Response of Resistant Pepper Varieties to Viruses in the Genus *Potyvirus*

by

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Abstract

Potyviruses are a persistent threat to bell pepper (*Capsicum annuum* L.) production worldwide. We have expended much effort to study the resistance response of pepper cultivars at cellular and whole plant levels. To evaluate the resistance response at the cellular level, mesophyll protoplasts are isolated and inoculated with viral RNA. An efficient isolation procedure was available but an inoculation procedure was needed that provided consistent and highly efficient inoculation. An electroporation inoculation procedure was developed by evaluation of key parameters that included voltage, number of pulses, time interval between pulses, viral RNA concentration and number of evaluated protoplasts. Consistent infection with the highest virus titer and protoplast viability resulted when 40 µg of virus RNA was used to inoculate 500,000 protoplasts using two 25-msec pulses of 200 volts each with a 10-sec time interval between pulses.

Two important sources of resistance were evaluated for their response to inoculation with four strains of *Tobacco etch virus* (TEV, genus *Potyvirus*). The resistant cultivars were CA4 and Dempsey which contain the *pvr1* and *pvr1*^{2} resistance genes, respectively. Both cultivars resisted infection by TEV strain NW; however, two CA4 plants inoculated with NW maintained in pepper became infected. When the infected CA4 plants were used as inoculum of additional CA4 plants, the newly inoculated plants developed systemic symptoms and accumulated virus in non-inoculated leaves more quickly than the originally infected CA4 plants. This new NW isolate, referred to as

NW-CA4 was tested extensively and shown to overcome the resistances expressed by both CA4 and Dempsey. The potyviral VPg is believed to be the determinant for *pvr1* and *pvr1*^{2} resistance genes, both of which are eIF4E encoding genes. The VPg amino acid sequence for NW-CA4 was determined and compared with that of NW isolates; two substitutions were found within regions of the VPg that were shown to be associated with overcoming eIF4E related resistances by other potyviruses.

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List of Abbreviations

CMV	*Cucumber mosaic virus*
dpi	days post-inoculation
eIF4E	Eukaryotic translation initiation factor 4E
ELISA	Enzyme-linked immunosorbent assay
msec	milliseconds
PEG	Polyethylene glycol
PepMoV	*Pepper mottle virus*
PVY	*Potato virus Y*
RNA	Ribonucleic Acid
SNP	Single-nucleotide polymorphism
TEV	*Tobacco etch virus*
TMV	*Tobacco mosaic virus*
VPg	Viral genome-linked protein

Chapter 1

Overview of plant virus infection process and resistance mechanisms

Plant viruses are among the most important agricultural pathogens because they are responsible for a significant proportion of plant diseases and are very difficult to combat due to the scarcity of effective countermeasures (Strange and Scott, 2005). Viral diseases can affect food quality as well as reducing yields. Food quality – may also affected by applications of pesticides used to manage virus vectors. A primary control strategy is the use of crop varieties that are genetically resistant. This is the most effective and reliable approach with no additional cost for the producer during the growing season and is environmentally friendly and safe for the consumer (Gomez et al., 2009; Kyrychenko et al., 2007). Resistant varieties carry heritable traits that are responsible for the suppression of virus multiplication or/and spread even under favorable conditions for virus infection (Kang et al, 2005b; Lewsey et al.,2009). In order to understand better the mechanisms of resistance, we should study first how the infection by a virus takes place in a susceptible plant.

1.1 Infection process

Plant viruses are intracellular pathogens that have a small genome relative to most other pathogens. As a result, several host factors are required for their replication. Viruses have evolved in such a way to make use of almost all the resources of its host, and hence interact with and manipulate the host pathways to transform their hosts into "virus factories" (Brizard et al., 2006; Culver and Padmanabhan, 2007; Nelson and Citovsky, 2005).

Successful infection of the host plant completely depends on the ability of progeny virus to move from the infected cell into neighboring cells and subsequently to the other parts of the plant (systemic infection). This infection process begins with the entry of virus particles into the plant cell cytoplasm, translation of viral proteins, genome replication and assembly of new virus particles. Progeny virus move cell to cell within the inoculated leaf from the initial cell to neighbor host cells through intercellular channels called plasmodesmata. Finally, the movement of progeny virus within the whole plant through the plant's nutrient transport system, the phloem (Culver and Padmanabhan, 2007; Hull, 2001; Hull, 2009; Nelson and Citovsky, 2005).

1.1.1 Entry of viruses to plant cells

Plant cell architecture represents a unique challenge for virus entry due to the presence of a rigid cell wall. Initial entry into the plant cell requires a non-lethal break in integrity of the cell wall and the plasmamembrane, most often produced by the virus' vector or by mechanical means (Hull, 2001). Thus, introduction into that initially

infected cell is a physical process performed by a vector or through mechanical means; however, it is essential that the process is non-lethal to the cell. Above ground, invertebrates such as aphids, leafhoppers, whiteflies and thrips, are known vectors for a number of plant viruses. Below ground, vectors are nematodes and fungi which feed on the root system of the plant (Andret-Link and Funchs, 2005; Campbell, 1996).

Mechanical damage to the plant's cell wall also can be a means of entry of plant viruses. This approach is used most often in experimental settings when the leaf surface is abraded prior to inoculation with a virus suspension, but also entry may happen as a result of agricultural applications, such as harvesting, vegetative propagation and grafting (Hull, 2001).

1.1.2 Plant virus gene expression, replication strategies and assembly of new virus particles

Given the relatively compact nature of their genomes, plant viruses have evolved a variety of strategies to expand their coding capacity and regulate gene expression while adhering to the rules for translation for mRNAs in their hosts. The majority (about 80%) of plant RNA viruses are non-enveloped and have single-stranded genomes with positive polarity (Zaccomer el at., 1995; Lewsey et al, 2009). All of the positive sense plant RNA viruses have genomes that behave like mRNAs and can be translated entirely or, in part, immediately after entrance to the cell. The newly synthetized viral RNA polymerase copies the viral genome to produce a complementary negative strand RNA (antisense intermediate). This antisense strand is used as template for synthesis of multiple viral

sense copies (Ahlquist et al., 2003; Mandahar, 2006). Plant viruses have to reconcile their genome structure with the requirements for monocistronic mRNAs in eukaryotic cells and to accomplish that, they use several strategies to regulate their genome expression at the level of transcription (e.g., through production of subgenomic RNA) and at the level of translation (through polyprotein synthesis, leaky ribosome scanning, read-through translation, ribosomal frameshift and internal ribosome entry) (Hull, 2001; Mandahar, 2006). Potyviruses and Cucumovirus are two examples of diverse strategies, Potyviruses have a single strand of single-stranded RNA that contains one ORF that encodes a polyprotein, whereas, Cucumoviruses have a tripartite genome, that contain five ORFs and synthesize two subgenomic RNAs (Adams et al, 2005a; Palukaitis and Garcia-Arenal 2003).

Potyvirus RNA genomes are about 10 kb in length, polyadenylated at their 3' ends, and covalently linked to a viral protein (VPg) at their 5' ends (Murphy et al., 1991; Urcuqui-Inchima et al., 2001). The viral genome is translated upon infection into a single polyprotein, which is processed into at least ten proteins by virally encoded proteases (Fig.1.1) (Adams, et al 2005a; Dougherty and Carrington, 1988; Revers et al., 1999; Urcuqui-Inchima et al., 2001).

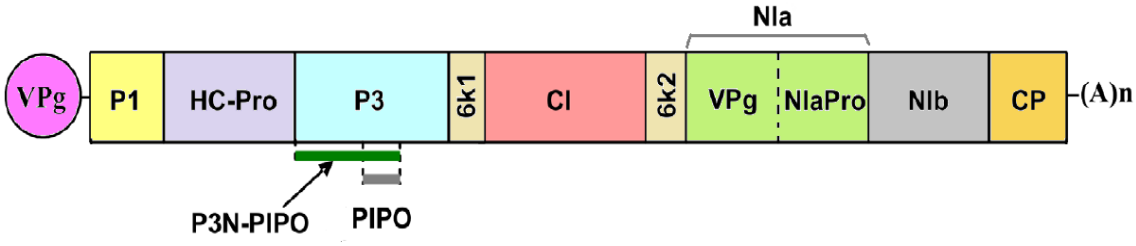

Figure 1.1 Schematic map of a Potyvirus genome. The genome contains one ORF, which is processed into ten mature proteins: P1, helper-component proteinase (HC-Pro), P3, 6K1, cylindrical inclusion (CI), 6K2, nuclear inclusion protein a (NIa- which is further cleaved into the VPg and the NIa-Pro), nuclear inclusion protein b (NIb), and coat protein (CP). The processing sites are indicated by vertical lines. VPg represented by the pink circle, is covalently attached to the 5' end of the viral RNA and the 3' end terminates with a Poly (A) tail. An additional ORF which is embedded in the P3 coding region encodes for the PIPO protein (short for pretty interesting potyvirus ORF) which is thought to be expressed as a fusion protein with the N-terminal portion of P3 (P3N-PIPO). These latter proteins are represented by the green and gray bars.

Cucumber mosaic virus (CMV) is the type member of the genus *Cucumovirus*, family *Bromoviridae* (Palukaitis and Garcia-Arenal 2003). The CMV genome is multipartite, organized into three single-stranded positive-sense genomic RNAs (RNAs 1, 2 and 3) and two major subgenomic RNAs (RNAs 4 and 4A, which serve for the expression of the 3'-proximal gene of RNAs 3 and 2, respectively). CMV genome encodes for five proteins: RNA 1 encodes protein 1a, which is necessary for viral replication. RNA 2 encodes protein 2a, the viral polymerase, and protein 2b, expressed from the subgenomic RNA 4A (Ding et al. 1994) and is involved in the suppression of gene silencing. RNA 3 encodes the 3a movement protein, which is responsible for the cell-to-cell movement of the virus (Li et al., 2001), and the coat protein (CP), which is expressed from subgenomic RNA 4 (fig.1.2) (Palukaitis and Garcia-Arenal 2003; Roossinck, 2002).

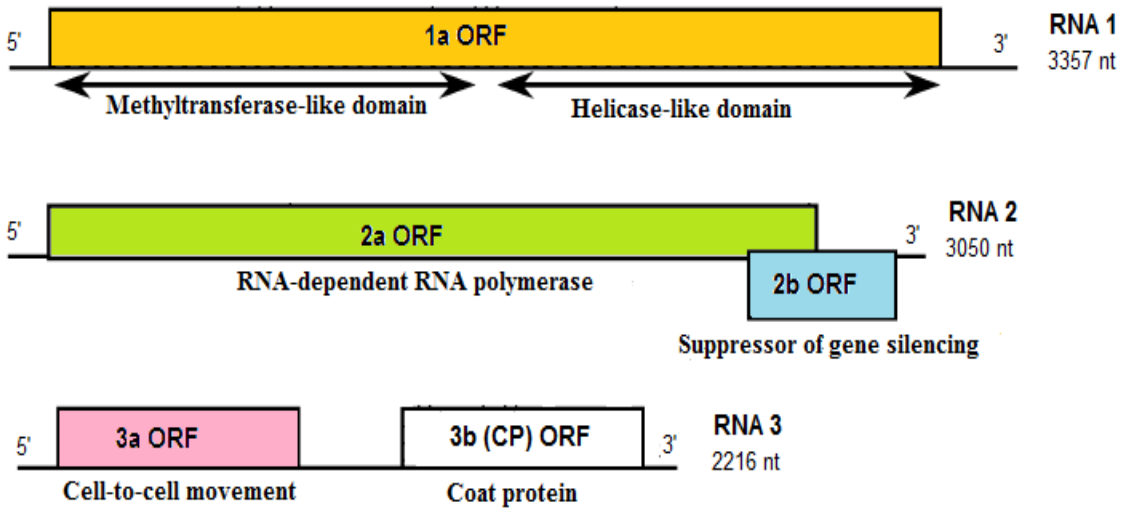

Figure 1.2 Schematic map of *Cucumber mosaic virus* (CMV) genome. CMV genome includes three RNAs that contain five ORFs. RNA1 encodes for protein 1a which has two domains methyltransferase and helicase, and is needed for genome replication. RNA 2 encodes the viral RNA polymerase and its subgenomic RNA 4A (sgRNA 4A) encodes protein 2b which functions as a gene silencing suppressor. The RNA 3 encodes the movement protein (3a) and its sgRNA 4 encodes the coat protein (CP). The CP of CMV is multifunctional and is involved in aphid transmission, as well as in cell-to-cell and systemic movement.

Large amounts of the viral proteins, especially the coat protein, are synthesized upon cell infection. Once the viral RNA and protein subunits have been synthesized, the two must aggregate to constitute the virus particles. Coat protein subunits fold in a specific manner because of their intrinsic properties and aggregate by themselves to form the capsid/capsid sub-assemblies in which genome molecule(s) must be incorporated to give the complete virus particle (Mandahar, 2006).

1.1.3 Cell to cell movement

Plant viruses face the barrier that is the plant cell wall not only for their entry into the cytoplasm but also their spread into neighboring host cells. Plant cells, although surrounded by walls, are connected by intercellular channels called plasmodesmata, through which, all plant cells are potentially in communication with each other through the passage of solutes and molecules (Lewsey et al.,2009). Therefore, although the cell wall represents a barrier, the plasmodesmata represent an opportunity for the virus to move directly from cell to cell without having to go through an extracellular phase (Lee and Lu, 2011).

All plant viruses encode a unique class of proteins namely movement proteins (MPs), that modify plasmodesma in a nondestructive way, increasing plasmodesmata size exclusion limit (Benitez-Alonso et al., 2010; Lewsey et al., 2009; Waigmann et al, 2004). Also, MPs may bind and unfold single-stranded RNAs to facilitate their cell-to-cell translocation by building protein-RNA transport complexes (ribonucleoprotein (RNP) complexes) in a fashion used by *Tobacco mosaic virus* (TMV) and other viruses that do

not require CP to move locally from cell to cell (Lucas et al., 2006). Through this mechanism, the virus spreads from the initially infected cell, which is usually epidermal or mesophyll, via bundle sheath cells, phloem parenchyma and companion cells to finally enter into phloem sieve elements (SE) where they are translocated to other parts of the plant. Several studies indicate that cell-to-cell and systemic virus transport follow different mechanisms, and there seem to be different viral and endogenous plant components involved (Lewsey, et al., 2009).

1.1.4 Movement through the phloem.

Plant viruses use the nutrient transport system of the plant, the phloem, to move out of the inoculated leaf to distant parts of the plant and effectively establish systemic infection (infection of the whole plant) (Waigmann et al., 2004; Scholthof, 2005). The entry into the phloem occurs through the companion cells connected to the sieve tubes and virus movement occurs with the flow of the photoassimilates from source tissues (where solutes are synthesized, e.g. photosynthetic leaves) to sinks (where transported substances are metabolized or stored, e. g. roots, actively growing tissues tubers and fruits) (Benitez-Alonso, et al., 2010; Lee and Lu, 2011). In the sink organs, the virus is unloaded from the phloem and then spreads progressively from cell to cell through the tissues. Few studies have focused on the mechanisms by which plant virus particles move out of phloem sieve elements to penetrate plant cells when invading a new tissue of the plant (unloading), but it is proposed that the CP in concert with MP, might act to dilate plasmodesmata at the boundary between vascular and non-vascular tissues in a mechanism distinct to the entry of virus into the phloem (loading) (Ueki and Citovsky,

2007). Evidence suggest that most plant viruses require, besides MPs, the CP for this long-distance movement, although whether this occurs in the form of stable assembled virions in all cases remains unknown (Ueki and Citovsky, 2007; Waigmann et al., 2004). Once the virus enters the phloem, movement can be very rapid. It takes some time before the middle-aged and older leaves become infected and this likely represents a cell-to-cell movement rather than phloem-based (Hull, 2001).

1.2 Natural defense mechanisms against viruses in plants

Resistance results from factors that delay or inhibit replication and systemic infection by a specific virus. These interferences may happen in any of the steps of the infection cycle, e.g., inhibition of replication, blockage of cell to cell movement or long-distance movement (Dawson and Hilf, 1992; Zaitlin and Hull, 1987). Numerous other types of resistance have been described but are not included in this review. For example, RNA silencing mechanisms operate to restrict viral replication at the single cell level and long-distance movement in several plant-virus interactions, (Diaz-Pendon and Ding, 2008; Dunoyer and Voinnet, 2008; Eamens et al., 2008; Mlotshwa et al., 2008; Giner et al, 2010). Induced resistance responses, which are governed by dominant R genes, are not reviewed in detail here, but have been broadly described in several recent papers (Caplan and Dinesh-Kumar, 2006; Fraile and Garcia-Arenal, 2010; Gilliland et al., 2006; Kang et al., 2005b; Lanfermejier and Hille, 2007; Maule et al., 2007).

1.2.1 Cellular resistance to plant viruses:

Resistance at the single cell level may be defined as a state where virus replication does not occur, or occurs at low levels in inoculated cells (Kang et al., 2005b; Narayanasamy, 2008). When virus replication is prevented, the plant's response is termed immunity (Bruening, 2006) and usually is assessed in isolated, single cells (protoplasts). If immunity occurs against all biotypes of a pathogen and in all cultivars or accessions of a particular plant species, the situation is called non-host resistance (Palukaitis and Carr, 2008).

Extreme resistance also operates at the cell level. This resistance is characterized by extremely low virus accumulation, not associated with cell death and complete prevention of virus spread (Palukaitis and Carr, 2008). Immunity and extreme resistance are difficult to differentiate when no highly sensitive detection techniques are employed. Extreme resistance is conferred by a dominant R gene, a well known example is the gene *Ry* from potato that restricts accumulation of the Potyviruses, *Potato virus Y* (PVY), *Potato virus A and Tobacco etch virus* (Flis et al., 2005; Hinrichs et al., 1998; Revers et al., 1999) in a mechanism very similar to the hypersensitive reaction (HR). Another well known example is the resistance of *Vigna unguiculata* cv. 'Arlington' to *Cowpea mosaic virus*, where a dominant resistant gene encodes a protease inhibitor that prevents virus polyprotein processing (Ponz et al, 1998). The *Tm1* gene of tomato is a third gene of this type in which the resistance is direct. It has been proposed that the product of *Tm1* confers resistance to TMV and *Tomato mosaic virus* by disrupting the function of the virus-encoded replicase component (Ishibashi et al., 2007; Watanabe et al., 1987).

1.2.2 Resistance to virus movement between cells.

In those instances where the virus is able to multiply at normal levels in the inoculated cells but it cannot move out from them, a subliminal infection is said to occur (Cheo, 1970; Hull, 2009; Sulzinski and Zaitlin, 1982). Resistance at this level can result from either failure of interactions between plant and viral factors necessary for cell-to-cell movement, or from active host defense responses that rapidly restrict virus spread (Fraser, 1992; Fraser, 2000; Narayanasamy, 2008).

1.2.3 Resistance to long-distance movement of virus within the plant

In susceptible hosts, plant viruses move cell-to-cell from the epidermal or mesophyll cells to the companion cells where the virus potentially has direct access to the sieve tube, the conducting element of the phloem that serves as the pathway for both nutrient and virus transport throughout the plant (Kang et al., 2005b). Entry into the sieve element-companion cell complex is currently thought to be the most significant barrier to long-distance movement. The result is either limited invasion of the plant, e.g., the virus remains in the lower portions of the plant, or may move systemically more slowly than in the susceptible host.

A specific example for this latter case, where systemic movement is delayed and reduced occurs when *Pepper mottle virus* (PepMoV) infects *Capsicum annuum* cv. Avelar. In this case, the virus accumulates in inoculated leaves and moves into the stem but does not enter the internal phloem for systemic movement to young tissues (Andrianifahanana et al., 1997). Interestingly, the infection with a second virus CMV

alleviates this restriction (Guerini and Murphy, 1999; Murphy and Kyle, 1995), which suggests that CMV is able to compensate the defect in the host, either by supplying a factor that facilitates movement of both viruses or alleviating the restriction for an unknown mechanism (Kang et al, 2005b; Ueki and Citovsky, 2006). A similar type of resistance was described for CMV in pepper cvs 'Milord' and 'Vania' whereby virus accumulated in external but not internal phloem in the petiole of the inoculated leaf and the lower part of the stem (Caranta et al, 2002; Dufour et al., 1989).

1.3 The research chapters

The primary focus of the research described in this dissertation is genetic resistance to viruses in the genus *Potyvirus*. While performing experiments to evaluate different sources of resistance in *Capsicum sp.*, a *Tobacco etch virus* (TEV, strain NW) isolate able to overcome two important resistances was identified and characterized (Appendix). Biological and molecular data indicate that this new isolate, referred to as TEV-NW-CA4, differs from the parent strain (Chu et al., 1995.) and the wild type strains maintained in our laboratory.

Plant resistance mechanisms and virus fitness studies are performed at whole plant and cellular levels. Cellular level studies often make use of plant protoplasts which offer unique *in vivo*/*in vitro* systems of evaluation, i.e., *in vivo* since they involve a living cell but *in vitro* since the protoplast has lost its important connection to neighboring cells and therefore is not a "normal" environment. In an effort to further our studies to understand the nature of *Capsicum* resistance and virus fitness, an efficient and highly

consistent protoplast inoculation procedure is needed. The ability to isolate pepper protoplasts is standard procedure in our laboratory; however, an efficient inoculation procedure for potyviruses was not available. A highly efficient electroporation-based procedure was developed that will be used further evaluate the biological and molecular characteristics of TEV-NW-CA4.

Chapter 2

Characterization of a TEV isolate that overcomes resistance in pepper

Abstract

Two important sources of resistance were evaluated for their response to inoculation with four strains of *Tobacco etch virus* (TEV, genus *Potyvirus*). The resistant cultivars were CA4 and Dempsey which contain the *pvr1* and *pvr1*^{2} resistance genes, respectively. Both cultivars resisted infection by TEV strain NW; however, two CA4 plants inoculated with NW maintained in pepper became infected. When the infected CA4 plants were used as inoculum of additional CA4 plants, the newly inoculated plants developed systemic symptoms and accumulated virus in non-inoculated leaves more quickly than the originally infected CA4 plants. This new NW isolate, referred to as NW-CA4 was tested extensively and shown to overcome the resistances expressed by both CA4 and Dempsey. The potyviral VPg is believed to be the determinant for *pvr1* and *pvr1*^{2} resistance genes, both of which are eIF4E encoding genes. The VPg amino acid sequence for NW-CA4 was determined and compared with that of NW isolates; two substitutions were found within regions of the VPg that were shown to be associated with overcoming eIF4E related resistances by other potyviruses.

2.1 Introduction

Tobacco etch virus (TEV) is a member of the *Potyvirus* genus and possesses a positive sense, single-stranded RNA genome of approximately 10 Kb (Allison et al., 1986; Chu et al., 1995). It is widely distributed in North, Central and South America, and most recently found in Europe and Asia (CABI, 2010). TEV has a moderately wide host range including 149 plant species in 19 families (Shukla et al., 1994; Edwardson and Christie, 1997), although most of them belong to the *Solanaceae*. For pepper, yield reductions range from 25 to 70% (Nutter et al., 1989; Rufty et al., 1989) with the potential for greater reductions in yield when plants are co-infected with TEV and, for example *Cuccumber mosaic virus* (CMV), *Pepper mottle virus* (PepMoV) or *Potato virus Y* (PVY) (Green and Kim, 1991; Roberts et al., 2004).

Since the original description of resistance to TEV in peppers (McKinney, 1952), much effort has been made to identify and introduce resistance genes into commercially acceptable varieties, with a particular interest given to breeding sources that display resistance for several potyviruses. Two such examples include *Capsicum annuum* cv 'Dempsey' and *C. chinense* PI 152934 (aka CA4) which harbor recessive genes *pvr1*^{2} and *pvr1*, respectively (Kyle and Palloix, 1997). Dempsey was obtained from a three way cross between PI 163192, PI 264281 and Jupiter (Lane et al., 1997) and was shown to be immune to infection by TEV strains -HAT, -NW and –Mex21, PepMoV strain California (PepMoV-CA) and PVY (Deom et al., 1997; Kang et al., 2005a; Lane et al., 1997; Yeam et al., 2005). CA4 has been used for genome mapping (Chaim et al, 2003; Livingstone, et al., 1999) and was shown to be resistant to PepMoV, PVY and TEV strains HAT, N and NW (Kang et al., 2005a; Murphy et al., 1998; Yeam et al., 2007).

CA4 *pvr1* and Dempsey *pvr1*^{2} were mapped to the same genetic locus on chromosome 3; they have a common degree of resistance which is considered (perhaps) operational immunity and; they encode for translation initiation factor eIF4E which interacts with a virus protein, VPg. The VPg (viral protein, genome-linked), is covalently linked to the 5' terminus of the potyvirus genome (Murphy et al.,1991; Riechmann et al., 1989; Roudet-Tavert et al.,2007) and is essential for potyvirus infectivity in pepper (Charron et al., 2008; Grzela et al., 2006; Kang et al., 2005a; Leonard et al., 2000; Ruffel et al., 2002; Yeam et al., 2007).

Resistance conferred by alleles of the *pvr1* locus are considered of medium to high durability (Janzac et al, 2009) and have been used for more than 50 years as a breeding source for several commercial cultivars. It was shown, however, that changes in at least one amino acid in the central region of the viral VPg can enable the virus to overcome this resistance, e.g., PVY strains and pepper alleles *pvr1*^{1}, *pvr1*^{2} and *pvr1*^{3} (Ayme et al., 2006; Ayme et al., 2007; Moury et al.,2004). The potential for a virus species to overcome these resistance alleles represents a serious threat to the pepper industry.

We have identified a TEV-NW isolate that systemically infects CA4 and Dempsey plants, both of which have been shown to resist TEV-NW infection (Kang et al., 2005a). In this report, this new TEV-NW isolate is evaluated for its ability to infect three sources of resistance and its VPg amino acid sequence is compared with that of other TEV strains.

2.2 Materials and methods

2.2.1 Plant growth conditions, experimental design and virus infection evaluation

TEV strains used in this study included HAT and NW which were originally obtained from Dr. T. Pirone, University of Kentucky. Each virus was maintained by mechanical passage in *Capsicum annuum* L. 'Early Calwonder' and *Nicotiana tabacum* 'Kentucky 14'.

The pepper cultivars used in this study included *Capsicum annuum* L. 'Dempsey', 'Early Calwonder' and 'Avelar', *C. chinense* PI 159234 (aka CA4) and *C. frutescens* 'Tabasco'. The cultivars CA4, Dempsey and Avelar contain the recessive resistance alleles *pvr1*, *pvr1*^{2}, and *pvr1*^{1} and *pvr3*, respectively (Charron et al., 2008; Kyle and Palloix, 1997; Lane et al., 1997; Murphy et al., 1998), Tabasco pepper is an indicator host for TEV strains; some induce severe wilt symptoms in Tabasco plants but no wilt results from TEV-NW infection. Early Calwonder was used as a susceptible control.

For all experiments, pepper seed was sown in Pro-Mix soilless potting medium (Premier Peat, Riviere-du-Loup, Quebec, Canada) in 72-well Styrofoam trays (Speedling, Inc., Bushnell, FL). Upon germination, seedlings were transplanted to 16.5 cm diameter round pots containing Pro-Mix supplemented with a controlled release fertilizer (18-6-12, Osmocote Classic, Scotts Company LLC, Marysville, OH).

Experiments were performed in a greenhouse at the Auburn University Plant Science Research Facility with mean temperatures of 24°C day and 20°C night. For each experiment, plants were arranged in a randomized complete block (RCB) design with

each block consisting of the appropriate pepper cultivars and virus treatments (details for each experiment are provided below).

Virus was applied to leaves 1 and 2 (the two oldest true leaves along the stem) by rub-inoculation when pepper plants were at the early 7 to 8 leaf stage of growth (as described by Andrianifahanana et al. 1997). Virus inoculum consisted of systemically infected Early Calwonder or Kentucky 14 leaf tissue ground in 50 mM potassium phosphate buffer, pH 8.

Virus infection was determined by visual assessment and virus detection by enzyme-linked immunosorbent assay (ELISA). For visual assessment, inoculated plants were monitored for virus-induced symptoms relative to mock-inoculated control plants. Virus was detected from non-inoculated leaf tissues using a commercial ELISA kit specific to TEV (Agdia Inc., Elkhart, IN). The ELISA procedure followed that recommended by the manufacturer with modifications. The primary, plate-coating, antibody step and the sample extract (antigen) step were each stored in a moist chamber at 4°C for at least 12 h. The alkaline phosphatase conjugated antibody step was incubated in a moist chamber at 37°C for 3 h. Leaf samples were ground with a motorized leaf squeezing apparatus with the addition of general extraction buffer (as described by the manufacturer) at a ratio of 1 g of tissue to 5 mL of buffer. Leaf extracts were added to microtiter plates at a final dilution of 1:20 (tissue:buffer). For each experiment involving ELISA, healthy control samples were included on each microtiter plate that consisted of at least three leaf samples comparable to those being tested for virus infection. Substrate (1 mg/ml of para-nitrophenylphosphate dissolved in 10% diethanolamine, pH 9.8) was added to microtiter plates and reactions were allowed to develop at room temperature for

30 to 60 min. In an effort to standardize the ELISA test among experiments, at least one sample of a known amount of purified virus was included on each microtiter plate. This sample was allowed to develop to an absorbance value of 1.0, at which time the ELISA absorbance values were determined using a Sunrise microtiter plate reader (Phoenix Research Product, Hayward, CA). Samples were considered positive for the presence of virus when the absorbance value was above the threshold, determined from the average ELISA absorbance value plus three standard deviations of the appropriate healthy control samples.

2.2.2 Identification and evaluation of A TEV-NW isolate that infects CA4.

Three TEV-NW isolates were used in most experiments in this study. TEV-NW (pepper) refers to the TEV-NW isolate maintained as the Early Calwonder source of inoculum. Likewise, TEV-NW (tobacco) refers to the TEV-NW isolate maintained as the Kentucky 14 tobacco inoculum. TEV-NW-CA4 refers to the TEV-NW isolate obtained from and maintained in CA4 plants. For simplicity, these isolates will be referred to as NW-pepper, NW-tobacco and NW-CA4, respectively.

Pepper cultivars CA4 and Dempsey were evaluated for their response to inoculation with two sources of TEV-NW, one maintained in the susceptible pepper cultivar Early Calwonder (NW-pepper) and the other maintained in Kentucky 14 tobacco (NW-tobacco). Previous experiments to evaluate a moderately resistant pepper genotype showed that the source of TEV-NW inoculum led to differences in the resistance response. The two sources of TEV-NW inoculum were therefore used to evaluate CA4 and Dempsey. Seven plants of each pepper cultivar, including the susceptible control

Early Calwonder, were inoculated with either NW-pepper or NW-tobacco. Non-inoculated young leaf samples were collected from each plant at 10 and 20 dpi and tested for virus infection (at the time of tissue collection) by ELISA.

The NW-pepper infected CA4 plants identified in the first experiment were used as inoculum to test infection of CA4 plants (and Early Calwonder). Virus treatments in this experiment included NW-CA4 (the source being CA4 plants that became systemically infected after inoculation with NW-pepper), NW-pepper and NW-tobacco. Eighteen plants of each pepper cultivar were inoculated with each virus treatment and arranged in a RCB design with six plants of each cultivar per virus treatment in each block. Plants were monitored for development of virus symptoms and young, non-inoculated leaves were tested for virus infection by ELISA at 15 dpi.

NW-CA4 was further evaluated by inoculation of CA4, Dempsey, Avelar and Tabasco plants. Three distinct resistance alleles are represented by CA4, Dempsey and Avelar. Tabasco is used as an indicator host for TEV strains; the strain name NW refers to a non-wilting systemic infection of Tabasco plants (Chu et al., 1995; Chu et al., 1997). The TEV strains tested include NW-CA4, NW-pepper, NW-tobacco and TEV-HAT isolates maintained in Early Calwonder pepper (HAT-pepper) and in Kentucky 14 tobacco (HAT-tobacco). HAT was included in this experiment as it induces severe wilting of Tabasco pepper plants. Ten plants of each pepper cultivar were inoculated with each virus treatment and arranged in a RCB design with five plants of each cultivar per virus treatment in each block. Plants were monitored for development of virus symptoms and young, non-inoculated leaves were tested for virus infection by ELISA at 20 dpi.

The discovery of NW-CA4 from CA4 plants inoculated with NW-pepper but not NW-tobacco led us to question whether the ability of NW to overcome CA4's resistance was related to the source of inoculums, i.e., pepper versus tobacco. In the first experiment, only seven plants were inoculated with each source of NW. In this experiment, 40 CA4 plants were inoculated with either NW-pepper or NW-tobacco. Four Early Calwonder plants were included as a susceptible control for each virus isolate. Plants were monitored for development of virus symptoms and non-inoculated leaves were tested for virus infection by ELISA at 30 dpi.

2.2.3 RNA isolation, reverse transcription-PCR amplification and sequencing of TEV-NW VPgs.

TEV (virion) was purified from systemically infected Kentucky 14 tissue as described previously (Murphy et al., 1990). Viral RNA was purified from the virion preparation using a RNeasy purification kit according to manufacturer's instructions (Qiagen, Valencia, CA). A pair of primers was designed to align outside the region of the TEV genome that encodes for VPg and amplify from nucleotide 5637 to 6484 (847 bp): TEV-VPg FW (5'-TGGATGCTTGCAACGTACT-3') and TEV-VPg Rv (5'-ATGAGGTGTTGTTGCAAAGT-3'). The purified viral RNA (100ng) was used as a template to synthesize cDNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA), with addition of 0.1 µM of reverse primer TEV-VPg Rv and 400 µM dNTPs. PCR reactions were made with 2 µL of cDNA added to a cocktail containing 1X HF buffer (as described by Finnzymes, Espoo, Finland), 2 mM $MgCl_2$, 0.2mM dNTP mix, 0.5 µM TEV-VPg Fw primer, 0.5 µM TEV-VPg Rv primer and 1U of Phusion DNA

Polymerase (Finnzymes, Espoo, Finland). Reactions were carried out using a Multigene Gradient thermal cycler (Labnet, Woodbridge, NJ), with initial denaturation at 94°C for 2 min, followed by 30 cycles (94°C for 60 sec, 50°C for 30 sec, and 72°C for 60 sec) and a final extension step at 72°C for 10 min.

RT-PCR products were sequenced either at the Auburn University Genomics and Sequencing Lab (GSL) or the Lucigen Corporation (Middletown, WI). When the PCR products were sequenced at the GSL, the samples were purified with QIAquick PCR purification kit (Qiagen, Valencia, CA), following manufacturer's instructions. The PCR products alone, without subsequent purification, were sent to Lucigen Corporation. The identity of the nucleotide sequences was assessed using the basic local alignment search tool (BLASTN) from the NCBI web page (Altschul et al., 1990), then translated to amino acid sequence and compared using the multiple alignment program ClustalW (Thompson et al., 1994) through BioEdit v 7.0.9. A further analysis to determine conserved amino acids and regions was made with Jalview (Waterhouse et al., 2009).

2.3 Results

2.3.1 A TEV-NW isolate overcame CA4 resistance:

Experiments were performed to evaluate different sources of resistance in pepper genotypes against several potyviruses. One genotype evaluated was CA4 which contained the recessive resistance allele *pvr1*. In the first experiment, plants of each genotype were inoculated with TEV-NW using two sources of inoculum for each virus

species, one being from systemically infected Kentucky 14 tobacco (NW-tobacco) and the other being from systemically infected Early Calwonder pepper (NW-pepper).

The average ELISA value for NW-pepper in CA4 plants at 20 dpi was above the healthy control threshold, an indication of positive detection of virus, although the ELISA value was significantly lower than observed for the Early Calwonder susceptible control plants (Fig. 2.1). Two of the seven NW-pepper inoculated CA4 plants were positive for virus in non-inoculated leaves with the average ELISA value from these two plants being 0.349 ± 0.183. In contrast, none of the NW-tobacco inoculated CA4 plants developed symptoms or contained virus in non-inoculated leaves when tested by ELISA at 10 or 20 dpi. In the same experiment, no NW infection (regardless of inoculum source) was identified in Dempsey plants, whereas 100% infection occurred in the susceptible genotype, Early Calwonder, with pronounced systemic symptoms and high levels of virus accumulation.

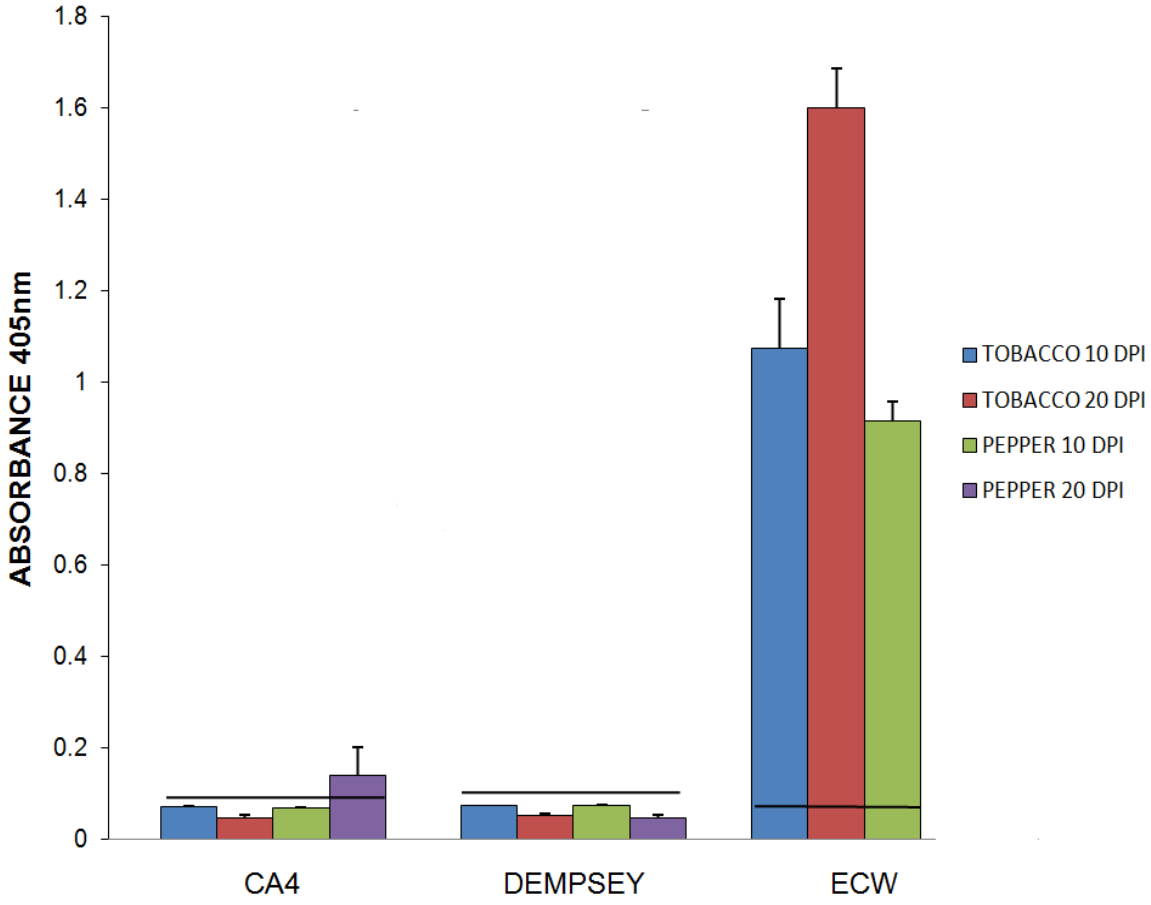

Figure 2.1 Response of Early Calwonder, Dempsey and CA4 to inoculation with *Tobacco etch virus* (TEV) strain NW from tobacco and pepper. Virus accumulation in non inoculated leaves was tested at 10 and 20 dpi by enzyme-linked immunosorbent assay (ELISA). Absorbance values above the horizontal threshold line are considered positive for virus infection.

The two CA4 plants infected with NW-pepper did not develop observable symptoms and virus was detected in non-inoculated leaves at 20 dpi but not 10 dpi, an indication of a delayed movement to and accumulation in young non-inoculated leaves. Infected young leaves from the CA4 plants were then used as inoculum for additional CA4 plants with comparative treatments including NW from pepper and tobacco. All 18 plants inoculated with NW-CA4 were systemically infected by 15 dpi with plants expressing systemic vein-clearing and mosaic symptoms on younger leaves (Fig. 2.2). The average ELISA value for NW-CA4-infected plants did not differ from that of the susceptible control Early Calwonder. In contrast, none of the 18 CA4 plants inoculated with either NW-pepper or NW-tobacco were infected at 15 dpi but all Early Calwonder plants were infected with relatively high average ELISA values (Fig. 2.2).

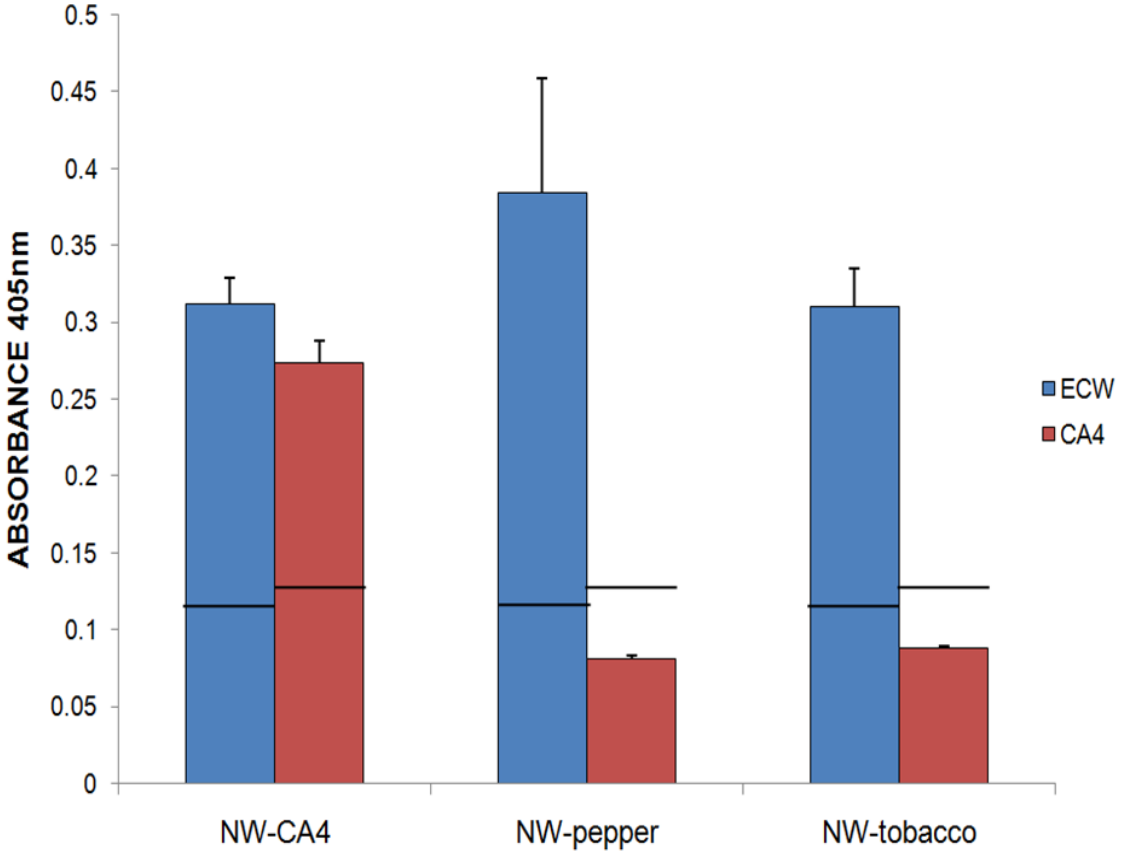

Figure 2.2 Response of Early Calwonder and CA4 to inoculation with NW-pepper, NW-tobacco and NW-CA4. Systemic infection was tested at 15 dpi by enzyme-linked immunosorbent assay (ELISA). Absorbance values above the horizontal threshold lines are considered positive.

2.3.2 Tobacco, as well as Early Calwonder pepper, is a source of NW-CA4 like isolates:

The original detection of NW-CA4 was from two of seven CA4 plants inoculated with NW-pepper but none of the CA4 plants became infected when using NW-tobacco. In an effort to challenge the possibility of pepper but not tobacco serving as a source of a NW-CA4-like isolate, 40 plants of CA4 pepper were inoculated with NW-pepper and NW-tobacco.

At 30 dpi, the average ELISA value for the 40 CA4 plants in each treatment was below the threshold for detection of NW; however, there were 3 of 40 CA4 plants and 12 of 40 CA4 plants that were infected from NW-tobacco and NW-pepper, respectively (Fig 2.3). The average ELISA value for those CA4 plants determined to be infected was 0.131 $\pm$ 0.027 for NW-tobacco inoculum and 0.119 $\pm$ 0.016 for NW-pepper inoculum. When the infected CA4 plants were tested at 35 dpi, (i.e., several non-inoculated leaves per plant tested by ELISA), all samples were again positive for virus infection with the average ELISA value being significantly greater than the previous test for the infected plants (Fig 2.3).

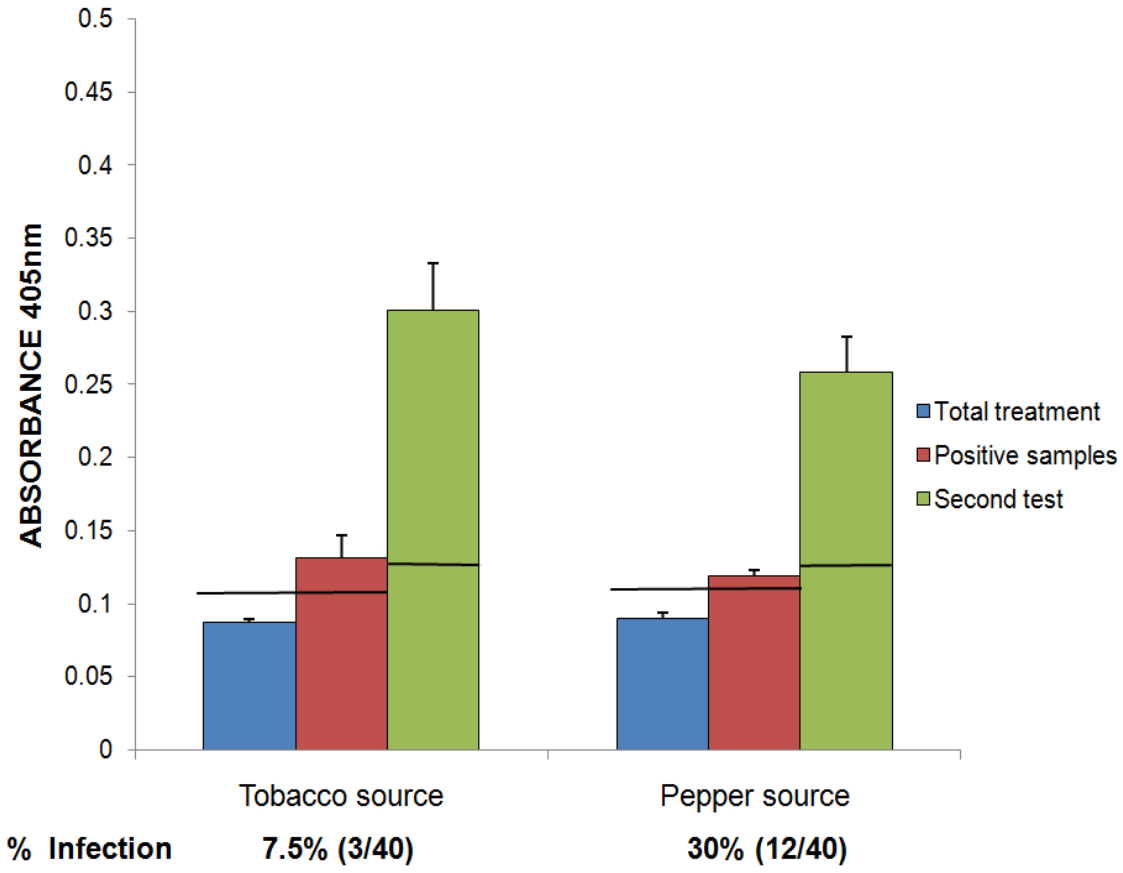

Figure 2.3 Evaluation of sources of inoculum pepper and tobacco to produce NW-CA4 like isolates. Accumulation of virus in non inoculated leaves of CA4 plants was tested at 30 dpi by enzyme-linked immunosorbent assay (ELISA). Absorbance values above the horizontal threshold lines are considered positive.

2.3.3 NW-CA4 infects Avelar, Dempsey and Tabasco.

The three NW isolates were tested with the resistant cultivars Avelar and Dempsey and the indicator host, Tabasco. Tabasco was used to determine whether each of the NW isolates induced wilting, i.e., NW stands for non-wilting. HAT isolates were used as a positive control for wilting phenotype.

In Tabasco pepper, all three NW isolates induced systemic mosaic symptoms with stunting but no wilting. In contrast, each of the HAT isolates induced severe wilting. These data indicate that NW-CA4 induces disease symptoms in Tabasco pepper characteristic of TEV-NW. All viruses accumulated to high levels in non-inoculated leaves (Table 2.1).

Avelar plants were susceptible to each of the NW isolates with strong positive average ELISA values, although for each NW isolate, the ELISA value was significantly less than obtained in Tabasco pepper (Table 2.1). The NW isolates induced more severe mosaic symptoms in Tabasco than Avelar plants. Also, vein clearing and mosaic appeared sooner in Tabasco (by 5 dpi) than Avelar (by 15 dpi).

Nine of the ten Dempsey plants inoculated with NW-CA4 developed mild mosaic systemic symptoms by 20 dpi with an average ELISA value of 0.711 ± 0.14 for the infected plants (Table 2.1). The treatment average ELISA absorbance value for NW-pepper and NW-tobacco inoculated Dempsey plants was below the healthy control threshold, although each virus treatment had 3 of 10 plants with an ELISA value above the threshold. For both virus treatments, the individual positive ELISA values were close to the healthy control threshold (0.140 ± 0.005, 0.165 ± 0.049 and 0.118 for NW-pepper, NW-tobacco and the healthy control threshold, respectively).

Table 2.1 Percent infection, average of enzyme-linked immunosorbent assay (ELISA) value and resistance or susceptibility response for pepper cultivars inoculated with *Tobacco etch virus* (TEV) strains NW, NW-CA4 and HAT

Genotype		NW-CA4	NW-Tob	NW-Pep	HAT-Tob	HAT-Pep
Dempsey	% infection	90[z]	30 (0.16)	30 (0.14)	NA[x]	NA
	ELISA(mean ± SD)	0.711 ± 0.14[y]	0.116 ± 0.042	0.101 ± 0.029		
	Interaction	S	R	R		
Avelar	% infection	100	100	100	NA	NA
	ELISA(mean ± SD)	0.43 ± 0.21	0.603± 0.219	0.542 ± 0.278		
	Interaction	S	S	S		
Tabasco	% infection	100	100	100	100	100
	ELISA(mean ± SD)	1.01 ± 0.171	1.295 ± 0.298	1.164 ± 0.412	1.677 ± 0.662	1.891 ± 0.264
	Interaction	S (NW[w])	S (NW)	S (NW)	S (W[v])	S (W)

[z] Percentage of infection was determined from the number of infected plants/the number of plants in the respective treatment. Virus infection was based on the occurrence of virus symptoms and detection of virus in leaf samples by ELISA.

[y] Average ELISA± stardard deviation for leaf samples in the respective treatment. The threshold for a positive ELISA was determined from the ELISA average plus 3 SD for healthy controls of each pepper genotype. Threshold values were: 0.117 for Dempsey, 0.118 for Avelar and 0.119 for Tabasco.

[x] NA= Not Applicable. Dempsey and Avelar plants were not inoculated with TEV-HAT.

[w] NW= No observation of wilting symptoms.

[v] W= Wilting symptoms observed.

2.3.4 VPg amino acid sequence comparisons

The VPg amino acid sequence was compared among NW-pepper, NW-tobacco and NW-CA4 isolates. The NW-pepper and NW-tobacco sequences were identical; however, two amino acids differed for NW-CA4: one at position 118 and a second at position 157 (Fig. 2.4). The first mutation (position 118) occurred in the central region of the VPg and corresponds to a serine to phenylalanine change (S118F). The second mutation (position 157) occurred in the C-terminal region of the VPg and corresponds to a tyrosine to phenylalanine change (Y157F).

NW-CA4 was used to inoculate a new set of CA4 plants, virus particles from these plants were purified and the VPg amino acid sequence obtained from these plants is referred to as NW-CA4-G2 (G2 meaning second generation). The G2 plants were used as inoculum to generate G3 plants (i.e., third generation passage through CA4 plants). This approach was performed two additional times to generate G4 and G5 generations in CA4 plants. Virus was purified from systemically infected tissues of G2, G3 and G5 CA4 plants, the respective RNA isolated and the VPg RT-PCR amplified and sequenced. After one passage of NW-CA4 through CA4 (NW-CA4-G2), an amino acid change was identified at position 162, which corresponds to an asparagine (N162) replaced by an aspartic acid (D). This mutation was maintained in the successive passages through CA4 plants (Fig. 2.4).

Sequence comparisons of VPgs from a representative collection of 21 potyviruses showed that S118 and N162 did not correspond to highly conserved amino acids, although asparagine N162 is embedded within a highly conserved 12 amino acid sequence (156GFPERE161 and 163ELRQTG168; data not shown). In contrast, our

"parent" NW strain (i.e., NW-pepper and NW-tobacco) has a tyrosine at amino acid position 157 (Y157), which differs from the other NW isolates as well as other potyviruses which have a phenylalanine at position 157. Surprisingly, the nucleotide sequence chromatogram for NW had a double peak at the middle of the codon responsible for Y157F change, indicating a single-nucleotide polymorphism.

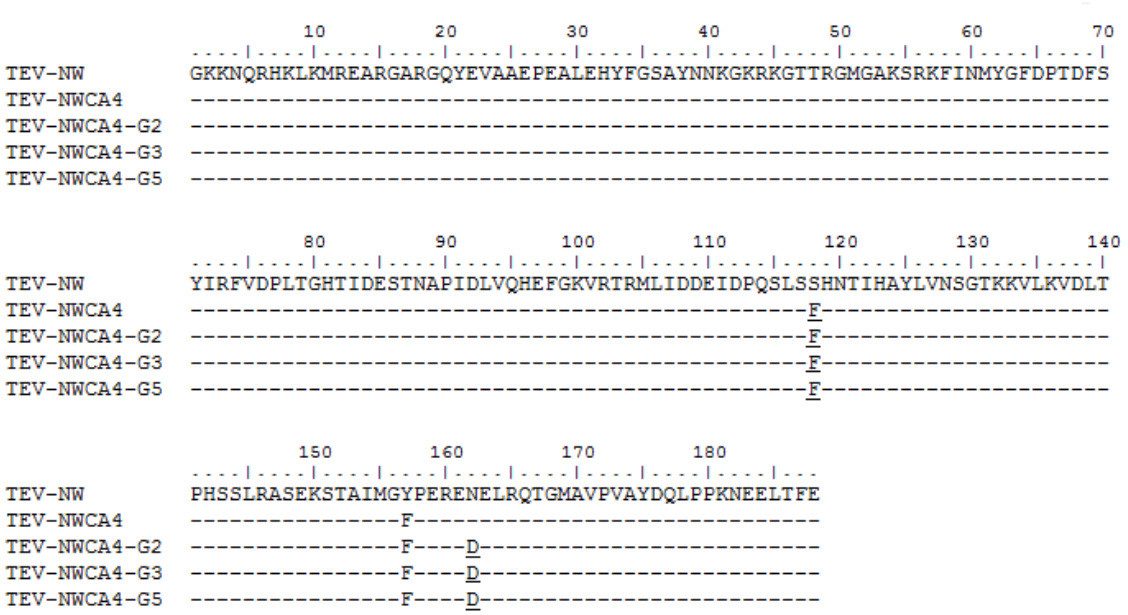

Figure 2.4 Amino acid sequence alignment of the viral genome-linked proteins (VPg) of the NW and NW-CA4 isolates. The derived isolates NW-CA4 G2, NW-CA4 G3 and NW-CA4 G5 correspond to successive passages of NW-CA4 through CA4 plants. Numbers represent amino acid residue positions of VPg. Dashes indicate identical amino acids. The S118F and N162D mutation positions are indicated in bold and underlined.

The potyviruses, *Potato virus A*, PVY and *Turnip mosaic virus* (TuMV), were shown to overcome resistances that involved eIF4E, with the determinant VPg amino acids mapped to 118 (PVA and PVY) and 162 (PVY and TuMV) or amino acids in close proximity to these positions (Table 2.2). No other amino acid changes within the polyprotein of these viruses determined to be involved in the resistance/susceptibility interaction. These findings further substantiate the likelihood of sequences within the NW-CA4 VPg as determinants for overcoming *pvr1* and *pvr1*^{2} resistances.

Table 2.2 Potyvirus VPg amino acid sequence substitutions related with overcoming resistances

Virus	VPg substitutions related to TEV	Host Resistance mechanism	Reference
TEV	S118F N162D	Pepper (*pvr1* and *pvr1*^{2}) Blocked virus replication	This work
TuMV	E114Q N162Y	Arabidopsis eIF(iso)4E knocked Blocked virus replication	Gallois et al., 2010
PVA	Y118H	*Solanum commersonnii* Reduced viral accumulation in infected cells	Rajamaki and Valkonen, 2002
PVA	R118H	*Lycopersicum hirsutun* (*pot1*) Blocked virus replication	Moury et al., 2004
PVY	T113M D117N/H/S S121N/K L147K N162E	Pepper (*pvr1*^{1} and *pvr1*^{2}) Reduced and blocked replication	Moury et al., 2004
PVY	T113K D117N S118N	Pepper (*pvr1*^{3}) Reduced virus replication	Ayme et al., 2006
PVY	T113M/V D117H H119N	Pepper *pvr1*^{1}, *pvr1*^{2} and *pvr1*^{3}	Ayme et al., 2007
PVY	D117G/A	Tobacco (*va* genes) Reduced efficiency cell to cell movement Reduced replication	Lacroix et al., 2011
PVY	K104E	Tobacco (*va* genes) Reduced efficiency cell to cell movement Reduced replication	Masuta et al., 1999

2.4 Discussion

TEV-NW was not able to infect pepper cultivars that contained the *pvr1* or *pvr1*^{2} gene (Kang et al, 2005a; Velasquez and Murphy, unpublished data). We have maintained this virus for years by mechanical passage in tobacco, and it has always been resisted by cultivars containing *pvr1* or *pvr1*^{2}. Similarly, we have used Early Calwonder or Calwonder pepper as a susceptible control or as a susceptible treatment for years. A recent seed lot of Calwonder obtained from a commercial vendor expressed varied levels of resistance to different potyviruses used in our program. This led to a process of evaluating the source of inoculum when testing resistant cultivars, i.e., each potyvirus was maintained by mechanical passage in tobacco or pepper (six generations for pepper) then used as inoculum. The identification of a NW isolate able to infect CA4 plants first occurred when using NW-pepper inoculum. A more extensive test using 40 plants from each inoculum source revealed CA4-infecting NW isolates from both NW-pepper and NW-tobacco. Significantly fewer CA4 plants became infected, however, when using NW-tobacco than NW-pepper. These findings suggest that within the population of NW isolates in pepper and tobacco, there are variants able to overcome the *pvr1* resistance gene. The greater percentage infection of CA4 plants resulting from NW-pepper than NW-tobacco suggests a host background effect on development of a virus population. Whether a virus population stabilizes during passage through some hosts, i.e., there is a lower rate of variant production, was not determined, although Sanjuán et al (2009) suggested a low mutation rate for TEV populations when passaged through tobacco. A second possibility may be simply that tobacco is further removed from CA4 than Early

Calwonder pepper as a replication milieu and, therefore, less likely to generate variants able to overcome resistances in pepper.

Passage of NW-CA4 through CA4 plants presented strong selection pressure for that NW isolate resulting in enhanced virulence with each subsequent passage through CA4 plants. The original CA4 plants shown to be infected did not develop symptoms; however, passage of virus from the infected CA4 plants to a new set of CA4 plants resulted in development of systemic vein-clearing and mosaic symptoms. In contrast, the amount of virus that accumulated from the first to the second set of CA4 plants did not appear to differ. This strong selection also operated to select NW-CA4 isolates able to systemically infect Dempsey plants. It is possible that the ability to overcome *pvr1* of CA4 plants accelerated the fixation of mutations required to overcome the $pvr1^2$ allele of Dempsey. This is similar to that observed by Ayme et al (2007) for PVY, whereby the viral determinants needed to infect cultivars with alleles $pvr1^1$ and $pvr1^3$ became fixed and allowed infection of $pvr1^2$ expressing plants.

For many plant–potyvirus interactions, amino acid changes in the VPg have been shown to be responsible for the ability of the virus to overcome eIF4E-mediated resistance (Ayme et al., 2006; Charron et al., 2008; Kang et al., 2005a; Leonard et al., 2000). The central region of the VPg of PVY was shown to be responsible for that virus' ability to overcome *pot1* and $pv1^2$ resistance genes in tomato and pepper, respectively (Moury et al., 2004). Chimeric TEV strains consisting of exchanged VPg coding regions demonstrated that the VPg alone was responsible for overcoming CA4 resistance (Perez et al., unpublished). For this reason, our sequence analyses focused on the VPg of the different NW isolates evaluated in this study.

We observed that mutation S118F in the VPg was present in the original NW-CA4 isolate and after a single passage through CA4 (NW-CA4 G2) another mutation was acquired: N162D. Since NW-CA4 G2 was used as inoculum to test the response of Dempsey plants, it is possible that N162D was the change acquired to overcome the *pvr1*^{2} (Dempsey).

Alleles *pvr1* (CA4) and *pvr1*^{2} (Dempsey) differ by only three amino acids from the susceptible allele *Pvr1*$^+$ i.e., with respect to *Pvr1*$^+$, the substitutions in *pvr1* are P66T, K71R and G107R and for *pvr1*^{2} the substitutions are V67E, L79R and D109N (Yeam et al., 2007; Kang et al, 2005a). The respective amino acid substitutions result in different profiles of susceptibility for TEV strains: TEV-Mex21 infects *pvr1* plants but is not able to infect *pvr1*^{2}, whereas TEV-N infects *pvr1*^{2} but not *pvr1* plants. As noted, NW-CA4 infects both *pvr1* and *pvr1*^{2} plants. This suggests that the interaction of VPg and its susceptible allele differs among these TEV strains with NW-CA4 representing a third and distinctly different category of VPg-eIF4E interaction.

An interaction of NW-CA4 VPg and the respective eIF4E encoded by *pvr1* and *pvr1*^{2} is plausible, although a susceptible response could occur by interaction of NW-CA4 VPg with (for example) eIF(iso)4E, which is present in both pepper cultivars (Kang et al., 2005a; Ruffel et al, 2004; Zhang et al., 2006). NW-CA4 might use an eIF4E and eIF(iso)4E independent pathway as it was shown for *Turnip mosaic virus* (TuMV) in *Arabidopsis thaliana* plants (Gallois, et al., 2010).

In summary, NW-CA4, is an isolate derived from TEV-NW that acquired the ability to overcome CA4 and Dempsey resistances and retained the ability to infect

Avelar and cause wilting in Tabasco. NW and NW-CA4 differ in their VPg amino acid sequence in two residues in the central and C-terminal regions of the VPg, the viral protein, shown to play a role in overcoming eIF4E related resistances.

Chapter 3

Electroporetic potyvirus transfection of pepper protoplasts

Abstract

Potyviruses are a persistent threat to bell pepper (*Capsicum annuum* L.) production worldwide. We have expended much effort to study the resistance response of pepper cultivars at cellular and whole plant levels. To evaluate the resistance response at the cellular level, mesophyll protoplasts are isolated and inoculated with viral RNA. An efficient isolation procedure was available but an inoculation procedure was needed that provided consistent and highly efficient inoculation. An electroporation inoculation procedure was developed by evaluation of key parameters that included voltage, number of pulses, time interval between pulses, viral RNA concentration and number of evaluated protoplasts. Consistent infection with the highest virus titer and protoplast viability resulted when 40 µg of virus RNA was used to inoculate 500,000 protoplasts using two 25-msec pulses of 200 volts each with a 10-sec time interval between pulses.

3.1 Introduction

Viruses in the genus *Potyvirus* infect a broad range of plants in most climatic regions, causing severe economic losses in many important crop species (Lopez-Moya and Garcia, 1999). The potyvirus virion consists of a single flexuous, filamentous particle containing a single strand of single-stranded messenger sense RNA of approximately 9,500 nucleotides (Dougherty and Carrington, 1988). The potyviral RNA has a 5' genome-linked protein (Murphy et al., 1991; Riechmann et al., 1989; Siaw et al., 1985) and a 3' poly A tail (Hari et al., 1979) and encodes a single polyprotein that is cleaved autocatalytically into at least ten proteins (Urcuqui-Inchima et al., 2001).

Studies on virus-host interactions have, more recently, added significant information on mechanisms by which plants resist virus infection (Boevink and Oparka, 2005; Culver and Padmanabhan, 2007; Nelson and Citovsky, 2005; Truniger and Aranda, 2009). Host resistance genes have been identified (Fraile and Garcia-Arenal, 2010; Kang et al., 2005b; Maule et al., 2007; Palukaitis and Carr, 2008) and their function determined in relation to the virus infection cycle (Diaz-Pendon et al., 2004; Lin et al., 2007; Soosaar et al., 2005). As these virus-host interactions are dissected in greater detail, there is a need for studies at the cellular level using plant protoplasts. The availability of an efficient protoplast inoculation system provides a synchronous infection process and greater quantitative accuracy during evaluations by testing known numbers of cells. Virus infection of plant protoplast studies allow determination of whether a plant's resistance mechanism is directed at replication or movement. For example, if virus titer in an inoculated leaf of a resistant host is low relative to a known susceptible host, the low titer could be due to limited replication or accumulation within individual cells or

limited movement to neighboring cells. In the latter case, the virus may have accumulated in individual cells to a similar level in both resistant and susceptible hosts; however, in the resistant host virus movement is limited and, therefore, fewer cells become infected leading to lower virus titers for those leaves. If the virus accumulates to relatively similar levels in protoplasts isolated from both susceptible and resistant hosts, this implies the resistance is not directed at replication and accumulation at the cellular level but a limitation on virus movement. For this type of study to be effective, an efficient and highly consistent protoplast infection system is needed.

The original viral RNA inoculation procedure developed for pepper protoplasts involved electroporation using the Hoefer Scientific's ProGenetorTM 1 (Murphy and Kyle, 1994). When this electroporation apparatus was no longer available, a polyethylene glycol (PEG) procedure (Loesch-Fries and Hall, 1980) was adapted for pepper protoplasts and used successfully in numerous virus-pepper studies (Deom et al., 1997; Guerini and Murphy, 1999; Turina et al., 2003.) The PEG procedure, however, had several drawbacks. Of primary concern, among others, was a lack of consistency within and between experiments due to complications with the PEG solution and the inoculation procedure. In an effort to obtain a high level of consistency among inoculations, an electroporation system was developed using a Gene Pulser Xcell Electroporation Apparatus. We report here, a systematic evaluation of parameters for electroporation and inoculation of pepper protoplasts that provide consistent successful infection using potyviral RNA.

3.2 Materials and methods

3.2.1 Virus isolates and their purification

Viruses used in this study included three *Potyvirus* species, *Tobacco etch virus* strain HAT (TEV), *Pepper mottle virus* strain Florida (PepMoV) and *Potato virus Y* strain NN (PVY). *Cucumber mosaic virus* strain Fast New York (CMV) was used as a control because successful infection of pepper protoplasts with CMV occurs with a broad range of electroporation conditions (Velasquez and Murphy, unpublished data). Each virus was maintained by mechanical passage in *Nicotiana tabacum* L. cv. Kentucky 14 in an insect-free, temperature controlled greenhouse (24 $\pm$ 4.5°C day/20 $\pm$ 3.5°C night) at the Plant Science Greenhouse Facility, Auburn University, AL (U.S.A.).

Each of the potyviruses, PepMoV, PVY and TEV, were purified from systemically infected Kentucky 14 tissue as described previously (Murphy et al., 1990). CMV was purified according to Roossinck and White (1998) with minor modifications. Viral RNA was isolated from each potyvirus preparation by treatment with Proteinase K and phenol, chloroform extraction as described by Guerini and Murphy (1999). CMV RNAs were isolated by several cycles of phenol, chloroform extraction according to Palukaitis and Zaitlin (1984). A sample of each viral RNA preparation was analyzed by electrophoresis through a 1% agarose gel, stained with ethidium bromide and visualized by ultraviolet light. Viral RNA concentration was measured using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE) and stored at -65°C.

3.2.2. Protoplast isolation

Capsicum annuum L. cv. Calwonder seeds (Harris® seeds, Rochester, NY, USA) were surface sterilized by treatment with 1% sodium hypochloride (Murphy and Kyle, 1994). Plants were grown in magenta boxes (77 mm wide x 77 mm long x 97 mm tall; Sigma-Aldrich, Inc., St. Louis, MO) in a temperature-controlled chamber (Percival Scentific, Inc. Perry,IA, USA) at 25°C under 12,000 lux illumination for 16 h and darkness for 8 h at 22°C. Calwonder leaf protoplasts were isolated as described by Murphy and Kyle (1994) and modified by Guerini and Murphy (1999). Protoplasts generated from these plants were washed and concentrated by three cycles of centrifugation at 294 X *g* at room temperature, and maintained in 0.42 M mannitol as osmoticum. Protoplast numbers were determined with a hematocytometer by light microscopy at 20x magnification.

3.2.3 Electroporation conditions

Initial efforts developed an electroporation procedure for inoculation (and infection) of pepper protoplasts with CMV. This procedure did not work, however, for potyviruses but was used as a basis for evaluation of electroporation parameters. A Gene Pulser Xcell Electroporation System (Bio-Rad Laboratories, Inc., Hercules, CA) was used for all experiments and, all experiments used square wave electroporation pulses. The standard procedure (developed for CMV) included inoculation of 0.5×10^6 protoplasts suspended in 0.42 M mannitol (molarity was appropriate for Calwonder protoplasts) and 3 mM $CaCl_2$ at a final volume of 800 µl in an electroporation cuvette that had a 0.4 mm gap (USA scientific, Inc., Ocala, FL, USA). Viral RNA (inoculum) was

added to the protoplast solution immediately before electroporation which involved two 5 msec pulses of 150 volts each pulse. The inoculated protoplasts were placed on ice for 15 min, centrifuged at ~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; Aoki and Takebe, 1969). An antibiotic mix containing carbenicillin, cephaloridine, and nystatin (Sigma Chemical, St. Louis, MO) was added at final concentrations of 100, 100, and 4 µg/ml, respectively. Protoplasts were placed in a Percival Growth Chamber with light of 12,000 lux for 16 h at 26°C and darkness for 8 h at 22°C.

The following electroporation parameters were evaluated for potyvirus infection of pepper protoplasts: pulse length, voltage, number of pulses and time between pulses, protoplast number at the time of inoculation and viral RNA concentration. The pulse length was tested using PVY RNA and the other parameters were tested using TEV RNA as inoculum. We chose to use TEV for subsequent experiments due to availability of TEV RNA and the use of this virus in related projects. The final procedure, identified after evaluation of each of the selected parameters, was tested for inoculation with TEV, PepMoV and PVY. Each experiment was performed three times and included inoculation of protoplasts with CMV (RNA inoculum) as a positive infection control, and a negative (mock) inoculation control inoculation of protoplasts that consisted of water.

3.2.4 Determination of infection by enzyme-linked immunosorbent assay (ELISA).

Protoplasts were counted at 24 hours post-inoculation (hpi) using a hemocytometer. Samples of 1×10^5 cells were collected, then pelleted by two pulse runs at 12,000 X *g* (Sorvall MC-12V table-top microfuge; Du Pont Co., Newton, CT) and resuspended in 100 µl of ELISA general extraction buffer (as described in the ELISA instructions, Agdia, Inc., Elkhart, IN). The resuspended protoplasts were lysed upon addition of the general extraction buffer and by repeated pipetting with a beveled pipette tip. Each sample was examined by light microscopy to determine efficient lysis of the protoplasts.

For detection by ELISA, a commercial ELISA kit (Agdia, Inc.) was used specific to each virus, and performed according to the manufacturer's instructions. The coating antibody and protoplast (virus) sample steps were each kept at 4°C for at least 12 h in a moist chamber. The alkaline phosphatase conjugated antibody step was incubated in a moist chamber at 37°C for 3 h. Substrate, 1 mg/ml para-nitrophenylphosphate in 10% diethanolamine, pH 9.8, reactions were allowed to develop at room temperature for 1 h and then recorded using a Sunrise microtiter plate reader (Phenix Research Products, Hayward, CA). A sample was considered positive for the presence of virus if the ELISA absorbance value at 405 nm was greater than the healthy control threshold. The healthy control threshold was determined from the average ELISA absorbance value plus three standard deviations of at least two samples of mock inoculated protoplasts.

3.3 Results

The procedure developed for successful infection of pepper protoplasts by CMV using the Gene Pulser Xcell Electroporation System did not lead to detectable infection of pepper protoplasts using potyviral RNA. Therefore, we systematically evaluated each inoculation parameter in an effort to obtain potyvirus infection of pepper protoplasts.

3.3.1 Electroporetic pulse length

The duration of time that the protoplasts are exposed to an electric pulse is an important parameter for successful virus infection (Saunders et al., 1989a). The effect of pulse duration on PVY and CMV infection of pepper protoplasts was tested using two 5 msec pulses, two 25 msec pulses or one pulse of 50 msec (Fig. 3.1). For each pulse treatment, a voltage of 150 was used and a time interval between pulses of 0.1 msec. PVY was detected, based on a positive ELISA value, in the treatment consisting of two 25 msec pulses only. The ELISA absorbance value for PVY treatments consisting of two 5 msec pulses or one pulse of 50 msec were not above the healthy control threshold. Successful infection of CMV occurred with each of the pulse treatments, although the ELISA absorbance value was significantly greater for the two 25 msec pulse treatment than the other two treatments, and a significantly greater CMV accumulation occurred in protoplasts subjected to two 5 msec pulses than those subjected to one pulse of 50 msec.

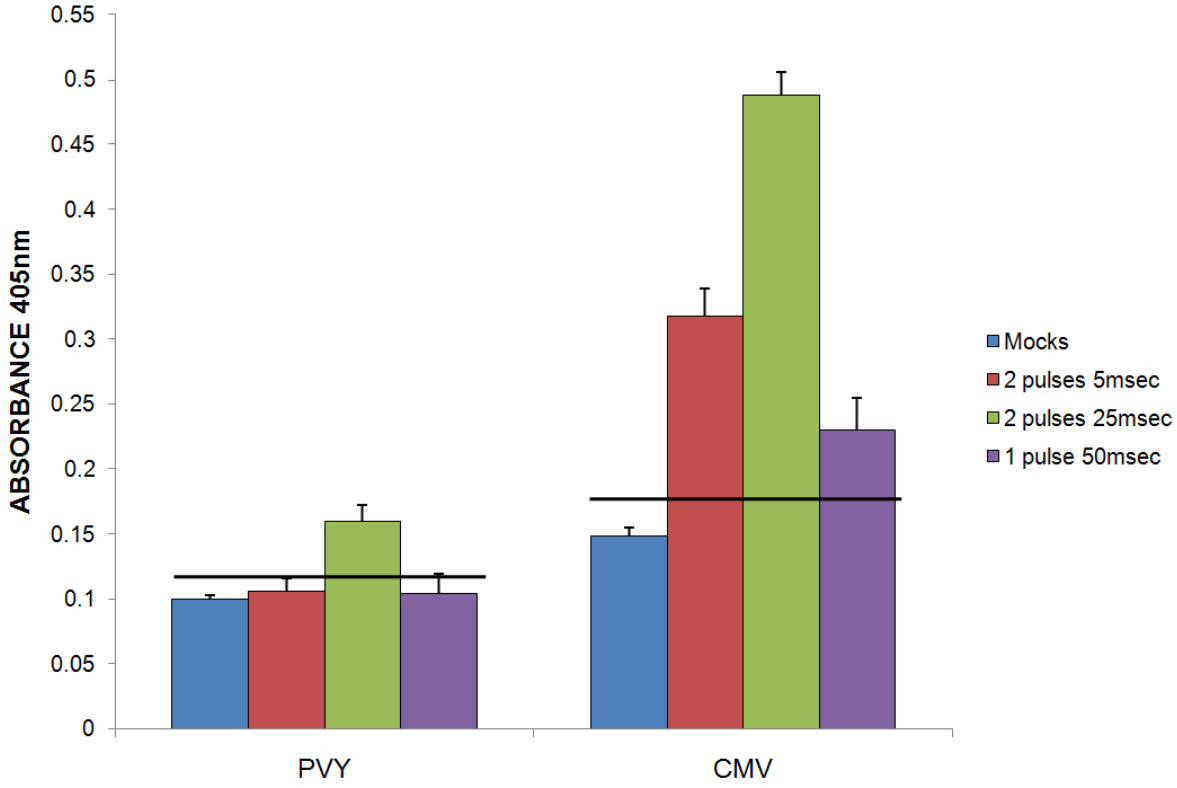

Figure 3.1 Effects of pulse length on accumulation of *Potato virus Y* (PVY) and *Cucumber mosaic virus* (CMV) in pepper mesophylI protoplasts electroporated with variable length and number of pulses of 150 volts. Enzyme-linked immunosorbent assay (ELISA) values above the horizontal threshold line are considered positive for virus infection.

3.3.2. Number of electroporetic pulses

The number of pulses was evaluated using parameters that included 150 volts, 25 msec for each pulse with a 0.1 sec interval between pulses. The viral RNA amount was increased to 40 µg and $5x10^5$ pepper mesophyll protoplasts were inoculated. A single pulse resulted in a borderline positive/negative ELISA absorbance value; however, a strongly positive ELISA value occurred with two, three and four pulses (Fig. 3.2A). Although three and four pulse treatments had significantly higher ELISA values than the two pulse treatment, protoplast viability was increasingly negatively affected with each additional pulse. We selected the two pulse treatment as a compromise for a strong ELISA reaction for virus accumulation and with a lesser effect on protoplast viability.

3.3.3. Voltage levels

The parameters used to evaluate electroporetic pulse number, including two 25 msec pulses, were used as a basis for evaluation of amount of voltage used during electroporation. Each voltage treatment, 150, 200, 250 and 300 volts, led to a positive detection of virus from protoplast samples (Fig. 3.2B). The ELISA absorbance value was significantly greater for the 200 voltage treatment than other treatments; the 250 voltage treatment had a significantly greater ELISA value than for the 150 and 300 voltage treatments; the 150 voltage treatment led to a significantly greater ELISA value than the 300 voltage treatment. With each increase in voltage, protoplast viability decrease by approximately 15% from one treatment to the next. These voltage data provided sound evidence that for TEV RNA electroporation, 200 volts resulted in the best infection while maintaining decent protoplast viability.

3.3.4 Interval time between electroporetic pulses

Experiments were performed to test the time interval between electroporetic pulses. These experiments used the base parameters of two 25 msec pulses of 200 volts each pulse. Inoculum consisted in 40 µg of TEV RNA that were used to inoculate $5x10^5$ pepper mesophyll protoplasts. The previously used time interval between pulses was 0.1 sec, which led to a detectable accumulation of TEV in protoplast samples; however, significantly greater amounts of TEV accumulated in protoplasts subjected to 5 and 10 second intervals between pulses (Fig. 3.2C). Longer time intervals of 20 and 40 seconds did not result in detectable amounts of virus from protoplast samples (Fig. 3.2C). Protoplast viability was lowest for the 0.1 sec time interval treatment with at least a 30% increase in viability with longer time intervals. The highest percent viability occurred with the 10 sec time interval treatment (Fig. 3.2C). The 10 sec time interval between electroporetic pulses was selected as a base parameter for subsequent experiments.

3.3.5 Viral RNA inoculum amount and number of protoplasts tested

The parameters identified from the previous experiments were used to evaluate the amount of TEV RNA inoculum and protoplast sample number needed to detect virus accumulation by ELISA. TEV was not detected from 50,000 or 100,000 protoplast samples when inoculum consisted of 10 µg of viral RNA (Fig. 3.2D). TEV was detected from both 50,000 and 100,000 protoplast samples when 20 µg and 40 µg of viral RNA inoculum was used, although the 50,000 sample for the 20 µg TEV RNA treatment was close to the healthy control threshold. The 40 µg TEV RNA treatment had significantly greater ELISA absorbance values for both 50,000 and 100,000 protoplast samples

compared with the 20 µg TEV RNA treatment. Furthermore, significantly more TEV was detected for the 40 µg TEV RNA 100,000 protoplast sample than the 50,000 sample (Fig. 3.2D).

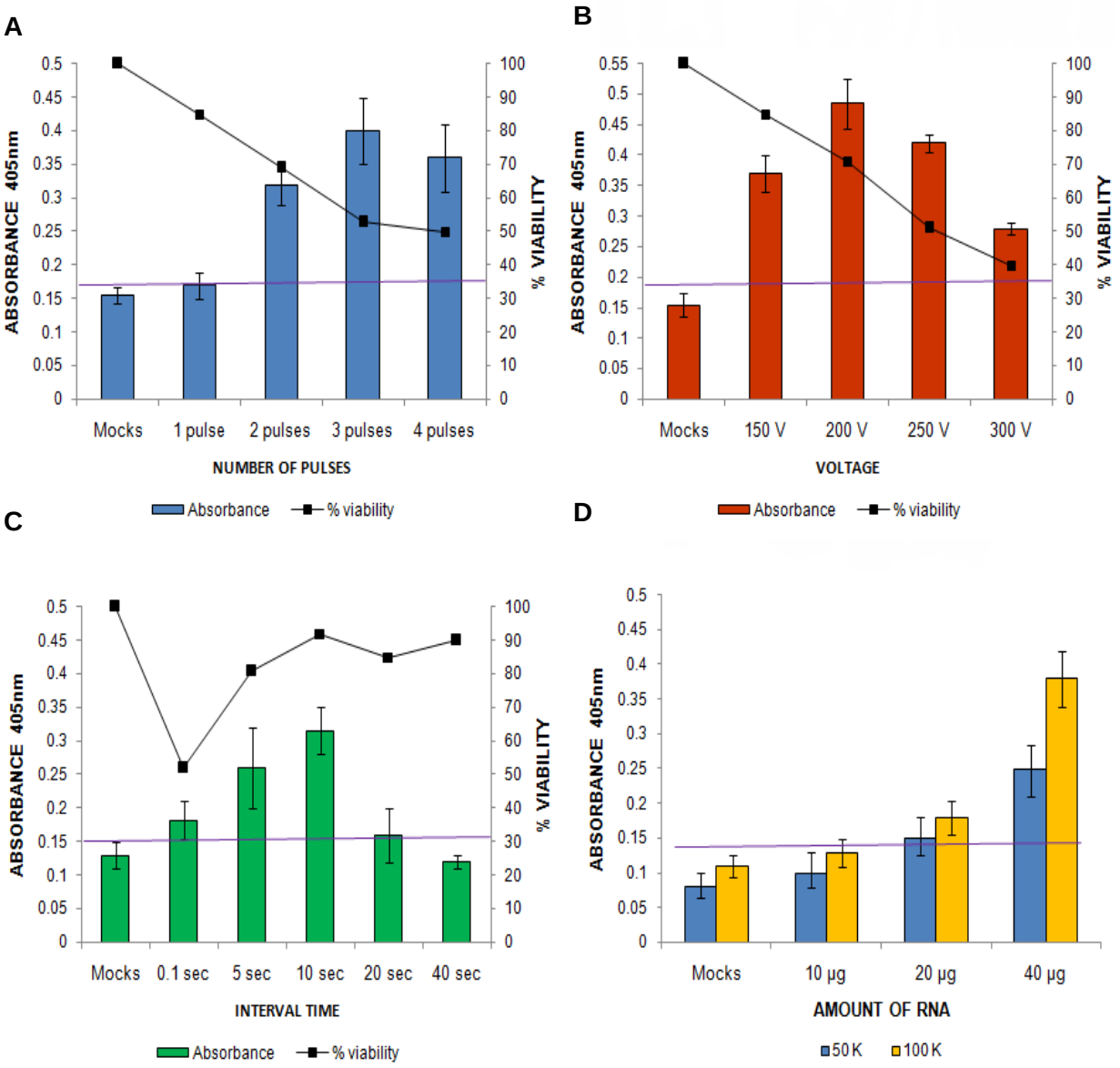

Figure 3.2 Effects of electroporation conditions on accumulation of *Tobacco etch virus* (TEV) HAT strain and pepper mesophyll protoplasts viability. (A) TEV accumulation (positive above horizontal threshold line) in relation to number of 25 msec pulses of 150 volts; (B) in relation to voltage used for electroporation with two 25-msec pulses; (C) in relation to time interval between two 25 msec pulses of 200 volts; (D) in relation to the amount of TEV RNA used as inoculum and number of protoplasts tested.

3.3.6 Validation of the electroporation procedure for infection of potyviruses.

The electroporation parameters developed for TEV inoculation of pepper mesophyll protoplasts (40 µg virus RNA was used to inoculate $5x10^5$ protoplasts using two 25-msec pulses of 200 volts each with a 10-sec time interval between pulses) were evaluated for the potyviruses, PepMoV-FL and PVY (Fig. 3.3). All three viruses accumulated to detectable levels as ELISA values were above the respective thresholds. Although it appears that the viruses accumulated to different levels, based on differing ELISA absorbance values, it was not determined whether these responses were due to differing levels of virus accumulation or sensitivities of the respective ELISA kit used for detection.

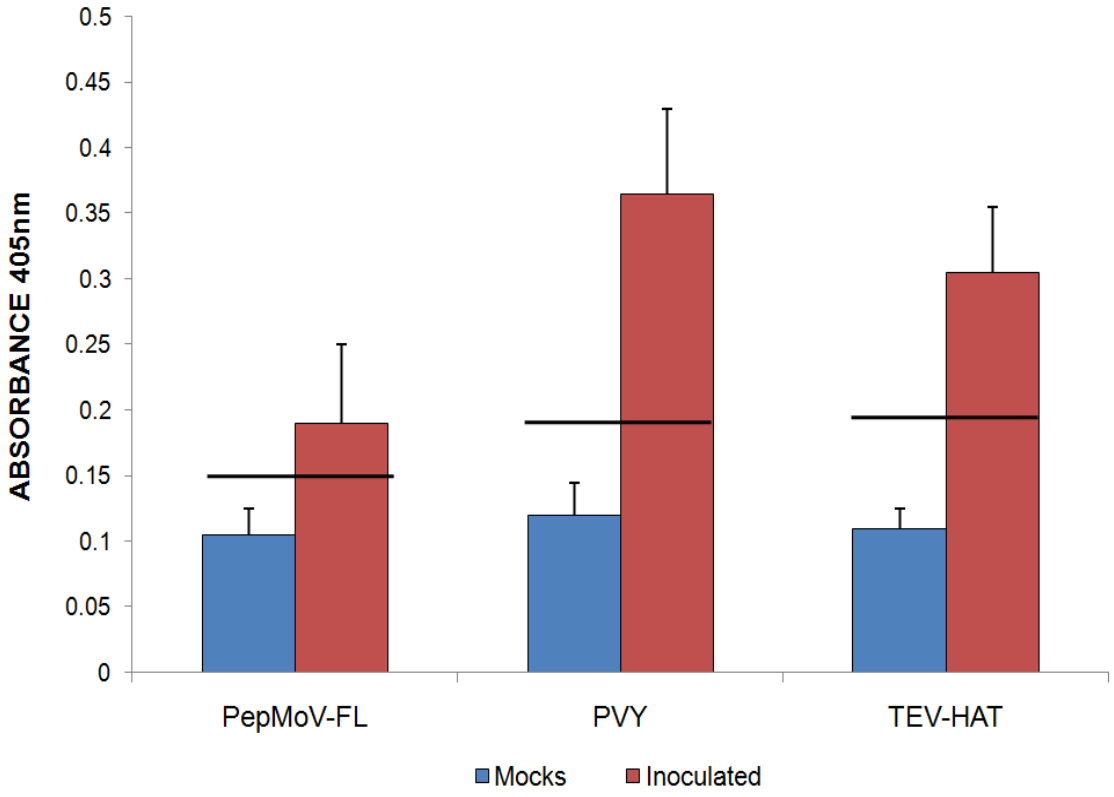

Figure 3.3 Evaluation of operational parameters used for *Tobacco etch virus* (TEV) for effective infection with Potyviruses, *Pepper mottle virus* (PepMoV-FL) and *Potato virus Y* (PVY). ELISA values above the horizontal threshold line are considered positive.

3.4 Discussion

Inoculation procedures involving PEG and electroporation have been used to successfully infect pepper protoplasts with potyviral RNA (Deom et al, 1997; Guerini and Murphy, 1999; Murphy et al, 1998; Murphy and Kyle, 1994). If available, an electroporation procedure can be more reliable and reproducible than PEG transfection (Bates, 1989; Hibi, 1989; Saunders et al., 1989b).

We initially evaluated two modifications to the electroporation protocol used for CMV inoculation of pepper protoplasts (Masiri et al., 2011) in an effort to obtain potyvirus infection: the amount of voltage and the amount of potyviral RNA used as inoculum. Neither parameter led to potyvirus infection of pepper protoplasts (data no shown). An increase in pulse length, however, did result in potyvirus infection (as determined by ELISA). Electroporetic pulse length was shown to be important for pore development and enlargement in the plasmamembrane of the protoplast (Weaver and Chizmadzhev, 1996). As the electric pulse increases, the pore size is thought to increase, which may lead to entrance of the potyviral RNA into the cell.

We evaluated numerous parameters in an effort to obtain successful potyvirus infection of pepper protoplasts. Each parameter revealed an improvement in the infection process, as measured by a greater level of virus accumulation in protoplasts. A peculiar outcome of our study is the extensive range of parameters that can be used with success for CMV infection which contrasts with our observations with potyvirus infection. The CMV genome consists of three RNA species with RNAs 1 and 2 able to replicate in the absence of RNA 3; however, all three RNA species are required for replication with coat protein accumulation (Palukaitis and Garcia-Arenal, 2003). In contrast, the potyviral

RNA is a single RNA species, although larger than each of the individual CMV RNA species (Adams et al., 2005b; Rajamäki et al., 2004; Shukla et al., 1994).

An explanation for the differences in electroporetic infection efficiency observed between CMV and potyvirus may reside in the overall charge of the viral RNAs. According to this model, the viral RNA becomes attached to the protoplast's surface prior to the formation of "electropores" in the protoplast membrane with subsequent movement into the protoplast (Krassowska and Filev, 2007; Pliquett et al, 2007; Sukharev et al, 1992). The membrane binding step is required for entry into the protoplast and correlates strongly with the charge of the RNA molecule (Xie and Tsong, 1993). Although we have no evidence to support this model for the CMV and potyvirus systems in pepper, the model offers a plausible component of the inoculation process.

Potyviral RNA has a genome-linked protein (VPg) covalently bound to the 5'-terminus of the RNA (Murphy et al.,1991; Riechmann et al., 1989) which was shown to be responsible for aggregation of RNA molecules (Guo et al., 2001; Luciano et al., 1991; Yambao et al., 2003). The aggregation of two or more potyviral RNA molecules could result in a complex too large for efficient inoculation of pepper protoplasts. This explanation corresponds with the greater level of each parameter needed for successful infection using potyvirus RNA as inoculum. The potyviral RNA used as inoculum in this study, however, was treated with Proteinase K during the extraction process. The Proteinase K treatment does not completely eliminate the VPg from the viral RNA 5'-terminus but leaves a small peptide still linked to the RNA (Murphy et al. 1991). We did not determine whether PVY or TEV RNAs aggregate with or without Proteinase K

treatment and, therefore, are unable to address this phenomenon in relation to electroporation, although it is a topic of interest for further study.

The complications encountered with efforts to obtain potyvirus infection in pepper protoplasts was consistent among each of the potyviruses tested, suggesting it is a potyvirus phenomenon. The seemingly unlimited ability to infect pepper protoplasts with CMV may be due to this virus' greater ability to establish an infection. Similar observations were made with CMV infection of tobacco protoplasts (Sanders et al., 1989a). In all experiments we have performed involving infection of pepper protoplasts with CMV versus a potyvirus, regardless of whether the inoculation method was PEG or electroporation, CMV accumulates to detectable levels sooner and to a greater degree than the potyvirus (Guerini and Murphy, 1999; Murphy, unpublished data). This was also observed in studies evaluating infection of whole plants whereby CMV accumulated throughout tissues of the stem of infected plants at a much greater rate and level of accumulation than a potyvirus (Guerini and Murphy, 1999). CMV may serve as an effective positive infection control for the sake of obtaining protoplast infection but, perhaps, it should be used with caution as a comparative treatment for potyvirus infection of pepper protoplasts.

We describe in this report, a reliable electroporation procedure for potyvirus infection of pepper protoplasts. This procedure will be useful for studies to evaluate potyvirus replication and accumulation at the cellular level and understand the basis for resistance mechanisms.

General conclusions

A TEV-NW isolate has been discovered that overcomes two genes that are used in breeding programs for pepper and have shown a broad spectrum of action against potyviruses: *pvr1* (from CA4) and *pvr1*^{2} (from Dempsey). This new isolate is referred to as TEV-NW-CA4.

TEV-NW and TEV-NW-CA4 differ in their VPg amino acid sequence in two residues within regions shown to be important for eIF4E-related resistances. These differences may account for NW-CA4's ability to infect CA4 and Dempsey.

A procedure for electroporetic transfection of pepper protoplasts with Potyviral RNAs was described. This procedure is highly efficient and consistent, and will serve an important role for future virus-host interaction studies, e.g., to study initial events of cell infection by potyviruses, to define mechanisms of resistance of pepper cultivars and to identify new sources of resistance to potyviruses in pepper.

Future perspectives

An interesting result of this work is the discovery of TEV-NW-CA4, an isolate that overcame the resistance conferred by two alleles of locus *pvr1*. It will be important to test the infectivity of TEV-NW-CA4 in other pepper varieties with different *pvr1* alleles, for example, Perennial (*pvr1*^{3}), Serrano Vera Cruz (*pvr1*^{7}) and Chile de árbol (*pvr1*^{9}).

In order to determine whether the identified substitutions in VPg of TEV-NW-CA4 are responsible for overcoming resistance conferred by *pvr1* and *pvr1*^{2}, an infectious clone of TEV-NW or TEV-NW-CA4 is required. Then, mutate the nucleotides on the specific codons to obtain the amino acid changes. Finally, inoculate protoplasts and plants of CA4 and Dempsey with the obtained viral genomes and evaluate accumulation of virus in each case.

Differences in percentage of infection of CA4 plants inoculated with TEV-NW from tobacco and pepper were observed in this study. This result may be due to differences in fitness of TEV-NW-CA4 in these hosts. This hypothesis could be tested by inoculation of pepper and tobacco plants with the same amount of TEV-NW-CA4 inoculum and then, accurately quantifying the accumulation of virus in non inoculated leaves at 7 dpi.

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Appendix

Evaluation of pepper resistance to potyviruses

Pepper cultivars CA4 and Dempsey were evaluated for their response to inoculation with seven potyviruses: *Pepper mottle virus* (PepMoV) strains FL and CA, *Potato virus Y* (PVY) strain NN and *Tobacco etch virus* (TEV) strains HAT, Mex21, N and NW. Seven plants of each pepper cultivar, including the susceptible control Early Calwonder, were challenged with inoculum from two sources for each potyvirus: one from systemically infected tobacco and the other from systemically infected Early Calwonder pepper. Non-inoculated young leaf samples were collected from each plant at 10dpi and tested for virus infection by enzyme-linked immunosorbent assay (ELISA). When the ELISA value was above the healthy control threshold, the result was considered positive for detection of virus. If a plant tested negative for virus infection at 10 dpi, it was evaluated again at 20dpi. (Table A1)

Each virus infected 100% of inoculated Early Calwonder plants (susceptible control) and virus accumulated to relatively high levels in non-inoculated leaves (determined by ELISA). Similar results occurred for Dempsey plants inoculated with PepMoV-FL and TEV-N, and for CA4 plants inoculated with TEV-Mex21 (Table A1). When the ELISA value for a plant was slightly above the threshold and the average ELISA value for the treatment was below the threshold, that plant was not considered infected and the response was considered resistant.

Table A1. Average enzyme-linked immunosorbent assay (ELISA) value for pepper cultivars inoculated with *Pepper mottle virus* (PepMoV) strains FL and CA, *Potato virus Y* (PVY) strain NN and *Tobacco etch virus* (TEV) strains HAT, Mex21, N and NW.

		10 DPI			20 DPI		
		ECW	DEM	CA4	ECW	DEM	CA4
	THRES	*0.07558*	*0.0792*	*0.0841*	*0.08229*	*0.0857*	*0.079*
PepMoV-FL	Tob	**100% (4/4)** **0.614± 0.062**	**100% (7/7)** **0.579± 0.068**	28% (2/7) 0.112 0.08± 0.013	**100% (2/2)** **0.572± 0.016**	NA	0% (0/7) 0.053± 0.007
	Pep	**100% (4/4)** **0.64± 0.064**	**100% (7/7)** **0.507± 0.144**	14% (1/7) 0.087 0.074± 0.006	**100% (2/2)** **0.581± 0.098**	NA	43% (3/7) 0.083 0.062± 0.022
PepMoV-CA	Tob	**100% (4/4)** **0.546± 0.044**	28% (2/7) 0.083 0.0754± 0.005	14% (1/7) 0.085 0.073± 0.006	**100% (2/2)** **0.565± 0.014**	14% (1/7) 0.108 0.081± 0.02	28% (2/7) 0.085 0.065± 0.01
	Pep	**100% (4/4)** **0.537± 0.016**	14% (1/7) 0.085 0.076± 0.002	43% (3/7) 0.088 0.084± 0.025	**100% (2/2)** **0.524± 0.053**	0% (0/7) 0.065± 0.005	28% (2/7) 0.088 0.07± 0.01
	THRES	*0.077*	*0.101*	*0.1035*	*0.111*	*0.102*	*0.096*
PVY	Tob	**100% (4/4)** **1.041± 0.271**	0% (0/7) 0.073± 0.002	0% (0/7) 0.073± 0.002	**100% (2/2)** **1.214± 0.381**	0% (0/7) 0.066± 0.006	0% (0/7) 0.065± 0.006
	Pep	**100% (4/4)** **1.203± 0.119**	0% (0/7) 0.074± 0.002	0% (0/7) 0.072± 0.006	**100% (2/2)** **1.355± 0.012**	14% (1/7) 0.105 0.072± 0.006	0% (0/7) 0.061± 0.013
	THRES	*0.0752*	*0.09584*	*0.08784*	*0.113*	*0.098*	*0.104*
TEV-HAT	Tob	**100% (3/4)** **1.064± 0.111**	0% (0/7) 0.072± 0.003	14% (1/7) 0.098 0.079± 0.003	**100% (2/2)** **1.332± 0.177**	0% (0/7) 0.057± 0.004	0% (0/7) 0.059± 0.005
	Pep	**100% (4/4)** **1.204± 0.305**	0% (0/7) 0.073± 0.003	0% (0/7) 0.076± 0.008	**100% (2/2)** **1.881± 0.081**	0% (0/7) 0.060± 0.007	0% (0/7) 0.063± 0.005
TEV-Mex21	Tob	**100% (4/4)** **1.071± 0.065**	0% (0/7) 0.076± 0.003	**100% (7/7)** **0.757± 0.061**	**100% (2/2)** **1.873± 0.055**	0% (0/7) 0.058± 0.008	NA
	Pep	**100% (4/4)** **0.787± 0.161**	28% (2/7) 0.097 0.089± 0.006	**100% (7/7)** **0.798± 0.107**	NA	0% (0/7) 0.055± 0.003	NA
TEV-N	Tob	**100% (4/4)** **1.271± 0.083**	**71% (5/7)** **0.315** **0.252± 0.347**	17% (1/6) 0.094 0.079± 0.011	**100% (2/2)** **1.332± 0.177**	NA	0% (0/7) 0.082± 0.011
	Pep	**100% (4/4)** **1.666± 0.849**	**86% (6/7) 0.61** **0.536± 0.277**	0% (0/7) 0.076± 0.003	NA	NA	0% (0/7) 0.06± 0.006
TEV-NW	Tob	**100% (4/4)** **1.075± 0.215**	0% (0/7) 0.074± 0.003	0% (0/7) 0.071± 0.001	**100% (2/2)** **1.601± 0.168**	0% (0/7) 0.053± 0.005	0% (0/7) 0.047± 0.01
	Pep	**100% (4/4)** **0.916± 0.084**	0% (0/7) 0.073± 0.008	0% (0/7) 0.068± 0.003	NA	0% (0/7) 0.047± 0.01	**28% (2/7)** **0.349** **0.141± 0.021**

In summary, Dempsey (*pvr1*^{2}) plants were resistant to five potyviruses and susceptible to PepMoV-FL and TEV-N. CA4 plants (*pvr1*) were resistant to six potyviruses and susceptible to TEV-Mex21 (Table A2).

Table A2. Summary of the response of Dempsey and CA4 plants to inoculation with potyviruses.

	Early Calwonder	Dempsey	CA4
PepMoV-FL	S	S	R
PepMoV-CA	S	R	R
PVY-NN	S	R	R
TEV-HAT	S	R	R
TEV-Mex21	S	R	S
TEV-N	S	S	R
TEV-NW	S	R	R

S= Susceptible, R= Resistant