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Age-related Resistance in Bell Pepper to *Cucumber mosaic virus*

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Summary

We demonstrated the occurrence of mature plant resistance in *Capsicum annuum* ‘Early Calwonder’ to *Cucumber mosaic virus* (CMV) under greenhouse conditions. When Early Cal wonder plants were sown at 10 day intervals and transplanted to 10-cm square pots, three distinct plant sizes were identified that were designated small, medium and large. Trials conducted during each season showed that CMV accumulated in inoculated leaves of all plants of each size category. All small plants (with the exception of the winter trial) developed a systemic infection that included accumulation of CMV in uninoculated leaves and severe systemic symptoms. Medium plants had a range of responses that included no systemic infection to detection of CMV in uninoculated leaves with the systemically infected plants being either symptomless or expressing only mild symptoms. None of the large plants contained detectable amounts of CMV in uninoculated leaves or developed symptoms. When plants were challenged by inoculation of leaves positioned at different locations along the stem or different numbers of leaves were inoculated, large plants continued

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to accumulate CMV in inoculated leaves but no systemic infection was observed. When systemic infection of large plants did occur, e.g. when CMV-infected pepper was used as a source of inoculum, virus accumulation in uninoculated leaves was relatively low and plants remained symptomless. A time-course study of CMV accumulation in inoculated leaves revealed no difference between small and large plants. Analyses to examine movement of CMV into the petiole of inoculated leaves and throughout the stem showed a range in the extent of infection. While all large plants contained CMV in inoculated leaves, some had no detectable amounts of virus beyond the leaf blade, whereas others contained virus throughout the length of the stem but with limited accumulation relative to controls.

Keywords: *Capsicum annuum*, mature plant resistance, *Pepper mottle virus*, virus movement

Introduction

Plants vary in their susceptibility to infection by certain pathogens due to their age or stage of development, a process often referred to as age-related resistance (Populer, 1978; Smith & Parlevliet, 1990; Leisner & Turgeon, 1993; DiFonzo *et al.*, 1994). Strategies to reduce losses caused by viral diseases have included age-related resistance whereby a crop is planted early or late in the season in an effort to avoid availability of young, highly susceptible plants at a time when insect vector densities achieve a specified threshold (Loebenstein, 1972; Walkey, 1991). This concept of age-related resistance is based on the fact that plants or plant parts change in their susceptibility to virus infection with age (Loebenstein, 1972; Fargette & Vie, 1994). In addition, for some virus-plant combinations it has been shown that mature plants resist or tolerate virus infection leading to increases in production of marketable fruit relative to plants that became infected at early stages of development (Knutson & Bishop, 1964; Ross, 1969; Loebenstein, 1972; Demski & Chalkley, 1974; Scott *et al.*, 1977; Rosenkranz & Scott, 1978; Thongmeearkom *et al.*, 1978; Pasko *et al.*, 1984; Agrios *et al.*, 1985; Avilla *et al.*, 1997; Bosque-Perez *et al.*, 1998; Lot *et al.*, 1998; Soler *et al.*, 1998; Sikora *et al.*, 1998).

Since 1992, a severe outbreak of *Cucumber mosaic virus* (CMV) has occurred in fresh-market tomato crops grown in northern counties of Alabama, U.S.A. (Sikora *et al.*, 1998). It was shown that cultivation practices used by local growers played an important role in the severity of the epidemic. Tomato seedlings were transplanted to the field every 2 wk beginning in early April and continued through early August. Consistently, large populations of cotton aphid (*Aphis gossypii*) were observed in the tomato crop in early July followed 10 to 14 days later by an extensive outbreak of CMV. The most severely affected tomato plants were those that were transplanted shortly before or after the appearance of the cotton aphids on tomato plants. Tomato plants transplanted early in the season, which were relatively mature at the time of the arrival of aphids, were shown to be infected by CMV, but these plants showed only mild symptoms, if any, and they sustained negligible yield losses (Sikora *et al.*, 1998). These observations led us to speculate that tomato plants transplanted to the field earlier in the season were less affected by CMV because of their mature stage of development at the time of infection. Similar observations occurred with bell pepper plants (*Capsicum annuum* L.) that, despite being grown in the same fields that contained severely affected tomato plants, expressed no CMV-like symptoms and did not contain detectable amounts of CMV when tested by enzyme-linked immunosorbent assay (ELISA) (J.F. Murphy, unpublished data). The explanation for the difference in response to CMV was not associated with resistance since none of the pepper and tomato varieties used in the area were considered as being resistant to CMV. However, cultivation practices differed in that pepper seedlings were transplanted to the field only a single time, in April, and thus, these plants were relatively mature at the time of the CMV outbreak in July.

Mature plant resistance is a well established phenomenon, and has been used to manage viral (Ferris & Jones, 1996; Madden *et al.*, 2000) and fungal diseases (Griffey *et al.*, 1993; Ma & Sing, 1996). Despite its recognition, little is known about the nature of this form of resistance. In this report, we demonstrate the occurrence of mature plant resistance of bell pepper plants to CMV under greenhouse conditions and show that this resistance appears to be directed at processes associated with virus movement.

Materials and Methods

Plant material, growth conditions and virus sources

All experiments were carried out in a temperature-controlled greenhouse at the Plant Science Greenhouse Complex at Auburn University, Alabama, USA. Ambient air temperatures in the greenhouse were maintained at 28°C day/21°C night throughout the year; however, day time temperatures during summer months sometimes exceeded 28°C. Supplemental metal-halide lighting was used for 14 h per day from September to April. The susceptible variety *C. annuum* L. 'Early Calwonder' was used for all experiments. Seeds were sown into Speedling trays containing Pro-Mix, a soil-less potting medium (Premier Peat, Rivière-du-Loup, Quebec, Canada). Upon germination, individual seedlings were transferred to 10-cm square pots containing Pro-Mix and slow release fertilizer (NPK ratio 25-4-12).

Early Calwonder seeds were sown at 10-day intervals over a period of 30 days, and upon germination were transplanted as described. Plants were grouped by age based on the number of days after germination. This grouping approach consistently resulted in plant size distinctions of small, medium and large plants that correlated with 24, 34, and 44 days after emergence, respectively (**Table 1**).

Table 1. *Capsicum annuum* 'Early Calwonder' plant size categories based on age, shoot height and number of leaves during different seasons

| <i>Season</i> | <i>Plant group</i> | <i>Days after emergence</i> | <i>Shoot height (cm)</i> | <i>Number of leaves</i> |
|---------------|--------------------|-----------------------------|--------------------------|-------------------------|
| Fall | Large | 48-51 | 16-21 | 19-27 |
| | Medium | 44 | 12-15 | 12-16 |
| | Small | 31-34 | 6-7 | 7-9 |
| Winter | Large | 44 | 21-24 | 20-28 |
| | Medium | 34 | 14-18 | 10-15 |
| | Small | 24 | 6-8 | 6-8 |
| Spring | Large | 44 | 16-24 | 18-30 |
| | Medium | 35-36 | 11-16 | 15-20 |
| | Small | 24-27 | 6-10 | 8-10 |
| Summer | Large | 39-41 | 21-24 | 23-30 |
| | Medium | 29-33 | 11-13 | 12-15 |
| | Small | 24 | 6-9 | 8-9 |

The emergence of leaves along the stem of Early Calwonder plants and their identification based on a defined numbering system was described by Andrianifahanana *et al.* (1997). Briefly, leaves emerged along the stem in pairs with individual leaves of a given pair occurring on opposite sides of the stem, the first pair of leaves (representing the oldest pair of leaves) was designated leaves 1 and 2 with each successive leaf (pair) occurring in order.

The KM isolate of CMV was used for all experiments (Guerini & Murphy, 1999). The Florida isolate of *Pepper motile virus* (PepMoV) was originally obtained from Dr T. Zitter, Cornell University. Both viruses were maintained in the greenhouse in *Nicotiana tabacum* cv 'Kentucky 14' and in Early Calwonder plants by mechanical passage.

CMV- or PepMoV-systemically infected Kentucky 14 leaf tissue was used as inoculum in each experiment. In experiments addressing inoculum source or type, inoculum consisted of either CMV-infected Early Calwonder tissue, -infected Kentucky 14 tissue or partially purified CMV (explained in greater detail below). When infected tissue was used as inoculum, approximately 1 g of tissue was ground in 10 ml of 50 mM potassium phosphate buffer, pH 7.0. All materials, e.g. buffer, mortar and pestle, were chilled prior to use and maintained on ice during the inoculation procedures.

Virus detection

Detection of virus in leaf samples was by indirect ELISA according to Voller *et al.* (1976) with the following modifications. Each sample was ground in 50 mM carbonate buffer, pH 9.6, using a motorized leaf squeezer and added to the microtiter plate at a final dilution of 1:25 (g tissue:ml buffer). Antigen was allowed to incubate for at least 12 h at 4°C. Anti-CMV and -PepMoV immunoglobulin (Ig) were purified by ammonium sulfate precipitation (Harlow & Lane, 1988) and used at 1.0 µg ml⁻¹ in phosphate buffered saline containing 0.5% Tween 20 (PBS-T). Ig-antigen interactions were allowed to occur for at least 12 h at 4°C. Goat anti-rabbit Ig conjugated to alkaline phosphatase (Sigma Chemical Company, St. Louis, MO) diluted to 1:6000 in PBS-T was allowed to incubate for 3–4 h at 37°C. Substrate (1.0 mg ml⁻¹ *p*-nitrophenylphosphate dissolved in 10% diethanolamine, pH 9.8) reactions developed at room temperature for 30 min and were recorded by a Dynatech MR700 microtiter plate reader at 405 nm.

Demonstration and challenge of mature-plant resistance in the greenhouse

In all experiments described below, plants were monitored on a daily basis for timing of appearance of symptoms, type and severity of symptoms. Virus accumulation in inoculated and uninoculated leaves was determined by ELISA while accumulation along selected portions of the petiole and stem were determined by immuno-tissue blot analysis (described below). Each experiment included uninoculated control plants representing each plant size treatment and a positive control treatment involving inoculation with PepMoV, which systemically infects Early Calwonder plants when inoculated at each of the different stages of development.

Treatments were arranged in randomized complete block and data analyzed as split-plot designs. Inoculation treatments represented the main plot including the number of leaves to which inoculum was applied and the position of the inoculated leaves along the stem, e.g. inoculation of leaves 1 and 2 vs leaves 1 through 6. The subplot consisted of plants of two (small and large) or three (small, medium and large) distinct growth stages (as outlined in Table 1). Thus, treatments consisted of a combination of plant size at the time of inoculation, and the number of leaves inoculated per plant or the leaf position along the stem. Three to six replications were included per treatment with three to 10 plants per replication. Data were analyzed using SAS (SAS Institute, Cary, N. C.) and means compared using the LSD test with ($\alpha=0.05$).

To demonstrate the occurrence of mature plant resistance under greenhouse conditions, Early Calwonder plants of each of the three stages of development were evaluated for their response to inoculation with CMV (along with the positive control, PepMoV). Small, medium and large Early Calwonder plants were inoculated with virus onto leaves 1 and 2 (oldest pair of leaves). Although this approach allowed comparison of inoculation of leaves of a similar position along the stem of plants of different growth stages, leaves 1 and 2 were not of a similar physiological state among the different treatments. Thus, for separate sets of large plants, virus was inoculated onto the third pair of leaves from the top of the plant (designated P1 and 2), which perhaps more accurately corresponded to the two oldest leaves of a small plant consisting of six leaves at the time of inoculation. In addition to inoculation of leaves 1 and 2 and P1 and 2, Early Calwonder

plants were evaluated for their response to inoculation of leaves 5 and 6 and 9 and 10 (the latter pair of leaves was for medium and large plants only). This experiment was initially carried out in a fall trial and was later repeated as winter, spring and summer trials.

Mature plant resistance of Early Calwonder plants to CMV was further evaluated by testing different amounts and types of inoculum. In the first set of experiments, inoculum dosage was examined by inoculation of either leaves 1 and 2 or leaves 1 through 6 of medium and large plants. Since small plants had only six leaves at the time of inoculation, either leaves 1 and 2 or all leaves were inoculated with CMV. In the second set of experiments, different types of CMV inoculum consisting of either systemically infected pepper leaves, tobacco leaves, or partially purified CMV (purified according to Palukaitis & Zaitlin, 1984) at 1 mg ml^{-1} were used to inoculate leaf 3 or leaves 1 through 6 of small and large plants (again, leaves 1 through 6 represented all leaves for small plants).

Nature of the resistance response

A series of experiments was carried out in an effort to further define the nature of the resistance expressed by large Early Calwonder plants to CMV. These experiments were designed to evaluate the general rate of virus accumulation in the inoculated leaf and the rate and extent of movement out of the inoculated leaf and into other parts of the plant. Each experiment was performed two times with consistent results between experiments.

To test the rate of virus accumulation in inoculated leaves, virus (CMV or PepMoV) was applied to leaf 3 or 5; the inoculated leaf was then tested using ELISA for levels of virus accumulation at 7, 14, and 21 day post-inoculation (dpi). Since CMV and PepMoV accumulated to comparable levels at each of their respective evaluation times, only data for 7 dpi are presented in Table 4.

In conjunction with these analyses, CMV movement to and accumulation in different locations along the petiole of inoculated leaf 3 and the stem were documented using immuno-tissue blot analysis (Andrianifahanana *et al.*, 1997; Guerini & Murphy, 1999). The relative locations along petioles and stems that were chosen to generate tissue prints are shown in **Figure 1**. Tissue prints were generated from three locations along the petiole of the inoculated leaf: (1) just below the leaf

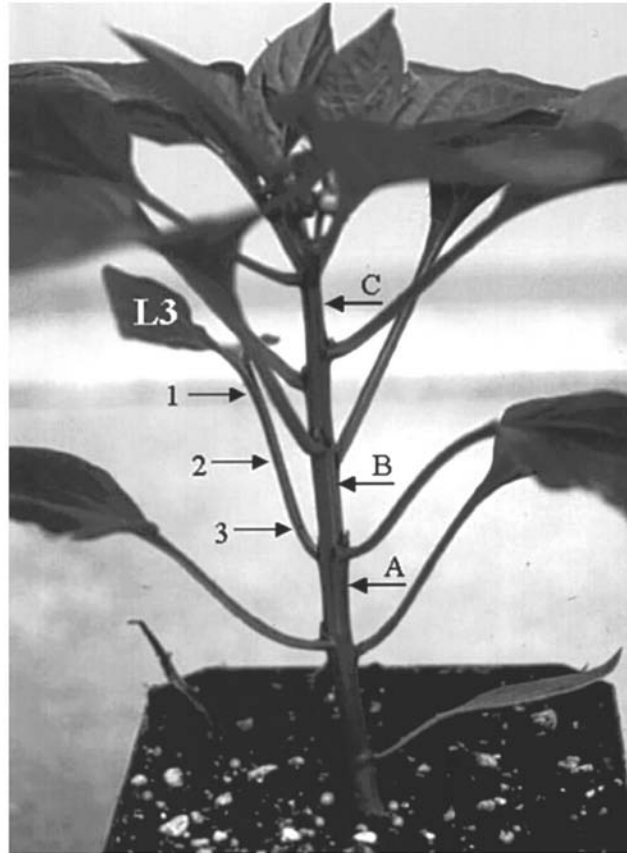


Fig. 1. A *Capsicum annuum* “Early Calwonder” plant showing the locations along the petiole of the inoculated leaf and the stem used to generate tissue prints for immuno-tissue blot analysis for detection of *Cucumber mosaic virus*.

blade, (2) in the middle of the petiole between the leaf blade and the stem, and (3) close to where the petiole adjoins the stem. Tissue prints representing stem segments included: (A) the internode immediately below inoculated leaf 3, (B) the internode immediately above inoculated leaf 3, and (C) the upper most internode along the main stem.

Results

Demonstration of mature plant resistance

There was variation in the growth and number of leaves of pepper plants from season to season (Table 1). Plants grew slower, and at specific times postemergence, had fewer leaves during experiments

carried out during the fall and winter trials compared with plants of the same age, i.e. measured as days after emergence, during the spring and summer trials.

In the initial trial carried out during the fall season, CMV infected inoculated leaves 1 and 2 of all plants in each size class (**Table 2**). Of the large plants inoculated onto leaves P1 and 2, CMV was detected in inoculated leaves of only 5 of 15 plants. Examination of ELISA values for inoculated leaves shown to be infected, i.e. the relative amount of CMV detected in these leaves, indicated that significantly more CMV accumulated in inoculated leaves 1 and 2 of large plants than in each of the other treatments. CMV accumulation in inoculated leaves 1 and 2 of medium plants was significantly greater than in inoculated leaves

Table 2. Response of *Capsicum annuum* 'Early Calwonder' plants of different age categories to inoculation with Cucumber mosaic virus (CMV)

| Season | Plant group | Leaves inoculated ¹ | Detection of CMV by ELISA in leaves ² | | Systemic Symptoms ³ | Latent period in days ⁴ |
|---------------|-------------|--------------------------------|--|-------------------------|--------------------------------|------------------------------------|
| | | | Inoculated | Uninoculated | | |
| Fall | Large | 1-2 | 1.458 ± 0.210 (15/15) a | (0/15) | (0/15) | — |
| | | P1-2 | 0.208 ± 0.023 (5/15) d | (0/15) | (0/15) | — |
| | Medium | 1-2 | 1.299 ± 0.035 (15/15) b | 0.258 ± 0.078 (9/15) d | (0/15) | — |
| | | Small | 1-2 | 0.564 ± 0.064 (15/15) c | 0.429±0.161 (15/15)c | (15/15) |
| Winter | Large | 1-2 | 0.694 ± 0.301 (4/4) b | (0/24) | (0/24) | — |
| | | P1-2 | 0.434 ± 0.128 (4/24) b | (0/24) | (0/24) | — |
| | Medium | 1-2 | 0.838±0.618 (6/12) b | (0/24) | (0/24) | — |
| | | Small | 1-2 | 0.434 ± 0.219 (14/24) b | 0.383 ± 0.193 (12/24) b | (9/24) |
| Spring | Large | 1-2 | 0.200 ± 0.180 (8/8) d | (0/8) | (0/8) | — |
| | Medium | 1-2 | 0.564 ± 0.187 (27/27) b | 0.866±0.119 (4/27) a | (3/27) | 20 |
| | Small | 1-2 | 0.770 ± 0.143 (26/26) a | 0.600 ± 0.280 (26/26) b | (26/26) | |
| 6-7 Summer | Large | 1-2 | 0.735 ± 0.124 (9/9) b | (0/9) | (0/9) | — |
| | | P1-2 | 0.393 ± 0.341 (9/9) d | (0/9) | (0/9) | — |
| | Medium | 1-2 | 0.970 ± 0.186 (6/9) a | 0.276 ± 0.000 (1/9) ce | (1/9) | 9 |
| | | Small | 1-2 | 1.123 ± 0.198 (9/9) a | 0.611 ± 0.199 (9/9) be | (9/9) |

1. Inoculated leaves and their position along the stem (as described in text).

2. Mean ELISA absorbance values of leaf samples determined to be positive for the presence of CMV ± standard deviation. An ELISA value was considered positive when greater than the mean plus three standard deviations of comparable healthy samples (e.g. the highest thresholds among all treatments for inoculated leaves was 0.060 for large plants in the spring trial, while for uninoculated leaves the highest threshold value was 0.075 for small plants in the spring trial). The numbers in parentheses are the number of CMV infected samples per number of samples tested. In the case of inoculated leaves 1 and 2 in the winter trial, inoculated leaves on most plants abscised prior to testing. Within each season, values with the same letter are not statistically different (LSD with $\alpha = 0.05$).

3. Number of plants expressing CMV induced systemic symptoms per number of plants inoculated.

4. Number of days between inoculation and the expression of systemic symptoms.

1 and 2 of small plants and leaves P1 and 2 of large plants. inoculated (and infected) leaves P1 and 2 of large plants had significantly less virus than in inoculated leaves of each of the other treatments.

In contrast to the 100% infection and high ELISA values observed in inoculated leaves 1 and 2 of large plants, none of these plants contained detectable amounts of CMV in uninoculated leaves, nor did any of the plants develop systemic symptoms during the course of the experiment (Table 2). Similarly, no systemic infection was detected in the large plants inoculated with CMV onto leaves P1 and 2. Nine of 15 medium plants inoculated onto leaves 1 and 2 with CMV became systemically infected; however, none of these plants developed systemic symptoms. In contrast, CMV systemically infected 100% of the small plants and developed typical systemic symptoms 9 to 12 days post-inoculation (dpi) that consisted of vein-clearing followed by a severe mosaic on young leaves. Examination of ELISA values indicated significantly more CMV accumulated in systemically-infected leaves of small plants than in comparable tissues of infected medium plants.

When this type of experiment was repeated in the winter, spring, and summer seasons, detection of CMV in inoculated leaves was fairly consistent; however, differences in the amount of virus detected varied from season to season (Table 2). The amount of CMV in inoculated leaves did not differ significantly among treatments in the winter trial, and with the exception that inoculated leaves of small plants consistently contained more virus, amounts of CMV in inoculated leaves were variable in the spring and summer trials. [Note that only four of 24 inoculated leaves of large plants were tested by ELISA in the winter trial due to abscission of inoculated leaves prior to testing.] Of particular importance, however, was the observation that no detectable systemic infection by CMV occurred in large plants regardless of which leaves were inoculated or the season in which the trial was conducted. A lack of systemic infection also occurred in medium plants during the winter trial, and only 4 of 27 and 1 of 9 plants developed a systemic infection in the spring and summer trials, respectively. All small plants developed a systemic infection in the spring and summer trials but only 12 of 24 did so in the winter trial. CMV-infected small plants always developed systemic vein clearing, mosaic and leaf distortion, regardless of the amount of time post-inoculation that symptoms developed. In contrast, those systemically infected medium plants that developed symptoms were delayed in the time of appearance of these

symptoms and the symptoms consisted of only a mild mosaic on the youngest leaves.

These results demonstrate that under greenhouse conditions, large plants consistently expressed what might be considered as mature plant resistance. These large plants were fully susceptible to CMV infection when considering infection and levels of accumulation in inoculated leaves. However, CMV was not detected by ELISA in young, uninoculated leaves of large plants and none of these plants developed symptoms. Additional experiments were carried out to examine if leaf position along the stem affected this resistance, *i.e.* did systemic infection occur when CMV was inoculated onto leaves 1 and 2, 5 and 6, or 9 and 10? As with previous experiments, CMV was not detected in young, uninoculated leaves and no symptoms developed regardless of the position of the inoculated leaves along the stem (data not shown).

Since previous work showed that mature Early Calwonder plants were not resistant to systemic infection by PepMoV (Andrianifahanana *et al.*, 1997), each of the trials in the current study included comparable treatments to those carried out with CMV but involving inoculation with PepMoV. In each treatment evaluating plant size at the time of inoculation and position of inoculated leaves along the stem, 100% of the plants (at least 15 in each treatment) developed a systemic infection. Symptom type was similar between treatments and included an initial appearance of systemic vein-clearing followed by a distinct mottle on younger leaves. The only difference observed among PepMoV-inoculated treatments was a 1 to 3 day delay in the timing of appearance of symptoms of large plants compared to medium and small plants.

Effect of inoculum dosage and type on mature plant resistance

Since systemic infection did not occur in large plants inoculated onto different sets of leaf pairs, an effort was made to overwhelm the resistance by an increase in inoculum dosage, *i.e.*, increase the number of leaves inoculated, and evaluate different sources of inoculum.

In this series of experiments, all inoculated leaves of small and large plants analyzed using ELISA were positive for CMV infection (**Table 3**). Inoculation of small plants with each of the CMV inocula (*i.e.* purified virus, or inoculum from systemically-infected tobacco or pepper) applied to either leaf 3 or leaves 1 through 6 resulted in 100%

Table 3. Evaluation of *Capsicum annuum* "Early Calwonder" plants to different amounts and types of Cucumber mosaic virus (CMV) inoculum

| Plant group | Source of inoculum | Inoculated leaves | Detection of CMV by ELISA in leaves ¹ | | SP ² | LP ³ |
|-------------|---------------------------------------|-------------------|--|------------------------|-----------------|-----------------|
| | | | Inoculated | Uninoculated | | |
| Large | Purified CMV (1 mg ml ⁻¹) | 3 | 0.895 ± 0.024 (15/15) a | (0/15) | (0/15) | — |
| | | 1-6 | 0.893 ± 0.043 (15/15) a | (0/15) | (0/15) | — |
| | Tobacco | 3 | 0.462 ± 0.13 (15/15) be | (0/15) | (0/15) | — |
| | | 1-6 | 0.889 ± 0.600 (15/15) a | 0.094 ± 0.000 (1/15) d | (0/15) | — |
| | Pepper | 3 | 0.877 ± 0.12 (15/15) a | 0.162 ± 0.108 (5/15) d | (0/15) | — |
| | | 1-6 | 0.624 ± 0.187 (15/15) b | 0.209 ± 0.086 (5/15) d | (0/15) | — |
| Small | Purified CMV (1 mg ml ⁻¹) | 3 | 0.853 ± 0.071 (15/15) a | 0.687 ± 0.07 (15/15) b | (15/15) | 5 |
| | | 1-6 | 0.641 ± 0.298 (15/15) b | 0.672 ± 0.21 (15/15) b | (15/15) | 5 |
| | Tobacco | 3 | 0.856 ± 0.053 (15/15) a | 0.797 ± 0.08 (15/15) a | (15/15) | 5 |
| | | 1-6 | 0.892 ± 0.010 (15/15) a | 0.841 ± 0.06 (15/15) a | (15/15) | 5 |
| | Pepper | 3 | 0.756 ± 0.079 (15/15) b | 0.670 ± 0.12 (15/15) b | (15/15) | 5 |
| | | 1-6 | 0.536 ± 0.046 (15/15) b | 0.462 ± 0.07 (15/15) c | (15/15) | 4 |

1. Mean ELISA absorbance values of leaf samples determined to be positive for the presence of CMV ± standard deviation. An ELISA value was considered positive when greater than the mean plus three standard deviations of comparable healthy samples (e.g. the highest thresholds for inoculated and uninoculated leaves of large plants were 0.042 and 0.053, respectively, while for small plants these values were 0.068 and 0.040). Numbers in parentheses are the number of CMV positive samples per number of plant samples tested. Within each column, values with the same letter are not statistically different (LSD with $\alpha = 0.05$). Inoculated leaf 3 was tested by ELISA for all plants at 7 dpi and uninoculated leaves were tested at 21 dpi.
2. Number of plants expressing CMV-induced systemic symptoms per number of plants inoculated (plants were monitored for symptoms up to 30 dpi).
3. Latent period: Days between inoculation and the expression of systemic symptoms.

systemic infection with development of severe systemic symptoms by 5 dpi (Table 3). Use of CMV-infected tobacco as inoculum resulted in significantly higher average ELISA absorbance values in uninoculated leaves of small plants compared to use of either purified CMV or CMV-infected pepper as inoculum. None of the large plants inoculated with purified virus, regardless of position or number of leaves, developed a systemic infection. Only a single large plant inoculated with CMV inoculum from tobacco onto leaves 1-6 contained detectable amounts of virus in uninoculated leaves, although the amount of virus in these tissues was low. When CMV-infected pepper was used as inoculum, five of 15 plants inoculated onto leaf 3 or leaves 1 through 6 contained detectable amounts of virus in uninoculated leaves; however, none of the plants developed observable symptoms and the amount of CMV detected by ELISA was significantly lower than observed in small plants. The difference observed in response to inoculation of large plants with the different inocula was not due to a difference in the amount of CMV

that occurred in the different inocula since a quantitative ELISA analysis indicated that the tobacco and pepper sap used as inocula each contained slightly more than 300 $\mu\text{g ml}^{-1}$ of virus (or more accurately CMV coat protein).

Nature of the resistance

The results presented thus far consistently showed that CMV accumulated to comparable levels in inoculated leaves of small, medium and large plants despite little or no detectable accumulation in uninoculated leaves of large plants. In an effort to more clearly define the nature of the resistance observed in large plants, two aspects of the infection process were examined: CMV accumulation through time in inoculated leaves, and CMV accumulation in inoculated leaf petioles and selected portions of the stem.

For the first series of experiments, we hypothesized that CMV accumulation in and subsequent movement out of the inoculated leaf of small plants may be a rapid process, whereas a delay in CMV accumulation in inoculated leaves of older plants may lead to restricted movement out of that leaf. When CMV was inoculated onto either leaf 3 or leaf 5 of small and large plants, virus was detected by 7 dpi in all inoculated leaves with similar levels of accumulation (**Table 4**). As in previous experiments, CMV was detected in uninoculated leaves of small

Table 4. ELISA absorbance values for the accumulation of Cucumber mosaic virus (CMV) and Pepper mottle virus (PepMoV) in inoculated leaves 3 or 5 of small and large *Capsicum annuum* 'Early Calwonder' plants at 7 dpi

| <i>Plant group</i> | <i>Leaf</i> | <i>Mean ELISA absorbance value</i> ¹ | |
|--------------------|-------------|---|-------------------------|
| | | <i>CMV</i> | <i>PepMoV</i> |
| Small | 3 | 0.503 \pm 0.099 (10/10) a | 0.383 \pm 0.094 (2/2) |
| | 5 | 0.576 \pm 0.115 (10/10) a | |
| Large | 3 | 0.482 \pm 0.089 (9/9) a | 0.646 \pm 0.025 (2/2) |
| | 5 | 0.520 \pm 0.098 (10/10) a | |

1. Mean ELISA absorbance values of leaf samples determined to be positive for the presence of CMV or PepMoV \pm standard deviation. An ELISA value was considered positive when greater than the mean plus three standard deviations of comparable healthy samples (*e.g.* the highest threshold for inoculated leaves of large plants was 0.028, while for small plants this value was 0.035). Numbers in parentheses are the number of CMV or PepMoV positive leaf samples per number of leaf samples tested. Means with the same letter are not statistically different (LSD with $\alpha = 0.05$).

plants but not in uninoculated leaves of large plants (data not shown). As a comparative test, plants were inoculated with PepMoV. In these plants, PepMoV was detected in inoculated (Table 4) and uninoculated (data not shown) leaves of small and large plants by 7 dpi.

The comparable accumulation of CMV in inoculated leaves of small and large plants over time suggested a block occurred in some subsequent step in the infection process. The occurrence of a block between the leaf blade and the petiole and stem was addressed by carrying out immuno-tissue blot analyses of the petiole of inoculated leaves and selected stem segments for detection of virus in tissues between the inoculated leaf and uninoculated leaves. This approach generates a greenish-colored print of the blotted stem segment with the colorimetric response of the virus-antibody reactions being brown. These colored differences are clearly observed in **Figure 2**, column 3. Each experiment also included a control treatment consisting of plants that were not inoculated with virus. In each case, a tissue print of the stem was clearly observed but with no colorimetric reaction indicating a lack of antigen.

In small plants inoculated with CMV, virus was detected in inoculated leaves of each plant, all tested segments of the petiole (data not shown) and in each segment of the stem by 7 dpi (Fig. 2, column 1). Based on the amount of labeling for each tissue print, the amount of CMV accumulation in each of the stem segments was extensive.

There was a range of responses in CMV-inoculated large plants. At one extreme, CMV was detected in inoculated leaves of all plants tested at 7 dpi but no virus was detected in petiole or stem segments. At the other extreme for the 7 dpi time analysis of large plants, CMV was detected in inoculated leaves, in all petiole segments tested and in the stem segment representing the internode below the inoculated leaf. At 21 dpi, some large plants contained no detectable amounts of CMV in the stem even though virus was detected in the inoculated leaf and in the petiole. When CMV was detected in the stem of large plants at 21 dpi, the extent of movement and accumulation within the stem varied from plant to plant. In some plants, CMV was detected only in lower stem segments, whereas other plants contained virus throughout the length of the stem (Fig. 2, column 2) but with dramatically reduced levels of accumulation compared with small plants, especially in upper portions of the stem (Fig. 2C). The CMV-infected plant presented in Fig. 2 (column 2) at 21 dpi represents one of the more extensive amounts of CMV accumulation observed among the samples

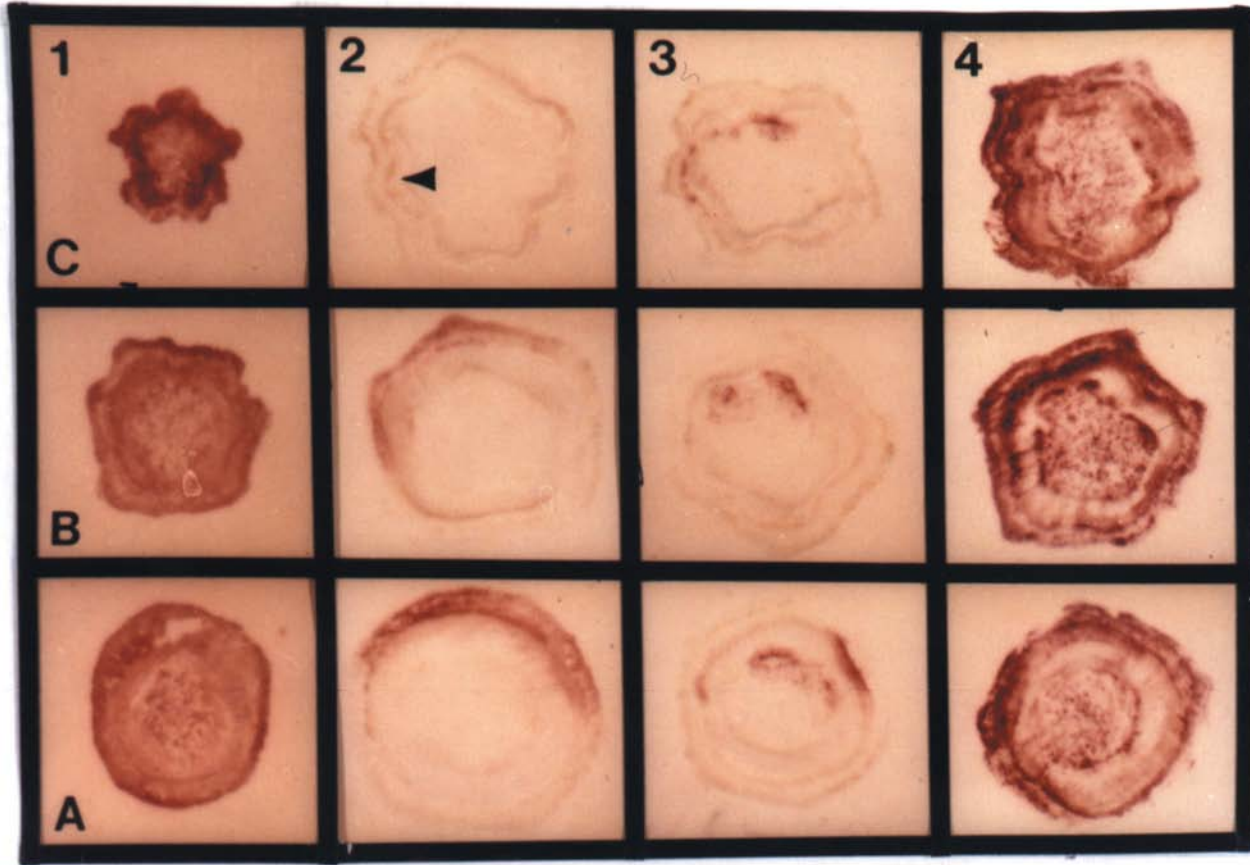


Fig. 2. Immuno-tissue blot analysis of Early Cal wonder stem segments for the presence of *Cucumber mosaic virus* and *Pepper mottle virus*. Prints in column 1 represent CMV-infected small plants at 7 dpi, column 2 represent CMV-infected large plants at 21 dpi, columns 3 and 4 represent PepMoV-infected large plants at 7 and 21 dpi, respectively. Tissue prints were generated from three locations along the stem: (A) the internode immediately below inoculated leaf 3, (B) the internode immediately above inoculated leaf 3 and (C) the upper most internode along the main stem. The arrowhead points to the location of a small amount of label representing detection of CMV in the upper internode of a large plant at 21 dpi.

tested. Regardless of the location along the stem in which CMV was detected, labeling always occurred along the side of the stem to which the inoculated leaf was attached and virus never occurred over the entire width of the stem as seen in small plants at 7 dpi.

In contrast to the extent of CMV movement and accumulation in stems of large plants, PepMoV was detected in all stem segments tested in large plants by 7 dpi (Fig. 2, column 3). By 21 dpi, PepMoV accumulated to high levels throughout the length and width of the stems of large plants (Fig. 2, column 4).

Discussion

We have shown that upon reaching a relatively specific stage of development, pepper plants grown in greenhouse conditions express mature plant resistance to infection by CMV. While the actual degree of this resistance was somewhat variable in its effect on CMV infection, the resistance was very consistent with regard to a lack of detection of CMV in young, uninoculated leaves and a complete lack of any apparent symptoms. This mature plant resistance occurred in tests carried out in different seasons and was not affected by inoculum dosage but was affected to a limited extent by the source of inoculum (e.g. pepper as illustrated in Table 3).

CMV consistently accumulated in inoculated leaves of large plants to levels comparable to those of small plants. The level of accumulation in inoculated leaves of large plants strongly suggests that CMV was not restricted in its ability to move from cell to cell within that leaf. Furthermore, time-course studies indicated that the relative timing of accumulation of CMV in inoculated leaves of large plants did not differ from that of small plants. Despite the apparent complete susceptibility of inoculated leaves of large plants, subsequent stages in the CMV infection process, i.e. movement into and through the stem and accumulation in uninoculated leaves, were severely affected.

The mature plant resistance against CMV appeared to be directed at movement of virus within and through the stem. While CMV accumulation in inoculated leaves, based on ELISA analysis of whole leaf tissues, occurred at the same relative rate in small and large plants, detection of virus and the extent of its movement within the stem were delayed when compared with small plants. In large plants, CMV moved through varying lengths of the stem, and regardless of the length of the stem traveled, accumulation remained fairly localized to the side of the stem to which the inoculated leaf was attached. In contrast, accumulation of CMV in small plants and PepMoV in large plants occurred throughout the width of the stem and appeared to include all tissue types (Andrianifahanana *et al.*, 1997; this report). Since CMV did accumulate in and move through the stem of some large plants, it would seem likely that subsequent steps in the systemic infection process, such as movement into and accumulation in young leaves, would also occur. However, based on detection of CMV by ELISA, infection of young leaves either did not happen or occurred at levels below the

limits of detection. It is possible that the mechanism(s) within the plant that limits the CMV infection process affects the ability of the virus to exit the phloem once in uninoculated leaves, restricts cell-to-cell movement in uninoculated leaves, reduces accumulation of virus at the cellular level or combinations thereof. The resistance in large plants may reflect a change in host physiology (Leisner *et al.*, 1993), an induced systemic resistance response (Naylor *et al.*, 1998; Hamerschmidt, 1999) or even post-transcriptional gene silencing (Smith *et al.*, 1994; Mueller *et al.*, 1995; Goodwin *et al.*, 1996; van den Boogaart *et al.*, 1998). However, if induced systemic resistance was responsible, one might expect a similar response to PepMoV. Since no resistance was observed against PepMoV, host physiology and induced systemic resistance (assuming the induced resistance is not to a specific virus) are unlikely to be key components in the mature plant resistance expressed in pepper plants against CMV. Interestingly, the two viruses used in this study, CMV and PepMoV, have been shown to suppress post-transcriptional gene silencing (Anandalakshmi *et al.*, 1998; Beclin *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau & Carrington, 1998). Whether the ability of PepMoV to systemically infect large Early Calwonder plants resulted from suppression of gene silencing, whereas CMV was not able to counteract the silencing mechanism was not determined but certainly would be a worthy study to pursue.

We did not evaluate the response of mature pepper plants to CMV infection at the cellular level (e.g. accumulation in protoplasts); however, the data suggest a restriction in virus movement. Plant resistance directed at virus movement is a common phenomenon (Lei & Agrios, 1986; Dufour *et al.*, 1989; Law *et al.*, 1989; Goodrick *et al.*, 1991; Nono-Womdim *et al.*, 1991; Simon *et al.*, 1992; Wilson & Jones, 1992; Nelson *et al.*, 1993; Murphy & Kyle, 1995; Schaad & Carrington, 1996; Derrick & Barker, 1997; Guerini & Murphy, 1999; Hämäläinen *et al.*, 2000). Several investigations have shown that virus is restricted in its ability to either enter phloem, exit phloem or combinations thereof (Dufour *et al.* 1989; Goodrick *et al.* 1991; Simon *et al.* 1992; Schaad & Carrington, 1996; Derrick & Barker, 1997).

A restriction in virus movement in pepper was described by Dufour *et al.* (1989), Nono-Womdim *et al.* (1991) and Guerini & Murphy (1999). Dufour *et al.* (1989) showed that in resistant pepper plants, CMV infection spread into the stem but remained in lower portions of the stem rather than moving up the stem to young tissues. Microscopy

analyses showed that CMV occurred in external but not internal phloem tissues in the petiole and stem (Dufour *et al.*, 1989). Non-Womdim *et al.* (1991) showed that CMV accumulated to similar levels in susceptible and resistant pepper varieties but systemic movement was limited to lower portions of the plant in the resistant variety. Guerini & Murphy (1999) described a similar phenomenon in a resistant variety of pepper where PepMoV was localized to lower portions of the stem; however, co-infection with CMV alleviated the restricted movement of PepMoV. They concluded that the block in movement for PepMoV was associated with entrance into internal phloem for rapid movement up the stem to young tissues (Guerini & Murphy, 1999).

Our results describing mature plant resistance in pepper to CMV are similar to the observations described by Dufour *et al.* (1989) and Guerini & Murphy (1999). A general lack of CMV movement into the stem, or upon reaching the stem, the spread of infection throughout the stem was slow and levels of accumulation were much less than in young plants or for PepMoV in mature plants. We have not been able to discriminate whether CMV accumulated in external versus internal phloem in the stem (Guerini & Murphy, 1999; this report) as reported for potyviruses (Andrianifahanana *et al.*, 1997) and as seen in this report for PepMoV. Whether the observed restriction in movement of CMV in mature pepper plants was due to virus occurring in external but not internal phloem was not determined. Such information may be important, however, since the type of phloem used by CMV to translocate the stem of large plants may help to explain mature plant resistance and further substantiate previous findings about the pathway of virus movement through the stem of pepper plants (Andrianifahanana *et al.*, 1997; Guerini & Murphy, 1999).

Mature plant resistance was identified as a key factor associated with reduced virus infection and yield losses in crops evaluated under field conditions (Knutson & Bishop, 1964; Ross, 1969; Loebenstein, 1972; Demski & Chalkley, 1974; Scott *et al.*, 1977; Rosenkranz & Scott, 1978; Thongmeearkom *et al.*, 1978; Pasko *et al.*, 1984; Agrios *et al.*, 1985; Avilla *et al.*, 1997; Bosque-Perez *et al.*, 1998; Lot *et al.*, 1998; Soler *et al.*, 1998). As a management tool, mature plant resistance has been incorporated into cultivation practices by altering the planting date of a crop in an attempt to avoid the occurrence of young plants in the field at the time of pathogen arrival. The basic premise being that plants that are young at the time of infection are more susceptible to

infection (Pasko *et al.*, 1984; Dogimont *et al.*, 1994) and often express a more severe disease, whereas mature plants often resist infection or express only mild disease symptoms (Agrios *et al.*, 1985; Sikora *et al.*, 1998). Our data argue against the concept of young plants being more susceptible, at least at the level of the inoculated leaf; however, our data strongly agree with observations related to disease severity. Our findings, in conjunction with studies showing that reduced virus titers in leaves result in a reduction in acquisition efficiency of aphid vectors (Stimmann & Swenson, 1967; Banik & Zitter, 1990; Gray *et al.*, 1993), and that plants that are symptomless or expressing only mild symptoms are less attractive to insect vectors (Power, 1992; Eckel & Lampert, 1996) support recommendations to integrate mature plant resistance into schemes to manage CMV in pepper.

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