

Plant Virus Diagnostics: Comparison of classical and membrane-based techniques for immunoassay and coat protein sequence characterization for *Cucumber mosaic virus* and three potyviruses

by

Peta-Gaye S. Chang

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Dr. Sue A. Tolin (Chair)

Dr. Erik L. Stromberg

Dr. Boris A. Vinatzer

Mrs. Mary-Ann Hansen

Dr. Richard E. Veilleux

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Blacksburg, Virginia

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Peta-Gaye S. Chang

Advisor: Dr. S. A. Tolin

Department of Plant Pathology, Physiology and Weed Science

ABSTRACT

Diagnostics is important in the development and implementation of pest management strategies. The virus diagnostic capabilities of several plant pathology collaborators within the Integrated Pest Management Collaborative Research Support Program (IPM CRSP) host countries were evaluated with the aid of a survey. Very few plant disease diagnostic clinics had funds to cover daily operations despite over half of the responding clinics receiving an operational budget. Academically and government affiliated clinics within the developing host countries had little access to molecular tools and equipment, relying mostly on biological and serological methods. Clinics affiliated with private companies and within the USA relied more upon molecular assays. Ten CMV isolates identified by tissue blot immunoassay (TBIA) were collected from a garden at the Historic Smithfield Plantation on the Virginia Tech campus, and from Painter, Virginia on the Eastern Shore. Three CMV isolates from Smithfield were biologically compared to six early CMV isolates stored since the 1970s, while all isolates were compared serologically and molecularly. Sequences obtained after reverse transcription-polymerase chain reaction (RT-PCR) assigned the CMV isolates into subgroups, with eleven to subgroup 1A and three to subgroup 2. The subgroup assignments were confirmed by TBIA using CMV subgroup-specific monoclonal antibodies (Agdia Inc). At Smithfield Plantation, another virus, *Turnip mosaic virus* (TuMV) was identified from Dame's Rocket (*Hesperis matronalis* L.). This is the first report of TuMV in Virginia. In TBIA virus-infected plant samples are blotted onto nitrocellulose membranes, dried, and processed. Membranes can be stored for long periods of time and transported safely across borders without risk of introducing viruses into new environments, but virus remains immunologically active for several months. Methods were developed with CMV and three potyviruses, using the same membranes, for detecting viral RNA by RT-PCR and direct sequencing of PCR products. Amplification by RT-PCR was possible after membrane storage for up to 15 months. The membranes also performed well with samples sent from IPM CRSP host countries and within the USA. This method should improve molecular diagnostic capabilities in developing countries, as samples can be blotted to membranes and sent to a centralized molecular laboratory for analysis.

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DEDICATION

To my parents: my mom Nerissa Chang, my dad Dennis Chang, and especially my sister Elise Chang for all the love and support they have shown me my entire life. I love you all.

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CHAPTER I

Introduction and Literature Review

Plant viruses are one of the leading causes of plant diseases in the world. Viral diseases result in billions of dollars lost per year by limiting plant produce quality and quantity (reviewed by Thresh, 2006; van der Vlugt, 2006). Symptoms of plant viruses can occur on leaves, stems, fruits or flowers and range from mild mosaic and mottling to severe distortion, stunting and, in very rare cases, death of the plant (reviewed by Agrios, 2005). However, not all viruses are capable of replicating in all plants. Since plants lack an immune system, they have developed defense mechanisms that detect and destroy a majority of invading pathogens including viruses. The evolution of plant viruses through genetic pressure and drift to infect particular crops has enabled them to overcome host plant defenses (reviewed by Garcia-Arenal and Fraile, 2008). In plants resistant to particular plant viruses, necrotic lesions are typically produced on inoculated leaves, and are indicators of the plant's defense response to virus replication. This response is called the HR or "hypersensitive response" and leads to programmed cell death (PCD) (Erickson et al., 1999).

Viral diagnostics is one of the most valuable tools for plant disease management. The control of plant viruses relies heavily on phytosanitary applications, insect vector control, and the use of cultivars resistant to specific plant viruses. To initiate effective control practices, viruses must first be accurately identified. In the last few decades diagnostic tools for virus identification and detection have become both available and affordable to diagnostic laboratories, research centers and universities. Virologists have

moved from the more traditional use of biological indicator hosts to molecular diagnostics and sequence data to establish relationships, groups, genera, and families among the ever growing list of new viruses (Jordan et al., 2008) or viruses on new hosts in new locations (Koike et al., 2008; Alfaro-Fernandez et al., 2008). In this review, the major diagnostic methods used for virus identification and detection are discussed, with special reference to one particularly important virus, *Cucumber mosaic virus* (CMV), the type member of the genus *Cucumovirus* (Family *Bromoviridae*).

CMV has been one of the most studied viruses in the world. Its host range extends to more than 1,200 plant species worldwide. The information pertaining to CMV is so vast that it would be almost impossible to cover every detail. Instead, this review highlights some of the important aspects as they relate to CMV and virus diagnostics. CMV is often known to co-infect plant hosts with other plant viruses, particularly members of the genus *Potyvirus*, as both are aphid-transmitted. Selected potyviruses and their interactions with CMV will thus also be reviewed.

Diagnostics

The identification, detection and diagnosis of plant viruses rely on biological, serological, and nucleic acid-based techniques, as well as determination of the physical and chemical properties of the virus. In the fight against plant viral diseases, these techniques have become the arsenals for many diagnosticians for aiding the production of disease-free crops as well as for disease management strategies in the event of virus introduction and detection. Plant virus diagnostics has grown steadily over the last few decades with an increasing repertoire of user-friendly molecular tools for the rapid detection of plant viruses that affect hundreds of plant crops. There are numerous reviews

and books on plant virus diagnostics (Cooper, 2006; van Regenmortel, 1992; Webster et al., 2004) that examine methodologies available to diagnostic laboratories, a few of which will be discussed here.

Symptomatology and Host Range

Diagnosing virus diseases begins with the proper identification of plant viruses associated with the disease. One of the earliest methods in plant virus diagnosis, which is still practiced today, is the differentiation of plant viruses using a range of symptom expressions and biological activities on inoculated indicator test plants. Plant viruses cause a wide variety of symptoms, including mosaic/mottling, stunting, leaf deformation, petal-color breaking, chlorotic and necrotic lesions and spots, ringspots, reduction in yield, wilting, and in many cases, combinations of these symptoms. The most commonly used indicator plants to distinguish plant viruses are from the families *Chenopodiaceae*, *Solanaceae*, *Cucurbitaceae* and *Fabaceae*. For instance, *Cucumber mosaic virus* (CMV) subgroups 1 and 2 can be separated using cowpea (*Vigna unguiculata* L. (Walp) cv. California Blackeye #5) based on lesion size. CMV subgroup 1 induces small necrotic lesions on inoculated leaves, whereas subgroup 2 induces minute, gray lesions on the leaf surface. However, not all CMV isolates produce lesions. The bean-infecting strain of CMV, CMV-B, infects cowpea systemically and is the only CMV strain described that does this. Virus strains can also react differently to different cultivars of a crop and vice versa. Strains are often defined by the reaction of a set of differential cultivars of the same crop species. For example, *Soybean mosaic virus* (SMV) strains can be characterized based on severity and pathogenicity on different soybean cultivars (Cho and Goodman, 1979; Ma et al., 2003). However, symptomatology and host range do not

give definitive answers on virus identification and must be used in conjunction with other diagnostic procedures. However, these biological approaches play a significant role by detecting differences between strains and pathotypes of plant viruses that may not be detected by other methods.

Serology

Serological techniques, which include enzyme-linked immunosorbent assay (ELISA) (Clark and Adams, 1977), tissue blot immunosorbent assay (TBIA) (Lin et al., 1990) and lateral flow devices (Tsuda et al., 1992), are powerful tools for the detection of plant viruses. These techniques are based on an antigen-antibody binding reaction between epitopes on the surface of virus particles and the binding sites of specific anti-virus antibodies (van Regenmortel, 1982). An antigen, which usually consists of purified virus nucleoprotein particles, is injected into an appropriate animal to induce the production of antibodies. Two types of antibodies can be made: polyclonal antibodies that consist of a population of antibodies that bind to different regions of the antigen protein (Ball et al. 1990), and monoclonal antibodies that consist of one type of antibody that binds to one specific region on the antigen protein (Jordan, 1990).

One of the first serological tests used to show identity between antigens was the Ouchterlony double diffusion test (Ball, 1990). The antigen-antibody binding reaction forms a white precipitate visible within the gel, and the precipitin patterns determine the relationship between adjacent antigen samples. Coalescence of the precipitin bands without deviation or alterations suggests close identity between the two antigens, while crossing of bands to form an 'X' suggests no identity and unrelatedness between the two antigens. Intermediate reactions also occur in which a 'spur' is formed. In these cases

both antigens share a common antigenic protein, but not all, suggesting a relatedness, but not identity (Ball, 1990; Walkey, 1991). The main drawback to this method is the high concentrations of antigen and antibody that are required. For optimum band formation, the concentration of virus should be between 1-2 mg/ml, a large amount if the virus titer within the tissue is low (Ball, 1990). The amount of antibody required is at least 20 μ l per Ouchterlony well of a dilution in the range of 1:32, depending on the antibody titer,.

The sensitivity of the antigen-antibody reaction can be greatly increased with the addition of a labeled probe. This was the premise for the enzyme-linked immunosorbent assay (ELISA) (Clark and Adams, 1977; Converse and Martin, 1990). The assay is conducted in microtiter plates, commonly with alkaline phosphatase and a substrate that is catalyzed to a yellow color relative to the amount of antigen present. There are two main types of ELISA, the 'double-antibody sandwich', in which the antigen is bound between the specific antibody and the enzyme conjugated antibody, the 'indirect ELISA', in which the antigen is bound only to the solid phase and rabbit antibody and the enzyme conjugated antibody is bound to the rabbit antibody. Antibody-coated multiwell kits have been developed against many different plant viruses and are commercially available (Agdia[®] Inc., Bioreba[®] Ag.) for large volume processing of samples. The disadvantages of ELISA are the incubation times required for samples and antibodies to adhere to microtiter wells, as well as the tissue extraction time, which can take between one and several hours.

The tissue blot immunosorbent assay (TBIA) was first used in the detection of several plant viruses in plant tissue by Lin et al. (1990), and has since become a widely used, sensitive, and reliable method for plant virus detection (Comstock and Miller, 2004;

Hsu and Lawson, 1991; Jonson et al., 2007). Virus particles are immobilized onto a nitrocellulose membrane by squashing or blotting infected plant tissue onto the membrane surface (Makkouk and Comeau, 1994), and the green pigment and other plant debris removed by washing with 5% Triton X-100, an anionic detergent (Srinivasan and Tolin, 1992). Homologous antibodies then detect and bind to the recognized virus particle. Alkaline phosphatase conjugated to the secondary antibody, which recognizes the homologous antibody, catalyzes the production of an insoluble purple precipitate upon the addition of a substrate, and is indicative of a positive reaction. TBIA has several advantages over ELISA. TBIA requires no tissue extraction and membranes can be blotted directly in the field. Additionally, samples blotted in the field can be processed at a later date (Makkouk and Comeau, 1994), removing the need for transport and storage of live plant specimens for serological analysis. The main disadvantage is that the test is qualitative, rather than quantitative.

Immunostrips[®] (Agdia[®] Inc.) and Agrostrips[®] (Bioreba[®] Ag.) are lateral flow devices (Tsuda et al., 1992) that give quick results and are simple to use. Infected tissue is ground in an extraction buffer and the tip of the strip is placed into the buffer. As the liquid moves up the wick, viral antigens are bound to gold flecks. As the infected sap continues to rise, the antigen is bound at an antibody line through antigen-antibody recognition binding. A positive result is displayed by the presence of two purple lines due to the accumulation of gold flecks at this antibody line and at the control line. Only one line, the control, is displayed for a negative result. The main advantage is time, as results are usually attained within 5-10 min. The disadvantage is the cost, so this technique is not recommended for large numbers of samples.

Nucleic acid-based techniques

Nucleic acid-based approaches are also used extensively for detection and identification of plant viruses, particularly since the advent of the polymerase chain reaction (PCR) (Saiki et al. 1988). Plant viruses with DNA genomes can be amplified directly using generic or gene-specific primers to the region of amplification. Reverse transcription (RT) of plant viral RNA genomes to a complementary DNA (cDNA) template and amplification by cloning has been done since the early 1980's.

Nucleic hybridization techniques are based on the recognition of target sequences, specific sequences within the nucleic acid, using specific probes to each sequence. If the target and probe are complementary in sequence, a duplex strand is formed. The techniques were originally designed for the detection of viroids, and have since been used for the detection of other virus-like pathogens and satellite RNAs, none of which can be detected by serological means (Nikolaeva, 1995). Nylon membranes are spotted with either sap from infected plants, treated with SDS to denature the virus particles (dot blot hybridization), or blotted directly with infected tissue (tissue print hybridization). Probes, which can be designed and purchased commercially (Agdia[®] Inc.), detect the specific nucleic acid sequence.

Polymerase chain reaction (PCR) is an extremely sensitive *in vitro* method that amplifies trace amounts of DNA to detectable levels using generic or gene-specific primers to the region of amplification, and Taq DNA polymerase (Saiki et al., 1988). PCR has numerous applications, including disease diagnosis, detection of plant pathogens (Vincelli and Tisserat, 2008), molecular characterization (Alfaro-Fernandez et al., 2008), DNA comparisons between related pathogen species (Kiss et al., 2008) and evolutionary

studies (Roossinck, 2001, 2002). PCR is used by many diagnostic and research laboratories worldwide. PCR is not used just for DNA pathogens. For RNA viruses, reverse primers to the RNA or poly(dT) oligonucleotides for RNA viruses with poly(A) tails, such as potyviruses, are used to initiate transcription of complementary DNA (cDNA). The cDNA is then used as a template in PCR reactions.

Initially, the source of viral RNA for cDNA synthesis and RT-PCR was from purified virus particles or total RNA extracted from infected plant tissue. Burgoyne (1996) patented the use of FTA[®] Cards for the collection and storage of DNA to be used either directly or indirectly in PCR. FTA[®] Plant Cards were later produced and commercialized by Whatman Inc. FTA[®] Cards are made from supported, cotton-based, cellulose fiber membranes to which infected plant tissue is blotted. As claimed in US Patent No. 6645717 (Smith et al., 2003), the fibers are “conditioned with chaotropic and other agents which lyse cells, and release and immobilize the genetic material while inhibiting their degradation”.

FTA[®] Plant Cards, and the methodology utilized with these cards, have proven useful for plant viruses (Ndunguru et al., 2005; Roy and Nassuth, 2005) and for plant gene expression studies (Roy and Nassuth, 2005). Virus-infected plant samples or healthy tissue are blotted onto the cellulose matrix and allowed to dry. The genomic DNA or RNA remains safely stored on the FTA[®] Cards at room temperature. Elution of the genomic DNA or RNA is accomplished simply by soaking a few discs removed from the area of blotted tissue in an extraction buffer and adding the extract directly into a (RT) PCR reaction mix. This method provides ample template for future reactions.

Diagnostics in developing countries

The role of plant clinics and diagnostic labs in any agricultural system is an important one, particularly when a majority of the economy is dependent on the exportation of its agricultural produce (Lawrence et al., 2005). In the United States, many plant clinics regularly use advanced diagnostic procedures, many of which require specific equipment and maintenance, and technical knowledge, in conjunction with the more traditional diagnostic methods. Many are further equipped with reference material, including computers to access the internet (Barnes, 1994), as well as access to scientific journals through university library subscriptions. Ausher et al. (1996) reported similar conditions in developing countries associated with international research centers or institutes. Most diagnostic clinics and research laboratories were adequately equipped with moderate to expensive equipment, qualified staff, scientific support, and in some cases reference material, including computer access to the internet. However, in laboratories not supported through international entities, the availability of equipment, infrastructure, and funding was low.

Now, in 2009, several developing countries are reportedly in financial debt. The demands for food crops are higher than ever, and some developing countries are on the brink of starvation, despite relief aid (FAO, 2009). As funds required for diagnostic laboratories are in short supply, many clinics rely on biological and serological techniques for diagnosis, as the cost of equipment for molecular analysis is too high. ELISA has been one of the most common serological techniques for virus detection since the late 1970s. Some countries, ignorant to the purpose of some reagents, tend to purchase entire kits to obtain a single buffer or antibody. This can become quite

expensive, as a simple 96-well plate ELISA kit can cost upwards of \$170 without shipping, while reagents alone would be upwards of \$80. An alternative to ELISA is TBIA. Srinivasan (1992) compared cost estimates for both TBIA and ELISA and found that, while the calculated cost to run 50 samples was the same for both methods, the initial start up cost for ELISA was much higher. Additionally, the antibodies used in TBIA can be reused up to 4 times when stored at 4°C or -20°C. ELISA antibodies can also be stored, but require individual removal from wells without damaging the well's surfaces. Finally, TBIA is not labor-intensive and results can be obtained within 3 hr, unlike ELISA, which can take up to 2 days. In developing countries, TBIA is an ideal method for virus diagnosis because it requires no expensive equipment.

Cucumber mosaic virus

CMV is an important plant virus, affecting hundreds of plant species and causing numerous diseases. Many reviews on CMV have been written in the last few decades, including those by Roossinck (2001, 2002), Palukaitis and Garcia-Arenal (2003), Palukaitis et al., (1992), Perry (2001), and Kaper and Waterworth (1981). These reviews cover a wide range of properties and characteristics of CMV, including genome and virus structures, transmission, strains, hosts, diagnostic and purification methods, and control. A brief description of several of these characteristics will be summarized herein.

CMV is one of the most economically important plant viruses in the world. CMV was first reported in 1916 as a pathogen affecting cucumber and muskmelon in Michigan (Doolittle, 1916). Since then, the recognized host range is one of the largest for any known plant virus, covering more than 1,200 plant species in over 100 plant families across monocotyledonous and dicotyledonous plants (reviewed by Edwardson and

Christie, 1997). CMV is the type member of the genus *Cucumovirus*, family Bromoviridae. Other members in this genus are *Peanut stunt virus* (PSV) and *Tomato aspermy virus* (TAV). In contrast to CMV, PSV and TAV have limited host ranges. PSV has been reported primarily in leguminous crops, although several species in the plant families Cucurbitaceae, Solanaceae and Chenopodiaceae are also susceptible (Miller and Troutman, 1966; Mink, 1980; Xu, et al., 1986). TAV is the least widely distributed species of the genus and is restricted to wherever *Chrysanthemum* spp. and some cucurbits and tomatoes are grown (Blencowe and Caldwell, 1949; Fauquet et al., 2005). Schmelzer (1971) and Phatak et al. (1976) identified two viruses, *Robinia mosaic virus* (RoMV) isolated from *Robinia* spp., and *Cowpea ringspot virus* (CpRSV) isolated from cowpea (*Vigna unguiculata* (L.) Walp.). The morphology and biology of both viruses suggested placement in the *Bromoviridae* family. The addition of sequence data later identified RoMV as a distinct strain of PSV (Kiss et al., 2008). CpRSV has remained a member of the Bromoviridae family despite several differences. including its having only 3 RNAs instead of 4 (lacking the sub-genomic RNA 4), very weak to no serological relationship to members of the family, and lack of transmission by aphids (reviewed by Edwardson and Christie, 1997).

***Cucumber mosaic virus* structure – physical and biochemical properties**

The CMV genome consists of three single-stranded ribonucleic acid (RNA) molecules housed in three separate protein capsids. With uranyl acetate-negative staining for electron microscopy, each capsid appears identical and icosahedral in shape, with what appears to be a hollow center. The particles regularly appear to be flattened and distorted (Tolin, 1977). The nucleoprotein capsids, approximately 28-31 nm in diameter,

are made up of 180 identical protein subunits in a $T = 3$ symmetry consistent with the pentamer-hexamer subunit clustering (Smith et al., 2000). The molecular weight of each protein subunit is between 24-25 kDa, depending on the strain. CMV has a sedimentation rate of 98-104 S (Svedbergs) with a particle density of approximately 1.36 g/cm^3 in CsCl when stabilized with formaldehyde (Symons, 1985). All CMV particles have the same density and sediment as single bands in sucrose gradients and are of equal density in CsCl. It was later concluded, using nucleotide data, that the distribution of the RNAs among the capsids balanced the particle weights (Kaper, 1975). The CMV capsid is stabilized by RNA-protein interactions and readily dissociates into its components, 18% RNA and 82% protein, in high alkaline pH and salts, and in low concentrations of the anionic detergent, sodium dodecyl sulfate (SDS) (reviewed by Kaper and Waterworth, 1981). CMV is also RNase-sensitive (Smith et al., 2000).

CMV is relatively unstable in extracted plant sap. The thermal inactivation point for CMV is 70°C for 10 min. and the dilution end point is 10^{-4} . Infectivity decreases and can be lost completely when stored at room temperature (reviewed Palukaitis and Garcia-Arenal, 2003). Stability of the virus in sap is greatly increased with the addition of antioxidants and/or storing the sap at temperatures close to freezing. For storage, CMV leaf samples must be frozen at temperatures at -70°C or dried and stored at 4°C , as freezing at -20°C inactivates the virus.

Cucumber mosaic virus genome structure and organization

The tripartite genome of CMV consists of three single-stranded, positive sense RNAs, two of which can be translated immediately by the host cell (Peden and Symons, 1973). The genome produces 5 messenger RNAs, 1, 2, 3, and 2 sub-genomic messengers

RNA 4 and 4a (Fig. 1.1). The two larger RNAs, RNA 1 and RNA 2, encode non-structural proteins required for viral replication. RNA 1 is approximately 3,357 nucleotides (nt) and codes for the 110 kDa 1a protein. The 1a protein contains two functional domains, methyltransferase in the N-terminal region, and helicase in the C-terminal region. RNA 2 is approximately 3,050 nt and encodes the 92 kDa 2a protein with one functional domain, the RNA dependent RNA polymerase. Together the replicase, helicase and methyltransferase, with cellular factors, enable RNA replication within the host cell.

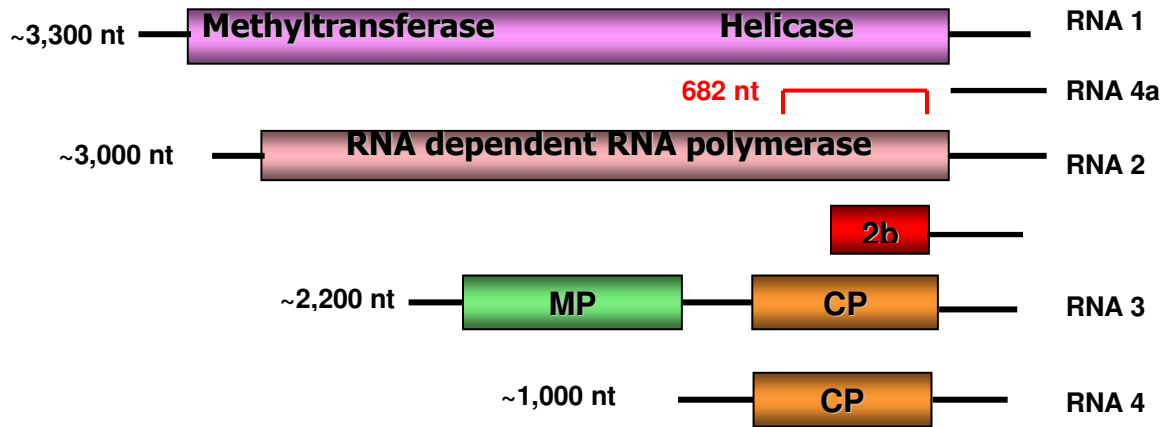
Also on the RNA 2, a subgenomic RNA 4a is formed and encodes a small 11 kDa 2b protein that influences the virulence of the virus by either facilitating long distance movement (Ding et. al., 1994) or silencing host defense systems (Brigneti et al., 1998). The RNA 4a is 682 nt long, with a sequence identical to the 3' terminus of the RNA 2. It was first identified in cucumber plants infected with CMV-Q, using western blotting (Ding et. al., 1994). Apart from the two features mentioned above, the 2b protein has also been implicated as a pathogenicity determinant of solanaceous plants. Ding et al. (1995) demonstrated that deletion of the 2b protein of the CMV RNA 2 resulted in no systemic infection in *Nicotiana glutinosa* tobacco plants, while the wild type strains produced severe mosaic, mottling and stunting. Further work by Du et al. (2007) using the *Ixora* strain of CMV demonstrated the role of 2b in virulence among different tobacco species.

The approximately 2,205 nt RNA 3 encodes for both a 32 kDa 3a protein and a 24 kDa coat protein (CP), both of which are required for short distance virus movement between plant cells through the plasmodesmata (Suzuki et al., 1991). Unlike RNAs 1 and 2, which are packaged individually in capsids, RNAs 3 and 4 are packaged together into

one capsid. The 3a protein, also known as the movement protein (MP), functions to regulate cell-to-cell movement of viral particles or ribonucleoproteins (positive sense RNA bound to MP) through plasmodesmata (Ding et al., 1995). The MP targets the plasmodesmata using tubules it forms through aggregates (Canto and Palukaitis, 1999; Ding, et al., 1994; Suzuki et al., 1991). Once at the plasmodesmata, the MP modifies the size exclusion limit to permit passage of the ribonucleoprotein (Vaquero et al., 1999) into the next cell.

The capsid or coat protein (CP) is encoded by RNA 3 and translated from the subgenomic coat protein messenger 4b, and is involved with short distance movement between cells and aphid-mediated transmission (Chen and Francki, 1990; Ding et al., 1995). The CP sequence, the most variable region of RNA 3, separates CMV into three subgroups, 1A, 1B and 2. The difference within each subgroup is only 2-3%, but up to 15% between subgroups 1A and 1B, and as much as 25% between subgroups 1 and 2 (Palukaitis et al., 1992; Roossinck et al., 1999).

Fig. 1.1 *Cucumber mosaic virus* genome



Modified from Fauquet et al., 2005

Cucumber mosaic virus infection cycle

Similar to many other plant viruses, CMV enters a host through mechanical wounds, or is introduced into the plant by insect or animal feeding. CMV is transmitted by many species of aphids in a non-persistent manner. Once inside the cell, the coat protein capsid disassociates, probably on cytoplasmic membranes, exposing viral RNAs. The membrane-bound RNA is translated by ribosomes to non-structural proteins and enzymes, the gene products of RNA 1 and 2, involved in virus replication (Nitta et al., 1988). These enzymes, RNA dependent RNA polymerase, helicase and methyltransferase, then act with host factors to synthesize a negative strand, a complementary copy of the viral RNA strand. At this point it is thought that both strands are temporarily bonded, forming double-stranded RNA molecules. Once copying is complete, the negative strand serves as a template for positive strand synthesis. With specific hybridization probes,

appreciable levels of RNA were detected within 15 hours post-inoculation (Gonda and Symons, 1979). When the copy number of positive-sense RNA reaches a threshold, a value not exactly known, translation of some RNA to produce protein begins (Agrios, 2005). Two important proteins formed are the movement protein, required for short and long distance movement within the plant, and the coat protein, used for short distance movement and protection of the viral RNA in capsids. At this point viral RNAs can be either encapsidated or form a ribonucleoprotein and move into adjacent cells through the plasmodesmata. Encapsidation is a self-assembly process that occurs between the RNA and the coat protein. There are no specific sites for binding, and instead, bonds are formed between the protein subunits and RNA. The complete virion remains trapped within the initial cell due to its size. Vaquero et al. (1994) demonstrated that, despite the MP ability to modify the size of the plasmodesmata when expressed transgenically, complete CMV particles, which are ~ 30 nm in diameter, could not enter the plasmodesmata, but ribonucleoproteins, with diameters of only 1.5-2 nm, could easily pass through.

The process of uncoating, replication, and encapsidation repeats in adjacent cells until the viral nucleic acids reach the vascular bundles. Whole particles enter the phloem and are transported with the photosynthates throughout the rest of the plant (Agrios, 2005).

CMV Diversity

Many RNA viruses have quasispecies, genetically diverse populations arising from an initial host, and CMV is no exception. Many evolutionary studies have been conducted on CMV (Bonnet et al., 2005; Roossinck, 2001, 2002), and all have observed

that the high rates of mutation, reassortment, and recombination of CMV have resulted in its highly diverse nature. Schneider and Roossinck (2000) provided correlative data between high rates of mutation of CMV and its increased host range. Initial passages into new hosts resulted in significant changes in diversity, or quasispecies cloud size, even in closely related species such as *Nicotiana tabacum* L. and *N. benthamiana* Domin (Schneider and Roossinck, 2001). With further passages within the same host, the number of mutations decreased rapidly, suggesting that CMV quickly attains and maintains variations specific to that particular host. What was interesting to note was that the mutations seen, even using identical CMV clones with the same host, were all unique and not completely random (Schneider and Roossinck, 2000). Observed mutations were distributed throughout the coat protein region on RNA 3 but had a higher bias towards untranslated regions, while no mutations were observed within the 'core' region of the coat protein, between nucleotide positions 1577 and 1846 of RNA 3.

The subgroup diversity of CMV can be determined molecularly by three methods. The first and most commonly used method is RNA sequencing. The amplified sequence, read from the original CMV RNA, can be compared to known strains and other isolate sequences, both at a nucleotide and an amino acid level. Several phylogenetic studies conducted with known strains of CMV displayed the same pattern of subgrouping using different RNA segments, suggesting that subgroup diversity is not determined by one RNA segment only (Bashir et al., 2006; Chen et al., 2007; Roossinck, 2002). In the second method, primers specific for the different subgroups in CMV are used. These specific primers are restricted only to the areas of the RNA that distinguish subgroups (Yanming et al., 1997). The final method, restricto-typing, involves the use of restriction

enzymes designed to cut at specific locations in the DNA sequence. One such enzyme, *MspI*, was highly successful in separating several CMV isolates into their respective subgroups based on the number of restriction fragment patterns observed upon cleavage of RNA 3. With the exception of the Ixora strain of CMV (subgroup IB), all of the CMV isolates and strains tested yielded two fragments if the CMV isolate was subgroup IA, four fragments if the CMV isolate was in subgroup IB, and five fragments of DNA for subgroup II (Bashir et al., 2006; Chen et al., 2007). Restriction-typing was also successful in determining reassortants of CMV (Chen et al., 2007). Reassortment is a natural occurrence in viruses containing multipartite genomes and is important in genetic variation and the development of new strains. In CMV, reassortment occurs more frequently between subgroups IA and IB than between subgroups I and II, possibly due to the higher percentage of similarity between IA and IB (Bonnet et al., 2005; Fraile et al., 1997).

The subgroup diversity of CMV strains can also be determined serologically. Hsu et al. (2000) developed monoclonal antibodies either for general CMV detection or specific antibodies for subgroups 1 and 2 by ELISA. For further differentiation between subgroups 1A and 1B, sequencing data must be obtained.

CMV Distribution

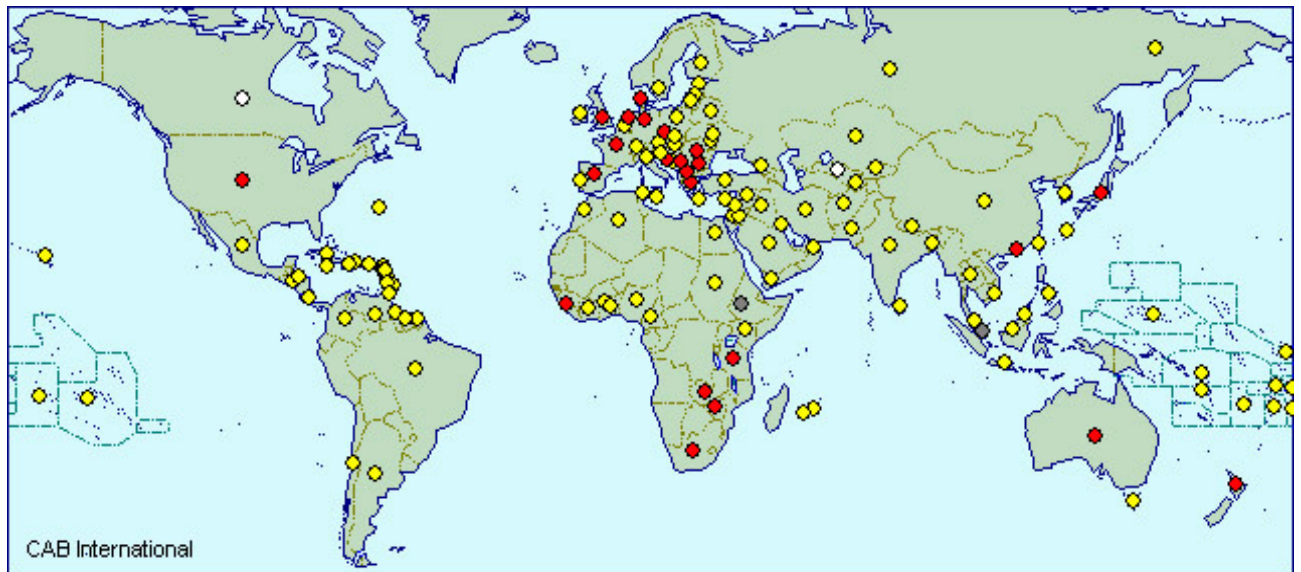
Every year, millions of dollars are lost because of severe diseases attributed to CMV. Diseases in tropical regions are especially serious and widespread because of constant warm temperatures, availability of crops year round, and ample rainfall resulting in high humidity, all favorable conditions for the replication and distribution of CMV by its aphid vector. Reoccurring epidemics of CMV (Albert et al., 1985; Grieco et al., 1997;

Kucharek et al., 1998) have prompted serological monitoring and development of several CMV-resistant crop cultivars.

The wide distribution of CMV (Fig. 1.2) is primarily attributed to its aphid vectors. Other methods of distribution include transportation of infected plant material and seed transmission. Several species of aphids are capable of acquiring CMV, the most common being the green peach aphid, *Myzus persicae* and the melon aphid, *Aphis gossypii* (Blackman and Eastop, 2000). A brief feeding time of less than one minute sufficiently insures acquisition of CMV and transmission to recipient plants by either aphid. However, the ability to transmit the virus is only temporary and retention of the virus is usually less than 1 hr from acquisition (Kucharek and Purcifull, 1997; Perry, 2001). Aphids are found worldwide, although a vast majority occurs in the Northern temperate regions (Dixon, 1998). It is interesting to note that almost all major aphid genera in the Northern temperate region are introduced Old World species, most likely occurring during the movement of food crops, ornamentals and other plants between countries (Blackman and Eastop, 2000).

Some of the more important crops affected by CMV include tomato, pepper, cucurbit species including pumpkin and watermelon, root crops such as dasheen, and tree crops such as banana. Several ornamental plants are also affected by CMV. In India, Chrysanthemums are important cut-flowers that can be affected by CMV and TAV through suckers, the primary method of propagation. Symptoms include stunting and flower quality and can result in severe losses (Verma et al., 2004).

Fig. 1.2 The global distribution of *Cucumber mosaic virus*



DISTRIBUTION

- ◆ present, no further details
- ◆ widespread
- ◇ present, localised
- ◆ distribution given on regional map
- ◆ confined and subject to quarantine
- ◆ occasional or few reports
- ◇ unconfirmed or uncertain
- absent: eradicated

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Control, Resistance and Management

Plant viruses cannot be treated using chemicals, and instead their control relies on several preventive measures. The effective control of aphid vectors, removal of infected plant material, and good cropping practices are just a few of the more commonly used measures. Control and management practices are employed to reduce plant disease, and include the use of pesticides for the control of aphid populations, barrier crops to protect susceptible hosts, late or early planting of crops to avoid vector movement into cropping area, and resistant cultivars (Gallitelli, 1998).

Breeding for Resistance

Breeding for natural resistance was the first method employed by farmers and scientists for introducing resistance genes into favorable crops. The process involves crossing of resistant and susceptible lines to introgress the resistant gene into the susceptible variety, and back-crossing to the susceptible parental variety to achieve a cultivar with both the resistance gene and the desired crop trait (Gallitelli, 1998). There had not been much success in breeding resistant cultivars to CMV until as recently as the last few decades when a CMV tolerant variety of pepper (Lapidot et. al., 1997), one of tomato (Stoimenova and Sotirova, 2000), and one of cucumber (Kherebah et al., 2009) were reported.

Induced Resistance

Cross-protection provided one of the early successes in the reduction of viral disease spread, and involved the inoculation of plants with mild strains of viruses in hopes of cross-protection against more virulent strains of the virus. Dodds (1982)

demonstrated that mixed mild and severe strains of CMV were antagonistic to each other, resulting in lower accumulation of virus particles over time.

Transgenic technologies have played a large role in development of resistance in plants. In transgenics, the plant itself is genetically modified by introducing a piece of a viral gene into the plant's genome (Gallitelli, 1998). In the mid 1980s, tobacco was the first plant to be transformed using the model virus *Tobacco mosaic virus* (TMV) CP. A delay in disease development was seen in the transformed tobacco when compared to the control (Abel et al., 1986). Cuozzo et al. (1988) expressed CMV CP in transgenic tobacco and tomato and found that delayed onset and fewer symptoms were seen on plants able to synthesize the CP compared to those that could not (control). Use of the anti-sense RNA for the CP also gave milder symptoms, but was easily overcome at higher concentrations of the virus. Cultivars of squash and melon have both been transformed using coat protein genes from three viruses, CMV (strain C), *Watermelon mosaic virus 2* (WMV 2) and *Zucchini yellow mosaic virus* (ZYMV), individually or together. Resistances to these viruses have been observed both in the laboratory and in field trials (Fuchs et al., 1995). Insertion of multiple coat protein genes into plant genomes conferred resistance to multiple viruses (Fuchs et al., 1998). Fuchs et al. (1998) demonstrated that insertion of one, two or all three CP genomes of CMV, ZYMV and WMV2 resulted in lower incidence of symptoms and higher yields when compared to non-transformed controls. Symptoms, when seen, were restricted to small dots on symptomatic leaves. It was hypothesized that this method would aid in the reduction of acquisition of virus during aphid feeding. The reduction in virus spread within a field supported this hypothesis

(Fuchs et al., 1998). Commercial crops have since been produced with resistance genes to several plant viruses using this technology.

Synergism

In the tropics, plant viruses cause damaging diseases on fruit and vegetable crops (Gallitelli, 2000; McLaughlin, 2003). In nature plants are usually bombarded by several viruses at once, and some viruses are able to facilitate systemic infection of other viruses that initially could not overcome the plant host's defenses. The combination of several viruses intensifies the reaction, enhancing disease severity with a greater virus accumulation within cells (Poolpol and Inouye, 1986). Recent work has been done involving cucurbits and the synergistic effects of more than one virus affecting a plant species (Murphy and Bowen, 2006). There have been several reports of CMV co-infecting plants with other viruses, most particularly with potyviruses (Murphy and Bowen, 2006; Pinto et al., 2008; Wang et al., 2002; Zeng et al., 2007). Choi et al. (2002), Guerini and Murphy (1999) and Wang et al. (2002, 2004) all reported enhanced movement and resistance-breaking by CMV when plants were co-infected with WMV. Because CMV and potyviruses are often found together in nature, it was important to review the potyviruses, their genomes, and characteristics. Potyviruses used in this study were *Tobacco etch virus* (TEV), *Turnip mosaic virus* (TuMV), and *Soybean mosaic virus* (SMV).

Potyviruses

The genus *Potyvirus* has been discussed in several reviews (Hollings and Brunt, 1981; Shukla et al., 1994), so only a few of the major characteristics will be highlighted

in this section. Potyviruses comprise a large group of plant viruses that belong to the genus *Potyvirus*, family *Potyviridae*. Several members are economically important plant pathogens, causing millions of dollars in crop loss worldwide. The *Potyvirus* genus has more than 400 members with a little over 50 tentative members (Fauquet et al., 2005). Like cucumoviruses, potyviruses are disseminated by several species of aphids in a non-persistent manner. But, unlike CMV, potyviruses require a helper component-proteinase (HC-Pro) for successful vector transmission (Blanc et. al., 1998). This virus-encoded, multi-functional protein has one motif that binds to the stylet of an aphid and another that binds to the N-terminus of the potyvirus CP (Hull, 2002). Three members of particular importance are *Tobacco etch virus* (TEV), *Turnip mosaic virus* (TuMV), and *Soybean mosaic virus* (SMV) as they can cause significant damage to crops. Many potyviruses have a narrow host range. SMV can only infect soybeans and other very closely related beans. In contrast, TEV has a wide host range, affecting crops in families such as *Cucurbitaceae* and *Solanaceae*, and is one of the leading causes in pepper decline in the tropics (Myers, 1996). TuMV has a natural host range restricted primarily to the family *Brassicaceae*, but affects several members within this family.

The *Potyvirus* genome and particle properties

Potyviruses are flexuous rods ~750 nm in length with a genome that consists of a positive-sense, single-stranded RNA of approximately 10,000 bases. Belonging to the picorna-like supergroup of viruses, potyviruses have a VPg covalently bound to the 5' end, and a poly(A) tail at the 3' end. During replication, the entire genome is translated to produce a large polyprotein, which is cleaved by proteases to give several proteins. The major, important proteins encoded include the P1-Protease, which cleaves the Tyr/Phe-

Ser and enhances amplification and movement of the virus, the Helper Component (HC-Pro) which aids in aphid transmission and acts as a silencing suppressor, the NIa and NIb, both of which are inclusion proteins with polymerase activity, the coat protein, which is important for the movement and protection of the viral RNA, and the VPg, which is a viral-linked protein required in the virus infection cycle (Shukla et al., 1994) (Fig. 1.3).

Fig. 1.3 General *Potyvirus* genome



Modified from Fauquet et al., 2005

Soybean mosaic virus

SMV occurs everywhere soybeans are grown worldwide. SMV is distributed primarily by the soybean aphid, *Aphis glycines* Matsumura, in a non-persistent manner, or through infected seed. The symptoms of SMV depend primarily on the host infected and can include mosaic, mottling, chlorosis, vein banding/clearing, stunting, seed coat mottling, necrosis and bud blight (Goodman, 1980).

Tobacco etch virus

TEV is a serious pathogen of pepper and tomato, as well as many members in the solanaceous group. TEV is distributed by at least 10 species of aphids in a non-persistent manner, and induces various symptoms including mosaic, stunting, and reduction in size of fruit and in yield. Early infection of young plants causes greater decrease in yield.

Turnip mosaic virus

TuMV is transmitted by at least 89 species of aphids to over 318 plant species, a majority of which belong to the family *Brassicaceae*. The virus causes mottling and mosaic patterns, necrotic and ring spots, distortion, reduction in fruit yield, and color-breaking on flowers. The distribution of TuMV has been reported worldwide particularly, wherever vegetable crops are grown (Shukla et al., 1994).

Objectives

Originally, the dissertation was focused primarily on CMV and the diagnostic techniques used for its detection and subgroup characterization, both locally in Virginia and globally, using a single, solid matrix. This method allowed for the safe transport of plant viruses across borders. Below are the original objectives of the dissertation.

- i) To analyze the political, economical and trade constraints to viral diagnosis in plant diagnostic and research laboratories in developed vs. developing countries.
- ii) To examine the diversity of CMV in Virginia and to validate the conventional methods used in molecular analysis.
- iii) To develop an affordable and rapid diagnostic and detection method for RNA plant viruses using CMV and paper-based technology.
- iv) To determine the diversity of CMV isolates in tropical and subtropical regions and compare this diversity to CMV isolates and strains reported in temperate regions.

The driving force behind the original dissertation – the application of diagnostic techniques for the detection and subgrouping of CMV, has remained the same. However, our discovery of the increased efficiency of NitroPure nitrocellulose membranes as sources of plant viral nucleic acids led us to revise the objectives. The direction of the dissertation is now focused specifically on diagnostics. The number of viruses was increased to include three potyviruses, one of which is a first report in Virginia. The new objectives are stated below:

- i) To characterize three recently acquired isolates of *Cucumber mosaic virus* (CMV) from Historic Smithfield Plantation using biological and molecular tools and applications.
- ii) To characterize *Turnip mosaic virus* isolated from *Hesperis matronalis* (L.) from Historic Smithfield Plantation.
- iii) To evaluate the virus diagnostic capabilities of collaborators in IPM-CRSP host countries.
- iv) To evaluate NitroPure nitrocellulose membranes for immunoassay, RT-PCR-based amplification and direct sequencing of *Cucumber mosaic virus* and potyvirus coat proteins
- v) To globally apply NitroPure nitrocellulose membranes for virus detection and identification.

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CHAPTER II

Biological and molecular characterization of *Cucumber mosaic virus* (CMV) from Virginia.

Abstract

In Virginia, *Cucumber mosaic virus* (CMV) was detected in ten samples collected from plants at Blacksburg and the Eastern Shore by tissue blot immunoassay (TBIA). Eight of the ten CMV isolates were examined further in this study. Three CMV isolates from the Historic Smithfield Plantation, Blacksburg, from bowl squash (*Cucurbita* sp.), flowering tobacco (*Nicotiana* sp.) and *Vinca minor*, common periwinkle, were cultured in Xanthi tobacco (*Nicotiana tabacum*) and used for biological and molecular studies. Each of these three isolates was compared with six CMV isolates collected and stored in the 1970's. Symptoms induced were similar to those reported for other strains of CMV, including a bean-infecting strain. There was very little change in a culture of CMV strain Y cultured continuously in the greenhouse, relative to the same strain stored for over 30 years. For molecular comparisons, the RNA from the three Smithfield Plantation isolates were obtained from purified virus dissociated with sodium dodecyl sulfate (SDS). For all remaining new Virginia isolates, the RNA source was obtained from nitrocellulose membranes from TBIA by a procedure described in Chapter V. The RNA of all CMV isolates was reverse transcribed using the reverse primers specific to either of the two subgroups of CMV: 1A/1B or 2. The cDNA was amplified by polymerase chain reaction (PCR) using primer pairs specific to the coat protein (CP) of each subgroup. Analysis of

sequences aligned by Cluster W from cleaned PCR products assigned eleven of the sixteen CMV samples to subgroup 1A, and three to subgroup 2. No samples aligned with subgroup 1B. Sequence data were not obtained for one sample from Smithfield and for one sample from the Eastern Shore that had tested positive by TBIA. The subgroup assignment of CMV isolates by CP sequence was confirmed by TBIA using monoclonal antibodies that reacted specifically with CMV subgroups 1 or 2.

Introduction

Cucumber mosaic virus (CMV; type species genus *Cucumovirus*, family Bromoviridae) is one of the most economically important plant viruses because of its enormous diversity and extensive host range. It infects more than 1,200 plant species in over 100 plant families, including both monocots and dicots. Symptoms caused by CMV vary in type and severity depending upon the host, strain, and environmental conditions, and include, but are not limited to, mosaic, stunting, wilting, chlorosis, vein-banding/clearing, fruit and leaf distortion, and reduction in yield.

CMV has a genome consisting of three single-stranded, positive-sense RNAs. RNAs 1 and 2 encode non-structural proteins involved in viral replication in the host cell. RNA 3 encodes both the 3a movement protein and the coat protein (CP), expressed from a subgenomic messenger. Both 3a and CP are required for short distance virus movement between plant cells through the plasmodesmata and vascular tissue (Canto et al., 1997; Suzuki et al., 1991). RNA 3 is also the most variable of the three RNA genomic species, as it has been demonstrated to determine the diversity of CMV strains and isolates. Based on serological relationships (Devergne and Cardin, 1973) and

comparative sequencing of RNA3, CMV strains are classified into three groups, subgroup 1A, 1B and 2 (Rizos et. al., 1992; Roossinck et al., 1999; Roossinck, 2002).

The recent recognition of CMV in a garden of a historic landmark in Blacksburg, Virginia prompted the re-examination of various CMV isolates from the Commonwealth. In the laboratory were various CMV isolates that had been collected more than 30 years previously during a project on *Peanut stunt virus* (PSV; genus *Cucumovirus*), and kept at 4°C in desiccated tissue. The objective of the current research was to compare the new and old CMV isolates using indicator hosts and test plants (Palukaitis et. al., 1992; Paradies et. al., 2000), using serology with polyclonal antibodies and subgroup specific monoclonal antibodies (Hsu et. al., 2000), and by molecular methods. Primers were designed from conserved regions identified near the coat protein encoding region of RNA3 and used in reverse transcriptase polymerase chain reactions (RT-PCR) that yielded high quality amplicons that were sequenced directly. Comparisons were made between sources of viral RNA for RT, namely: purified virus (Lot et. al., 1972) or partially purified virus (Lane, 2003) treated with a low concentration of detergent to release RNA (Boatman and Kaper, 1976), and a new immunoassay-RT-PCR protocol developed in this dissertation and described in Chapter V.

Materials and Methods

Collection and maintenance of field isolates

Leaves from plants displaying virus-like symptoms were collected from two locations in Virginia. The first was a garden of heirloom plants at the Historic Smithfield Plantation in Blacksburg. In this garden, symptoms included severe stunting, mosaic and leaf curl on flowering tobacco (*Nicotiana* sp.); mosaic, leaf curl and stunting of the apical

meristem of a bowl gourd (*Cucurbita* sp.); and mosaic and deformation of leaves on common periwinkle (*Vinca minor* L.). Leaves were collected and used to inoculate *N. tabacum* cv. Xanthi in the greenhouse, establishing isolates designated T05, from flowering tobacco, MO5 from bowl gourd, and V06 from Vinca, respectively.

The second location was the Eastern Shore Agricultural Research and Extension Center (ESAREC) field station and surrounding farmers' fields. The primary crops located at the station were cucurbits and legumes. The samples were stored in labeled plastic bags at 4°C. Representative leaves from each sample were blotted onto NitroPure nitrocellulose (NPN) membranes and allowed to dry overnight at room temperature (26°C ± 2°C). The remainder of the leaf samples were stored until the virus identities could be ascertained by TBIA. Samples testing positive for CMV were stored at both -80°C and desiccated over silica gel at 4°C.

CMV cultures were maintained on greenhouse plants by inoculating to new hosts at six week intervals. Inoculum was prepared by grinding infected leaf tissue in 0.01M sodium phosphate buffer, pH 7.0 (1:10, w:v) in a cooled, sterile mortar and pestle. The pestle was used to rub the sap onto hosts previously dusted with silicon carbide powder (carborundum). Excess carborundum and sap were removed from leaves by rinsing with tap water. All new CMV isolates testing positive for CMV were maintained on *N. tabacum* cv. Xanthi in the greenhouse with an average temperature of 28°C (± 2°C).

Viability and maintenance of stored samples

Seven CMV isolates (Table 2.1) were removed from storage and their viability determined by inoculation to host plants. The plants were originally stored dried at 4°C. Cultures CMV strain Y (# 108) was provided by J. L. Troutman (1967), CMV strain B (#

837) by R. Provvidenti (New York, 1977), CMV isolate 1/78 Wells Lupine (# 925) by Wells and Demski (Georgia, 1978), CMV isolate BBS (# 220) isolated from squash (Virginia, 1972), CMV strain S (# 926) isolated from squash (Virginia, 1976), CMV strain N (# 961) and CMV isolate CVG (# 969) both isolated in Virginia 1978. For re-activation, 0.1 – 0.2 gm of dried tissue was re-hydrated in 2 ml of 0.01M phosphate buffer, pH 7.0, ground using a mortar and pestle, and rubbed onto healthy hosts dusted with silicon carbide powder, grit 600 (Buehler[®], Illinois USA). The development of virus-like symptoms and newly infected hosts testing positive for CMV demonstrated viability. All successfully re-activated stored samples were kept in the greenhouse on hosts similar to those in which they had been stored.

Detection of *Cucumber mosaic virus* using Tissue blot immunoassay (TBIA)

Presence of CMV was confirmed using TBIA protocols modified from Lin et al. (1990) and Srinivasan and Tolin (1992). The dried membrane was treated for 10 min with 5% Triton X-100 to remove the residual chlorophyll and other leaf debris. The membrane was rinsed for 3 min in potassium phosphate buffered saline (KPS) (0.02 M K₂HPO₄, 0.15 M NaCl, pH 7.4) containing 0.05% Tween-20. The membrane was blocked for 20 min with 5% non-fat dry milk (Nestle Carnation, Nestle USA Inc., Solon OH) and 0.5% bovine serum albumin (BSA) (Sigma-Aldrich[®] Inc., Missouri) in KPS. The membrane was then placed for 90 min into either a combined anti-virus monoclonal antibody and rabbit anti-mouse-IgG-alkaline phosphatase (RAM-AP), both at a dilution of 1:15,000 in KPS, or a combined anti-virus polyclonal antibody and goat anti-rabbit (GAR-AP), both at a dilution of 1:10,000 in KPS.

Three monoclonal antibodies were used: ID#: 10F10F9 which detected all CMV subgroups, ID#: 44E9A7 which detected CMV subgroup 1, and ID#: 6D11D2 which detected CMV subgroup 2 (Hsu et al., 2000). Monoclonal antisera were a gift from Agdia[®] Inc. and the RAM-AP conjugate was purchased commercially (ImmunoResearch Laboratories Inc., West Grove, PA). Polyclonal antisera, aCMV-S, was previously prepared the Tolin laboratory, and the GAR-AP conjugate was purchased commercially from Sigma-Aldrich[®]. After incubation, the membrane was rinsed in Tris buffered saline (TBS) (0.05 M Tris base, 0.15 M NaCl, pH 7.6) containing 0.05% Tween-20 for 10 min, followed by two additional 5 min rinses.

Finally, the membrane was immersed in substrate, which consisted of a combination of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Zymed Laboratories Inc, Invitrogen Corporation, Carlsbad CA), and incubated for 5 – 10 min. The development of purple precipitate on blotted areas was indicative of viral antigen presence. The membrane was rinsed thoroughly in de-ionized water and left to air dry. All procedures were conducted at room temperature with constant, gentle agitation using a MS1 Minishaker (IKA[®] Wilmington, NC) set at 200 rev/min. All reagents used were obtained from Fisher Scientific Inc., USA unless otherwise stated.

Comparison of host range and symptomatology

All isolates of CMV were mechanically transmitted to the following indicator host plants: *Nicotiana tabacum* L. cv. Xanthi, *Nicotiana benthamiana* L., *Chenopodium quinoa* Willd., pumpkin (*Cucurbita moschata* Duchesne) cv. Small Sugar, zucchini (*C. pepo* L.) cv. Black Beauty, cucumber (*Cucumis sativus* L.) cv. National Pickling, melon (*C. melo* L.) cv. Hale's Best Jumbo, tomato (*Solanum lycopersicum* L.) var. Rutgers,

cowpea (*Vigna unguiculata* (L.) Walp) cv. California Blackeye #5), pepper (*Capsicum annum* (L.) cv. California Wonder, and bean (*Phaseolus vulgaris* L. cv. Bush Blue Lake). Three or more plants of each host were inoculated with each CMV isolate. Symptoms were recorded every three days for up to 8 weeks after inoculation. The experiment was repeated with a second set of plants, inoculated 8-10 days after source virus transfer to new hosts.

Virus Purification

Two purification methods were used. The purification procedure according to Lot et al. (1972) produces a highly concentrated suspension of purified virus and uses no NaCl, which has been found to inactivate certain strains of CMV. Minipurification is a partial purification procedure designed, according to Lane et al. (2003), for the identification of plant viruses based on coat protein size determined by polyacrylamide gel electrophoresis. This procedure requires less time than virion purification. The final suspension concentrations were determined by either the Bradford Reagent assay, a colorimetric assay for protein, or ultraviolet absorption, using an extinction coefficient of 5 for CMV (Lot et. al., 1974) at 260 nm for a 1 mg/ml concentration of viral nucleoprotein. In the Bradford assay, red Coomassie dye changes to a blue color when bound to proteins. The absorbance of the dye at 595 nm was compared to that of the Bovine Serum Albumin (BSA) standard. The absorbance is a linear function proportional to the increase in protein (concentration) in the suspension (Bradford, 1976). Detailed descriptions of both purification procedures are documented in Appendix A for the procedure according to Lot et al. (1972), and Appendix B for the procedure according to Lane et al. (2003).

Protein and virus concentration

For protein concentration using the Bradford Reagent, two fold serial dilutions of a 2 mg/ml BSA (bovine serum albumin) were prepared in microtiter plate wells (Nunc, Thermo Fisher Scientific, Rochester, NY USA) to produce the standard protein curve. A similar two-fold dilution series of a 1:10 dilution of each purified or partially purified virus particle suspension and healthy sap were placed in wells in line with the BSA serial dilutions. The negative control was 100 μ l of de-ionized water in plate wells. To each well, 25 μ l of Bradford Reagent was added and the plate incubated at 25°C for 30 min in The Jitterbug (Boekel Scientific, Feasterville, PA USA). The absorbance at wavelength 595 nm was recorded with a Spectra Max Plus spectrophotometer (Molecular Devices, Sunnyvale, CA USA). The concentration (mg/ml) of protein in the purified virus preparations was determined from the BSA standard curve.

For nucleoprotein concentration, the absorbance of a 1:10 dilution of each virus suspension was read directly with the spectrophotometer at wavelengths of 260 and 280 nm. The absorbance at 260 nm times the dilution factor and divided by the extension coefficient for CMV gave the virus concentration in mg/ml. The virus suspension was stored at 4°C.

Sucrose density gradients

To six centrifuge tubes, four concentrations of sucrose solution (Sigma, USA), 40%, 30%, 20% and 10%, in 0.005 M citrate buffer, pH 7, were layered from bottom to top and allowed to diffuse overnight at 4°C. In the first tube, 1 ml of a 1:20 dilution of 8 mg/ml purified virus was layered onto the top of the sucrose gradient (Fig. 2.2A). In two tubes, 1 ml of a second virus, *Brome mosaic virus* (BMV), concentration 2 mg/ml, also in

family *Bromoviridae*, was added as a sedimentation standard. In another tube, 1 ml of a third virus, *Tobacco mosaic virus* (TMV), concentration 1.5 mg/ml, was added as the second sedimentation constant. The tubes were placed in a SW28 swinging bucket rotor and centrifuged (Beckman L8-80, Beckman Coulter, CA USA) at 24,000 rpm for 3 hr. The tubes were removed and visualized with a bright beam of light shone vertically downward through the gradient. The resulting virus band was extracted using a glass pipette and stored at 4°C. The solution was diluted with an equal volume of 0.01 M phosphate buffer and inoculated to healthy tobacco and *Chenopodium quinoa* plants lightly dusted with carborundum to confirm virus presence.

RNA analysis

Primer design

Two sets of primers for amplification of the coat protein region of CMV were designed using sequences obtained from the National Center for Biotechnology Information (NCBI) database (see Appendix C). RNA 3 and coat protein sequences were aligned using MegAlign, an alignment program in sequence software DNASTAR Lasergene 8 (DNASTAR Inc., Madison, WI USA) and the general consensus was taken just within the coat protein. The first set of primers: PfcMVCP4 forward, 5` - GACAAATCTGAATCAACCAGTGC – 3`, and PrCMVCMVCP618 reverse, 5` - CTCGACGTCAACATGAAGTA – 3`, were designed to amplify a DNA product 614 bp in length within the coat protein genome. The second set of primers: PfcMVRNA31163 forward, 5` - ATGCTTCTCCRCGAGATT – 3` and PrCMVRNA32034 reverse, 5` - GTAAGCTGGATGGACAAC – 3`, were designed to amplify the entire CP genome including flanking 5` and 3` regions for a DNA product of 871 bp for subgroup 1.

A third set of primers: CMVSeo2FPr forward, 5` - TTCTCCGCGAGTTAGC – 3` and CMVSero2RPr reverse, 5` - CGTAAGCTGGATGGAC – 3`, were from Yanming et. al. (1997) and were designed to amplify the entire CP genome including flanking regions for the specific detection of subgroup 2. These primers amplified a DNA product of 860 bp.

RNA preparation and Reverse transcription

cDNA synthesis from purified virus was performed using the Reverse Transcription System kit (Promega Corporation, Madison WI USA). To a 200 µl RT tube, 1 µl of 8 mg/ml purified virus nucleoprotein (0.4 mg/ml final concentration) and 2 µl 0.1% sodium dodecyl sulfate (SDS) (0.01% final concentration) for protein disassociation (Boatman and Kaper, 1976) were directly added to a 20µl reaction mix containing the following reagents: 4 µl 25 mM MgCl₂, 2 µL 10X Reverse Transcription buffer, 2 µl 10 mM dNTP mix, 0.5 µl 40 U/µl Recombinant RNAsin® Ribonuclease Inhibitor, 0.6 µl 25 U/µl AMV reverse transcriptase, 1 µl 10 mM CMV reverse primer PrCMVCP618, and nuclease-free water up to 20µl. Optimization of SDS % in RT reactions were performed using one RT reaction mix contained virus with no SDS and another contained virus with 2 µl 1% SDS (final concentration of 0.1%: high concentration). Healthy plant extracts were used as negative controls for the experiment.

Alternatively, RNA was obtained from NitroPure nitrocellulose membranes for the CMV samples used in this study,. Following the procedure described in Chapter V, a disc from infected tissue blotted onto a membrane was removed, cleaned and placed in a 20 µl RT mix.

For, RT was performed in a PCR Sprint Thermal Cycler (Thermo Electron Corporation, Bioscience Technologies, Milford MA USA). The RT protocol for the two RNA template preparation methods, using primer sets 1 and 2, was as follows: 25°C for 10 min, 42°C for 15 min, 95°C for 5 min, and 4°C for 5 min (Madhubala et. al., 2005). The RT protocol for CMV subgroup 2, using only the membrane method, was as follows: 42°C for 60 min (Yanming et al. 1997). This protocol was also used for positive and weakly positive samples by TBIA that could not be cultured

Polymerase chain reaction

The polymerase chain reaction step was performed using the PCR Core System II kit (Promega Corporation, USA). To a 200µl PCR tube, 3 µl of cDNA template were added to a 50 µl reaction mix containing the following reagents: 4 µl 25 mM MgCl₂, 1 µl 10 mM dNTP mix, 10 µl 10X Colorless Flexi GoTaq® Reaction Buffer, 0.25 µl 5 U/µl GoTaq® DNA Polymerase (Promega Corporation, USA), 1 µl 10 mM primer set, and 29.75 µl nuclease-free water. PCR was performed in a PCR Sprint Thermal Cycler. The protocol for CMV primer set 1 was as follows: 95°C for 2 min, [94°C for 30 sec, 50°C for 60 sec, and 72°C for 60 sec] for 35 cycles, and 72°C for 5 min (modified from Madhubala et. al., 2005). The protocol for CMV subgroup 1 was as follows: 95°C for 2 min, [94°C for 30 sec, 46°C for 60 sec, and 72°C for 60 sec] for 35 cycles, and 72°C for 5 min (modified from Madhubala et. al., 2005). The protocol for CMV subgroup 2 was as follows: 94°C for 2 min, [94°C for 45 sec, 45°C for 45 sec, and 72°C for 60 sec] for 35 cycles, and 72°C for 5 min (modified from Yanming et. al., 1997). The PCR products were analyzed in a 2% agarose gel electrophoresis system using Tris-acetate-EDTA

(TAE) buffer (0.04M Tris-acetate, 0.1 mM EDTA, pH 8.0), stained with ethidium bromide, and viewed using a UV transilluminator (UVP Inc., San Gabriel CA).

Preparation of PCR products and sequence analysis

PCR products were cleaned either from the 2% agarose gel with QIAquick Gel Extraction Kit or directly with QIAquick PCR Purification Kit (Qiagen Inc., USA) as per the manufacturer's instructions. The concentration of the products was measured using a NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE) and diluted to a concentration of 10 ng/μl for sequencing at the Virginia Bioinformatics Institute (VBI) at Virginia Tech (Blacksburg, VA).

The EditSeq program of DNASTAR Lasergene was used to remove any high background signals at the starts and trim existing sequences obtained from GenBank. The Cluster W program in MegAlign was used to align all sequences and produce a phylogenetic tree comparing to CMV sequences submitted to GenBank.

Results

Collection of field isolates and tissue blot immunoassay

Three samples collected in Blacksburg, Virginia tested positive by TBIA for CMV. The source plants were an ornamental tobacco, an heirloom gourd, and *Vinca minor* L., all of which were located in the gardens and surrounding areas of the Historic Smithfield Plantation. Symptoms on the naturally infected plants included vein banding, leaf deformation, vein clearing, chlorosis and mosaic. CMV was weakly detected in hops (*Humulus lupulus* L.) by TBIA, suggesting a different, but related, virus. However, the virus could not be transferred to new hosts. Other plants at the Historical Plantation exhibited virus-like symptoms but did not test positive for CMV. These included

Hesperis matronalis L. (Dame's Rocket), ornamental tobacco, and *Dipsacus* sp. (Teasel). The Dame's Rocket tested positive for *Turnip mosaic virus* (TuMV), as described in Chapter III, and the tobacco tested positive for TMV. CMV was also detected in *Magnolia stellata* on the Virginia Tech campus in Blacksburg. The infected leaf was collected as part of a disease notebook collection by an undergraduate student at Virginia Tech. However, at the time of receipt from the teaching assistant of Pathology course, the state of the tissue and the unavailability of tobacco Xanthi plants for transfer did not permit culturing.

Crop trials at the Eastern Shore Agricultural Research and Extension Center (AREC), Virginia, and four fields belonging to local farmers surrounding the main AREC station were inspected for virus symptoms. Crops included watermelon, pumpkin, pepper, tomato, soybean and snap beans. CMV tested weakly positive in pumpkin, pepper, snap bean legumes and tomato, and, except the pumpkin and tomato, samples were not transferred. Attempts at transferring the pumpkin and tomato samples to Xanthi tobacco plants were unsuccessful, and the remaining leaf samples were discarded. Watermelons tested positive for *Watermelon mosaic virus 2* (WMV2) and had a high incidence of downy mildew. A complete list of all plants, their symptoms, and presence of CMV is shown in Table 2.2.

Stored samples

Only one of the seven isolates removed from storage, CMV isolate CVG (accession # 969), was not viable, as repeated transfers to cucumber or tobacco were unsuccessful. The seven isolates and their viability on new hosts are shown in Table 2.1. CMV Blacksburg isolate, BBS (accession # 220), exhibited a low fitness level. At

temperatures 30-35°C and above, the virus was lost and the host plant fully recovered. TBIA consistently failed to detect this CMV culture after the third transfer to new hosts at these temperatures. Infection of other hosts was similarly not as robust. Symptoms, if seen, developed within the first 2 weeks after inoculation. TBIA using tissue from inoculated leaves gave relatively weak positive responses. CMV strain Y (accession # 108) displayed bright yellow mosaic patterns on newly emerging leaves, as typical for that strain. CMV strains B and N (accession # 837 and # 961) and a Georgia isolate Lupin (accession # 925) produced systemic mosaic and slight stunting on cowpea and Xanthi tobacco respectively.

Comparison of host range and symptomatology

Symptoms induced by both field and re-activated isolates were similar to those previously reported for CMV (Palukaitis and Garcia-Arenal, 2003). Table 2.3 summarizes the symptoms on 11 host plants covering three families: *Solanaceae*, *Cucurbitaceae* and *Leguminaceae*. Symptoms of the three field isolates collected at Smithfield induced very similar results, differing only slightly on melon, zucchini, tomato and *N. benthamiana*. CMV-B completely differed in symptom responses from the other isolates, particularly on legumes. Similar to the literature, CMV-B was systemic on all legumes used, demonstrating that CMV-B was a legume-infecting strain. The remaining isolates and strains produced small necrotic lesions on each legume. Comparison of the continuously cultured CMV strain Y (CMV-Y-CC) and the stored CMV strain Y culture (CMV-Y-108) showed very little difference in symptoms suggesting that strain Y had changed little in the 30 years of culturing since storage. CMV isolate BBS induced very little symptoms on any of the host plants in this study, and symptoms induced, for

instance on tobacco species, were very mild. It was noticed that this isolate was also intolerant to wide changes in temperature. Temperatures falling below or going above 5°-10°C from room temperature (26°C) partially inactivated the virus as no symptoms were observed at these temperature ranges. The symptomatology of remaining isolates varied among the different hosts, as shown in Table 2.3.

Virus Purification

The protein and nucleoprotein concentrations of each purified or partially purified virus were obtained with the Bradford or ultraviolet absorption methods, respectively. The concentration of each virus was adjusted to give a final concentration of 0.4 mg/ml in the RT reaction mixture.

A sucrose gradient is a common procedure for the final purification step for plant viruses and demonstration that purification has been successful. It can also be used to estimate the sedimentation coefficient of a virus. Two viruses, BMV and TMV, with known sedimentation values of 78-79 S and 194 S were used as comparisons against the sedimentation of the new CMV isolates. CMV has a reported sedimentation value of 98-104 S (Palukaitis and Gracia-Arenal, 2003). The CMV band was seen at a distance of 3.8 cm from the top of the solution, just below the band of BMV (Fig. 2.2B) at 2.9 cm, and above the band for TMV at 4.5 cm (figure not shown). The band sedimented to within the estimated depth for CMV, using BMV and TMV as reference points. To confirm that the band observed was made by virus particles, it was extracted using a glass pipette, diluted with an equal volume of 0.01M phosphate buffer, and inoculated to three plant hosts: Xanthi tobacco, *N. benthamiana* and *C. quinoa*. Systemic mosaic, mottling and leaf curling were observed on both tobacco plants (data not shown), while on *C. quinoa* 60-

100 chlorotic lesions (Fig. 2.2C) were observed. This confirmed that the band produced in sucrose centrifugation contained virus particles. The tobacco plants, when processed by TBIA, were positive for CMV.

RNA release and reverse transcription-polymerase chain reaction

The most common way of releasing nucleic acids from purified virus is the phenol/chloroform extraction method (Logemann et. al., 1987). The hazards of this procedure prompted research in alternative methods. Sodium dodecyl sulfate (SDS) is a common anionic detergent which denatures secondary and tertiary structures. Boatman and Kaper (1976) demonstrated that minute amounts of SDS resulted in the dissociation of CMV particles. In the RT reaction, 0.01% SDS was sufficient to release RNA from 0.4 mg/ml of virus suspension into the reaction mix allowing for transcription, as shown by the amplification of cDNA, viewed on a 2% agarose gel. Only PCR reactions for which virus particles were present and had SDS added to the RT reaction showed amplicon bands (data not shown). The concentration of SDS influenced the intensity of the amplicons. In the same cDNA mixture, addition of virus with SDS at a higher concentration (0.1%), resulted in lower amplicon intensity than seen with 0.01% SDS,

Nitrocellulose membranes as sources of viral RNA

As described in Chapter V, discs from membranes positive for CMV were removed, cleaned and subjected to RT-PCR. Weakly positive CMV samples from the Eastern Shore and the *Magnolia* were also processed by this method. Depending upon the set of primers used, the amplicons fell within the expected size range for the corresponding CMV strain or isolate. Amplification from RNA transcribed from bound

virus particles from processed membranes was also observed from the four samples (tomato, pepper, pumpkin and *Magnolia*) that could not be cultured (data not shown).

PCR purification and sequence analysis

Sequence analysis placed all but three samples within subgroup 1A (Fig. 2.3a) with sequence identities between 93.5-94.8% at the nucleotide level with the three reported subgroup 1A CMV strains Fny, Sny and C. All CMV isolates in this study had over 97% sequence identity with each other. The three remaining samples: CMV-S (#926), CMV-Lupin (#925), and CMV-BBS (#220) were placed with subgroup 2, with identities between 96.4-97.5% with the two reported subgroup 2 strains, Kin and Trk. Sequence identities remained within the same range when compared with each other (Fig. 2.3b). For the three CMV isolates from the Smithfield Plantation, sequence identities for the gourd isolate were 93.9-94.5% identical to the *Vinca minor* and tobacco isolate, which had 98.3% identity with each other. This suggested that, although all three isolates were collected from the same location, the tobacco and *Vinca* isolate were possibly from the same original source. The sequence data classification was confirmed using CMV subgroup specific monoclonal antibodies (Fig. 2.4)

Discussion

CMV is one of the most diverse plant viruses in the world. Its economic importance is reflected by its ability to affect more than 1,200 plant species. Over 50 strains and isolates of this virus have been reported from various temperate and tropical regions (Palukaitis and Gracia-Arenal, 2003). In Virginia, CMV has been identified, isolated and stored, but never characterized to subgroup. Strains from surrounding states have also been collected and stored, but never characterized. The recent detection of three

CMV isolates at Virginia Tech's Historic Smithfield Plantation in Blacksburg, VA, has prompted renewed interest in strain characterization, particularly with previously stored isolates. The newly detected isolates were from tobacco and winter gourd, both heirloom varieties grown in the vegetable garden in back of the plantation house, and *Vinca minor*, an evergreen, perennial, creeping plant surrounding the plantation were compared biologically and molecularly, and assigned to subgroup.

Six of the seven strains of CMV removed from storage after 30 years were viable (Table 2.1) and had originally been collected from Virginia, New York, and Georgia, desiccated, and stored at 4°C. On hosts, the reactivated viruses had symptoms ranging from mild mosaic to chlorotic spots to severe stunting and leaf deformation. Eleven indicator hosts were used based on previous characterization studies for CMV (Raj et. al., 2002; Valveri and Boutsika, 1999; Wahyuni et. al., 1992). CMV-Y/108 and CMV-Y-CC (continuous culture) were from the same initial source. The similarities between symptoms on hosts indicated that the continuously cultured CMV-Y had not changed significantly in these properties since it was first put in storage 30 years ago.

Comparisons of all CMV isolates and strains used in this study are summarized in Table 2.3. CMV-B (accession # 837) was the only CMV strain to systemically infect bean. This result is consistent with previous reports pertaining to CMV-B (Edwards et al., 1983) Symptoms displayed were similar to those reported in literature. Bos and Maat (1974) and Schmelzer and Schmidt (1975) first reported a CMV bean-infecting strain that induced severe, systemic mosaic and mottling of bean and peas. In recent years, the incidence of bean-infecting CMV has increased in midwestern and northeastern states of USA, affecting snap bean production and yield (Shah et al., 2006). The remaining CMV

isolates induced necrotic lesions on both cowpea and Bush Blue Lake bean. Cowpea, *Vigna unguiculata*, is an indicator host used to separate CMV isolates into subgroups 1 or 2 (Palukaitis and Garcia-Arenal, 2003).

Sequences obtained were clean and were adequate for use in the subgroup characterization study. Sequence analysis of all samples are shown in Fig. 2.3a and b. All but three CMV isolates: Lup-CMV, CMV-S and BBS-CMV, were aligned with reported CMV subgroup 1A strains from GenBank with sequence identities between 93 and 95% with subgroup 1A reported strains Fny, Sny and C, and over 97% sequence identity with each other. The three CMV isolates that were not aligned to subgroup 1A, were determined to be in subgroup 2. These isolates had sequence identities between 96 and 97.5% with the two reported subgroup 2 strains, Kin and Trk. Sequence identities remained within the same range when compared with each other (Fig. 2.3b). For M05-CMV, T05-CMV and V06-CMV, the three isolates from Smithfield Plantation, there was a higher percentage identity between T05-CMV and V06-CMV than of either isolate with the M05-CMV isolate. This suggests two possible explanations. The first is that the tobacco and *Vinca* strains were initially from the same host, while the gourd was from a different host, or that all three arrived from the same original host, but the rate of mutation during initial passage through gourd was higher than that through tobacco or *Vinca minor* (Schneider and Roossinck, 2001). It is also possible that the source of the gourd isolate was from the seed of this heirloom variety, as there were no other plants with virus symptoms testing positive for CMV with TBIA.

In this study, several isolates of CMV collected from two locations in Virginia were characterized to subgroup. Of the samples collected, three were isolated from a

garden surrounded by relatively undisturbed woodland. Biological characterization revealed the presence of one bean-infecting strain, strain B, and the significant difference in symptoms for CMV Blacksburg isolate (accession # 220). Sequence data showed that all CMV isolates obtained in Blacksburg between 2005 and 2006 were within subgroup 1A, while a 1970's isolate from Blacksburg (220) belonged to subgroup 2. Four additional CMV isolates were obtained in Virginia, but could not be transferred to maintenance hosts. The method described in Chapter V allowed molecular analysis of these isolates, all of which fell within subgroup 1A (Fig. 3 a and b). This new methodology shows the significant contributions nitrocellulose membranes can make to molecular characterizations. TBIA was sufficient sensitive to detect low levels of CMV in some of the infected tissue collected from the crop plants growing on the Eastern Shore. Previously, without fresh tissue, there was no possibility of obtaining nucleotide sequence data. Membranes showing weakly positive TBIA reactions, however, yielded PCR products and sequence data, permitting molecular characterization of CMV without their isolation and culture. The methodology of using membranes as sources of RNA is elaborated further in Chapter V.

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Table 2.1 Hosts used for determining viability of stored *Cucumber mosaic virus* (CMV) isolates

Isolate Code	Strain/Isolate	Plant host used (and originally stored on)	Date stored	Viable
108	CMV-Y	<i>Nicotiana tabacum</i> – ‘Xanthi’ tobacco	02/20/68	Yes
220	CMV-BB-VA	<i>Nicotiana tabacum</i> – ‘Burley 21’ tobacco	02/16/73	Yes
837	CMV-B-NY	<i>Cucurbita pepo</i> – ‘Black Beauty’ zucchini squash	08/24/77	Yes
925	CMV-Lupin-Wells	<i>Nicotiana tabacum</i> – ‘Xanthi’ tobacco	02/02/78	Yes
926	CMV-S	<i>Nicotiana tabacum</i> – ‘Xanthi’ tobacco	02/09/78	Yes
961	CMV-N	<i>Cucumis sativus</i> – ‘National pickling’ cucumber	04/11/78	Yes
969	CMV-CVG	<i>Cucumis sativus</i> – ‘National pickling’ cucumber	04/13/78	No

Table 2.2 Plants tested for the presence of *Cucumber mosaic virus* with Tissue blot immunoassay

Host	Origin ^a	Field Symptoms ^b	CMV Present
<i>Hesperis matronalis</i> (L.) – Dame’s Rocket	SP	MM, PCB	No
<i>Nicotiana</i> sp. (L.) – Tobacco ornamental	SP	MM, SSt, DF	Yes
<i>Nicotiana</i> sp. (L.) – Tobacco ornamental	SP	MM, St, DF	No
<i>Vinca minor</i> (L.) – Periwinkle	SP	MM, DF	Yes
<i>Cucurbita</i> sp. – heirloom gourd	SP	DF, MM, St	Yes
<i>Humulus lupulus</i> (L.) – Hops	SP	MM	Yes
<i>Dipsacus fullonum</i> (L.) – Teasel	SP	MM, Br, DF	No
<i>Citrullus lanatus</i> – Watermelon	ESAREC	MM	Weak
<i>Cucurbita moschata</i> – Pumpkin	ESAREC	MM	Weak
<i>Solanum lycopersicum</i> – Tomato	ESAREC	Mild MM	Weak
<i>Magnolia stellata</i>	BB	VC	Yes
<i>Echium vulgare</i> (L.) – Viper’s Bugloss	SP	CS, DF	No
<i>Asclepias syriaca</i> (L.) – Milkweed	SP	MM, CS	No
<i>Conyza canadensis</i> (L.) Cronquist – Horseweed	SP	MM	No
<i>Glycine max</i> (L.) – Soybean	ESAREC	MM	No
<i>Phaseolus vulgaris</i> (L.) – Snap bean	ESAREC	MM	Weak
<i>Capsicum annum</i> – Pepper	ESAREC	VC	Weak

^a Origin of plants detected for the presence of *Cucumber mosaic virus*. SP – Smithfield Plantation, Blacksburg VA, ESAREC – Eastern Shore AREC, VA, BB – Blacksburg

^b Virus-like symptoms observed on plants tested for the presence of *Cucumber mosaic virus*. MM – mosaic/mottle, VC – vein clearing, PCB – petal color-breaking, DF – deformed leaves, St – stunting, SSt – severe stunting, Br – bronzing of leaves, CS – chlorotic spots

Table 2.3 Host range and symptomatology of new and old isolates and strains of *Cucumber mosaic virus*

	M05-CMV	T05-CMV	V06-CMV	CMV-Y	CMV-Y	BBS-CMV	CMV-B	Lup-CMV	CMV-S	CMV-N
	New	New	New	CC	108	220	837	925	926	961
Cucumber	MM, VB ^a	MM, VB	MM, CS	MM	MM, DF	CS	MM, CS	MM, CS	CS	MM, VC, DF
Melon	MM, VB, CS	VB	MM, VB, CS	CS	VC	NS	MM	NS	-	-
Pumpkin	VB	VB	VB	VC	VC	NS	MM, VB, CS	NS	VB, CS, RS	-
Zucchini	MM, VB	NS	VC, CS	VC	-	NS	MM, VC, DF, CS	VB, CS	-	CS
Pepper	NRL	NRL	-	MM, VC, DL,CS	MM, CS	NS	St	VB, DF, St	MM	MM, DF, CS, St
<i>N. benthamiana</i>	MM, VB, DF	MM, VB, DF	MM, DF, St	MM, DF, St	MM, DF, St	MM, DF	MM, DF	MM, DF	MM	MM, DF
Tomato	CS	CS	MM, DF, CS, St	MM, DF, CS, St	MM, CS	MM, DF	MM, VB, CS, St	MM, DF	VB	MM, VB, DF, CS, St
Xanthi tobacco	MM, VB	MM, VB	MM, DF	MM, VC	MM, CS, VC	MM, RS	MM	MM, VB, RS, CS	MM, RS	VB
Bush Blue Lake bean	NL	NL	NL	NL	NL	-	MM, VB, DF, St	NL	NL	NL
Blackeye cowpea	NL	NL	NL	NL	NL	NL	MM, NL, St, W	NL	NL	NL
<i>Chenopodium quinoa</i>	CS	CS	CS	CS	CS	CS	CS	CS	CS	CS

Table 2.3 (continued)

^a Symptoms observed on indicator hosts inoculated with different strains and isolates of *Cucumber mosaic virus*. MM – mosaic, mottle, W – wilting, (–) – not tested, RS – ringspot on inoculated leaf, St – stunting, DF – deformed leaves, CS – chlorotic spots, NRL – necrotic ringspot lesions, VB – vein banding, NL – necrotic lesions, NS – no symptoms, VC – vein clearing

Fig. 2.1 Symptoms representative of different indicator plants inoculated with various *Cucumber mosaic virus* isolates and strains in host range and symptomatology tests.

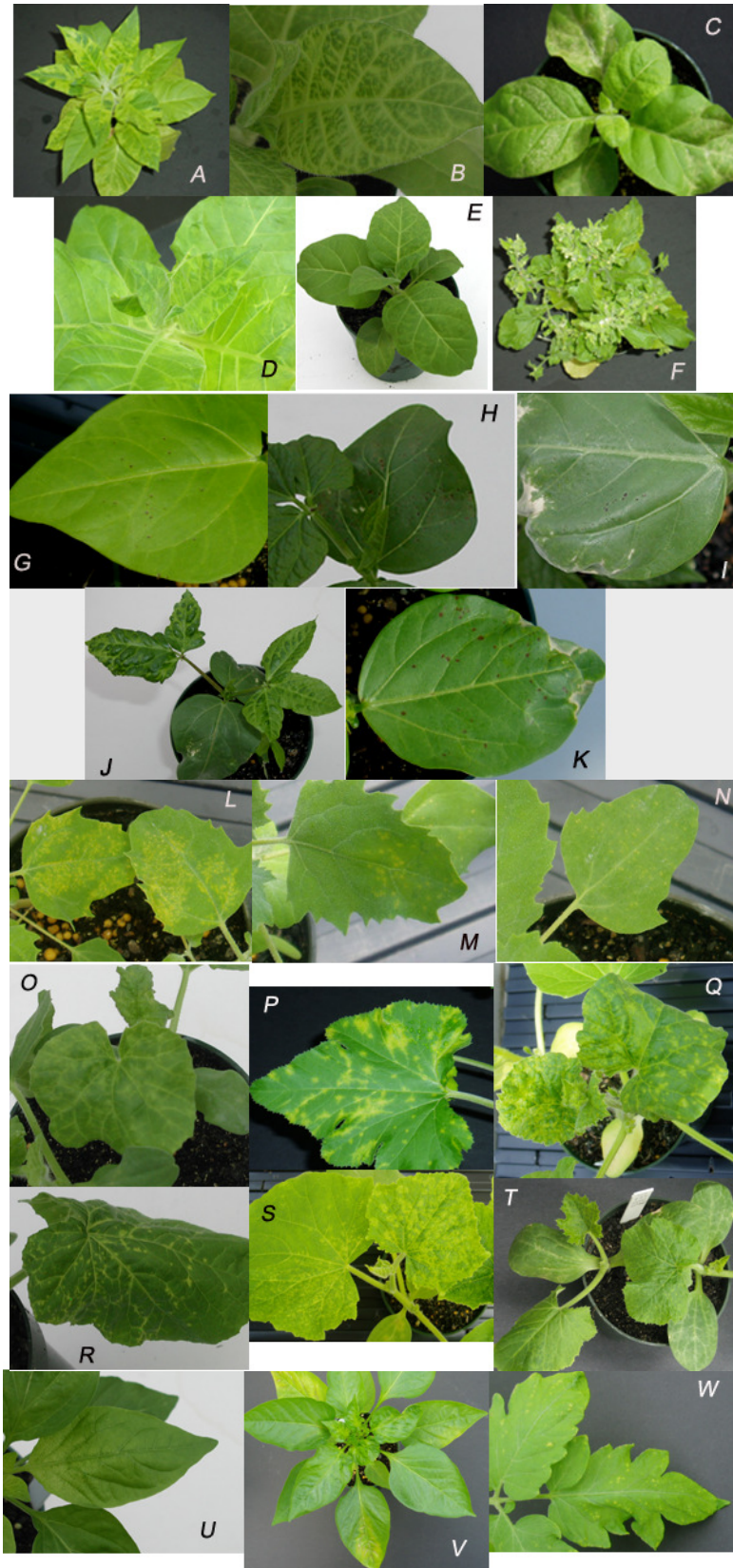
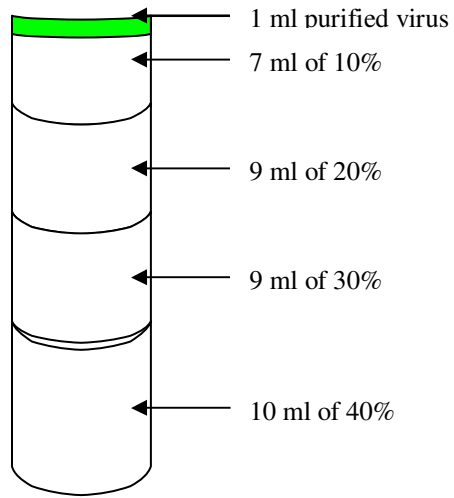


Fig. 1	Caption	days post inoculation
A	CMVY-CC on Xanthi tobacco	60
B	CMVY-108 on Xanthi tobacco	14
C	M05-CMV on Xanthi tobacco	14
D	T05-CMV on Xanthi tobacco	10
E	V06-CMV on Xanthi tobacco	12
F	T05-CMV on <i>N. benthamiana</i>	60
G	M05-CMV on cowpea	6
H	T05-CMV on cowpea	7
I	V06-CMV on cowpea	6
J	CMVB-837 on cowpea	9
K	CMVY-108 on cowpea	6
L	M05-CMV on <i>C. quinoa</i>	7
M	T05-CMV on <i>C. quinoa</i>	7
N	V06-CMV on <i>C. quinoa</i>	7
O	V06-CMV on melon	11
P	V06-CMV on zucchini	12
Q	M05-CMV on melon	10
R	CMVB-837 on pumpkin	8
S	M05-CMV on cucumber	12
T	CMVB-837 on zucchini	9
U	CMVB-837 on pepper	14
V	M05-CMV on pepper	22
W	M05-CMV on tomato	30

Fig. 2.2 Sucrose gradient showing *Brome mosaic virus* (BMV) and *Cucumber mosaic virus* (CMV) sedimentation bands



A



B

C



Fig. 2.3a. Dendrogram obtained from the alignment of CMV samples isolated in Virginia with selected sequences published in GenBank

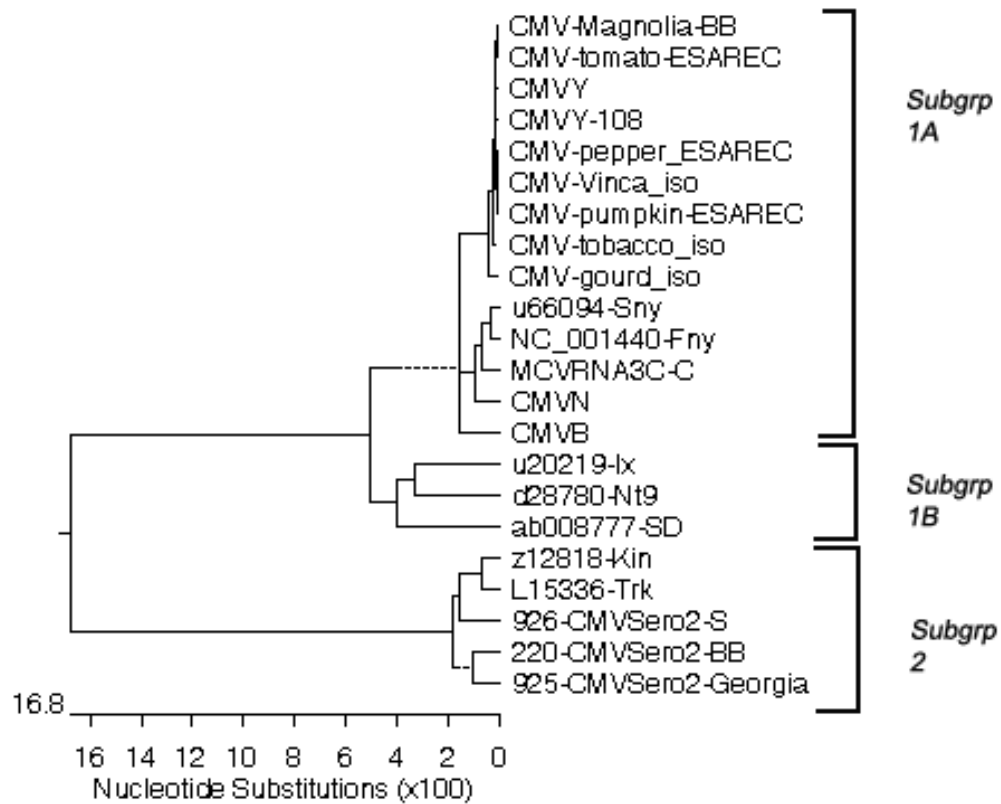


Fig. 2.3b Percent Identity and Divergence of CMV samples isolated in Virginia compared to sequences published in Genbank

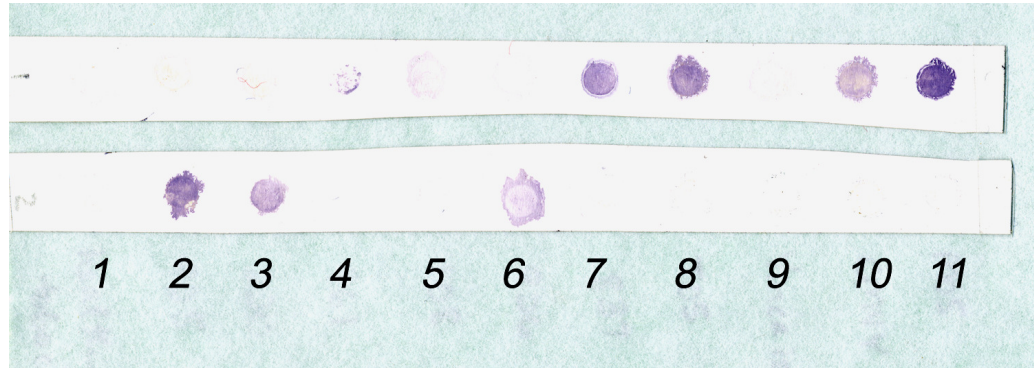
		Percent Identity																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
Divergence	1	■	98.8	70.3	70.9	70.0	71.1	71.7	71.5	97.4	96.9	97.5	72.9	72.5	73.4	73.4	72.5	73.0	73.2	72.9	72.7	77.0	72.8	1	z12818-Kin
	2	1.2	■	70.0	70.7	69.7	70.8	71.4	71.1	96.5	96.2	96.4	72.6	72.2	73.1	73.0	72.3	72.7	72.9	72.6	72.4	76.5	71.8	2	L15336-Trk
	3	38.1	38.7	■	88.8	89.2	88.9	93.7	91.7	72.9	72.9	72.8	89.1	88.4	89.6	89.4	88.5	89.1	89.3	88.9	89.2	91.1	87.9	3	u20219-Ix
	4	37.1	37.5	12.2	■	98.6	99.4	90.8	90.4	73.7	73.2	73.1	94.0	93.5	94.6	94.4	93.3	94.5	94.4	93.9	94.7	97.8	94.4	4	u66094-Sny
	5	38.5	39.1	11.9	1.4	■	98.7	91.6	91.1	73.8	73.2	73.2	94.1	93.6	94.7	94.5	93.5	94.6	94.5	94.0	94.8	97.4	93.8	5	MCVRNA3C-C
	6	36.9	37.3	12.1	0.6	1.3	■	91.1	90.6	74.1	73.5	73.5	94.4	93.8	95.0	94.7	93.7	94.8	94.7	94.3	95.0	97.9	94.4	6	NC_001440-Fny
	7	35.8	36.2	6.6	9.9	9.1	9.6	■	93.4	74.2	74.0	74.1	90.7	90.0	91.5	91.4	90.3	91.0	91.0	90.6	90.7	93.7	90.5	7	d28780-Nt9
	8	36.0	36.8	8.9	10.3	9.6	10.1	6.9	■	74.5	74.3	74.4	89.2	88.7	90.1	90.0	88.8	89.7	89.7	89.2	89.3	92.8	88.9	8	ab008777-SD
	9	2.6	3.6	34.2	32.8	32.7	32.2	32.0	31.4	■	96.9	97.8	72.9	72.1	72.7	72.7	72.1	72.3	72.9	72.7	73.1	76.6	72.4	9	926-CMVsero2-S
	10	3.2	3.9	33.9	33.6	33.5	33.0	32.2	31.7	3.1	■	97.9	72.5	71.7	72.1	72.4	71.7	71.9	72.6	72.2	72.5	77.3	71.8	10	220-CMVsero2-BB
	11	2.6	3.7	34.2	33.7	33.6	33.1	32.1	31.6	2.2	2.1	■	72.7	72.1	72.7	72.7	72.1	72.3	72.8	72.8	72.8	77.1	72.1	11	925-CMVsero2-Georgia
	12	31.2	31.8	9.5	3.8	3.7	3.4	7.9	9.5	32.0	32.0	32.1	■	97.8	98.4	98.1	98.1	98.4	98.1	98.4	97.8	95.7	94.0	12	CMVY-108
	13	31.2	31.8	9.8	3.9	3.8	3.5	8.1	9.7	32.7	32.8	32.3	0.2	■	98.0	98.3	98.3	98.1	98.0	98.1	97.3	95.7	93.9	13	CMV-Magnolia-BB
	14	31.0	31.5	9.3	3.5	3.4	3.1	7.4	8.8	32.4	32.9	32.4	0.1	0.5	■	99.0	98.3	98.7	98.9	98.1	98.1	95.7	94.0	14	CMV-pumpkin-ESAREC
	15	30.8	31.4	9.5	3.8	3.6	3.4	7.4	8.8	32.4	32.4	32.3	0.1	0.5	0.1	■	98.3	98.3	98.9	98.0	97.7	95.7	93.8	15	CMV-pepper-ESAREC
	16	31.5	31.8	9.8	4.1	4.0	3.8	8.1	9.6	32.8	32.9	32.5	0.2	0.0	0.4	0.4	■	98.2	98.2	98.3	97.5	95.7	94.2	16	CMV-tomato-ESAREC
	17	30.7	31.3	9.1	2.9	2.8	2.6	7.3	8.6	32.4	32.5	32.2	0.0	0.5	0.0	0.0	0.4	■	98.3	98.1	97.9	95.9	94.5	17	CMV-tobacco_iso
	18	31.2	31.7	9.5	3.6	3.5	3.2	7.7	9.1	32.3	32.4	32.5	0.1	0.4	0.1	0.0	0.4	0.0	■	98.1	97.6	95.7	93.9	18	CMV-Vinca_iso
	19	31.5	31.8	9.7	3.9	3.8	3.5	7.9	9.5	32.2	32.7	32.0	0.1	0.1	0.2	0.2	0.1	0.1	0.4	■	97.5	95.7	94.0	19	CMVY
	20	31.0	31.5	9.1	2.7	2.6	2.4	7.4	8.9	32.3	32.3	32.0	0.5	0.7	0.6	0.9	0.9	0.2	0.4	0.5	■	96.8	94.7	20	CMV-gourd_iso
	21	27.7	28.4	9.6	2.3	2.6	2.1	6.7	7.6	28.2	27.2	27.4	3.7	3.7	3.7	3.7	3.7	3.4	3.5	3.5	3.2	■	97.1	21	CMVB
	22	30.6	31.0	9.0	1.5	2.2	1.5	6.3	8.0	31.5	32.3	31.5	2.8	3.0	2.9	3.0	2.7	2.6	2.8	2.6	2.7	2.8	■	22	CMVN
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22			

Selected GenBank subgroup 1A sequences– MCVRNA3C-C; NC_001440-Fny; u66094-Sny

Reported subgroup 1B sequences from GenBank – u20219 – Ix; d28780-Nt9; ab008777-SD

Reported subgroup 2 sequences from GenBank – z12818-Kin; L15336-Trk

Fig. 2.4. Tissue blot immunoassay demonstrating specificity of *Cucumber mosaic virus* (CMV) monoclonal antibodies to subgroup 1 (top) and 2 (bottom)



Legend: 1 – Healthy tobacco, 2 – Lup-CMV (accession # 925), 3 – BBS-CMV (accession # 220), 4 – CMVN (accession # 961), 5 – CMVY (accession # 108), 6 – CMVS (accession # 926), 7 – CMVB (accession # 837), 8 – T05-CMV, 9 – V06-CMV, 10 – CMVY-CC, 11 – M05-CMV

CHAPTER III

First occurrence of *Turnip mosaic virus* in Virginia, United States

Turnip mosaic virus (TuMV) is a member of the genus *Potyvirus*, family *Potyviridae*.

The virus is transmitted by at least 89 species of aphids to over 318 plant species

(Edwardson and Christie, 1991), a majority of which belong to the family *Brassicaceae*

Cruciferae. During a study of new isolates of *Cucumber mosaic virus* (CMV) at the

Smithfield Plantation garden in Blacksburg, VA, several flowers of the invasive weed

Dame's Rocket (*Hesperis matronalis* L.) displayed bright, petal color-breaking

symptoms. Upon closer inspection, mosaic patterns were noticed on its leaves (Fig. 3.1).

Symptomatic leaves were collected from several plants and tested against antibodies to

CMV and TuMV. TuMV is the only plant virus reported naturally infecting Dame's

Rocket (Brunt et al., 1996). All samples tested positive for TuMV but negative for CMV

using tissue blot immunoassay (TBIA). To establish an isolate and determine host range

and symptomatology, the leaf tissue was ground in 0.01 M neutral phosphate buffer and

mechanically transferred to seven test plants. Symptoms observed correlated with those

reported in literature (Ford et al., 1988), and included systemic mosaic on turnip

(*Brassica rapa* L. subsp. *rapa*) cv. Green Seven Top (Fig. 3.2) and *Nicotiana*

benthamiana L.; necrotic lesions on *Chenopodium quinoa* and *Gomphrena globosa*; and

no symptoms on cucumber (*Cucumis, sativus* L.) cv. National Pickling, pepper

(*Capsicum frutescens* L.) or tomato (*Solanum lycopersicum* L.) cv. Rutgers. RNA was

obtained (as described in Chapter V) and reverse transcription-polymerase chain reaction

(RT-PCR) performed using TuMV coat protein-specific primers (Sanchez et al., 2003) with an expected size of 986 bp. The PCR products were cleaned using the Qiagen QIAquick PCR purification kit and sequenced at the Virginia Bioinformatics Institute at Virginia Tech. Sequence comparisons of TuMV isolated in Virginia to several isolates reported in Genbank revealed a 95-96% sequence identity to one Polish isolate CAR39, a 93-94% identity to US isolate USA1 and Kenyan isolate KEN1, and a 94% identity to Chinese and Canadian isolates CHN1 and Q-Ca. TuMV maintained in turnip plants was inoculated to healthy Dame's Rocket plants. Symptoms developed approximately 16 days post inoculation and, depending on age of the plants when inoculated, the mosaic patterns on the leaves varied. Younger plants had more severe mosaic and chlorotic leaves than older inoculated plants, which displayed mild mosaic patterns. In all inoculated plants, petal color-breaking symptoms were reproduced, similar to those on the naturally infected plants. These symptoms were compared to mock inoculated and healthy plants which had solid purple flowers only (Fig. 3.3). Leaves and petals from all plants were tested with TBIA, and TuMV detected from both mosaic leaves and striped petals. To our knowledge, this is the first report of *Turnip mosaic virus* in the Commonwealth of Virginia and the first report of an association of TuMV with petal breaking in *Hesperis*.

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Sanchez, F., Wang, X., Jenner, C. E., Walsh, J. A., and Ponz, F. 2003. Strains of *Turnip mosaic* potyvirus as defined by the molecular analysis of the coat protein gene of the virus. Virus Research 94:33-43.

Fig. 3.1. Mosaic (left) and petal color-breaking (right) symptoms on Dame's Rocket naturally infected by *Turnip mosaic virus*.



Fig. 3.2. Systemic mosaic symptoms on a turnip leaf inoculated with *Turnip mosaic virus*.



Fig. 3.3. Petal color-breaking symptoms on Dame's Rocket inoculated with *Turnip mosaic virus* (left) compared with a mock inoculated plant (right).



CHAPTER IV

Evaluation of Virus Diagnostic Capabilities of Collaborators in IPM CRSP Host Countries

Abstract

Plant virus diagnostics plays an integral part in the development and implementation of pest management strategies for the control of plant viruses. In this study, the diagnostic capabilities of several plant pathology collaborators within the Integrated Pest Management Collaborative Research Support Program (IPM CRSP) host countries and global theme projects were evaluated by survey on their capacity to perform virus diagnostics. Several criteria, including viral diagnostic capabilities, personnel, infrastructure, and funding, were reported through the use of a survey. Of the 14 surveys collected, 20 of which was disseminated, only ten reported performing virus diagnostics. Serology and biological assays using indicator hosts were the primary diagnostic approaches used for virus identification. Very few clinics were capable of performing molecular assays due in part to funding, availability of specialized equipment, and technical personnel. Clinics capable of performing virus diagnostics were either privately owned or academically affiliated. All government affiliated clinics, and one academically affiliated clinic, did not do molecular diagnostics of plant viruses despite some clinics having access to PCR machines and imagers. Clients of clinics participating in this study varied depending on the affiliation of the lab. Privately owned clinics received a majority of their samples from seed companies and other large corporate bodies. Academically

affiliated clinics also dealt with companies but also had samples from researchers and extension agents. Government affiliated clinics received a majority of their samples from large and small farmers and extension agents. Of the samples submitted, more than 50% of the clinics reported virus diseases on three main crops – tomatoes, peppers and potatoes. Clinics were also asked to rate themselves in diagnostic capabilities. Many clinics rated average to above average in the areas of infrastructure, funding, equipment, facilities and personnel knowledge in diagnostics. Only two clinics rated themselves as poor due to lack of equipment, trained personnel and proper, working infrastructure. These constraints, just some of many, were shared by several clinics. The information gathered in this survey can help diagnostic clinics in developing countries to make international programs, such as the IPM CRSP, aware of their deficiencies and to direct assistance where possible. This type of targeted effort could greatly increase the diagnostic capabilities of clinics through capacity-building programs for staff, and alternative procedures to extensive chemical controls, which can be extremely costly and environmentally hazardous.

Introduction

Plant viruses are one of the leading causes of plant diseases in the world. They cause a wide range of symptoms, including mosaics, mottling, stunting, and most importantly, reduction in crop yield. The development of integrated pest management strategies for agricultural pests has become increasingly important globally due to the current food shortage, and the increased dangers to health and the environment with the constant use of pesticides to control these pests. The IPM CRSP is a program funded by the U.S. Agency for International Development (USAID) to address the health, economic

and environmental issues through IPM interventions. The current phase of the IPM CRSP has specific thematic programs on plant disease diagnosis and on the assessment and management of viruses.

The most important step in developing IPM procedures for plant viruses is the proper identification of these viruses, and determining their distribution and alternate hosts located adjacent to crops of interest. Plant clinics and diagnostic laboratories (both terms will be referred to interchangeably within this chapter) play a crucial role in the identification and detection of plant viruses. Numerous techniques are currently in use for the detection of plant viruses, including serology with enzyme-linked immunosorbent assay (ELISA) (Clark and Adams, 1977), tissue blot immunoassay (TBIA) (Lin et al., 1990) and lateral flow devices, such as Immunostrips (Tsuda et. al., 1992), and nucleic acid-based procedures, including the polymerase chain reaction (PCR) (Saiki et. al., 1988), hybridization (Nikolaeva, 1995) and molecular arrays. In countries where the economy is primarily dependent upon agricultural production both for local consumption and export, the need for fully functional plant clinics and capable diagnosticians is of paramount importance (Lawrence et. al., 2005). As part of a study on the educational value and diagnostic capabilities of plant clinics in developing countries, Ausher et al. (1996) conducted a survey to evaluate plant clinics within developing countries. They reported that the capabilities of plant clinics varied depending upon several factors, including laboratory affiliations to international companies and institutions. The affiliated clinics had access to both moderately expensive and expensive equipment and, due to this association, had access to specialized personnel, several of whom also worked within the clinics. The highest educational levels of personnel working in these clinics, namely

doctorate and masters level, were uniformly represented within developing countries. Personnel had access to consistent ‘in-house training’ for increased capacity building and attendance at workshops and technical meetings on a regular basis. Despite several constraints including funding and inadequate facilities, Ausher et al. stated that many developed countries had shown marked improvement in diagnostic capacity and the acquisition of trained and qualified staff compared to that reported by Black (1993) and Black and Sweetmore (1995).

In this study the virus diagnostic capabilities of plant clinic collaborators within the IPM-CRSP project were evaluated based on several criteria, including access to standard and specialized equipment, personnel, virus and general diagnostics, and funding. The evaluation was performed by the dissemination of surveys to developing countries within the Caribbean, Latin America and Africa.

Methodology

Training

Before dissemination of the survey, a mandatory, on-line ethics course on “Relating to Human Subjects and Ethics” was taken by all developers of the survey. The ethics course was provided by the Virginia Tech Office of Research and Compliance (ORC) Institutional Review Board (IRB), <http://www.irb.vt.edu/>. The survey and the protocol were submitted to the chairman of IRB for review.

Survey

Information about the diagnostic capabilities of collaborating countries within the IPM-CRSP was obtained through a survey with an accompanying letter attached. The

information was subdivided into categories for easier comparisons between the developing host countries. The categories were as follows:

- i) Virus diagnostic capabilities: methods and approaches employed in the identification and detection of plant viruses. This section also included the use of diagnostic kits for plant viruses.
- ii) Clients: researchers, home owners and other interested parties who use the diagnostic facilities on a regular basis for virus diagnostics.
- iii) Crop submission: the types of crops frequently submitted to plant clinics for plant disease diagnosis, and the number of suspected virus-infected samples.
- iv) Self evaluation of respective clinics
- v) Personnel and capacity building: the number of trained and/or qualified personnel in virus diagnostics.
- vi) Funding and purchasing: the ability of the clinic to generate adequate funding for equipment and reagent purchase, maintenance, and payment of staff.
- vii) Overall constraints with performing viral and general diagnostics.

A copy of the survey is exhibited in Appendix D

Regions within developing countries were separated into 3 subgroups: The Caribbean, Latin America, and Africa. Areas within the survey that were not answered were considered either void or taken as a zero rating.

Results

Training

Upon passing the examination, a Certificate of Completion was awarded. A copy of the approval letter, granting exemption from further IRB review, is exhibited in Appendix E.

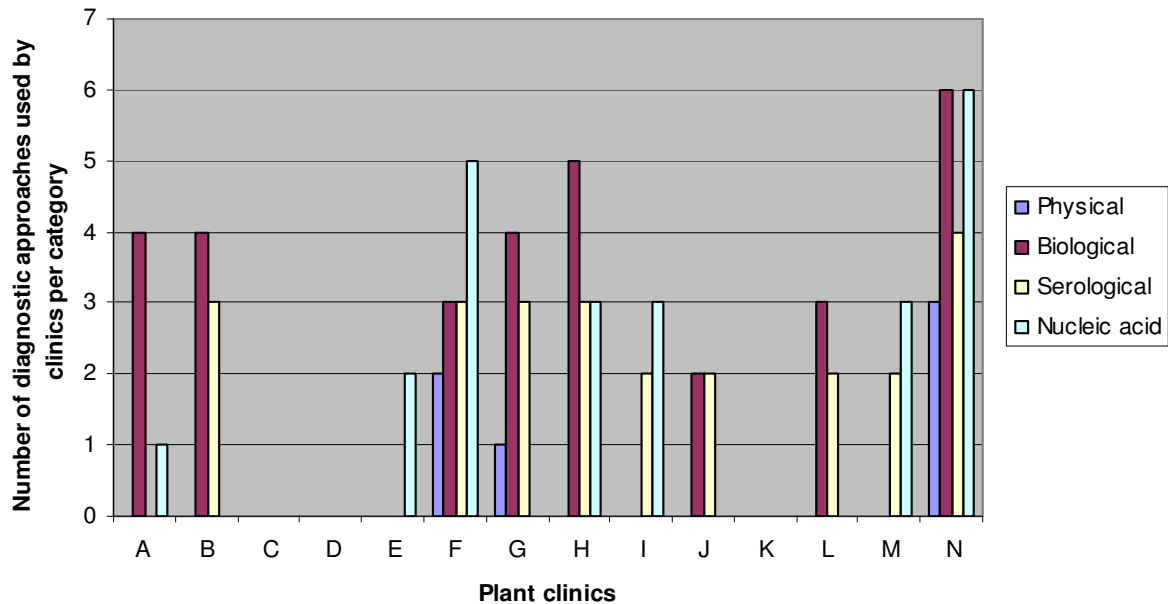
Survey

Of the twenty surveys disseminated, fourteen were returned and included representative clinics from each of the three regions: Latin America, the Caribbean, and Africa. The clinics were grouped and reported accordingly: A – F were clinics within East Africa, G – I were clinics within the Caribbean, and J – N were clinics within Latin America, specifically Central America. The clinics were laboratories with government or academic affiliations, or were privately owned by agricultural companies.

Virus diagnostic capabilities

Several methods can be applied for the identification and detection of plant viruses. These methods were categorized into four main groups: physical properties of virus particles, biological properties such as symptomatology on indicator host plants, serological approaches, and nucleic acid-based approaches. Fig 4.1 represents the total number of methods and/or approaches used in plant virus diagnostics by clinics within each region.

Fig. 4.1 Virus diagnostic approaches used by responding plant clinics



Of the fourteen clinics, four clinics – C, D, E and K, responded that they do not perform plant virus diagnostics as part of their routine diagnostic tests and, as such, did not complete this section of the questionnaire. Clinic D expressed an interest in performing viral diagnostics but did not have the capability. Clinic E indicated that they could perform PCR-real time and used PCR purification kits for product cleaning for outside sequencing. Of the remaining ten clinics, the most common diagnostic approaches were biological and serological. Among the biological approaches, mechanical transmission onto indicator hosts was the main method that was performed. Observation of symptoms helped to determine the general viral grouping, as well as demonstrating mechanical transmission of a pathogen.

Eight of the ten clinics also performed serological assays for confirmation of the biological assays. The clinic which did not use serology, clinic A, performed reverse

transcription-polymerase chain reaction (RT-PCR) as the confirmatory test. The primary serological test performed by developing countries was the enzyme-linked immunosorbent assay (ELISA), reagents for which were purchased as kits. Four clinics also used Immunostrips[®] purchased from Agdia[®], and another four clinics used tissue blot immunoassay. Only clinic A used RT-PCR for virus diagnosis. Clinic I used only serological and molecular assays.

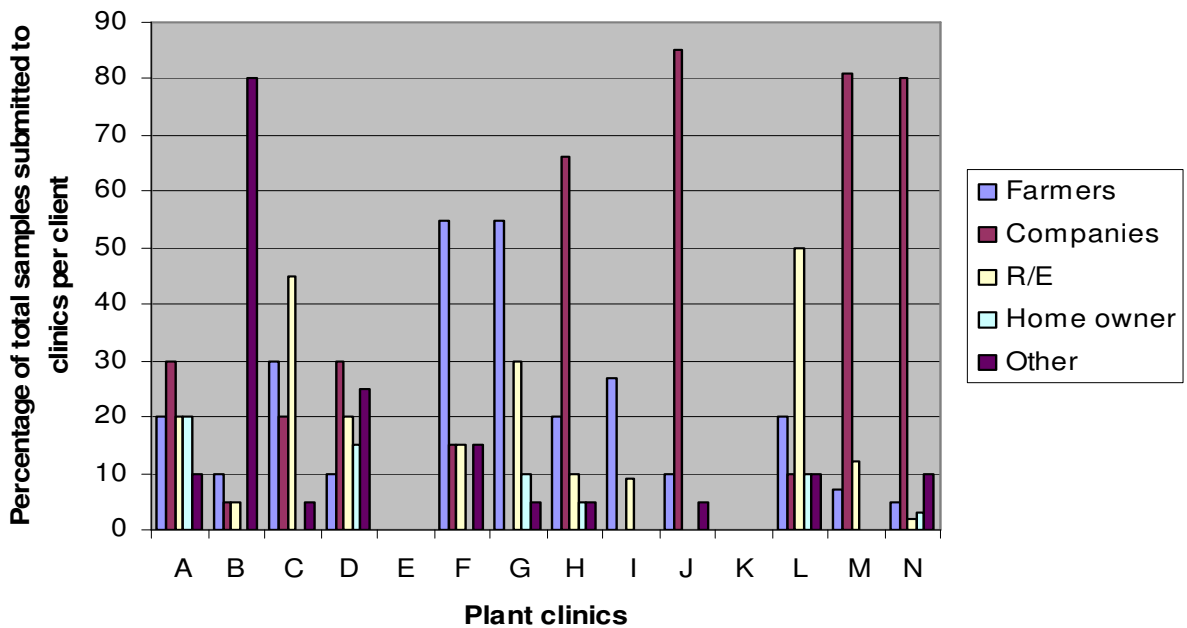
The capability to perform nucleic acid-based tests was variable across the developing countries, and was primarily confined to end point RT-PCR, gel electrophoresis, and observation of amplicons using a transilluminator and imager. Five of the clinics were capable of extracting nucleic acid, and those clinics (F, H, I, M and N) had fully functional laboratories with academic affiliations or were privately owned. The clinics that reported no nucleic acid extraction procedures were, with one exception, government affiliated.

Clients and crop submission

Users of the plant clinic services were divided into five categories: farmers, companies, researchers and extension officers (R/E), homeowners, and other, which covered consultants and anyone else that did not fit the profiles of the previous categories. The percentage of samples from each category that was received by each plant clinic is shown in Fig. 4.2. These percentages constitute the total number of diseased plants received by each clinic. The percentage of diseased plants diagnosed as viral-infected ranged from as low as 2% for clinic D, which had minimal to no equipment for virus diagnostics, to as high as 80% for clinic F, which was a fully functional diagnostic clinic. Of the fourteen clinics, only two, E and K, did not complete the 'Client and Crop

section'. Both of these labs are privately owned by commercial companies. Other privately owned clinics, J and M, receive more than 81% of their samples from companies, 16% and 25% of which were viral-infected, respectively. Clinics H and N also receive 60% and 80% of their samples from companies, with 50% and 56% viral-infected, respectively. Clinics F and G received approximately 55% and clinic I approximately 27% of the annually submitted samples from both small and commercial farmers, whereas clinic L received up to 50% of their samples from extension officers and fellow researchers.

Fig. 4.2 Percentage of samples received by each clinic



Of the many crops submitted, the most common crops with virus-like symptoms were tomato, pepper, maize, root crops, such as sweetpotato, Irish potato, and cassava, ornamentals, tree crops, citrus, banana, and pumpkin. Over 50% of the clinics reported

that tomato, potato and pepper were among the top five reported crops infected with a viral disease.

Self evaluation of diagnostic clinics

Plant clinics and diagnostic laboratories were asked to rate their labs on a scale of 1 (inadequate) to 5 (excellent) in the following categories: equipment, facilities, funding, infrastructure, knowledgeable staff in virus diagnostics, and reagent availability for diagnostics. The self evaluation ratings for each plant clinic are tabulated in Table 4.1.

Table 4.1 Self-ratings of plant clinics participating in the study

Plant clinics	Equipment	Facilities	Funding	Infrastructure	Knowledge	Reagent availability
A	2	1	1	2	1	1
B	3	3	4	3	3	4
C	3	2	1	3	1	1
D	1	1	3	1	1	1
E	3	3	4	4	3	3
F	4	3	3	5	4	3
G	3	3	1	3	4	3
H	4	4	3	3	3	3
I	4	3	3	4	4	3
J	2	3	4	3	3	2
K	2	NI	NI	3	NI	NI
L	5	5	5	3	3	2
M	4	3	3	4	3	1
N	4	3	2	4	3	2

NI – not indicated

The most frequent self-rating of clinics was 3 in infrastructure, knowledge and facilities. Two clinics, A and D, rated themselves inadequate (1) in all categories except equipment and infrastructure (A) and funding (D). Both clinics were from Africa and were government affiliated. Clinic A, which performs virus diagnostics, reported access to basic equipment such as microscopes, cameras and refrigerators, and adequate

greenhouse and land space. Clinic D, which does not do virus diagnostics, had no equipment and severely limited reference material. Research land space was the only asset in large supply. The remaining clinics were adequately outfitted with basic, moderately expensive and expensive equipment, regardless of whether they performed virus diagnosis as part of their diagnostics protocol. Equipment included different microscopes, pH meters, centrifuges, vortex/mixers, magnetic stirrers/hotplate, cameras and electrophoresis units with power supplies. Many respondents had adequate research land space, although less than 50% had fully functioning greenhouses. All clinics, with the exceptions of A and D, reported access to reference material, including computer access with internet capabilities.

Personnel and capacity building

Education levels and training of staff within the participating laboratories are summarized in Table 4.2. There appeared to be very little variation in the distribution of qualified staff among the different countries and regions. All clinics except D had at least one member of staff with a second degree, whether it was at the Master's or Doctorate level. Clinic D's highest levels were technicians, although there were a reported four certificates among the technicians.

The total number of years of experience varied both among diagnostics labs within the same region, and between different regions. In Africa, the total years in virus diagnostics ranged from as high as 12 years (clinic B), to no experience (clinic C). Region wide, clinics H, M and N had 28-35 years of experience, and clinic K had the greatest combined experience of 60 years. Only clinic D, which expressed a desire to do viral diagnostics, had staff with at least 1 year of experience in virus diagnostics.

Several clinics reported that outside speakers or presenters for training of staff in virus diagnostics had not been invited. One clinic commented that it was an issue of funding, as costs to conduct such a training course and support participants were high. All participating laboratories within Latin America (J – N) and a few in Africa (B, D, and F) have conducted workshops and invited outside speakers for capacity-building exercises for staff. The remaining clinics send staff for outside training.

Table 4.2 Education and total years of service of staff in participating plant clinics

Plant Clinics	Education of staff				Total yrs of experience		
	Total Doctorate /clinic	Total MSc/ clinic	Total BSc/ clinic	Other degrees	Virus diagnostics	General diagnostics	Virus training courses
A	2	1	0	1	6	> 35	0
B	0	1	0	1	12	9	6
C	0	1	1	1	0	45	2
D	0	0	0	4	1	3	0
E	NI	NI	NI	NI	NI	NI	NI
F	2	0	0	1	6	9	6
G	2	1	0	3	35	43	8
H	0	1	0	0	0	3	0
I	0	1	0	0	3	0	1
J	1	0	2	0	< 3	38	9
K	0	0	0	1	0	20	2
L	0	1	2	3	60	59	23
M	2	0	1	1	28	47	10
N	1	1	6	5	35	70	32

NI – not indicated

Funding and purchasing

Funding is a major requirement for the smooth day-to-day running of diagnostics and operations for laboratories and plant clinics. With funding, acquisition and maintenance of equipment, purchase of reagents, payment of staff, and research into the implementation of better IPM strategies for virus disease management can all be

accomplished. Of the fourteen clinics surveyed, all reported collaborations with other entities, including government and academic institutions, and private companies, that augmented their resources.

Funding is received through a number of routes (Table 4.3). For instance, all but three plant clinics, clinics A, I and J, receive an operational budget, whether on a yearly basis or periodically every few years. Similarly, all clinics except clinic D obtain a portion of their funding through their collaborations with institutional entities or companies. Several clinics also charge for their diagnostic services. All clinics that were academically affiliated, and several government and privately owned clinics, charged a service fee. Only four clinics – D, E, G and L, did not charge for their diagnostic services, and only four clinics – H, J, K, and L, were able to generate enough income for the daily operations of their clinics.

Table 4.3 Funding status as described by the participating plant clinics

Budget Category	No. responses	Yes	No	At times
Allocated an operating budget	14	7	3	4
Grant funding dependent	14	7	5	2
Funding via collaborations	14	10	1	3
Fee charged for diagnosis	14	8	4	2
Ability to maintain lab operations	13	4	9	0

Depending upon the reagents and chemicals needed, the items were acquired within the home country of the clinic. However, a majority of the responding clinics could obtain little or no reagents within their countries, and instead relied on purchases

from other countries, primarily the United States. Some clinics reported difficulties in purchasing equipment and reagents due to high costs and to importation fees. Clinics responding from the Latin America and Caribbean regions required intermediary companies for the purchase of reagents and chemicals from other countries. These intermediary companies also dealt with shipping and handling of these reagents between companies and clinics, usually at a high cost.

Constraints

One of the major constraints reported by many plant clinics was the lack of trained virologists and the lack of knowledge of the newer techniques for identifying and detecting plant viruses. Other constraints reported by clinics included: time required for importation of equipment and reagents purchased internationally, the high cost of diagnostic kits, reagents, equipment, the requirement for importation fees and taxes associated with international orders, and the cost of maintaining infrastructure. Other constraints reported were the lack of available literature on new viruses and new diagnostic procedures now being used, and the inability to attend useful diagnostic workshops.

Discussion

Plant viruses cause some of the world's leading plant diseases, resulting in billions of dollars lost annually, yet viruses are one of the most difficult-to-detect plant pathogens. Implementation of proper pest management strategies to control virus diseases is primarily dependent upon accurate identification of plant viruses. Plant clinics play a vital role in the detection and identification of plant viruses using a number of diagnostic

tools currently available. These tools can be categorized into four main groups: identification of plant viruses using physical properties, biological characterization using symptomatology on indicator hosts, serological detection using specific antigen-antibody recognition, and molecular characterization using virus nucleic acids.

Ausher et al. (1996) conducted a survey among developing countries to evaluate their diagnostic capabilities. The survey showed that, despite constraints experienced by these clinics, which included funding, infrastructure and specialization of personnel devoted purely to diagnostics, several clinics had made vast improvements in plant diagnostics since the report of Black and Sweetmore (1995). Today, with an increase in the use of molecular techniques in viral diagnostics, it was important to determine how developing countries performed. Unlike Ausher et al. (1996), our study concentrates primarily on the capabilities of several plant clinics within the IPM CRSP to perform viral diagnostics, and included equipment, personnel, and other items required for proper diagnosis. Of the twenty surveys disseminated, fourteen clinics responded, four of which did no virus diagnosis. One of these four clinics, clinic D was unable to perform virus diagnosis because of funding constraints. They reported that the government procurement team for the clinic had deemed that there was no urgency for virus diagnostics and thus had not allocated funds to increase the diagnostic capabilities of the clinic.

In our survey, the most common diagnostic approaches used were biological and serological assays (Fig. 4.1). Several clinics used combinations of both of these assays for the detection and identification of the plants viruses. Of the ten clinics, five clinics reported the capability to perform molecular analysis. These five clinics were, with one exception, either privately owned or academically affiliated. The remaining clinics, all

government affiliated, relied on serological techniques including ELISA, TBIA, and Immunostrips[®].

For virus detection and identification, specialized equipment is required to perform several assays, particularly with molecular analyses. Clinics that were privately owned or academically affiliated had well equipped laboratories, including expensive equipment such as electron microscopes, centrifuges, and PCR machines, and adequate facilities and infrastructure. This was in comparison to government affiliated clinics where only three out of the six had well equipped labs. The remaining three clinics had non-functioning greenhouses, poor infrastructure which affected the procurement of particular equipment, and little to no equipment available for basic diagnostics.

Clinic affiliation also affected the clients that use their services. Privately owned clinics processed samples primarily from seed companies and other corporations. Academically affiliated labs processed samples primarily from companies, researchers and some extension officers and government affiliated clinics worked almost exclusively with farmers and extension officers. The number of virus infected samples varied per year, but over 50% of the responding clinics stated that tomato, pepper and potato were the most submitted samples containing viruses.

Clinics were asked to self-rate their diagnostic laboratories in each of five categories (Table 4.1). The most frequent self ratings among the clinics was average (3) throughout most of the categories. Two clinics A and D, rated themselves inadequate (1) in almost every category, reporting several issues with the availability of equipment, reagents, trained personnel, reference material including computers, and funding for kits and other diagnostic tools required for virus detection. Again clinics privately owned or

academically affiliated rated themselves above average (4) to excellent (5) in several categories. Clinic L, although rating itself excellent in equipment, facilities and funding, fell short within infrastructure and knowledge. In examining the listing of available equipment, thermocyclers and imagers necessary for amplification of DNA, were not present. This was reflected in clinic L's ability to perform molecular diagnostics, resorting instead to biological and serological techniques for the identification and detection of plant viruses (Fig. 4.1).

Several clinics reported the lack of trained personnel in both virus identification, modern detection and molecular techniques. While there appeared to be very little variation in the distribution of qualified staff in general diagnostics (Table 4.2), for virus diagnostics there appeared to be a higher concentration of knowledgeable personnel within the Latin American region. Three of the five clinics in Latin America (clinics L – N) had at least 4 members of staff with upwards of 60 years of experience in virus diagnostics. This was in sharp contrast to clinics within the African region where staff was at a minimum of 2 years with at most 12 years of experience. Within the Caribbean, clinic G had up to six staff members with a total of 35 years of experience combined. These results can be explained by the amount of training and capacity building within these countries. Clinics L – N host several 'in-house' training and capacity building workshops on a yearly basis. Other clinics have also reported training for staff, but the number of years of virus experience could suggest that these workshops have been more geared towards general diagnostics. Among the clinics that do not host workshops, clinic G has the most experience in virus diagnostics. Several members of staff were actually trained as plant virologists.

From our survey, it seemed that government clinics fared the worst when it came to molecular diagnostics. Virus diagnostics was restricted to biological and serological techniques as many of these clinics either lacked equipment or the infrastructure to support such equipment. It is clinics like these that work directly with the farming community for which the IPM CRSP can make a large contribution to the economy and the nation of that country as a whole. Assistance in capacity building through training workshops for staff, and development of IPM strategies designed to increase the productivity of our farmers while reducing the cost of pesticides for the treatment and/or control of plant diseases.

Although the equipment or infrastructure is not present, these clinics can also make a contribution to the molecular detection and diversity of viruses through the use of simpler methods for virus identification and characterization. In the following chapters we present an alternative use of nitrocellulose membranes, the solid matrix used in TBIA. We focus on using this membrane to not only detect plant viruses with specific antibodies, but to also use this membrane as a source for viral RNA for molecular studies. Membranes would allow for the easy transport of samples to different countries for molecular analysis without any special storage conditions and without the possibility of introducing viruses into new locations. The ability to obtain sequence data for further studies would contribute significantly, not just to the scientific community, but by increasing the exposure of this laboratory through publications, attracting international institutions for collaborations and possibly source funding for a more self-sufficient clinic.

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CHAPTER V

Immunoassay, RT-PCR and direct sequencing of *Cucumber mosaic virus* and potyvirus coat proteins from the same NitroPure nitrocellulose membrane

Abstract

NitroPure nitrocellulose (NPN) membranes are an effective solid matrix for the detection and identification of plant viruses by tissue blot immunosorbent assay (TBIA). This study reports an additional use of nitrocellulose membranes as a source of viral RNA for three potyviruses, *Tobacco etch virus* (TEV), *Soybean mosaic virus* (SMV), and *Turnip mosaic virus* (TuMV), and two cucumoviruses, *Cucumber mosaic virus* (CMV) and *Peanut stunt virus* (PSV). Discs from dried membranes blotted with symptomatic tissue were minimally cleaned and placed directly into RT reactions with oligo-dTs or reverse primers to initiate cDNA synthesis. An aliquot used directly in PCR resulted in amplicons of the expected sizes for each virus only with its specific primers. The intensity of the PCR-amplified bands from cDNA transcribed from either non-processed and TBIA-processed nitrocellulose membranes compared favorably with those from FTA[®] Plant Cards. The quantity of amplification product, estimated from band intensity on gels, decreased over time in membranes stored for several months, but could be increased by increasing the number of PCR cycles. The PCR products were of high quality and could be sequenced directly. Thus, nitrocellulose membranes can be used to

collect and store samples that retain immunologically active virus particles, as well as viral RNA that can be amplified by RT-PCR, for more than a year at room temperature.

Introduction

Serological techniques, including enzyme-linked immunosorbent assay (ELISA) (Clark and Adams, 1977), lateral flow devices (Tsuda et. al., 1992), and tissue blot immunosorbent assay (TBIA) (Lin et al., 1990) are powerful tools for the detection and diagnosis of plant viruses. These techniques are based on an antigen-antibody binding reactions between epitopes on virus coat proteins and the binding sites of specific anti-virus antibodies (van Regenmortel, 1982). Although several variations of ELISA are used for plant viruses, all are performed in microtiter plates and require extraction of virus from fresh tissue and several hours to complete. Immunostrips[®] (Agdia, Inc.) are lateral flow devices that are quick and simple to use, but are costly when testing a large number of samples. In contrast, TBIA requires no tissue extraction, as plant leaves are pressed or blotted directly onto a nitrocellulose membrane without mechanical disruption of tissue. The method was first described for detection of several plant viruses by Lin et. al. (1990), and has since become more widely used as a sensitive and reliable method for plant virus detection (Comstock and Miller, 2004; Hsu and Lawson, 1991; Jonson et. al., 2007; Langham et al., 2007; McDonald et al., 2004). TBIA is less costly than immunostrips for high numbers of samples and can be completed in less time than ELISA. A distinct advantage of TBIA is that leaves can be blotted onto membranes directly in the field and processed at a later date (Makkouk and Comeau, 1994).

Nucleic acid-based approaches are also used extensively for detection and identification of plant viruses, particularly since the advent of the polymerase chain

reaction (PCR) (Saiki et. al. 1988). Plant viruses with DNA genomes can be amplified directly using generic or gene-specific primers for the region of amplification. Reverse transcription (RT) of plant viral RNA genomes to a complementary DNA (cDNA) template and amplification by cloning has been done since the early 1980's (Owens and Cress, 1980; Goulet, et al. 1982). Until recently, the source of viral RNA for cDNA synthesis and RT-PCR was from purified virus particles or total RNA. Burgoyne (1996) patented the use of FTA[®] Cards for the collection and storage of DNA to be used either directly or indirectly in PCR. The cards were then modified and distributed by Whatman[®] Inc. (Whatman, 2006) to produce FTA[®] Plant Cards. The matrix is a cotton-based cellulose fiber membrane on a supported backing, to which infected plant tissue is blotted. As claimed in US Patent No. 6645717 (Smith et. al. 2003), "the fibers are conditioned with chaotrophic and other agents which lyse cells, and release and immobilize the genetic material while inhibiting their degradation". These plant cards, and the methodology utilized with these cards, have proven useful for plant viruses (Ndunguru et. al., 2005; Roy and Nassuth, 2005) and plant gene expressions (Roy and Nassuth, 2005).

Because both FTA[®] Plant Cards and NitroPure nitrocellulose (NPN) membranes are solid matrices, and TBIA is used extensively in our laboratory, it was hypothesized that NPN could be used as a source of plant viral RNA for RT-PCR. Three potyviruses, *Tobacco etch virus* (TEV), *Turnip mosaic virus* (TuMV), and *Soybean mosaic virus* (SMV) (Genus *Potyvirus*, Family *Potyviridae*) and two cucumoviruses *Cucumber mosaic virus* (CMV) and *Peanut stunt virus* (PSV) (Genus *Cucumovirus*, Family *Bromoviridae*) were used to test this hypothesis. These genera are often found in mixed

infections in several plants including cucurbits (Pinto et. al., 2008; Wang et. al., 2002) and ornamentals (Arneodo et. al., 2005). Both genera are economically important and widespread. Even though all five viruses are spread by aphids, their host ranges vary from very narrow, with SMV affecting only soybeans, to very wide, with CMV affecting more than 1200 plant species (Palukaitis and Garcia-Arenal, 2003).

The objective of this study was to establish the versatility of NPN not only for binding proteins of plant viruses in TBIA, but also as a source of viral RNA for RT-PCR. The protocol required no mechanical tissue maceration, no elution of virus particles or RNA from the membranes, and yielded good sequence information for virus identification. Sequences obtained from PCR products enabled accurate separation of the five viruses into the expected taxa. NPN membranes were comparable to FTA[®] Plant Cards in efficiency and archival storage, and also provided a confirmation of immunological assays.

Materials and Methods

Virus cultures and maintenance

All viruses used, with the exception of TEV, were isolated in Montgomery County, VA. TEV was isolated in Scott County, VA, from burley tobacco and was maintained in tobacco (*Nicotiana tabacum* L. cv. Burley 21) or hot pepper (*Capsicum chinense* Jacq. cv. Scotch bonnet), seeds of which were obtained from Bodles Research Station of the Ministry of Agriculture and Lands, St. Catherine, Jamaica, West Indies. SMV, strain G1, was from soybean (*Glycine max* (L.) Merrill) cv. Lee and was maintained in either cultivar Essex or Lee 68 soybean (Hunst and Tolin, 1982). TuMV was isolated from Dame's Rocket (*Hesperis matronalis* L.) and maintained in turnip

(*Brassica rapa* L. subsp. *rapa*) cv. Green Seven Top (Ferry-Morse Seed Co., Fulton, KY) or *Nicotiana benthamiana* Domin. CMV was isolated from flowering tobacco (*Nicotiana* sp.) from the Historic Smithfield Plantation and maintained on *Nicotiana tabacum* L. cv. Xanthi. PSV was isolated from bean (*Phaseolus vulgaris* L.) cv. Bronco and maintained on bean. All virus cultures were maintained on greenhouse-grown plants by periodic transfer to new susceptible hosts by preparing sap in 0.01 M phosphate buffer, pH 7.0, and rubbing onto young plants dusted with silicon carbide powder, grit 600 (Buehler[®], Lake Bluff, IL). For long term storage, leaf pieces from infected plants were stored at -70° to -80° C or as desiccated tissue at 4° C.

Solid Matrices and Virus Deposition

Two paper matrices were tested. The nitrocellulose membrane was NitroPure, a supported nitrocellulose membrane, 0.45 μ pore size, (GE Osmonics Labstore, Minnetonka, MN). Pieces of desired sizes were cut and taped by the edge to index cards for support. A template with 6 mm diameter holes, made from the membrane's protective paper, was taped over the NPN membrane for positioning tissue blots in an array and for protecting non-blotted areas of the membrane. Leaf tissue from plants non-infected or infected with each of the viruses was rolled into a tight coil. The rolled leaf was torn in half, and the freshly torn edges were gently pressed onto the membrane surface until uniformly green (Fig. 5.1, panel A). The second matrix was the FTA[®] Plant Saver Card that uses patented Whatman[®] technology to collect, transport, store and purify plant DNA (Whatman, 2006). Leaf tissue was blotted to FTA[®] Cards in the same manner, except that no templates were used. After sample application, cards and membranes were allowed to air dry a minimum of 2 hours at room temperature (26 \pm 3°C) before continuing.

Tissue Blot Immunoassay

Virus identity and presence were confirmed by TBIA using a protocol modified from Srinivasan and Tolin (1992) and McDonald et al. (2004). The dried membranes were placed in 5% Triton X-100 for 10 min to remove plant debris and green color from the membranes. Each NPN membrane was rinsed for 3 min in potassium phosphate buffered saline (KPS) (0.02 M K_2HPO_4 , 0.15 M NaCl, pH 7.4) containing 0.05% Tween-20 (Lin et al., 1990), then blocked for 20 min with 5% non-fat dry milk (Nestle Carnation, Nestle USA Inc., Solon OH) and 0.5% bovine serum albumin (BSA) (Sigma-Aldrich[®] Inc., Missouri) in KPS. The membranes were then placed for 90 min into a combined primary antibody specific for each virus and a secondary enzyme-labeled anti-animal antibody, at dilutions of 1:10,000 in KPS for polyclonal antibodies, and 1:15,000 in KPS for monoclonal antibodies. After antibody incubation, membranes were rinsed for 10 min in Tris buffered saline (TBS) (0.05 M Tris base, 0.15 M NaCl, pH 7.6) containing 0.05% Tween-20, followed by two additional rinses each for 5 min. Finally, membranes were immersed in substrate, nitroblue tetrazolium (NBT) / 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Zymed Laboratories Inc., Invitrogen Corporation, Carlsbad CA), for 5 – 10 min until a purple precipitate was visible on positive samples, then rinsed thoroughly in de-ionized water and left to air dry. All procedures were conducted at room temperature with constant, gentle agitation using a MS1 Minishaker (IKA[®] Wilmington, NC) set at 200 rev/min. All chemicals were purchased from Fisher Scientific Inc., USA unless otherwise stated.

Antibodies for PSV, SMV, TuMV or TEV were polyclonal rabbit antiserum. Antisera to SMV and PSV were prepared in-house (Hunst and Tolin, 1982; Tolin and Boatman, 1972). Antisera to TEV and TuMV were from G. V. Gooding (Raleigh, NC)

and T. P. Pirone (Lexington, KY), respectively. The secondary antibody was goat anti-rabbit IgG (whole molecule) conjugated with alkaline phosphatase (GAR-AP), purchased from Sigma Chemical Co. (St. Louis, MO). Primary and secondary monoclonal antibodies for CMV were from Agdia[®] Inc. (Elkhart, IN), and were used at 1:15,000 dilution.

NPN membrane preparation for RNA

Discs of nitrocellulose were cut from plant cards or membranes, either before or after TBIA processing, with a 3 mm Harris Micro-Punch on a cutting mat (Whatman[®]). A single disc was placed in a 1.5 ml microcentrifuge tube and rinsed with 200 µl of either FTA[®] purification reagent (Whatman[®]) or 5% Triton X-100 in deionized water. Tubes were vortexed at 1,000 rpm for 5 sec and allowed to stand 5 min at room temperature. The reagent or detergent was then removed, and the treatment repeated two additional times. The disc was then rinsed twice with 200 µl TE buffer (10 mM Tris-HCl, 0.1 mM EDTA disodium salt, pH 8), and allowed to air dry for a minimum of 1 hr at room temperature.

The Micro-Punch was cleaned between samples by placing the core end of the punch in 10% household bleach (0.5% sodium hypochlorite) for 60 sec with gentle agitation. The punch was rinsed with de-ionized water for a further 60 sec and blotted dry, both inside and out of the core. This differed from the manufacturer's recommendation of cleaning in 90% ethanol and punching a blank card between samples.

RT-PCR and gel electrophoresis

The RT step was performed with the Reverse Transcription System kit (Promega Corporation, Madison WI). To a 200 µl PCR tube, the single sample disc was added to a

20µl reaction mix containing: 4 µl 25 mM MgCl₂, 2 µL 10X Reverse Transcription buffer, 2 µl 10 mM dNTP mix, 0.5 µl 40 U/µl RNAsin[®], 0.6 µl 25 U/µl AMV reverse transcriptase (high conc.), 1 µl 10 µM primer, and 9.9 µl nuclease-free water. The primers in RT were the reverse primers to the CMV and PSV coat protein genomes (Table 5.1), and oligo(dT)₁₅ to complement the polyA tail of the potyviruses. The RT protocol used for each virus is as follows: TEV => 42°C for 60 min, 95°C for 5 min and 4°C for 5 min; SMV and TuMV =>42°C for 30 min; PSV and CMV => 25°C for 10 min, 42°C for 15 min, 95°C for 5 min and 4°C for 5 min. The cDNA, still containing the disc, was stored at -20°C until required. Both RT and PCR were performed in a PCR Sprint Thermal Cycler (Thermo Electron Corporation, Bioscience Technologies, Milford MA).

The PCR step was performed with the GoTaq[®] DNA Polymerase kit (Promega) and cDNA from the RT step. To a 200 µl PCR tube, 2µl of each cDNA template were added to a 25µl reaction mix containing the following reagents: 2 µl 25 mM MgCl₂, 1 µl 10 mM dNTP, 5µl 10X Colorless Flexi GoTaq[®] Reaction Buffer, 0.25 µl *Taq* (*Thermus aquaticus* strain YT1) polymerase (Promega), 1 µl of 10 µM forward and reverse primers specific to each virus (Table 1) and 12.75 µl nuclease-free water. The protocol used for each virus is as follows: TEV => 95°C for 5 min, [95°C for 60 sec, 50°C for 60 sec, 72°C for 2 min] for 35 cycles, and 72°C for 15 min (modified from McLaughlin, unpublished); SMV and TuMV => 95°C for 4 min, [94°C for 20 sec, 47°C for 30 sec, 72°C for 60 sec] for 35 cycles and 72°C for 5 min (modified from Sanchez, et al., 2003); CMV => 95°C for 2 min, [94°C for 30 sec, 46°C for 60 sec, 72°C for 60 sec] for 35 cycles, and 72°C for 5 min (modified from Madhubala et al., 2005); and PSV => 95°C for 2.5 min, [94°C for 30 sec, 46°C for 45 sec, 72°C for 90 sec] for 30 cycles, and 72°C for 5 min (modified

from Naidu et al., 1995). The specificity of the primers was tested by conducting PCR with each cDNA and heterologous primers from the same virus genus. The PCR products were electrophoresed in 2% agarose gel in Tris-acetate-EDTA (TAE) buffer (0.04M Tris-acetate, 0.1 mM EDTA, pH 8.0), stained with ethidium bromide, and observed on a UV transilluminator (UVP Inc., San Gabriel CA) and AlphaInnotech Imager (San Leandro, CA). A low range exACTgene[®] DNA ladder was included in all gels. At least ten replications of RT-PCR were performed for each virus with both FTA[®] plant cards and NPN processed and non-processed by TBIA.,

Sequence data

The remaining PCR products were cleaned with either the QIAquick PCR purification kit (Qiagen, Valencia, CA) or with shrimp alkaline phosphatase/ exonuclease 1 (SAP/EXO1) (usb-Affymetrix, Cleveland, OH) and sequenced at the Virginia Bioinformatics Institute at Virginia Tech, Blacksburg VA, or the CRC-DNA Sequencing Facility at the University of Chicago (Chicago IL), respectively. These sequences were aligned with reported sequences from GenBank for each of the five viruses using the Cluster W multiple alignment program in MegAlign, Lasergene. PCR products were sequenced at least three times for each virus except for PSV which was not sequenced.

Longevity of virus and RNA on NPN membranes:

Longevity of virus on membranes was tested by infectivity assay on plants. SMV, CMV, and healthy soybean and tobacco plants (negative controls) were blotted onto NPN membranes 6, 5, 4, 2, 1 day and 2 hr before inoculation preparation. Six 6 mm circular blots were removed from the membrane and soaked in 1 ml 0.01 M sodium phosphate buffer, pH 7.0, for 30 min with gentle agitation every 10 min. Cotton tipped applicators

(Puritan, Hardwood Products Company, Guilford ME) were used to inoculate SMV to 9 soybean cv. Essex plants, and CMV to 6 tobacco var. Xanthi and 9 cowpea (*Vigna unguiculata* (L.) Walp. cv. Black-eye) plants for each time period. Negative and positive (fresh sap from infected leaves) controls for each virus were transferred to 2 plants each. Longevity of viral RNA on membranes, as detected by successful amplification of PCR products, was examined by sampling tissue blots from infected tissue at known intervals of storage at room temperature.

Results

Viral antigen detection on NPN membranes and FTA[®] Plant Cards

Several NPN membranes were blotted with each virus and processed against antibodies to each virus (Fig. 5.1, panels B-F). In all cases, the antibodies detected the appropriate, homologous virus on membrane strips, as indicated by the development of a purple precipitate. All heterologous antibody-virus combinations within the same genus, and non-infected controls, remained negative as no purple precipitate was observed (Fig. 5.1).

FTA[®] plant cards were also blotted and tested for viral antigen by TBIA. Cards blotted with TEV were cut into strips and processed using the standard procedure with antibody to TEV. The deposition of purple precipitate demonstrated that viral antigen was bound to the FTA[®] plant cards (Fig. 5.2). However, the location of the precipitate was not restricted to the areas originally blotted with virus infected tissue, but instead spread to non-blotted areas along the strip. The increased possibility of obtaining false positives negates the use of FTA[®] Plant Cards for possible use in serology. The localized

and highly intense purple precipitate on NPN membranes suggests a greater capacity for binding viral protein, presumably as intact virions.

Cleaning FTA[®] Plant Cards for viral RNA recovery

Following manufacturer's instructions, discs from Plant Cards were treated with Whatman FTA[®] purification reagent to remove plant debris, chlorophyll and any other PCR inhibitors, leaving clean nucleic acid for PCR and downstream applications. For the three host plants tested, soybean for SMV, pepper for TEV, and turnip for TuMV, green residues were nearly lacking in color with two rinses with the FTA[®] purification reagent if blots were processed within 1 week. Amplicons of the expected size 956 bp for TEV (Fig. 5.3A, lane 1), 904 bp for SMV (Fig. 5.3A, lane 3), and 986 for TuMV (Fig. 5.3A, lane 5) were observed. Older blots required additional rinse treatments to remove green color over blotted areas. Removal of all plant residue and green color, however, was not necessary for successful amplification (data not shown).

To determine if the FTA[®] purification reagent was necessary for cleaning discs from FTA[®] Plant Cards, it was substituted with 5% Triton X-100. This anionic detergent is used in the initial step of TBIA to remove interfering green color from the NPN membrane, and it was observed to remove green color from FTA[®] plant cards when they were used in TBIA. Amplicons from potyvirus cDNA obtained from FTA[®] plant card discs cleaned with Triton X-100 were quite similar in band intensity to those obtained from Reagent-cleaned discs (data not shown). As with the FTA[®] purification reagent, the number of Triton X-100 washings required to remove the green color depended on the age of the blot, but had no effect on RNA recovery (data not shown).

Since NPN membranes are presumed to bind intact virions, discs on which each of the RNA viruses and healthy tissue were blotted were prepared from NPN membranes. Discs from FTA[®] plant cards were similarly prepared. Each disc was cleaned as above with FTA[®] purification reagent then used in RT-PCR. All five viral RNAs were successfully transcribed, synthesized, and amplified, producing the expected size amplicons (Fig. 5.3A). The band intensities from RT-PCR products from several different discs were quite similar with either FTA[®] Plant Cards or non-processed NPN membranes as sources of RNA for TEV (Fig. 5.3A, lanes 1 and 2), SMV (Fig. 5.3A, lanes 3 and 4), TuMV (Fig. 5.3A, lanes 5 and 6). Similar results were achieved with CMV and PSV (data not shown).

Because Triton X-100 was a successful alternative to the FTA[®] purification reagent for plant cards, this reagent was tested for the cleaning of NPN membrane discs for RT. The Triton X-100 rinses visibly removed more green color and plant residues from membrane discs in the same number of washings as did the reagent. Amplicon bands from potyvirus cDNA obtained from NPN membrane discs cleaned with Triton X-100 (Fig. 5.3B lanes 2, 4, 6) were similar in intensity to those from FTA[®] purification reagent-cleaned discs (Figure 5.3B, lanes 1, 3, 5). Results were similar with CMV and PSV for Triton X-100 cleaned NPN membranes (Fig. 5.3E, lanes 1 and 5).

To determine if the washing step with the FTA[®] purification reagent was necessary, 3 mm discs were removed from both FTA[®] plant cards and non-processed NPN membranes and added directly into the RT mix. Amplification within the expected size range was observed from discs removed from the membrane but not from the plant card, suggesting that the chemicals reportedly impregnating the cellulose fibers of the

plant card (Martin et. al., 2003) inhibit the transcription of cDNA, and must first be removed before RT.

TBIA-processed NPN membranes as sources of RNA

To determine whether TBIA processing interfered with RT-PCR from NPN membranes, the amplification was repeated using discs from TBIA-processed NPN membranes. The expected sized amplicons of all three potyviruses were observed on the agarose gels with little difference in band intensity (data not shown). Band intensities of CMV and PSV amplicons were greater from non-processed NPN membranes (Fig. 3E, lanes 1 and 3) than from TBIA-processed NPN membranes (Fig. 3E, lanes 2 and 6). A minimum of five replications were done for each virus. In all replications, the negative controls were discs removed from blots of virus-free plant tissue on plant cards and NPN membranes. These negative controls produced no amplicons when subjected to RT-PCR using each set of virus primers (Fig. 5.3A-C, lane 7; Fig. 5.3E, lane 9).

The intensity of the bands from processed membranes was slightly lower than from unprocessed membranes for the cucumoviruses, suggesting that some virus particles may have been removed or dissociated during TBIA and/or during the cleaning of the discs for RT, resulting in fewer particles and less RNA template for the RT reaction. To test whether reduction in template affected amplicon intensity, three different size discs were removed from a NPN membrane blotted with CMV-infected leaf tissue, then cleaned and subjected to RT-PCR. Results shown in Figure 5.4 indicate little to no difference in the band intensities for the 3 mm, 2 mm or 1.25 mm diameter discs (Fig. 5.4, lanes 1, 2 and 3, respectively). This suggested that the amount of transcribed cDNA does not depend solely on the amount of RNA available.

To demonstrate that samples blotted onto membranes processed against heterologous antibodies could still be used as sources of RNA without interference, discs were removed from samples testing negative against heterologous antibodies (no purple precipitate). The discs were subjected to the same cleaning procedure, followed by RT-PCR with homologous primers. Amplicons of the expected sizes were generated for each virus (Fig 5.3C and Fig. 5.3E, lanes 3 and 7) and there was little difference in band intensities when compared to blots processed using homologous antibodies in TBIA.

To determine if the rinsing steps with the FTA[®] purification reagent or Triton X-100 were necessary after TBIA, several discs from processed membranes were removed and the number of washing steps varied, from 0 to 3. The intensity of the PCR products amplified from membranes washed only in TE buffer were low, whereas the intensity of the amplicon bands was higher for two and three washes with the FTA[®] purification reagent or Triton X-100 (data not shown). Based on these results, discs from membranes processed by TBIA were rinsed in the Reagent or Triton X-100 not less than twice, while discs from Plant Cards and membranes not processed by TBIA were rinsed 3 to 4 times, depending on the age of the blot.

Specificity of primers

The sequence diversity of viruses, particularly within a genus, is often in the coat protein genome. In many analyses, primers are often designed to amplify the coat protein. To confirm the specificity of the five sets of primers used in the current study, cDNA from membrane discs processed by TBIA was used. The RT step, as before, used polydT for the three potyviruses, and specific reverse primers for CMV and PSV. Within each genus, cDNA was paired with heterologous primers in the PCR reaction. The PCR

protocols corresponded to the respective primer sets used (Table 5.2). Homologous primers paired with cDNA were the positive controls, and discs from membranes blotted with healthy plants paired with each primer set were negative controls. Amplicons within the expected size range were observed for all positive controls (Fig. 5.3D, lanes 1, 4 and 7 for TuMV, SMV and TEV, respectively (Fig. 5.3E, lanes 2 and 6 for CMV and PSV, respectively). No amplification was observed in the negative controls (Fig. 5.3E, lane 9 represents the various negative controls). In all cases, the heterologous primers did not amplify the cDNA in PCR, demonstrating the specificity of the primers within each genus (Fig. 5.3D, lanes 2, 3, 5, 6, 8, 9; Fig. 5.3E, lanes 4, 8). Primers were not tested across the different genera.

Infectivity of viruses on NPN membranes

Tobacco and soybean infected with CMV and SMV, respectively, and corresponding healthy tissue were blotted to NPN membranes on 6 consecutive days (except T=3 days), and at 2 hr pre-inoculation. Tobacco plants inoculated with 6 CMV blots extracted in phosphate buffer showed no symptoms for any storage time period used in the experiment. TBIA of the leaves from the tobacco plants also gave negative results for CMV. Soybean plants inoculated with SMV blots extracted in 0.01 M phosphate buffer displayed characteristic symptoms of SMV, including mosaic and slight stunting, at the storage times of 2 hr, 1 and 2 days pre-inoculation. The number of plants showing symptoms was 7/9 for time=2 hr, 6/9 for time=1 day, and 6/9 for time=2 days. No symptoms developed on soybean plants inoculated with blots stored for 4, 5 or 6 days. Presence or absence of SMV was confirmed by TBIA. The soybean and tobacco plants

inoculated with blots from healthy tissue showed no symptoms and were negative by TBIA. None of the other viruses used in this research were tested.

Micro-punch cleaning and maintenance

The recommended cleaning procedure by Whatman[®] Inc. (2006) is to swirl the core of the Punch in 70% ethanol, blot dry, and punch blank discs between samples. Unfortunately, all blank discs removed by this method had sufficient RNA to be amplified and produce PCR products, even if two blank discs were taken in succession. Different methods were explored to clean the core including the use of full strength (100%) ethanol, 10% household bleach (0.5% sodium hypochlorite) and RNase AWAY (Molecular BioProducts Inc., San Diego, CA) with treatment times of 30 sec, 45 sec, and 60 sec, before and after a blank disc. Ethanol, even at full strength, did not degrade RNA left on the core of the Punch, resulting in the amplification of PCR products from blank discs. With 10% bleach and RNase AWAY no amplification from carry-over RNA was observed, regardless of time and without punching a blank disc,. The reproducibility of these results led to the modification of the cleaning procedure to use bleach, which is more cost-effective than RNase AWAY.

Sequences

The sequences obtained from cleaned PCR products aligned each virus with the two or three representatives from each species or genus reported in GenBank (Fig. 5.5). CMV had a 94.7-95% sequence identity to U.S. CMV strain Fny, TEV had a 95.6% identity to U.S. TEV strain TEV-GEN, SMV had a 96% identity to U.S. SMV strain G7, and TuMV had a 93% identity to U.S. TuMV strain USA1. No attempt was made to obtain sequences for the PSV isolate.

Discussion

The objective of this study was to develop a method for the rapid immuno-detection and molecular characterization by RT-PCR of plant viruses using a single solid matrix, NitroPure nitrocellulose membranes. The development of paper-based technologies has greatly improved our ability to identify, detect and characterize plant viruses and their genomes without the use of nucleic acid extraction and virus purification methods. It has been demonstrated that Whatman FTA[®] Plant Cards were a suitable matrix as sources of nucleic acids for five RNA viruses: three potyviruses, TEV, SMV and TuMV, and two cucumoviruses, CMV and PSV. Viral cDNA was generated from RNA assumed to be encapsidated in virus particles bound to the FTA[®] plant cards. Earlier reports of FTA[®] plant cards focused on detection of the DNA of geminiviruses, including *Cassava mosaic virus*, *Maize streak virus*, and *Tomato yellow leaf curl virus* (Ndunguru et. al., 2005), of plant genes (Drescher and Graner, 2002; Karle et al. 2004), and plant gene expression (Roy and Nassuth, 2005).

The use of FTA[®] Plant Cards with RNA viruses has not been as extensively studied. Ndunguru et. al. (2005) used *Tobacco mosaic virus*, TEV, and *Potato virus Y* and demonstrated the effective retrieval of viral sequences through RT-PCR from RNA eluted from FTA[®] Cards. Rogers and Burgoyne (2000), with *Coxsackievirus B4* (CVB-4), added cleaned discs directly into RT reaction mixes (or one-step RT-PCR reaction mixes) instead of concentrating RNA eluted from several discs. The direct amplification of RNA by RT-PCR was further demonstrated with various plant viruses and plant genes (Nischwitz et al., 2007; Roy and Nassuth, 2005) from tissue blotted onto FTA[®] plant cards.

In this study NPN membranes were demonstrated to be an excellent source of plant viral RNA. Tissue blot immunoassays demonstrate that virus particles from leaf tissue bind to membranes. In addition, NPN is known to bind nucleic acids as well as protein (GE Osmonics, Minnetonka, MN). Logic dictated that viral RNA is present and might be accessible as a template for RT-PCR. This hypothesis was tested by removing a 3 mm disc from NPN membranes blotted with symptomatic leaves and subjected to FTA[®] purification protocols, before placing the disc in RT. Transcription of cDNA was demonstrated by PCR amplification of TEV, SMV, TuMV, CMV and PSV. All PCR amplicons produced from NPN membranes were of the expected size range, and were of similar band intensity compared to RNA obtained from FTA[®] Plant Cards. There was little difference in the intensity of the bands from PCR products obtained from NPN before or after processing by TBIA (Fig. 5.3). The amplification of cDNA obtained from both processed and non-processed NPN membranes were somewhat of a surprise since US Patent No. 6645717 (Smith et. al., 2003) claimed that the FTA[®] plant card's cellulose fibers were "conditioned with chemicals that lysed cells and released RNA, while at the same time immobilizing and preventing degradation of the exposed RNA". NPN lacked these chemicals, but amplification was nevertheless observed. One possible explanation could be the exposure to higher temperatures during reverse transcription, which could destabilize virus particles, exposing RNA for primer recognition. Cucumovirus and potyvirus particles are known to be temperature sensitive, losing infectivity after treatment in sap for 10 min at 55-70° and 50-55°C respectively (Palukaitis and Garcia-Arenal, 2003; Shukla et. al., 1994). We suggest that, at temperatures of 42°C and higher for a minimum of 30 min, virions are partially dissociated, exposing RNA to the primers

and reverse transcriptase in the RT reaction mix, and initiating cDNA transcription. Alternatively, non-encapsidated RNA may have been bound to the NPN. The sequences obtained from cleaned PCR products were comparable regardless of which solid matrix was used as the original source of RNA (data not shown). The viruses were easily assigned to their respective species based on these sequences.

Triton X-100 was demonstrated to be an alternative to the FTA[®] purification reagent for cleaning plant cards. A non-ionic detergent, Triton X-100 (Octylphenolpoly(ethyleneglycolether)x, is commonly used to solubilize membrane proteins. It apparently does not affect viral coat proteins because there are reports of its use in plant virus purification (Lot et al., 1972; Han et al., 1999) and in reducing background in dot-blot immunoassays (Abdel-Salam, 1999). Apart from removal of more green color by Triton X-100 than by the FTA[®] purification reagent, there was no difference in band intensity using cDNA from discs treated with either solution. However, the intensity of bands decreased markedly with discs not cleaned with either reagent.

NPN membranes have the advantage of being a good matrix for immuno-detection of several plant viruses and now, as a source of RNA for sequence analysis, molecular characterization and further downstream applications. The similarities between FTA[®] plant cards and membranes prompted the investigation into whether FTA[®] plant cards could be used for immuno-detection. With TBIA, infected tissue blotted onto the plant cards formed a purple precipitate indicative of a positive reaction, demonstrating that the plant card binds whole virions. However, unlike NPN, the purple precipitate was also observed in regions beyond the blot site, suggesting movement of the virus particles on the cards. One possibility is that cellulose fibers, to which the virions had bound, were

dislodged during TBIA processing. Deposition of these fibers, and thus the virions, to other regions on the cards resulted in false positives in non-blotted regions.

Since the results indicated that adequate amounts of RNA are preserved on NPN after TBIA, the virus particle integrity, and hence encapsidated RNA, after processing with non-matching antibodies was tested. Discs were removed from blots on NPN testing negative by TBIA, cleaned, and subjected to RT-PCR using homologous primers. Amplicons within the expected size ranges were obtained for each virus with little observed difference between the intensities of the bands for the different viruses (Fig. 5.3C). The availability of virus particles on discs affecting band intensity was investigated using three different size discs: 3 mm, 2 mm and 1.25 mm diameter. All three sizes gave similar band intensities (Fig. 5.5) suggesting the number of virus particles is not the sole determinant in the amount of cDNA produced during RT. Unfortunately, virus particles cannot be measured quantitatively on membranes to confirm these findings.

The primers used to amplify the coat protein genome were from published papers or were designed from sequences in GenBank . To demonstrate the specificity of the primers within each virus species, cDNA from each virus was added to PCR reaction mixes with pairs of primers designed for different viruses. Amplification of cDNA was only observed using primers designed on the same virus. Not only did this demonstrate the specificity of the primers, but it also reinforced the cleaning procedure of the MicroPunch in eliminating contamination between samples.

One of the main attributes of TBIA is the ability to store plant viruses on membranes over long periods of time at room temperature. Once infected tissue is blotted

and dried, the viruses remain immunologically active for more than 1 year. Biological activity of plant viruses on the membrane depended on the stability of the virus. CMV was quickly inactivated, losing infectivity after 2 hr dried on the membrane, while SMV, which is more stable in sap than CMV (Palukaitis and Garcia-Arenal, 2003), was inactivated after 4 days on NPN. CMV and SMV, as well as TuMV, PSV, and TEV, could still be detected by TBIA after 10 months. Demonstration of inactivation of virus on membranes confirms the lack or risk of pathogen introduction during transport of samples between different parts of the world. Since the virus remained immunologically active, the integrity of its RNA after long periods of storage was also tested. Amplification of cDNA was obtained from membranes blotted with CMV and TEV up to 15 months, either processed by TBIA or not, has been observed (Chapter VI). The PCR products also yielded good sequences which were used for molecular studies.

NPN membranes offer a quick and simple way to store plant viruses long term for serological and molecular diagnostics without the need for storage of leaf tissue. The membranes can be easily transported over long distances without the risk of new pathogen introduction in new locations, but yet remain immunologically active for several months. Here, we have shown an additional use for NPN as a source of plant viral RNA for direct addition to RT-PCR. The membranes can be used either before or after TBIA processing, and in both cases give quality PCR products suitable for molecular analysis without costly or extensive RNA extraction methods.

In the following chapter, results of testing use of NPN on a global scale for the detection and subgroup classification of CMV within the Integrated Pest Management Collaborative Research Support Program host countries will be demonstrated. This was

done by disseminating sample cards containing NitroPure nitrocellulose membranes to collaborators. Once the global application of NPN membranes has been established, it will allow for a greater generation of molecular data from countries lacking molecular capabilities, as samples can be collected on membranes and shipped to diagnostic laboratories equipped for collecting molecular data and bioinformatics.

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<http://www.whatman.com/FTANucleicAcidCollectionStorageandPurification.aspx>

Table 5.1. Primers used for each virus in the polymerase chain reaction procedure and the expected amplicon size.

Virus	Primer	Primer sequence 5' – 3'	Product size (bp)	Reference
TEV	PfTEVCP8429	TCAGCACGGAACAAACTCTG	956	McLaughlin, (unpublished)
	PrTEVCP9385	CAAGTCCCACTGTGAATAGC		
SMV	PfSMVCP8475	AGATATCTTGAAGTGCTGGATTT	904	Qusus et al., 1995
	PrSMVCP9379	ATAAAGCGACCCGAAATGAT		
TuMV	PfTuMVCP8705	CAAGCAATCTTTGAGGATTATG	986	Sanchez et al., 2003
	PrTuMVCP9690	TATTTCCCATAAGCGAGAATAC		
CMV	PfCMVRNA31163	ATGCTTCTCCRCGAGATT	871	This paper
	PrCMVRNA32034	GTAAGCTGGATGGACAAC		
PSV	PfPSVRNA31178	AGRATGCTTATCCYGAAC	876	This paper
	PrPSVRNA32037	TTAGCCGAWAGCTGGATG		

Fig. 5.1 NitroPure nitrocellulose membranes with five viruses (*Turnip mosaic virus* (TuMV), *Tobacco etch virus* (TEV), *Soybean mosaic virus* (SMV), *Cucumber mosaic virus* (CMV), and *Peanut stunt virus* (PSV)). Panels A-D are NPN membranes blotted with known infected leaf tissue (lanes 1=TuMV; 2=TEV; 3=SMV) or healthy tissue (column 4) onto membranes. Panel A, is before TBIA. Panels B – D, after TBIA with the matching antibody, showing a positive purple precipitate. Panels E-F are blotted with healthy tissue (lane 1, 3), (PSV; lane 2); and *Cucumber mosaic virus* (CMV; lane 4) processed with CMV (panel E) or PSV (panel F) antibodies.

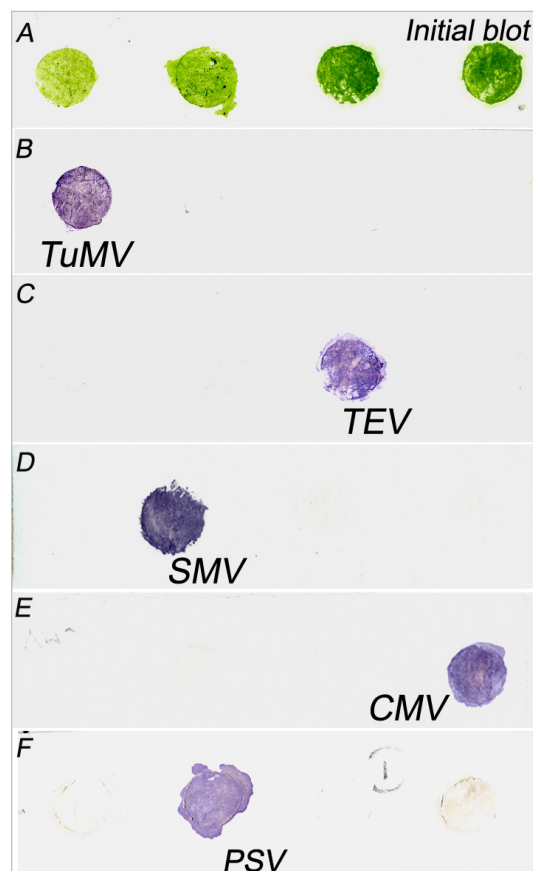


Fig. 5.2 Comparison of NPN membranes (A) and FTA[®] Plant Cards (B) in tissue blot immunosorbent assay processed with antibody to *Tobacco etch virus* (TEV). Blots on the NPN membrane were (from top to bottom): TEV, *Soybean mosaic virus*, *Cucumber mosaic virus*, and TEV. Blots on Plant Card were both TEV.

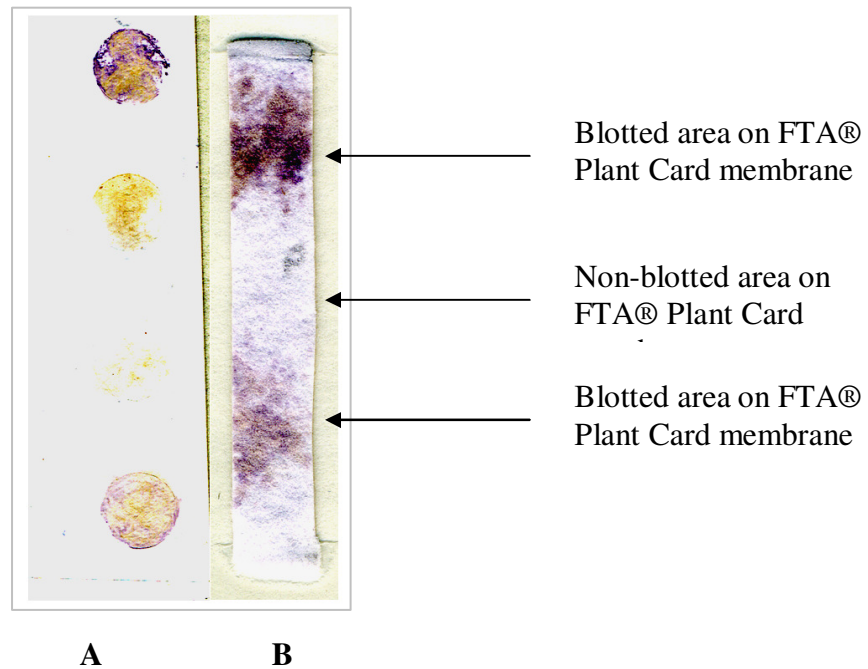


Fig. 5.3 PCR products of *Tobacco etch virus* (TEV), *Soybean mosaic virus* (SMV) and *Turnip mosaic virus* (TuMV) transcribed from cDNA, and viewed on an ethidium bromide stained 2% agarose gel. The initial lane contains the low range exACTgene® DNA ladder. All amplicons fell within the 800 and 1000 bp ladder range as indicated by the arrows.

A is the comparison of discs removed from FTA® Plant Cards and membranes used as sources of RNA for cDNA amplification by RT-PCR: a) TEV amplified from RNA bound to discs from Plant Card (lane 1) and membrane (lane 2), b) SMV amplified from Plant Card (lane 3) and membrane (lane 4), c) TuMV amplified from Plant Card (lane 5) and membrane (lane 6) and d) Healthy control (lane 7). **B** is the comparison between the FTA® purification reagent and Triton X-100 in the disc cleaning procedure using TBIA processed membranes: a) TEV amplified from discs treated with FTA® Reagent (lane 1) and 5% Triton X-100 (lane 2), b) SMV amplified from discs treated with FTA® Reagent (lane 3) and 5% Triton X-100 (lane 4), c) TuMV amplified from discs treated with FTA® Reagent (lane 5) and 5% Triton X-100 (lane 6) and d) Healthy control (lane 7). **C** shows the amplification of cDNA from RNA on discs obtained from membranes processed with heterologous antibodies: a) TEV amplified from RNA on discs processed against TuMV (lane 1) and SMV (lane 2) antibodies, b) SMV amplified from RNA on discs processed against TuMV (lane 3) and TEV (lane 4) antibodies, c) TuMV amplified from RNA on discs processed against SMV (lane 5) and TEV (lane 6) antibodies, d) Healthy control (lane 7). **D** shows the specificity of the potyvirus primers within the *Potyvirus* genus: a) TuMV cDNA with homologous TuMV primers (lane 1), b) SMV cDNA with heterologous

TuMV primers (lane 2), c) TEV cDNA with heterologous TuMV primers (lane 3), d) SMV cDNA with homologous SMV primers (lane 4), e) TuMV cDNA with heterologous SMV primers (lane 5), f) TEV cDNA with heterologous SMV primers (lane 6), g) TEV cDNA with homologous TEV primers (lane 7), h) TuMV cDNA with heterologous TEV primers (lane 8), i) SMV cDNA with heterologous TEV primers (lane 9). **E** summarizes the amplification of the two cucumoviruses on NitroPure nitrocellulose membranes: a) CMV amplified from RNA on unprocessed membranes (lane 1), and membranes processed with CMV (lane 2) and PSV (lane 3) antibodies, b) CMV cDNA with heterologous PSV primers (lane 4), c) PSV amplified from RNA on unprocessed membranes (lane 5), and membranes processed with PSV (lane 6) and CMV (lane 7) antibodies, d) PSV cDNA with heterologous CMV primers (lane 8), e) Healthy control. The healthy control throughout these experiments represents healthy tissue blotted to membranes and used in various RT-PCR reactions with all conditions and primers mentioned above.

Fig. 5.3

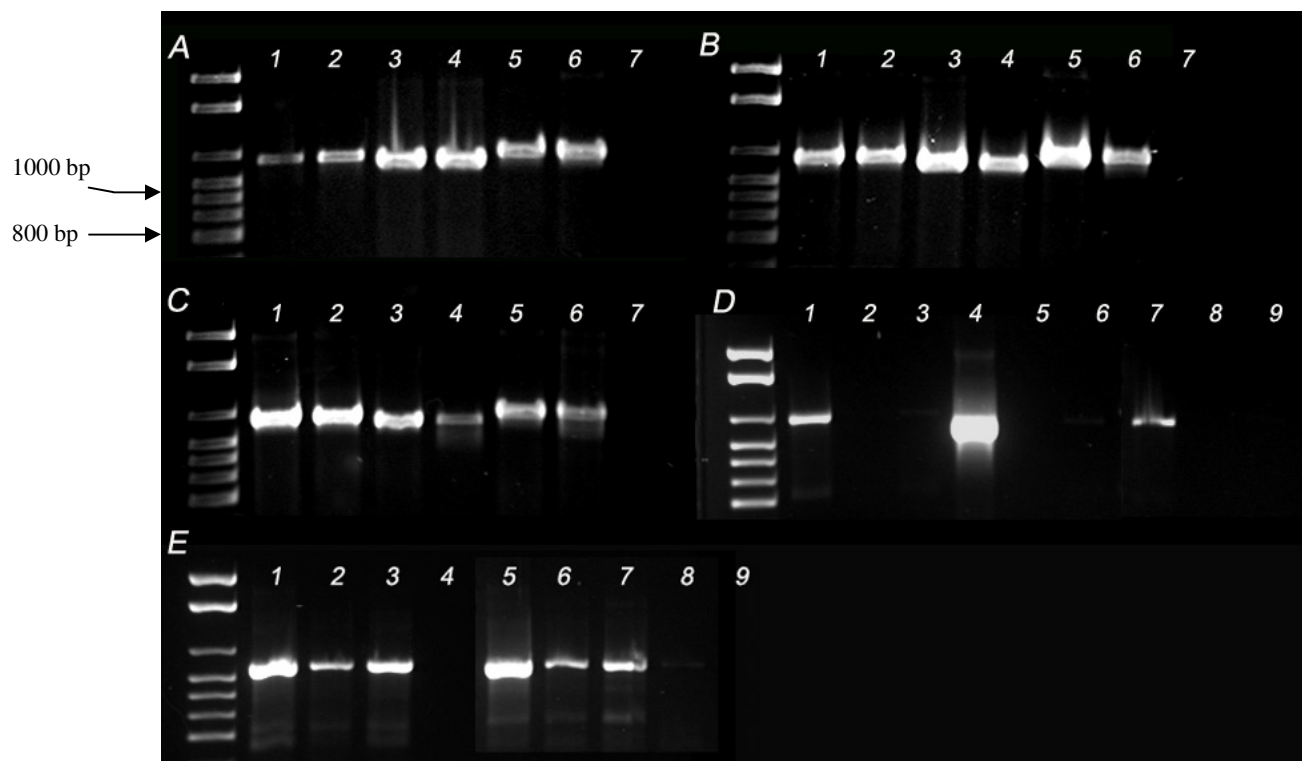


Fig. 5.4. Dendrogram obtained from the alignment of cucumovirus and potyvirus samples with reported sequences from GenBank representing each group. The isolates for each virus used in this study are indicated by an arrow.

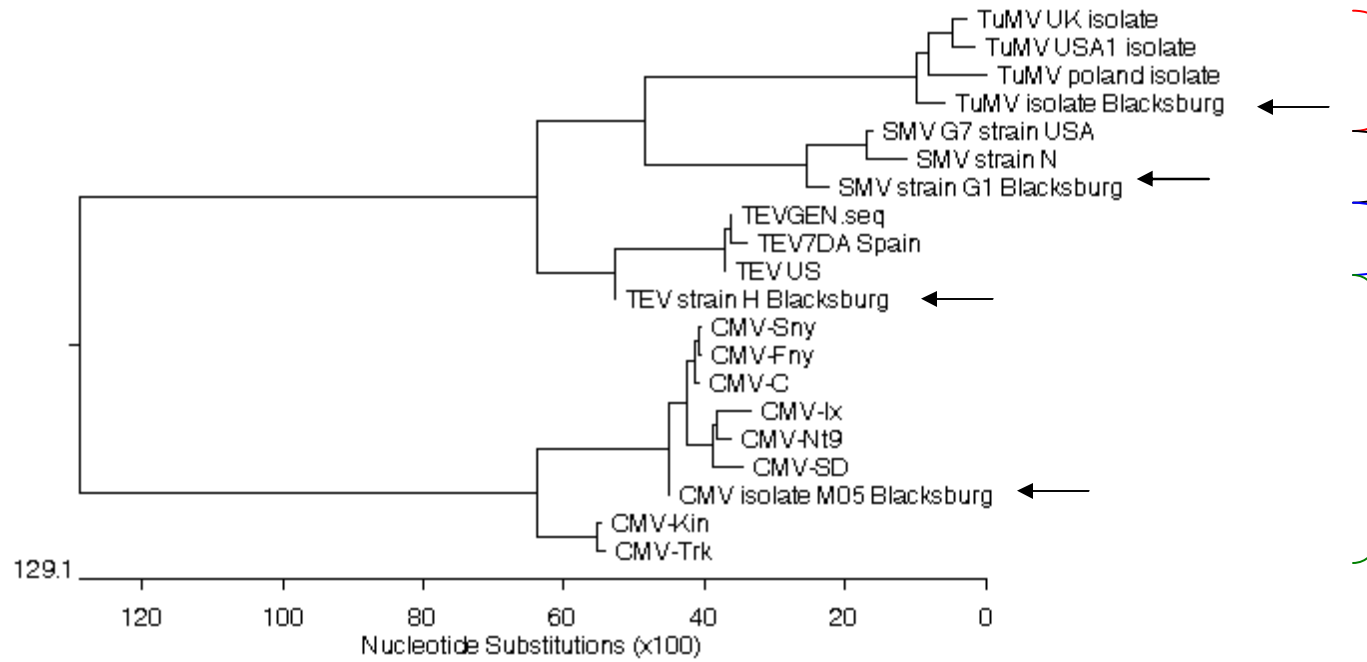
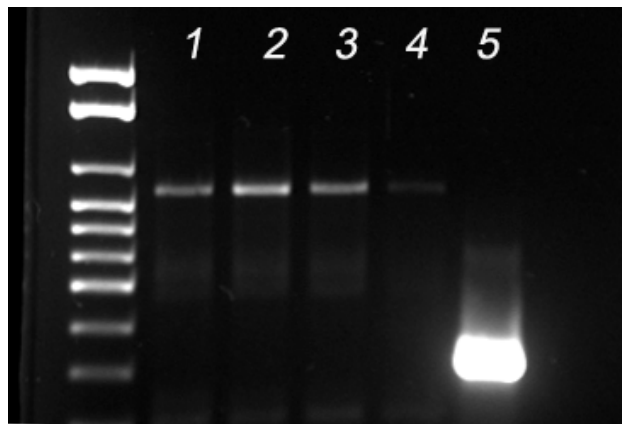


Fig. 5.5 Comparison of RNA template size with intensity of amplicon bands. Lanes 1-3 represent three disc diameter sizes, 3 mm, 2 mm and 1.25 mm respectively, removed from a positive CMV blot processed by TBIA. A 3 mm disc was removed from healthy tissue also processed with CMV antibodies (lane 4) for the negative control and the PCR kit positive and the positive control (lane 5)



CHAPTER VI

Global application of NitroPure nitrocellulose membranes for virus detection and identification

Abstract

NitroPure nitrocellulose (NPN) membranes have proven to be an effective source of plant viral RNA. Since plant viruses are inactivated after drying, membranes offer an easy way of transporting plant viruses over long distances without the risk of introducing viruses to new areas. This study reports the use of NPN for the global collection, detection, characterization and sample databasing of plant viruses.

Plant cards were disseminated to collaborators within the IPM CRSP for the collection of plant virus samples. The cards were returned and processed using tissue blot immunoassay (TBIA) and antibodies to *Cucumber mosaic virus* (CMV) and *Tobacco etch virus* (TEV). Discs from dried membranes were cleaned and added directly into reverse transcription reactions. Aliquots of the cDNA were added to PCR reactions to obtain the expected size amplicons. The PCR products were cleaned and sequenced. The coat protein sequences obtained placed all but two CMV positives into subgroup 1A. Discs were also obtained from 15 month old CMV positives collected through the Legume PIPE project processed by TBIA. PCR protocols were modified from 35 cycles to 40 to compensate for the age of the samples. Sequences of PCR products placed all US samples tested also within subgroup 1A. Membranes offer a quick, simple and safe way

of collecting samples from long distances and storing these virus samples for several months.

Introduction

NitroPure nitrocellulose membranes (NPN) were shown in the Chapter V to enable both the detection and molecular characterization of four plant RNA viruses using a single membrane. In this study, the membranes were field tested by dissemination to collaborators in the Integrated Pest Management Collaborative Research Support Program (IPM CRSP) host countries (Table 6.1). Membranes were blotted and shipped back to the Virology lab at Virginia Tech for processing. Samples were also taken from previously TBIA processed cards through the 2007 Legume Pest Information Platform for Extension and Education (PIPE) project.

Materials and Methods

Preparation and dissemination of sample cards to IPM CRSP collaborators

NitroPure nitrocellulose membranes were cut into 70 x 120 mm strips and the edges taped to a folded 5" x 8" index card printed with sampling instructions. A template with 6 mm diameter holes, made from the membrane protective paper, was taped over the NPN membrane for positioning tissue blots in an array and for protecting non-blotted areas of the membrane (Fig. 6.1 a-c). The cards were labeled numerically and distributed to collaborators in selected IPM CRSP host countries in West Africa, Latin America and the Caribbean (Table 6.1).

Instructions to samplers were to select symptomatic plants, select a leaf and roll it into a tight coil, and pull to tear it in half. The torn edges were to be blotted onto five

spots in a row on the membrane as indicated by the arrow in Fig. 6.1. A total of nine samples were blotted per card. Once sampling was complete, the card(s) was shipped to the Tolin Virology lab at Virginia Tech, Blacksburg, VA, by regular mail.

Samples on BioRad nitrocellulose membranes were also received from the Dominican Republic. This provided a comparison between two types of membranes in this study.

Immunological assay for plant viruses from collected sample cards

Cards returned by each host country were either not processed by TBIA or were processed for the detection of *Cucumber mosaic virus* (CMV) or *Tobacco etch virus* (TEV). For unprocessed cards, the template was removed and strips of the membrane were cut lengthwise. Two strips, each blotted with a representative of the nine samples, were developed with TBIA using antibodies against CMV and TEV using the procedure described in the previous chapters. The remaining three strips were recovered with the template and stored at room temperature for later testing with other antibodies.

Collection of Cucumber mosaic virus samples within the United States

CMV positive samples were obtained from several states (Table 6.1) participating in the Legume Pest Information Platform for Extension and Education (PIPE) (Langham et al., 2007). TBIA sampling cards submitted to Dr. Sue Tolin, Virginia Tech. Using the method described in the previous chapter, one 3 mm disc was removed from each of the samples testing positive for CMV. All cards had been processed by TBIA at least 15 months prior to disc sample removal.

Membrane preparation of RNA and RT-PCR

Membrane preparation of RNA and RT-PCR was conducted as described in the previous chapter for CMV and TEV samples. Briefly, discs, 3 mm in size, were removed from all positive samples from the U.S. Legume PIPE cards and from representative CMV positive samples from each of the collaborating host countries' cards. Due to time constraints, only selected samples were cleaned and directly sequenced. The discs were cleaned and added directly to two-step RT-PCR with virus specific primers used as described in the previous chapter.

To compensate for the age of the U.S. samples, the CMV PCR protocol described in the previous chapter was modified by increasing the number of cycles from 35 to 40. The PCR products were observed in an ethidium bromide-stained 2% agarose gel with a UV transilluminator (UVP Inc., San Gabriel CA) and AlphaInnotech Imager (San Leandro, CA). The remaining products were cleaned with either the QIAquick PCR purification kit (Qiagen, Valencia, CA) or with shrimp alkaline phosphatase/ exonuclease 1 (SAP/EXO1) (usb-Affymetrix, Cleveland, OH), and sequenced at the CRC-DNA Sequencing Facility at the University of Chicago, IL.

Analysis of sequence data

The EditSeq program of DNASTAR Lasergene software (Madison, WI) was used to remove background signals at the starts and modify existing sequences obtained from NCBI. The Cluster W program in MegAlign, DNASTAR (Madison, WI) was used to align all sequences to produce a phylogenetic tree. A total of eight coat protein sequences were used from GenBank for the comparison with the sequences from samples received from collaborators. Three sequences from CMV strains Fny, Sny, and C, all U.S. isolates, represented CMV subgroup 1A; three sequences, Ix, SD and Nt9, all

isolated from Asia, represented CMV subgroup 1B; and 2 sequences, Kin and Trk7, from Scotland and Hungary, respectively, represented CMV subgroup 2. All sequences have been used previously in evolutionary studies of CMV (Roossinck, 1999).

Results

Immunological assay from collaborator sample cards

A total of 362 samples were obtained from the IPM CSRP collaborating host countries. A summary of the results is shown in Table 6.1. A majority of the samples received were blotted from different varieties of pepper and tomato, particularly from countries in West Africa and Latin America. Several mixed infections of CMV and TEV were observed from peppers in Dominican Republic, as indicated by TBIA (Fig. 6.2). Several samples were negative to both CMV and TEV, even though plants were showing symptoms of viral disease. From the Jamaica, up to 90% of the samples were blotted from infected cucurbits including watermelon, pumpkin and squash. Three samples blotted in Jamaica from pumpkin displaying mosaic patterns on leaves tested positive for *Watermelon mosaic virus* (WMV2) but were negative *Peanut stunt virus*, and for all potyviruses using a general potyvirus monoclonal antibody.

U.S. isolates of CMV

A total of 24 positive CMV samples were selected from Legume PIPE cards from a total of 7 locations in 5 states, namely, New York, Wisconsin, Michigan, North Carolina and Minnesota. No other participating state had samples that were positive for CMV. Discs were taken from a range of TBIA positive samples, from purple specks on blotted areas to deep purple spots, which indicated a high concentration of virus particles.

RT-PCR and sequence data

Based on sequences obtained, all Legume PIPE samples were aligned in subgroup 1A, with percentage identities between 93 and 96%. Sequences obtained from the Dominican Republic samples gave variable identity percentages, but most were also aligned with subgroup 1A with 87 to 92% identities (Fig. 6.3a and b). Direct observations of chromatograms of the Dominican Republic samples revealed multiple Ns within the genome sequences resulting from several overlapping peaks. This was noticed especially in samples from Ocoa (from CMV card 96) and Paya (CMV card 97), the two outliers in the phylogenetic tree (Fig. 6.3a). These samples share only 50-70% sequence identity with the other samples and CMV sequences from GenBank. Many plants were co-infected with TEV. In the pepper field from which the samples were obtained 100% of the plants showed severe symptoms, including extreme mottling and stunting.

Two types of membranes were used in this research, the NitroPure nitrocellulose membranes commonly used in the Tolin lab, and the Bio-Rad pure nitrocellulose membranes (Hercules, CA) used by collaborators in the Dominican Republic. With TBIA, the Bio-Rad membranes had a higher background, making it difficult to determine weak positives of CMV and TEV from negatives in TBIA. There was no difference between the membranes when they were used as sources of RNA for RT-PCR as the series of Ns in the Dominican Republic samples was not associated the type of membrane.

Discussion

As shown in Chapters II, III and V, NitroPure nitrocellulose membranes have made an invaluable contribution to the detection and molecular characterization of four

economically important plant viruses in two different virus families. The use of membranes as sources of RNA, regardless of whether the membrane has been processed by TBIA or not, eliminate the need for the collection and storage of fresh tissue, and the extensive and often laborious task of RNA extraction.

Our data demonstrate that NPN can be used to store virus samples for long periods of time without any special storage conditions, even after TBIA processing. Previous experience with TBIA had shown that membranes remained immunologically active for up to 1 year (data not shown). This led to the assumption that if intact virus particles were still bound to the membranes, RNA should be available as a template for RT-PCR. Using membranes from the 2007 Legume PIPE, cDNA was amplified from samples that had tested positive for CMV by TBIA performed 15 months previously. Amplification of cDNA by PCR, and sequencing of PCR products, demonstrated that RNA was still available for molecular analysis after 15 months.

In Chapter IV, the viral diagnostic capabilities of several developing countries within the IPM CRSP were investigated. Despite the availability of thermocyclers and imagers, molecular diagnosis of viruses was not practiced by more than 50% of the clinics that responded to the questionnaire. We assumed that obtaining reagents and specialized equipment could be the cause of the lack of molecular analysis. For clinics that cannot do molecular analysis but need to have these data for diagnostics or publication, the development of a centralized hub lab for molecular analysis for surrounding clinics is an option. We tested NPN for the ease of transport of plant viral material between countries to replicate countries submitting virus samples to a centralized clinic for molecular diagnostics. Virus samples blotted onto NPN in sample

cards were received from collaborating countries within the IPM CRSP. Our results show that samples blotted to NPN can be sent between countries and used to obtain nucleic acid sequences.

Samples from the Dominican Republic had lower percentage identity to representative CMV sequences for each CMV subgroup. A closer look at the sequences of these samples showed several N's, primarily due to several overlapping peaks. Since these samples had been obtained from an old, infested field (Fig. 6.2), the possibility of multiple strains of CMV within one plant exists. We suggest that sampling younger plants might give cleaner sequences, as there would be less chance of mixed infection or mutation.

Finally, we have shown that virus samples can be collected safely and easily on membranes and sent to other laboratories or countries for molecular testing. It was also demonstrated that NPN can be used as a databasing matrix for storing non-infectious plant viruses for several months at room temperature. Whether or not membranes had been processed by TBIA, both old and newly blotted membranes were successfully used as sources of viral RNA for molecular characterization of CMV coat protein. Further work using a wider range of plant viruses and testing within other developing countries would broaden the application of these methods

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Table 6.1. Total number of CMV and TEV samples collected from collaborating host countries

Participating host country	Total no. of samples	No. CMV positives	No. TEV positives
Jamaica (Caribbean)	87	0	0
Guatemala (Latin America)	9	4	6
Honduras (Latin America)	27	0	0
Baguineda, Mali (Africa)	79	0	0
Bamako, Mali (Africa)	8	0	0
Sotuba, Mali (Africa)	5	0	0
Koulikoro, Mali (Africa)	37	0	0
Dominican Republic (Caribbean)	71	11	48
Dominican Republic (BioRad)	39	10	21
Michigan (USA)	5	5	0
North Carolina (USA)	2	2	0
Minnesota (USA)	2	2	0
New York (USA)	6	6	0
Wisconsin (USA)	9	9	0
TOTAL SAMPLES	386	49	75

Fig. 6.1. Sample cards for the collection of plant viruses from collaborators within the IPM CRSP host countries. One plant sample is blotted in the five spaces per row, as indicated by the arrow.

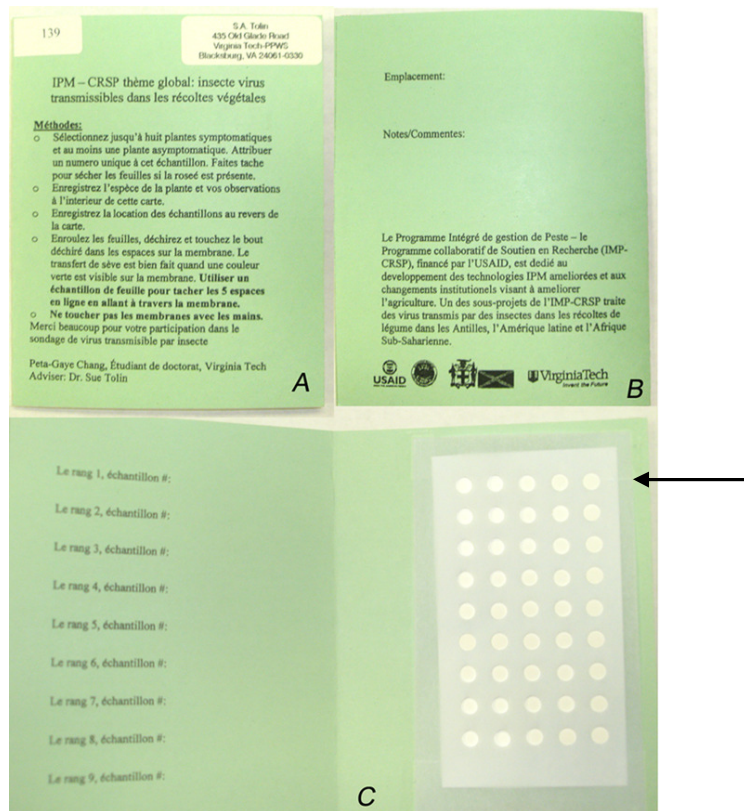


Fig. 6.2. Samples from Paya, Dominican Republic were blotted onto NitroPure nitrocellulose membranes and strips processed with tissue blot immunoassay against antibodies to *Cucumber mosaic virus* and *Tobacco etch virus*. Mixed infections of both viruses are indicated by arrows on the right

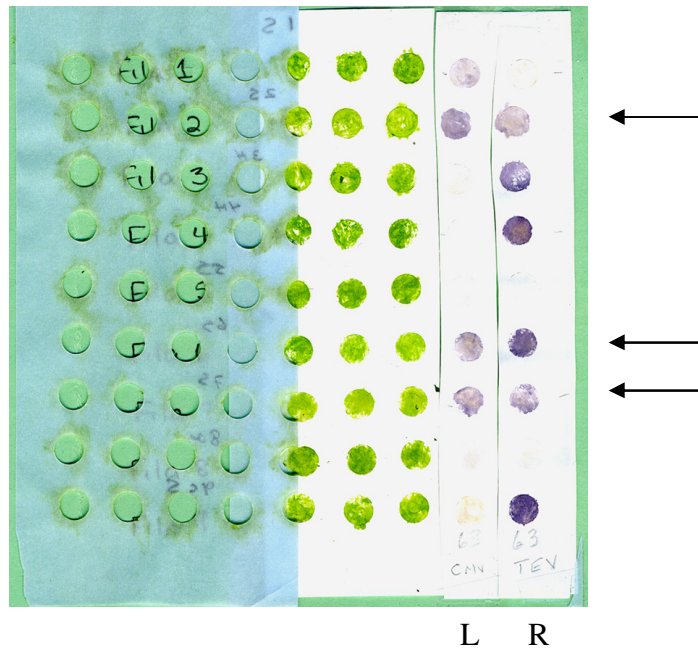


Fig. 6.3a. Dendrogram obtained from the alignment of *Cucumber mosaic virus* samples amplified from samples collected through IPM CRSP collaborators and the Legume PIPE project with sequences published in GenBank (indicated by arrows).

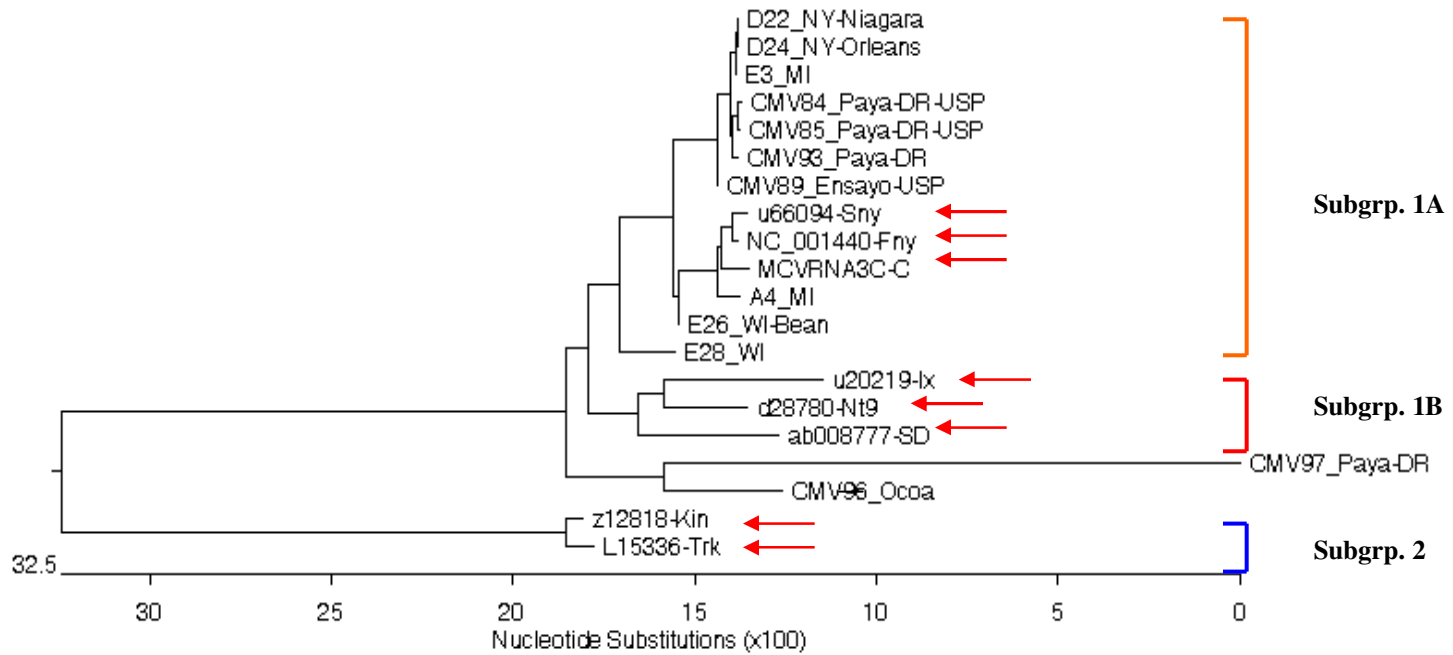


Fig. 6.3b Percent identity and divergence of *Cucumber mosaic virus* samples amplified from samples collected through IPM CRSP collaborators with sequences published in GenBank

		Percent Identity																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
Divergence	1	█	98.8	71.5	72.2	71.6	72.3	73.2	72.8	72.7	72.7	71.1	71.2	71.2	70.4	40.9	67.6	70.2	68.7	70.4	58.5	1	z12818-Kin
	2	1.2	█	71.1	72.1	71.4	72.2	72.8	72.2	72.2	72.6	71.0	71.1	71.3	70.3	40.8	67.1	70.1	68.3	70.2	58.2	2	L15336-Trk
	3	36.2	37.0	█	88.0	87.8	88.1	93.7	91.7	87.9	89.5	87.4	87.9	87.9	86.7	51.4	82.4	86.0	83.6	86.4	70.0	3	u20219-Ix
	4	35.0	35.2	13.1	█	98.6	99.4	90.2	89.9	92.2	96.1	93.1	93.5	93.5	93.0	52.4	87.4	91.1	88.4	91.7	69.3	4	u66094-Sny
	5	36.0	36.3	13.4	1.4	█	98.7	90.5	90.2	92.1	95.7	93.2	93.6	93.6	92.9	52.0	87.5	91.2	88.5	91.6	68.9	5	MCVRNA3C-C
	6	34.9	35.1	13.0	0.6	1.3	█	90.5	90.0	92.5	96.2	93.4	93.9	93.9	93.1	52.4	87.6	91.3	88.6	91.9	69.4	6	NC_001440-Fny
	7	33.4	34.0	6.6	10.5	10.2	10.2	█	93.4	89.5	91.3	89.3	89.7	89.8	88.9	51.8	84.0	87.9	85.3	88.1	70.3	7	d28780-Nt9
	8	34.1	35.0	8.9	10.9	10.6	10.7	6.9	█	89.6	90.3	88.4	88.5	88.5	88.0	51.3	82.9	86.6	84.4	86.9	68.9	8	ab008777-SD
	9	29.6	30.4	9.2	4.2	4.4	3.8	7.4	7.2	█	93.3	92.5	92.5	92.8	92.9	53.7	85.2	89.8	87.7	90.1	70.3	9	E28_WI
	10	31.3	31.4	8.6	1.2	1.6	1.1	6.7	7.7	4.1	█	94.3	94.4	94.8	94.3	53.9	89.0	92.3	89.7	93.1	70.7	10	A4_MI
	11	32.6	32.8	10.0	3.4	3.3	3.1	7.8	8.8	4.8	3.3	█	98.5	98.3	94.1	54.1	91.5	95.7	91.6	96.0	70.4	11	E3_MI
	12	33.2	33.3	10.0	3.6	3.5	3.2	8.1	9.3	4.8	3.3	0.0	█	98.7	94.3	54.0	91.1	95.6	92.0	95.9	70.2	12	D22_NY-Niagara
	13	32.9	32.9	9.9	3.5	3.4	3.1	7.8	9.2	4.7	3.3	0.0	0.0	█	94.3	53.9	91.3	95.5	91.1	95.5	70.1	13	D24_NY-Orleans
	14	31.2	31.3	8.6	1.3	1.5	1.1	6.1	7.1	3.7	1.4	1.4	1.4	1.4	█	53.8	87.5	91.3	89.3	92.6	70.3	14	E26_WI-Bean
	15	51.0	51.7	24.5	22.6	23.4	22.6	24.2	25.0	22.6	23.1	22.2	22.2	22.2	21.8	█	53.9	55.8	54.0	55.1	55.3	15	CMV97_Paya-DR
	16	32.5	32.6	9.8	3.6	3.7	3.4	8.0	9.2	5.2	3.5	0.3	0.5	0.5	1.5	23.5	█	93.7	90.7	92.9	69.9	16	CMV84_Paya-DR-USP
	17	32.4	32.4	9.7	3.8	3.6	3.5	7.7	8.9	5.1	3.5	0.3	0.4	0.4	1.9	22.2	0.1	█	93.8	97.0	72.5	17	CMV85_Paya-DR-USP
	18	30.7	31.1	8.9	2.8	2.8	2.5	7.0	7.7	4.4	2.8	0.4	0.4	0.4	1.2	21.7	0.3	0.5	█	93.4	70.7	18	CMV89_Ensayo-USP
	19	32.5	32.6	9.6	3.5	3.5	3.2	7.8	9.1	5.2	3.4	0.4	0.3	0.4	1.8	21.7	0.4	0.4	0.4	█	71.5	19	CMV93_Paya-DR
	20	29.7	30.3	10.2	11.1	11.6	10.9	9.8	11.6	10.8	10.8	11.4	11.1	11.2	9.2	19.1	10.9	11.1	10.1	11.1	█	20	CMV96_Ocoa
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20			

Reported subgroup 1A sequences from GenBank – MCVRNA3C-C; NC_001440-Fny; u66094-Sny

Reported subgroup 1B sequences from GenBank – u20219 – Ix; d28780-Nt9; ab008777-SD

Reported subgroup 2 sequences from GenBank – z12818-Kin; L15336-Trk

CHAPTER VII

Conclusions and Recommendations

Diagnostics plays an important role in any agricultural system. There are four general approaches to virus diagnosis, including i) biological, ii) physical, iii) serological, or iv) molecular techniques. Three of these approaches were used in the identification and characterization of three recent isolates of *Cucumber mosaic virus* (CMV; Genus *Cucumovirus*; Family *Bromoviridae*) from the garden at the Historic Smithfield Plantation in Montgomery County, VA. The CMV isolates were obtained from flowering tobacco (*Nicotiana* sp. L.), bowl gourd (*Cucurbita* sp. L.) and periwinkle (*Vinca minor* L.). Symptoms included mosaic and leaf deformation of varying severities. Positive reactions were obtained in tissue blot immunoassay (TBIA) using CMV polyclonal antibody from the Tolin lab (code HCV-7409), and monoclonal antibodies donated by Agdia Inc. The isolates were compared to CMV isolates collected in the 1970s and stored as dry tissue for over 25 years. Biological characterization using indicator hosts induced symptoms similar to those described for CMV (Palukaitis and Garcia-Arenal, 2003). CMV RNA was first obtained from purified CMV particles through SDS release. The cDNA was amplified with CMV coat protein specific primers, and the PCR products cleaned and sequenced. All CMV sequences were aligned using the Clustal W program in the Lasergene software (Madison, WI), which produces a phylogenetic tree (Fig. 2.3a). The sequences of the new CMV isolates were 94.5% identical to representative sequences for CMV subgroup 1A from GenBank. Three of the old isolates were classified as subgroup 2, which was not previously reported to occur in Virginia.

Another virus, *Turnip mosaic virus* (TuMV; genus *Potyvirus*, family *Potyviridae*), was isolated from Dame's Rocket (*Hesperis matronalis* L.) located along the periphery of the Smithfield Plantation gardens. The plant displayed symptoms of mosaic on leaves and petal color-breaking in flowers. TuMV was detected using TBIA with antibodies donated from T. P. Pirone (Lexington, KY). Symptoms on indicator plants and sequence data confirmed the identity of TuMV. Petal color-break symptoms were reproduced upon inoculation of young Dame's Rocket plants. This is the first reported case of TuMV in Virginia.

Because diagnostics is especially important in developing countries where the export of agricultural produce contributes heavily to the economy (Lawrence et. al., 2005), the capabilities of clinics in developing countries to perform viral and general diagnostics were investigated. Ausher (1996) previously reported that the diagnostic capabilities in developing countries were often limited by funding and the availability of trained diagnosticians. We conducted a survey to look more closely at the current viral diagnostic capabilities of several clinics in developing countries, collaborators of the IPM CRSP. Clinics privately owned or affiliated with academic institutions had better equipped laboratories than clinics that were affiliated with the government. Clients that used clinic services varied, depending on the affiliations to the different institutions. Privately owned clinics obtained a majority of their samples from companies and corporations, academically affiliated clinics obtained samples from corporations and extension agents, and clinics affiliated with the government received samples primarily from farmers and extension agents. It was interesting to note the contrasting diagnostic approaches employed by these various clinics. Unlike clinics privately owned or

affiliated to academic institutions that used molecular diagnostics, government affiliated labs employed biological and serological approaches for virus diagnosis. The unavailability of specialized equipment and personnel, reagents and kits has hampered the use of molecular techniques in virus diagnosis.

For laboratories that require molecular data for quarantine and phytosanitary purposes, characterization studies and virus identification but are unable to perform such tasks, samples can be collected and shipped to diagnostic laboratories. However, because of state, nation, and International Plant Protection Organization (IPPO) regulations, special permits have to be obtained for the importation of any pathogen into a locality or political division where the pathogen has been either controlled or is not present. Presented with this issue, our laboratory turned to NitroPure nitrocellulose (NPN) membranes as a solid matrix on which pathogens are non-infectious and can be transported without permit. These membranes, used in TBIA, bind whole virus particles to its surface. Our hypothesis was that if whole particles are present on membranes, then RNA will also be present and might be available for molecular studies. We proved this hypothesis using five viruses in two genera, CMV and *Peanut stunt virus* (PSV) in the genus *Cucumovirus*, and *Soybean mosaic virus*, *Tobacco etch virus*, and *Turnip mosaic virus* in the genus *Potyvirus*. Amplicons of the expected sizes for each virus were produced from viruses blotted to NPN membranes. CMV had a 94.7-95% sequence identity to U.S. CMV strain Fny, TEV had a 95.6% identity to U.S. TEV strain TEV-GEN, SMV had a 96% identity to U.S. SMV strain G7, and TuMV had a 93% identity to U.S. TuMV strain USA1.

A limited trial of the international application of this procedure was conducted in several countries in West Africa, Latin America and the Caribbean. Special sampling cards were designed, and distributed to collaborators with instructions for blotting with plant tissue displaying virus-like symptoms. Non-processed membranes received from collaborators in these countries were developed by TBIA using monoclonal antibodies to CMV and a polyclonal antibody to TEV (#601). No CMV or TEV positive samples were obtained from West Africa or Jamaica. Samples positive to CMV and TEV were obtained from the Dominican Republic, but the possible presence of two or more strains of CMV within the samples, shown as multiple peaks in the chromatograph, interfered with a good sequence reading. The samples were, however, approximately 70-80% identical to CMV subgroup 1A sequences, compared to 60% to subgroup 1B and 30% to subgroup 2.

Diagnostics is important for the management of plant viruses. However, without the right tools, equipment and knowledge, proper management practices cannot be accomplished. Our work has compared both the classical approaches to plant virus diagnostics and a new, simple method for both detecting plant viruses and obtaining sequence data from one solid matrix, nitrocellulose membranes. To further explore membrane applications, we propose studies into the use of membranes as sources of nucleic acid for other plant RNA and DNA viruses. It is possible also to explore the use of membranes for other pathogens, including bacteria, as bacteria has been detected using TBIA protocols (Lin et al., 1990)

I would also like to use this method to survey the current virus diversity in Jamaica and, if possible, in surrounding islands as well. Nitrocellulose membranes would allow for the collection of several viral samples from various locations. With the

knowledge of the types of viruses found within the region, islands would be better able to develop management strategies that would benefit farmers, both large, and small for increased productivity within agriculture.

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<http://www.dpvweb.net/dpv/showadpv.php?dpvno=400>

APPENDICES

Appendix A

Virus purification according to Lot et. al. (1972)

Six virus isolates: M05-CMV, T05-CMV, CMVY-CC, V06-CMV, CMVY-108 and CMVB were each inoculated to six 8-week old Xanthi tobacco plants. Each plant had four to six fully expanded leaves. Eight days after inoculation, the leaves from all plants were harvested for a combined weight of 20 – 30 gm. The leaves were cooled at 4°C for 2 hr and then homogenized in a Waring blender with 0.5M citrate buffer, pH 6.5 (sodium citrate – citric acid), containing 0.1% thioglycolic acid at a rate of 2 ml/gm of tissue. The tissue was macerated for 10 sec at low speed. Cold chloroform, at a rate of 2 ml/gm of tissue, was added to the crude sap and homogenized at high speeds for 30 sec. The homogenate was quickly moved to a chilled beaker, mixed, and centrifuged at 7500 rpm for 10 min. The aqueous layer was decanted into a cooled beaker. To the total volume of the decanted layer, PEG 6000 (10% w:v) was added and magnetically stirred for 15 min at 4°C. The suspension was allowed to stand in an icebath at 4°C for 40 min, and then centrifuged at 10,000 rpm for 20 min. The supernatant was discarded and the pellet re-suspended in 0.05 M citrate buffer, pH 7, containing 2% (v:v) Triton X-100, at a rate of 0.5 ml/gm of original weight of leaves. To ensure maximum re-suspension, the pellets were magnetically stirred at 4°C overnight. The suspension was centrifuged at 7500 rpm for 20 min. The supernatant was centrifuged using a Type 65 rotor (Beckman L8-80 Ultracentrifuge (Fullerton, CA. USA), for 2 hr at 40,000 rpm. The supernatant was discarded and the pellets re-suspended in distilled water, with gentle agitation, overnight. After low speed centrifugation of 13,600 g (Microcentrifuge Model 235B, Fisher

Scientific) for 2 min, the supernatant was removed and made up to 0.5 M in citrate buffer.

The purified virus was stored at 4°C.

Appendix B

Virus purification according to Lane et. al. (2003)

Three CMV isolates: M05-CMV, T05-CMV and V06-CMV, were each inoculated to two 8-week old Xanthi tobacco plants. Eight days after inoculation, a combined weight of 1 gm of infected leaf tissue was harvested from both plants and stored at 4°C for 2 hr. The leaves were homogenized in a small Waring blender at high speed with 22 ml cold sodium citrate buffer, pH 6.5, 150 µl 0.5 M sodium diethyldithiocarbamate (DIECA) (Acros Organics, Geel Belgium) and 125 µl 0.2 M Iodoacetamide (Sigma-Aldrich, St. Louis, MO USA). The mixture was expressed through a damp cheesecloth into ultracentrifuge tubes and centrifuged at 40,000 rpm for 10 min using a Type 65 rotor. The supernatant was decanted into new ultracentrifuge tubes. Eight drops of 5% Triton X-100 were added, and the supernatant mixed before centrifugation at 40,000 rpm for 40 min. The resulting supernatant was discarded and the pellets rinsed with de-ionized water. The pellets were left to air dry for 15 min, re-suspended in 50 µl 0.05 M sodium phosphate, and stored at 4°C.

Appendix C

Cucumber mosaic virus sequences used in primer design

NCBI Code	bp #	Subgrp	RNA 3/CP	Location	Host	Strain	Isolate
EF153734	2220	1b	3	India	Tomato		Ts
EF153739	657	1b	CP	India	Jatropha		Jatropha
EF050074	899	2	CP	Iran	Lepidium		Ld
DQ302717	784		CP	China	Pepper		Bt
EF088683	657			China	Echinacea		2/1/2002
AB261173	657		CP	Japan	Alstroemeria		GT
AB250962	657			Philippines	abaca		aP81
DQ914877	657			India	rauvolfia		rauvolifa
DQ873558	657			china			BG_18_4
DQ873527	657			China	BG 2-1		
DQ910858	657			India	banana		Lucknow
DQ885291	828			China	lily		
AY450854	657			Greece	tobacco	G2	
DQ459482	657			China	squash	YN	
AB109908	657	2	CP	Korea	paprika	PaFM	
DQ141675	657			India	Tomato		
AY690621	657			India	Pepper		
AY380533	657			Brazil	chrysanthemum		
AY374328	657			Brazil	lilium		
AB109909	657	1	CP	Korea	water chickweed	Sa	
AF523341	657			USA			
D00462	1255		CP	USA	tobacco	C	
U32859	862		CP	Columbia	banana		
U32858	864		CP	Columbia	plantain		
CMU22821	1033		CP	Australia		Ny	
M98501	657		CP	Puerto Rico			
M98500	657		CP	Puerto Rico			
M98499			CP	Puerto Rico			
AB069971	842		CP	Tokyo	banana		B2
S70105	1360	2	CP			WL	
AY871070	841	1	CP	Iran	cucumber		
AF172841	502		CP	Brazil	Tomato	S	
AF368192	657		CP	China	passiflora	Pf	

AJ239098	657		CP	China		YN	
AJ271416	657		CP	USA	for aphid trans.		
AM183119	2192		3	Spain	Tomato	Ri-8	
AY611027	657		CP	China	melon	99/10	
DQ018288	895	1	CP	Poland	cucumber	M	
DQ302719	784		CP	China	Tomato		Gf
DQ302721	784		CP	China	Tomato		Sf
MCVYCNS	2217		3	China		Y	
NC_001440	2216		3	USA		Fny	
D10538	2216		3	New York		Fny	
						Sny-	
U66094	2216		3	New York	pumpkin	CMV	
				United			
D10539	2215		3	Kingdom		M	
D42080	2214		3	Japan		E5	
D12499	2217		3	Japan		Y	
AB042294	2214		3	Indonesia		IA	
U20219	2216		3	USA	tomato	Ix	
D16405	2213		3	Japan	legume	Leg	
AF127976	2198		3			Ls	
AJ276481	2214	1a	3	South Korea		Mf	
D28780	2214		3	Taiwan		Nt9	
AF063610	1468		CP	South Africa		S	
M21464	2197		3	Australia		Q	
D28486	2218		3	Japan		N	
U43888	957		CP	Israel	banana	Ban	
				United			
D10545	657		CP	Kingdom		P6	Price's 6
D10544	657		CP	United States		FC	Fulton's
L15336	2209		3	Hungary		Trk	
AF127977	2219		3	China		K	
Z12818	2199		3	Scotland		Kin	
AB046951	2219		3	Indonesia	banana	B2	
Y18138	2206		3	France		R	
U31219	657		CP	Hawaii	banana	Hi	
D00385	2217		3	Japan		O	
D00463	1267		CP			WL	

Appendix D

Dissertation diagnostic survey in conjunction with the IPM-CRSP IPDN and Insect-Transmitted Viruses Global Theme Projects

This survey is part of a PhD dissertation designed to assess the capability of plant diagnostic laboratories to identify, detect and perform molecular analyses on plant viruses in developed and developing countries.

Name of Diagnostic or Research Lab: _____

Country: _____

Please choose one: Academic: _____ Government: _____ Private: _____

Section 1 – Viral Diagnostics

1. Does your laboratory perform viral diagnostics? _____

- If no (which suggests you depend on outside sources for viral identification and detection), please disregard question 2.

2. If yes, please mark an X for all methods and approaches used by your lab for viral diagnosis.

Physical properties

Inclusion bodies in cells _____
Protein assays (size) _____
Sedimentation properties _____
Virus morphology _____
Virus purification _____

Biological

Differential hosts _____
Host plant symptoms _____
Indicator hosts _____
Transmission – graft _____
Transmission – insect _____
Transmission – mechanical _____

Serological methods

ELISA – direct _____
ELISA – indirect _____
Immunostrips _____
Ouchterlony _____
Tissueblot immunoassay _____

Nucleic acid methods

Cloning and sequencing _____
Gel electrophoresis _____
PCR – end point _____
PCR – Real Time _____
Reverse transcription – PCR _____
RNA or DNA extraction _____
PCR purification kits _____

Do you use kits purchased from companies (e.g. Agdia, Bioreba)? _____

Do you prepare your own buffers? _____

Please indicate the source of your antibodies with an X:

- a. Prepared in-house _____
- b. Donated _____
- c. Purchased _____
- d. Purchased as part of a kit _____

Diagnostics and Clients

3. Approximately how many samples do you receive in an average year? _____

4. Approximately how many samples received by your lab are caused by, or suspected to be caused by, viruses (per year)? _____

5 Please approximate the percentage of your total samples that are received from each of the types of clients listed below in an average year.

Clients	% of samples submitted yearly
Farmers	
Companies (includes certification programs and nurseries)	
Researchers/Extension officers	
Home owners/home growers	
Other (including consultants, quarantined plants)	

6. Please list up to 5 of the most common plants received by your lab that are diagnosed with a viral disease.

- 1. _____
- 2. _____
- 3. _____
- 4. _____
- 5. _____

Section 2 – General Laboratory

7. How would you rate your laboratory in the following areas, based on a scale of 1 (inadequate) to 5 (excellent)?

- Equipment (own or have access to necessary equipment) _____
- Facilities (functional and reliable) _____
- Funding (from grants, etc., covers all expenses) _____
- Infrastructure (see list below) _____
- Knowledge of staff about viral diagnostics _____
- Reagent availability (ease in acquiring supplies) _____

Equipment, Supplies and Infrastructure

8. Please mark an X for all items your lab uses, or has access to, for plant disease diagnosis.

Major (expensive) Equipment

- Centrifuge – refrigerated low speed _____
- Centrifuge – ultra high speed _____
- Camera-mounted microscope _____
- DNA sequencer _____
- Growth chambers _____
- Laminar flow hood _____
- Microscope – electron _____
- Microscope – compound _____
- Microscope – dissecting _____
- Thermocycler – end point _____
- Thermocycler – Real Time _____
- Transilluminator & imager _____

Expendable (< \$2000) Equipment

- Balances _____
- Camera – digital/35mm _____
- Electrophoresis unit and power supply _____
- Hot plate _____
- Magnetic stirrer _____
- Microcentrifuge (tabletop) _____
- Micropipettes _____
- Microwave _____
- pH meter _____
- Shaker _____
- Vortex/mixer _____
- Water-bath _____

Infrastructure – building

- Autoclave _____
- Bench top workspace _____

Disposables

- Burners _____
- Glassware/plasticware _____

Consistent electrical supply _____
 Fume hood _____
 Gas supply _____
 Generators (backup) _____
 Greenhouse _____
 Research land space _____

Glass slides and cover slips _____
 Magnet stir-bars _____
 Graduated cylinders _____
 Micropipette tips (various) _____
 Tubes – microcentrifuge _____
 Tubes – PCR _____

Infrastructure – other

Computers _____
 Internet access _____
 Reference literature _____
 Programs (analyses, statistical, etc.) _____

Cold Storage

4°C Refrigerator _____
 - 20° Freezer _____
 - 70° Ultra low freezer _____
 Walk-in Cold Room _____

Personnel and Capacity Building

9. Please describe all staff in your diagnostic lab who are involved in viral diagnostics.

Staff member (position/function)	Level of Education	No. years in viral diagnostics	No. years in general diagnostics	No. of viral training courses attended
<i>e.g. Diagnostician</i>	<i>M.S.</i>	<i>3</i>	<i>5</i>	<i>5</i>
<i>e.g. Research assistant</i>	<i>B.Sc.</i>	<i>< 1</i>	<i>1</i>	<i>0</i>
Technicians	certificate			

10. Do you invite outside speakers from different laboratories to conduct viral training sessions?

Yes _____
 No _____
 Occasionally _____

Funding and Purchasing

11. Please mark an X in the appropriate box, indicating your answer to each of the following questions:

Your Lab	Yes	No	Sometimes
Does your lab receive a fixed annual operating budget?			
Does your lab depend heavily on grant(s) money?			
Does your lab collaborate with other entities (institutions, companies, etc.)			
If so, is funding also obtained through these entities?			
Do you charge for your diagnostic services?			
Is all the income/funding generated enough to:			
○ Run the general functions of the lab			
○ Purchase and maintain equipment			
○ Maintain infrastructure and			
○ Pay staff on an annual basis?			
Can reagents/chemicals be readily purchased in your country?			
If not, is it difficult to obtain these reagents and chemicals from another country (considering taxes, shipping, financial approval, etc.)			
Is an intermediary company necessary to purchase/receive your reagents/chemicals from other countries?			

Section 3 – Constraints

13. Please list any constraints experienced by your lab in viral diagnostics.

14. Please list any constraints experienced by your lab in general plant disease diagnostics.

15. For IPM-CRSP demographic purposes are you

- a. Male _____
- b. Female _____

Thank you very much for your participation.

Peta-Gaye Chang

PhD student

Dr. Sue Tolin (advisor)

Professor/Virologist

Virginia Tech

Appendix E

Approval letter from the International Review Board granting permission to disseminate survey



Office of Research Compliance
Carmen T. Green, IRB Administrator
2000 Kraft Drive, Suite 2000 (0497)
Blacksburg, Virginia 24061
540/231-4358 Fax 540/231-0959
e-mail ctgreen@vt.edu
www.irb.vt.edu
FWA00000572(expires 1/20/2010)
IRB # is IRB00000667

DATE: January 23, 2009

MEMORANDUM

TO: Sue A. Tolin
Peta-Gaye Chang

FROM: Carmen Green 

SUBJECT: **IRB Exempt Approval:** "Survey of Plant Virus Diagnostic Laboratory Capabilities",
OSP #425634, IRB # 09-038

I have reviewed your request to the IRB for exemption for the above referenced project. The research falls within the exempt status. Approval is granted effective as of January 23, 2009.

As an investigator of human subjects, your responsibilities include the following:

1. Report promptly proposed changes in the research protocol. The proposed changes must not be initiated without IRB review and approval, except where necessary to eliminate apparent immediate hazards to the subjects.
2. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

cc: File
OSP

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