Resistance in *Cucumis melo* to Watermelon Mosaic Virus 2 Correlated with Reduced Virus Movement Within Leaves

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ABSTRACT

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A resistance that involves suppression of the levels of watermelon mosaic virus 2 (WMV 2) was investigated in systemically infected muskmelon tissue. Differential temperature manipulation of WMV 2-infected whole plants increased the synchrony of systemic infection and facilitated studies on the kinetics of capsid protein accumulation in resistant and susceptible plants. Virus was extracted from 5-mm-diameter leaf disks collected from a single leaf at various times after virus replication began and quantified from immunoblots. Host tissue type did not affect extraction or quantification of the protein. Differential temperature treatment of susceptible plants increased the amount of capsid protein detected early in the infection but did not affect the maximum level accumulating in the leaves. Similar treatment of resistant tissue caused an increase in the intensity of the symptoms induced by WMV 2, but the symptom type, discrete chlorotic areas, was not changed. An increased level of capsid protein was associated with the increase in symptom severity in the resistant tissues. The resistance mechanism(s) does not affect vascular transport of the virus but appears to reduce cell-to-cell movement of the virus within leaves.

There have been few studies on the temporal accumulation of plant viral proteins in whole plants (2,3,16). Interpretation of results from these types of studies is confounded by the asynchronous infection of leaf cells by the virus, making it difficult to identify the sequential events in virus replication and viral protein synthesis. Another disadvantage of asynchronous infections is the difficulty in identifying viral polypeptides that may be present in low amounts or at specific times during replication. The use of protoplasts may increase the synchrony of infection in groups of individual cells; however, the physiological differences between protoplasts and cells of intact plant tissue have a differential effect on virus pathogenesis and confound interpretation of the data (12). The response of protoplasts from resistant plants to virus infection is often different than that of whole plants (17,19), particularly if the resistance is to a viral process other than replication. Studies of temporal accumulation of viral gene products in resistant hosts are further confounded because of the reduced accumulation of the products and the uneven distribution of virus-infected tissue (e.g., 11). A system is needed that allows the repeated sampling of synchronously infected whole plant tissue over time to study the quantitative and qualitative effects of resistant plant genotypes on viral pathogenesis.

Procedures that manipulate systemic virus infection of whole plants using differential temperatures approach synchronous virus replication in intact leaves (4). Cells of young leaves maintained at low temperatures (e.g., 5 C) become infected by virus from mechanically inoculated older leaves maintained at temperatures permissive for virus replication (e.g., 25 C). The low temperature inhibits virus replication; however, raising the temperature to 25 C allows replication to begin in all infected cells at approximately the same time. Differential temperature manipulation of systemic infection in several virus-host systems enhances the uniformity of virus replication by increasing the initial number of infected cells (5,14,18). This results in uniform symptom distribution in systemically infected tissue (14) and an increase in detectable nonstructural virus proteins that normally occur in low levels at specific times during pathogenesis (16).

A form of resistance to watermelon mosaic virus 2 (WMV 2) that involves suppression of the levels of virus occurs in the muskmelon accession 91213 (11). WMV 2 induces distinct, small chlorotic areas in systemically infected tissue of the resistant plants. The chlorotic areas expand slowly; however, they remain discrete and nonuniformly distributed in the leaf. The predominant symptom exhibited by susceptible plants is a uniform mottling of all systemically infected leaves. The present paper reports the use of differential temperatures to manipulate the systemic infection in resistant plants. The plant response to the virus (i.e., symptom type) was not altered, but the number of primary infection sites was increased, resulting in a uniform distribution of symptoms in systemically infected tissue. The kinetics of capsid protein accumulation was measured in single, systemically infected leaves of resistant and susceptible plants.

MATERIALS AND METHODS

Plant material and virus strain. Plants were grown in a temperature-controlled greenhouse (26 C, 14-hr day/22 C, 10-hr night) under natural lighting conditions. Top Mark, a commercially available cultivar of *Cucumis melo* L., was the virus-susceptible standard. The *C. melo* accession 91213, previously described, possesses suppressive virus resistance to WMV 2 (11,15). The strain of WMV 2 used in these experiments was isolated from field squash (11). Virus was purified from muskmelon (cultivar Top Mark) by the method of Purcifull and Hiebert (13) and stored as 10-μg samples at −80 C to avoid multiple cycles of freezing and thawing.

Antisera production and purification. Antisera to purified WMV 2 or to SDS-PAGE purified capsid protein (9) were produced in rabbits given both toe-pad and intramuscular injections at four weekly intervals. Each injection contained

0.1–0.5 mg of virus or protein in 0.5 ml of PBS (8.2 mM sodium phosphate, pH 7.4, 140 mM NaCl, 3.0 mM KCl, 0.02% sodium azide) emulsified with 0.5 ml of Freund's incomplete adjuvant. Immunoglobulins were precipitated from whole sera by adding an equal volume of 36% (w/v) sodium sulfate at room temperature. The precipitate was washed twice in 18% sodium sulfate. The final pellet was resuspended in PBS and dialyzed overnight against the resuspension buffer. The IgG fraction was isolated by Protein A Sepharose affinity chromatography (Pharmacia, Uppsala, Sweden)(8).

Coat protein assays. A single, frozen leaf disk (5 mm) was homogenized in TSE buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM disodium ethylenediaminetetraacetate, 10 mM sodium diethyldithiocarbamate, 0.1 mM phenylmethylsulfonyl-fluoride, 0.5% NP-40) (1:20, w/v) and chloroform (1/3 total volume). Leaf tissue was disrupted in a 500-μl microfuge tube with a ground glass rod and the extract clarified by centrifuging 10 sec at 12,700 *g* (Eppendorf centrifuge, model 5415, Brinkman Instruments, Westbury, NY). The supernatant was transferred to another 500-μl microfuge tube. The chloroform phase was removed and discarded, and the interphase was suspended the original volume of TSE buffer and reclarified. The supernatants were combined, and the virus selectively concentrated by immunoprecipitation by adding 0.5 volume of antiserum (1.0 mg/ml), incubating at 37 C for 2 hr, and centrifuging at 14,000 *g* for 20 min. Pellets were resuspended in 5-μl of 2 × dissociation buffer (10). Precipitation of the virus and capsid protein from the clarified supernatant was also attempted by the addition of 8% polyethylene glycol or 60% ammonium sulfate followed by a 60- or 15-min incubation at 4 C, respectively. The precipitate was collected by centrifugation at 8,000 *g* for 15 min and resuspended in 2 × dissociation buffer (10).

Electrophoretic blotting procedure. Immunoprecipitated samples were electrophoresed in 10% acrylamide-bisacrylamide gels (60 × 80 × 0.8 μm) (10) and transferred to nitrocellulose filters (Schleicher and Schuell, Keene, NH) (20). The filters were soaked in TBS (20 mM Tris-Cl, pH 7.5, 150 mM NaCl) containing 3% bovine serum albumin (BSA) for 2 hr to saturate additional protein binding sites. This and subsequent incubations were done at room temperature. The filters were rinsed briefly in distilled water and incubated overnight in primary antiserum (anti-WMV 2) appropriately diluted in TBS and 1% BSA. The filters were washed (3 × 10 min) in TBS containing 0.5% Tween 20 (TTBS) and incubated for 1 hr with biotin-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:1,000, v/v, in TBS and 1% BSA. The filters were washed as described above and incubated for 1 hr with horseradish peroxidase (HRP)-conjugated streptavidin (BRL, Gaithersburg, MD) diluted 1:1,000, v/v, in TBS and 1% BSA followed by additional TTBS washes. The immunodetectable proteins were visualized by soaking the filters for 2 min in a solution containing 60 mg of HRP color development reagent (Bio-Rad, Richmond, CA) dissolved in 20 ml of ice-cold methanol and mixed with 100 ml of TBS containing 60 μl of cold hydrogen peroxide. The reaction was stopped by rinsing the filters in deionized water. The immunoblots were immediately photographed with a direct positive film (LPD-4, Kodak). The relative absorbance of each protein band image on the film was measured at 575 nm and plotted with Gilson (model 2600) Spectrophotometer and plotter. The area under the curve of the absorbance scan was calculated and used as a relative measure of the amount of protein present in that band. Known amounts of purified capsid protein were included as standards on each immunoblot.

To ensure there were no differential effects of host tissue on extraction or quantification of the capsid protein, known amounts of purified WMV 2 were added before and after homogenization of uninoculated tissue samples from Top Mark and 91213. The capsid protein was reextracted from these samples and quantified as described above.

Differential temperature manipulation of the systemic infection. The lower three leaves of resistant and susceptible plants with primary stems 20–25 cm long were mechanically inoculated with WMV 2. The remaining three to five fully expanded leaves and all secondary stems were removed. Plants were immediately placed in a styrofoam chamber with the terminal bud and one or two immature leaves extending out of the chamber. The entire styrofoam chamber was placed in a plant growth chamber. Lower leaves were maintained at temperatures permissive for viral replication and systemic movement (22 ± 4 C) by two 40-watt light bulbs controlled by a thermostat. Upper leaves were maintained at colder temperatures (7 or 9 C) known to be permissive for systemic invasion, but restrictive for replication in other virus systems (6). Plants whose upper leaves were maintained at 7 or 9 C were kept at the differential temperatures for 3 and 5 days, respectively (7 C, 3 day; 9 C, 5 day). Plants were then removed from the styrofoam chamber and transferred to a growth chamber maintained at 25 C with a 12-hr photoperiod. Control plants were treated in the same manner as described above, except after inoculation they were transferred directly to the growth chamber maintained at 25 C.

Sampling procedures. Leaf disks (5 mm) were collected from all plants at 24-hr intervals for up to 18 days from day 0 (where day 0 was the time of inoculation for the control plants maintained at 25 C and the time when the differential temperature-treated plants were transferred to the 25 C chamber). The same leaves were repeatedly sampled for the 18-day period. Because of plant growth characteristics, leaf positions sampled differed between control plants and plants exposed to differential temperatures. Growth was halted during the 3- or 5-day differential temperature treatment, whereas control plants continued to grow. The immature leaf immediately below the apical bud at the time of inoculation and the first leaf developing from the apical bud after the plant was transferred to 25 C were sampled on plants exposed to differential temperatures. Leaves sampled on the control plants were generally the second, third, or fourth leaves developing from the apical bud after the plants were inoculated. Leaves sampled on all plants were the first two systemically infected leaves showing symptoms. Samples were stored at −80 C until all samples from a leaf could be assayed at once. The virus was extracted from each of the samples collected from a single leaf in one experiment and quantified on a single gel.

RESULTS

Extraction, concentration, and detection of WMV 2. The amount of virus in the pellets (discarded) was one-third to one-fifth of that in the supernatant. It was minimized by completely homogenizing the tissue and reextracting the plant material that composed the interphase from the first clarification. The efficiency of virus extraction and recovery was similar from both resistant and susceptible tissue.

Immunoprecipitation was the most efficient method to concentrate WMV 2 from the clarified homogenate. Immunoprecipitated samples were easily resuspended in 5 μl of dissociation buffer so that the entire sample could be loaded in a single well of the small polyacrylamide gels. Samples nonspecifically concentrated either by polyethylene glycol or ammonium sulfate were contaminated with host proteins. Resuspension of the relatively large final pellets required a volume of dissociation buffer greater than 30 μl, preventing the entire sample from being analyzed in a single gel lane. The banding pattern of immunoreactive products prepared by either of these methods was distorted because of large amounts of host contaminants making quantification of the capsid protein difficult.

The two antisera (prepared against purified WMV 2 or SDS denatured capsid protein) did not differ significantly in their ability to precipitate capsid protein from infected tissue samples or uninoculated tissue samples spiked with purified virions. However, the latter antiserum increased the sensitivity of detection of capsid protein on immunoblots. The lower limit of detection of capsid protein in uninoculated tissue spiked with purified virions was approximately 400 picograms, with antiserum to the SDS-degraded capsid protein and approximately 1 ng using antiserum to intact WMV 2 virions. Antiserum to SDS degraded capsid protein was used in subsequent experiments.

The relationship between amount of purified virus added to uninoculated tissue and levels of capsid protein detected was similar for both Top Mark and 91213 (Fig. 1). Host genotype did not influence extraction or quantification of capsid protein in these experiments.

Differential temperature manipulation of the systemic infection. The temporal pattern of capsid protein levels in the susceptible Top Mark tissue was altered by the differential temperatures (Fig. 2). Capsid protein was detectable 1 day after the shift to 25 C in the differential temperature-treated plants, whereas 3 days were required for capsid protein to reach detectable levels in control plants inoculated and maintained at 25 C. Maximum levels of capsid protein accumulation were not different between differential temperature-treated and control plants. The rate of accumulation of capsid protein, once detected, was greater in control plants. Capsid protein was not detected in systemically infected leaves of differential temperature-treated plants before the plants were moved to the permissive temperature (25 C).

Differential temperature manipulation of the systemic infection of resistant 91213 plants increased the rate of invasion and amount of tissue initially infected by the virus (Fig. 3). Capsid protein was detected 1 or 4 days (9 C, 5-day or 7 C, 3-day treatment, respectively) after the shift to 25 C in the treated plants, whereas capsid protein was not detected in control plants until day 8. Levels of capsid protein were increased three- to sixfold in the differential temperature treated 91213 plants and approached levels of capsid protein extracted from susceptible Top Mark plants.

Capsid protein accumulated in susceptible plants throughout the test period, but did not accumulate beyond 10–13 days in the differential temperature-treated 91213 plants. The 91213 plants were sensitive to the 7 C temperatures, and 45% of the plants died as a result of this treatment. Survival of the plants was increased to 80% by raising the nonpermissive temperature to 9 C and increasing the length of the DTT to 5 days. The Top Mark plants were tolerant of either temperature regime.

The detection of capsid protein in both genotypes was closely associated with the appearance of symptoms on the sampled leaves. The symptom type or intensity was not appreciably altered in the Top Mark plants. Symptoms appeared very rapidly throughout a systemically infected leaf on either a treated or control leaf and consisted of a uniform mottling of the leaf. Uniform levels of capsid protein were detected from numerous samples collected from various locations throughout single leaves, indicating a uniform distribution of virus within leaves from differential temperature-treated and control Top Mark plants.

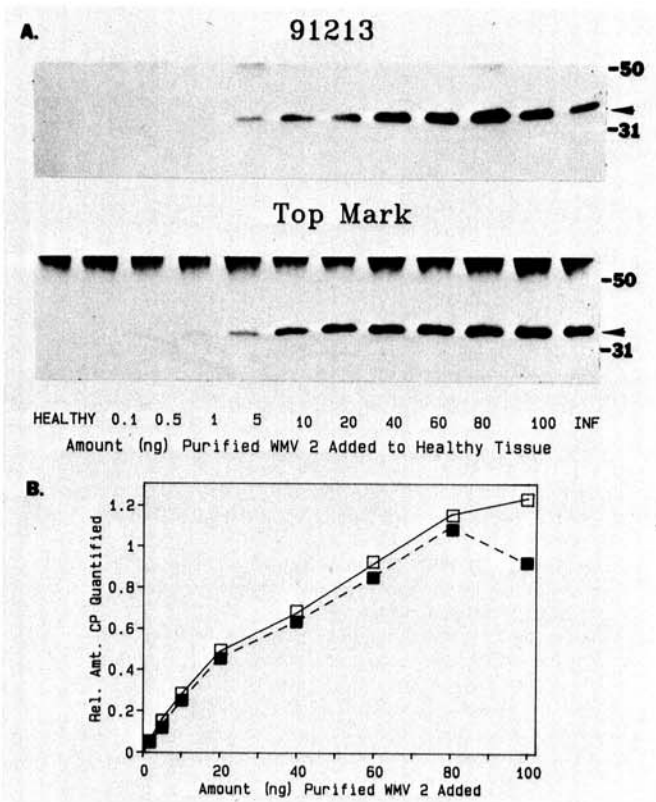

Fig. 1. A, WMV 2 capsid protein (←) detected on immunoblots from samples of healthy *Cucumis melo* 'Top Mark' and '91213' tissue extracts with known amounts (0.1–100 ng) of purified virus added. Samples from a healthy plant and an infected plant (INF) were included on both immunoblots. Electrophoretic mobility of two molecular weight markers (31 and 50 Kd) are indicated at right. **B,** Relationship between the amount of purified watermelon mosaic virus 2 (WMV 2) added to extracts of healthy tissue from susceptible (Top Mark) (□—□) and resistant (91213) (■---■) *C. melo* plants and the relative amount of WMV 2 capsid protein quantified from the immunoblots shown in A.

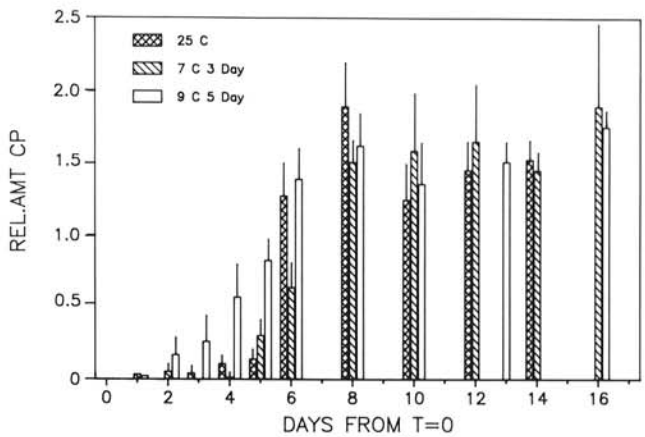

Fig. 2. Relative levels of watermelon mosaic virus 2 capsid protein (CP) in tissue samples (5 mm diameter) collected from the first symptomatic, systemically infected leaves of susceptible Top Mark plants. Plants were either inoculated and maintained at 25 C (25 C), inoculated and subjected to a 3-day 7/22 C differential temperature treatment (7 C 3 Day), or inoculated and subjected to a 5-day 9/22 C differential temperature treatment (9 C 5 Day). $T = 0$ is the time of inoculation for the plants inoculated and maintained at 25 C and the time at which the differentially temperature treated plants were transferred to 25 C (i.e., 3 or 5 days after inoculation). The standard error (n = 4, 5, or 6) is represented as a line extending from each bar.

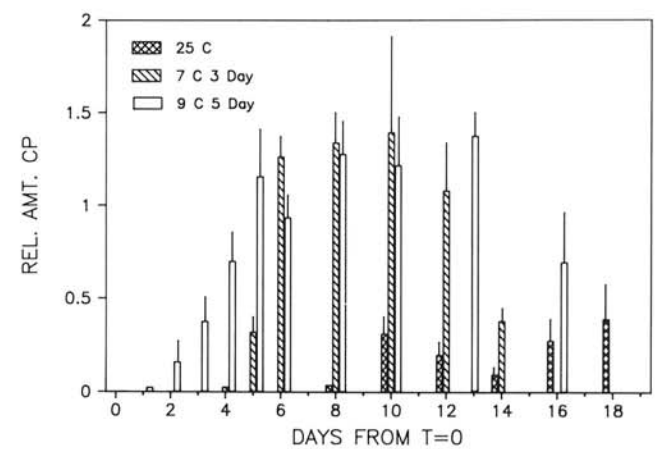

Fig. 3. Relative levels of WMV 2 capsid protein (CP) in tissue samples (5 mm diameter) collected from the first symptomatic, systemically infected leaves of resistant 91213 plants. Plants were either inoculated and maintained at 25 C (25 C), inoculated and subjected to a 3-day 7/22 C differential temperature treatment (7 C 3 Day), or inoculated and subjected to a 5-day 9/22 C differential temperature treatment (9 C 5 Day). $T = 0$ is the time of inoculation for the plants inoculated and maintained at 25 C and the time at which the differentially temperature treated plants were transferred to 25 C (i.e., 3 or 5 days after inoculation). The standard error (n = 4, 5 or 6) is represented as a line extending from each bar.

In the systemically infected leaves of the 91213 control plants the virus induced discrete, circular, chlorotic lesions that were not uniformly distributed in the leaf. The chlorotic lesions, which eventually became necrotic, were initially <0.5 mm in diameter and slowly enlarged over time. In the younger leaves of older plants, the symptom type changed to a mosaic pattern, and leaf morphology became distorted. Capsid protein could only be detected from the chlorotic areas of the symptomatic leaves and not from the unaffected green areas. The distribution of virus within 91213 leaves was therefore not uniformly distributed and caused a sampling problem in early experiments when a single 5-mm leaf disk was used to determine the capsid protein level in the leaf. This problem was overcome by the use of multiple leaf disks.

The symptom type on the systemically infected leaves of the differential temperature treated 91213 plants was not altered, but the number of chlorotic lesions was increased (Fig. 4). There was a significant increase in levels of virus extracted from all differential temperature-treated leaves with increased symptom severity. The increase in number of chlorotic lesions occurred only in the immature leaves adjacent to the treated apical bud and the first (and occasionally second) leaf developing from the apical bud following the differential temperature treatment. Symptoms on subsequently developing leaves reverted to the milder symptoms typical of the 91213 control plants. The distribution of the symptoms and virus extractible from the two to three leaves showing increased symptom severity was uniform and sampling of the leaves over time was done by taking a single 5-mm leaf disk on each sample date.

DISCUSSION

The process of vascular transport of plant viruses is distinct from cell-to-cell movement (1); therefore, it should be possible to affect short distance (i.e., cell-to-cell) movement without affecting vascular transport. Inoculation of the *C. melo* accession 91213 by WMV 2 resulted in an uninhibited systemic infection; however, detectable virus was restricted to small distinct chlorotic areas within leaves.

Differential temperature manipulation of the systemic infection in 91213 plants did not alter the response of the plant to the virus (i.e., virus movement was restricted within leaves), but the number of chlorotic areas and the total capsid protein content in treated leaves increased.

We have concluded that the resistance mechanism was not overcome by the differential temperatures. Rather, growth of these plants was inhibited at the cold temperature and apical leaves became a sink for virus transported out of lower inoculated leaves. Restoring normal growth by shifting plants to 25 C resulted in the infection (symptoms and capsid protein content) in new tissue being similar to systemically infected leaves in control 91213 plants. Only systemically infected tissue present on plants during differential temperature treatment showed increased symptom severity.

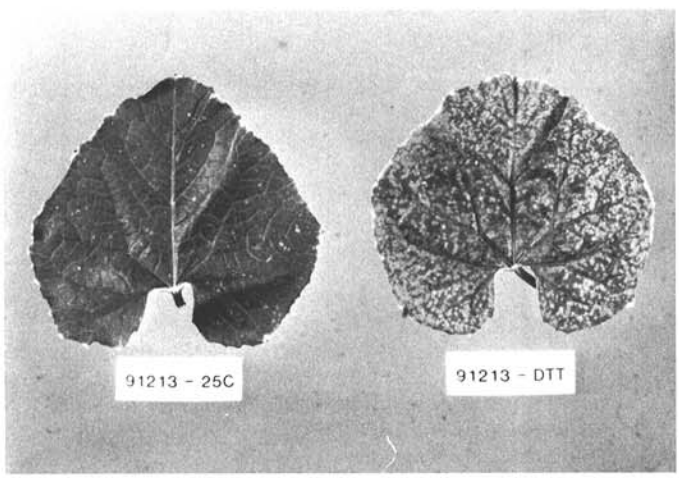

Fig. 4. Symptoms induced by watermelon mosaic virus 2 (WMV 2) on resistant 91213 plants that were either inoculated and maintained at 25 C (91213 25 C) or subjected to a 5 day 9/22 C differential temperature treatment (91213 DTT). WMV 2 capsid protein and symptom expression was first detected in both leaves 2 days before taking the photograph.

Virus or capsid protein detected in systemically infected leaves of actively growing plants represents virus transported into the leaf and virus replicated within the leaf both in a continual, nonsynchronous manner. Use of differential temperatures separated the processes of viral invasion and virus replication within a leaf and allowed the events of viral infection, e.g., movement and viral product accumulation, to be characterized in whole plants. Results of this study suggest that cell-to-cell movement of the virus is reduced, although equal amounts of the virus can systemically invade leaf tissue. The kinetics of capsid protein accumulation are not significantly different in resistant and susceptible plants. The decline in capsid protein in 91213 tissue after 13 days (Fig. 3) can be attributed to a reduction in the number of new cells becoming infected. Previous studies using greenhouse grown plants identified a two- to fourfold and a six- to 12-fold reduction in capsid protein and infectious virus, respectively, in 91213 relative to Top Mark. Virus acquisition by aphids was also reduced by 80% when 91213 was used as a source (15). The differential effects by the resistant 91213 plant on the above-mentioned virus characteristics suggest that multiple resistant mechanisms may be operating, one of which is a reduction or inhibition of cell-to-cell movement of virus within leaves. Additional studies of virus acquisition efficiency by aphids, infectious virus levels, and WMV 2 nonstructural protein levels over time in differential temperature treated tissue, known to contain levels of capsid protein equal to those found in susceptible tissue, will provide information on additional resistance mechanisms operating in 91213 plants.

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