**The Nature of Cucumber Mosaic Virus-Induced Symptoms
in Bell Pepper (*Capsicum annuum* L.)**

by

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Abstract

We determine phenotypic and cytological effects on pepper plant growth and development in response to systemic invasion by CMV and evaluate a CMV strain, mutated in the gene silencing suppressor gene, *2b*, for its ability to infect pepper plants. We show that symptom development in CMV susceptible 'Calwonder' pepper plants occurred in three phases, each associated with a different host plant developmental stage. The initial expression of systemic symptoms occurred in young leaves in the form of a chlorosis over the basal half of the leaf at ca. 7 dpi. As new leaves emerged, ca. 12 dpi, these leaves developed a mosaic symptom expressed over the entire blade. Leaves expressing both symptom phases had high CMV titers. When the primary stem branched into two secondary stems, the leaves that developed on each secondary stem had a dull green appearance with varying degrees of distortion. These leaves accumulated significantly less CMV than leaves in the early symptom phases. Leaves that emerged after the leaf distortion phase were smaller and had a dull green color relative to comparable leaves on healthy plants. These newly emerging leaves had little or no detectable amounts of CMV. The apparent recovery from CMV infection in young leaves coincided with dramatically reduced and localized accumulations of CMV throughout the stem. CMV-infected plants were stunted relative to healthy plants. We show that the stunting occurred only along the primary stem during and shortly after chlorosis and mosaic symptom phases but not in secondary stems in the leaf distortion phase. The

CMV 2b mutant was able to accumulate in inoculated leaves of pepper plants but did not establish a systemic infection. The accumulation of this mutant was significantly less in inoculated leaves than for the wild type parent. Mesophyll protoplasts isolated from 'Calwonder' leaves were inoculated with RNA isolated from the CMV 2b mutant or its wild type. Less CMV coat protein accumulated in CMV 2b mutant-inoculated protoplasts compared with those inoculated with the wild type. These findings indicate that the CMV 2b gene/protein is required for systemic infection of pepper plants and accumulation (translation/replication) at the cellular level.

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List of Abbreviations

CMV   Cucumber Mosaic Virus

ELISA   Enzyme-Linked Immunosorbent Assay

PTGS   Post-Transcriptional Gene Silencing

PCR   Polymerase Chain Reaction

RT-PCR   Reverse Transcription-Polymerase Chain Reaction

1   Literature Review

1.1   The pepper plant

Peppers are a commonly-grown crop worldwide due to their ability to grow and yield fruit in a wide range of climates (Smith and Heiser, 1951). Peppers belong to the *Solanaceae* family and the genus *Capsicum* which are known to include 25 species (Pernezny et al., 2003). Most pepper cultivars commercially cultivated in the United States belong to the species *C. annuum* L. and *C. frutescens* L. which are also the most economically important species in the world (Bosland et al., 1996). The bell pepper belongs to the species *C. annuum* and is considered one of the most economically important and widely cultivated species in the United States with more than twice the acreage planted to sweet pepper types as to those considered pungent. According to Bosland et al. (1996), the name bell pepper refers to its blocky shape, although it is not known when the name was applied. There are many bell pepper cultivars with most baring fruit that are sweet and used as fresh produce as opposed to the pungent species, *C. frutescens*. Bell pepper fruits are characteristically 3 to 4 inches in diameter and 4 to 5 inches long. In the United States, fruit shape consisting of four lobes are commercially preferred (Bosland, 1996). The color of the fruit can be green, orange, purple, red or yellow with a 75% increase in consumption of orange, purple, red and yellow fruits over the green bell in the United States (Bosland, 1996; Bosland et al., 1996; Smith and Heiser, 1951).

The bell pepper is a warm-season, shallow-rooted crop that can be grown as an annual or perennial (Pernezny et al., 2003). A deep, well-drained, medium-textured loam or sandy loam with a pH of 7.0-8.5 is considered the best soil type to grow peppers (Pernezny et al., 2003).

According to Edwardson and Christie (1997), peppers grown worldwide are susceptible to plant viruses. They indicated (at that time) that at least 99 viruses were reported to infect pepper. Fifty-six of the 99 viruses infecting peppers are virus species representing 24 genera of nine families, while 29 species occur in genera not yet assigned to families, and 15 viruses infecting peppers have not been assigned to either genera or families. A more recent listing grouped viruses by their vector and mode of transmission (Pernezny et al., 2003).

1.2   Plant virus infection

Plant viruses containing a positive-sense RNA genome share similarities in their infection process: (*i*) translation of viral-encoded proteins; (*ii*) replication of the viral genome; and (*iii*) movement to (and infection of) neighboring cells (Fig. 1.1). These processes are affected by the compatibility of the virus and its host, as well as by environmental conditions such as temperature and light (Beemster, 1987).

Plant virus infection can have dramatic effects at the cellular level, leading to host physiological alterations such as decreased photosynthesis, increased respiration, accumulation of nitrogen compounds, and expanded oxidase activities (Culver and Padmanabhan, 2007; Lewsey and Carr, 2009). Plant virus infections are often referred to

as local or systemic. Local infections typically refer to either a single cell or group of cells in which virus replicates and accumulates or, for example, within the inoculated leaf. Systemic infections typically refer to the spread of virus away from the leaf or tissue that was originally infected. The degree or extent of systemic infection can vary, e.g., to lower or upper portions of the plant (in the case of herbaceous host) or throughout the plant (White and Antoniw, 1991).
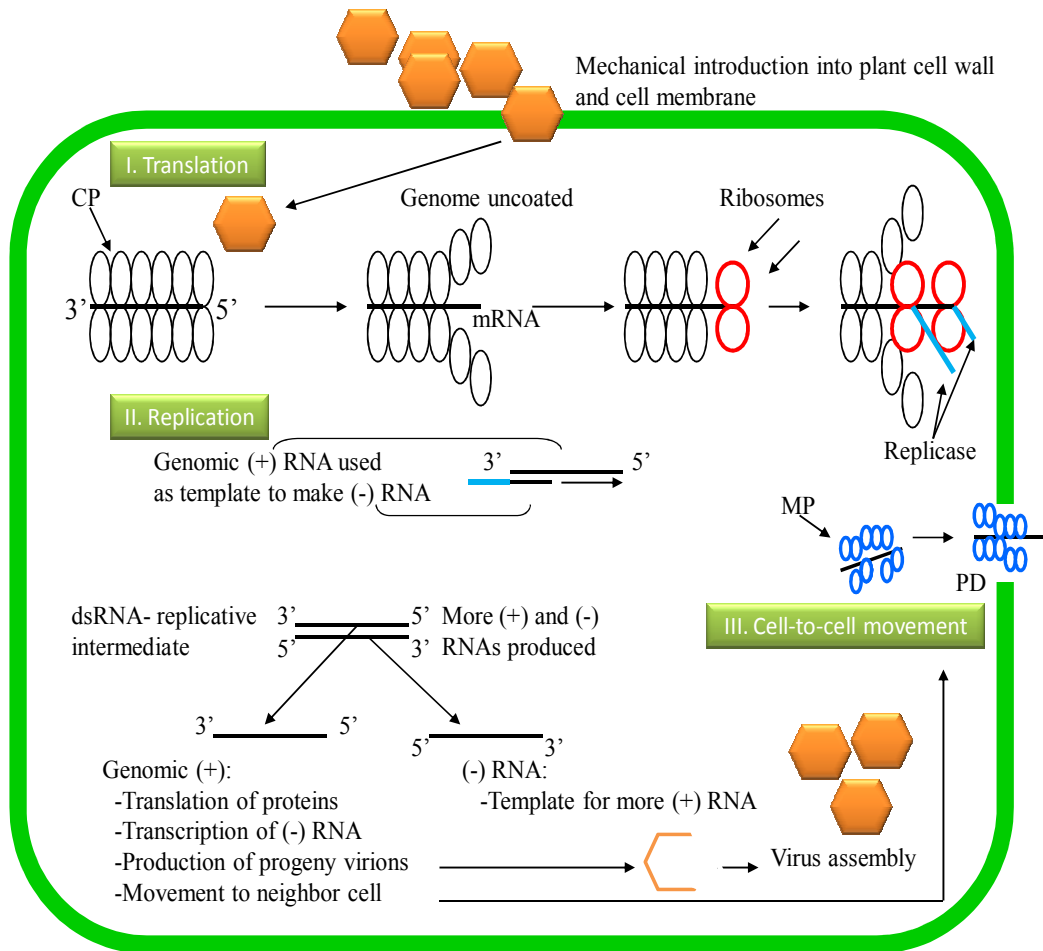
Figure 1.1 Schematic diagram of infection cycle of a plant virus with a positive-sense RNA genome.

Translation of viral-encoded proteins and replication of the viral genome

The infection process begins when a virus particle, i.e., the viral nucleic acid and its protective coat protein, is introduced into a cell through injury or by a vector (Siegel and Zaitlin, 1964). In the case of viruses with a RNA genome (the focus of this report), the viral RNA or a portion of the viral RNA is translated by host ribosomes to produce one or more viral proteins. One or more of these proteins is involved in replication of the viral genome. Viral polymerase(es) uses the viral genome as a template to synthesize negative sense viral RNA, a non-translatable complimentary strand to the genomic RNA. The negative sense RNA is then used as a template for generation of genomic-sense RNA. Nagy (2008) proposed that during genome replication, the steps of a single replication cycle of (+) RNA viruses include the following: "(*i*) the recruitment/selection of the viral (+) RNA template for replication, including a requirement for switching of the genomic RNA from translation to replication; (*ii*) targeting of viral replication proteins to the site of replication; (*iii*) preassembly of the viral replicase components; (*iv*) activation/final assembly of the viral replicase complexes containing the (+) RNA template on intercellular membraneous surfaces; (*v*) synthesis of the viral RNA progeny by the replicase complexes, including minus- and plus-strand synthesis; (*vi*) release of the viral (+) RNA progeny from the replicase complexes to the cytosole; and (*vii*) disassembly of the viral replicase complexes.".

An essential next step in the infection cycle of RNA viruses in plants is the assembly process of mature virions for movement in the vascular tissues of the plant (shown to be the case for some viruses) and for further infections outside the plant (Atabekov and Dorokhov, 1984; Noueiry and Ahlquist, 2003). The assembly of

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infectious progeny virion is referred to as packaging or encapsidation of the viral genome by structural protein components. Rao (2006) stated that "once viruses have been assembled, the resulting virion architecture has three primary roles: (*i*) to be stable enough to survive as an infectious particle in the external environment required for transmission between host individuals; (*ii*) to be flexible enough to disassemble upon entering uninfected host cells facilitating release of the infectious genome; and (*iii*) to provide an optimal interaction with the host machinery for the inception of successful infection.".

Cell-to-cell movement

An important component of a successful infection by a virus depends on its capacity to move within a plant. In susceptible hosts, especially, the plant becomes systemically infected when the virus replicates and moves from the initially infected cells to surrounding cells, and then to other distant tissues.

Cell-to-cell movement of virus occurs via plasmodesmata found in different types of plant tissues (Hull, 1989; Maule, 1991; Waigmann et al., 2004). These cytoplasmic bridges connecting adjacent cells are the passageway in which virus (i.e., its infectious nucleic acid or virion, depending on the virus) moves from the initially infected cell to surrounding cells (Lucas and Gilbertson, 1994). From the infection site in an epidermal cell, the virus must move from cell to cell sequentially into mesophyll, bundle sheath, and then phloem parenchyma and companion cells (Carrington et al., 1996). Plant viruses encode one or more nonstructural proteins whose function is directly tied to virus movement within its host. Both nonstructural and structural viral proteins are often

required for the various stages of movement through the plant (Hull, 2002). For *Cucumber mosaic virus* (CMV), for instance, movement involves the nonstructural 3a movement protein, as well as the coat protein (Boccard and Baulcombe, 1993; Canto et al., 1997; Ding et al., 1995a; Kaplan et al., 1995; Li and Palukaitis, 1996; Suzuki et al., 1995; Taliansky and Garcia-Arenal, 1995). Some reports have indicated involvement of replication-associated proteins (Ding et al., 1995b; Gal-On et al., 1994).

The 3a protein encoded by CMV RNA 3, is a movement protein in all CMV hosts. It cooperatively binds to the viral RNA and forms a complex capable of increasing the permeability of mesophyll and epidermal plasmodesmata allowing the infectious particles to move from cell to cell until infection reaches the vascular tissues (Ding et al., 1995a; Kaplan et al., 1995; Li and Palukaitis, 1996; Palukaitis and Garcia-Arenal, 2003). Tubules composed of the 3a movement protein were identified on the surface of CMV-infected plant protoplasts (Kaplan et al., 1998; Palukaitis and Garcia-Arenal, 2003). However, the capacity to form such tubules appears to be required for cell-to-cell movement between particular cell types such as epidermal tissues. These tubule formations may be only transient structures in cells where they were observed (Astier, 2007), and they may also be required to promote movement within certain host tissues (Canto and Palukaitis, 1999).

In addition to the 3a movement protein, CMV also requires the coat protein for efficient movement (Boevink and Oparka, 2005; Canto et al., 1997; Carrington et al., 1996; Kaplan et al., 1998; Ryabov et al., 1999). A deletion of either the CMV 3a movement protein or the coat protein did not negatively affect virus replication in protoplasts; however, virus was unable to move in plants, indicating a specific

compatibility of both proteins that affect cell-to-cell movement (Palukaitis and Garcia-Arenal, 2003).

For many viruses, CMV for example, a ribonucleoprotein complex that contains the viral RNA, coat protein and movement protein rather than the virions is the infectious form involved in movement (Blackman et al., 1998; Ding et al., 1995a). CMV virions were not observed inside plasmodesmata connecting infected cells (Ding et al., 1995a). The virions were not even seen inside plasmodesmata between companion cells and sieve elements (Blackman et al., 1998).

Phloem-dependent or long-distance movement

As virus infection spreads from cell to cell in the initially infected leaf, the virus eventually encounters vascular tissue resulting in (typically) a rapid form of movement throughout the plant referred to systemic movement (Atabekov and Dorokhov, 1984). Cell-to-cell movement of virus leads to infection of cells within the vascular bundles with systemic infection through the plant occurring via the phloem, often referred to as phloem-dependent or long-distance movement. The movement of virus from the infected leaf to other leaves, roots and organs occurs through vascular parenchyma or companion cells, specialized parenchyma cells located in the phloem cell, with rapid movement occurring in phloem sieve elements. The general pattern of movement through the plant occurs as a source-to-sink pattern (Carrington et al., 1996; Leisner et al., 1992; Turgeon, 1989) which follows the movement of photoassimilates from mature, exporting tissue as sources to immature, developing tissues that exert a carbon demand as sinks.

In the case of CMV, at least one protein encoded by each of the CMV RNA species has been reported to be involved in movement. The coat protein is required for cell-to-cell and systemic movement (Boccard and Baulcombe, 1993; Suzuki et al., 1995; Taliansky and Garcia-Arenal, 1995). CMV coat protein-mediated movement has host-specific determinants for systemic movement and is involved in passage of virus through the bundle sheath-intermediary cell interface boundary and entrance into the phloem for systemic transport (Palukaitis and Garcia-Arenal, 2003). The 1a replicase protein was involved in the differential rate of systemic symptom development which resulted from a difference in the rate of movement rather than the rate of replication of the virus in zucchini squash (Gal-On et al., 1994). The 2b protein was shown to be involved with systemic movement in cucumber (Ding et al., 1995b), although it isn't clear whether this protein is actually involved in movement or facilitated movement as a gene silencing suppressor.

In contrast to cell-to-cell movement in which viral RNA associated with one or more movement proteins is believed to be the infectious form for movement, CMV, as well as other viruses, are thought to move as intact virion through phloem tissues for systemic infection (Palukaitis and Garcia-Arenal, 2003; Seron and Haenni, 1996).

Plant-to-plant movement

Acquisition and dissemination of a plant virus to new hosts occurs by a wide range of biological vectors such as aphids, leaf hoppers, nematodes, mites or fungi (Astier et al., 2007; Chen and Francki, 1990; Chen et al., 1995; Rao, 2006). Some viruses may be transmitted on or through seed, by pollen or through vegetative tissues (Dawson

and Hilf, 1992). Entry of a virus into the plant cell mainly occurs through a non-lethal wound created by a vector during feeding on host plant tissues. For experimental purposes, many viruses are applied to leaves by wiping inoculum across the surface of the leaf. An artificial wounding process is created by addition of an abrasive, such as carborundum, which creates non-lethal lesions through which virus enters to initiate infection. Transmission of positive-sense RNA viruses by insect vectors requires one or more viral proteins with involvement of coat protein being essential in all cases since virion is the infectious entity acquired and transferred by insect vectors (Lewsey and Carr, 2009). In the case of CMV, the coat protein is the sole determinant for transmission by aphid vectors (Chen and Francki, 1990; Ng et al., 2005; Pirone and Perry, 2002).

1.3   Virus-host interactions result in visible symptoms

Virus-induced symptom expression in plants varies with the virus, host and environment conditions, to name just a few factors involved in this complex interaction. Symptoms associated with the site of infection include chlorotic or necrotic lesions which appear two or more days after the infection is initiated (Roberts et al., 1997; Siegel and Zaitlin, 1964). Systemic infections may lead to chlorosis, mosaic or mottle patterns on leaves, stunting and dwarfing of the entire plant or portions of the plant, ringspots on leaves, stems and fruit, necrosis on leaves, stems and fruit, leaf and stem deformation, as well as developmental abnormalities of flowers, fruit, seed and pollen (Culver and Padmanabhan, 2007; Walkey, 1991).

According to Culver et al. (1991), chlorosis and necrosis are the most common symptoms associated with viral infections. Chlorotic symptoms are usually associated with a disruption of chloroplast in terms of their structure, function, and development leading to inhibition of photosynthetic activity. The symptoms usually appear as yellowed areas in expanded leaves, or as a mosaic pattern of dark green mixed with light green, yellow, or white areas in leaves that developed after infection. Leaves with mosaic symptoms consist of normal appearing dark green tissue containing little or no virus, surrounded by light green tissue containing high virus titers. These dark green islands appear to be resistant to infection of the same or closely related viruses (Lewsey and Carr, 2009) and a proportion of plants regenerated from protoplasts isolated from this tissue were virus-free (Dawson, 1999). Chlorosis is the main symptom associated with the economically important yellowing viruses: beet yellows virus, barley and cereal yellow dwarf viruses.

A necrotic local lesion develops from a hypersensitive response by the host to the pathogen. This response restricts virus movement to the vicinity of the lesion, although in at least one case, it was shown that the restricted movement did not result from the necrotic response but a distinct resistance mechanism (Culver et al., 1991; Kim and Palukaitis, 1997; Lewsey and Carr, 2009). When a necrosis-inducing virus is not limited to the initially infected area but able to move to other parts of the plant, vascular necrosis typically results. This systemic necrosis develops in response to the presence of the virus and, therefore, develops throughout the plant as the virus spreads often leading to plant death (Culver et al., 1991; Walkey, 1991).

Systemic infection of a virus may cause developmental abnormalities such as stunting of the plant or portions of the plant and various leaf deformations. These striking

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symptoms appear to result from disruption in the distribution of growth regulators such as

auxins and cytokinins (Culver and Padmanabhan, 2007; Lewsey and Carr, 2009).

2 Cucumber Mosaic Virus (CMV)-Induced Symptoms in Bell Pepper Plants (*Capsicum annuum* L.)

2.1 Introduction

Plant virus diseases cause considerable economic losses in crop plants worldwide. Vegetable crops are persistently threatened by viruses, although the prevalence and severity of their outbreaks can vary from year to year. Plants infected by viruses show a reduction in plant growth and vigor, resulting in a decline in quality or market value of plant organs, in particular, fruits and seeds (Hull, 2002; Hull, 2009). The most common apparent effects of virus infection are plant stunting. The plants not only have a reduction in overall plant size but also have smaller leaves, flowers, fruits and roots than those of a healthy plant. The stunting symptom typically results from shortening of stem internodes and leaf petioles. In addition, induction of mosaic patterns and related symptoms can also lead to poor fruit quality and marketable yield.

Cucumber mosaic virus (CMV) is one of the most common viruses found in vegetable crops. CMV is the type member of the genus *Cucumovirus* in the family *Bromoviridae* (International Committee on Taxonomy of Viruses, 2002). It is considered a potential threat to a large variety of crop plants in tropical and temperate climates due, in part, to its extensive natural host range and its ability to be transmitted by more than 80

species of aphids in a non-persistent manner (Edwardson and Christie, 1997; Kaper and Waterworth, 1981; Palukaitis and Garcia-Arenal, 2003; Palukaitis et al., 1992). CMV induces a variety of symptoms, depending on host plant species and virus strain, although two highly characteristic symptoms include leaf deformation (often referred to as strap-leaf or shoestring symptoms) and stunting of plant growth (Hellwald et al., 2000; Palukaitis et al., 1992; Sclavounos et al., 2006; Szilassy et al., 1999).

CMV causes an abnormal distribution of chlorophyllous pigments in the leaves, often associated with an alteration of the structure of chloroplasts referred to as mosaics (Hull, 2002; Hull, 2009). This irregular coloration is particularly clearly visible on young leaves and is sometimes accompanied by leaf deformations. Various types of mosaic can be seen; however, typically light green or yellow is interspersed with dark green areas over portions of or the entire leaf (Astier, 2007; Hull, 2009; Walkey, 1991). Growth reduction induced by CMV is accompanied by small leaves and short internodes, as well as small or few flowers, fruits, or seeds. CMV is also responsible for deformation of leaves, resulting in a characteristic filliform or shoe-string shape that can occur in numerous plant species, including pepper, tobacco and tomato. Typical symptoms induced by CMV which have been reported in different crop plants worldwide are mosaic in cucumber, melon and other cucurbits; blight in spinach; mosaic, fern leaf and systemic necrosis in tomato; mosaic and ringspot in pepper; mosaic and stunting in clover, lupins and lucerne; stunting in soybean; mosaic, chlorosis and heart rot in banana; and mosaic and dwarfing in many other species of dicotyledonous and monocotyledonous plants (Kaper and Waterworth, 1981; Palukaitis et al., 1992).

There are two subgroups of CMV strains, I and II, which are categorized based on their distribution, virulence and/or host range. Generally, subgroup I strains may be more heterogeneous and more virulent than subgroup II strains. They may also have different host ranges from subgroup II strains (Palukaitis et al., 1992; Zhang et al., 1994). Subgroup I can be further divided into subgroups IA as well as IB (Roossinck et al., 1999).

In pepper plants, varying degrees of severity of mosaic symptoms induced by CMV occurred in young leaves, whereas older leaves expressed large necrotic rings (Agrios and Walker, 1985). The appearance of conspicuous yellow concentric ringspots, as well as fruit distortion, was observed for infected pepper plants (MacNab et al., 1983). CMV caused distinct phases of symptom expression that coincided with host plant developmental stages in 'Early Calwonder' pepper plants (Murphy and Bowen, 2006). This included symptom remission in later stages of plant growth. In tobacco plants infected with the CMV Fast New York strain (-Fny, subgroup IA), a cyclical infection occurred in which some leaves expressed typical symptoms with high virus titers, whereas other leaves were symptomless with little or no virus (Gal-On et al., 1995). This cyclical infection phenotype did not occur with a closely related CMV strain. It is not uncommon for plants to recover, to varying degrees, from virus infection (Baulcombe, 2004; Kalantidis et al., 2002; Ratcliff et al, 1997; Xin and Ding, 2003), a response thought to result from post-transcriptional gene silencing (PTGS) which functions as a natural defense mechanism against virus infection in plants (Carrington et al., 2001; Covey et al., 1997; Lindbo et al., 1993; Lucy et al., 2000; Vance and Vaucheret, 2001; Voinnet, 2001).

In this study, we demonstrate that systemic invasion induced by CMV is responsible for phenotypic and cytological changes on Calwonder pepper plant growth and development. We analyzed foliar symptoms as well as the accumulation of the virus at different times after inoculation in leaves and stems of the plant and examined plant growth characteristics as disease assessments. We also show that anatomical and immunohistochemical effects appear to be directly correlated with each type of symptom and the amount of virus in different parts of Calwonder plants.

2.2   Materials and Methods

Phenotypic effects on pepper plant growth and development in response to systemic invasion by CMV.

Plant material and growth conditions. All experiments (spring, summer, and fall 2007) were performed in the Plant Science Research Facility, a temperature controlled greenhouse (mean temperatures of 24°C day and 20°C night throughout the year), located on the Auburn University campus. *Capsicum annuum* L. cv. 'Calwonder', a susceptible host to CMV, was used for all experiments. 'Calwonder' seeds were sown in 72-well Styrofoam trays (Speedling, Inc., Bushnell, FL) in Pro-Mix, soil-less potting medium (Premier Peat, Riviere-du-Loup, Quebec, Canada). Upon germination to the early two-leaf stage, seedlings were transplanted to one-gallon pots (one plant per pot) containing Pro-Mix. The growth medium was supplemented with a controlled release fertilizer 18-6-12 (Osmocote Classic, Scotts Company LLC, Marysville, OH). Plant growth stage designations for inoculation and sampling were as described by

Adrianifahanana et al. (1997). Briefly, leaves emerged in pairs on opposite sides of the stem with each successive leaf pair emerging nearly perpendicular to the previous leaf pair. The oldest leaf along the stem was designated leaf 1 with each subsequent leaf numbered sequentially.

Experimental design and statistic analysis. Experiments were carried out in a randomized complete block design. The two treatments, CMV-inoculated and mock-inoculated, were arranged randomly in four blocks. Each block consisted of 20 and 10 plants of CMV-inoculated and mock-inoculated, respectively. Thus, a total of 80 CMV-inoculated and 40 mock-inoculated plants were used for each experiment. The ELISA absorbance values, plant height, and total fresh weight of aboveground tissues were analyzed with analysis of variance (SAS, Cary, NC) and for significant ANOVA treatment means separated using LSD mean separation tests.

Viral strain and inoculation procedures. The CMV Fny strain (subgroup I) was kindly provided by Dr. P. Palukaitis (Cornell University, Ithaca, NY). Virus was maintained by mechanical passage in tobacco (*Nicotiana tabacum* L.) cv. 'Kentucky 14' and squash (*Cucurbita pepo* L.) cv. 'Dixie' grown in an environmentally-controlled greenhouse at the Plant Science Research Facility. CMV inoculum consisted of systemically infected 'Kentucky 14' or 'Dixie' leaf tissues ground in 50 mM potassium phosphate buffer, pH 7.3, containing 10 mM sodium sulfite. A mock-inoculated treatment was performed using buffer alone. All materials used for the inoculation process, such as buffer, mortars, and pestles, were chilled before use and kept on ice during inoculation. 'Calwonder' plants were inoculated with CMV by mechanical-rub inoculation using inoculum-saturated cheesecloth. Plants were inoculated when at the

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early 7 to 8 leaf stage in the spring 2007 trial and early 5 to 6 leaf stage in the summer and fall 2007 trials. For each experiment, virus was applied to leaves 1 and 2.

Virus infection evaluations. CMV-inoculated plants were monitored daily for development of symptoms. Time of symptom appearance, and severity were recorded throughout the course of each of the experiments. Symptoms varied with the stage of pepper plant growth at the time of symptom expression, as documented previously (Murphy and Bowen, 2006). Symptom description provided in this report follows a similar format.

Growth measurements consisted of plant height and aboveground fresh weight. Plant height (cm) represented the difference between the initial height determined the day of inoculation and height at five-day intervals with the final measurement taken at 35 days post-inoculation (dpi). Height was measured from stem base at the soil line to the apical bud. The total number of leaves per plant and total fresh weight of aboveground tissues (g), excluding fruit, was measured at 40 dpi. Additional growth parameters included time and leaf number to initiation of stem branching, time to first flower and fruit development.

Virus accumulation in the stem was determined by immuno-tissue blot analysis (Andrianifahanana et al., 1997; Guerini and Murphy, 1999). Tissue prints were generated by pressing the cut surface of stem and petiole sections directly onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH) that were treated with 0.2 M $CaCl_2$ prior to blotting (Holt and Beachy, 1991). Three CMV-inoculated plants and one mock-inoculated plant were tested for each sampling period. Each stem segment used to

generate a tissue print was from a central position of the selected internode and included internodes immediately below the inoculated leaves and each internode above the inoculated leaves. Tissue prints were also generated from the petiole of inoculated and non-inoculated leaves. For the spring 2007 trial, tissue prints were generated at 3, 4, 5 and 7 dpi and at 5, 10, 15, 20, 25, and 30 days after the onset of symptoms. The summer and fall 2007 experiments were conducted based on occurrence of distinct symptom phases as described by Murphy and Bowen (2006). Tissue prints were allowed to dry at room temperature and then stored at 4°C until serological analysis.

The detection of CMV in tissue prints was by immune-tissue blot analysis as described by Guerini and Murphy (1999). Membranes were incubated in 5% powdered milk dissolved in Tris-buffered saline (TBS; 50 mM Tris-HCl at pH 7.4, and 200 mM NaCl) at room temperature for 1 hr with gentle shaking. Following several rinses with TBS, membranes were incubated in anti-CMV immunoglobulin (Ig) at 1.0 µg/ml in TBS at room temperature for at least 12 hr. The membranes were subjected to three 10 min washings in TBS and were then treated with TBS-powdered milk containing goat anti-rabbit Ig conjugated to alkaline phosphatase (Sigma Chemical, St. Louis, MO) diluted 1:5000 for 3 hr in the dark at room temperature. After three 10-min washings with TBS, substrate [50 mg nitroblue tetrazolium (Fisher Scientific, Fair Lawn, NJ) dissolved in 1 ml 70% N, N-dimethyl formamide (Fisher Scientific Co.), 5 mg 5-bromo-4-chloro-3-indolylphosphate (Sigma Chemical Co.) dissolved in 1 ml N, N-dimethyl formamide, as well as 3 ml of 10X alkaline phosphatase buffer (1.0 M Tris-HCl, pH 9.5, 1.0 M NaCl, and 50 mM $MgCl_2$)] was added and reactions were allowed to develop at room temperature and stopped by rinsing with cold water.

'Calwonder' plants used for immuno-tissue blot analysis were also used to determine CMV accumulation in leaves by antigen plate-coating, indirect enzyme-linked immunosorbent assay (ELISA) (Garcia-Ruiz and Murphy, 2001; Voller et al., 1976). Samples collected from each plant that consisted of individual symptomatic non-inoculated leaves from the upper to the lower canopy were ground individually with a motorized leaf squeezing apparatus (Piedmont Machine and Tool, Six Mile, SC) in 50 mM carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ at pH 9.6) to a final dilution of 1:20 (g tissue/ml buffer). Negative control samples from mock-inoculated plants (comparable to those leaves collected from CMV-inoculated plants), as well as a known positive control of purified CMV, were added to each microtiter plate and incubated in a moist chamber at 4°C for at least 12 hr. After incubation, the microtiter plate was rinsed three times, 3 min each, with phosphate buffered saline (PBS-T; 137 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl at pH 7.4, with 0.05% Tween 20). Anti-CMV Ig was added at 1.0 µg/ml in PBS-T and incubated in a moist chamber at 4°C for at least 12 hr. After rinsing with PBS-T, goat anti-rabbit Ig conjugated to alkaline phosphatase (Sigma Chemical Co.) diluted 1:5000 in PBS-T was added and allowed to incubate in a moist chamber for 3 hr at 37°C. Microtiter plates were rinsed with PBS-T and substrate [1 mg/ml para-nitrophenylphosphate (Sigma Chemical Co.) in 10% diethanolamine, pH 9.8], was added and reactions were allowed to develop at room temperature for 30 to 60 min. A Sunrise microtiter plate reader (Phoenix Research Product, Hayward, CA) was used to record the absorbance value at 405 nm. Samples were considered positive for the presence of CMV when the ELISA absorbance value was greater than the mean plus three standard deviations for comparable healthy control samples.

Cytological effects on pepper plant growth and development in response to systemic invasion by CMV.

General anatomy. Leaf and apical bud samples were collected from CMV-infected and mock-inoculated plants at each of the three different stages in symptom development. Leaf segments (approximately 3×4 mm) were taken from symptomatic areas with comparable samples taken from mock-inoculated plant leaves. Approximately 2-3 mm long apical buds were taken from the stem tip. Tissue fixation and dehydration procedures were performed as described by Johansen (1940) with minor modifications. The segments were fixed under vacuum in a freshly prepared mixture of 2.5% (w/v) glutaraldehyde and 4.0% (w/v) paraformaldehyde in 0.2 M phosphate buffer, pH 7.2 (Garcia-Castillo et al., 2001; Karnovsky, 1965) for 30 to 60 min. After infiltration, fixation was allowed to proceed for at least 12 hr at 4°C. The samples were washed three times, 1 hr each washing, in 0.2 M phosphate buffer, pH 7.2, and dehydrated in a graded ethanol series (10, 20, 30, and 40% for 1 hr each, and 50% for 2 hr) under vacuum. Tissue samples were then subjected to a graded tertiary butyl alcohol (TBA) series at room temperature consisting of ethanol:TBA at ratios of 40:10% for 1 hr, 50:20% at least 12 hr, 50:35% for 1 hr, 40:55% for 1 hr, and 25:75% for 1 hr. Dehydrated samples were incubated in 100% TBA two times each for 2 hr at ~25°C. The final step involved incubation in a 1:1 mixture of TBA and paraffin oil for at least 12 hr at ~25°C, followed by an infiltration in paraffin (Polysciences, Inc., Warrington, PA) for at least 12 hr at 58°C.

Infiltrated tissues were embedded in paraffin using Tissue-Tek II 4603 (Division Miles Laboratories, Westmont, IL). Transverse sections, 10 μm thick, were cut with a

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sharpened microtome knife on a Leitz 1512 rotary microtome (Leitz Wetzlar GmbH, Germany) and adhered to glass slides coated with Haupt's adhesive (Carolina Biological Supply Co., Burlington, NC) (Jensen, 1962). Sections were stained with 0.5% aqueous toluidine blue O as described by O'Brien et al. (1964) and rinsed with deionized water, followed by a 4% formalin rinse. Tissues were dehydrated for at least 12 hr at 40°C. The stained sections were then treated with Hemo-De (Fisher Scientific Co.) two times for 5 min each to remove the paraffin and mounted on slides using Permount (Fisher Scientific Co.) with at least 12 hr incubation at 40°C. Samples were viewed by light microscopy (Nikon Biophot, Tokyo, Japan) and micrographs were taken with a digital camera (Nikon D70, Tokyo, Japan).

Immunohistochemistry. Tissue samples, the same as those described for general anatomy, were taken from comparable tissues of CMV and mock-inoculated plants. Selected tissue sections were adhered to glass slides coated with 0.1% poly-l-lysine (Sigma Chemical Co.) by heating at 40°C for at least 12 hr. The tissue was treated with Hemo-De two times for 5 min each to remove paraffin and then rehydrated by passage through a decreasing ethanol series from absolute ethanol to water for 5 min each (100%, 95%, 70%, 50%, 30% ethanol, followed by two passages to water). Rehydrated tissues were treated with PBS three times for 5, 10, and 15 min each, and then treated with 5% bovine serum albumin (BSA) in PBS for 20 min at room temperature. Tissue samples were treated with anti-CMV Ig (1 μg/ml) in PBS containing 1% BSA for at least 12 hr at 4°C. After four 5-min rinses with PBS, goat anti-rabbit Ig conjugated to alkaline phosphatase (Sigma Chemicals Co.) diluted 1:5000 in PBS containing 1% BSA was added and allowed to incubate for 1 hr at 4°C. The samples were subjected to four 5-min

rinses with PBS, followed by a single rinse step for 15 min in equilibration buffer (1M Tris-HCl at pH 9.5, 1M NaCl, 50 mM MgCl$_2$).

For colorigenic detection, tissues were incubated in a substrate solution consisting of 10 mg nitroblue tetrazolium (Fisher Scientific Co.) dissolved in 1 ml 70% N, N-dimethyl formamide (Fisher Scientific Co.), 5 mg 5-bromo-4-chloro-3-indolylphosphate (Sigma Chemical Co.) dissolved in 1 ml N, N-dimethyl formamide, as well as 3 ml of 10X alkaline phosphatase buffer (1.0 M Tris-HCl, pH 9.5, 1.0 M NaCl, and 50 mM MgCl$_2$). Tissue samples were incubated in substrate solution at 37°C for 30 to 60 min (Garcia-Castillo et al., 2001), followed by a water rinse to stop the reaction. The stained tissues were then dehydrated through a graded ethanol series for 5 min each (30%, 50%, 70%, 95%, and two times 100%). Dehydrated samples were immersed in Hemo-De two times for 5 min each and mounted on slides using Permount. Sections were then examined and photographed with a Nikon D70 Digital Camera, using a light microscope (Nikon Biophot, Japan).

2.3   Results

Characterization of the symptom expression induced by CMV. The CMV-induced systemic symptoms expressed in inoculated Calwonder plants were consistent with those described by Murphy and Bowen (2006). We describe these symptoms as three symptom phases which varied with leaf position along the stem and developmental stage of the plant (Fig. 2.1). The CMV-induced symptoms were consistent and predictable among experiments.

When plants at the early five- to six-leaf stage were inoculated onto leaves 1 and 2, systemic symptoms in the form of chlorosis over the basal portion of leaves 6, 7 and 8 developed at approximately 7 dpi. No symptoms occurred on inoculated leaves or leaves 3-5. This initial systemic phase of symptom expression will be referred to in this report as the chlorosis phase. As new leaves emerged, typically leaves 9-11 at approximately 12 dpi, a chlorotic mosaic symptom occurred over the entire blade of these newly emerged leaves. This symptom will be referred to as the mosaic phase. During the mosaic phase, leaves 6, 7 and 8 remained in the chlorosis phase with older leaves remaining symptomless. The mosaic phase (and associated leaf tissues) occurred prior to the onset of branching of the primary stem. Leaves that emerged during mosaic phase were smaller than comparable leaves of the control.

At the 11-12 leaf stage (~21 dpi), the primary stem branched into two secondary stems. A single leaf developed on each secondary stem (leaves 13 and 14). As these leaves expanded, they had a dull green appearance (as opposed to a dark, waxy appearance of healthy leaves), abnormally elongated and downward cupped with the interveinal lamina appearing sunken with protrusion of the main vein. Plants in this phase will be referred to as the leaf distortion phase. As new leaves emerged (above leaves 13 and 14), they also had a dull green appearance but without mosaic or distortion and were smaller than comparable leaves on healthy control plants. As subsequent leaves emerged, they became increasingly less symptomatic and more similar to leaves on healthy control plants. Secondary branches branched into clusters of tertiary branches above leaves 13 and 14, sometimes above leaves 15 and 16. Older leaves, 6-8 and 9-11,

which had expressed chlorosis and mosaic symptom phases, respectively, had only mild

symptoms or had become asymptomatic.

Figure 2.1   Systemic symptoms induced by *Cucumber mosaic virus* (CMV) in Calwonder plants expressing three distinct symptom phases: **A,** chlorosis (7 dpi); **B,** mosaic (12 dpi); and **C,** leaf distortion (25 dpi).

Anatomical features. Leaf samples in the chlorosis, mosaic and leaf distortion symptom phases were collected and processed for microscopy analysis along with comparable tissues from healthy control plants (Fig. 2.2). Leaf cross sections taken from healthy control samples (comparable to leaves in the chlorosis symptom phase) had an organized cell pattern consisting of a clearly defined layer of adaxial epidermal cells over a clearly defined layer of palisade cells over numerous layers of relatively organized spongy parenchyma cells (Fig. 2.2A). The final organized layer consisted of abaxial epidermal cells. In addition to the layered, well organized cellular content of healthy control leaf tissues, the phenotypic appearance of healthy control leaves was a smooth, waxy surface with minor indentations along veins. In contrast to the healthy control leaf tissues, leaves expressing the chlorosis symptom phase had a disorganized cellular profile (Fig. 2.2B). An adaxial epidermal layer was apparent; however, palisade and spongy parenchyma cells had no apparent layering in cellular organization. The nuclei within these cells were not as visually apparent as in healthy cells. The abaxial surface was convoluted with what appeared to be swollen epidermal cells. This convoluted appearance correlated with a puckered, swollen appearance of leaves in the chlorosis symptom phase.

Healthy control samples, comparable to leaves selected for the mosaic symptom phase had an organized cell pattern that included an adaxial layer of epidermal cells, a well defined layer of palisade cells over spongy parenchyma cells and finally a layer of abaxial epidermal cells (Fig. 2.2C). The spongy parenchyma cells were less well defined and they tended to be more circular than observed for healthy leaves representative of the chlorosis symptom phase. CMV-infected leaf cross sections taken from samples during

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the mosaic phase revealed an adaxial epidermal layer but no clear indication of a layer of palisade cells (Fig. 2.2D). Spongy mesophyll cells were disorganized with many large intercellular spaces throughout. An organized abaxial epidermal layer was apparent but tended to be highly convoluted. Leaf sections in the mosaic phase always appeared swollen or larger in diameter than comparable healthy control leaves. The lack of chlorophyllous palisade and spongy cells correlated with a faint green color appearance of leaves in the mosaic symptom phase.

Cell content and organization did not differ between leaves in the leaf distortion symptom phase (Fig. 2.2F) and comparable healthy controls (Fig. 2.2E). CMV-infected leaves were always thinner than healthy leaves which corresponded with the sunken interveinal lamina for leaves in this symptom phase.
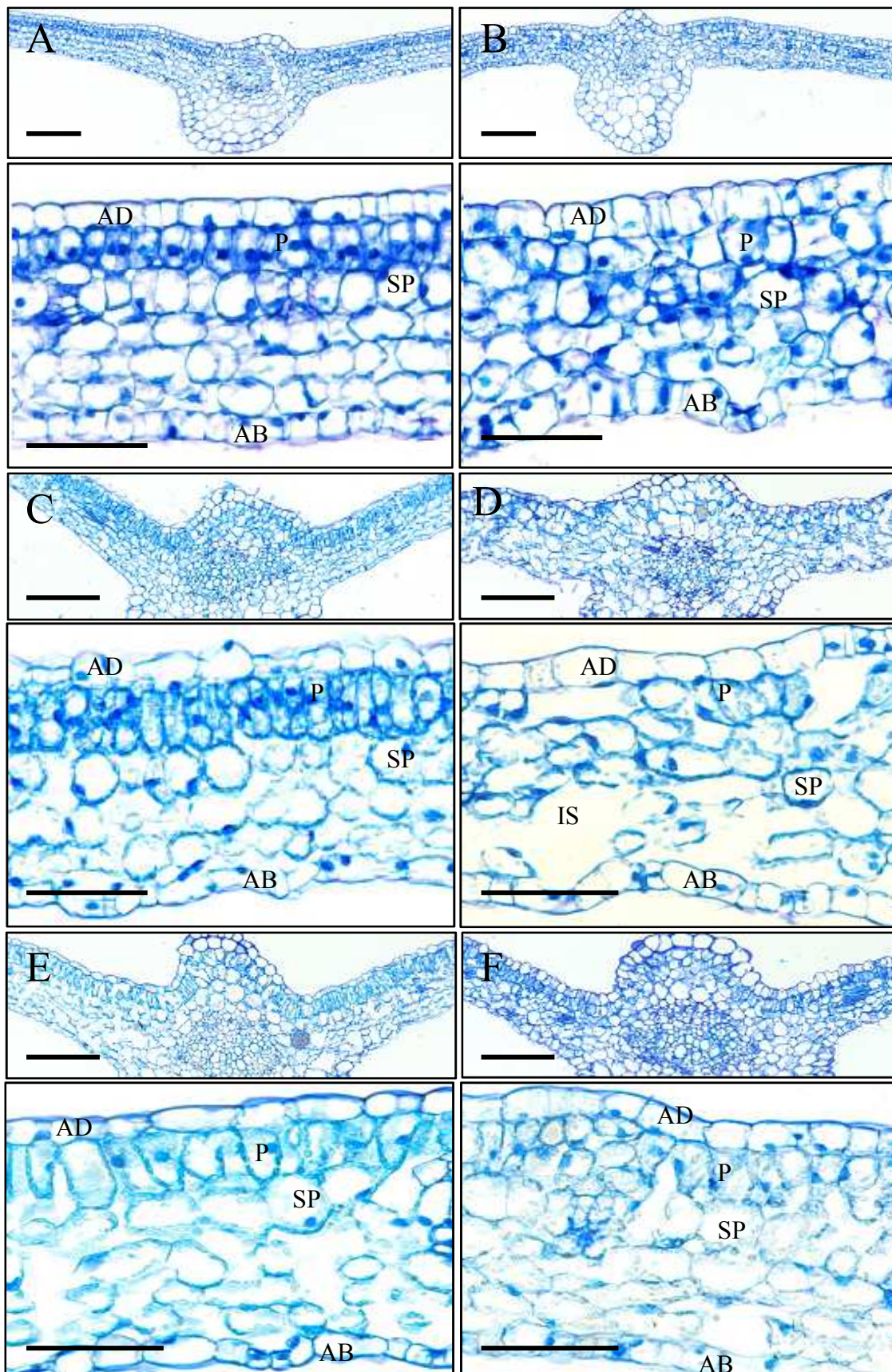
Figure 2.2   Transverse sections of leaf collected from the *Cucumber mosaic virus* (CMV)-infected Calwonder plants stained with Toluidine blue O during expression of the chlorosis **(B)**, mosaic **(D)**, and leaf distortion **(F)** symptom phases relative to comparable healthy leaf sample **(A, C,** and **E)**. Bottom row of each symptom phase represents higher magnification views. AB, abaxial (lower) epidermis. AD, adaxial (upper) epidermis. P, palisade parenchyma. SP, spongy parenchyma, IS, intercellular space. Scale bars = 50 μm.

CMV accumulation in non-inoculated leaves.   Initial efforts (spring trial) to correlate virus accumulation in leaf tissues with symptom phases involved testing non-inoculated leaves for CMV by ELISA at specific times post-inoculation (Fig. 2.3). CMV was first detected at 3 dpi with increased amounts of virus detected in non-inoculated leaves through 12 dpi. ELISA absorbance values began to decline between 12 and 17 dpi and continued to decline through the remainder of the experiment. This trend, whereby CMV titers in non-inoculated leaves increased in time followed by a decline in titer, was highly consistent among experiments. CMV accumulation in non-inoculated leaves was highest from 7-17 dpi which coincided with the chlorosis and mosaic symptom phases and reduced dramatically between 22-32 dpi, coinciding with the leaf distortion phase. As newly emerged leaves showed greater recovery from CMV infection with little or no symptoms, CMV titers declined to levels just above the healthy control threshold.

For the next two trials (summer and fall), CMV accumulation in non-inoculated leaves was tested by ELISA at specific symptom phases rather than sequential days post-inoculation (Fig. 2.4). Leaves in chlorosis and mosaic symptom phases had high CMV titers, which did not differ from each other.   In contrast, leaves in the leaf distortion

symptom phase had significantly less CMV than those in chlorosis and mosaic symptom phases.
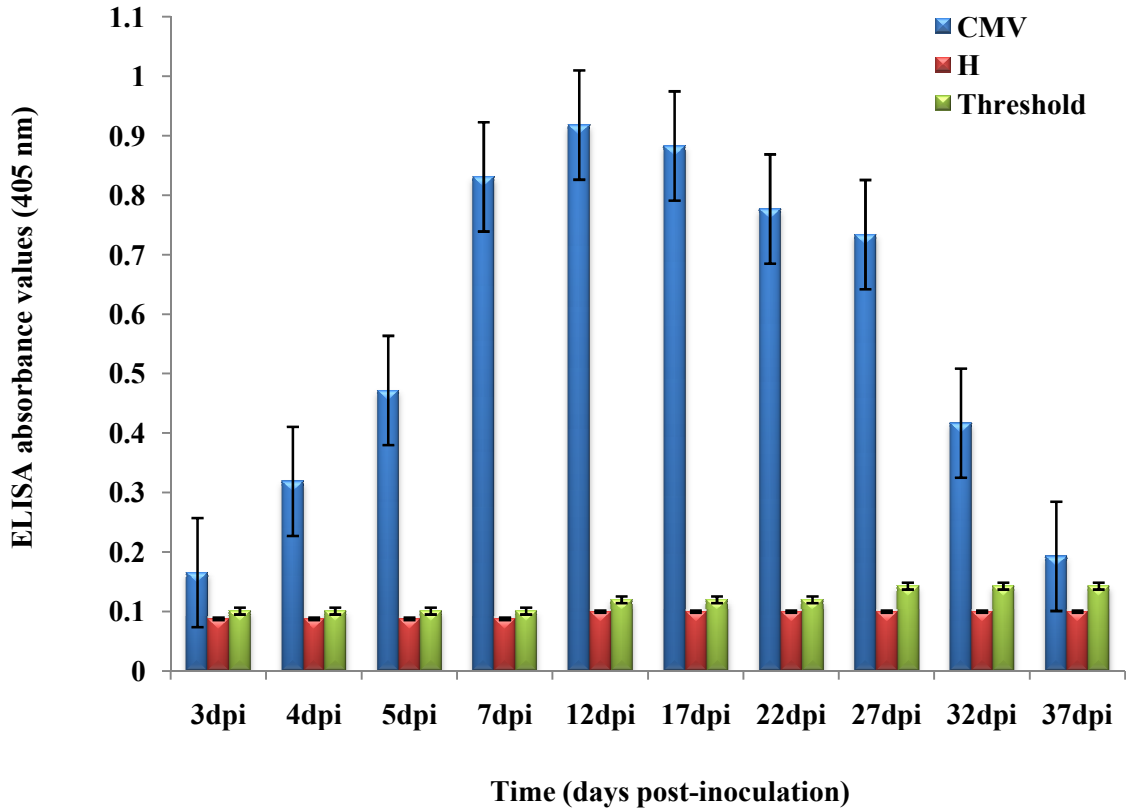
Figure 2.3   Evaluation of *Cucumber mosaic virus* (CMV) in non-inoculated leaves at specific days post-inoculation (dpi) in the spring trial. Virus titers were determined by antigen plate-coating, indirect enzyme-linked immunosorbent assay. Samples considered positive for CMV infection were above the respective threshold. The bars represent the mean value from 20 infected and 10 healthy plants. The threshold bar represents the mean plus three standard deviations for comparable healthy control samples. Error bars refer to standard error of the mean.
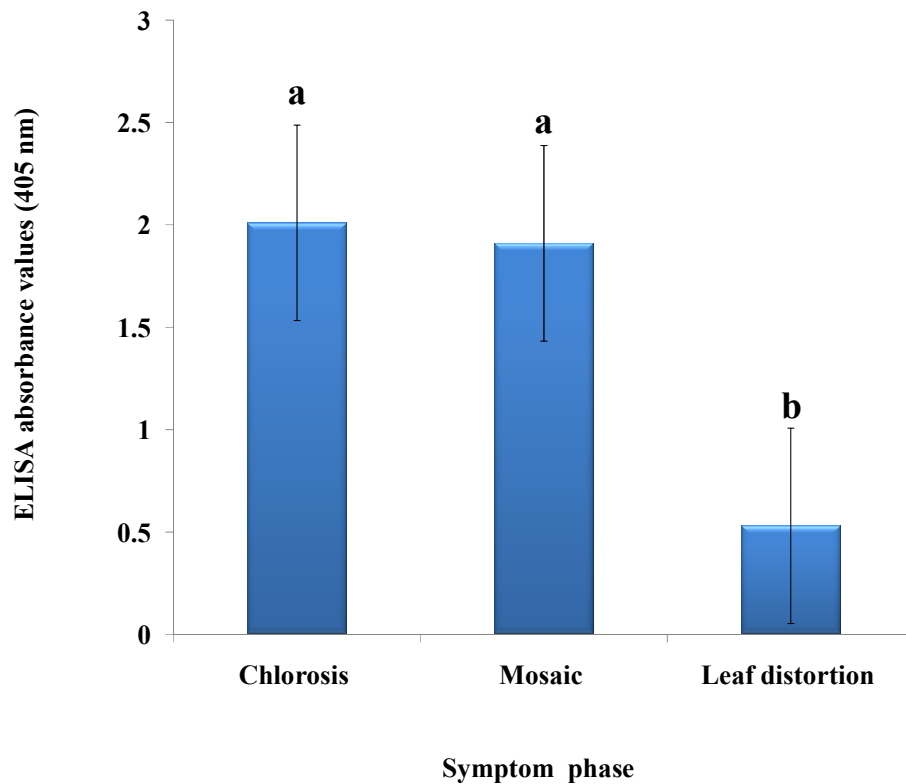
Figure 2.4   *Cucumber mosaic virus* (CMV) accumulation in leaves collected during the chlorosis (7 dpi), mosaic (12 dpi) and leaf distortion symptom phases (25 dpi) in the fall trial. Virus titers were determined by antigen plate-coating, indirect enzyme-linked immunosorbent assay. The bars represent the mean value from 20 infected plants. Error bars refer to standard error of the mean. Means with the same letter are not significantly different ($P \leq 0.05$) using LSD mean separation tests.

Immunohistochemical features. CMV accumulation in leaf tissues representing each symptom phase was analyzed by immunolabeling of transverse sections and viewed by light microscopy (Fig. 2.5). CMV was detected in all cell types throughout evaluated sections for leaves in chlorosis and mosaic symptom phases (Fig. 2.5A and C, respectively). In contrast, CMV detection was localized to epidermal and spongy mesophyll cells in leaves expressing the leaf distortion symptom phase (Fig. 2.5E). No labeling was observed in respective comparable healthy plant tissues (Fig. 2.5B, D, and F).

Figure 2.5 Immunohistochemical analysis of transverse sections of leaf samples collected from *Cucumber mosaic virus* (CMV)-infected Calwonder plants expressing chlorosis **(A)** mosaic **(C)** and leaf distortion **(E)** symptom phases. Blue color indicates

CMV coat protein-antibody reactions. No immunolabeling occurred in respective comparable healthy leaf samples **(B, D,** and **F)**. AB, abaxial (lower) epidermis. AD, adaxial (upper) epidermis. P, palisade parenchyma. SP, spongy parenchyma. IS, intercellular space. Scale bars = 50 μm.

Leaves previously expressing chlorosis or mosaic symptoms were tested for virus accumulation by ELISA and immunolabeling microscopy at a time, 30 dpi, when plants were recovering from CMV-induced symptoms. Plants in the recovery phase had reduced amounts of CMV accumulation in young leaves and throughout the length of the stem. We wanted to determine if leaves shown previously to have high levels of virus, i.e., leaves with chlorosis and mosaic symptoms, also expressed a recovery from infection. CMV titers in those leaves, according to ELISA, declined to levels just above the healthy control threshold or, for some samples below the threshold (data not shown). The immunolabeling microscopy experiment agreed with the ELISA; there was much less CMV detected in these leaves in the recovered plant than observed in leaves when originally expressing chlorosis and mosaic symptoms. CMV accumulation appeared confined to palisade and spongy mesophyll cells of leaves that previously expressed the chlorosis symptom phase (Fig. 2.6A), whereas virus was localized to spongy parenchyma cells in leaves that previously expressed the mosaic symptom phase (Fig. 2.6C).
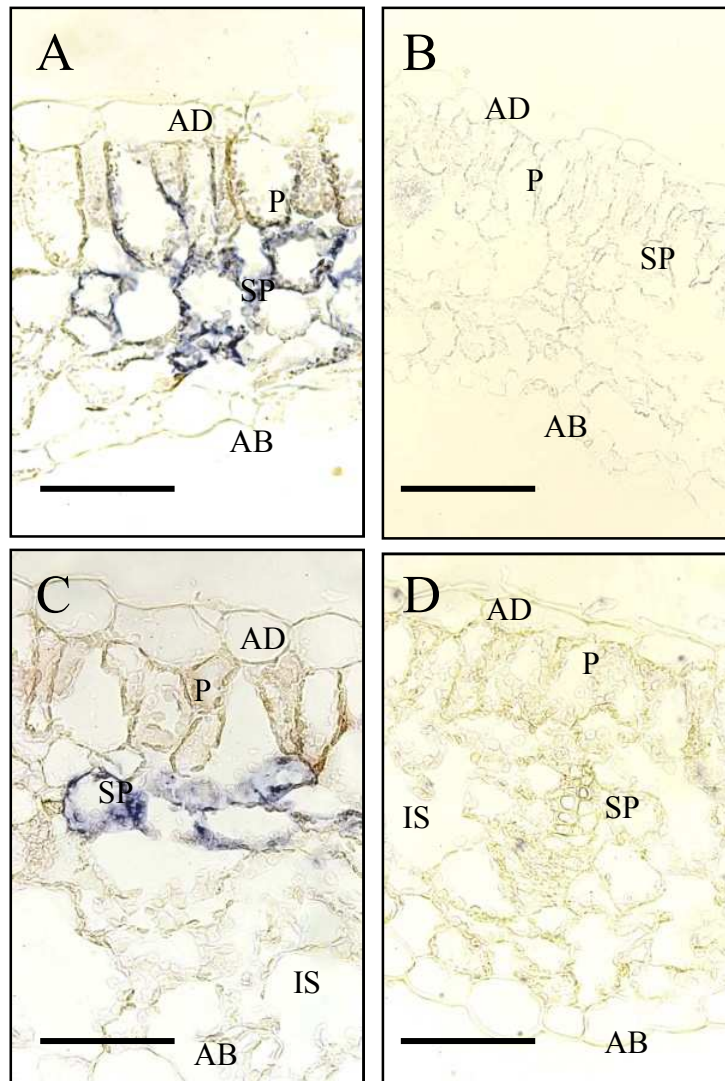
Figure 2.6 Immunohistochemical analysis of transverse sections of leaf samples from *Cucumber mosaic virus* (CMV) infected plants. Samples were taken at 30 days post-inoculation from leaves identified previously to express chlorosis **(A)**, and mosaic **(C)** symptoms. Respective comparable healthy leaf samples are in **B** and **D**. Blue color indicates CMV coat protein-antibody reactions. AB, abaxial (lower) epidermis. AD, adaxial (upper) epidermis. P, palisade parenchyma. SP, spongy parenchyma. IS, intercellular space. Scale bars = 50 μm.

CMV accumulation in the stem and leaf petiole sections. Symptom phases and ELISA tests to determine CMV accumulation in non-inoculated leaves, representing a systemic infection, indicated a rapid increase in virus accumulation at ca. 7 dpi which continued through 17 dpi but then markedly declined in leaves in the leaf distortion phase and beyond. At later stages of infection, CMV accumulation was reduced close to or below the threshold of detection by ELISA. We next determined whether the decline in accumulation occurred in the stem and leaf petioles using immuno-tissue blot analysis (Fig. 2.7).

Stem tissue prints generated at 7 dpi, a time representing the onset of the chlorosis symptom phase, indicated high levels of CMV accumulation throughout the length and width of the stem and in all tissue types including epidermis, cortex, vascular tissue, and pith (Fig. 2.7A). Petioles of non-inoculated leaves showed similar amounts and extent of CMV accumulation. Essentially identical results were obtained for plants through 12 dpi with the mosaic symptom phase. When the main stem of Calwonder plants began to branch, which coincided with the leaf distortion phase and a decline in CMV accumulation in young expanding leaves, CMV accumulation throughout the stem decreased dramatically and was localized to vascular tissues (Fig. 2.7B). This localized accumulation occurred over the entire length of the stem and in petioles of non-inoculated leaves for the remainder of the experiment. No immuno-staining occurred in mock inoculated plants (Fig. 2.7C).
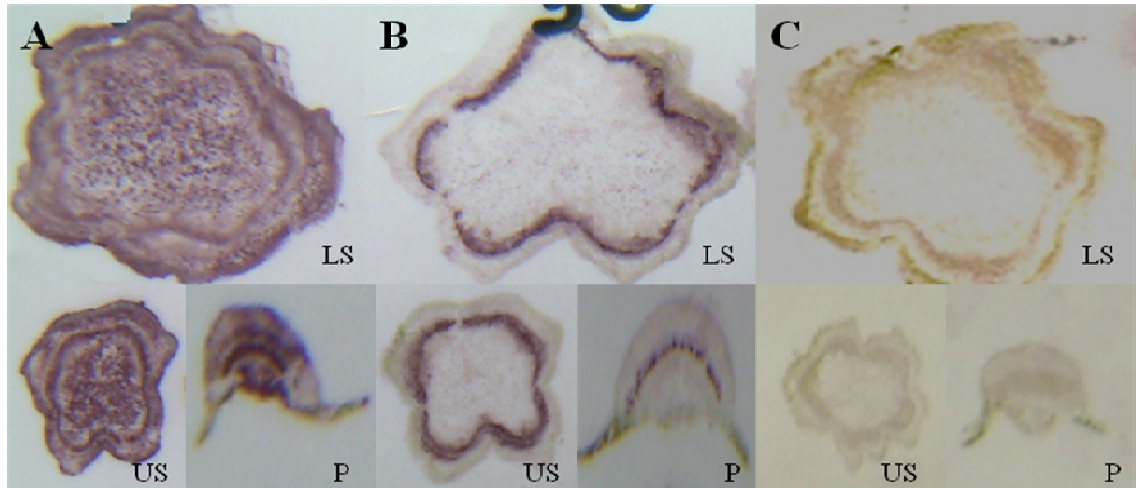
Figure 2.7 Detection of *Cucumber mosaic virus* (CMV) coat protein in Calwonder stems and leaf petioles of CMV-infected plants at different symptom phases by immuno-tissue blot analysis. **A,** tissue prints during chlorosis phase (7 dpi); **B,** leaf distortion phase (25 dpi); and **C,** from a healthy control. LS, lower stem; US, upper stem; and P, petiole of non-inoculated leaf. The brown stain indicates presence of CMV coat protein.

Microscopy analysis of apical bud region. The apical bud region was examined microscopically at times correlating with each leaf symptom phase. There were no apparent differences in cell content or organization for the apical bud region from each symptom phase of CMV-infected or healthy plants (data not shown). Sections were then tested for CMV accumulation by immunolabeling. Sections taken from the apical bud when plants were expressing the chlorosis and mosaic symptom phases showed widespread occurrence of CMV in the epidermis, hypodermis, cortex, vascular tissue (vascular cambium, phloem, and xylem), and pith (Fig. 2.8A-B). In contrast, CMV was localized to epidermal, hypodermal and vascular cambium in apical bud tissues collected during the leaf distortion symptom phase (Fig. 2.8C-D). No labeling was observed in respective comparable healthy plant tissues (Fig. 2.8E-F).
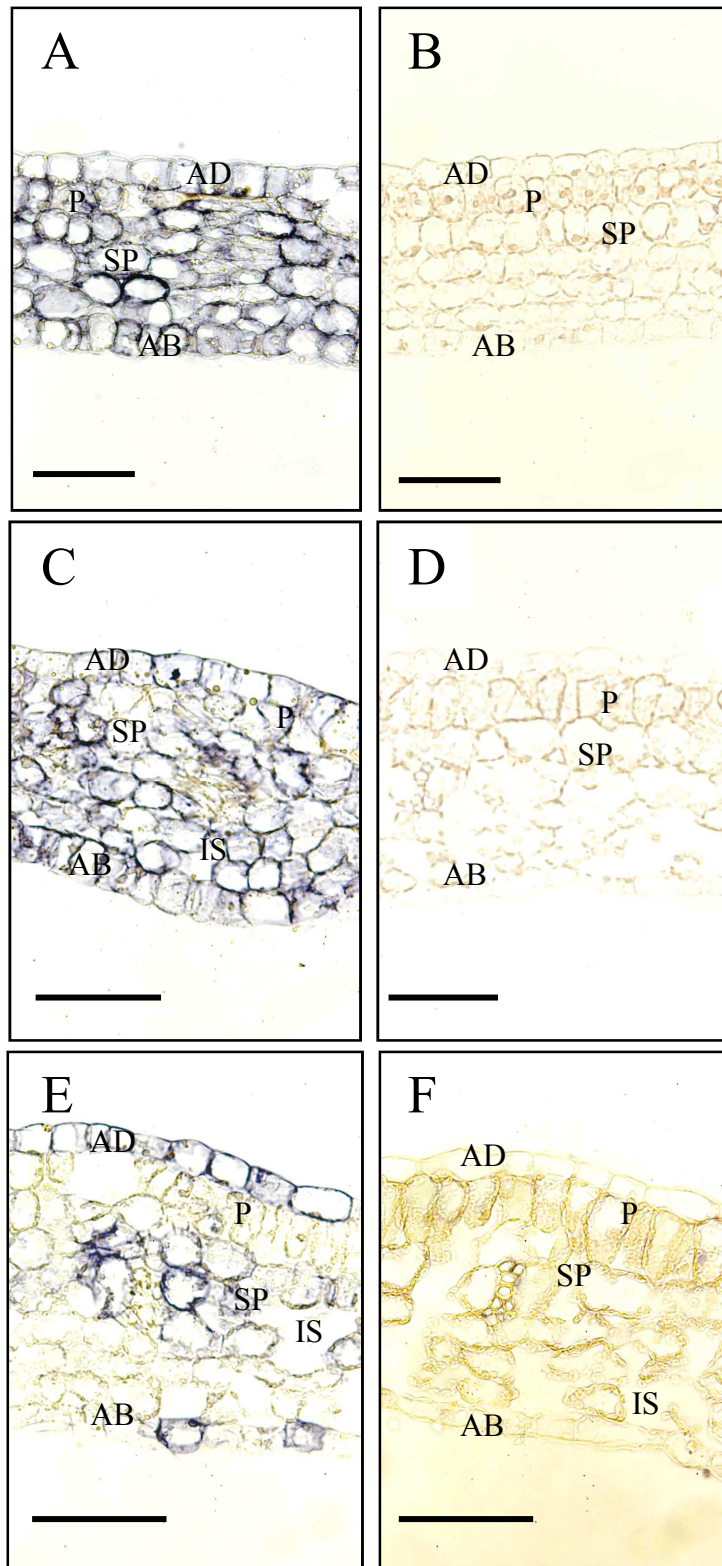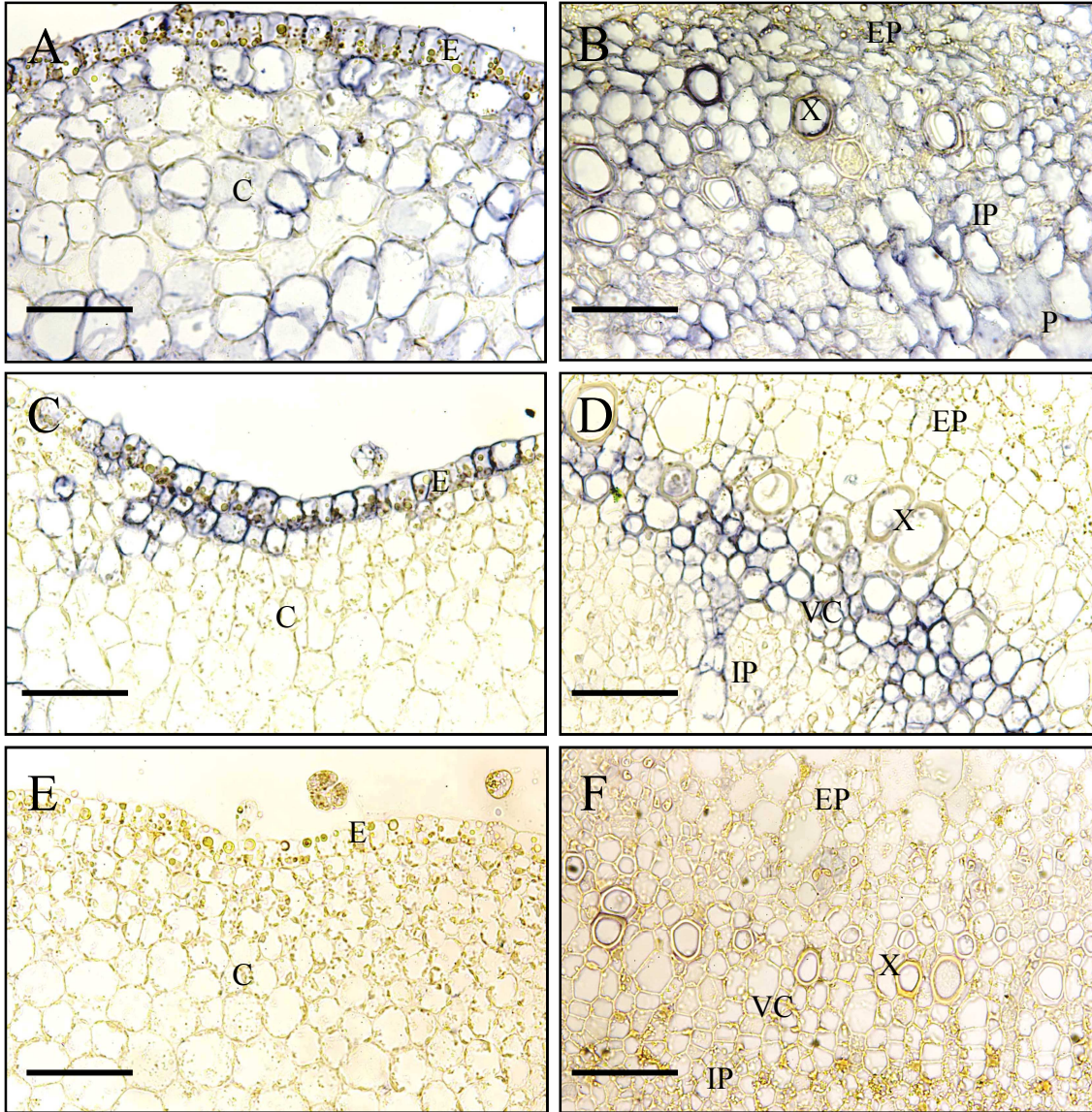
Figure 2.8   Immunohistochemical analysis of transverse sections of apical bud samples collected from *Cucumber mosaic virus* (CMV)-infected Calwonder plants expressing chlorosis **(A** and **B)** and leaf distortion **(C** and **D)** symptom phases. Blue color indicates CMV coat protein-antibody reactions. No immunolabeling occurred in respective comparable healthy leaf samples **(E** and **F)**. Left column represents a view of outer cell structures, whereas the right column represents a view of vascular system. E, epidermis. C, cortex. VC, vascular cambium. X, xylem. EP, external phloem. IP, internal phloem. P, pith. Scale bars = 50 µm.

Evaluation of plant growth characteristics.   In the spring trial, aboveground plant height did not differ between CMV-infected and mock-inoculated control plants through 10 dpi, at which time, plants were in the chlorosis symptom phase (which began at 7 dpi) (Fig. 2.9). Aboveground plant height was significantly less for CMV-infected plants than mock-inoculated control plants from 10 to 30 dpi. This period of time spanned all three symptom phases. Although CMV-infected plants expressed varying degrees of recovery from infection at the leaf distortion phase (25 dpi), aboveground plant height remained significantly less than for controls. Differences in aboveground plant height were not observed among treatments at 35 dpi, at which time plants were in a nearly complete recovery from virus infection.

In the summer and fall trials, similar trends occurred as described for the spring trial; however, because plants were inoculated at the early 5 to 6 leaf stage (as opposed to the early 7 to 8 leaf stage in the spring trial), systemic symptoms occurred sooner after inoculation, symptoms were more severe and recovery occurred later. Although the chlorosis phase first occurred two days earlier than in the spring trial, a significant difference in aboveground plant height was observed at 10 dpi (data not shown). As occurred for the spring trial, significant differences in aboveground plant height among treatments occurred from 10 to 30 dpi. While these plants recovered from infection, i.e., reduced symptom severity of older leaves and little or no symptoms of young leaves, aboveground plant height remained significantly less for CMV-infected plants until 40 dpi.

Aboveground fresh weight, excluding fruit, was evaluated at 40 dpi. In contrast to reductions in aboveground plant height for CMV-infected plants, no differences occurred

for aboveground fresh weight between CMV-infected and mock control plants (Fig. 2.10). In addition, total leaf number did not differ among treatments (data not shown). CMV- infected plants were delayed in time to initiation of stem branching, time to first flower and fruit development, relative to the mock-inoculated plants. Flowering and subsequent fruit development did not occur for CMV-infected plants until after the leaf distortion symptom phase.
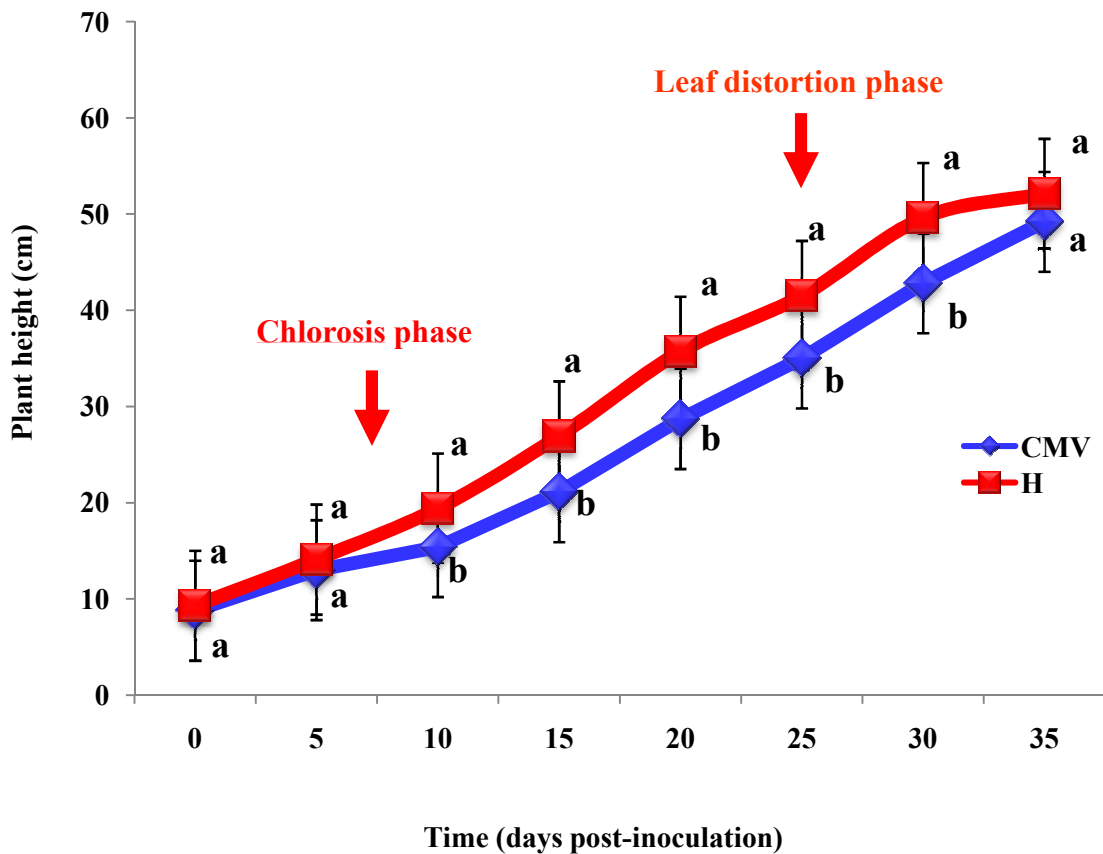
Figure 2.9   Growth in height (cm) of *Cucumber mosaic virus* (CMV)-infected and mock (H)-inoculated plants in the spring trial measured at specific days post-inoculation. The lines represent the mean value from 20 infected and 10 healthy plants. Means with the same letter are not significantly different ($P \leq 0.05$) using LSD mean separation tests.
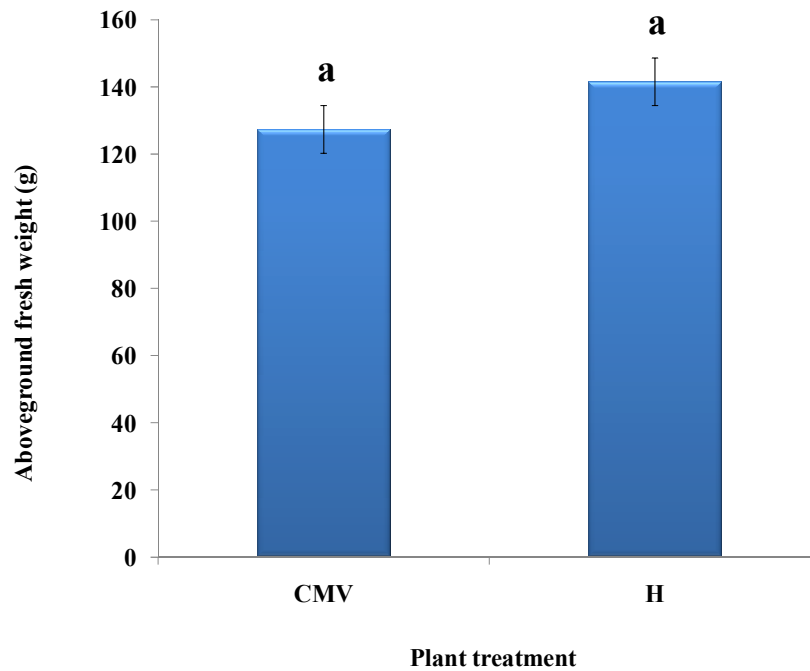
Figure 2.10 Total fresh weight of aboveground tissues (g) of *Cucumber mosaic virus* (CMV)-infected and mock (H)-inoculated plants in the spring trial measured at 40 dpi. The bars represent the mean value from 20 infected and 10 healthy plants. Error bars refer to standard error of the mean. Means with the same letter are not significantly different ($P \leq 0.05$) using LSD mean separation tests.

Stem stunting and its relationship to virus accumulation and foliar symptoms. CMV was detected in the stem and non-inoculated leaves of infected Calwonder plants between 3 and 5 dpi. The first systemic symptom, chlorosis phase, did not occur until 7 dpi. When CMV-infected plants expressed chlorosis phase symptoms, no stunting of the stem was apparent relative to healthy control plants. In this first phase of systemic symptom development, CMV accumulation was extensive through all tissues of the stem with dark immune-staining, an indication of high levels of virus accumulation. Leaves expressing chlorosis phase symptoms also accumulated high levels of CMV. As newly emerging leaves developed with mosaic phase symptoms, CMV titers remained high in the mosaic leaves and throughout the stem. It was during the mosaic symptom phase that plant stunting was first observed. The stem of CMV-infected plants had reduced internode length for all leaves along the stem at the time of the mosaic symptom phase (for plants in Fig. 2.11A, this included leaves 1 through 8). Leaves that expressed chlorosis and mosaic symptom phases always occurred along the primary stem. The primary stem developed into two secondary stems, most often above leaves 9 and 10. The development of secondary stems correlated with the leaf distortion symptom phase (typically leaves 11 and 12 if secondary stems developed above leaf 10) and a reduction in CMV accumulation in distorted leaves as well as in the stem. In addition, stem extension above the branching point was similar or equal to that of the healthy control. As new leaves emerged and secondary stems branched, CMV accumulation dropped below the positive threshold in young leaves and, interestingly, CMV accumulation throughout the stem, this included the entire length of the stem from soil line to apical buds, was nearly eliminated with the exception of the vascular ring which retained a

strong immune-stain reaction. Thus, CMV-induced stunting occurred along the primary

stem in association with the initial flush of infection and symptoms with plants appearing

to recover from infection at and times after branching of the primary stem.
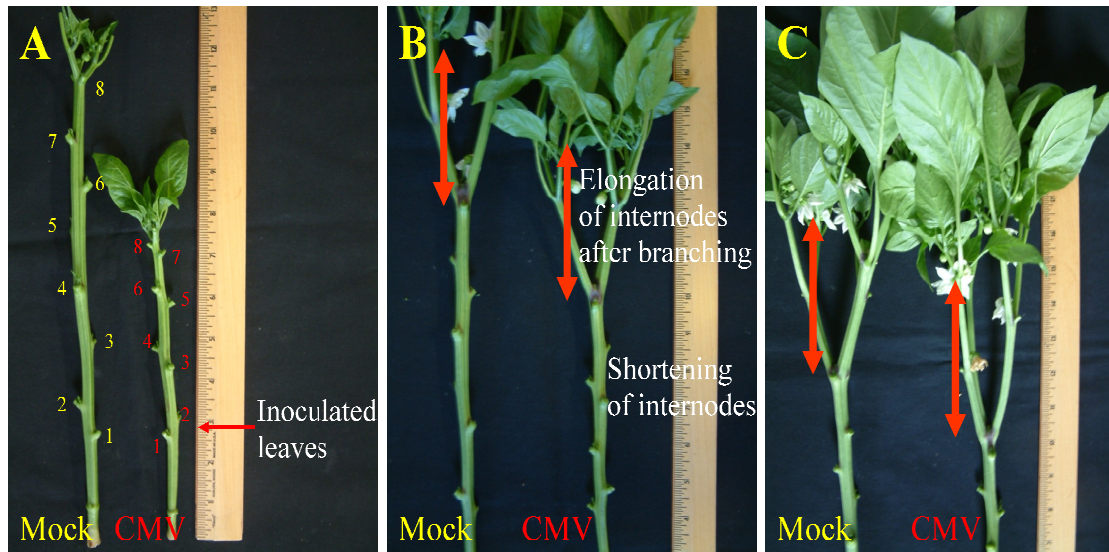
Figure 2.11　The effect on stem growth by *Cucumber mosaic virus* (CMV) infection in Calwonder pepper plants. **A,** a plant in the mosaic symptom phase (12 dpi) illustrating reduced extension of internodes for leaves 1 through 8; **B,** a plant in the leaf distortion phase (25 dpi) and **C,** following the leaf distortion phase illustrating normal stem elongation (no internode stunting) for secondary stems. Each example includes a comparable healthy control plant as a measure of normal growth.

2.4   Discussion

We evaluated disease symptoms, virus accumulation and effects on plant growth of plants infected with CMV. Three distinct foliar symptom phases were identified and designated chlorosis, mosaic and leaf distortion phases. A fourth phase could be included for times following the leaf distortion phase when newly emerged leaves are essentially symptomless. We also correlated each foliar symptom phase with CMV accumulation in the respective leaves and stem. CMV accumulated to high levels in chlorosis and mosaic symptom leaves and throughout the stem with a significant decrease in CMV accumulation in leaves of the leaf distortion phase and all subsequent leaves. Moreover, CMV accumulation decreased or was not detectable in all tested stem segments, with the exception of vascular bundle tissues, at times after the leaf distortion phase. Similar symptom phases and associated levels of CMV accumulation were described by Murphy and Bowen (2006), although designated phases 1, 2 and 3, respectively. While their work focused on quantifying a synergistic disease response to two viruses, recovery of pepper plants to CMV infection was noted and reported as a reduction in disease severity rating.

CMV's rapid and extensive movement to young developing tissues of pepper plants preceded the first phase of systemic symptoms. It was not until after the second phase of systemic symptoms, the mosaic symptom phase, however, that affects on stem elongation were observed. At that time, it was apparent that internode extension was reduced between all leaves along the primary stem. In contrast, stem length extension was reduced only slightly or not at all after the primary stem branched into two secondary stems which coincided with a significant reduction in CMV accumulation. We view the reduction in CMV accumulation as two distinct responses: the reduction of virus in

developing leaves and stems above the branching point represent an inhibition of CMV infection in these tissues, whereas the reduction of CMV in lower portions of the stem which previously had high virus titers represent a recovery from CMV infection. This was illustrated most clearly in the primary stem which contained extensive amounts of CMV during the first two symptom phases but recovered with little or no virus except in the vascular ring.

The recovery of plants to virus infection has been studied in numerous virus-host systems. Plants infected by viruses in the genus *Nepovirus* experience a "shock and recovery syndrome" where an initial severe systemic infection is followed by recovery in newly emerging tissues (Hull, 2009; Ratcliff et al., 1997). A recovery response of infected plants has been described for CMV in tobacco (Gal-on et al., 1996) and pepper (Carrillo-Tripp et al., 2007; Yamamoto et al., 2000). A recovery phenotype likely results from the plant's ability to silence specific genetic elements, a process referred to as gene silencing (Baulcombe, 2004; Dougherty et al., 1994). Gene silencing against CMV infection was studied in numerous host stems and shown to function as a natural defense mechanism against virus infection in plants (Carrington et al., 2001; Lucy et al., 2000). Many viruses express one or more proteins that suppress the host's gene silencing system (Carrington et al., 2001; Li and Ding, 2001; Vance and Vaucheret, 2001). The CMV 2b protein was identified as a gene silencing suppressor (Brigneti et al., 1998; Ding et al., 1995; Li et al., 1999; Mayers et al., 2000; Shi et al., 2003) and shown to target suppression of gene silencing only in newly emerging tissues that develop after infection (Brigneti et al., 1998). This suggests that the CMV 2b protein does not function in pepper plants since plants recovered from infection.

Of particular interest in our study was the shift from severe infection to recovery in association with stem branching. Gal-On et al. (1996) indicated that two closely related strains of CMV, Fny and Sny (Fast New York and Slow New York, respectively), induced different disease phenotypes due to the slower rate of systemic infection of Sny. CMV-Fny was able to rapidly invade young developing tissues of tobacco plants resulting in a severe disease that was cyclical with recovery from infection in some subsequently developed leaves. In contrast, CMV-Sny systemic infection was slower with no cyclical disease event. Our findings are similar to those described for tobacco, i.e., CMV-Fny rapidly invades young developing tissues with extensive accumulation which is followed by a shut-down in the infection process upon branching of the primary stem. Three intriguing questions arise from this study. What physiological change is associated with branching that initiates silencing against CMV infection? What is the nature of the vascular bundle in the primary stem and CMV accumulation in these tissues that separates it from the recovery response? Will virus that resides in the vascular bundle of the recovered stem be a source of inoculum for a later cyclical stage of infection? Future studies will attempt to address these questions, some of which will involve physiology-based analyzes of plant development in response to virus infection as well as evaluation of selected CMV strains or mutants that do not induce a shock phase systemic infection with little or no plant recovery response.

3   A Cucumber Mosaic Virus 2b-Deficient Mutant Is Not Able to Establish a Systemic Infection in Bell Pepper (*Capsicum annuum* L.)

3.1   Introduction

The complex relationships between the host plant and a virus are the major factors in symptom expression upon infection. Infection of plant virus is a result of the ability of a virus to move locally via plasmodesmata and systemically through the phloem, leading to a disruption of the development of plant tissues and organs. The silencing, the major plant defense system referred to as RNA silencing, of a virus can spread systemically throughout the plant after induction in the initially infected cell (Hull, 2002; Hull 2009). Many plant viruses, therefore, have developed a range of mechanisms to overcome this defense system that functions to silence infection. Those mechanisms include a suppression of the RNA-silencing by encoding a protein that interferes with the silencing pathway or through a nucleic acid mediated mechanism (Hull, 2009). The *Cucumber mosaic virus* (CMV) 2b is one of the suppressors of RNA silencing that has been well characterized in the virus-host plant systems (Brigneti et al., 1998; Ding et al., 1995b; Li et al., 1999; Mayers et al., 2000; Shi et al., 2003).

CMV is the type member of the genus *Cucumovirus* in the family *Bromoviridae* (International Committee on Taxonomy of Viruses, 2002). It has a tripartite genome

consisting of single-stranded messenger sense RNAs and an encapsidated subgenomic RNA from which the coat protein is produced. A complete virion consists of three icosahedral particles each approximately 29 nm in diameter and containing 180 coat protein subunits. The genomic RNAs are 3300-3400 nucleotides (nts), 3000 nts, and 2100-2200 nts for RNAs 1, 2, and 3, respectively. There are at least five open reading frames (ORFs) that encode five distinct viral proteins. RNA 1 encodes the ~112 kDa replication protein, referred to as 1a, and this protein contains methyltransferase and helicase domains (Allison et al., 1988; Hayes and Buck, 1990; Nitta et al., 1988). RNA 2 encodes two proteins: the 93-97 kDa 2a protein, a RNA-dependent RNA polymerase required for replication (Allison et al., 1988; Bruenn, 1991; Habili and Symons, 1989; Nitta et al., 1988; Poch et al., 1989), and the ~13 kDa 2b protein which is translated from subgenomic RNA 4A. RNA 3 encodes two proteins: a 31 kDa 3a protein which is required for virus cell-to-cell movement (therefore, the 3a protein considered a movement protein) (Boccard and Baulcomb, 1993; Canto et al., 1997; Suzuki et al., 1991), and the 24 kDa coat protein which is expressed from the subgenomic RNA 4 (Habili and Francki, 1974; Schwinghamer and Symons, 1975). The coat protein is the sole structural protein and is required for virus movement and aphid transmission (Allison et al., 1990; Canto et al., 1997; Ng and Perry, 1999; Suzuki et al., 1991)

CMV occurs worldwide and invades more than 1200 plant species in over 100 families, including both dicotyledonous and monocotyledonous plants. Induction of a variety of symptoms by CMV depends on the host plant species and cultivar, and the virus strain (Palukaitis and Garcia-Arenal, 2003). Additional important factors affecting the symptom expression include plant age or stage of growth at the time of inoculation as

well as environmental conditions (Kaper and Waterworth, 1981). Transmission of CMV in nature occurs, primarily, by more than 80 species of aphids (33 genera) in a non-persistent manner (Palukaitis and Garcia-Arenal, 2003). Two ubiquitous aphids species recognized as the most efficient vectors of CMV are *Myzus persicae* Sulzer and *Aphis gossypii* Glover. Some aphid species, however, are more or less efficient vectors of some CMV strains (Edwardson and Christie, 1997).   Dissemination of CMV from one plant to another also occurs through seed in more than 20 plant species including pepper (*Capsicum annuum* L.) (Ali and Kobayashi, 2009; Palukaitis et al., 1992)*.* The specific seed tissues shown to contain CMV are the embryo, endosperm, and seminal integuments; CMV may also be present on pollen (Yang et al., 1997). Experimentally, CMV is easily transmissible by mechanical inoculation of infected plant sap.

Severe symptoms induced by CMV, including mosaic, plant stunting, and leaf deformation, have been attributed to the *2b* gene (Ding et al., 1995b; Ding et al., 1996; Lewsey et al., 2007; Shi et al., 2002; Zhang et al., 2006). The CMV 2b mutant in which an insertion of a termination codon in the *2b* ORF or a deletion of the *2b* ORF was introduced into the CMV RNA 2 has been extensively investigated for its direct and indirect roles in suppressing RNA silencing. The lack of the gene encoding the 2b protein from CMV-Q (subgroup II strain) and CMV-Fny (subgroup IA strain) yielded mutants CMV-QΔ2b (Ding et al., 1995b) and CMV-FnyΔ2b (Ryabov et al., 2001), respectively. The mutants from both subgroups of CMV strains were less virulent than the wild-type. CMV-QΔ2b systemically infected *Nicotiana glutinosa* plants, inducing very mild symptoms; however, it failed to systemically infect cucumber (*Cucumis sativus*) plants

(Ding et al., 1995b; Ding et al., 1996). CMV-FnyΔ2b infections were symptomless in tobacco (*N. tabacum*) (Soards et al., 2002; Ziebell et al., 2007).

RNA silencing against CMV infection and CMV 2b suppressor capabilities in pepper have not been investigated, despite reports on symptom and disease remission or recover in pepper (Carrillo-Tripp et al., 2007; Masiri et al., 2008; Murphy and Bowen, 2006; Yamamoto et al., 2000).

In this study, we evaluated a 2b mutant for its ability to infect pepper plants as well as other CMV host species. The effects of the 2b protein were assessed based on characterization of symptom expression and virus accumulation in inoculated and non-inoculated tissues relative to CMV-Fny. CMV-FnyΔ2b accumulation, particularly in pepper plants, was also examined relative to CMV-Fny at the cellular level.

3.2   Materials and Methods

Plasmid DNA preparation, purification, and transcription. Full-length infectious CMV clones were provided by Dr. J.P. Carr (University of Cambridge, Cambridge, UK) as stab cultures of *Escherichia coli* harboring plasmids pFny109, pFny209, pFny309, and pFny209/M3 encoding CMV-Fny RNA 1, CMV-Fny RNA 2, CMV-Fny RNA 3, and CMV-Fny RNA 2 with a 294 bp deletion, respectively. This resulted in the complete deletion of the *2b* ORF (nucleotides 2419-2713 of the CMV-Fny RNA 2) (Ryabov et al., 2001). In this report, the wild type parent will be referred to as CMV-Fny and virus consisting of CMV-Fny RNAs 1 and 3 and Δ2b RNA 2 will be referred to as CMV-FnyΔ2b. Each plasmid in *E. coli* culture was streaked on LB

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(Sambrook et al, 1989) agar containing 50 μg/ml of ampicillin and 40 μg/ml of x-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) and incubated at 37°C for at least 12 hr. A single (white) colony was selected and cultured in LB medium containing 50 μg/ml ampicillin under vigorous shaking at 37°C for 12-16 hr. Each plasmid was extracted and purified from the respective bacterial culture using a Miniprep kit according to the manufacturer's instructions (Miniprep, Qiagen Inc., Valencia, CA). The resulting DNA preparations were analyzed by agarose gel electrophoresis and quantified using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE). Plasmid DNA (1 μg/μl) was linearized by treatment with *Pst* I (Invitrogen, Carlsbad, CA). The linearized plasmid DNAs were subjected to Klenow fragment of DNA polymerase I (Promega, Madison, WI) treatment to create blunt end before use as templates for *in vitro* transcription with a T7 mMessage mMachine Kit (Ambion Inc., Austin, TX) according to manufacturer's instructions. The resulting RNA transcripts were quantified using a NanoDrop 1000 Spectrophotometer.

Inoculation of plants. Capped *in vitro* transcripts generated from pFny109, pFny209, pFny309, and pFny209/M3 were used to inoculate three different host plants; tobacco (*Nicotiana tabacum* L.) cv. Kentucky 14, zucchini squash (*Cucurbita pepo* L.) cv. Dixie, and pepper *(Capsicum annuum* L.) cv. Calwonder. Three tobacco plants were inoculated onto leaves 1 and 2 when each was 3-4 cm in length. Three squash plants were inoculated onto each cotyledon when leaf 1 was 1-2 cm in length. Three pepper plants were inoculated onto leaves 1 and 2 when plants were at the early 5 to 6 leaf stage. All plants were placed in the dark for 24 prior to inoculation. Transcripts (an amount of 1.0 μg each) were combined in the appropriate combinations to generate CMV-Fny and

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CMV-FnyΔ2b. To each set of CMV RNA combinations, a 3X strength RNA inoculation buffer [3% (w/v) Celite, 110 mM $Na_4P_2O_7$, 3 mM EDTA (pH 9.0)] was added to a 1X final strength. The RNAs were then rub-inoculated onto the appropriate leaves. Inoculated plants were later tested by ELISA for CMV infection and the presence of wild type or Δ2b RNA 2 was confirmed by reverse transcription-PCR amplification and analysis by agarose gel electrophoresis with ethidium bromide staining.

CMV-Fny and CMV-FnyΔ2b accumulation in leaf tissues. CMV accumulation in leaf tissues was determined by double antibody sandwich enzyme-linked immunosorbent assay (ELISA) using a commercial kit specific to CMV, performed according to the manufacturer's instructions (Agdia, Inc., Elkhart, IN). Leaf samples collected for ELISA tests included inoculated leaves 1 and 2 and all non-inoculated leaves, collected and tested as individual samples. Each leaf sample was ground on a weighted basis at a 1:5 ratio [tissue (g): ml general extraction buffer (detailed by the manufacturer)] using a motorized leaf squeezing apparatus (Piedmont Machine and Tool, Six Mile, SC). Sap extracts were added to microtiter plates at a final dilution of 1:20 (g tissue/ml buffer). Comparable leaf samples were collected from CMV-Fny, CMV-FnyΔ2b and mock-inoculated plants. The primary antibody coating step and sap extraction step were incubated in a moist chamber at 4ºC for at least 12 hr. The conjugated antibody step was incubated in a moist chamber for 2-3 hr at 37ºC. Substrate reactions [1.0 mg/ml para-nitrophenylphosphate (Sigma Chemical Co.) in 10% diethanolamine, pH 9.8] were allowed to develop at room temperature for 30 to 60 min. A Sunrise microtiter plate reader (Phoenix Research Product, Hayward, CA) was used to record the absorbance value at 405 nm. Samples were considered positive for the

presence of CMV when the ELISA absorbance value was greater than the mean plus three standard deviations for comparable healthy control samples added to each microtiter plate.

Evaluation of CMV-Fny and CMV-FnyΔ2b in pepper plants. Two experiments were carried out, each using a randomized complete block design, in a temperature controlled greenhouse at the Plant Science Research Facility, Auburn University with the average temperatures of 24°C and 20°C during days and nights, respectively. Each experiment consisted of three treatments: CMV-Fny, CMV-FnyΔ2b and mock-inoculated plants. Each block (three in total) consisted of ten plants CMV-Fny as well as CMV-FnyΔ2b and three mock-inoculated plants. Thus, a total of thirty of each CMV-Fny-inoculated and CMV-FnyΔ2b-inoculated, as well as nine mock-inoculated plants were used for each experiment.

Susceptible *C. annuum* L. cv. Calwonder seeds were sown in 72-well Styrofoam trays (Speedling, Inc., Bushnell, FL) in Pro-Mix, soil-less potting medium (Premier Peat, Riviere-du-Loup, Quebec, Canada). Upon germination to at approximately the early two-leaf stage, seedlings were transplanted, one plant per pot, to one-gallon pots containing Pro-Mix which was supplemented with a controlled release fertilizer 18-6-12 (Osmocote Classic, Scotts Company LLC, Marysville, OH). Plant growth stage designations for inoculation and sampling were as described by Adrianifahanana et al. (1997). Briefly, leaves emerged in pairs on opposite sides of the stem with each successive leaf pair emerging nearly perpendicular to the previous leaf pair. The oldest leaf along the stem was designated leaf 1 with the oldest pair of leaves designated leaves 1 and 2.

CMV-Fny and CMV-FnyΔ2b were maintained by mechanical passage in tobacco (*N. tabacum* L.) cv. Kentucky 14 grown in an environmentally-controlled greenhouse at the Plant Science Research Facility. Each type of CMV inoculum consisted of systemically infected Kentucky 14 leaf tissues ground in 50 mM potassium phosphate buffer, pH 7.3, containing 10 mM sodium sulfite, whereas buffer alone was used to perform a mock-inoculated treatment. During the inoculation process, buffer, mortars, and pestles were chilled before use and kept on ice during inoculation. Calwonder plants at the early 5 to 6 leaf stage were inoculated onto leaves 1 and 2 with each CMV inoculum by mechanical-rub inoculation using inoculum-saturated cheesecloth. CMV-inoculated plants were monitored daily for development of symptoms as documented previously (Murphy and Bowen, 2006).

The accumulation of CMV-Fny and CMV-FnyΔ2b in pepper leaves was determined by ELISA according to the manufacturer's instructions (Agdia, Inc.). The accumulation of CMV-Fny and CMV-FnyΔ2b in the stem of inoculated plants was determined by immuno-tissue blot analysis (Andrianifahanana et al., 1997; Guerini and Murphy, 1999). One CMV-Fny inoculated plant, ten CMV-FnyΔ2b inoculated plants and one mock-inoculated plant were tested for each sampling period based on occurrence of distinct symptom phases in CMV-Fny infected plants (Masiri, 2010; Murphy and Bowen, 2006). Positions along the stem chosen to generate tissue prints included the internode immediately below the inoculated leaves and each internode above the inoculated leaves. Tissue prints were allowed to dry at room temperature and then stored at 4°C until serological analysis.

The detection of CMV in tissue prints was by Western analysis as described by Guerini and Murphy (1999). Membranes were incubated in 5% powdered milk dissolved in Tris-buffered saline (TBS; 50 mM Tris-HCl at pH 7.4, and 200 mM NaCl) at room temperature for 1 hr with gentle shaking. Blocking membranes were incubated in anti-CMV immunoglobulin (Ig) at 1.0 µg/ml in TBS at room temperature for at least 12 hr and were then treated with TBS-powdered milk containing goat anti-rabbit Ig conjugated to alkaline phosphatase (Sigma Chemical, St. Louis, MO) diluted 1:5000 for 3 hr in the dark at room temperature. Substrate [50 mg nitroblue tetrazolium (Fisher Scientific, Fair Lawn, NJ) dissolved in 1 ml 70% N, N-dimethyl formamide (Fisher Scientific Co.), 5 mg 5-bromo-4-chloro-3-indolylphosphate (Sigma Chemical Co.) dissolved in 1 ml N, N-dimethyl formamide, as well as 3 ml of 10X alkaline phosphatase buffer (1.0 M Tris-HCl, pH 9.5, 1.0 M NaCl, and 50 mM $MgCl_2$)] was added and reactions were allowed to develop at room temperature and stopped by rinsing with cold water.

Detection of CMV-Fny and CMV-FnyΔ2b in plant tissue samples by RT-PCR. Total RNA in leaf tissues from the CMV-Fny-inoculated plants and CMV-FnyΔ2b-inoculated plants were purified using a RNeasy Plant Mini Kit according to manufacturer's instructions (Qiagen, Valencia, CA). RNA concentration was determined by a Nanodrop 1000. Total RNA was then used for RT-PCR, followed by PCR to detect the presence of CMV-Fny and CMV-FnyΔ2b or stored at -80°C. The primers were designed to amplify a region of RNA 2 sequence (nt 2367-3031) flanking the *2b* ORF of CMV-Fny as well as the corresponding region in the RNA 2 of CMV-FnyΔ2b containing a deletion in the *2b* ORF (Ryabov et al., 2001). Reverse transcription was carried out using the reverse primer (5'-CCACAAAAGTGGGGGGCACCCG-3'), followed by PCR

using the reverse transcription reverse primer and forward primer (5'-AGTACAGAGTTCAGGGTTGAGCGTG-3') (Ziebell et al., 2007). PCR reaction conditions consisted of denaturation at 94°C for 5 min; 30 cycles at 94°C for 30 sec, 65°C for 30 sec and 72°C for 90 sec; and a final extension at 72°C for 7 min. PCR products were analyzed on 1% (w/v) agarose gel.

Virus purification. CMV-Fny and CMV-FnyΔ2b were propagated in *N. tabacum* cv. Kentucky 14 maintained in an environmentally-controlled greenhouse at 24°C day and 20°C night with 10 hr light and 14 hr dark cycles. Leaf tissues at approximately 100-200 g from CMV-Fny-infected plants, as well as CMV-FnyΔ2b-infected plants were collected for virus purification by differential centrifugation (Palukaitis and Zaitlin, 1984). Virus concentration (mg/ml) was determined using a Nanodrop 1000 Spectophotometer. The purified virus for CMV-Fny and CMV-FnyΔ2b were stored at 4°C.

Accumulation of CMV-Fny and CMV-FnyΔ2b in pepper mesophyll protoplasts. Isolation and incubation of Calwonder pepper protoplasts was carried out according to Murphy and Kyle (1994) as modified by Guerini and Murphy (1999). The plant growth and development was maintained in an environmentally-controlled chamber at 25°C under 12,000 lux illumination of 16-hr light and 8-hr dark cycles. Isolated protoplasts were collected by low-speed centrifugation using Eppendorf 5810R (GMI, Inc, Ramsey, MN) at 1450 rpm (~294 X g) at 25°C for 3 min. Protoplasts were subjected to three washing steps with 0.42 M mannitol. The pellets were resuspended in 0.42 M mannitol and counted using hematocytometer.

Inoculation of Calwonder mesophyll protoplasts was by electroporation using Gene Pulser Xcell Electroporation System (Bio-Rad Laboratories, Inc., Hercules, CA). The same concentration (12.5 ng/µl) for CMV-Fny and CMV-FnyΔ2b RNAs was used. Chilled cuvettes were used for each inoculation. Inoculation solutions consisted of 0.42 M mannitol (400 µl), 8 µl of 0.3 M $CaCl_2$, $0.5×10^6$ protoplasts (in 400 µl) and viral RNA (10 µg in no more than 10 µl; 10 µl of sterile water for the mock sample). Inoculation consisted of two 5-msec pulses of 150 volts. The protoplast solution was then placed on ice for 15 min. Protoplasts were centrifuged at 1,250 rpm (~218 X g) for 2 min and resuspended in incubation medium containing 0.2 mM KH_2PO_4, 1.0 mM KNO_3, 0.1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1.0 µM KI, and 0.01 µM $CuSO_4$ (Murphy and Kyle, 1994). Antibiotic mix containing carbenicillin, cephaloridine, and nystatin (Sigma Chemical, St. Louis, MO) was added at concentration of 1 µg/ml. Protoplasts were maintained in a controlled chamber at 25°C with a 16 hr photoperiod supplied with fluorescent lights for 24 hr. Protoplasts were counted at 24 hr post-inoculation and samples of $0.05×10^6$, $0.1×10^6$, and $0.2×10^6$ protoplasts were collected and tested for infection by ELISA (Agdia, Inc.).

3.3   Results

Evaluation of CMV infectious transcripts. Gel electrophoresis analysis indicated the presence of high quality plasmid DNAs containing full length cDNA copies of CMV-Fny (Fig. 3.1A). Plasmid pFny109 was the largest in size, followed by pFny209. Plasmid pFny309 was the smallest relative to other plasmids. In addition, plasmid

pFny209/M, with a deletion of 294 bp, was smaller in size than plasmid pFny209. The decrease in size of plasmids pFny109, pFny209, pFny209/M, and pFny309 corresponded to nucleotide numbers for CMV RNAs RNA 1, RNA 2, RNAΔ2b, and RNA 3, respectively. The circular plasmids were linearized after digestion by *Pst*I, resulting in single bands for each plasmid that corresponded to the anticipated respective sizes (Fig. 3.1B).
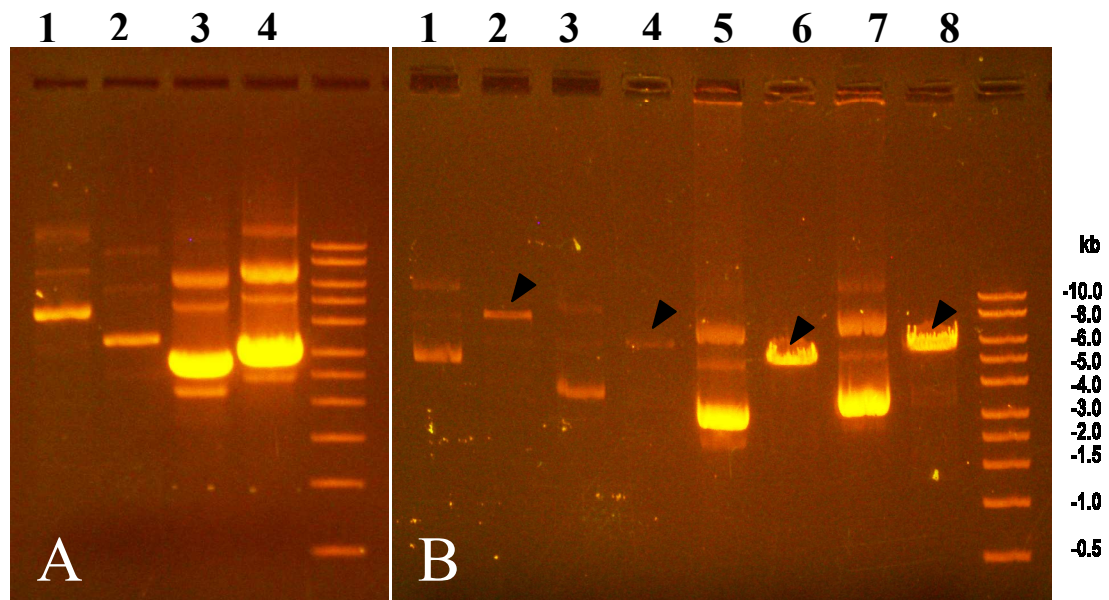
Figure 3.1   Agarose gel electrophoresis analysis of purified plasmids containing full-length cDNA copies of *Cucumber mosaic virus* strain Fny RNAs. **A,** plasmids pFny109, pFny209, pFny309, and pFny209/M3 encode RNA 1, RNA 2, RNA 3, and RNA 2 with a 294 bp deletion, respectively (lane 1, 2, 3, and 4). **B,** linearized plasmids pFny109, pFny209, pFny309, and pFny209/M3 (lane 2, 4, 6, and 8; arrowheads) compared with non-linear plasmids (lane 1, 3, 5, and 7).

Characterization of the symptom expression induced by CMV-Fny and CMV-FnyΔ2b transcripts. CMV-Fny- and CMV-FnyΔ2b-induced symptoms in inoculated tobacco, zucchini squash, and pepper plants varied among host plants (Fig. 3.2). In tobacco, CMV-Fny-induced systemic symptoms were first detected at approximately 10 days post-inoculation (dpi) with a mosaic pattern on young emerging leaves (Fig. 3.2A) which continued as new leaves emerged (Fig. 3.2D). Squash leaves expressing systemic infection had more of a chlorotic mosaic and downward cupping at approximately 7 dpi (Fig. 3.2B) with newly emerging leaves emerging in a cupped formation but later expanding into mosaic patterns (Fig. 3.2E). Pepper plants developed the three systemic symptom phases described previously: chlorosis by 7 dpi (Fig. 3.2C) and mosaic by 12 dpi (Fig. 3.2F) with leaf distortion and recovery in newly developed leaves from secondary and tertiary stems (Masiri, 2010, Murphy and Bowen, 2006). In contrast to CMV-Fny, pepper, squash and tobacco plants inoculated with CMV-FnyΔ2b were symptomless through 14 dpi (Fig. 3.2G-K). Squash and tobacco plants remained symptomless throughout the remainder of the experiment, whereas CMV-FnyΔ2b-inoculated pepper plants developed a mild chlorotic vein-clearing on older, non-inoculated leaves by 15 dpi but young leaves remained symptom-free (Fig. 3.2L).

CMV-Fny
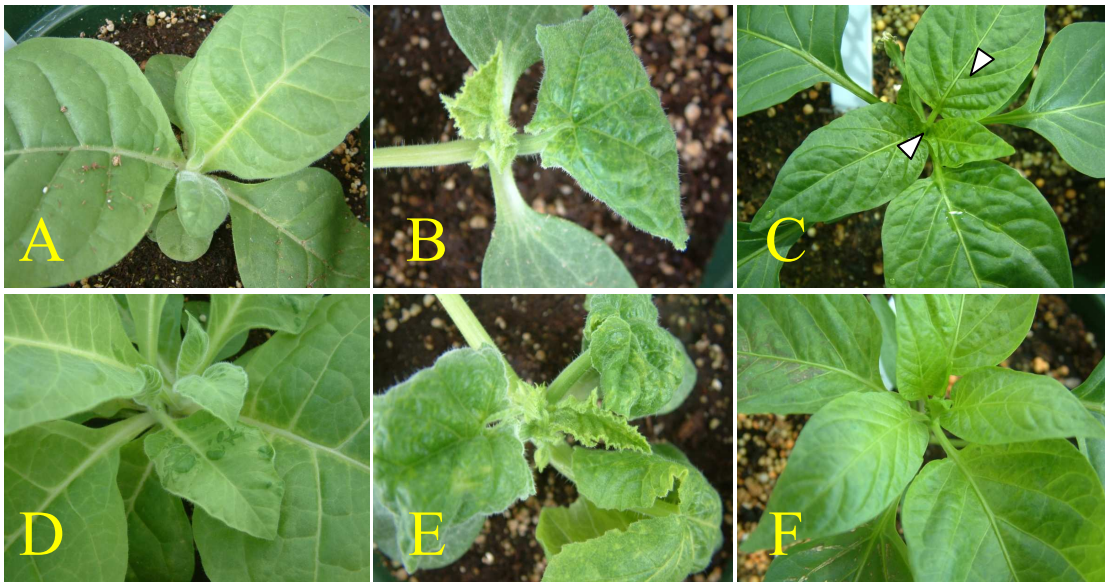
CMV-Fny△2b
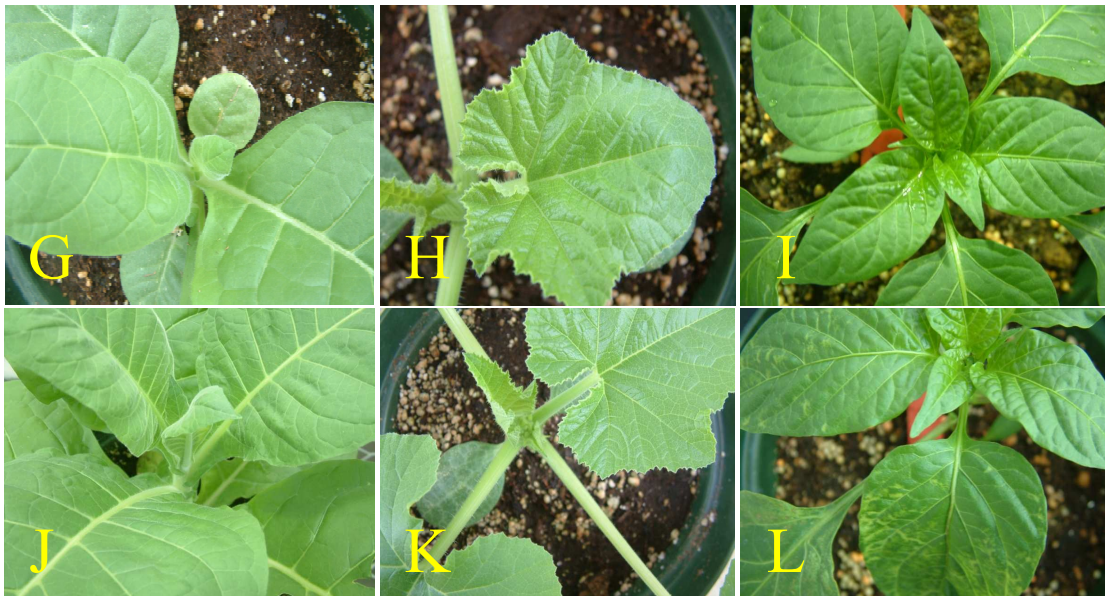
Figure 3.2 Characterization of the symptom expression induced by CMV-Fny (Row 1 and 2) and CMV-FnyΔ2b (Row 3 and 4) on different host plants. **A, B,** and **C,** mosaic symptoms occurred in tobacco and squash, and a chlorosis symptom at the basal portion of pepper's leaves (arrowheads) induced by CMV-Fny at 10, 7, and 7 days post-inoculation (dpi), respectively; **D, E,** and **F,** a mosaic and leaf distortion in tobacco, a mosaic and leaf deformation in squash, and a chlorotic mosaic over the entire leaf blade in pepper induced by CMV-Fny at 18, 15, and 15 dpi, respectively; **G, H,** and **I,** comparable to wild type, asymptomatic leaf tissues of the CMV-FnyΔ2b-inoculated tobacco, squash, and pepper plants, at 10, 7, and 7 dpi, respectively; **J, K,** and **L,** leaf tissues that remained asymptomatic on the CMV-FnyΔ2b-inoculated tobacco, squash, and pepper plants at 18, 15, and 15 dpi, respectively. Bottom row represents the CMV-Fny-infected (bottom pot) and the CMV-FnyΔ2b-infected (top left pot) relative to the mock-inoculated (top right pot) observed in **M,** tobacco; **N,** squash; and **O,** pepper plants at 12 dpi.

CMV-Fny and CMV-FnyΔ2b accumulation in inoculated and non-inoculated leaves. In an attempt to correlate virus accumulation in infected leaf tissues with systemic symptoms for CMV-Fny, as well as in asymptomatic leaf tissues for CMV-FnyΔ2b, the process involved testing inoculated leaves and individual non-inoculated leaves from the lower to the upper canopy for CMV by ELISA (Fig. 3.3). In tobacco plants, CMV accumulation was detected in inoculated and non-inoculated leaves for both CMV-Fny-infected plants and CMV-FnyΔ2b-infected plants. In zucchini squash plants, CMV titers both in inoculated and non-inoculated leaves were highly positive for CMV-Fny, but with no detection for CMV-FnyΔ2b both in inoculated and non-inoculated leaves. The virus titers in both categories of leaves were at levels below the healthy control threshold. The same trend detected in tobacco and squash plants for CMV-Fny, with high increase in CMV accumulation, also detected in pepper plants. In contrast, CMV accumulation only in inoculated leaves was detected for the CMV-FnyΔ2b-inoculated peppers.

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RT-PCR confirmed the presence of CMV RNA 2 for CMV-Fny and CMV-FnyΔ2b in leaf tissue of inoculated tobacco and pepper plants with no contamination from the other (Fig. 3.4).
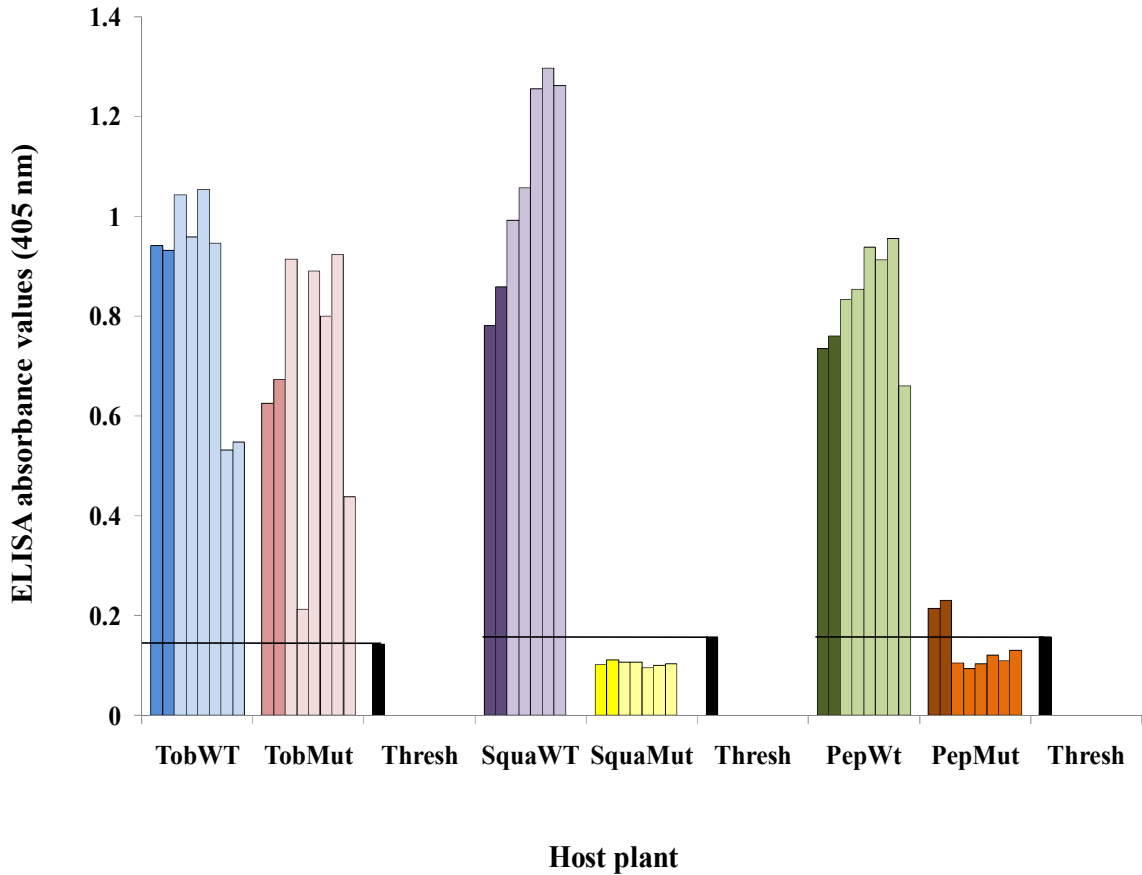
Figure 3.3 Evaluation of *Cucumber mosaic virus* accumulation in leaf tissues on CMV-Fny-inoculated tobacco (TobWT), squash (SquaWT) and pepper (PepWT) and CMV-FnyΔ2b-inoculated tobacco (TobMut), squash (SquaMut) and pepper (PepMut) at 12 days post-inoculation. Virus titers were determined by double antibody sandwich enzyme-linked immunosorbent assay (ELISA). The color bars represent the mean value from 3 inoculated plants, whereas the black bars represent that from 1 healthy plant. The bars in each category of plants darker in color represent the inoculated leaves, whereas those with lighter color represent individual non-inoculated leaves from the lower to the upper canopy. The positive ELISA value threshold (Thresh) representing the mean plus three standard deviations for comparable healthy control samples.
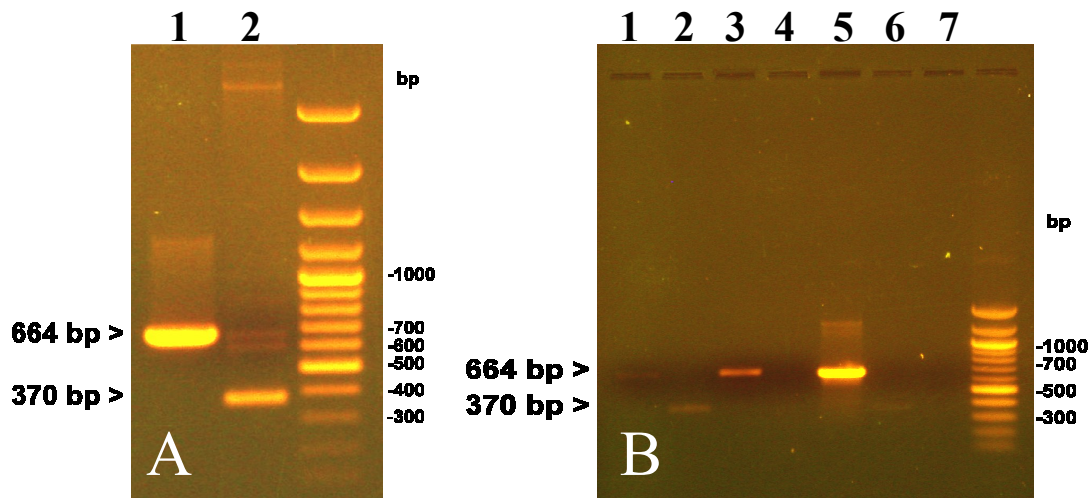
Figure 3.4 The presence of RNA 2 for CMV-Fny and CMV-FnyΔ2b in non-inoculated leaves of host plants. **A,** CMV-Fny and CMV-FnyΔ2b in tobacco at 12 dpi (lane 1, and 2, respectively); **B,** CMV-Fny in tobacco, in pepper, and in the positive control of RNA 2 (lane 1, 3, and 5, respectively) and the comparable CMV-FnyΔ2b (lane 2, 4, and 6, respectively) relative to the negative control (lane 7) at 15 dpi.

CMV-Fny and CMV-FnyΔ2b accumulation in Calwonder pepper plants.

Typical systemic symptoms including chlorosis, mosaic, and leaf distortion were occurred in young, non-inoculated leaves of pepper (cv. Calwonder) by CMV-Fny. As observed in the previous experiment, each of the three symptom phases occurred, i.e., chlorosis, mosaic and leaf distortion. In addition, stunting of the primary stem occurred during the chlorosis and mosaic symptom phases. In contrast, CMV-FnyΔ2b-inoculated plants were symptomless with no observable stunting of the primary stem (Fig. 3.5).

ELISA was used to determine virus accumulation in leaf tissue from plants inoculated with CMV-FnyΔ2b or CMV-Fny. Plants were tested when CMV-Fny-infection was expressing each of the systemic symptom phases (Fig. 3.6). As shown

previously (Masiri, 2010; Murphy and Bowen, 2006), CMV-Fny accumulated to high levels in inoculated and non-inoculated leaves in chlorosis and mosaic phases with a decline in the leaf distortion phase. In contrast, CMV-FnyΔ2b was detected in inoculated leaves at each testing date with no virus detected in non-inoculated leaves, with the exception of one of the older non-inoculated leaves in the chlorosis phase.

Immuno-tissue blot analysis further illustrated a lack of systemic infection by CMV-FnyΔ2b in Calwonder plants (Fig. 3.7). None of the tissue prints had detectable amounts of CMV-FnyΔ2b, regardless of the symptom phase during which the prints were generated. In contrast, CMV-Fny accumulated to high levels through the stem of infected plants with an apparent reduction in accumulation in the leaf distortion phase.

Figure 3.5 Evaluation of CMV-FnyΔ2b (third row) and CMV-Fny (second row) in Calwonder plants during expression of chlorosis, 7 dpi (first column), mosaic, 12 dpi (second column), and leaf distortion, 25 dpi (third column) symptom phase relative to the mock controls (bottom row). Top row represents growth in height of CMV-FnyΔ2b (middle), CMV-Fny (left), and the mock control (right) **A,** during the chlorosis; **B,** mosaic; and **C,** growth in height of CMV-FnyΔ2b (left), CMV-Fny (middle), and the mock control (right) during the leaf distortion symptom phase.
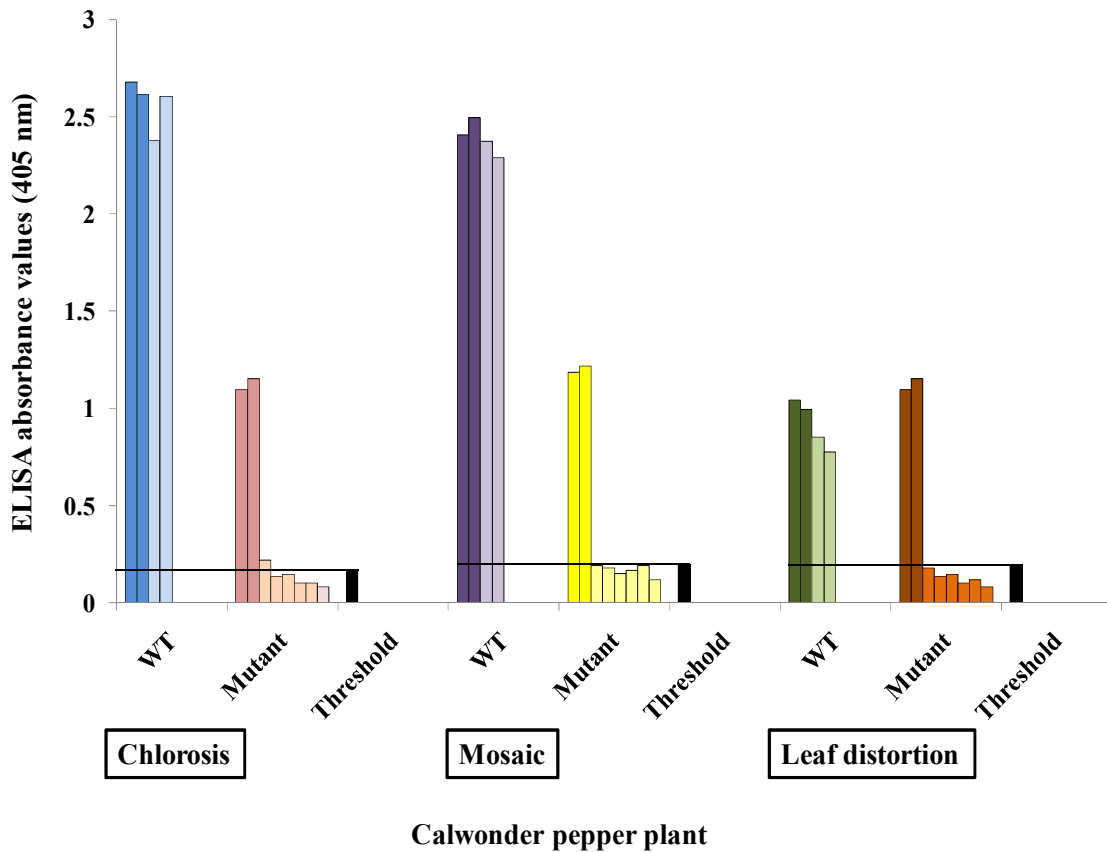
Figure 3.6 Evaluation of *Cucumber mosaic virus* in individual asymptomatic leaf tissues for CMV-FnyΔ2b (Mutant)-infected Calwonder plants relative to symptomatic leaf tissues for CMV-Fny (WT)-infected plants during the chlorosis (7 dpi), mosaic (12 dpi) and leaf distortion (25 dpi) symptom phases. Virus titers were determined by double antibody sandwich enzyme-linked immunosorbent assay (ELISA). The color bars represent the mean value from 10 infected plants, whereas the black bars represent that from 3 healthy plants. The bars in each category of plants darker in color represent inoculated leaves, whereas those with lighter color represent individual non-inoculated leaves from the lower to the upper canopy. The positive ELISA value threshold (black bars) representing the mean plus three standard deviations for comparable healthy control samples.
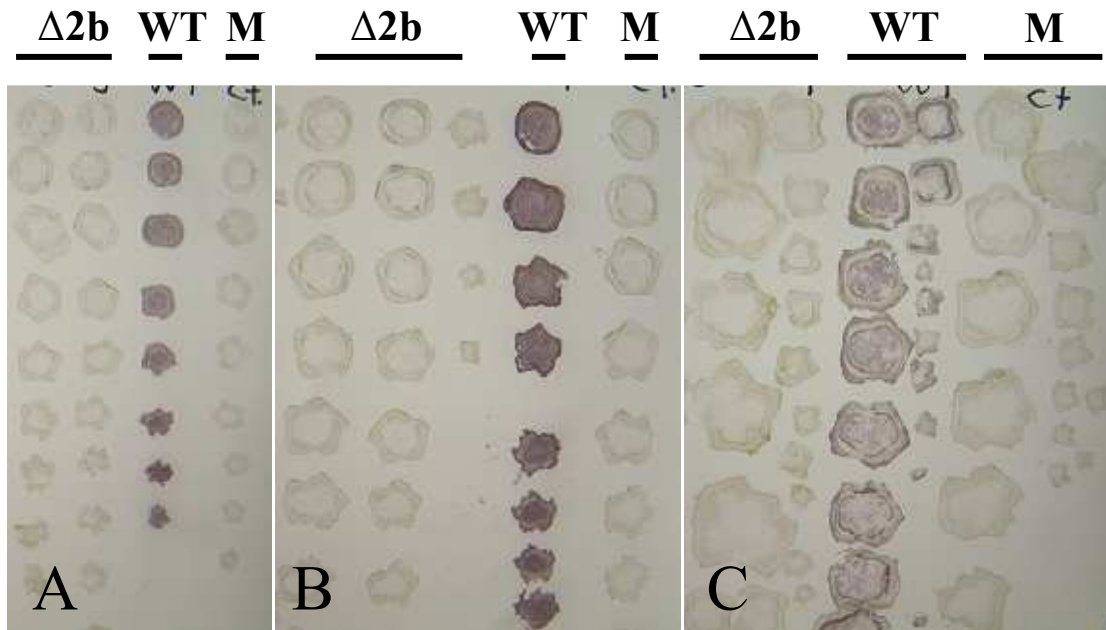
Figure 3.7 Detection of *Cucumber mosaic virus* coat protein in stem sections for CMV-FnyΔ2b (Δ2b)-infected Calwonder plants and CMV-Fny (WT)-infected plants during expression of each symptom phase relative to the mock (M)-inoculated plants by immuno-tissue blot analysis. **A,** tissue prints at the chlorosis (7 dpi); **B,** mosaic (12 dpi); and **C,** leaf distortion (25 dpi) symptom phases. The brown stain indicates presence of CMV coat protein.

CMV-Fny and CMV-FnyΔ2b accumulation in mesophyll protoplasts. CMV accumulation by ELISA analysis was to determine the accumulation for CMV-Fny and CMV-FnyΔ2b at the cellular level. As shown in Fig. 3.8, CMV-Fny and CMV-FnyΔ2b were detected from protoplast samples at 24 hr post-inoculation. CMV-FnyΔ2b accumulation (based on ELISA absorbance values) was always less than that for CMV-Fny. This experiment was repeated one time with similar results (data not shown).
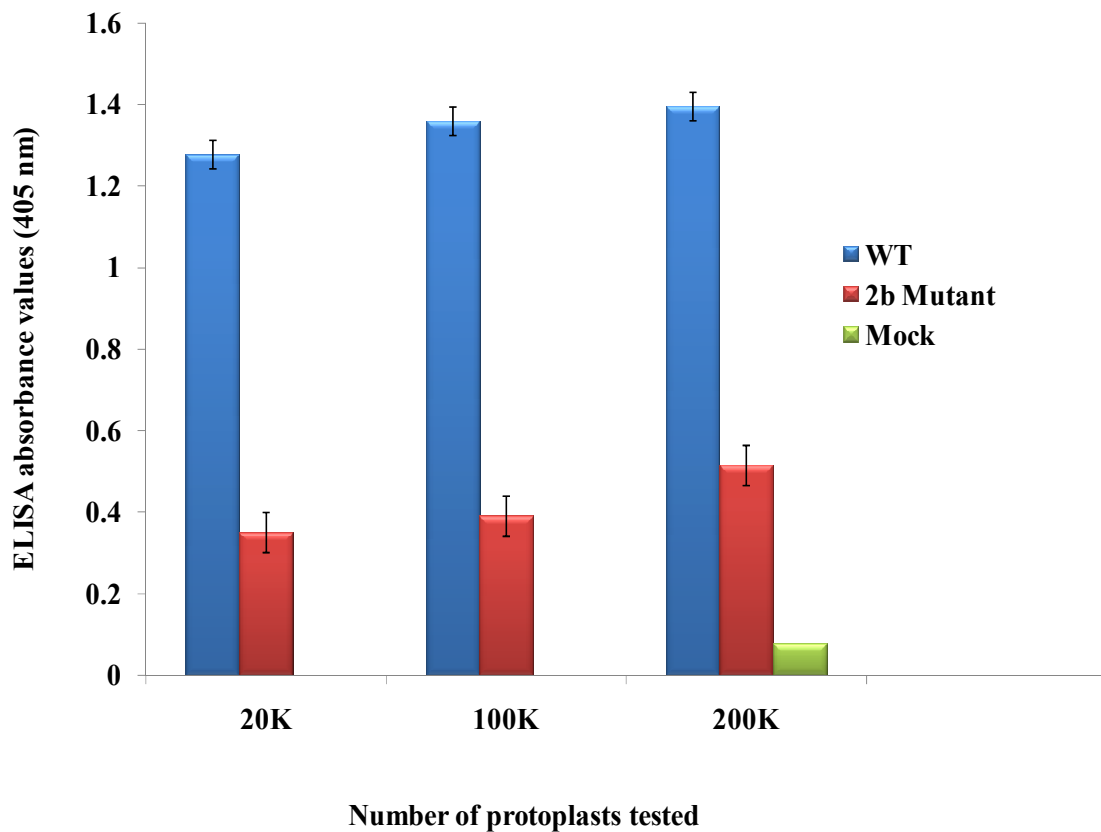
Figure 3.8 The accumulation of *Cucumber mosaic virus* in CMV-Fny- and CMV-FnyΔ2b-inoculated Calwonder protoplasts at 24 hours post-inoculation. Virus titers were determined by double antibody sandwich enzyme-linked immunosorbent assay (ELISA). Error bars refer to standard error of the mean.

3.4 Discussion

This study has provided a genetic basis of CMV on symptom induction in pepper plants that assessed through the effects of the 2b protein of Fny strain of CMV RNA 2. Based on the results, we consider it likely that CMV 2b is the symptom determinant in different solanaceous host species, including peppers, based on induction of symptom expression and its accumulation both in inoculated and non-inoculated tissues.

The transcripts of a mutant of the CMV subgroup IA strain Fny lacking the gene encoding the 2b protein (CMV-FnyΔ2b) exhibited differently among the three different host plant species examined. In Calwonder pepper plants, CMV-FnyΔ2b caused a mild chlorotic vein-clearing on older leaves, but no symptoms were detected in young developing leaves. CMV accumulation in inoculated tissues as well as those showing mild symptoms was detected by ELISA; however, the accumulation appeared to be more slowly than that for CMV-Fny. A chlorotic vein-clearing symptom, however, disappeared after propagating CMV-FnyΔ2b by a mechanical passage in healthy pepper plants, causing no visible symptoms on a whole plant associated with no detection of CMV in upper, non-inoculated leaf tissues by ELISA and RT-PCR analyses. The accumulation of CMV in mesophyll protoplasts was consistent with the detection in leaf tissues by ELISA and the stem by immuno-tissue blot analysis. CMV accumulation in CMV-FnyΔ2b- was 3 to 4-fold lower than that in CMV-Fny-infected protoplasts. This result strongly agreed with a similar work reported by Soards et al. (2002) indicating an initially slower rate of replication of CMVΔ2b-GFP-infected than that of CMV-GFP-infected tobacco protoplasts. However, the relative amounts of virus replication revealed

no significant differences among protoplasts infected with CMV-GFP and CMVΔ2b-GFP by 20 hr post-electroporation.

In tobaccos (*N. tabacum*), CMV-FnyΔ2b induced a symptomless systemic infection. These CMV-FnyΔ2b- were distinguished from CMV-Fny-infected plants by using RT-PCR. Symptomless systemic infection coincided with a lower accumulation for CMV-FnyΔ2b both in inoculated and non-inoculated tissues than that for CMV-Fny. This part of our findings appears to be consistent with a previous study by Soards et al. (2002) in which a systemic spread of CMV-FnyΔ2b in tobacco plants was slower than that for CMV-Fny. This finding is also supported by the recent observation by Ziebell et al. (2007) indicating that CMV-FnyΔ2b infections in tobacco were symptomless and they continued to be symptom-free even after superinfection on the same leaf was challenged by CMV-Fny. Alternatively, as reported by Ji and Ding (2001), CMV-FnyΔ2b was unable to establish systemic infections when older *N. glutinosa* seedlings or young seedlings pretreated with salicylic acid were used for infection. This suggests that the 2b protein is able to inhibit various defense reactions. Rather, by suppression of the post-transcriptional gene silencing (PTGS) mechanism (Brigneti et al., 1998; Guo and Ding, 2002; Li et al., 1999), in this case, it acted by interference with the salicylic acid-mediated response, an important component in the signal transduction pathway that leads to systemic acquired resistant (SAR) (Raskin, 1992; Ryals et al., 1996).

In squash (*C. pepo*) plants, however, no infections occurred in view of the fact that the accumulation of CMV-FnyΔ2b both in inoculated cotyledons and upper, non-inoculated leaves was below the healthy control threshold. This suggests the restriction of further spread antiviral silencing after the initial cell-to-cell movement as documented in

the past by Guo and Ding (2002). Contrary to our results, a previous study has been documented in cucumber (*Cucumis sativus*) plants by Ding et al. (1995b) indicating that although CMV-FnyΔ2b failed to spread systemically, it was able to accumulate in the inoculated cotyledons. Thus, it seems unclear whether different species of cucurbitaceous host plants respond differently on the mechanism of RNA silencing antiviral defense system. Taken together, our observation alone would be consistent with a conclusion that differential virulence of CMV appears to be attributable to its 2b protein in certain host species as has been broadly documented (Beclin et al., 1998; Diaz-Pendon and Ding, 2008; Ding et al., 1995b; Ding et al., 1996; Du et al., 2007; Du et al., 2008; Lewsey et al, 2007; Lewsey et al., 2009; Shi et al., 2002; Shi et al., 2003; Soards et al., 2002; Ziebell et al., 2007; Ziebell et al., 2009).

In summary, it is apparent that a deficiency of 2b, a gene encoding silencing suppressor protein or a viral counter-defense factor that interferes with the establishment of virus-induced gene silencing in plants tissue, could be another option for generating mild or non-symptom-inducing strain for investigating in the system of plant-virus interactions, such as cross protection, in certain host plant species.

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