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Potato Leafroll Virus Stimulates Systemic Movement of Tobacco Mosaic Virus in Tobacco Plants Carrying N Gene

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Abstract: Leaves of young *Nicotiana glutinosa* and *Nicotiana tabacum* var. Xanthi nc plants were rub inoculated with tobacco mosaic virus (TMV) before and after aphid inoculation with potato leafroll virus (PLRV). Local necrotic lesions developed on all leaves inoculated with TMV. After 4-6 weeks, systemic necrotic lesions developed on leaves, stems and tips of plants inoculated with both TMV and PLRV but not in plants inoculated with either virus individually. TMV could be rub transmitted from the systemic necrotic lesions of dually infected plants, but is could not be transmitted from non-necrosis caused collapse of some dually infected plants. Re-growth from the base of such plants was initially non-necrotic but a few necrotic lesions developed on young leaves after plants reached the 10-15 leaf stage. TMV could not be rub transmitted from non necrotic re-growth but could be graft transmitted to healthy gene tobacco scion. TMV could not be transmitted by rub inoculations or by graft transmission from non-inoculated leaves and stems of plants inoculated with TMV only. Concentration and distribution of PLRV was not affected by dual infection with TMV.

Key words: Mixed infection, movement, viruses, tobacco, N gene, PLRV,TMV, PVX, PVY

Introduction

Nicotiana glutinosa is a local lesion host of tobacco mosaic virus (TMV). A single dominant gene, N (Holms, 1938) (in the presence of N gene, TMV remains confined to the local lesions), controls the local necrotic lesion reaction. This gene has been introduced into cultivars of *Nicotiana tabacum* by inter-specific hybridization (Holms, 1938; Takahashi, 1956). Following rub inoculations of N gene plants, TMV initially multiplies at initial infection sites at the same rate as in n gene plants (in n gene hosts, TMV spreads to all plant parts) and virus moves to several layers of adjoining cells. Soon thereafter, the central area of infected N gene tissue collapses and local necrotic lesions develop. Virus remains restricted to a few cells at the edge of the lesion, and systemic infection does not occur.

Potato leafroll virus (PLRV), apparently lacks the capacity for cell-to-cell movement in parenchyma cells. This may be the reason it is limited to phloem tissue (Anonymous, 1986., Arai *et al.*, 1969; Barker *et al.*, 1986; Kojima *et al.*, 1969). For successful infection PLRV must be deposited directly into the phloem by its aphid vectors (Hull, 1989). PLRV has been transmitted into isolated mesophyll protoplasts (Barker *et al.*, 1982) and tobacco necrotic dwarf virus, a phloem-limited virus, into mesophyll cells but it appears to be confined to only infected cells (Kubo *et al.*, 1979). In contrast, TMV moves to all locations in susceptible plants (Murayama *et al.*, 1965). It has been demonstrated that cell-to-cell movement of TMV in controlled by a specific viral coded protein named 30-KDa protein (Doem *et al.*, 1987., Meshi *et al.*, 1987). It seemed possible that the transport protein of TMV could complement cell-to-cell movement of PLRV.

The movement of phloem limited viruses into the parenchyma cells can be complemented by an appropriate virus. Atabekov *et al.* (1984) reported that PLRV antigen accumulated in some parenchyma cells in addition to phloem cells of *Datura stramonium* also infected with potato virus X (PVX). Similar observations have been made in bean plants co-infected with TMV and bean golden mosaic virus (BGMV) (Kubo *et al.*, 1979) and in potato plants co-infected with both PLRV and potato virus X (PVX), and PLRV and potato virus Y (PVY) (Barker, 1987, Jayasinghe *et al.*, 1989). These studies did not determined whether the antigen in parenchyma cells was viable virus and nor did they determined whether it originated within the parenchyma cells or moved there from other cells.

Infection with PVX after inoculations with PLRV did not affect the initial virus infection or the concentration of PLRV antigen in any of the 12 tested potato clones (Ahmed *et al.*, 1999).

The initial objectives of this study were to determine whether co-infections with TMV could facilitate the escape of PLRV from the phloem and cell-to-cell movement in parenchyma cells.

Materials and Methods

Plants: We used *N glutinosa* and *N. tabacum var.* Xanthi nc, both of which contain the N gene. Seeds of these varieties were sown in 10 cm plastic pots containing a mixture of sand, loam, and peat moss. When seedlings were 2-3 cm tall, they were transplanted to 12.5 cm diameter clay pots with the same potting mix. These plants were kept in a glass greenhouse with supplementary light provided as needed to maintain 16 hour day-length by rotary, low pressure, 1000 watt, sodium lamps (lux 70,000). The temperature was maintained between 20-24°C. Liquid fertilizer consisting of 6% nitrogen, 6% phosphorus, and 6% potassium diluted 1:200 was continuously added in irrigation water. Plants 8-10 cm tall and with 4-5 fully expanded leaves were used for inoculations. Virus for TMV antibody production was propagated in systemic Xanthi.

Virus isolates: TMV isolate UI, the wild type strain was maintained in tomato grown at 20-24°C. Potato leafroll virus isolate No.7 was maintained on *Datura tatula* kept in an insectory at 22-25°C with aphids, *Myzus persicae*.

Virus inoculations: Plants were inoculated with PLRV using aphids (*M. persicae*), reared on infected *D. tatula* plants. Aphids, 10-15 per plant, were allowed to feed for 48 hours

and then were killed using nicotine sulfate fumigation at the end of transmission period. TMV was rub-inoculated on carborundum-dusted (600 mesh) lower leaves of each plant with a cheesecloth pad moisten in inoculum. TMV inoculum was prepared by grinding fresh diseased leave tissue diluted 1:10 w/v in 50 mM potassium phosphate buffer, pH 7.00, using a mortar and pestle.

Purification of TMV: Young leaves of TMV inoculated *N. tabacum* var. Xanthi these plants were harvested 10 days after inoculation. Virus was purified according to the method of Wojciech Kaniewski (personal communication). Infected leaves were triturated in 2 volume (w/v) in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.05 M ascorbic acid and 0.02 M EDTA. The triturate was emulsified in a volume of chloroform:butanol (1:1) equal to one-half of the initial buffer volume and was clarified by centrifuging for 15 minutes at 6,000 rpm in Beckman rotor JA-10. The upper aqueous phase was centrifuged in Beckman rotar 45 Ti for 2 hours at 28,000 rpm at 4°C. Pellets were re-suspended in 0.01 M potassium phosphate buffer, pH 7.00, containing 0.001 M EDTA. One-fifth of the initial buffer volume was added to the pellet in each centrifuge tube. The contents were stirred slowly overnight at 4°C. The low and high speed centrifugation steps were repeated and the pellet loaded on a 10-40% sucrose density gradient (SDG) prepared with the same buffer and centrifuged at 23,000 rpm for 4 hours in a Beckman SW 25.1 rotor at 4°C. The gradient tubes were photographed, fractionated into 1.2 ml fractions, and analyzed by ultra violet (U.V.) light (256 nm) absorbance monitor. After SDG centrifugation, virus was further purified on 45-7% Cs Cl gradient using 0.05 M citrate buffer, pH 7.0, containing 0.005 M
EDTA. The tubes were centrifuged at 21,000 rpm for 12 hours at 4°C in a Beckman SW 25.1 rotor. Tubes were photographed and virus bands were collected using an ISCO model 640 fractionator. Virus containing fractions were dialyzed against 0.01 M potassium phosphate buffer, 0.001 EDTA, pH 7.0.

Production of TMV antibodies: Purified virus was treated with formaldehyde (1% v/v of a 37% solution was added to the virus suspension) and divided into aliquot containing 2 mg of virus, and held frozen until injected. A rabbit was injected intramuscularly with 2.0 mg of virus emulsified in Freund's incomplete adjuvant at weekly intervals for the first 4 weeks and at 2 weeks intervals thereafter. Blood was harvested twice a week beginning 2 weeks after the first injection. Antibodies used in these experiments were prepared from antiserum of the third bleeding. Its titer was 1:512 determined by the precipitin tube test. The antibodies of PLRV used in this study were previously produced in our laboratory using the same method and also had a titer of 1:512.

Virus detection and assay: PLRV antigen was detected in systemic tissue by using a two step (Keniewski *et al.*, 1988) modification of the double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) described by Clark and Adams (Clark *et al.*, 1977). Indirect ELISA procedures (Converse, 1990) detected TMV with much greater sensitivity than DAS-ELISA and were used for the detection of TMV antigen in these studies. Samples ere diluted 1:50 for PLRV detection, and 1:25 for TMV detection. The tissue from the original diseased source, used for inoculations was used as a diseased control. ELISA results were read as optical density at 405 nm using a Gilford model EIA manual reader.
A tissue blot method was developed to determine the specific locations of PLRV and TMV antigen in infected plants. This is described in section 3 of this dissertation. Briefly, freshly cut free hand cross sections of infected or healthy plants were pressed lightly on nitrocellulose membrane for 30 seconds. These membranes were processed to produce an insoluble reaction product at sites where antigen was bound. The results were observed and photo-micrographed with a binocular stereoscope.

Results

To determine whether co-infections with TMV would induce escape of PLRV from phloem tissue and cell-to-cell movement in parenchyma tissue, we inoculated *N. glutinosa* plants with TMV before and after inoculations with PLRV. In the initial experiments, four plants were inoculated first with TMV followed by inoculations with PLRV four days later, after TMV local lesions had developed. Two control plants were inoculated with TMV only and two with PLRV only.
Concurrently, four plants were inoculated first with PLRV followed by TMV inoculations 7-10 days later. Two control plants were inoculated with PLRV only and two with TMV only. This basic experiment was repeated a number of times under various conditions and a number of parameters were measured as described below.

Effects of TMV on PLRV movement: Results of tissue blot assays confirmed that PLRV is routinely confined to the phloem tissue of PLRV infected tobacco plants (Fig. 1). The results also showed that co-infection with TMV did not cause large scale movement of PLRV antigen from phloem tissue. Although, a few instances were detected in co-infected plants in which PLRV had moved from the phloem to an adjoining cell, further cell-to-cell movement did not occur.

Effects on virus concentration: To determine the effects of mixed infection on the concentration of PLRV antigen, plants infected with both TMV and PLRV and plants infected with PLRV only were assayed using ELISA. No difference in the concentration of PLRV antigen was observed in co-infected and in plants infected with PLRV only (Fig. 2).

Effects of PLRV on TMV movement: Plants inoculated only with PLRV developed distinct yellow symptoms and produced no necrotic reactions. Plants inoculated only with TMV developed local necrotic lesions on rub-inoculated leaves. Plants inoculated with both viruses initially developed the local necrotic lesions of TMV on rub-inoculated leaves and the systemic yellow symptoms typical of PLRV. However, new leaves emerging 4-6 weeks after the last inoculations were curled and distorted (Fig. 3). Small necrotic lesions developed on these leaves and grew to a diameter of 2-4 mm. These lesions were always associated with veinal tissue, either the midrib or with secondary leaf veins (Fig. 4). In some plants necrotic lesions developed in the apical regions of the stem. These

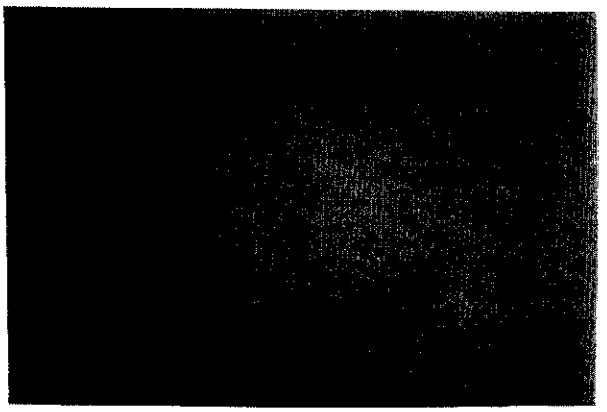

Fig. 1: Immunological localization of potato leafroll virus antigen on nitrocellulose membrane blotted with stem section of *Nicotiana tabacum* Xanthi c. Purple dots (circled) indicated the presence of potato leafroll virus antigen. On Nitrocellulose membranes it is easy to distinguish between purple reaction product and chlorphyll but on black and white photographs it is not clear

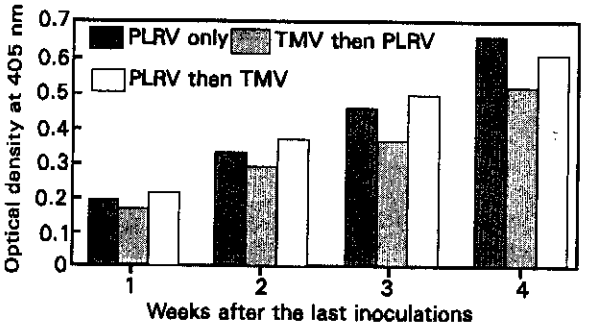

Fig. 2: Effect of TMV on ELISA detectable PLRV in *Nicotiana glutinosa* plants

Fig. 3: Curling and idstrotion caused by the tobacco mosiac virus infection on newly developed leaves of Nicotiana tabacum cultivar Xanthinc. Co-infected with potato leafroll virus

Fig. 4: Symptoms of tobacco mosiac virus: left-typical systemic symptoms on a leaf of *Nicotiana tabacum* cultivar Xanthi; center-healthy leaf o *Nicotana tabacum* Xanthi nc; and right-systemic necrotic lesions developed on a leaf of *Nicotiana tabacum* Xanthi nc. Plant co-infected with potato leafroll virus

Fig. 5: Apical and stem necrosis developed by tobacco mosaic virus on Nicotiana glutinosa plants co-infected with potato leafroll virus. Re-growth from the base of plant is initially non-necrotic

plants developed apical necrosis after 2-3 weeks.

Necrotic and non necrotic tissue from plants co-infected with both viruses and from plants individually infected with each virus were assayed for viral content by ELISA and by local lesions assay on Xanthi nc tobacco (Table 1). All necrotic lesions, including the systemically developed lesions on young leaves of co-infected plants always assayed positive for TMV by both ELISA and local lesion assays. Non necrotic areas, including areas between systemic or local TMV lesions always assayed negative for TMV by both assay methods.

Seven to 10 weeks after the last inoculations, the vascular system of some co-infected plants collapsed resulting in death of the foliage (Fig. 5). New shoots that grew from base of such plants had mild PLRV symptoms but contained no necrotic tissue characteristic of TMV infection until they reached the 10-15 leaf stage. Subsequently, a few lesions that contained TMV, developed on their re-growth. The initial re-growth on these plants assayed positive for PLRV

Table 1: Detection of tobacco mosaic virus and potato leafroll virus from Nicotiana glutinosa plants by enzyme-linked immunosorbent assay (ELISA) and local lesion assay

Inoculation Category	Assay methods					
	ELISA				Local lesion assay	
	Non Nec		Nec		Non Nec	Nec
	TMV	PLRV	TMV	PLRV	TMV	TMV
TMV only	-	NA	+	NA	-	+
PLRV only	NA	+	NA	+	NA	NA
PLRV and TMV	-	+	+	+	-	+

Nec : Necrotic tissue = Non Nec : Non necrotic tissue = NA : Not applicable + : Positive =- : Negative

antigen by ELISA but negative for TMV by both ELISA and local lesion assay. After 3-4 weeks, typical TMV mosaic symptoms developed on newly expanded leaves of n gene tobacco scions grafted at the tops of these plants. The n gene tobacco scions assayed positive for TMV both by ELISA and local lesion assay. In parallel tests, n gene tobacco scions that were grafted on Xanthi nc plants, which were inoculated with TMV only, did not develop any symptoms and they also assayed negative for TMV by both ELISA and local lesion assay.

A study was conducted to determine whether the systemic movement of TMV in co-infected plants could be accounted for by a change in the TMV isolate or the selection of a special strain that would not be restricted to local lesions on rub-inoculated N gene plants. N. glutinosa plants were rub-inoculated with systemic necrotic tissue from co-infected plants. Typical TMV local lesions developed on inoculated leaves and no systemic spread of virus occurred. After 4-6 weeks, n gene tobacco scions were grafted on the tops of these plants. No TMV symptoms developed in the scions, and the scions assayed negative for TMV by both ELISA and local lesion assay.

Effects of time of the year: We repeated the initial experiments, which indicated that TMV, could be induced to move systemically in N gene tobacco by co-infections with PLRV at different times of the year (fall, winter, spring and summer). In all of our experiments, similar results were obtained. Systemic TMV necrotic lesions developed only on plants co-infected with PLRV, and TMV remained confined in necrotic lesions associated with the phloem. Co-infections with PLRV always induced long distance movement of TMV in N gene plants. Season of the year did not affect the influence of TMV on PLRV. PLRV remained confined to the phloem tissue of co-infected plants in all experiments. Season did not affect the size, color or shape of necrotic lesions.

Effect of temperature: Results obtained in the glass greenhouse were compared with those in growth chambers at control temperatures at 18 and 26 °C. Results were the same at the two temperatures and were the same as results in the glass greenhouse. Systemic necrotic lesions developed only on co-infected tobacco plants and not on plants which were rub-inoculated with TMV only.

Effects of plant species: After initial experiments showed that PLRV induced systemic movement of TMV in N. glutinosa , the experiment was repeated using N. tabacum cv. Xanthi nc, which also carries N gene. The same results were achieved. PLRV induced systemic, long distance movement in N gene tobacco plants. TMV infection did not affect movement of PLRV.

Discussion

Our results indicate that co-infection with TMV will not induce movement of PLRV from phloem to parenchyma tissue. Co-infection with TMV also did not change the symptoms caused by PLRV and did not affect PLRV concentration in infected tissue.

In contrast to our results, some earlier reports indicated that co-infection of potato virus X (PVX) and/or potato virus Y (PVY) with PLRV causes increased susceptibility of plants of PLRV and also increased concentration of PLRV in plants (Anonymous, 1987; Barker, 1989; Jayasinghe *et al.*, 1989). Ahmed and Thomas (Ahmed *et al.*, 1999) reported that essential function of PLRV inhibited in resistant potato genotypes is at least partially complemented by active function of PVX and PVY. Barker (Barker, 1987., Barker, 1989) observed PLRV movement from phloem tissue to parenchyma cells of N. clevelandii plants that were co-infected with either Potato virus S or PVY. He also observed a 10 fold increase in the concentration of PLRV in these plants. Similarly, TMV induced escape of BGMV from phloem tissue to non phloem tissue in co-infected bean plants (Carr *et al.*, 1983). However, cucumber mosaic virus, alfalfa mosaic virus, broad bean mottle virus, parsnip yellow fleck virus, tomato black ring virus, raspberry ring spot virus, and potato virus S showed no effect on the concentration of PLRV in N. Clevelandii and in potato plants (Barker, 1989; Hull, 1989). It is interesting to note that all viruses reported to increase the concentration of PLRV, have elongated particles, but not all viruses with elongated particles have increased the concentration of PLRV.

Although, TMV did not affect PLRV, co-infection with PLRV had a marked influence on movement of TMV in tobacco containing the N gene. TMV remained localized in necrotic lesions on the rub-inoculated leaves in plants inoculated with TMV only. When TMV inoculated plants were co-infected with PLRV, systemic TMV lesions developed, particularly in new leaves of the plants. The virus was confined to these lesions and the lesions were clearly associated with veins in the leaves.

These observations are explicable on the basis that PLRV facilitated the escape of TMV from the primary local lesions and movement in the phloem. The virus apparently did not cause necrosis as long as it remained confined to the phloem, but at locations where the virus moved out of the phloem, it produced necrotic lesions similar to primary local

lesions.

Dodds and Hamilton (Dodds *et al.*, 1972) reported similar results in barley co-infected with TMV and barley stripe mosaic virus (BSMV). In the presence of BSMV, TMV moved systemically. Our studies provide no evidence concerning the mechanism by which PLRV influenced the movement of TMV. It has been established that a virus coded 30-KDa protein (Doem *et al.*, 1987; Meshi *et al.*, 1987) governs cell-to-cell movement of TMV. There is evidence that cell-to-cell movement of most (if not all) viruses is governed by the virus genomes themselves (Atabekov *et al.*, 1984; Atabekov *et al.*, 1990).

There is good evidence that phloem limited viruses are deficient in cell-to-cell movement function. Classical studies have indicated that phloem transport is passive (Bennett, 1937) and therefore, movement function is not required for long distance movement in the phloem. However, some recent studies suggest that in long distance movement, both the coat protein with the ability to assemble into virus particles and the assembly origin in the genomic RNA are involved (Saito *et al.*, 1990).

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