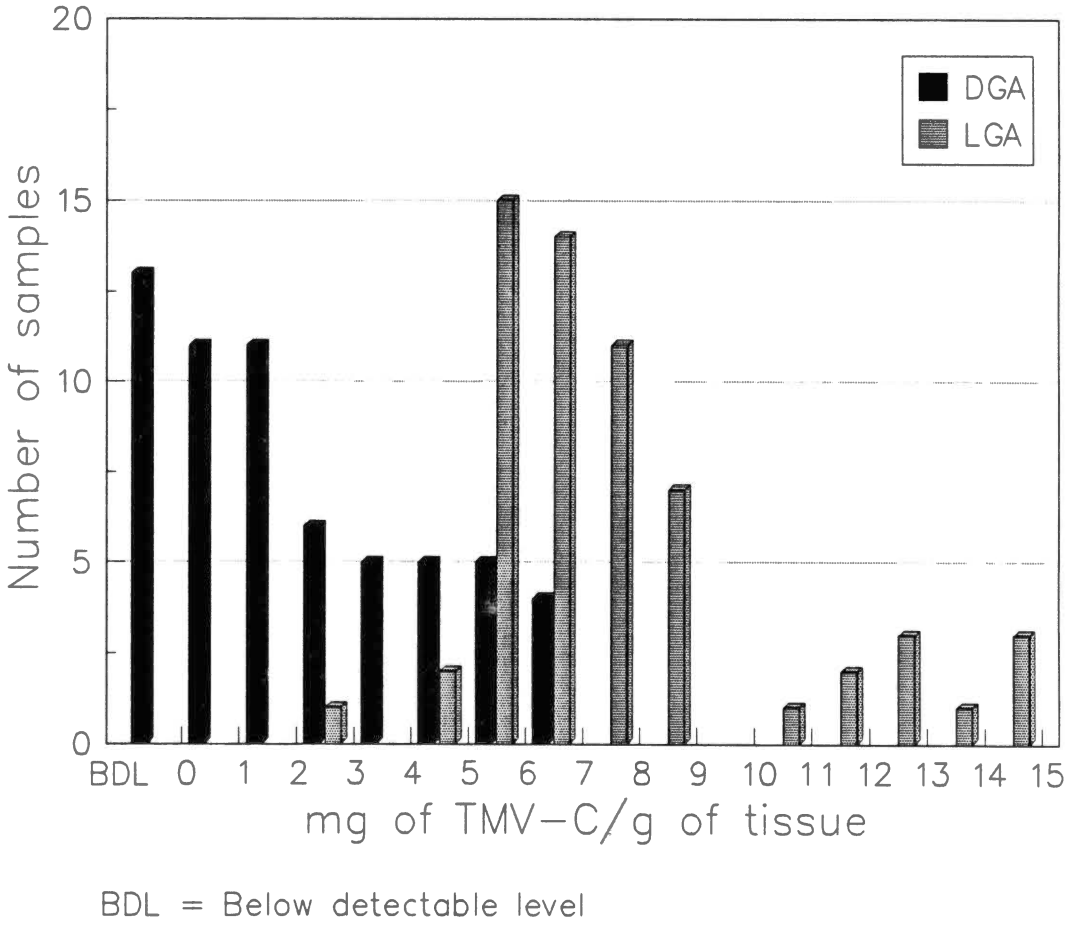

FIGURE 3. Concentration of the common strain of tobacco mosaic virus (TMV-C) in leaf discs from dark green areas (DGA) and light green areas (LGA) of Nicotiana tabacum cv. Xanthi.

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