Alterations in the accumulation of the viral proteins and RNAs in tomato under mixed infection with *Potato X potexvirus* and *Tobacco mosaic tobamovirus*.

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Abstract

The acute stage (between 5 and 12 days postinoculation) of the synergistic disease, induced in cultivar Fukuju ♯2 tomato (a tobacco mosaic tobamovirus (TMV)-susceptible, common Japanese cultivar), which had their primary leaves inoculated at the five-true leaf stage by mixed infection with PVX and TMV, is characterized by the significant enhancement of the concentration of PVX in the systemically infected upper leaf positions 5 or 7, as determined by direct antibody sandwich- enzyme linked immunosorbent assay (DAS-ELISA). Further evaluation of the accumulation dynamics of the coat protein of PVX and its 25 -kDa movement protein, encoded by the ORF2 (triple gene block), by SDS-PAGE followed by Western blot analysis revealed their pattern of accumulation as following those of the virion, being relatively faster and higher in doubly than in singly infected plants. On the other hand, both the movement into, and accumulation of the TMV coat protein in the same systemically mixed infected leaves were considerably lower compared to that in singly infected plants. Northern hybridization analysis of samples, on a time series basis, using an RNA probe complementary to 350 nucleotides (nts) of the PVX coat protein gene (inserted into T7 plasmid vector and labeled with digoxigenin (DIG)), showed that the genomic RNA of PVX was also relatively enhanced in the systemically mixed infected leaves compared to the singly infected ones.

Keywords: SDS-PAGE, Western blot analysis, Northern hybridization, and RNA probe.

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INTRODUCTION

Pairs of related and unrelated viruses can often replicate in the same cells and may interact synergistically or antagonistically (Otsuki and Takebe, 1976) while the concentration of one or both viruses may significantly increase. Goldberg and Brakke (1987) reported a 5.4-fold increase in the concentration of maize chlorotic mottle virus (MCMV) in a mixed infection with the strain B of maize dwarf mosaic virus (MDMV-B) but there was no corresponding increase in MDMV-B concentration.

According to Scheets (1997), however, while the MCMV concentration in corn plants in a mixed infection with wheat streak mosaic rymovirus (WSMV) increased 3.3- to 11.2-fold compared to the average for plants inoculated with MCMV alone, the WSMV concentration was also enhanced at least 2.1 fold higher than in singly infected plants. In a mixed infection involving PVX and PVY, PVX was reported (Vance, 1991) as being enhanced while PVY remained unchanged. Similar trends in soybean under mixed infection with the soybean mosaic potyvirus and the other nonpotyvirus member (Calvert and Ghabrial, 1983; Anjos et al., 1992) had also been reported. The titer of the bean pod mottle comovirus (BPMV), as determined by enzyme–linked immunosorbent assay (ELISA) varied with leaf position and correlated well with symptom severity in singly and dually infected soybean (Calvert and Ghabrial, 1983). The co-infection of Japanese radish plants with turnip mosaic virus (TuMV) and cucumber mosaic virus (CMV) led to increased concentration of the latter (Sano and Kojima, 1989). As measured by indirect ELISA, the increase was most significant in the upper systemically infected leaves, producing severer symptoms than that in TuMV only-infected ones. Plants with CMV only, however, did not display any symptoms.

In a mixed infection involving CMV and TuMV, the enhancement of CMV concentration was largely attributable to an increase in the number of CMV-infected cells (Ishimoto et al., 1990). Yet in some instances, the increase had been attributed to enhanced transport of virus (Barker, 1987) and in others to both cases as in the enhancement of CMV multiplication in cucumber plants co-infected with zucchini yellow mosaic virus (Poolpol and Inouye, 1986). Vance (1991) reported alteration in the replication of PVX RNA leading to higher accumulation of anti-sense RNA of PVX in co-infections with PVY.

This study aimed at further elucidating the host-pathogen, and pathogen-pathogen interactions that are involved in the synergistic disease being studied. It addressed not only the questions of whether the components of PVX i.e. the coat protein, movement protein and the RNAs are also enhanced in line with the virion, but also determined the TMV accumulation dynamics in a PVX-enhancing situation.

MATERIALS AND METHODS

Plant Propagation and virus inoculation. Cultivars Fukuju # 2 (a common Japanese cultivar), tomato plants that were used in the various experiments were raised under normal greenhouse conditions. Maximum temperature was 32 ^{o}C while the minimum was 18 ^{o}C. Sandy loam soil was steam sterilized at 121°C for 30 min and vermiculite including fertilizer was supplemented at planting. Inoculation of plants at the 5-leaf stage with each of the viruses alone, and simultaneously with a mixture of equal quantities of PVX and TMV was by rubbing the carborundum-dusted primary leaves; i.e. leaves 1 and 2 with 0.2 mg/ml of any one virus. Plants were washed immediately with water. Mock-inoculated plants served as control.

Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis.

Protein fractionation and western blot analysis for the assay of virus specific proteins accumulation in infected plants were carried out essentially by the standard method (Sambrook et al., 1989). Known quantities (0.1 g to 0.3 g) of leaf samples collected from same positions of infected or healthy plants were macerated, with pre-cooled mortars and pestles, in homogenizing buffer (0.05 M Tris-HCl, pH 6.8 containing 0.1 % PMSF or 0.1 % 2-mercaptoethanol) at a ratio of 1 g to 10 ml of buffer. An equal volume of 2 x SDS gel loading buffer (100 mM Tris–HCl, pH, 6.8; 200 mM dithiothreitol (DTT); 4 % SDS, 0.2 % bromophenol blue and 20 % glycerol (Sambrook et al., 1989), was then added and mixed thoroughly before boiling for 8 min at 100 °C to denature the protein. Samples were loaded at 10 µl per lane on 15 % mini gels and resolved at 30 mA until the dye head reached the bottom of the gel.

For western blot analysis, the fractionated proteins were electrophoretically transferred to hybond-C extra nitrocellulose membrane (Amersham life science) at a constant current of 180 mA for 30 min. Antiviral antibody and anti-PVX's 25 kDa antibody were used at 5 µg/ml of blocking buffer (5 % skim milk in phosphate buffered saline (PBS) containing 0.05 % Tween 20 and 0.02 % NaN₃). Goat anti-rabbit antibody-alkaline phosphatase conjugate (Biosource International, Camarillo Ca. USA) was used as secondary antibody at 1: 1,000 dilution. Visualization of virus-specific protein bands was achieved in 15 ml of alkaline phosphatase buffer i.e. substrate solution (100 mM Tris-HCl, pH 9.5; 100 mM NaCl; and 5 mM MgCl₂) containing 100 µl of 50 mg of nitro blue tetrazolium (NBT) per ml of 70 % dimethyl formamide, and 50 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) per ml of dimethyl formamide. The reaction

was terminated as soon as the desired intensity was attained, usually within 5 min for coat proteins, with PBS containing 1mM EDTA i.e. 200 µl of 0.5 M EDTA, pH.8.0, per 50 ml of PBS.

RNA analysis

Isolation and purification of total RNA Total RNA was isolated from leaf samples of both mock inoculated and infected plants according to the method of Logemann et al. (1987). Leaf tissues were frozen and then homogenized in liquid nitrogen immediately with mortar and pestle to a fine powder. Further homogenization was carried out by addition of 2 vol. of guanidine buffer (8 M guanidine hydrochloride, 20 mM Mes (4-morpholineethansulfonic acid), 20 mM EDTA, and 50 mM mercaptoethanol at pH 7.0. After centrifugation for 10 min at 10,000 rpm, the homogenate was extracted with 1 vol. phenol/chloroform/isoamyl alcohol (25:24:1). RNA was extracted from the aqueous phase by mixing with pre-cooled 0.7 vol of ethanol and 0.2 vol. of 1 M acetic acid and keeping at –70° C for 1 h. The pelleted RNA was washed twice with 3 M sodium acetate, pH 5.2, and a final wash with 70 % ethanol was done to remove the salt. The RNA pellet was dissolved in sterile DEPC-treated water. Aliquots of 10µl were quantified spectrophotometrically using the Pharmacia/Biotech's Ultrospec 3000 while the rest were kept at –70 ° C until used.

Northern blot hybridization For analysis of the relative abundance of the genomic and subgenomic RNAs, equal quantity of total RNA (20µg) of each treatment sample was fractionated on 1.2 % formaldehyde agarose gel, and transferred by capillary elution overnight to hybond nylon membrane in 20 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). An RNA probe, complementary to 350 nts of the PVX coat

protein gene, inserted into T7 plasmid vector and labeled with digoxigenin (DIG) (Boehringer Mannheim) according to the manufacturer's instructions, was hybridized to the total RNA. Prehybridization, hybridization and washing were carried out using the standard procedures (Sambrook et al., 1989; Ausubel et al., 1995). Prehybridization in formamide prehybridization /hybridization solution (5 x SSC, 5 x Denhardt's solution, 50 % (W/V) formamide, 0.5 % SDS, and 100 µg/ml sonicated salmon sperm DNA, denatured just before use) was carried out at 45 ^{o}C for 6 h and hybridization in the same buffer was carried out at 60 ^{o}C overnight. Washing was carried out using 2 x SSC/0.1% SDS for 5 min at room temperature twice, followed by use of 0.2 x SSC/0.1 % SDS, also for 5 min with rotation at room temperature, twice (low stringency wash). High stringency wash was then carried out using pre-warmed 0.1 x SSC/0.1% SDS, for 15 min at 68 ^{o}C, twice. The immobilized PVX RNAs were then immunodetected using anti-DIG alkaline phosphatase conjugate and color development was carried out using NBT/BCIP at twice the concentration for normal protein detection, in alkaline phosphatase buffer.

RESULTS

Analysis of viral proteins accumulated in singly and doubly infected plants.

Western blot analysis of SDS-PAGE fractionated proteins of leaf samples were carried out to evaluate the accumulation patterns of both TMV and PVX coat proteins and the PVX ORF2- encoded 25 kDa protein (movement protein) as regards time of appearance and the concentration in both inoculated as well as systemically infected leaves of cultivar Fukuju # 2 tomato plants under single and double infections, at different times postinoculation.

Coat proteins accumulation patterns

The coat proteins of TMV and that of PVX were detectable in the inoculated primary leaves of 5- leaf tomato plants, 24 h after inoculation though those of the former group were apparently more concentrated than that of PVX under single and double inoculation.
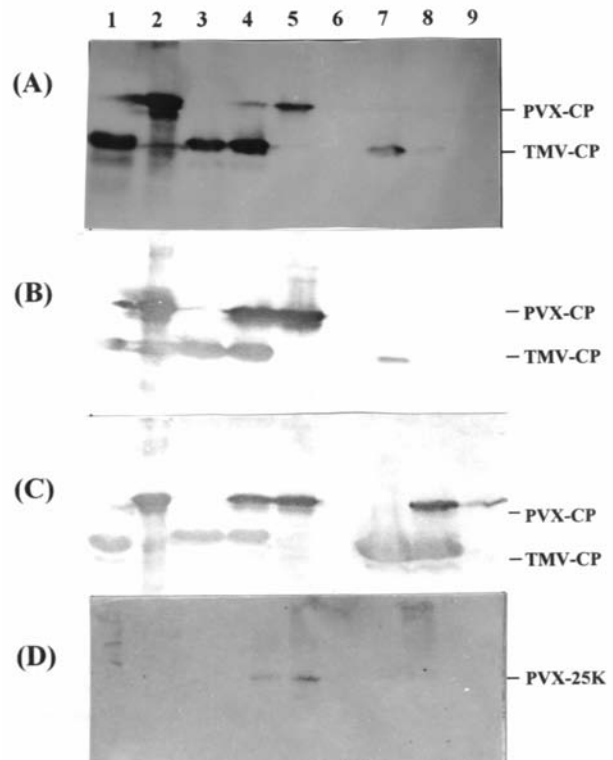

Plate 1: Western blot analysis of the accumulated virus-specific proteins, in the inoculated and systemically infected leaves of cv Fukuju # 2 tomato plants at different days after inoculation of the primaty leaves with PVX and TMV-L at the 5-leaf stage.
(A): Coat proteins in the inoculated leaf, and in the systemically infected leaf no. 5, at 3 days post inoculation (dpi) (B): Coat proteins in the inoculated leaf, and in the systemically infected leaf no. 3 at 5 dpi. (C): Coat proteins in the inoculated leaf, and in the systemically infected leaf no. 5, at 5 dpi. (D): PVX 25 kDa TGB protein, in the inoculated and in the systemically infected leaf no. 5 at 5 dpi. In (A), (B), and (C) lane 1= Purified TMV-L, and in (D) Prestained protein marker. In all, lane 2= Purified PVX. Lane 3= TMV-L alone, 4= PVX +TMV-L, 5= PVX alone, 6= Control (mock inoculation) respectively in the inoculated leaf. Lane 7= TMV-L alone, 8= PVX+TMV-L, 9= PVX alone in the systemically infected leaf. All coat protein bands in A, B, and C were detected with a mixture of antisera against PVX and TMV-L. The PVX 25 kDa TGB protein in (D) was detected by using antibody against the fusion protein, expressed in E-coli M 15.

TMV-L was detectable in the upper leaves no. 3 and 5 of singly inoculated plants by 72 h pi increasing to substantial levels by 120 h. Plate 1A shows variations in the concentration of the accumulated CPs of PVX

and TMV at 3 dpi in both the inoculated and the systemically infected upper leaf 5. TMV, the first to be detected in the upper leaf also had substantially higher level in singly infected than in mixed infected leaves. Plate 1 B and C show accumulated CPs in the inoculated leaf and systemically infected leaves no. 3 and 5 respectively, at 5 dpi. They show that whereas in leaf 3 TMV was detectable only in singly infected plants, in leaf 5, it accumulated to substantial levels in both singly and doubly infected plants indicating that TMV was faster in singly inoculated plants than in mixed infected ones, at least initially.

PVX, on the other hand, was detected at substantial level under mixed infection but faintly in singly infected ones. The enhancement by TMV of PVX coat protein was manifest. Although the quantity of TMV in the inoculated leaves remained at the same levels, for both single and double inoculations, by 5 and 7 dpi, the concentration in doubly infected upper leaf no. 5 remained substantially lower in the doubly infected plants than in singly infected ones. Generally, PVX coat protein concentration from day 5 through 12 continued to increase in the doubly infected upper leaves compared to singly infected ones. The levels of both PVX and TMV in leaf 3 was still very low as at 7 dpi (Plate 2 A). Plate 3A shows the accumulated CPs in leaf 5 and leaf 7 at 12 dpi. It shows clear reduction in the level of TMV CP under mixed infection relative to single infection in the upper leaf 5 but not in leaf 7. Nevertheless, both upper leaves also contained considerably higher PVX levels than singly infected ones.

The PVX 25 kDa protein accumulation

The accumulated PVX ORF2-encoded 25 kDa movement protein was first detected 48 h after inoculation in the inoculated primary leaves. It increased with time, but was at about the same levels in both singly and doubly inoculated leaves. Though the level in the

doubly inoculated leaf, as shown in Plate 1 D, was relatively high compared to the low level (weak signal) in leaf no. 5 at 5 dpi, comparison between the levels in the inoculated leaf and the systemically infected leaf 5 at 7 dpi showed about the same level (Plate 2 B). The level in leaf no. 3 (i.e. the leaf just above the inoculated leaves), however, remained undetectable following the pattern of the coat protein accumulation.
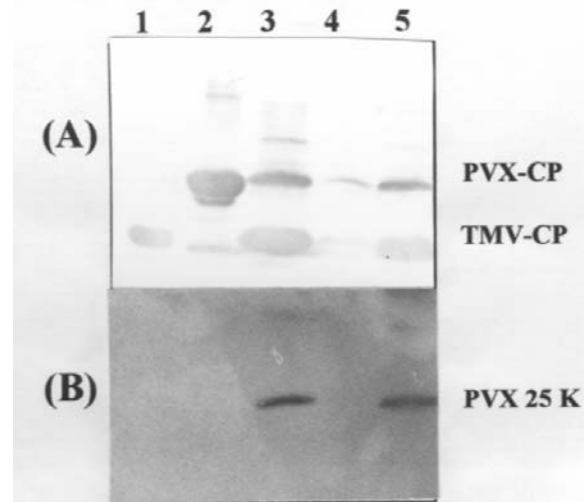

Plate 2: Western blot analysis of the accumulated virus-specific proteins, at different leaf positions of cv Fukuju # 2 tomato plants, 7 days after simultaneous mixed inoculation with PVX and TMV-L. (A) Coat proteins of PVX and TMV-L detected by using a mixture of antisera against PVX and TMV-L. PVX 25 kDa protein detected with antibody against PVX 25 kDa protein expressed in *E-coli* M 15. For both (A) and (B), lanes 1 and 2 = Purified TMV-L and PVX virion respectively. Lanes 3 to 5 are PVX+TMV-L in the inoculated leaf, leaf no. 3, and leaf no. 5 respectively

Plate 3 B shows the relative levels of the 25 K, TGB protein, in leaf 5 and leaf 7 at 12 dpi. There was a substantial increase in the level under mixed infection than that under single infection at both leaf positions.

Analysis of PVX RNA accumulated in singly and mixed infected plants

Northern blot hybridization analysis of total RNA fractionated on 1.2% agarose gel were carried out using RNA probes derived from a
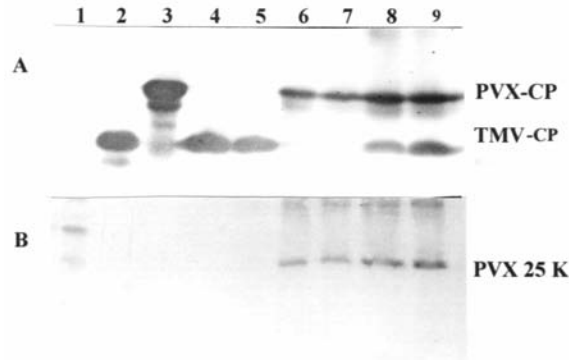
Plate 3: Western blot analysis of accumulated viral proteins in leaves no. 5 and 7 of singly and doubly infected cv Fukuju # 2 tomato plants, 12 days following inoculation with PVX and TMV-L.

(A) Coat proteins of PVX and TMV detected by usinfg amixture of antisera against PVX and TMV-L. **(B)** PVX 25 kDa protein detected with antibody against PVX 25 kDa protein expressed in *E-coli* M 15.

For both (A) and (B) Lanes 1 to 3 areprestained protein marker, purified TMVand PVX virions respectively. Lanes 4 and 5 = TMV-L alone in leaf 5 and 7 respectively, lanes 6 and 7= PVX alone in leaf 5 and 7 respectively, whilelanes 8 and 9 = PVX plus TMV-L in leaf 5 and 7 respectively. The positions of PVX and TMV bands are as indicated.
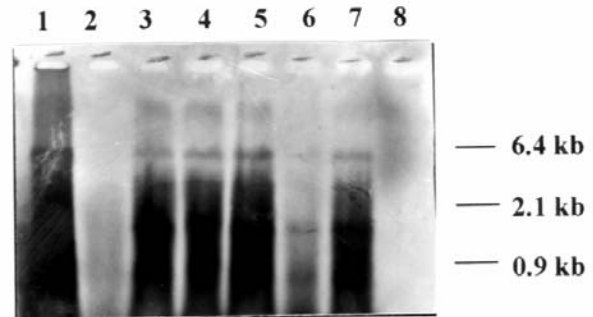
Plate 4: Northern blot analysis of the accumulated PVX RNAs in systemically infected upper leaf no. 5 of Fukuju # 2 tomato plants at different times after inoculation with PVX and TMV-L.
Lane 1=Purified PVX virion RNA, while lanes 2, 4, and 6 are PVX alone, at 5, 9, and 12 dpi respectively. Lanes 3, 5, and 7 are PVX plus TMV-L at 5, 9, and 12 dpi respectively. Lane 8 = TMV-L alone. PVX RNA were hybridized with a cDNA probe prepared from a 350 nts portion in the coat protein gene of PVX and labeled with Digoxigenin (DIG). The hybridized RNAs were immunodetected using anti DIG –alkaline phoosphatase conjugate. The position of the genomic and subgenomic RNAs, as shown, were determined from preliminary experiments and by comparison with standard texts.

350 nts coat protein section of the PVX genome cloned into a T7 plasmid vector and in-vitro labeled with digoxigenin (DIG) according to the manufacturers' instructions. It specifically hybridized the genomic as well as the sub-genomic RNAs, that were then immunodetected using anti-DIG antibody labeled with alkaline phosphatase, as earlier confirmed in preliminary experiments. A time-course analysis of leaf position 5 from day 5 through 12 revealed the gradual accumulation of both the genomic and subgenomic RNAs in both singly as well as doubly inoculated plants. The trend was as for virion and CP accumulation. By 5 dpi, whereas the hybridization signal was almost undetectable in PVX alone it was very clear in the mixed infected plants. Both the genomic and subgenomic RNAs accumulated to substantially higher levels in doubly infected than in singly infected plants (Plate 4).

DISCUSSION

Alterations in the levels of virus specific proteins accumulated under single and mixed infections by the different interacting viruses were observed in this study with mixed infection invariably leading to higher accumulation of PVX components than that observed in singly infected plants. The enhancement phenomenon is believed to play significant role in the subsequent tomato symptom, growth and yield responses.

The position of the leaf on the plant and the mode of entrance of the virus into it appeared to be crucial to the accumulation of the viral components and also the interactions leading to the enhancement of the PVX components in tomato. For instance, while no significant enhancement of PVX by TMV was noticeable in the inoculated leaf, systemically infected upper leaves supported enhancement to different levels. In this regard, the results are in line with those of some earlier workers

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(Calvert & Ghabrial, 1983; Vance, 1991) who had also reported lack of significant enhancement of any of the interacting viruses in the inoculated leaves. They also reported no change in the concentration of the enhancing virus, in the upper leaf position that supported enhancement. In this study, however, in the acute stage leaf position no. 5 of tomato that supported high enhancement of PVX, the accumulation of the coat protein of TMV-L was relatively lower than that in comparable leaves of singly infected plants (Plate 3 A).

That there was relative increase in the PVX 25 kDa movement protein and the CP accumulated in the mixed infected cv Fukuju #2 tomato plants as with the viral particle for instance, could portend the need to play some significant roles in the host–viral interactions that may lead to enhanced viral accumulation. A facilitated cell-to-cell movement in infected cells through the process of increasing the plasmodesmal size exclusion limit (SEL) of such cells is among such possibilities. After all, the ability of PVX to increase the plasmodesmal SEL of infected tobacco cell has been attributed (Angell et al., 1996) to the 25 kDa TGB protein although other viral proteins may also be involved. However, that alone cannot explain the resulting higher concentration of PVX in the systemically infected leaves of tomato supporting enhancement. It is apparent that there are differences in the mechanisms for enhancement in inoculated and systemically invaded leaves.

It is plausible to think of movement enhancing by TMV, as one of the possible mechanisms employed in the overall accumulation-enhancement of PVX, in the upper leaf portion of the mixed infected tomato plants. The western blot analysis results that showed that the systemic movement of TMV-L as well as its concentration is slowed significantly under simultaneous mixed infection with PVX while that of PVX increased, suggest that possibility (see plate 1A,B & C). A slowing-down-strategy induced by some factors involved in PVX replication which then enabled TMV to facilitate the systemic movement of PVX in doubly infected upper leaves to the initial detriment of TMV among other possibilities may be the reason. A transgenic tobacco plant expressing the ORF2 protein of PVX has been reported (Ares et al., 1998) as showing substantial resistance to tobacco mosaic virus.

The considerable low level of PVX genomic RNA observed during the acute stage of disease (see Plate 4) could be responsible for sustained low synthesis of virion and hence the attendant low concentration of PVX that were invariably observed in the singly infected plants. In the same vein, the high level in doubly infected plants led to more synthesis and higher accumulation of virion. A change in the regulation of PVX RNA replication in doubly infected cells was also credited with the synergistic increase in PVX accumulation in a mixed infection with PVY (Vance, 1991) and this invariably was associated with increased pathogenicity in tobacco plants.

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