MOLECULAR ANALYSES OF PAPAYA VIRUSES IN BANGLADESH: DETECTION, CHARACTERIZATION, AND DISTRIBUTION

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI‘I AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
TROPICAL PLANT PATHOLOGY

MAY 2019

BY

ISLAM HAMIM

DISSERTATION COMMITTEE:
JOHN S. HU, CHAIRPERSON
MICHAEL J. MELZER
BRENT S. SIPES
MICHAEL SHINTAKU
RICHARD M. MANSHARDT

ACKNOWLEDGMENT

This dissertation is the outcome of approximately four years of work, during which I have been accompanied and supported by different people. It is with pleasure that I now have the opportunity to express my gratitude to these great people.

I would like to express my sincere and deep gratitude to my Advisor and principal investigator (PI) of plant virology laboratory at University of Hawaii at Manoa Dr. John S. Hu for providing guidance, advice, constructive criticism, suggestions, and discussion about my PhD dissertation research. I thank him for giving me the opportunity to be a part of his research team. He is the most energetic advisor and one of the smartest people I know. I greatly appreciate his unique thinking about science and his ability to collaborate with other scientists in different disciplines, and for sharing with me his experiences and ideas about science. He taught me consciously and unconsciously, how a good experiment should be accomplished. Dr. Hu is someone you will instantly like and never forget once you work with him.

I also would like to express my gratitude to all my committee members, Drs. Brent Sipes, Michael J Melzer, Richard M Manshardt and Michael Shintaku for their willingness to serve on my PhD committee, and for their encouragement and suggestions throughout this process. Dr. Melzer has been supportive and given me direct suggestions in pursuit of my various PhD projects. His delight and enthusiasm for his own research were contagious and motivational. I wish to extend my thanks and gratitude to Dr. Sipes, Dr. Manshardt, and Dr. Shintaku for their advice and instruction and many insightful discussions and suggestions.

I would like to extend my gratitude to all my plant virology lab members and collaborators, past and present. I would specifically like to thank Dr. Wayne Borth for his guidance and pep talks, which helped me both professionally and personally. With my lack of

background in molecular virology, my work in the laboratory would have been more difficult without Wayne's continuous help. He was an amazing and passionate guide throughout my PhD project. I wish to thank the other members of the plant virology lab, especially Nancy, Wang Da, James, Adriana and Xupeng for their support whenever I needed it. Thanks to all my colleagues and friends in the Department of Plant and Environmental Protection Sciences, University of Hawaii at Manoa, and Bangladesh Agricultural University.

For financial assistance I would like to thank Borlaug Higher Education for Agriculture and Research development (BHEARD) for providing me fund from the United States Agency for International Development, as part of the Feed the Future initiative, under the CGIAR Fund, award number BFS-G-1100002 and the predecessor fund of the Food Security and Crisis Mitigation II grant, award number EEM-G-0004-00013.

Finally, I take this opportunity to thank those to whom I am indebted forever, my mom, dad, and younger sister. I would like to dedicate this dissertation to my beloved parents, whose role in my life is always special. The last word of acknowledgement I have reserved for my dear wife, Papia, for her support, inspiration, patience and unwavering love. I wish to give her very special thanks.

ABSTRACT

Aphid-transmitted PRSV is the greatest disease threat to commercial papaya production worldwide. Specific, ultrasensitive assays are important for the early detection of PRSV in the field. I developed a single-tube nested PCR (STNP) assay to address this need. Two nested PCR primer sets were designed to target the P3 gene of the virus. The concentrations and annealing temperatures of both primer pairs were optimized to avoid potential competition between primer sets during STNP. The assay was more sensitive than regular RT-PCR as determined by serial dilutions of cDNA and RNA templates and sample extracts from infected plants. RT-PCR and ELISA were capable of detecting PRSV 14 to 21 days post-inoculation, whereas STNP detected PRSV in plants 7d post-inoculation. This new STNP assay also detected PRSV from virus-infected asymptomatic plants. This system could assist epidemiological studies in the field and in quarantine protocols by enabling early detection of very low PRSV titers in the field and in imported plant samples. As in other countries, PRSV is the major limitation to papaya production in Bangladesh. Full-length coding genomes of PRSV strains from severely infected papaya plants were determined using the Illumina NextSeq 500 platform, followed by Sanger DNA sequencing of viral genomes obtained by reverse transcriptase polymerase chain reaction (RT-PCR). The genome sequences of two distinct PRSV strains, PRSV BD-1 (10,300 bp) and PRSV BD-2 (10,325 bp) were 74% and 83% identical to each other at the nucleotide and amino acid levels, respectively. PRSV BD-1 and PRSV BD-2 were 74 to 75% and 79 to 88% identical to other full-length PRSV sequences at the nucleotide level, respectively. Based on phylogenetic analysis, PRSV BD-2 was most closely related to PRSV-Meghalaya (MF356497) from papaya in India. PRSV BD-1 formed a distinct branch from the other PRSV sequences by nucleotide as well as amino acid sequence comparisons. Comparisons of the genome sequences of these newly identified PRSV strains with other sequenced PRSV genomes indicated two putative

recombination events in PRSV BD-2. One recombinant event contained a 2,766-nucleotide fragment with the highest identity to PRSV-Meghalaya (MF356497) and the other contained a 5,105-nucleotide fragment with the highest identity to PRSV-China (KY933061). The occurrence of PRSV BD-1 and PRSV BD-2 in the sampled areas of Bangladesh was approximately 19% and 69%, respectively. Plants infected with both strains (11%) exhibited more severe symptoms than plants infected with either strain alone. The new full-length genome sequences of these PRSV strains from Bangladesh and their distribution provide important information on the dynamics of papaya ringspot virus infections in papaya in Bangladesh. Forty-five papaya samples with severe leaf curl symptoms were tested by PCR using a degenerate primer set for virus species in the genus *Begomovirus*. Of these, 29 were positive for tomato leaf curl Bangladesh virus (ToLCBV). The complete genome sequences of ToLCBV (GenBank accession no. MH380003) and its associated tomato leaf curl betasatellite (ToLCB) (MH397223) from papaya isolate Gaz17-Pap were determined and characterized. Defective betasatellites were found in ToLCBV-positive papaya isolates Gaz19-Pap, Gaz20-Pap and Gaz21-Pap. This study confirmed that papaya is a host of ToLCBV, ToLCB, and other defective and recombinant DNA satellites in Bangladesh. The complete genome sequence of tomato leaf curl Joydebpur virus (ToLCJoV) and its associated tomato leaf curl Joydebpur betasatellite (ToLCJoB) were determined and characterized from papaya isolate J1-Pap. ToLCJoV infecting papaya was most closely related to ToCJoV reported from Gazipur, Bangladesh, causing tomato leaf curl disease of tomato. ToLCB infecting papaya had the highest homologies at nucleotide and amino acid levels to ToLCB from tomato in India. The complete genome sequences of the DNA A and DNA B components of tomato leaf curl New Delhi virus (ToLCNDV) were determined and characterized from papaya isolate ND-71. ToLCNDV infecting papaya was most closely related

to ToLCNDV reported from Gazipur, Bangladesh, causing tomato leaf curl disease of tomato. DNA B of ToLCNDV infecting papaya had the highest homologies at nucleotide and amino acid levels of DNA B of ToLCNDV from tomato in India.

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

CHAPTER I

INTRODUCTION AND REVIEW THE OF LITERATURE

Management of papaya ringspot disease: challenges and solutions

Papaya (*Carica papaya* L.) is a unique edible fruit in the family Caricaceae. Although it originated in the Americas, it is now popular in tropical and subtropical areas worldwide (Badillo 1993; Gonsalves 2002; Hamim et al. 2014). Papaya is eaten ripe as a dessert fruit or unripe as a vegetable and is a good source of nutrients. It is also used by some for its medicinal properties (Chan and Tang 1978; Sturrock 1940; USDA/ARS 2001; Ye and Li 2010), as the leaves are used to make tea to treat malaria (Titanji et al. 2008). In India, China, the Philippines, Brazil, Mexico, Australia, Thailand, South Africa and Indonesia, papaya is an established cash crop. The countries of India, Brazil, Indonesia, and Mexico together grow 10 million tons per year, valued at more than $200 million (USD) (FAOSTAT 2014). Papaya is one of the largest agricultural crops in Hawaii, USA, but only contributes 0.1% of the world's papaya production. However, the development of transgenic cultivars resistant to papaya ringspot virus (PRSV) has made Hawaii a pioneer in the papaya industry (Gonsalves 2002). Many pests and diseases affect papaya production but viruses are one of the greatest concerns, potentially causing a 100 % loss in yield (Purcifull et al. 1984; Ye and Li 2010). Papaya ringspot disease, caused by PRSV, is the most important viral disease of papaya worldwide. Other viruses that infect papaya include: papaya leaf curl virus (PLCV), papaya mosaic virus (PapMV), papaya leaf distortion mosaic virus (PLDMV), papaya meleira virus (PMeV) and papaya lethal yellowing virus (PLYV) (Paolla et al. 2015). These viruses are also of international importance, significantly reducing yield and fruit quality (Paolla et al. 2015).

PRSV was first reported in Hawaii in 1945 (Jensen 1949; Ferreira et al. 1992) and since then has become the primary concern of Hawaii's papaya industry. The incidence of PRSV in the field can reach 80 to 100% (Ventura 2004; Ye and Li 2010; Hamim, *personal observation*). Papaya is often found growing as a "volunteer" along roadsides and in wild areas where it is a source of PRSV inoculum (Alabi et al. 2016).

PRSV is in the family *Potyviridae,* genus *Potyvirus* and has linear, flexuous rod-shaped virions (Purcifull et al. 1984; Yeh et al. 1992). The single-stranded, positive sense RNA genome is about 10,000 nucleotides in length and encodes a single large polyprotein. This protein is cleaved into eight smaller functionally active proteins: P1, HC-Pro, P3, CI, 6K, NIa-Pro, NIb and CP. Moreover, some suggest another protein P3N-PIPO is produced from a different small ORF overlapping the with P3 coding region (Chung et al. 2008; Fermin et al. 2015). There are two types of PRSV: the P-type that infects papaya, and the W-type that only infects the cucumber family (Adams et al. 2005; Yeh et al. 1992).

PRSV is spread from diseased to healthy plants in a non-persistent manner by several species of aphids (Jensen 1949; Purcifull et al. 1984; Tripathi et al. 2008) such as *Aphis nerii* (Boyer de Fonscolombe), *A. gossypii* (Glover), *A. spiraecola* (Patch), *Myzus persicae* (Sulzer), *Toxoptera aurantii* (Boyer de Fonscolombe), *A. craccivora (*Koch), and *Rhopalosiphum maidis* (Fitch) (Namba and Higa 1981; Prasad and Sarkar 1989; Wang 1981).

The virus systemically infects its host, causing severe mosaic symptoms on leaves and water-soaked streaks on leaf petioles and the trunk of the plant (Fig 1.1). In severe cases, plants have distorted shoestring-like leaves, reduced photosynthetic capacity, and sometimes develop systemic necrosis and wilting (Purcifull et al. 1984). When PRSV infects papaya at an early

stage, plants are stunted and do not develop fruit. Infected mature plants typically produce poor-quality fruit with bumps or ring-like spots and a low sugar concentration (Gonsalves 1998).

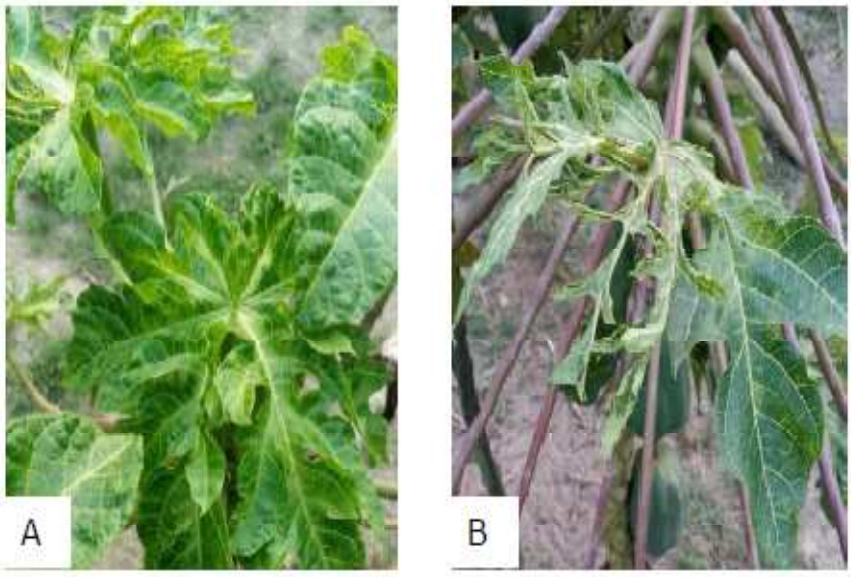


Fig ure1.1. Papaya plants severely affected by PRSV. (A) Leaf mosaic and chlorosis; (B) narrow, distorted, ‘shoestring-like’ leaves

Many efforts have been made to manage papaya ringspot disease, including: vector control by chemical or biological agents, disease-tolerant varieties, cross-protection, planting in virus-free areas, rouging, planting times during low populations of winged aphids, inter-cropping with barrier crops, and growing papaya inside insect exclusion nets. None of these methods have proven effective in the field (Paolla et al. 2015; Yeh et al. 2010; Swain and Powell 2001; Yeh and Gonsalves 1994; Azad et al. 2013; Tripathi et al. 2008). Cross-protection was attempted on the island of Oahu and in Taiwan with a mild form of nitrous acid mutant from PRSV strain HA 5–1 from Hawaii (Yeah and Gonsalves et al. 1994; Yeh et al. 2014**)**. This method did not provide long-lasting economic benefits, especially since it required continuous production of mild strains and inoculation of plants. Furthermore, the mild strain caused significant symptoms on some commercial cultivars including the popular 'Sunrise' **(**Ferreira et al. 1992**)**. Therefore, cross-protection was not used widely because of its shortcomings, initiating transgenic papaya research in 1985 (Gonsalves et al.2004; Gonsalves 1998).

In the 1980s, Roger Beachy's research group reported that transgenic tobacco plants expressing the coat protein gene of tobacco mosaic virus (TMV) postponed the expression of TMV (Abel et al. 1986). This discovery motivated research in the fields of agriculture and plant science to apply the approach to the development of virus-resistant transgenic plants for commercial crops. The approach was initially called "parasite-derived resistance" (Sanford and Johnston 1985) and later changed to pathogen-derived resistance (PDR). In PDR, genes from the pathogen are inserted into the desired plants to develop resistance to a pathogen and those closely related to it (Baulcombe et al. 1996; Beachy et al. 1997). PDR in transgenic papaya governs gene expression through post-transcriptional gene silencing (PTGS) (Tennant et al. 2001; Ruanjan et al. 2007). Generally, gene silencing operates either at transcriptional or post-

transcriptional stages. It employs genes that have the regions of maximum similarity to the targeted genes and is evident by decreased amounts of steady-state messenger RNA (mRNA) of the transgene and targeted gene (Fagard and Vaucheret 2000; Matzke and Matzke, 1998). Transcriptional gene silencing results in reduced transcription due to termination of the promoter. Transcription occurs with PTGS, but mRNA is degraded prior to translation, resulting in reduced levels of gene product in the cytoplasm. Between the two gene silencing systems, PTGS (de Carvalho et al. 1992) has been linked to RNA-mediated transgenic resistance (Baulcombe 1996). Therefore, the resistance is referred to either as homology-dependent PTGS or homology-dependent PDR (Baulcombe 1996) to define the specific resistance mechanism. Furthermore, scientists have confirmed post-transcriptional gene silencing in transgenic virus-resistant papaya by the presence small interfering RNA (siRNA) (Ruanjan et al. 2007), a hallmark of the PTGS mechanism.

Investigations by Beachy's group encouraged the research group of Dr. Gonsalves from Cornell University and Dr. Manshardt at the University of Hawaii at Manoa to collaborate on the development of PRSV CP-transgenic resistance in papaya in 1986 (Gonsalves 2002). The Hawaiian papaya industry was in a great crisis at that time. Gonsalves and his collaborators used the mild Hawaiian PRSV strain HA 5-1 as the source of the CP gene for the transgene construct since the aim was to generate transgenic resistant papaya resistant to Hawaiian PRSV strains (Quemada et al. 1990). The transgene was designed to facilitate the CP-gene translation, as it was thought that the CP was necessary for PDR (Table 1.1). When the CP of PRSV is generated through post-translational protease cleavage, there are no indigenous translation signals specifically available for the CP sequence. Thus, a chimeric gene was used to translate signals present in the leader sequence of the cucumber mosaic virus (CMV) CP gene linked in frame to

the sequence of the PRSV CP (Ling et al. 1991). In 1988, papaya researchers started using the biolistic approach to transform embryogenic cultures of papaya (Fitch et al. 1992). They focused on transforming the Hawaiian cultivars Sunrise, Sunset, and Kapoho. These commercial cultivars breed true to type and are exclusively grown in the main papaya production region of Hawaii. The results of their transformation with the transgene were excellent. Fitch and associates (1992) pioneered the transformation of papaya using the biolistic approach by targeting embryogenic cultures, creating 17 independently transformed cultivars (Fitch et al.1990). They immediately tested the resistance of the R0 lines against a severe Hawaiian strain called PRSV HA 5-1 (Fitch et al.1992; Tennant et al.1994, 2001). In 1991, they identified a single resistant line designated '55-1' that was resistant to PRSV HA in greenhouse experiments (Fitch et al. 1992). This line was the red-fleshed cultivar Sunset, a less desirable fruit than the yellow-fleshed 'Kapoho.' In 1992, '55-1' was also found to be resistant to PRSV in a field test at the Waimanalo Field Station of the University of Hawaii. In addition, two new transgenic cultivars, SunUp and Rainbow were developed. Transforming the red-fleshed 'Sunset' with the coat CP gene produced the homozygous 'SunUp' and the hemizygous 'Rainbow' wasan F1 hybrid developed by crossing 'SunUp' with the yellow-fleshed nontransgenic 'Kapoho' (Manshardt 1998).

The trial in 1992 validated the PRSV resistance of transgenic plants in the field. In 1995, a crucial field trial of 'Rainbow' and 'SunUp' papaya was conducted in the Puna District of Hawaii Island where most commercially papaya is grown. The results conclusively displayed that the transgenic 'SunUp' and 'Rainbow' were resistant, of acceptable commercial quality and had superior yields compared to nontransgenic 'Kapoho' (Ferreira et al. 2002). Moreover, the yellow- fleshed 'Rainbow' bore mature fruit earlier than the 'Kapoho,' impressing the papaya growers of Puna. Within four years of the development of these varieties, papaya production in

Hawaii had returned to pre-PRSV levels. In 1998, another popular papaya variety, 'Kamiya,' was transformed with the CP gene (untranslatable) derived from HA5-1 or its replicase gene. Transformants generated using both genes were immune to PRSV in laboratory and greenhouse trials (Fitch 2002). Another complex hybrid is between F2 plants of 'Kamiya' and 'Rainbow,' which was patented as 'Laie Gold' showed PRSV resistance in a field trial (Fitch et al. 2010). Resistance to plant viruses through the development of transgenics is free from the problems of cross-protection, such as inoculation costs, possible mutation of the cross-protecting strain to a more virulent strain, movement of the mild strain of the virus to other crops, and adverse effects of attenuated strains on papaya plants (Yeh and Gonsalves 1984). Therefore, the Hawaiian scenario confirmed that use of the PRSV-resistant transgenic papaya was a realistic solution for the control of PRSV. Transgenic papaya cultivars have been grown commercially in Hawaii since 1998 and to date maintain resistance to PRSV (Tripathi et al. 2008; Gonsalves 1998; Stokstad 2008; Gonsalves 2002; Yeh et al. 2010; Hu, *personal observation*).

PRSV isolates from different geographical regions of the world are genetically diverse (Tripathi et al. 2008). Zhao and associates (2017) inoculated transgenic papayas from Hawaii with a PRSV strain from Hainan and observed the typical development of PRSV symptoms Therefore, the Hawaiian CP-transgenic cultivars may not be useful in regions outside of Hawaii (Azad et al. 2013). The success of transgenic papaya in Hawaii has encouraged other papaya-cultivating states of the U.S. and other countries to develop transgenic papayas resistant to their local PRSV isolates. Resistant transgenic varieties of papaya have been developed in Florida (US), Brazil, China, Jamaica, Indonesia, Malaysia, Thailand, Venezuela, the Philippines and Australia (Bau et al. 2003; Cheng et al. 1996; Davis and Ying 2004; Lines et al. 2002; Tripathi et al. 2008).

Davis and Ying (2004) reported transformation of the papaya cultivar 'L. cv. F65' by *Agrobacterium*-mediated transformation with four different constructs: the unmodified or modified CP gene, i.e. sense orientation (S), antisense orientation (AS), sense orientation with a frame-shift mutation (FS), or sense orientation mutated with three in-frame stop codons (SC) of Florida PRSV isolate H1K. They obtained 256 putative transgenic lines with the CP constructs and challenged these lines with PRSV H1K. Highly resistant lines were found in the different transgene groups (Table 1.1). These lines were crossed with six different genotypes of papaya. The transgenic lines derived from the FS/CP and SC/CP transgene groups showed high fertility, but the plants from the S/CP and AS/CP transgene groups were infertile. In a field test, 23.3% of the fertile transgenic plants became infected with PRSV, whereas 96.7% of the non-transgenic control plants became infected (Davis and Ying 2004).

Zimmerman et al. (2007) transformed somatic embryos of papaya lines 'Washington' and 'Yuen Nong' with *A. tumefaciens* containing the CP gene of a PRSV strain from the Virgin Islands (Table 1.1). Regenerated plants were resistant to PRSV.

Lines and associates (2002) reported the development of two Australian transgenic papaya cultivars that were immune to infection with PRSV (Table 1.1). Somatic embryos from the commercially grown 'GD3-1–19' and 'ER6-4' in Queensland were transformed with a particle inflow gun using a construct containing an untranslatable PRSV CP-coding region. Two transgenic lines demonstrated immunity to PRSV following repeated inoculations in the greenhouse and field.

In the 1990s, transgenic papayas resistant to PRSV in Brazil were developed in the U.S. by visiting Brazilian scientists M. Souza and associates (2005, Table 1.1). They were later

transferred to the Brazilian Agricultural Research Corporation (EMBRAPA) under a technology transfer program. A particle bombardment gun was used to insert translatable or non-translatable CP genes of the Brazilian PRSV strain into somatic embryos derived from 'Sunrise' and 'Sunset.' A greenhouse study tested 26 regenerated lines containing the translatable CP gene and 28 regenerated lines containing the untranslatable CP gene. They were inoculated with three different virus isolates from Brazil, Hawaii and Thailand and revealed mixed resistant reactions (mono-, double- and triple-resistance) (Souza et al. 2005).

Chen et al. (2001) introduced a viral replicase (RP) gene conferring transgenic resistance to PRSV in papaya (Table 1.1). Embryogenic calli of the cultivar Tai-nong-2 were transformed by *A. tumefaciens* harboring the pRPTW vector. This vector was constructed orientating the 3′-truncated and 5′-extended RP gene fragments under the control of the CaMV35 S promoter and nonpaline synthase gene (NOS) termination sequence in the mini Ti plasmid vector pRok. PRSV inoculation tests indicated that the RP gene conferred resistance to PRSV in regenerated transgenic papayas. In 2006, the People's Republic of China deregulated this PRSV-resistant transgenic papaya, 'Huanong No. 1,' for commercial production (Guo et al. 2009). This cultivar elicits PDR, but uses the replicase gene from southern China instead of the CP gene (Azad et al. 2013; Tripathi et al. 2008). There was no loss of resistance observed in the transgenic papaya plants with the replicase gene for the first 5 to 6 years (Mendoza et al. 2008). However, an example of a loss of resistance in 'Huanong No. 1' has been observed in recent years.

In Jamaica, transgenic papaya lines containing either the translatable or non-translatable CP genes were tested for PRSV resistance in the field (Tennant et al. 2005) (Table 1.1). Transgenic lines with the translatable CP gene had a much higher field resistance (80%) than lines with the non-translatable CP gene (44%).

Scientists at the University of the Philippines, Los Banos, initiated the development of transgenic papaya with resistance to a virulent strain of PRSV isolated from Cavite, Philippines (Villegas et al. 2001) (Table 1.1). The transgenic lines were moderate to highly susceptible to PRSV in a field evaluation.

In Taiwan, embryogenic tissues from immature zygotic embryos of the Tainung No. 2 cultivar were used for *Agrobacterium*-mediated transformation using the binary vector pBGCP (Cheng et al. 1996) containing the CP gene of the PRSV YK strain, a severe virus strain from Taiwan (Table 1.1). Yeh and colleagues (1998) tested 38 transgenic lines for PRSV resistance: two lines demonstrated immunity, nine lines showed high resistance, and eight lines produced moderate resistance (Yeh et al. 1998). Three transgenic lines were selected for evaluation under field conditions. Less than 0.2% of the transgenic lines were infected with PRSV 12 months after planting, while 100% of the control plants were infected 8 months after planting. To deal with the emerging problem of papaya leaf distortion mosaic virus (PLDMV) infection, dual resistance was attempted using a transgenic papaya carrying a chimeric construct containing partial CP genes of PRSV and PLDMV (Bau et al. 2008). A transgenic papaya line was also developed using an untranslatable construct targeting the PTGS-suppressor protein (HC-Pro) of PRSV superstrain 5-19, which provided resistance to PRSV strains in Taiwan and other geographical locations (Yeh et al. 2010; Kung et al. 2015).

Thai scientists at Cornell University (USA) in 1995 transformed ‘Khak Dum’ and ‘Khak Nual’ using microprojectile bombardment (Gonsalves 1998) (Table 1). The transformed lines were then to Thailand for virus resistance evaluations. The transformed ‘Khak Nual’ showed excellent field resistance to PRSV (97% to 100%) and had a higher yield than non-transgenic

papaya (Sakuanrungsirikul et al. 2005). Another transgenic line 'Khakdam,' had 100% field resistance and higher yields than the non-transgenic variety (Phironrit et al. 2005).

In collaboration with Dennis Gonsalves, the first transgenic papaya in Venezuela was developed in 1993 by the University of the Andes. They used the CP gene from two different PRSV strains isolated from local papaya cultivars and *Agrobacterium*-mediated transformation. Regenerated plants and their progenies were resistant to local PRSV strains in glasshouse testing (Fermin et al. 2004).

Despite these successes, there have been reports of failed transgenic resistance of papaya in China and Taiwan (Tripathi et al. 2008; Kung et al. 2015). Different factors may be involved in this loss of resistance, including: loss of sequence identity between the transgene and the virus population, emergence of new viruses or strains, gene silencing suppressors, transgene dosage, plant developmental stage, and growing temperature during the cropping season. I discuss these factors, including ways to establish a durable, nonspecific system to develop transgenic resistance in papaya. Of note, it is work with the Hawaiian transgenic papaya and transgenic papaya developed in other regions that has contributed to a better understanding of the factors affecting transgenic virus resistance in papaya. Therefore, knowledge from this review will give researchers further direction for effective management of PRSV.

Table 1.1. Transgenic lines developed in different countries to date and their resistant properties

Country	**Cultivar**	**Virus protein used in the construct**	**Transformation mechanism**	**Resistant properties**	**References**
Australia	Local variety	Translatable CP	Biolistics	Immune to infection with PRSV	Lines et al. 2002
Brazil	Sunrise or Sunset	Translatable or Untranslatable CP	Biolistics	Mixed resistant reactions to isolates from Brazil, Hawaii and Thailand	Souzaet al. 2005
China	Tai-nong-2	3′-truncated and 5′-extended RP gene fragments	*Agrobacterium tumefaciens*	Initially resistant to local PRSV isolates, but recently loss in resistance observed to newly emerged PRSV strains	Chen et al. 2001
	Sunrise	A 544-bp conserved region of the PRSV CP gene	Biolistics	Resistance to PRSV local strains	Jia et al. 2017
Florida (US)	CV.F65	Translatable or Untranslatable CP	*Agrobacterium tumefaciens*	Resistant to Florida isolate H1K of PRSV	Davis et al. 2004
Hawaii(US)	Sunset	Translatable CP	Biolistics	Resistant to a severe Hawaiian strain called PRSV HA 5-1	Tennant et al.2005; Ferreira et al.2002
Jamaica	Sunrise	Untranslatable CP or Translatable CP	Biolistics	Transgenic lines with the translatable CP gene showed higher field resistance compared to lines with the non-translatable CP gene	Tennant et al.2002;Tennant et al. 2005
Taiwan	Tainung no. 2	Translatable CP	*Agrobacterium tumefaciens*	Initially showed high resistance to PRSV YK strain, later PLDMV overcame the resistance	Yeh et al. 1998, Yeh et al. 2010
	Tainung no. 2	Untranslatable chimeric construct containing partial CP genes of PRSV and PLDMV	*Agrobacterium tumefaciens*	Initially resistant to PLDMV and PRSV-YK, later PRSV super strain 5-19 weakened the resistance	Yeh et al. 2010; Kung et al. 2009

Country	Cultivar	Type of virus protein used in the construct	Transformation mechanism	Resistance properties	References
	Tainung no. 2	Untranslatable construct targeting PRSV HC-pro of PRSV superstrain 5-19	*Agrobacterium tumefaciens*	Resistance to PRSV strains in Taiwan and other geographical locations	Yeh et al. 2010; Kung et al. 2015
Thailand	Khak Dum and Khak Nual	Translatable CP	Biolistics	Excellent field resistance	Sakuanrungsirikul et al. 2005; Phironrit et al. 2005
Venezuela	Thailand red	Translatable CP	*Agrobacterium tumefacien*	Resistant to local PRSV strains	Fermin et al. 2004
Virgin Islands	Washington and Yuen Nong	CP gene of PRSV strain from the Virgin Islands	*Agrobacterium tumefaciens*	Resistance to PRSV	Zimmermanet al. 2007

I. Factors affecting loss of resistance in transgenic papaya

1. Sequence homology between transgene and an infecting PRSV CP gene

To express successful resistance in transgenic papaya, high nucleotide homology is required between the transgene and the challenged virus CP gene. Transgenic virus resistance in papaya is sequence specific and provides resistance to only closely related strains of the virus (Baulcombe 1996; Tennant et al. 2001). The transgenic lines developed in Hawaii are highly resistant to local PRSV strains. These lines were obtained by transferring the CP gene of mild Hawaiian strain HA 5-1 into papaya and no loss of resistance has been observed in more than two decades. (Yeh et al. 2010; Gonsalves 2002; Fitch et al. 1992; Lius et al. 1997; Fuchs and Gonsalves 2007; Hu, *personal observation*). However, these transgenic lines are susceptible to PRSV isolates from outside of Hawaii (Tennant et al. 1994). The continued use of these transgenic cultivars in Hawaii relies on their resistance to Hawaii's PRSV strains and the absence of foreign strains that may enter Hawaii. Thus, Dr. Tennant and her colleagues (2001) showed that PRSV resistance in commercial transgenic papayas is affected by the sequence homology of the invading strains to the PRSV transgene (Tennant et al. 2001). They provided experimental evidence that the PRSV HA 5-1 strain from Hawaii could not overcome the resistance of 'Rainbow' or 'SunUp.' However, a recombinant virus of PRSV HA could infect both of these cultivars (Chiang et al. 2001). In this recombinant virus, the CP gene was replaced with the PRSV YK-CP gene. The CP of the resulting virus has less than 89% sequence homology to the original CP transgene.

Detailed comparative studies were made of CP-gene sequences from various PRSV isolates from different countries and the resistance responses of transgenic papaya cultivars (Tennant et al. 2001; Tripathi et al. 2008). Transgenic resistance was positively correlated with the degree of homology between the CP of the infecting PRSV strain and the transgene. PRSV isolates from

Hawaii had 97 to 100% sequence homology to the transgene CP, but isolates from other regions had only 89 to 93% CP sequence homology. Tennant et al. (2001) compared the nucleotide identities of the isolates from Hawaii (HA, OA, KA and KE), Jamaica (JA), Brazil (BR), and Thailand (TH) with the sequence of the severe Hawaiian strain PRSV HA 5-1 (Quemada et al. 1990) used in screening resistant lines in Hawaii. The identities ranged from 89.5% to 99.8%. Isolates from Hawaii had the highest sequence identities with PRSV HA 5-1 (96.7 to 99.8%). Interestingly, some of the isolates from Hawaii (OA, KA and KE) that had the lowest sequence homology to the transgene had the highest incidence of infection on hemizygous. The most distantly related isolate to the transgene was PRSV isolate TH, which caused severe symptoms on all transgenic papaya plants. As expected, the core region of the CP gene was most conserved between isolates with percent similarities from 97 to 99% between the Hawaiian isolates and PRSV HA 5-1, and 90 to 95% between PRSV HA 5-1 and isolates outside of Hawaii. Similarly, the C terminus of the CP gene was highly conserved among the isolates (91 to 100%). The most variable region was the N terminus. Percent similarities at the N terminus among isolates outside of Hawaii and PRSV HA 5-1 were 83.7%, 84.4%, and 89.3% for isolates from Thailand, Brazil, and Jamaica, respectively. In contrast, similarities of 95.3 to 99.3% were observed among Hawaiian isolates for the N terminal region (Tennant et al. 2001).

2. Emergence of recombinant strains

Plant viruses may undergo recombination and produce new strains expressing distinctly different disease symptoms. These recombination events can occur between similar viruses co-infecting a non-transgenic plant or between an infecting virus and a transgenic plant expressing mRNA with sequences from the target virus (Bau et al. 2003, 2008; Kung et al. 2009, 2015). These recombination events can initiate the emergence of new PRSV strains (Chaves-Bedoya

and Ortiz-Rojas 2015; Mangrauthia et al. 2008, 2010; Valli et al. 2007) which are important events in PRSV evolution (Mangrauthia et al. 2008; Ohshima et al. 2007). The emergence of more virulent recombinant strains is a concern when growing transgenic papaya cultivars with PRSV resistance. Chiang and colleagues (2001) reported that the infectivity of recombinant PRSV is influenced more by positional effect than by the degree of sequence identity between the recombinant CP gene and the transgene. They demonstrated that recombinant strains of PRSV can overcome the resistance of transgenic papaya cultivars, producing mild to severe symptoms corresponding to the region of substitution within the CP gene. Furthermore, transgenic 'Rainbow' was resistant to PRSV HA but produced severe symptoms when challenged with PRSV YK from Taiwan or with a recombinant PRSV HA containing the CP gene of YK. PRSV HA recombinants with fewer CP gene segments from PRSV YK induced milder symptoms on 'Rainbow' than those of the entire gene. Interestingly, an HA recombinant containing a segment of the YK CP gene from the 5' region, which had the lowest comparative nucleotide sequence homology to the transgene, induced very mild PRSV symptoms compared to recombinants containing a segment of the YK CP gene from mid- and 3'-end region (Chiang et al. 2001). This suggested that a lack of sequence homology was not the only factor in overcoming resistance.

The PRSV recombinant YK/AS contained a 263-nucleotide (nt) YK segment that had 82 to 92% sequence identity to the corresponding region of the transgene. It induced very mild symptoms on 'Rainbow' in contrast to the more prominent symptoms induced by two other recombinants, YK/SE and YK/EN, which have 89-95% and 82-100% nt similarity respectively to the transgene. Moreover, the length of the YK replacement segment did not account for the different symptoms induced, since the YK segments in the YK/EN and YK/AS recombinants

were similar in length. The variable symptoms that the PRSV recombinants produced on ‘Rainbow’ were evidently not due to their inherent capability to replicate, as all the recombinants induced severe symptoms on non-transgenic papaya plants. Therefore, recombination is another factor apart from sequence homology in reducing transgenic resistance. There is a possibility of resistance-breaking PRSV strains emerging through recombination events between PRSV strains from Hawaii and transgenic papaya recombination events between PRSV strains from Hawaii and transgenic papaya that express the CP or other genes of PRSV strains that could overcome the resistance of transgenic papaya in Hawaii (Chiang et al. 2001).

The transgenic lines targeting the YK CP-3’ untranslated region (UTR) developed in Taiwan showed resistance to PRSV strains from Hawaii, Mexico, and Thailand (Bau et al. 2003). However, to evaluate transgenic resistance, Kung et al. (2015) constructed a virulent recombinant PRSV by replacing the PRSV HC-Pro region with the PRSV 5-19 HC-Pro region to evaluate transgenic resistance. The newly constructed PRSV recombinant strain decreased transgenic resistance in a sequence-homology independent manner. Interestingly, the sequences of the transgene transcript shared 97±1% nt identity with the genome of the infecting virus (Bau et al. 2003, 2004; Tripathi et al. 2008; Bau et al. 2008). This indicated that the resistance-evading phenomena did not depend on a difference in sequence divergence between the YK CP-3’UTR transgene sequence from the transgenic papaya lines and the respective PRSV strain (Tripathi et al. 2004; Kung et al. 2015). It has been suggested that CP-transgenic plants may also promote the development of mutants or variants within the natural PRSV population (Kung et al. 2015).

The transgenic papaya ‘Huanong No. 1’ developed in China maintained PRSV resistance for several years in the field (Mendoza et al. 2008), but a reduction in resistance against field isolates of PRSV has recently been observed. Zhao et al. (2015) determined the complete genome

sequence of PRSV isolate KF791028 from transgenic 'Huanong No. 1,' which had the maximum sequence similarity (92%) to the other Hainan PRSV isolates EF183499, HQ424465, and KF734962 and possessed the least similarity (81%) to the Hawaiian isolate EU126128 (Lu et al. 2008; Zhang et al. 2014). It is assumed that this new PRSV variant evolved in transgenic papaya through recombination within Hainan's PRSV population (Kung et al.2015; Zhao et al. 2015).

3. Virus-encoded PTGS suppressors

Viruses with moderate to large genomes encode functionally distinct, highly diverse proteins. Some of these proteins help suppress the PTGS pathway (Dıaz-Pendon and Ding 2008; Ding and Voinnet 2007). Many scientists accept that these suppressor proteins interfere with the biogenesis of siRNAs. The potyviral HC-Pro, for example, explicitly inhibits accumulation of secondary siRNAs, but not primary siRNAs (Dıaz-Pendon and Ding, 2008). Several investigators agree that sequence homology of the virus to the transgene is not the only viral factor employed in defeating transgenic resistance produced by PTGS (Pruss et al. 1997; Anandalakshmi et al. 1998; Kasschau and Carrington 1998). They consider viral suppressors as important factors in the failure of virus-resistant transgenic plants, along with increasing virus pathogenicity and enhanced synergism between viruses.

The HC-Pro gene of potyviruses changes the deposition of endogenous micro RNAs (miRNAs) indicating that symptoms expressed by viruses might be the abnormal miRNA metabolism (Mallory et al. 2002). Mangrauthia and colleagues (2009) reported that PRSV-resistant transgenic plants face a crucial barrier in obtaining resistance due to the involvement of PRSV-HC-Pro as a suppressor in the RNA silencing mechanism. HC-Pro binds miRNA, interfering with miRNA-directed regulatory pathways of plants and affecting their

developmental biology. The suppressor HC-Pro facilitates establishment of the infecting PRSV and also has a strong positive synergism with other heterologous viruses. PRSVHC-Pro does not bind dsRNA but has a strong affinity toward miR171 duplexes. This distinct feature indicates that HC-Pro might use a sequestration model to operate a suppressor function (Merai et al. 2006; Lakatos et al. 2006), in which viral suppressor proteins prevent siRNA from assembling with the RNA-induced silencing complex (RISC) responsible for PTGS. HC-Pro is a strong silencing suppressor and could reduce PRSV resistance in transgenic papaya (Tripathi et al. 2004; Ruanjan et al. 2007). The resistance of transgenic YK CP-3'UTR lines was overcome by the virulent strains 5-19, CS and TD2, due to the silencing suppressor gene HC-Pro (Kung et al. 2015). In a separate study, these researchers investigated the mechanism of the homology-independent breakdown of CP-transgenic resistance by strain 5-19 (Kung et al. 2015). Analyses of the reactions of single-virus PRSV-resistant and double-virus PRSV+PLDMV-resistant lines to the transgene-donating strain YK, the resistance-evading strain 5-19, and their recombinants have been completed. The results of the recombinant analysis indicated that 5-19 HC-Pro was a PTGS suppressor and may that may have suppressed the RNA silencing mechanism in the YK CP-3'UTR transgenic lines resulting in the loss of PTGS-mediated resistance (Kung et al. 2015).

Kung and colleagues (2015) also evaluated the gene-silencing suppression mechanism of the HC-Pro gene of strain 5-19 in a transient expression system developed in the model plant, *Nicotiana benthamiana*. They determined that 5-19 HC-Pro was a stronger suppressor of gene-silencing than YK HC-Pro. The 5-19 strain might have emerged in non-transgenic plants and then been transmitted to transgenic papayas by aphids. The superior ability of the 5-19 strain to suppress PTGS allowed it to overcome CP-transgenic resistance in a sequence-homology independent manner. Gene silencing suppressors can disrupt PTGS pathways at multiple points,

inhibiting the plant's defense against their actions. This blocking or attenuation inhibits host defense responses by acting against essential elements of the cellular silencing system and over-simulating their normal cellular activities (Mangrauthia et al. 2008, 2009).

4. Plant developmental stage

Different aspects of plant physiology can influence PTGS in the field. Sometimes, a temporary loss of resistance in transgenic plants appears during certain stages of growth and development (Davis and Ying 2004). Studies with papaya plants displaying RNA-silencing-mediated resistance suggest an influence of plant developmental stage on expressed resistance. Transgenic papaya plants were susceptible to inoculation with PRSV at a younger stage, but resistant at an older stage (Tennant et al. 2001). Some studies support the idea that younger transgenic plants accumulate fewer transgene-specific siRNAs than older plants, or correspondingly accumulate greater amounts of transgene-specific transcripts. These findings suggest that transgenic resistance against plant-infecting viruses is less efficient in younger plants (Kalantidis et al. 2002).

5. Transgene copy number

The number of transgene copies plays a role in the level of virus resistance in transgenic papaya cultivars. Cultivars with a single transgene copy show less resistance to target viruses than cultivars with multiple copies (Tennant et al. 2001). This effect is also found with PTGS-mediated silencing and PRSV resistance in the original Hawaiian transgenic papayas (Lines et al. 2002). The R1 transgenic papaya of line 55-1 is resistant to PRSV isolates from Hawaii but susceptible to foreign strains. Line 55-1 R1 plants like 'Rainbow' are hemizygous for the CP transgene and have a narrow, specific resistance to certain PRSV strains. When 'Rainbow' was inoculated with PRSV strains from Mexico in greenhouse trials it was resistant, but strains from

Thailand, Australia, and Brazil produced symptoms of ringspot disease. Increasing the transgene copy number expanded resistance to foreign PRSV strains. 'SunUp' is homozygous for the CP gene and resistant to some, but not all, PRSV strains outside of Hawaii (Tennant et al. 1994, 2001). Line 63-1 has a double insertion of the CP gene and is resistant not only to isolates of PRSV from Hawaii, but also to those from Jamaica, Thailand and Brazil (Tennant et al. 2005; Souza et al. 2005). McDonald et al. (1997) reported that homozygous transgenic tobacco lines with the transgene of potato virus Y strain N (PVYN) were resistant to three different strains, PVY, PVYN, and PVY-36, whereas hemizygous transgenic plants were less resistant. Transgenic plants resistant to lettuce mosaic virus were of homozygous progenies conferring resistance to three lettuce mosaic virus isolates, LMV-1, LMV-E, and LMV-13. However, their hemizygous progenies were not virus resistant (Dinant et al. 1997).

6. Environment

Environmental temperature influences virus resistance in transgenic plants. Ye and Li (2010) reported that transgenic papaya with a high resistance to PRSV showed mosaic symptoms during the colder temperatures of early spring. Symptom expression and virus accumulation in papaya cultivar Pusa Nanha were greatest at temperatures between 26 and 31^{o}C (Mangrauthia et al. 2009). However, there was a marked reduction in virus accumulation and symptom expression at 10^{o}C above or below the 26 to 31^{o}C range. The HC-Pro protein of PRSV showed a temperature-related affinity for small RNAs (sRNAs). In one assay, recombinant PRSV HC-Pro bound to 21-nt double-stranded miRNAs more efficiently at ambient temperatures (25^{o}C) than at high (35 to 45^{o}C) or low (15^{o}C) temperatures (Mangrauthia et al. 2009). A mobility shift of sRNAs was not detected during incubation with PRSV HC-Pro at either high or low temperatures. This suggested a plausible role of HC-Pro in temperature-directed, host–virus interactions in papaya

(Mangrauthia et al. 2009). Vela´zqueza and associates (2010) observed that an immediate shifting of temperature from 25^{o}C to 40^{o}C drastically reduced ssRNA synthesis. But, the changes in temperature occasionally affected synthesis of dsRNA replication or intermediate forms until the synthesis of ssRNA had shut down. Higher amounts of dsRNA deposition after an increase in temperature could initiate PTGS.

7. Emergence of new viruses

The introduction of a new virus into a region may create a synergistic interaction or an additive effect with an existing virus and may overcome single-copy transgene resistance in papaya (Bau et al. 2003, 2008; Kung et al. 2019, 2015). Multiple viral infections that may result in synergism or an additive effect frequently are found in nature, with an unpredictable lossof transgenic virus resistance. Synergistic interactionsarea result of mixed infections among two or more viruses in a plant. They may result in an increase in the titers of one or more of the viruses and result in enhanced symptoms (Karyeija et al. 2000; Pruss et al. 1997). Several scientists found that the introduction of a new virus results in an additive effect on the existing virus through a dramatic increase in virus titers and disease symptom expression (Calvert and Ghabrial 1983; Savenkoy and Valkonen 2001). In a complex situation, a pronounced synergistic effect could result in a drastic reduction in plant height, weight, and yield, with severe disease symptoms leading to plant death (Murphy and Bowen 2006). Multiple infections of PRSV and one or more viruses, such as PLCV, PapMV, PLDMV, PMeV or PLYV, can increase virus accumulation and symptom expression and reduce resistance in transgenic papaya (Bau et al. 2008; Daltro et al. 2012; Noa-Carrazana et al. 2006; Ventura et al. 2003; Kung et al. 2015). In Taiwan, a transgenic papaya with broad resistance was developed using CP-3'UTR from local PRSV strains. Infection by both PLDMV and PRSV interfered with plant resistance in the transgenic line and caused

severe leaf distortion in both transgenic and non-transgenic papayas (Bau et al. 2008; Kung et al. 2009). Both viruses cause similar symptoms in papaya, such as mosaic and leaf discoloration, distortion of leaves, water-soaked oily patches on petioles and ring-like spots on fruits (Bau et al. 2008, Cruz et al. 2009).

II . Potential ways to develop durable, broad-spectrum transgenic resistance against PRSV

1. Use of transgenes from local or resistance-breaking PRSV strains

PRSV-CP-mediated transgenic resistance relies on the homology between the transgene and the coat protein of the virus. Using the CP gene of a native, widespread strain of PRSV is a requirement for potent resistance in a specific geographic area (Gonsalves 2002). Successful use of the CP gene of a native PRSV strain to transform indigenous commercial papaya cultivars has been reported from several countries. An untranslatable PRSV CP-coding region used as a transgene to generate two transgenic papaya cultivars in Australia displayed immunity to the native PRSV isolate (Lines et al. 2002). Fermin and colleagues (2004) constructed PRSV-resistant transgenic papaya plants by transforming them with the CP genes of PRSV isolates from two separate locations in Venezuela. All these transgenic lines and their progenies showed various levels of resistance to PRSV isolates from Hawaii and Thailand. Scientists in Florida, U.S.A developed PRSV-resistant papaya lines using the CP gene of a local strain (Davis and Ying 2004). The resulting transgenic resistance was transferred to popular papaya cultivars through a traditional breeding program. Additionally, the researchers used the truncated replicase gene (RP) of PRSV as a transgene and developed a PRSV-resistant transgenic papaya using an *Agrobacterium*-mediated transformation system (Davis and Ying 2004). In 2015, scientists from Taiwan developed transgenic papaya plants using untranslatable constructs of HC-Pro from a highly virulent PRSV strain. The plants were resistant to the newly emerging, more virulent virus

strains and recombinant strains known to overcome CP-mediated resistance in transgenic papaya plants (Kung et al. 2015).

2. Use of multiple virus genes or virus segments

Several viruses, or strains of the same virus, can threaten papaya production in a specific area. Therefore, new transgenic papaya lines would include resistance to these viruses. Gene pyramiding inserts multiple virus transgenes into the same plant, producing a plant resistant to these viruses (Yeh et al. 2014). A research group from Taiwan used chimeric constructs to develop resistance to two different potyviruses, PRSV and PLDMV (Kung et al. 2009). This approach may protect papaya from new strains or non-target viruses if mRNA expressed by the transgenes can fold into hairpin structures that induce gene silencing (Schumann et al. 2013; Agrawal et al. 2003). Fermin and Gonsalves (2001) created tospovirus-resistant *Nicotiana benthamiana* by inserting a chimeric transgene consisting of a 200-bp N-gene segment from the tospovirus.

Another method of developing broad resistance in transgenic papaya is to use synthetic transgenes with a high nucleotide similarity to the targeted viruses (Fermin-Munoz 2002; Fermin and Gonsalves 2004). This novel approach does not use existing CP genes or chimeras and avoids multiple transformations with the desired sequences.

Fermin and Gonsalves (2001) developed transgenic plants using a synthetic transgene with 90% homology to tomato spotted wilt virus and groundnut ringspot tospovirus and the plant was resistant to both viruses. The reduction in sequence homology between the transgene and the invading virus or viruses resulted in a lower percentage of resistant lines. The Gonsalves group is refining this approach to provide papaya in Hawaii with a more durable resistance to PRSV

(Fermin and Gonsalves 2004). Another group from Hawaii used an untranslatable, synthetic CP gene with more than 94% similarity to citrus tristeza virus (CTV) strains from Hawaii and other countries to transform lime to generate transgenic resistant lines. The selected lines are currently being evaluated for resistance to CTV transmitted by brown citrus aphids (*Toxoptera citricida* Kiek) infecting citrus (Melzer and Hu, *unpublished*).

Bonfim and colleagues (2007) developed a transgenic line of common bean using transgenes containing virus sequences in an inverted orientation. This orientation delayed and attenuated golden mosaic symptoms following achallenge with viruliferous whiteflies. Their idea was to use a construct with RNA interference that silences the viral replication protein gene (AC1) and produces highly resistant transgenic common bean plants. Transgenic lines were obtained with an intron-hairpin construction and induced PTGS against the AC1 gene. Line -5.1 was highly resistant to repeated inoculation by viruliferous whiteflies at a very early stage of plant development. Transgene-specific siRNAs were detected in transgenic plants.

Jia and colleagues (2017) used RNA interference (RNAi) approach to target a conserved CP region of PRSV during virus replication anddevelop broad-spectrum resistance to PRSV isolates from Hainan, China (Table 1.1). By analyzing PRSV isolates from Hainan, they found a 544-bp region of the CP gene that shared 97 to 100% nucleotide identity among all isolates. The result was a transgenic line that produced siRNAs and hadresistance to local strains of PRSV in greenhouse test. This research indicates that the newly developed transgenic papaya line using the RNAi approach targeted a conserved CP gene region of PRSV andhad a useful application against PRSV in the major papaya growing area

3. Increasing gene dosage

Increasing the transgene dosage strengthens and broadens virus resistance in papaya plants against both homologous and heterologous PRSV isolates (Souza et al. 2005). It may be a factor in the homozygous 'SunUp' having a greater level of PRSV resistance than hemizygous 'Rainbow' (Gonsalves et al. 2004).

Despite virus resistance of 'SunUp' to local and to some exotic PRSV strains, farmers prefer the agronomic characteristics of 'Rainbow.' The Rainbow cultivar is resistant only to a narrow range of PRSV strains, but has higher quality fruit, the preferred yellow flesh, earlier maturity, and a greater market demand in Hawaii (Gonsalves 2004). Pyramiding transcriptionally active CP transgenes in the genome of a transgenic papaya plant by super-transformation could be a potential option to increase the resistance in 'Rainbow.' Transgene management by recurrent selection is helpful ingenerating a transgenic papaya plant highly resistant to many PRSV isolates (Souza et al. 2005).

4. Development of virus-resistant papaya using gene editing technology

The novel CRISPR/Cas9 technology can expedite the development of virus-resistant papaya either by targeting the virus directly, or editing one or more host genes essential for virus replication. There is no complete resistance to PRSV in any known papaya cultivar. CRISPR/Cas9-mediated mutations of host plants can confer resistance against many invading viruses (Green and Hu 2017). The CRISPR/Cas9 system causes a sequence-specific double-stranded break (DSB) to modify the targeted DNA sequences. It uses a single guide RNA (sgRNA) that directs a Cas9 nuclease (Hsu et al.2014). Chandrasekaran and others (2016) used the CRISPR/Cas system to introduce targeted mutations in the eukaryotic translationinitiation factor (*eIF4E gene*) of cucumber (*Cucumis sativus)*. The homozygotic T3 generation plants were immune to cucumber vein yellowing virus and resistant to two other potyviruses, zucchini

yellow mosaic virus and PRSV-W (Chandrasekaran et al. 2016). A CRISPR/Cas9 system has been inserted successfully into *A. thaliana* to create mutations in *eIF(iso)4E*, an isoform of *eIF4E*. The insertion provided high levels of resistance to turnip mosaic virus (TuMV) (Pyott et al. 2016). RNA viruses that infect plants, like PRSV, require the host factors *eIF4E* or *eIF(iso)4E* (Lellis et al. 2002; Nicaise et al. 2003; Ruffel et al. 2006) to continue their life cycle as it interacts with viral protein genome –linked (VPg) in the hosts. The *eIF4E* complex binds to the potyviral 5′ m7G cap structure and 3′ polyA tail of mRNA for translation. Disruption of this interaction by either mutagenesis or silencing stops further virus infection (Léonard et al. 2000; Sanfaçon 2015; Jiang and Laliberté 2011; Duprat et al. 2002; Lellis et al. 2002; Rodríguez-Hernández et al. 2012; Sato et al. 2005). The CRISPR/Cas9 system was used to knockout the target gene from host factors in different plants. Broad-spectrum resistance to RNA viruses has been demonstrated by silencing or mutations of the *eIF4E* and *eIF(iso)4E* genes in various crops such as tomato and melon (Mazier et al. 2011; Rodríguez-Hernández et al. 2012; Gómez et al. 2009).

CRISPR/Cas9 can also be used to specifically target the dsDNA of a geminivirus (Ji et al. 2015). The circular single-stranded DNA (ssDNA) of geminiviruses replicates within the nuclei of plant cells and can cause serious production losses for many dicotyledonous crop plants (Hsu et al. 2014; Manssor et al. 2003; Moffat et al. 1999). The ssDNA is converted to a double-stranded DNA (dsDNA) intermediate during geminivirus replication, from which new ssDNA is generated by rolling-circle replication. These dsDNAs can then be specifically targeted by CRISPR/Cas9 to inhibit virus replication and confer virus resistance to host plants (Ji et al. 2015). CRISPR/Cas9 systems have also been used in *A. thaliana* and *N. benthamiana* to confer resistance to geminiviruses (Hanley-Bowdoin et al. 2013). In transgenic *N. benthamiana*,

reduced virus accumulation and symptom expression were produced in plants challenged with bean yellow dwarf virus and beet severe curly top virus (Baltes et al. 2015; Ji et al. 2015). Ali and colleagues (2015) reported that transgenic *N. benthamiana* engineered with a CRISPR/Cas9 system targeting tomato yellow leaf curl virus (TYLCV) slowed virus production or decreased its titer of the TYLCV and eliminated or substantially reduced symptoms.

This technology has benefits over the commonly used non-specific integration methods of *Agrobacterium*-mediated transformation or microprojectile bombardment. CRISPR/Cas9 technology can be used, for example, to integrate transgenes into selected loci, avoiding undesired positional effects and averting the interruption of innate gene activities. The widespread application of CRISPR/Cas9 to plant disease problems is an indicator of its future potential in this field (Green and Hu, 2017).

5. Strengthening quarantine systems and field monitoring

Strengthening quarantine procedures can help reduce the introduction of foreign viruses or new virus strains into an area. Detection techniques with greater sensitivity, like single-tube nested PCR, can improve quarantine efforts and disease monitoring. They also can provide an early warning of emerging viruses or virulent strains in transgenic papayas (Hamim et al. 2017; Dey et al. 2012; Kung et al. 2015; Tripathi et al. 2008; Yeh et al. 2014; Hu, *personal observation*). It is also important to regularly survey papaya-producing regions to identify existing viruses and virus strains and monitor their movement to other papaya-producing areas (Paolla et al. 2015). ‘Rainbow’ and ‘SunUp’ have proven, durable transgenic resistance to PRSV in Hawaii. The emergence of new viral strains locally or by the introduction of foreign divergent strains could overcome this resistance. Therefore, it is essential to monitor the diversity and the arrival or the emergence of new and more virulent strains in the PRSV population. The

recent advances in research on PRSV gene sequences in different geographical regions will be helpful for devising effective PRSV management strategies, including designing the most effective transgene for achieving broad resistance to PRSV isolates in specific regions (Tripathi et al. 2008, Wang et al. 2017a, b). Monitoring and quarantine should be implemented on a local, national, and international level.

6. Integrating transgenic technology with Integrated Pest Management (IPM)

To date, transgenic papaya cultivars are used commercially in Hawaii and other countries, but their use in an IPM program has been limited. This is the consequence of the gap between the reductionist approaches of biotechnology and the holistic approaches of IPM (Kos et al. 2009). PRSV-resistant transgenic papayas have claimed to be the sole solution to the PRSV problem, whereas proponents of IPM strategies have found that a single solution is not sufficient to provide robust PRSV management. Indeed, current transgenic papaya based on PTGS can solve PRSV problems in a region for a certain period, but introduction of new or foreign strain of PRSV can reduce this resistance.

Some scientists have demonstrated that IPM components work successfully to reduce PRSV infection in papaya. The use of insecticides is unlikely to effectively control PRSV because of the erratic and unpredictable non-persistent transmission of the virus by aphids. Fermin et al. (2010) reported that silver reflective plastic mulches in the papaya orchard effectively repelled aphids from young papaya plants, either reducing or delaying virus infection. This approach helps protect plants when they are most susceptible and has worked effectively until expanding foliage covers the reflective surface. Another successfully adopted cultural practice in Colombia and Brazil is eradication or rouging of curcurbits and weeds from inside and around the borders of papaya orchards. These plants served as aphid hosts allowing

populations to build (Fermin et al. 2010). Considering transgenic papayas as an important component of IPM of PRSV, we believe that existing and future transgenic papaya cultivars will have extensive potential when incorporated into IPM systems.

Detection of papaya ringspot virus in papaya

RNA and DNA viruses pose a serious threat to papaya production in tropical and subtropical countries (Tripathi et al. 2008; Maoka et al. 1996; Wang et al. 2013; Chang et al. 2003; Sing-Pant et al. 2012; Yue et al.2009). PRSV is the most widespread papaya virus and causesa destructive, commercially important ringspot disease (Tripathi et al. 2008; Lu et al. 2008; Purciful et al. 1984). PRSV infection causes mosaic, chlorosis, mottling, vein clearing and distortion of leaves, typical “ringspot” and streaking offruits, and water-soaked streaks on stems and petioles (Tripathi et al. 2008; Gonsalves et al. 2008). The emergences of new PRSV isolates are threats to commercial papaya production and its international trade (Bau et al. 2008; Shen et al. 2014; Tuo et al. 2013). An accurate and effective assay system is crucial for monitoring early PRSV infections, minimizing economic losses, and preventing introduction of new PRSV isolates to a country or region.

Different assays have been developed to detect PRSV over the past few decades. Enzyme-linked immunosorbent assay (ELISA) is a widely used serological assay. However, the sensitivity of ELISA to detect PRSV is low compared to molecular diagnostic methods. Polymerase chain reaction (PCR), real-time (quantitative) PCR, loop-mediated isothermal amplification (LAMP) and molecular hybridizations are commonly used molecular methods (Ling et al. 1991; Chiang et al. 2001; Noa-Carrazanaet al. 2006; Cruz et al. 2009; Chiang et al. 2001; Shen et al. 2014a, b; Usharani et al. 2013). The highly sensitive real-time PCR needs

sophisticated, expensive instruments, chemicals, and complicated protocols that limit its use by laboratories in developing countries (Shen et al. 2014a, b). LAMP is a comparatively new, rapid detection assay. However, it requires complex reagents, protocols, and skill in order to use it reliably. These limitations make these assays prohibitive for many laboratories worldwide.

Another very sensitive assay, two-step nested PCR, has been used in laboratories worldwide for the sensitive detection of PRSV (Huo-gen et al. 2000; Jeong et al. 2014). It needs comparatively less expensive equipment and fewer chemicals, and is easy to operate compared to real-time PCR or LAMP (Jeong et al. 2014; Dey et al. 2012). However, nested PCR requires the manipulation of previously amplified material and involves the transfer of DNA from one PCR tube to another, increasing the risk of cross-contamination (Jeong et al. 2014; Dey et al. 2012; Lin et al. 2010). This potential problem can be overcome by using a single-tube nested polymerase chain reaction (STNP) assay capable of reliably detecting low-titer viral targets (Dey et al. 2012).

For decades, serological and molecular methods have been used to detect PRSV in papaya samples collected from the field. The assays currently available to assess PRSV infections can be used to confirm PRSV infection in symptomatic samples (Hamim et al. 2018). However, most of these assays are not able to consistently detect low levels of PRSV that occur early in field or in nursery infections (Jeong et al. 2014). In the early stages of infection, PRSV occurs in low titers and is unevenly distributed within papaya tissues, often before the development of any symptoms, or with symptoms similar to those produced by abiotic stressors (Jeonget al. 2014; Hamim, *personal observation*). Because early detection and careful monitoring of symptoms are essential to reduce the spread of viruses to new areas or hosts

(Jeong et al. 2014; Llop et al. 2000), improved methods to detect PRSV with enhanced reliability and sensitivity in asymptomatic samples are crucial.

Nested PCR assays are capable of detecting extremely low titers of PRSV in diseased plants and with greater sensitivity than other molecular and serological methods (Huo-gen et al. 2000). However, conventional nested PCR is prone to cross-contamination during manipulation when amplified PCR products from the first-round are transferred to the second-round of PCR (Dey et al. 2012; Llop et al.2000).These limitations can be overcome by using STNP assays (Jeong et al. 2014; Dey et al. 2012; Anderson et al.2003). Llop and colleagues (2000) used STNP todetect *Erwinia amylovora* in infected plants at rates 20% higher than conventional PCR and the sensitivity of the assay was comparable to nested PCR using sequential reactions.

Shen et al. (2014b) developed an RT-LAMP assay capable of detecting PRSV in about 1.0 pg of total RNA extracted from PRSV-infected papaya. Huo-gen et al. (2000) compared the detection of PRSV from PRSV-inoculated plants using conventional nested PCR, immunocapture-PCR, ELISA-PCR, and ELISA. The nested PCR assays were the most sensitive and able to detect virus at 3 days after inoculation.

Genome characterization of papaya ringspot virus in Bangladesh

Papaya ringspot disease caused by PRSV is the primary constraint to papaya production in Bangladesh (Jain et al. 2004). PRSV is spread by aphids in a nonpersistent manner, although seed and mechanical transmission have also occurred (Bayot et al. 1990; Gonsalves et al. 2010). The extended host range of PRSV makes disease management of this virus challenging, as alternate hosts can act as reservoirs and drivers of the evolution of this virus (Alabi et al. 2017).

As in other papaya-growing countries, PRSV is the major limitation to its cultivation in all regions of Bangladesh throughout the year. PRSV outbreaks were first reported from Bangladesh in 2004 (Jain et al. 2004) and since then disease incidences as high as 100% have been reported (Hamim et al.2018c; Hamim et al. 2018d).

The PRSV genome encodes a single large polyprotein. This polyprotein is cleaved by virus-encoded proteases into eight smaller, functionally active proteins: P1, HC-Pro, P3, CI, 6K, NIa-Pro, Nib, and CP (Yeh et al. 1992). There is another protein,'P3N-PIPO,' that is derived from a different small ORF and overlaps the P3 coding region (Chung et al. 2008; Fermin and Randle 2015). The complete genome sequences of thirty-one PRSV isolates were reported from Taiwan, Brazil, India, China, Thailand, South Korea, Venezuela, Hawaii, and Mexico (Ortiz-Rojas and Chaves-Bedoya 2017; Mishra and Patil 2018). To date, the full-length genome sequence of PRSV isolates from the Indian subcontinent are all from India. Preliminary characterization of PRSV isolates in Bangladesh using partial coat protein (CP) gene sequence comparisons indicated that these isolates shared amino acid sequence identities of 86 to 95% with Asian isolates and 83 to 93 % with American isolates. Amino acid sequence divergence among the partial CPs of PRSV isolates from Bangladesh was up to 14% (Akter et al. 2013).

Virus diseases are a major threat to papaya production worldwide, and several different viruses have been reported. Among these PRSV, a dominant and widespread virus, was first reported from Bangladesh in 2004 (Jain et al. 2004). Recombination within single-stranded RNA viruses is a major evolutionary means for viruses to adapt to new hosts and environments (Noa-Carrazana et al. 2007). Recombination occurred frequently in the evolution of potyviruses (Hamim et al. 2018b). A previous study showing that the C1 and CP regions are prone to recombination events in the PRSV genome (Noa-Carrazana et al. 2007).

Detection and characterization of begomoviruses in papaya

Leaf curl disease and associated begomoviruses are an emerging threat to global papaya production. Their incidence may be increasing due to the broad host range of begomoviruses, the polyphagous nature of their whitefly (*Bemisia tabaci)* vectors, and the use of mixed cropping systems (Singh-Pant et al. 2012). Additionally, the high rates of mutation and recombination among begomoviruses may stimulate the evolution of new species and their associated subviral DNAs (Singh-Pant et al. 2012). Leaf curl symptoms in papaya have been reported from India, Pakistan, Nepal, Iran, and China, where monopartite begomoviruses are known to infect papaya (Singh-Pant et al. 2012; Bananej et al. 2016). Papaya plants infected by begomoviruses suffer serious production losses and can act as sources of virus inoculum for transmission by whitefly vectors (Guo et al. 2015). Singh-Pant and colleagues (2012) have reported infections by chili leaf curl virus (ChiLCuV) and papaya leaf crumple virus in papaya. Other begomoviruses found in papaya include: papaya leaf curl Guandong virus, papaya leaf curl China virus, malvastrum leafcurl virus, okra enation leaf curl virus,cotton leaf curl Multan virus, ageratum yellow vein virus, tomato leaf curl Joydebpur virus (ToLCJoV) and tomato leaf curl New Delhi virus (ToLCNDV) (Bananejet al. 2016; Guo et al. 2015; Raj et al. 2008; Maruthi et al. 2007; Shahid et al. 2013; Sinha et al. 2013; Cai et al. 2005; Wang et al. 2004).

Begomoviruses, in the family *Geminiviridae,* have circular, single-stranded DNA (ssDNA) genomes that are grouped into monopartite and bipartite viruses. Monopartite begomoviruses have genomes that are homologs of the DNA-A components of bipartite begomoviruses, and include V1, C1, C2, C3, and C4 genes that respectively encode the capsid protein (CP), replication-associated protein (Rep), transactivator protein (TrAP), replication enhancer protein (REn), and C4 proteins (Harrison and Robinson 1999; Saunders and Stanley

1999). Monopartite begomoviruses are often associated with subviral DNA molecules such as alphasatellites, betasatellites, and defective satellites. These satellites depend on the begomovirus for replication, encapsidation, insect transmission, and movement within plants (Briddon and Stanley 2006; Saunders and Stanley 1999). DNA-A components occur with bipartite begomoviruses, along with a second component of similar genomic size that encodes a movement protein and a nuclear shuttle protein (Saunders and Stanley 1999).

DNA A components occur in bipartite begomoviruses along with a second component of similar genomic size that encodes a movement protein and a nuclear shuttle protein. DNA-A encodes two genes on the virus strand, the coat protein (AV1 or CP) and AV2. It also encodes four genes on the complementary strand: the replication-associated protein (AC1 or Rep), transcriptional activator protein (AC2 or TrAP), replication enhancer protein (AC3 or REn), and AC4. The virus and complementary strands of DNA-B each contain one gene, the nuclear shuttle protein (BV1 or NSP) and movement protein (BC1 or MP), respectively. A common region (CR) sequence of near identity shared between DNA A and DNA B contains promoters and iteron sequences involved in virus replication (Raj et al 2008).

There are reports of a host range expansion of ToLCBV to *Gaillardia pulchella* and *Gomphostemma niveum* in India (Datta et al. 2015; Mahatma et al. 2012). Sing-Pant and colleagues (2013) reported that the ToLCB satellite from the unrelated virus ChiLCuV could infect papaya plants in India. Synergistic interactions between a particular virus and an unrelated satellite could alter the host range or increase the pathogenicity of the virus (Saunders et al. 2008).

These defective satellites retained a portion of the satellite conserved region (SCR) but lacked the βC1 coding sequence. The SCR helps defective small DNA satellites associate with monopartite begomoviruses (Huang et al. 2013). *Ageratum conyzoides* with symptoms of yellow vein disease can also contain recombinants between a helper virus and a betasatellite (Saunders et al. 2001; Stanley et al. 1997). Furthermore, Saunders and others (2001) demonstrated that co-inoculation of *A. conyzoides* with ageratum yellow vein virus and defective recombinant DNA satellites induced the disease phenotype (Saunders et al. 2001). Defective satellites contained parts of a betasatellite, so the defective satellites could have the ability to induce disease symptoms (Saunders et al. 2001).

In many regions of Bangladesh, begomoviruses and associated betasatellite molecules are emerging as serious pathogens infecting vegetables and weeds. Previously, tomato leaf curl New Delhi virus (ToLCNDV) was the only begomovirus from papaya reported in Bangladesh (Maruthi et al. 2007). However, begomoviruses other than papaya leaf curl virus (PLCV) also have been reported on papaya from India, Nepal, and the USA. Other studies from India indicate that papaya is also a host of tomato leaf curl Joydebpur virus (ToLCJoV), ToLCNDV, and betasatellites (Shahid et al. 2013).

The high genetic diversity of tomato-infecting begomoviruses and their recombination in papaya in Bangladesh was expected, because such variability has been documented in neighboring India. Higher rates of mutation and recombination among begomoviruses may stimulate the evolution of new species and different groups of associated satellites in papaya (Singh-Pant et al. 2012).

In the last decade, a large number of alphasatellites and betasatellites have been found associated with begomoviruses causing severe diseases of crop plants. Interactions of helper

begomoviruses with their cognate betasatellites play a significant role in symptom induction and severity in the hosts due to the direct synergistic interaction between PTGS suppressors. ToLCJoV does not require a betasatellite to infect and produce disease symptoms in indicator hosts or the primary host, tomato (Tiwari et al. 2013). Maruthi et al. (2005) reported that an earlier ToLCJoV infection produced faint to complete yellowing, mild leaf curling, and slightly stunted growth on tomato. However, Tiwari et al. (2013) found a contrasting disease reaction by a ToLJoV isolate on tomato of severe leaf curl, deformation, and stunting. The symptoms were severe, even when DNA A alone was used for inoculation. This isolate is more closely related to a chili isolate.

ToLCNDV occurs on various crop plants in India, Pakistan, Thailand, and Bangladesh (Fauquet et al. 2008). It was reported for the first time in India on tomato and requires both DNA-A and DNA-B for symptom development (Padidam et al. 1995). ToLCNDV was later found on various crops and weeds, such as chili (Hussain et al. 2004), cucurbits (Ito et al. 2008), potato (Usharani et al. 2004), papaya (Raj et al. 2008), bittergourd (Tahir and Haider 2005), and cotton (GenBank acc.no. EF063145), and *Solanum nigrum* (GenBank acc. no. AJ620187).

Molecular analyses of viruses infecting papaya in Bangladesh and sustainable disease management

, Papaya is grown on large commercial farms and by small subsistence farmers and is one of the largest agricultural crops in Bangladesh (BBS 2015). Many pests and diseases affect papaya production, but viruses are one of the greatest concerns (Purcifullet al. 1984; Ye and Li 2010). Papaya ringspot disease, caused by papaya ringspot virus, is a widely distributed, severe disease of this crop (Hamim et al. 2018c). Another emerging viral leaf curl disease of papaya irregularly

observed in Bangladesh (Hamim et al. 2019). Both viral diseases have international importance, reducing yield and fruit quality wherever they are found (Paolla et al.2015; Hamim et al. 2018a).

Papaya trade networks in Bangladesh are expanding and increase the risk of spreading these two diseases. Papaya producers and stakeholders require rapid, flexible, accurate management technologies to mitigate the spread of viruses associated with these two diseases to ensure the safety of papaya production.

Papaya viruses are chief antagonists to papaya production in Bangladesh and can destroy entire crops, jeopardizing the food security system. These viral diseases have not been fully reported or characterized in Bangladesh, so control measures are practically nonexistent (Milkovich 2017). Based on recent observation of the disease symptoms on papaya plants in Bangladesh, my hypothesis is that a strain or strains of PRSV or other viruses are infecting papaya.

Molecular analysis of viruses in plants is increasingly being used for detection, characterization, epidemiology, and management of viruses.The research on molecular detection, distribution and characterization of papaya viruses in Bangladeshis needed to help preserve this important, nutritious crop in Bangladesh. This research will also contribute to an important goal of USAID:to encourage people to consume a wider array of nutrient-rich foods in a country heavily reliant on rice (Milkovich 2017).

OBJECTIVES

Based on disease symptoms in the papaya fields, my dissertation has the following three objectives and hypotheses:

(i) Detection of PRSV using STNP (hypothesis: STNP will detect very low titers of PRSV);

(ii) Characterize the genome of PRSV from *Carica papaya* in Bangladesh (hypothesis: PRSV strains from Bangladesh are distinct from PRSV strains reported in other countries); and

(iii) Molecular detection and characterization of viruses associated with leaf curl diseases of papaya in Bangladesh (hypothesis: Different begomoviruses are associated with leaf curl symptoms in papaya in Bangladesh).

CHAPTER II

DETECTION OF PAPAYA RINGSPOT VIRUS USING SINGLE-TUBE NESTED PCR

Introduction

Papaya (*Carica papaya* L.) is a widely grown fruit crop in tropical and subtropical areas and is valued for its nutritional, digestive, and medicinal properties (Manshardt 1992). Several RNA and DNA plant viruses pose a serious threat to papaya production (Tripathi et al. 2008; Maoka et al. 1996; Wang et al. 2013; Chang et al. 2003; Sing-Pant et al. 2012; Yue et al.2009). Among these, aphid-transmitted *Papaya ringspot virus* (genus *Potyvirus*, family *Potyviridae*) causes the most widespread, destructive, and commercially important disease and results in serious economic and agronomic impacts on papaya production worldwide (Tripathi et al. 2008; Lu et al 2008; Purciful et al. 1984). PRSV is divided into two types, PRSV-P and PRSV-W, which are serologically indistinguishable but have different host specificities. PRSV-P infects papaya and cucurbits, while PRSV-W infects only cucurbits (Purciful et al. 1984; Yeh et al. 1984; Chen et al. 2008). PRSV-P causes mosaic, chlorosis, mottling, vein clearing and distortion of the leaves, typical "ringspot" and streaking on fruits, and water-soaked streaks on stems and petioles (Tripathi et al. 2008; Gonsalves et al. 2008). The international trade of papayas threatens commercial papaya production, especially with the potential emergence of new PRSV isolates (Bau et al. 2008; Shen et al. 2014; Tuo et al. 2013). Therefore, the accurate and effective diagnosis of PRSV is crucial for monitoring early infections, minimizing economic losses, and detecting new PRSV isolates arising from worldwide trade.

Over the past few decades, various diagnostic methods have been developed to detect PRSV including both serological and molecular-based methods. Enzyme-linked immunosorbent

assay (ELISA) is widely used to detect PRSV, but the sensitivity of this method is less than for molecular diagnostic methods such as polymerase chain reaction (PCR), real-time (quantitative) PCR, loop-mediated isothermal amplification (LAMP) and molecular hybridizations (Ling et al. 1991; Chiang et al. 2001; Noa-Carrazana et al. 2006; Cruz et al. 2009; Chiang et al. 2001; Shen et al. 2014a,b; Usharani et al. 2013). However, highly sensitive real-time PCR requires sophisticated and expensive instruments, chemicals, and complicated protocols that limit its use by many of laboratories in developing countries (Shen et al. 2014 a, b). LAMP is a comparatively new and rapid detection assay, but requires complex reagents and protocols, and also considerable skill to use reliably. Therefore, these molecular assays are prohibitive for many laboratories worldwide.

Another very sensitive molecular technique is two-step nested PCR. It has been used in laboratories globally for sensitive detection of viruses including PRSV (Huo-gen et al. 2000; Jeong et al.2014). This method does not require costly equipment or chemicals like real-time PCR, or the use of complex protocols like LAMP (Jeong et al 2014, Dey et al. 2012). However, nested PCR requires the manipulation of previously amplified material and involves the transfer of DNA from the initial PCR tube to another tube for the second round of PCR, raising the risk of cross-contamination (Jeong et al. 2014; Dey et al.2012, Lin et al. 2010). This potential problem can be overcome by using a STNP assay capable of reliably detecting very low viral titers (Dey et al. 2012).

We have developed an ultra-sensitive, single-tube nested PCR (STNP) assay to detect PRSV, and compared its sensitivity to regular PCR and ELISA. The papaya industry and government plant quarantine offices could use this new method to reliably and sensitively detect very low titers of PRSV.

Materials and Methods

Sample collection, RNA extraction and cDNA synthesis

Papaya leaves with symptoms of papaya ringspot were collected from the island of Oahu in Hawaii, USA (Figure 2.1) and used to mechanically inoculate PRSV onto 4-week-old healthy papaya cv. Sunset for greenhouse studies (Tennant et al., 1994). Total cellular RNA was extracted from systemically infected leaves using the RNeasy® Plant Mini Kit (Qiagen, Inc.) according to the manufacturer's protocol. RNAs were eluted into 80 μL of RNase-free water and stored at -80°C until use**.** The yield and quality of RNA samples were monitored by electrophoresis in 1% agarose gels and on a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). cDNAs were synthesized in reverse transcription (RT) reactions of total RNAs as described previously (Dey et al. 2012) with minor modifications.The reaction mixture consisted of 2 μL RNA, 1 μL random hexamer primers (50μg/ml) and 6.5 μL RNase-free H_2O. The reaction was incubated for 10 min at 72°C and quickly chilled on ice. A cocktail of 5 μL dNTP mixture (2.5 mM each), 4 μL 5x MMLV reaction buffer, 1 μL MMLV reverse transcriptase (200U/μL) and 0.5 μL RNase inhibitor (40 U/μL) was added. The RT reaction was incubated for 10 min at 25°C followed by 50 min at 42°C. The products were chilled on ice and stored at −20°C until RT-PCR and STNP reactions were performed.

Primer design

Two nested primer sets (outer and inner) were designed to target the P3 gene of PRSV using Primer 3® software (http://frodo.wi. mit.edu/) (Shen et al, 2014 a,b; Dey et al. 2012; Lin et al. 2010). In designing the inner primers, we established a GC content of 40 to 50%, a melting temperature (Tm) of 53 to 57°C, primer lengths from 18 to 22 bp, and predicted amplicon sizes

from 120 to 200 bp. In designing, the outer primers we selected a GC content similar to the inner primers but the melting temperatures needed to be at least 10°C higher than the melting temperatures of the inner primers. Lengths for the outer primers were similar to that of the inner primers, but were chosen to produce amplicon sizes from 300 to 500 bp (Figure 2.2) (Dey et al. 2012; Lin et al. 2010). All designed primers were checked for their uniqueness to PRSV against the available microbial and papaya sequences in GenBank for their uniqueness to PRSV (Lin et al. 2010).

Figure 2.1. PRSV-infected papaya samples collected from the island of Oahu, Hawaii

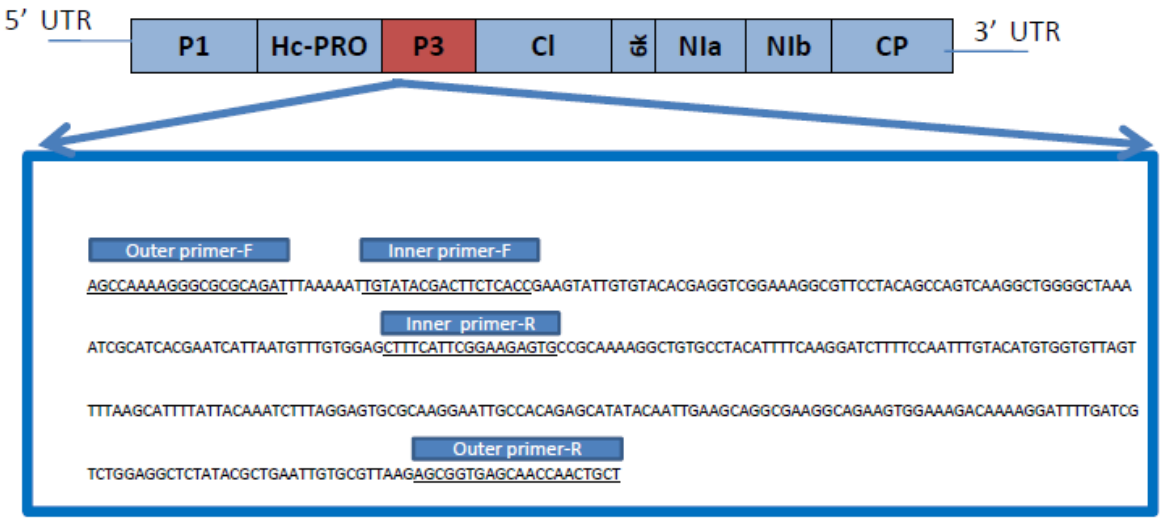


Figure 2.2. Primers used in the single-tube nested PCR (STNP) assay for the detection for PRSV. F, sense primer; R, antisense primer.

Sensitivity of STNP

Sensitivity of the STNP assay was compared to conventional RT-PCR using the inner primer set of cDNAs generated from 130 ng of cellular RNAs. They were serially-diluted in nuclease free water from 0 to 10^7-fold and then used as templates. We also evaluated STNP sensitivity using similar RNA dilutions of cellular RNAs extracted from PRSV-infected papaya leaves in both assays. Serially diluted RNAs (100 ng to 100 fg) were converted into cDNAs and the STNP and RT-PCR assays were performed as described above.

The sensitivity of STNP was further compared to results from RT-PCR and ELISA using serial dilutions of extracts from infected papaya leaf tissues (Table 2.1). ELISA was performed according to the manufacturer's (Agdia) instructions and absorbance at 405 nm measured with a Bio-Rad Model 680 Microplate reader. Absorbance ratios of infected (I) and healthy tissues (H)

were calculated from mean values of absorbance for each extract dilution. A positive/negative threshold was set for each plate at two times the absorbance of the healthy controls (I/H=2) for each dilution (Sreenivasulu et al. 2010).

Inoculation of papaya plants to monitor PRSV infection

We mechanically inoculated four-week-old papaya plants of cv. Sunset with PRSV inoculum prepared from PRSV-infected papaya plants. To prepare the virus inoculum, leaves of papaya plants six-week post-inoculated with symptoms of PRSV were ground in 10 volumes (vol/wt) of inoculation buffer (0.01 M potassium phosphate, pH 7.4) (Tennant et al. 1994). The inoculum was then applied to the leaves of six papaya plants dusted with carborundum. The inoculum were gently rubbed on the leaf surface with a glass rod and then rinsed in dH_2O. Inoculated plants were kept in the greenhouse for symptom development. Young leaves were collected from the inoculated plants at 7-day intervals, beginning 7 days after inoculation (DAI) and continuing until 42 DAI. STNP, RT-PCR, and ELISA were used to detect PRSV from the inoculated samples at each time point. The experiment was repeated twice.

Detection of PRSV from symptomatic and asymptomatic samples

A total of 52 papaya leaf samples were collected for diagnosis of PRSV from the island of Oahu. Of these 52 samples, 26 displayed typical PRSV symptoms, including mosaic, ring spots, and leaf distortions of leaves, and water soaked streaks on petioles. The remaining samples were asymptomatic. An STNP assay was performed as described above and its detection limits compared to those generated by RT-PCR and ELISA.

Results

Sensitivity of single-tube nested-PCR (STNP)

The STNP assay was designed based on similar assays reported previously (Dey et al. 2012; Lin et al. 2010). For the inner and outer primers, annealing temperatures were optimized at 55°C and 65°C, respectively. The optimal amounts of the outer and inner primers were 0.1pmol and 10 pmol, respectively.

The detection limit of the STNP assay was determined in replicated experiments using serially-diluted cDNAs as templates. The cDNAs were generated from PRSV RNAs previously isolated from the infected plants. The STNP assay was able to detect cDNA templates diluted up to 10^7-fold, whereas the detection limit of conventional PCR was 10^3-fold, as determined by the presence of the predicted 128-bp amplicon by gel electrophoresis (Figure 2. 3).

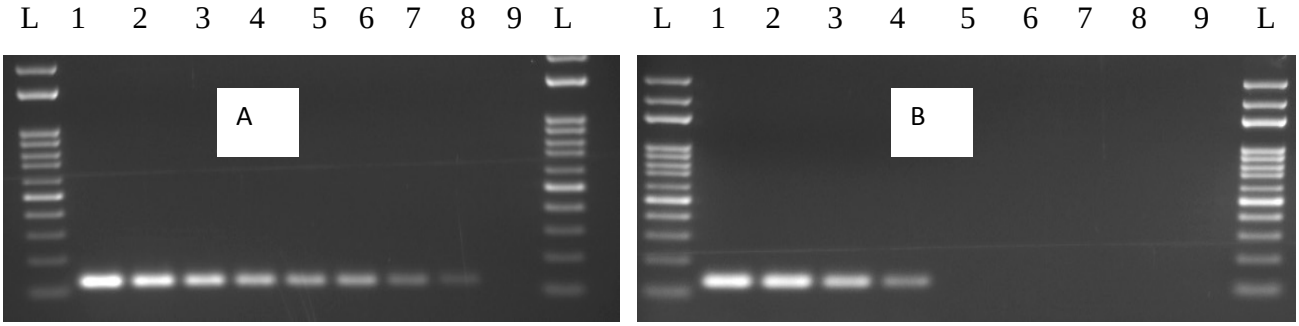


Figure2.3. Sensitivity of STNP (panel A) compared to RT-PCR (panel B). Serially diluted cDNAs from PRSV-infected plant RNAs were used as templates. Lanes 1 to 8 correspond to cDNA dilutions from 0 to 10^7 fold. Lane 9 is cDNA from healthy control plants. L is a 100-bp ladder. Expected bands are 128 bp.

The sensitivities of the newly developed PRSV-specific STNP and RT-PCR assays were also compared using total RNAs from PRSV-infected leaves serially diluted from 100 ng to 10 fgRNA. cDNAs were reverse transcribed from diluted total RNAs and used as templates in STNP and conventional PCR. STNP was able to detect the template at concentrations of 100 fg per reaction (Fig. 2.4A), one-thousand-fold greater than in RT-PCR, which could not detect PRSV at less than 100 pg per reaction (Figure 2.4B).

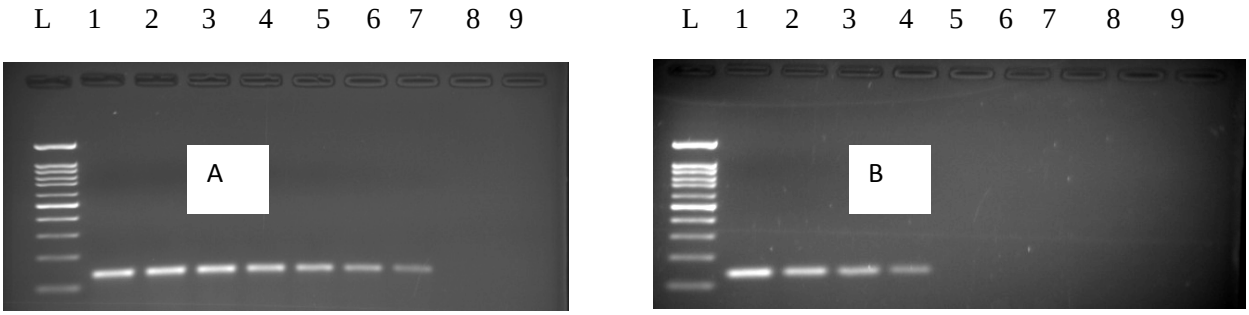


Figure 2.4. Sensitivity of STNP (A) compared to RT-PCR (B) using total RNAs serially diluted from 100 ng to 10 fg. cDNAs were reverse transcribed from diluted total RNAs. Lanes 1 to 8 correspond to 100 ng to 10 fg total RNA from papaya leaves. Lane 9 is RNA from healthy control plants. L is a 100-bp ladder. Expected bands are 128 bp.

The sensitivity of the STNP assay was also compared to the sensitivities of RT-PCR and ELISA assays using serial dilutions of extracts from PRSV-infected papaya leaf samples (Table 2.1). STNP detected PRSV targets in samples at dilutions of at least 1:81,920, whereas PRSV targets could only be detected at dilutions of 1:10,240 and 1:320 by RT-PCR and ELISA, respectively (Table 2.1).

Table 2.1. Comparison of sensitivities of single-tube nested-PCR with ELISA and conventional RT-PCR for the detection of PRSV in serial dilutions of extracts from infected papaya leaf samples.

Dilution (w/v)	**ELISA**	**RT-PCR**	**STNP**
1:10	+[a]	+	+
1:20	+	+	+
1:40	+	+	+
1:80	+	+	+
1:160	+	+	+
1:320	+	+	+
1:640	-[b]	+	+
1:1280	-	+	+
1:2560	-	+	+
1:5120	-	+	+
1:10240	-	+	+
1:20480	-	-	+
1:40960	N.T[c]	-	+
1: 81920	N.T	-	+

a- PRSV positive, b- PRSV negative, c- Not tested

Diagnostic evaluations

To evaluate the suitability of STNP for routine assays, two independent experiments were conducted comparing it to conventional RT-PCR and ELISA. In the first experiment, healthy plants were inoculated with PRSV and young leaves were collected for PRSV detection by STNP, RT-PCR, and ELISA at 7, 14, 21, 28, 35, and 42 DAI. STNP was the most sensitive assay, capable of detecting PRSV 7DAI from 16% of the PRSV-inoculated plants. PCR and ELISA could only detect the virus at 14 and 21 days, respectively. At 21 DAI, RT-PCR and STNP detected PRSV-positive plants in 50% and 83% of the inoculated plants, respectively. At 28 DAI, PRSV was detected by STNP in 100% of the inoculated samples, whereas ELISA and RT- PCR detected PRSV in only 65% and 80% of samples, respectively (Figure 2.5).

Fifty-two symptomatic and asymptomatic papaya leaf samples were collected on Oahu, Hawaii and assayed for PRSV using by STNP, RT-PCR and ELISA for PRSV infection. All of the symptomatic samples tested positive for PRSV by each of these three techniques. When asymptomatic plant samples were tested, however, PRSV could be detected by STNP in 20/26 (77%), by RT-PCR in 12/26 (46%), and by ELISA in only 3/26 (12%) of the plants (Table 2). These results confirmed that STNP was the most robust and sensitive assay.

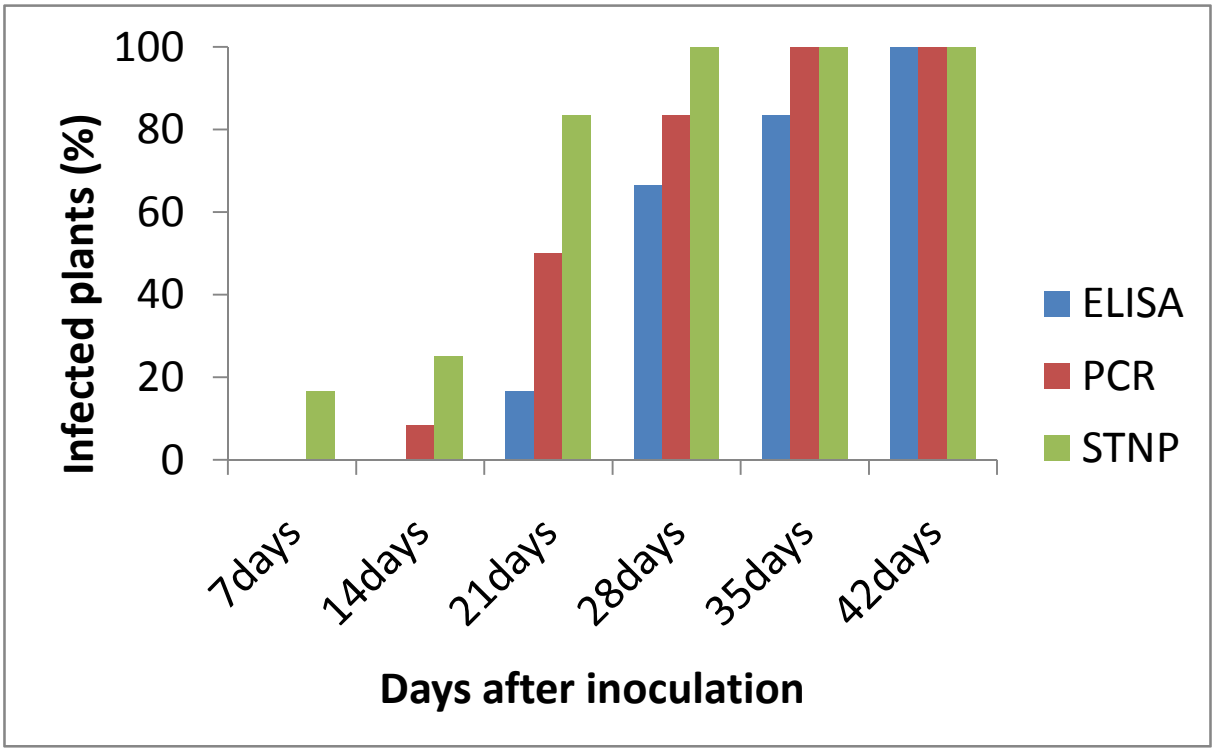


Figure 2.5. Comparison of sensitivities of STNP to ELISA and RT-PCR for the detection of PRSV infection in inoculated plants.

Table 2.2. Detection of PRSV by STNP, RT-PCR and ELISA from symptomatic and asymptomatic samples

Type of samples	**Number of Positive Samples (total number of samples tested)**		
	ELISA	**RT-PCR**	**STNP**
Symptomatic	26 (26)	26(26)	26 (26)
Asymptomatic	3 (26)	12 (26)	20 (26)

Discussion

For decades, serological and molecular methods have been used to detect PRSV in papaya samples collected from the field. The assays currently available to assess PRSV infections can be used to confirm PRSV infection in symptomatic samples (Hamim et al. 2018).However, most of these assays are not able to consistently detect the low levels of PRSV that occur in early infections, either in fields or nurseries (Jeong et al. 2014). In the early stages of infection, PRSV occurs at low titers and is unevenly distributed within papaya tissues. Often, symptoms have yet to develop, or symptoms may be due to abiotic stress (Jeonget al. 2014; Hamim, *personal observation*). Early detection of plant viruses and careful monitoring of symptoms are essential to reduce the spread of viruses to new areas or hosts (Jeong et al. 2014; Llop et al. 2000). Therefore, improved methods to detect PRSV in asymptomatic samples with enhanced reliability and sensitivity are crucial.

Nested PCR assays are capable of detecting extremely low titers of PRSV in diseased plants and with more sensitivity than other molecular and serological methods (Huo-gen et al.

2000). However, conventional nested PCR, is prone to cross-contamination during manipulation when the amplified first-round PCR products are transferred for the second-round of PCR (Dey et al. 2012; Llop et al.2000). These limitations can be overcome by the STNP assay (Jeong et al. 2014; Dey et al. 2012; Anderson et al. 2003). Llop and colleagues (2000) used a STNP assay to detect *Erwinia amylovora* in infected plants at rates 20% higher than conventional PCR. They also found that the sensitivity of this assay was comparable to nested PCR using sequential reactions.

To improve the detection limits of the PRSV assay and avoid problems associated with cross-contamination, we developed an ultra-sensitive STNP assay. It has the capability of detecting PRSV with high sensitivity in serially diluted cDNA, RNA, and plant extracts. This superior sensitivity was greater than conventional RT-PCR. Shen et al. (2014b) developed an RT-LAMP assay capable of detecting PRSV in about 1 pg of total RNA extracted from PRSV-infected papaya. This is about 10-fold less sensitive than our newly developed PRSV STNP assay. Huo-gen et al.(2000) compared the detection of PRSV in PRSV-inoculated plants using conventional nested PCR, immunocapture-PCR, ELISA-PCR and ELISA. They reported that nested PCR assays were the most sensitive of these techniques, able to detect virus at 3 DAI. In our study, STNP was the most sensitive assay evaluated, allowing reliable detection of PRSV at 7DAI.

This ultrasensitive STNP was capable of detecting low virus titers in asymptomatic PRSV–infected papaya, and could identify PRSV in a higher percentage of field samples than either RT-PCR or ELISA assays. STNP provided an ultrasensitive and robust method to diagnose PRSV infection. To our knowledge, this is the first report of the use of this method to detect potyvirus infections in any plant host. This novel technique eliminated the potential of

cross-contamination by eliminating the transfer of amplification products from one PCR tube to another during two-tube nested PCR. This STNP assay was also more sensitive than conventional RT-PCR or ELISA, and could detect PRSV in asymptomatic plants. This new robust assay will facilitate epidemiological studies of PRSV and lead to improved government quarantine systems by detecting extremely low infection levels of PRSV in papaya material from various sources.

CHAPTER III

GENOME CHARACTERIZATION OF DISTINCT PAPAYA RINGSPOT VIRUS STRAIN (S) FROM *Carica papaya* IN BANGLADESH

Introduction

Papaya (*Carica papaya* L.) is a highly nutritious, economically valuable fruit crop extensively cultivated in Bangladesh (Akhter et al. 2013; Hamim et al. 2014a). Native to the Americas, the fruit is consumed fresh, in salads or with curry, and is also widely used in the leather, pharmaceutical, cosmetic, juice processing, and food industries (Rodriguez et al. 2013). Several pests and diseases affect papaya and can significantly impact production (Hamim et al. 2018a). Plant viruses in diverse genera have been reported in papaya. Among these are: the potyviruses, papaya ringspot virus (PRSV) and papaya leaf distortion mosaic virus (PLDMV); the potexvirus,papaya mosaic virus (PapMV); the begomoviruses, papaya leaf curl virus (PLCV) and tomato leaf curl virus (ToLCV); and the tospovirus, tomato spotted wilt virus(TSWV) (Hamim et al. 2018a; Raj et al. 2008). Of these, PRSV, the cause of papaya ringspot disease, is the primary constraint to papaya production in Bangladesh (Jain et al. 2004). PRSV outbreaks were first reported from Bangladesh in 2004 (Jain et al. 2004) and since then disease incidences as high as 100% have been reported (Hamim et al.2018c; Hamim et al. 2018d).

PRSV is spread by aphids in a nonpersistent manner, although seed and mechanical transmission have also occurred (Bayot et al. 1990; Gonsalves et al. 2010). The extended host range of PRSV makes disease management challenging, as alternate hosts can act as reservoirs and drive evolution of this virus (Alabi et al. 2017).

The PRSV genome encodes a single large polyprotein. This polyprotein is cleaved by virus-encoded proteases into eight smaller, functionally active proteins: P1, HC-Pro, P3, CI, 6K, NIa-Pro, NIb and CP (Yeh et al. 1992). There is another protein, P3N-PIPO, derived from a different small ORF and overlapped with the P3 coding region (Chung et al. 2008; Fermin and Randle 2015). The complete genome sequences of thirty-one PRSV isolates have been reported from Taiwan, Brazil, India, China, Thailand, South Korea, Venezuela, Hawaii, and Mexico (Ortiz-Rojas and Chaves-Bedoya 2017; Mishra and Patil 2018). To date, all full-length genome sequences of PRSV isolates from the Indian subcontinent are from India. Preliminary characterization of PRSV isolates in Bangladesh using partial coat protein (CP) gene sequence comparisons indicated that these isolates shared amino acid sequence identities of 86 to 95% with Asian isolates and 83 to 93% to American isolates. Amino acid sequence divergence based on the partial CP of PRSV isolates from Bangladesh was up to 14% (Akter et al. 2013).

In this report, both high-throughput sequencing (HTS) of total RNAs and Sanger sequencing of RT-PCR products were used to determine the full-length coding sequences of two PRSV isolates from infected papaya plants in Bangladesh. Specific RT-PCR assays were used to determine the distribution of two newly identified PRSV strains in papaya plants in Bangladesh.

Material and methods

Source of virus material/isolate collection

We collected 118 leaf samples from papaya plants with virus-like symptoms from December 2016 to January 2017. We surveyed locations in eight districts of Bangladesh: Pabna (24.070032°N, 89.113417°E), Rajshahi (24.366346°N, 88.642614°E), Chapai Nawabganj(24.607635°N, 88.290519°E), Chandpur (23.348015°N, 90.708153°E), Munshiganj

(23.550215°N, 90.534806°E), Gazipur (23.998997°N, 90.419802°E), Tangail (24.643032°N, 90.055994°E), and Mymensingh (24.746026°N, 90.373906°E) (Figure 3.1). Disease incidence was assessed at each of the locations (Hamim et al. 2014a). The collected leaves were immediately stored in RNA*later* solution (Qiagen, Inc., USA) and brought to the plant virology laboratory of the University of Hawaii at Manoa, USA, under USDA PPQ 526 permits (P526-160413-009 and P526P-16-03662). After arrival of leaf samples at the plant virology laboratory, they were stored at -80°C until total RNA extractions were concluded.

Preliminary detection of PRSV

The 11 papaya samples with virus-like symptoms were tested withPRSV-specific single-tube nested PCR and ELISA (Agdia, Inc., Elkhart, IN, USA) as previously described (Hamim et al. 2018d). Total RNA was extracted from the leaf samples using the RNeasy®Plant Mini Kit (Qiagen, Valencia, CA, USA) and cDNAs were synthesized according to a method described previously (Hamim et al. 2018b). RNA purity as assessed by A260/A280 ratios ranged from 1.9 to 2.2. Previously identified PRSV-infected and PRSV-free leaves were included as positive and negative controls, respectively.

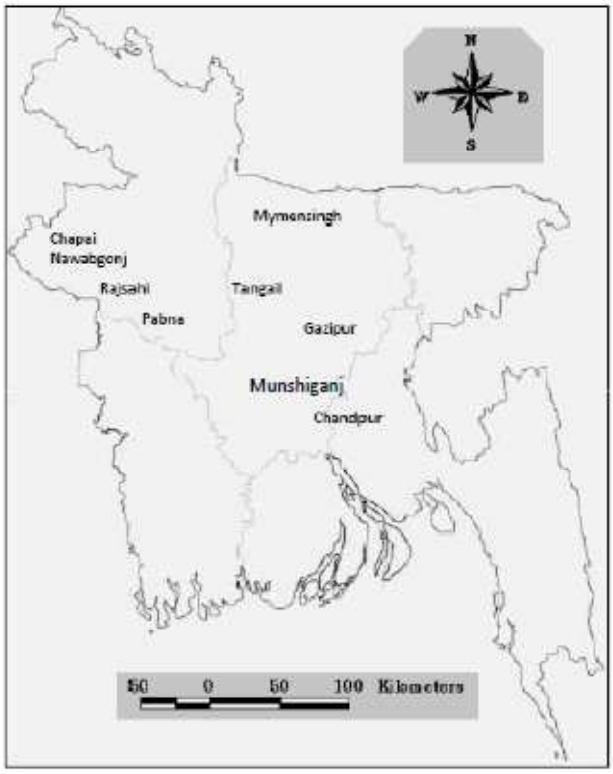


Fig.3.1. Map of the districts in Bangladesh, where virus infected papaya samples were collected

High throughput sequencing (HTS) of total RNAs from PRSV-infected composite samples

The 11 leaf samples with virus-like symptoms were divided into two separate composite samples for HTS at Foundation Plant Services, Davis, CA, USA. Composite sample pap-1 consisted of six papaya samples with virus-like symptoms: CD-141, Gaz-30, Gaz-52, Tang-62, Tang-66, and Tang-72. Composite sample pap-2 consisted of five papaya samples with virus-like symptoms: CD-112, CD-125, CD-129, Gaz 34, and Gaz-39. Each of the composite samples was prepared by aliquoting 10 µg of total RNA from each sample and combining them. The combined RNA aliquots were subjected to ribosomal RNA (rRNA) depletion and a cDNA library was constructed from the RNA template using TruSeq Stranded Total RNA with a Ribo-Zero Plant Kit (Illumina, Inc., San Diego, CA, USA). Sequencing was performed at Foundation Plant Services using the Illumina NextSeq 500 platform and the raw HTS reads analyzed as described by Al Rwahnih et al. (2018).

RT-PCR-Sequencing to validate the HTS results

To validate the results of HTS, specific primers (Table 3.1 and Table 3.2) were designed to amplify DNA fragments of the virus genome by RT-PCR. cDNA templates were constructed from the isolated combined RNA from the HTS samples using Qiagen RNeasy® Kits (Qiagen, Hilden, Germany). The total RNAs extracted from each plant were quantified as described above and cDNAs synthesized as described previously (Hamim et al. 2018b). Conditions for the PCR amplification were: 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50 to 65°C depending on the primers, 2 min at 72°C, and followed by final elongation at 72°C for 7 min. The PCR products were resolved on 1% agarose gel in 1% TAE buffer for 60 min at 100 V. The gels were stained with ethidium bromide and visualized in a UVP transilluminator. PCR products

Table 3.1. Primers used to amplify the complete genome of the PRSV BD-1 strain

SL.No	Primer ID	Sequence (5'-3')	Bp	°C	Product(bp)
1	S11F	CTCAACACAACATAATTCAAAGCAATCTACA	1-31	55	
	S11R	TCGAAGTTGCCTGCTTGATACCA	1,232-1,254		1,254
2	S12F	GAAATTTCTGTCCCACTCTTGGTGATTA	714-741	55	
	S12R	TACACGTTATCTTGTGACAAGG	1,848-1,869		1,155
3	S13F	CACGGGTCGAGTGGTCTAATTTACA	1,551-1,575	55	
	S13R	TCTTTTGTTTGTGGTCAACGAGAATCCTT	2,918-2,946		1,395
4	S14F	AAC AGA TGG TGG AGC ATA ATA TTG A	2,494-2,518	53	
	S14 R	TCA ATT GGT CCG CGA TTA AT	4,344-4,363		1869
5	S15F	AGA GGC TGT TGA AAA AAC CTA C	3,521-3,542	55	
	S15R	CGA ATT CAA ACC AGT ACA ACT GGT CAT TG	5,959-5,987		2466
6	S16F	CATTAAGGATGTTCC AGA GAG GTT GTA	5,750-5,776	55	
	S16R	CGA CTC CCA CTC TGC TCT ATA ACA T	7,612-7,636		1886
7	S17F	GATTTCGGGAGTCACTGGATCTCA ACT A	7,317-7,344	59	
	S17R	ACT GTG ATT GAG TGG CAC GAG TGT	9,538-9,561		2244
8	S18F	ACG AGT CAA AAA GCT TAG GAG ATT ACC AA	9,172-9,200	55	
	S18R	CTC TCA TTC TAA GAG GCT CAG ATA G	10,276-10,300		1128

Table 3.2. Primers used for amplification of the complete genome of PRSV BD-2 strain

SL.No.	Primer ID	Sequence (5'-3')	Postion (bp)	°C	Product size (bp)
1	S21F	CAT AAA CGA TGC CGA CTC AAC AC	1-23	55	
	S21R	CCA ACT TCC AGT GCC AGA T	884-904		904
2	S22F	ACACCATCTGAGATTGATTCAATCG	517-541	55	
	S22R	AAAACTCTGTTCAGTTGATCAAGCA	2,027-2,051		1534
3	S23F	AAGCCGAAGTTTGGTGATAAAATAG	1,606-1,630	53	
	S23R	TTTCTGAGCAAATCTCGAAGTTCGT	3,170-3,194		1588
4	S24F	AAGACTCCAACCAAGAATCACAT	2,656-2,678	54	
	S24 R	TTGTCCTCATAGAAGTCCTGAATGTC	4,309-4,334		1678
5	S25F	CAGAGAGATTTTGATCGGTTAGAA	3,985-4,008	52	
	S25R	TTTCACTCTCAATCAGTTTGTCG	5,943-5,965		1980
6	S26F	TGCACTGTACGGCAAGCAC	5,524-5,542	57	
	S26R	AGTGGACTACCACAATCACCATCAT	7,376-7,400		1876
7	S27F	GGATAAAGTTTGCATGATAGGTG	7,257-7,279	51	
	S27R	TTAACATCTCTATCTCTCTCTCCA	9399-9422		2165
8	S28F	GGCTCCTTATGTCTCTGAAGTT	9069-9090	55	
	S28R	CTCTCATTCTAAGAGGCTCAAATATCA	10299-10325		1256

of the predicted sizes were purified, cloned into the PGEM-T Easy® cloning vector (Promega, Madison, WI, USA) and sequenced at the University of Hawaii's Advanced Genomic and Sequencing Services and at Genwiz, CA (Wang et al. 2017). The sequences were assembled with the Cap3 sequence genome assembly (http://doua.prabi.fr/software/cap3). The complete genome sequences of all PRSV strains were deposited in GenBank.

Analysis and characterization of the genome sequences of PRSV strains in Bangladesh

We used BLASTn and BLASTx analysis to compare all sequences with those available in GenBank. The open reading frames (ORFs) and their predicted aa sequences were analyzed with the ORF finder (www.ncbi.nlm.nih.gov/projects/gorf/). We then used the sequence demarcation tool (SDT) with default parameters to obtain the specific nucleotide sequence identity of the PRSV strains and compared their sequences to PRSV strains from different countries and hosts (Muhire et al. 2017). Multiple sequence alignment was done with Clustal W (www.ebi.ac.uk), followed by phylogenetic analysis with MEGA 7.0 (www.megasoftware.net). Phylogenetic trees were constructed by the neighbor joining method (NJ), bootstrapped with 1,000 replicates (Thompson et al. 1994; Kumar et al. 2016). Further recombination analyses were made to identify potential recombinants and identify likely parental sequences and recombination break points. For this we used the RDP4 package (version 4.72) with RDP, GENECOV, Bootscan, Max Chi, Chimara, Siscan, and 3Seq methods (Martin et al. 2010). A multiple comparison corrected P-value cutoff of 0.05 and default settings were used and only events detectable by at least four different methods were considered for further analysis (Martin et al. 2010).

RT-PCR screening of field samples

The distribution and frequency of occurrence of the two PRSV strains were determined by RT-PCR with purified RNAs isolated from leaves of symptomatic, PRSV-infected papaya plants. Specific diagnostic primers were designed based on the sequence of PRSV strains found in Bangladesh and used for routine diagnosis (Table 3.3). Total RNAs were extracted using the RNeasy® Plant Mini Kit (QIAGEN, Valencia, CA, USA) and synthesis of cDNAs performed as described above. To detect PRSV strains, the thermal cycler program was: 94°C for 5 min, followed by 35 cycles of amplification at 94°C for 30 s, 57°C (PRSV BD-1) or 58°C (PRSV BD-2) for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min.

Table 3.3. Detection primers for PRSV BD-1 and PRSV BD-2 strains used in this study

Primer ID	Sequence (5'-3')	Tm (°C)	Product size (bp)	Purpose
BD -1F	GTCATCGTAAACCAACTGACCACA	57	446 bp	Detection of PRSV BD-1
BD-1R	AATGCCAACGAACGATCTCTCGTAA	58		
BD-2F	AACACAGAAAGCCAACAGATCACGTG	60	446 bp	Detection of PRSV BD-2
BD-2R	GATGCCATCTCACAATTTCTCGAAGG	58		

Results

Preliminary detection of PRSV in papaya plants with virus-like symptoms

During a survey from December 2016 to January 2017 in Bangladesh, 90 to 100% of the papaya trees were severely infected with papaya ring spot disease (Figure 3.2). Infected trees had striking symptoms of leaf mosaic, distortion, vein clearing, yellow mottling, brittleness, as well as water-soaked streaking of petioles and trunks. Papaya trees with severe virus infections exhibited shoestring-like leaves, occasional systemic necrosis, and wilting. In some orchards, trees were stunted and bushy due to extreme disease pressure during early growth stages and bore no fruit. Many mature, infected plants produced poor-quality fruit with ring-like spots characteristic of the disease. Occurrence of PRSV-like symptoms by region was: was 100% (345/345), 91% (10/11), 99% (165/167), 97% (192/168), 92% (12/13), 90% (124/137) and 94% (15/16) in the respective regions of Pabna, Rajshahi, Chandpur, Gazipur, Chapai Nawabgonj, Tangail, Mymensingh, and Munshigonj. For preliminary confirmation of PRSV in papaya, PRSV-specific, STNP and ELISA were used (Hamim et al. 2018b) to test the 11 selected samples with virus-like symptoms. All samples were positive for PRSV in both assays.

Identification of two distinct PRSV strains, BD-1 and BD-2.

HTS performed on an Illumina® HiSeq platform generated a total of 80,648,570 and 61,307,471 reads from composite samples pap 1 and pap-2, respectively (Table 3.4). Following *de novo* assembly using CLC Bio Genomic Workstation v8.5.1 (Qiagen, Hilden, Germany), 134 and 79 plant virus contigs were determined from pap-1 and pap-2, respectively. Composite samples pap-1 and pap-2 generated 127 and 74 PRSV-specific contigs, respectively. The size of the contigs in pap-1 and pap-2 varied from 200 to 10,282 bp and 200 to 3,484 bp, respectively.

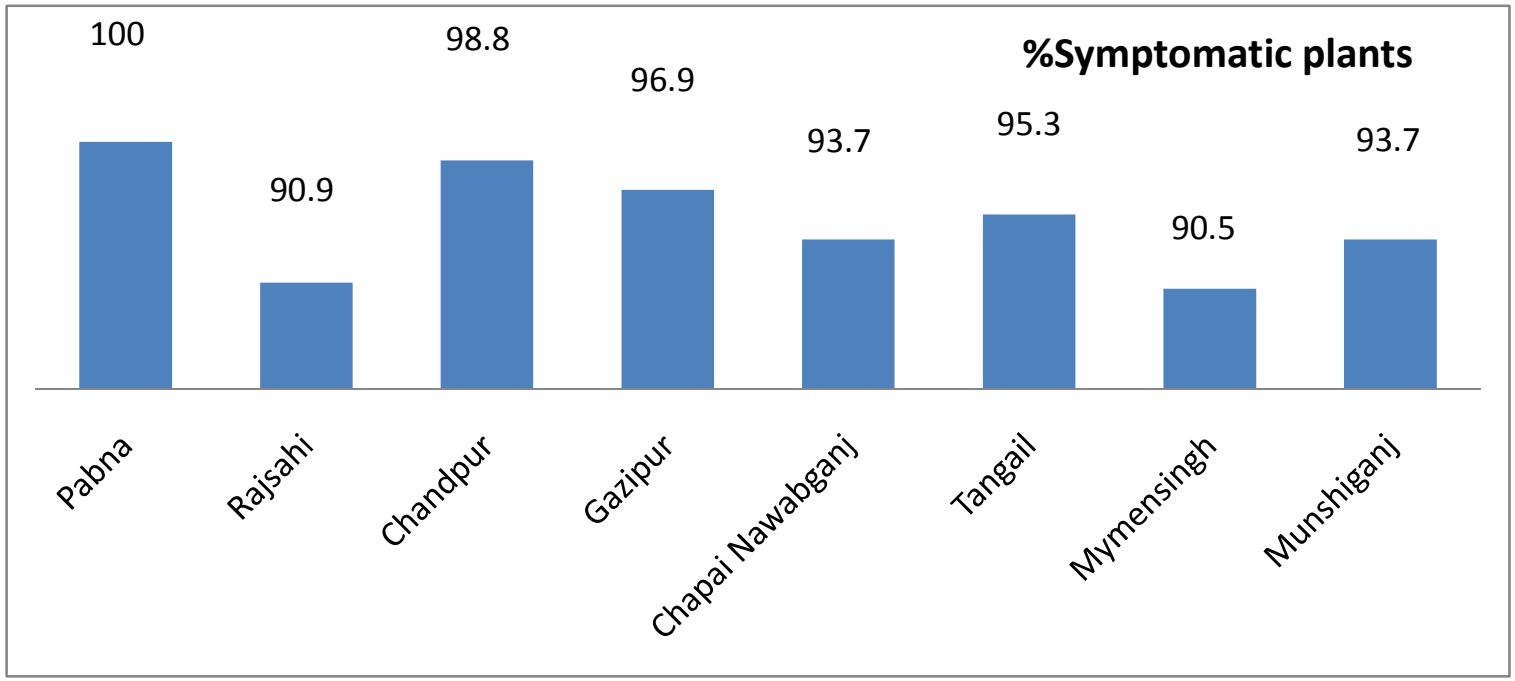


Figure 3.2. Incidence of virus-symptomatic plants in surveyed districts of Bangladesh from December 2016 to January 2017.

The contigs in pap-1 and pap-2 were respectively 52 to 100% and 64 to 100% identical to PRSV isolates for which sequence information is available in GenBank. Distinct strains of PRSV were identified from pap-1 (PRSV BD-1) and pap-2 (PRSV BD-2). The full-length coding sequences of PRSV genomes BD-1 and BD-2 were further verified by Sanger DNA sequencing and primer-walking of PCR-amplified, overlapping DNA fragments obtained by virus-specific primers designed from the HTS-derived sequences (Table 3.1 and Table 3.2).

The complete genome sequence of PRSV BD-1 (MH644652) was amplified from papaya sample GAZ-52 (Table 1) and PRSV BD-2 (MH397222) was amplified from papaya sample CD-129 (Table 3.4). PRSV BD-1 (10,300 nt) and PRSV BD-2 (10,325 nt) had polyproteins of 3,339 and 3,342 aa residues, respectively. The cleavage sites were identical in both strains, except for the cleavage sites between P1 and HC-pro, and CI and 6K2 (Figure 3.5) (Alabi et al.2017; Ortiz-Rojas and Chaves-Bedoya 2017).

Sequence alignment and phylogenetic analyses

The identities between PRSV BD-1 and BD-2 strains were 74% and 83% at the nucleotide and amino acid levels, respectively. The genome sequence of PRSV BD-1 had identities of 74 to 75% to the complete genome sequence of PRSV strains available in GenBank (Table 3.6). PRSV BD-1 exhibited the lowest identity (82%) to PRSV-Meghalaya (MF356497) at the amino acid level and the highest amino acid identity (86%) to PRSV-SK (KY996464). The genome sequence of PRSV BD-2 had identities of 79 to 88% at the nucleotide level to PRSV strains in GenBank (Table 3.6). At the amino acid level, PRSV BD-2 had the lowest identity (87%) to PRSV-SK (KY996464) and the highest identity (90%) to PRSV-Meghalaya (MF356497) (Table 3.7). The P1 protein was the most variable in both strains (Table 3.5). The

3’-UTR regions of PRSV BD -1 and -2 were highly conserved, with sequence identities of 90 to 96% compared to other PRSV isolates.

Table 3.4. Assembly of HTS data from PRSV-infected, composite papaya samples in Bangladesh

Composite sample ID	**pap-1**	**pap -2**
Total reads	80,648,570	61,307,471
No. of non-virus contigs	906	1043
No. of plant virus contigs	134	79
No. of PRSV-specific contigs	127	74
Sequence length of PRSV contigs	200 -10,282 bp	200-3,484 bp
Percentage of identical matches to reported isolates	52-100%	64-100%
Identified strains	PRSV BD strain-1 (10,300 bp)	PRSV BD strain-2 (10,325 bp)

Table 3.5. Nucleotide and amino acid identities in particular coding regions of PRSV BD-1 and PRSV BD-2 compared to other reported PRSV isolates

Coding regions	**PRSV strain BD-1**		**PRSV strain BD-2**	
	nt (%)	aa(%)	nt(%)	aa(%)
P1	66-71	55-66	67-82	60-74
HC-Pro	75-78	86-94	84-91	86-98
P3	73-77	79-82	82-91	89-93
6K1	77-84	87-96	84-93	92-98
CI	76-78	90-95	81-91	95-97
6K2	71-76	74-80	82-91	90-98
VPg	73-75	88-92	80-89	91-94
NIa-Pro	74-79	85-89	81-93	92-97
Nib	76-78	83-90	80-90	89-94
CP	89-90	89-97	86-97	86-97
PIPO	86-92	71-80	90-94	87-96

Table 3.6 Percentage of the genomic nucleotide and amino acid sequence identities of PRSV BD-(MH444652) compared to other PRSV strains

Virus Name	%Nucleotide identity	% Amino acid identity
PRSVBD – 2 (MH397222)	74	83
PRSVW-38NT- cucurbit-Australia (KX655872)	75	85
PRSV-PG-Hawaii-Papaya-EU126128	75	85
PRSV-PTX-Papaya-USA-KY271954	74	84
PRSV-Papaya-Taiwan(X67673)	75	84
PRSV-P-5-19-Papaya- Taiwan-EU882728	75	84
PRSV-HYD-India-Papaya(KP743981)	74	84
PRSVP-DEL-Papaya-India-EF017707	74	84
PRSV-Meghalaya–Papaya-India-MF356497	75	82
PRSV-Hn-Pumpkin-China-MF074214	75	85
PRSV-XM-Papaya-China-KY933061	74	84
PRSVP-pfT3-AX-D-Ph-Papaya-Taiwan-JX448372	74	84
PRSV-SK- Cucurbita pepo-South Korea(KY996464)	75	86
PRSV-E2-Cucurbita pepo-France-KC345609	75	85
PRSV-Mex-Vrpro-Papaya-AY231130	75	84
ZTMV-Momordicacharantia-France-NC_023175	67	73
MWMV-zucchini-Tunisia-NC_009995	59	57
DMV-Daphne mezereum-Czech Republic-NC_008028	50	43
PLDMV-China-Papaya-JX974555	50	43

Table 3.7. Percentages of the genomic nucleotide and amino acid sequence identities of PRSVBD-2 (MH397222) compared to other PRSV strains

Virus Name	**%Nucleotide identity**	**% Amino acid identity**
PRSVBD-1 (MH444652)	74	83
PRSVW-38NT- cucurbit-Australia (KX655872)	81	88
PRSV-PG-Hawaii-Papaya-EU126128	82	88
PRSV-PTX-Papaya-USA-KY271954	82	88
PRSV-Papaya-Taiwan(X67673)	82	88
PRSV-P-5-19-Papaya- Taiwan-EU882728	81	88
PRSV-HYD-India-Papaya(KP743981)	81	88
PRSVP-DEL-Papaya-India-EF017707	82	87
PRSV-Meghalaya–Papaya-India-MF356497	88	90
PRSV-Hn-Pumpkin-China-MF074214	81	89
PRSV-XM-Papaya-China-KY933061	80	88
PRSVP-pfT3-AX-D-Ph-Papaya-Taiwan-JX448372	81	88
PRSV-SK- Cucurbita pepo-South Korea(KY996464)	79	87
PRSV-E2-Cucurbita pepo-France-KC345609	82	88
PRSV-Mex-Vrpro-Papaya-AY231130	82	88
ZTMV-Momordicacharantia-France-NC_023175	67	72
MWMV-zucchini-Tunisia-NC_009995	59	43
DMV-Daphne mezereum-Czech Republic-NC_008028	50	42
PLDMV-China-Papaya-JX974555	50	43

The phylogenetic tree (Figure 3.3) was based on nucleotide sequences of the newly identified PRSV BD strains and PRSV strains worldwide reported in GenBank. The PRSV clusters were divided into two major phylogroups, A and B. PRSV BD-1 was in phylogroup B, while phylogroup A included PRSV BD-2 and the other reported PRSV isolates. Phylogroup A was divided into five minor phylogroups (i-v). PRSV BD-2 was in phylogroup (iv) with the Indian PRSV isolate, PRSV-Meghalaya (MF356497). However, two other Indian isolates, PRSV-HYD (KP743981) and PRSVP-DEL (EF017707), were clustered in phylogroup (iii) and closely related to American and Australian PRSV isolates found in phylogroups (i) and (ii), respectively. All Chinese isolates formed a distinct phylogroup (v) with Taiwanese and Korean strains. A second phylogenetic tree (Figure 3.4) generated from amino acid sequence comparisons of PRSV strains and other potyviruses was constructed. Results of this analysis also supported the phylogenetic relationship of PRSV BD-1 and PRSV BD-2 with other PRSV strains and potyviruses in Figure 3.3. As in the phylogenetic tree based on nucleotide sequence comparisons, PRSV BD-1 was in a distinct major phylogroup (B) and PRSV BD-2 formed a minor phylogroup (v) with PRSV-Meghalaya (MF356497). Thus, phylogenetic analysis of PRSV populations revealed that PRSV BD-1 and PRSV BD-2 were located in two distinct, major phylogroups.

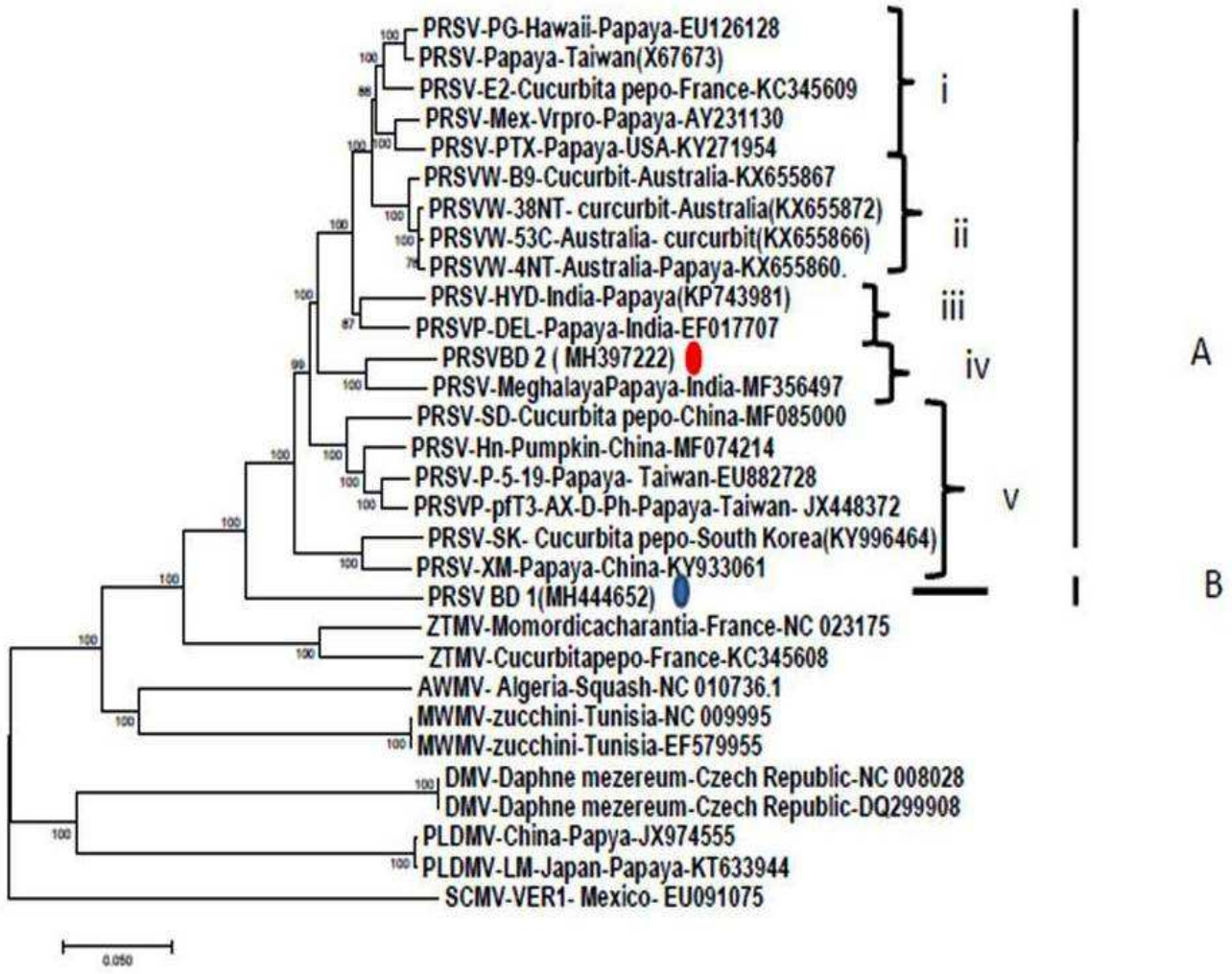


Figure 3.3. Neighbor-joining phylogram obtained from the alignment of nucleotide (nt) sequences of PRSV BD-1 and BD-2 with other PRSV strains and potyviruses obtained from GenBank. The tree was constructed in MEGA 7.0.14 using Clustal W with 1,000 replicates. Bootstrap values are percentages with only values over 75% shown at the nodes. The tree was rooted with SCMV-Ver1-Mexico nt sequence (EU091075). The symbols show two newly sequenced strains from Bangladesh, PRSV BD-1 (marked by a blue oval) and PRSV BD-2 (marked by a red oval).

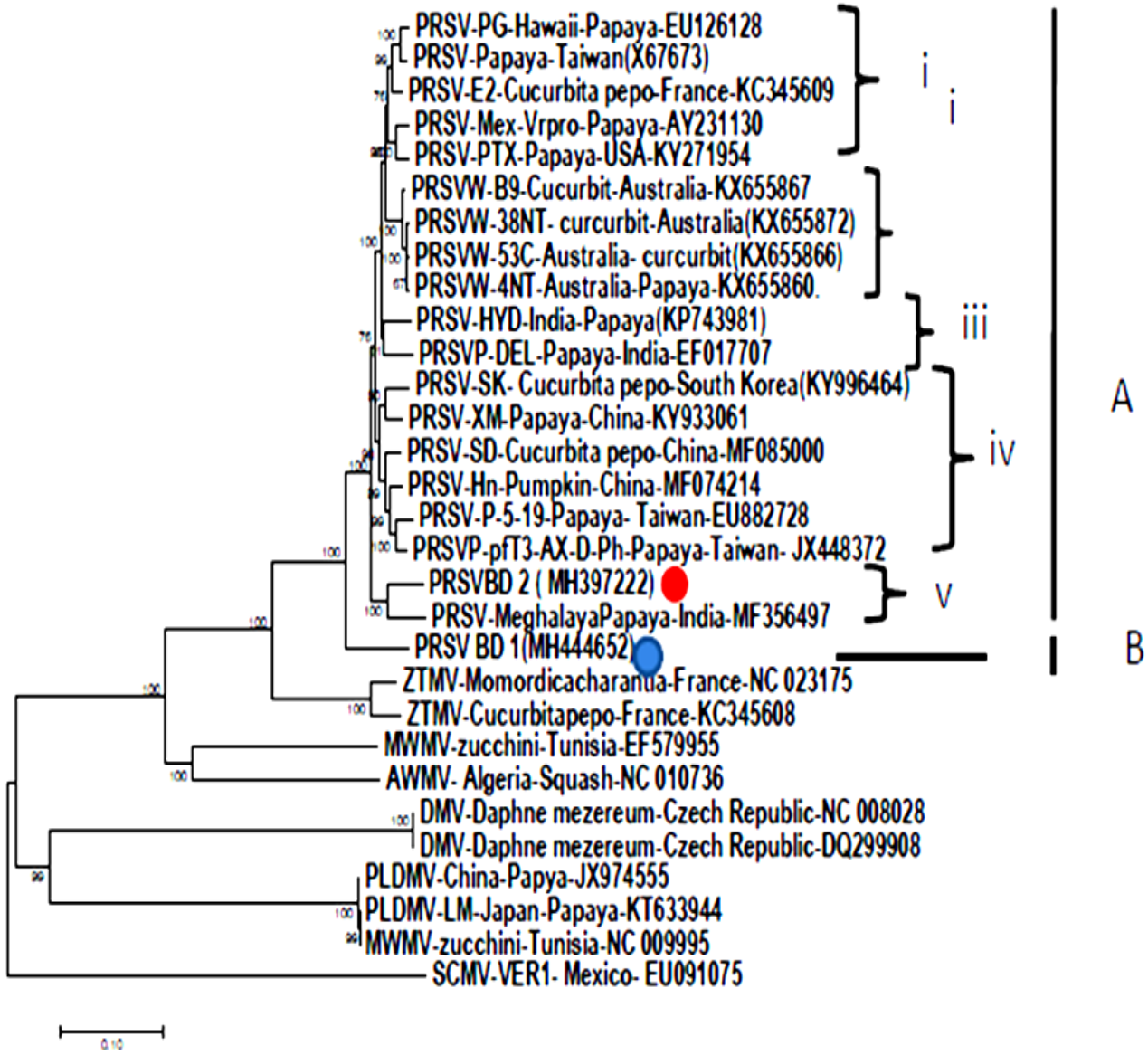


Figure 3.4. Neighbor-joining phylogram obtained from the alignment of amino acid (aa) sequences of PRSV BD-1 and BD-2 with other PRSV strains and potyviruses obtained from GenBank. The tree was constructed in MEGA 7.0.14 using Clustal W with 1,000 replicates. Bootstrap values are percentages with only values over 65% shown at the nodes. The tree was rooted with SCMV-Ver1-Mexico aa sequence (EU091075). The symbols show two newly sequenced strains from Bangladesh PRSV BD-1 (marked by a blue oval) and PRSV BD-2 (marked by a red oval).

Recombination analysis

We used the recombination analysis program RDP4 to screen for possible recombination events within the genome sequences of PRSV BD-1 and PRSV BD-2 (Table 3.8 and Figure 3.5) using sequences of other PRSV isolates from GenBank. PRSV BD-1 was not a recombinant strain, but analysis revealed that PRSV BD-2 was and included two unique recombination events, E1 and E2. Recombination event E1 spanned the region from 1,302 nt to 4,068 nt and E2 spanned the region from 4,095 nt to 9,200 nt (Table 3.8 and Figure 3.4). BD-2 was the recombinant of a minor parent, PRSV-XM (KY933061), and a major parent, PRSV-Meghalaya (MF356497). PRSV-Meghalaya (MF356497) contributed the larger region of the sequence to PRSV BD-2 in both recombination events, whereas PRSV-XM (KY933061) contributed a smaller region of sequences in E1 and/or E2.

Distribution of PRSV BD-1 and PRSV BD-2

Two pairs of specific primers, BD-1F (1,779 to 1,802 nt)/ BD-1R (2,201 to 2,225 nt) and BD-2 F (1,779 to 1,804 nt)/ BD-2R (2,200 to 2,225 nt) were used to develop RT-PCR detection assays for PRSV BD-1 and BD-2, respectively (Table 3.9). PCR assays specific for strains PRSV BD-1 and BD-2 produced amplicons of the expected 446-bp size. Sequence analysis of the amplicons from each strain-specific assay confirmed the specificity of the primers for PRSV BD-1 and PRSV BD-2, as all amplicon sequences were identical to the corresponding region of the assembled strain genomes. The incidence of PRSV BD-1 among the 118 papaya samples with typical virus symptoms collected from Pabna, Rajshahi, Chapai Nawabganj, Chandpur, Munshiganj, Gazipur, Tangail, and Mymensingh was respectively 6% (1/17), 0% (0/5), 0% (0/4), 33% (11/33), 22% (2/9), 36% (8/22), 0% (0/13) and 0% (0/15)(Table 3.9). The incidence of

PRSV BD-2 among the 118 papaya samples with typical virus symptoms collected from Pabna, Rajshahi, Chapai Nawabganj, Chandpur, Munshiganj, Gazipur, Tangail and Mymensingh was respectively 100% (17/17), 60% (3/5), 75% (3/4), 73% (24/33), 56% (5/9), 68% (15/22), 77% (10/13) and 73% (11/15). The overall percentage of samples positive for PRSV BD-1 and BD-2 in the surveyed districts was 19% (22/118) and 75% (88/118), respectively. PRSV BD-1 had a mean incidence of 12% in all districts sampled, while PRSV BD-2 occurred in all districts with an overall average incidence of 73% (Table 3.3). In the surveyed districts, 11% (13/118) of the symptomatic papaya plants were infected by both PRSV BD-1 and BD-2.

Discussion

Virus diseases are a major threat to papaya production worldwide and several different viruses have been reported. Among these PRSV, a dominant and widespread virus, was first reported from Bangladesh in 2004 (Jain et al. 2004). Incidence of PRSV-like disease symptoms occurred in 90 to 100% of the plants surveyed in this study. Most of the symptomatic plants were severely affected, with stunted growth and severe reductions in fruit yield and quality.

To detect the presence of different strains of PRSV in Bangladesh and obtain their full-length sequences, we used total RNAs extracted from infected papaya tissue to construct cDNA libraries, followed by HTS and conventional Sanger sequencing (Hamim et al. 2018c). The sequence of two distinct PRSV strains, PRSV BD-1 (10,300 bp) and PRSV BD-2 (10,325 bp) represent the first complete genomes of PRSV strains from Bangladesh. These strains were 74% and 83% identical to one another at the nucleotide and deduced amino acid levels, respectively. PRSV BD-1 was 74 to 75% and BD-2 79 to 88% identical to other full-length PRSV isolates from around the world. Alignment of the coding regions of the PRSV strains from Bangladesh with the available PRSV genomes in GenBank indicated that the CP regions and 3’-UTRs were

relatively conserved and the P1 regions were the most variable. Based on phylogenetic sequence analyses, PRSV BD-2 was most closely related to PRSV-Meghalaya (MF356497) from papaya in India. PRSV BD-1, however, was on a distinct branch from the other PRSV sequences based on nucleotide and amino acid sequence comparisons. PRSV BD-1 was only distantly related to PRSV populations from other countries, supporting the hypothesis that PRSV BD-1 emerged in Bangladesh, rather than being introduced from other countries. PRSV BD-2 was phylogenetically close to a PRSV isolate from Meghalaya, in northeast India, with which it formed a minor phylogroup based on both nucleotide and amino acid sequences.

According to the current International Committee on Taxonomy of Viruses (ICTV), the species demarcation criteria for different species within the genus *Potyvirus*, the complete genomic nucleotide sequence identity must be <76% and the amino acid sequence identity <82% (Stephen et al. 2017). Based on the sequences obtained in this study, PRSV BD-1 and PRSV BD-2 were divergent strains of PRSV at the nucleotide level with identities of 74-75 % and 79-88%, respectively, over the complete viral genome compared to other complete genomes of PRSV worldwide. At the aa level, PRSV BD-1 exhibited 82 to 86% identities and PRSV BD-2 exhibited 87 to 90% identities to other full-length coding regions of PRSV strains reported globally.

Recombination within single-stranded RNA viruses is a major evolutionary means for viruses to adapt to new hosts and environments (Noa-Carrazana et al. 2007). Recombination has occurred frequently in the evolution of potyviruses (Hamim et al. 2018b). Computer analysis detected the likely occurrence of recombination within PRSV BD-2, but not the PRSV BD-1 genome. Comparisons of the genome sequences from the newly identified PRSV strains to other sequenced PRSV genomes detected two putative recombination events in PRSV BD-2 based on

the presence of a 2,766-nucleotide fragment identical to PRSV-Meghalaya (MF356497) and a 5,105-nucleotide fragment identical to PRSV-China (KY933061). This agrees with a previous study showing that the C1 and CP regions are prone to recombination events in the PRSV genome (Noa-Carrazana et al. 2007). Furthermore, PRSV BD-2 was not found to be a parent of other recombinants. The presence of two recombination events in PRSV BD-2 originating from PRSV strains from India and China may reflect the increasingly frequent agricultural exchanges between the two countries. Our results add new knowledge on PRSV genomes in Bangladesh and facilitate further study of their genetic variation, and, development of more accurate diagnostic assays to monitor spread of the virus.

A possible natural means for the spread of PRSV is by the annual, strong monsoonal winds that travel from India towards Bangladesh. PRSV is transmitted nonpersistently by aphids, which may be spread by strong monsoon winds (Coutts et al. 2011). The spread of other potyviruses by similar means has been reported (Coutts et al. 2011).

In this study, we developed specific RT-PCR assays capable of identifying two strains of PRSV in the same papaya plant. PRSV BD-1 and BD-2 in papaya tissue can be detected and distinguished from other isolates using the specific RT-PCR assays developed. To our knowledge, this is the first report of specific primers used to differentiate strains of PRSV occurring in the same papaya plant. Our RT-PCR assays provide a way to detect and monitor PRSV BD-1 and BD-2 in papaya orchards and may help manage this disease in the field. The relative occurrence of PRSV BD-1 (19%) and PRSV BD-2 (69%) was based on 118 randomly sampled papaya plants with virus-like symptoms in Bangladesh. Our results indicate that mixed infections of these two strains can occur in single papaya plants as 13 papaya samples out of 118 were co-infected by both strains. We also analyzed 28 samples with virus-like symptoms that

tested negative for PRSV BD-1 and BD-2, suggesting that there are unknown isolates of PRSV or other viruses infecting papaya in Bangladesh.

Our finding that two distinct strains of PRSV could occur in the same plant, together with data from the survey, suggest genetic diversity in the Bangladesh PRSV population. We plan to screen papaya plants and their associated weeds growing in other districts of Bangladesh for the presence of PRSV, since weeds and cucurbit hosts may contribute to the molecular diversity of PRSV. The sequencing of additional PRSV strains, along with studies to link various symptoms with specific virus populations are valuable areas for future research.

Transgene-derived resistance is currently the only method for successfully managing PRSV in papaya (Gonsalves 1998; Hamim et al. 2018b). PRSV-resistant transgenic papaya cultivars are highly specific to local virus strains (Hamim et al. 2018b). Our study showed that PRSV strains in Bangladesh were very diverse compared to PRSV strains from other geographical locations. Therefore, characterization of local PRSV populations and identification of conserved regions in the Bangladesh strains is needed to develop transgene-derived, PRSV-resistant papaya for Bangladesh.

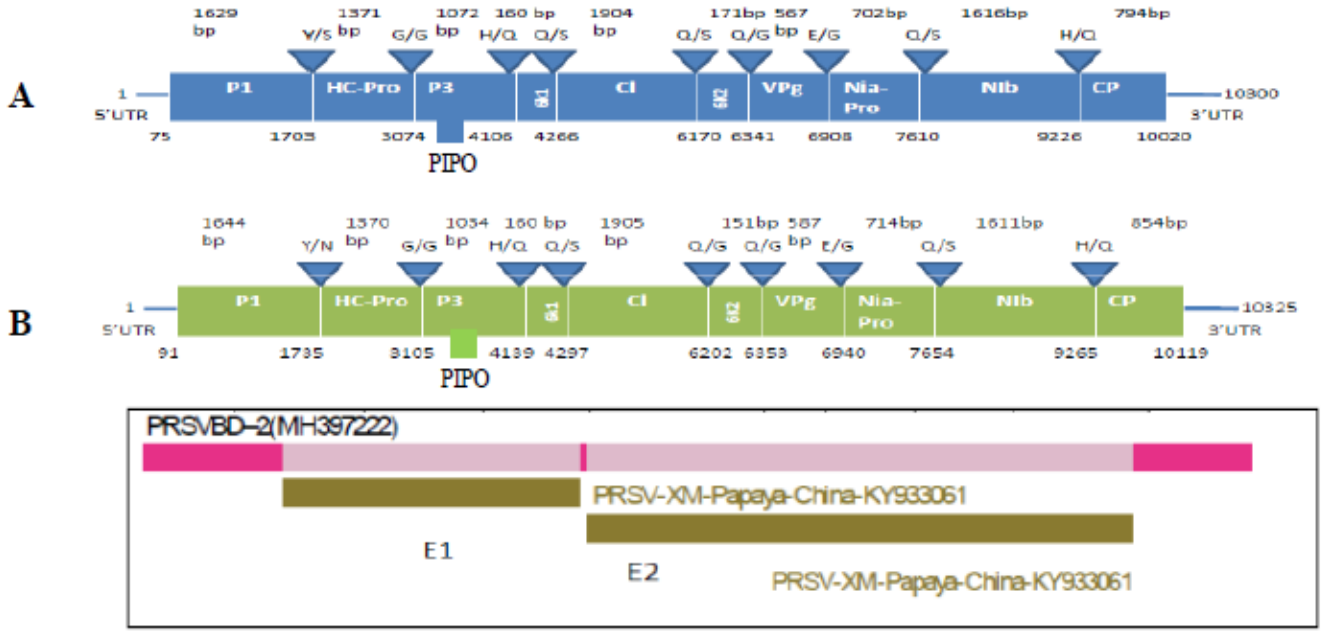


Figure 3. 5. Genome organization of PRSV BD-1 and BD- 2. A: Genome organization of PRSV BD-1 determined from Sanger DNA sequencing of PCR DNA fragments by primer-walking. B: Genome organization of PRSV BD- 2 determined from Sanger DNA sequencing of PCR fragments by primer-walking. Two recombination events detected in PRSV BD-2 by the RDP4 package: RDP, Maxchi, Chimaera and 3Seq. Minor parental strain=PRSV-XM-Papaya-China-KY933061, major parental strain=PRSV-Meghalaya–Papaya-India-MF356497

Table 3.8. Information about recombinant events identified in the PRSV BD -2 strain as analyzed by RDP4

Recombination event	Breakpoint positions		Recombinant sequence(s)	Minor parental strain	Major parental strain	Detection methods (P value)			
						RDP	Maxchi	Chimaera	3Seq
E1	1313	4079	PRSVBD–2(MH397222)	PRSV-XM-Papaya-China-KY933061	Unknown (PRSV-Meghalaya–Papaya-India-MF356497)	$4.2x10^{-52}$	$5.4x10^{-40}$	$2.8x10^{-37}$	$3.2x10^{-13}$
E2	4106	9211	PRSVBD–2(MH397222)	PRSV-XM-Papaya-China-KY933061	Unknown (PRSV-Meghalaya–Papaya-India-MF356497)	$2.6x10^{-48}$	$2.1x10^{-50}$	$2.83x10^{-25}$	$4.30x10^{-13}$

Minor parental strain: this strain contributed a small sequence fraction in the recombinant strain

Major parental strain: this strain contributed a large sequence fraction in the recombinant strain

Table 3.9. Occurrence and distribution of PRSV BD-1 and PRSV BD-2 of papaya ringspot virus (PRSV) on papaya in Bangladesh

Districts	**Number of plants tested**	**PRSV BD-1 positive**	**PRSV BD-2 Positive**	**Mixed infection of PRSV BD-1 and PRSV BD-2**
Pabna	17	1	17	1
Rajshahi	5	0	3	0
Chapai Nawabganj	4	0	3	0
Chandpur	33	11	24	8
Munshiganj	9	2	5	1
Gazipur	22	8	15	3
Tangail	13	0	6	0
Mymensingh	15	0	8	0
Total	118	22	81	13

CHAPTER IV

DETECTION AND CHARACTERIZATION OF TOMATO LEAF CURL BANGLADESH VIRUS AND ASSOCIATED SUBVIRAL DNA MOLECULES IN PAPAYA IN BANGLADESH

Introduction

Leaf curl disease and associated begomoviruses are an emerging threat to papaya production worldwide. Their incidence may be increasing due to the broad host range of begomoviruses, the polyphagous nature of their whitefly vectors, and the use of mixed cropping systems (Singh-Pant et al. 2012). Additionally, the high rates of mutation and recombination among begomoviruses may stimulate the evolution of new species and their associated subviral DNAs (Singh-Pant et al. 2012). Leaf curl symptoms in papaya have been reported from India, Pakistan, Nepal, Iran and China, where monopartite begomoviruses are known to infect papaya (Singh-Pant et al. 2012; Bananej et al. 2016). Papaya plants infected by begomoviruses suffer serious production losses and can act as sources of virus inoculum for transmission by whitefly (*Bemisia tabaci)* vectors (Guo et al. 2015). Singh-Pant and colleagues (2012) reported infection by chili leaf curl virus (ChiLCuV) and papaya leaf crumple virus in papaya. Other begomoviruses found in papaya include: papaya leaf curl Guandong virus, papaya leaf curl China virus, malvastrum leaf curl virus, okra enation leaf curl virus, cotton leaf curl Multan virus, ageratum yellow vein virus, tomato leaf curl Joydebpur virus (ToLCJoV) and tomato leaf curl New Delhi virus (ToLCNDV) (Bananej et al. 2016; Guo et al. 2015; Raj et al. 2008; Maruthi et al. 2007; Shahid et al. 2013; Sinha et al. 2013; Cai et al. 2005; Wang et al. 2004).

Begomoviruses, in the family *Geminiviridae*, have circular single-stranded DNA (ssDNA) genomes grouped into monopartite and bi-partite viruses. Monopartite begomoviruses have genomes homologous to the DNA-A components of bipartite begomoviruses, and include V1, C1, C2, C3, and C4 genes that respectively encode the capsid protein (CP), replication-associated protein (Rep), transactivator protein (TrAP), replication enhancer protein (REn), and C4 proteins (Harrison and Robinson 1999; Saunders and Stanley 1999). Monopartite begomoviruses are often associated with subviral DNA molecules such as alphasatellites, betasatellites and defective satellites. These satellites depend on the begomovirus for replication, encapsidation, insect transmission, and movement within plants (Briddon and Stanley, 2006, Saunders and Stanley 1999). DNA-A components occur with bipartite begomoviruses, along with a second component of similar genomic size that encodes a movement protein and a nuclear shuttle protein (Saunders and Stanley 1999).

Severe leaf curl symptoms were observed on papaya during surveys of seven districts in Bangladesh. Incidence of plants affected by leaf curl disease was less than 10% of those plants infected by papaya ringspot disease, but the symptoms on these plants were very severe (Hamim et al. 2019). It is possible that changes in agricultural practices and high populations of *Bemisia tabaci* species that were also present have facilitated infection of papaya by begomoviruses and led to more severe disease symptoms. In this paper, we report the molecular detection and characterization of tomato leaf curl Bangladesh virus (ToLCBV) and its associated subviral DNA molecules in papaya in Bangladesh. Findings from this study will assist development of strategies to sustainably manage papaya leaf curl disease and its associated virus complexes in Bangladesh.

Materials and methods

We surveyed the incidence of leaf curl diseases of papaya from December 2016 to January 2017 in seven districts of Bangladesh: Pabna (24.0700°N, 89.1134°E), Rajshahi (24.3636°N, 88.6241°E), Chapai Nawabganj (24.7413°N, 88.2912°E), Chandpur (23.2513°N, 90.8518°E), Gazipur (24.0958°N, 90.4125°E), Tangail (24.3917°N, 89.9948°E), and Mymensingh (24.7471°N, 90.4203°E). During the survey, 45 papaya samples with distinct leaf curl symptoms were collected (Figure 4.1) and immediately stored in RNA*later*® (Qiagen, Inc., USA) for later analyses.

Figure 4.1 Papaya plants in Bangladesh with symptoms of leaf curl disease, including: severe leaf curling, swollen veins, and twisted petioles

Total genomic DNA was extracted from the 45 symptomatic and two asymptomatic leaves of papaya. These DNA samples were used as templates for the preliminary detection of begomoviruses by PCR with the primer pair MKBEGAF4/MKBEGAR5 (Table 4.1) (Usharani et al. 2013). The target PCR products corresponded to a 1,300-bp region of the DNA-A component of begomoviruses. The partial begomovirus PCR products were cloned and sequenced. We amplified the full-length genome sequence of ToLCBV-[BD-Pap-17] (2,760 bp) from the Gaz17-Pap isolate obtained from Gazipur, Bangladesh by PCR using primer pairs MKBEGAF4/MKBEGAR5, PA/ToLCBV39R2, ToLCBV-DNA2F/PB, ToLCBV-DNAA1F/ToLCBV39R2 and ToLCBV-DNA2F/ToLCBV39R1 (Table 4.1). The same Gaz17-Pap isolate was used to amplify the ToLCBV cognate betasatellite ToLCB-[BD-Pap-17] (1,367 bp) using primer sets Beta 01/Beta 02 (Briddon et al. 2002) and ToLCBbetaPaF/ToLCBbetaPaR (Table 4.1). Betasatellite-specific primers were also used to amplify the small defective betasatellites- DefS39 (564bp), DefS40 (731bp), and Defs43 (738bp) from ToLCBV-positive samples Gaz19-Pap, Gaz20-Pap, and Gaz21-Pap, respectively. The amplified PCR products were cloned, sequenced, and assembled using the CAP3 genome assembly program (Huang and Madan 1999). Genome sequences were compared to those in GenBank by BLASTn analysis (Altschul et al. 1990). Open reading frames (ORFs) and their predicted amino acid sequences were analyzed with the ORF Finder tool in Geneious®Pro (Kearse et al. 2012). Begomovirus and satellite sequences were aligned with ClustalW in Geneious®Pro (Kearse et al. 2012) and the Sequence Demarcation Tool (SDT) version 1.2 with default parameters was used to obtain percent pairwise nucleotide sequence identity scores of the ToLCBV and DNA satellites (Muhire et al. 2014). Phylogenetic trees were constructed using the neighbor joining method (NJ) with a

maximum-likelihood algorithm of 1,000 bootstrapped replicates in MEGA 7.0 (Kumar et al. 2016).

Results

Papaya plants with leaf curl disease had pronounced leaf curling, swollen veins, and twisted petioles (Figure 4.1). Some of the diseased plants were stunted and failed to produce fruit. The incidence of diseased plants varied from 1.9 to 8.8% across the areas surveyed (Figure 4.2). In the preliminary detection of begomoviruses in symptomatic papaya leaves, 43 samples were positive for begomovirus (Table 4.2 and Table 4.3). Of these, 29 (67%) samples contained sequences of ToLCBV, the remainder contained sequences of ToLCJoV or ToLCNDV (Table 4.2 and Table 4.3). BLASTn analysis revealed that at the nucleotide level the ToLCBV isolates were 97 to 100% identical to one another and 97 to 99% identical to ToLCBV (KM383761) that infects tomato in Gazipur, Bangladesh. ToLCJoV isolates from papaya were 95 to 98% identical to ToLCJoV (KM383749), and ToLCNDV isolates from papaya were 97 to 98% identical to ToLCNDV (KM383742) at the nucleotide level.

The size, number, and arrangement of the ORFs and intergenic region (IR) of ToLCBV-[BD-Pap-17] (MH380003) were identical to ToLCBV isolates that infect tomato. ToLCBV-[BD-Pap-17] had the highest nucleotide identity (98%) and a close phylogenetic relationship to ToLCBV (KM383761) that infects tomato in Bangladesh (Table 4.4 and Figure 4.3). The CP of ToLCBV-[BD-Pap-17] also had the highest amino acid sequence identity (99%) and a close phylogenetic relationship to the CP of ToLCBV (KM383761) (Table 4.4 and Figure 4.3b). These results suggested that ToLCBV-[BD-Pap-17] from papaya is a variant of the ToLCBV isolates previously reported from tomato (Brown et al. 2015). ToLCB-[BD-Pap-17] (MH397223) had

typical structural features shared by other tomato-infecting betasatellites. It had the highest sequence identity (92%) to ToLCB (JN638434) detected from tomato in India and clustered with betasatellites JN638434, AJ542489 and JN663872 (Table 4.4 and Figure 4.4a) in phylogenetic analyses.

Table 4.1 Primers used to detect begomoviruses in papaya and characterize the complete genomes of tomato leaf curl Bangladesh virus (ToLCBV) and its associated DNA satellites

Primer ID	Primer sequence (5'-3')	Annealing temperature	Target	Product size
MKBEGAF4	ATATCTGCAGGGNAARATHTGGATGGA	56	646bp-1945bp	1300 bp
MKBEGAR5	TGGACTGCAGACNGGNAARACNATGTGGGC			
PA	TAATATTACCKGWKGVCCSC	53	2754bp-1417bp	1424 bp
ToLCBV39R2	CAAGCCGAGAATGGCGTGTATATG			
ToLCBV DNA2F	AGCTGCAGTGATGAGTTCC	55	1440bp-528bp	1849bp
PB	TGGACYTTRCAWGGBCCTTCACA			
ToLCBVDNA A1F	ACCGGATGGCCGCGATTT	57	1bp-1417bp	1417bp
ToLCBV39R2	CAAGCCGAGAATGGCGTGTATATG			
ToLCBV DNA2F	AGCTGCAGTGATGAGTTCC	55	1440bp-2760bp	1321bp
ToLCBV39R1	AATATTAGTGCGGATGGCCGCTTT			
Beta 01	GGTACCACTACGCTACGCAGCAGCC	60	1303bp-1327bp	1367bp
Beta 02	GGTACCTACCCTCCCAGGGGTACAC		1309bp-1284bp	
ToLCBbetaPaF	ACCGTGGGCGAGCGGGGTTTTTGGC	65	1bp-25 bp	1367 bp
ToLCBbetaPaR	AATATTAGAACGTGGGCGAGCTAAG		1367bp-1343bp	

Based on the species demarcation threshold of <91% sequence identity at the nucleotide level for a distinct betasatellite, ToLCB-[BD-Pap-17] was classified as a variant of ToLCB (JN638434)that infects tomato in India [23]. ToLCB-[BD-Pap-17] contained an ORF (βC1) that encoded a protein with an important role in pathogenicity, based on a function determined for homologous protein C1 of tomato leaf curl Ranchi betasatellite (AGA60247). The βC1 protein of ToLCB-[BD-Pap-17] had an amino acid identity of 86 to 89% to that of betasatellites (JN663872, JN638434 and AJ542489) infecting tomato in Bangladesh and India. However, it had the highest amino acid identity (89%) to the βC1 protein of tomato leaf curl Ranchi betasatellite (JN663872) that infects chili pepper in India (Table 4.4). Except for the present study, there have been no other reports of betasatellites associated with begomoviruses that infect papaya in Bangladesh.

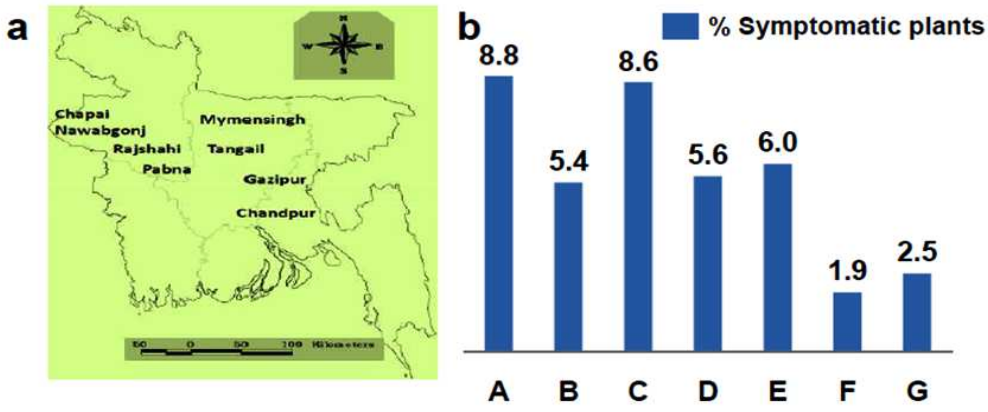


Fig 4.2. Distribution of papaya plants with leaf curl disease symptoms. a. Map of the districts in Bangladesh, where papaya leaf curl disease was found. b: Percentage of leaf curl disease in seven districts of Bangladesh recorded during a survey from December 2016 to January 2017. A=Pabna, B=Rajshahi, C=Chandpur, D=Chapai Nawabgonj, E=Gazipur, F=Tangail, G=Mymensingh

BLASTn analysis of the defective betasatellites revealed that nucleotide positions 1 to 178 nt of DefS39, DefS40, and DefS43 (MH473589, MH507169, and MH507170) were 98 to 99% identical to the satellite-conserved region (SCR) of ToLCB (MH397223) (Figure 4.5a). Nucleotide positions 407 to 564 nt of DefS39, 584 to 731 nt of DefS40, and 580 to 738 nt of DefS43 contained the conserved sequence of the stem–loop structure with 93 to 100% identities to the corresponding region of ToLCB (MH397223) (Figure 5c). In addition to being defective (lacking βC1), they also appeared to be recombinant, containing sequences derived from the helper virus ToLCBV (Figure 4.5b). The nucleotide positions 266 to 405 nt of DefS39, 266 to 405 nt of DefS40, and 351 to 494 nt of DefS43 were 92 to 100% identical to corresponding regions of ToLCBV (MH380003) (Figure 4.5). All defective betasatellite molecules had the ALl binding-specific iteron GGTACTCA (Figure 4.5b). One iteron upstream and one downstream of the Rep promoter TATA box, plus a possible second iteron, were absent. Repeated PCR-sequence analyses confirmed the absence of any betasatellites in papaya samples Gaz19-Pap, Gaz20-Pap, and Gaz21-Pap and the absence of any defective satellites in Gaz17-Pap.

Table 4.2 Distribution of tomato leaf curl Bangladesh virus (ToLCBV) in papaya in Bangladesh

District	**Samples tested**	**Samples positive for begomovirus**	**ToLCBV**	**ToLCJoV**	**ToLNDV**
Pabna	8	7	3	3	1
Chapai Nawabgonj	4	4	1	0	3
Rajshahi	4	4	4	0	0
Gazipur	10	9	8	1	0
Tangail	4	4	3	1	0
Chandpur	10	10	5	5	0
Mymensingh	5	5	5	0	0
Total	45	43	29	10	4

Table 4.3 GenBank accession numbers for the partial sequences of 43 begomovirus isolates: ToLCBV, ToLCJoV and ToLCNDV

	Sample	**Begomovirus**	**Location**	**Accession nos.**
1	Pab 16-Pap	ToLCBV	Pabna	MK172053
2	Pab2-Pap	ToLCBV	Pabna	MK172054
3	Pab4-Pap	ToLCBV	Pabna	MK172055
4	Chapa10-Pap	ToLCBV	Chapai	MK172056
5	Raj11-Pap	ToLCBV	Rajshahi	MK172057
6	Raj13-Pap	ToLCBV	Rajshahi	MK182574
7	Raj14-Pap	ToLCBV	Rajshahi	MK185017
8	Raj12-Pap	ToLCBV	Rajshahi	MK185018
9	Gaz21-Pap	ToLCBV	Gazipur	MK202759
10	Gaz17-Pap	ToLCBV	Gazipur	MK202760
11	Gaz 18-Pap	ToLCBV	Gazipur	MK202761
12	Gaz19-Pap	ToLCBV	Gazipur	MK202762
13	Gaz20-Pap	ToLCBV	Gazipur	MK202763
14	Gaz22-Pap	ToLCBV	Gazipur	MK202764
15	Gaz23-Pap	ToLCBV	Gazipur	MK202765
16	Gaz24-Pap	ToLCBV	Gazipur	MK238042
17	Tang25-Pap	ToLCBV	Tangail	MK240348
18	Tang27-Pap	ToLCBV	Tangail	MK240349
19	Tang28-Pap	ToLCBV	Tangail	MK241556
20	Chand31-Pap	ToLCBV	Chandpur	MK213916
21	Chand33-Pap	ToLCBV	Chandpur	MK213917
22	Chand 38-Pap	ToLCBV	Chandpur	MK213918
23	Chand 39-Pap	ToLCBV	Chandpur	MK213919
24	Chand 40-Pap	ToLCBV	Chandpur	MK213920
25	Mym41-Pap	ToLCBV	Mymensingh	MK213921
26	Mym42-Pap	ToLCBV	Mymensingh	MK213922
27	Mym43-Pap	ToLCBV	Mymensingh	MK213923
28	Mym44-Pap	ToLCBV	Mymensingh	MK213924
29	Mym45-Pap	ToLCBV	Mymensingh	MK213925
30	J1-Pap	ToLCJoV	Pabna	MK213927
31	J2-Pap	ToLCJoV	Pabna	MK213926
32	P3-Pap	ToLCJoV	Pabna	MK213928
33	G4-Pap	ToLCJoV	Gazipur	MK213928
34	T5-Pap	ToLCJoV	Tangail	MK213930
35	C85-Pap	ToLCJoV	Chandpur	MK213931
36	C86-Pap	ToLCJoV	Chandpur	MK213932
37	C87-Pap	ToLCJoV	Chandpur	MK213933
38	C88-Pap	ToLCJoV	Chandpur	MK213934
39	C102-Pap	ToLCJoV	Chandpur	MK213935
40	Nd56-Pap	ToLCNDV	Chapai	MK213937
41	Nd61-Pap	ToLCNDV	Chapai	MK213938
42	Nd62-Pap	ToLCNDV	Chapai	MK238041
43	Nd71-Pap	ToLCNDV	Pabna	MK213936

Discussion

ToLCBV-[BD-Pap-17] (MH38003) is a variant of the previously reported tomato-infecting ToLCBV, suggesting that the virus may have been introduced to papaya from ToLCBV-infected tomatoes in Bangladesh. In support of result, others have reported the host range expansion of ToLCBV to *Gaillardia pulchella* and *Gomphostemma niveum* in India (Datta et al. 2015; Mahatma et al. 2012). Similarly, the ToLCB satellite associated with ToLCBV-[BD-Pap-17] had high homology to a betasatellite (JN638434) isolated from tomato in India. Sing-Pant and colleagues (2013) also reported that the ToLCB satellite from the unrelated virus ChiLCuV could infect papaya plants in India. Synergistic interactions between a particular virus and an unrelated satellite could alter the host range or increase the pathogenicity of the virus (Saunders et al. 2008).

ToLCBV-associated defective betasatellites MH473589, MH507169, and MH507170 were distinct from the ToLCBV-associated betasatellite (MH397223), and less than half the size of the full-length betasatellites associated with other begomoviruses. These defective satellites retained a portion of the satellite conserved region (SCR) but lacked the βC1 coding sequence. The SCR helps defective, small DNA satellites associate with monopartite begomoviruses (Huang et al. 2013). Finally, the defective betasatellite sequences were not only betasatellite-derived, they also contained helper virus-derived sequences. Alignment of the missing corresponding sequences of the betasatellite indicated the recombination sites and betasatellite sequences immediately adjacent to the virus-derived sequence (Figure 5b). *Ageratum conyzoides* with symptoms of yellow vein disease can also contain recombinants between the helper virus and betasatellite (Saunders et al. 2001; Stanley et al. 1997). Furthermore, Saunders and others

(2001) demonstrated that co-inoculation of *A. conyzoides* with ageratum yellow vein virus and defective recombinant DNA satellites induced the disease phenotype.

Our results further confirmed that defective satellites do not occur with betasatellites in the same papaya plant. Defective satellites contained parts of a betasatellite, so the defective satellites could induce disease symptoms (Saunders et al. 2001). Papaya plants associated with a defective or recombinant betasatellite developed slightly milder disease symptoms than those associated with a native betasatellite. ToLCBV and its associated subviral DNA molecules were identified in papaya for the first time in this study. Their distribution and any subsequent yield losses in papaya in Bangladesh remain to be determined. More work is needed to establish if synergistic effects among ToLCBV-subviral DNA molecule complexes and PRSV could occur.

Table 4.4 Pairwise identity (%) of ToLCBV [MH38003] compared to other reported begomoviruses and ToLCB [MH397223] compared to other reported begomovirus-associated satellites

Begomoviruses	ToLCBV [MH38003]		Begomovirus-associated satellites	ToLCB [MH397223]	
	Complete genome identity (%nucleotide)	Coat protein (CP) identity (%amino acid)		Complete genome identity (%nucleotide)	Beta C1(βC1) identity (%amino acid)
ToLCBV:BD:Tom (KM383761)	98	99	ToLCB:Ind:Tom(JN638434)	91	86
ToLCBV:BD:Tom (AF188481)	95	99	ToLCB:BD:Tom (AJ542489)	90	87
ToLCBV:BD:Tom (KM383762)	91	98	ToLCRnB:Ind:Chi(JN663872)	86	89
ToLCRnV:Ind: Tom(GQ994095)	90	98	ToLCB:Ind:Pap(HM143902)	86	76
TbCSV:Ch:Wat(KU198364)	88	98	ToLCBB:Ind:Chi(HM007118)	86	74
AEV:Ind:Ag(JQ911767)	87	99	ToLCB:Ind:Mb(JQ654464)	85	77
ChiLCV :Ind:Chi(DQ673859)	87	97	ChLCB:Ind:Pap(HM143904)	85	75
ChiLCV :Pak:Chi (LN886651)	86	96	ChLCB:Ind:Ok (KJ614228)	82	73
ToLCJV:BD:Tom(KM383750)	86	97	ToLCBB:Ind:Bri (JX311469)	85	72
ToLCNDV:BD:Tom(KM383738)	73	92	ToLCBB:Ind:Chi (JN663876)	85	72

ToLCBV=tomato leaf curl Bangladesh virus, BD=Bangladesh, Tom-tomato; ToLCRnV=tomato leaf curl Ranchi virus, Ind=India, TbCSV=tobacco curly shoot virus, Wat= watermelon, Ch=China, AEV= ageratum enation virus, Ag-=*Ageratum* sp.*; ChiLCV*= chili leaf curl virus, Chi= Chili,Pak= Pakistan, ToLCJV= tomato leaf curl Joydebpur virus, ToLCNDV= tomato leaf curl New Delhi virus, Pap=papaya

ToLCB=tomato leaf curl beta-satellite, Ind= India, Tom= tomato, BD= Bangladesh, ToLCRnV= tomato leaf curl Ranchi beta-satellite, Ind= India, ToLCBB= tomato leaf curl Bangladesh beta-satellite, Chi= chili, Pap= papaya, Mb= mung bean, Ok= okra, Bri= brinjal, Sau= Saudi Arabia.

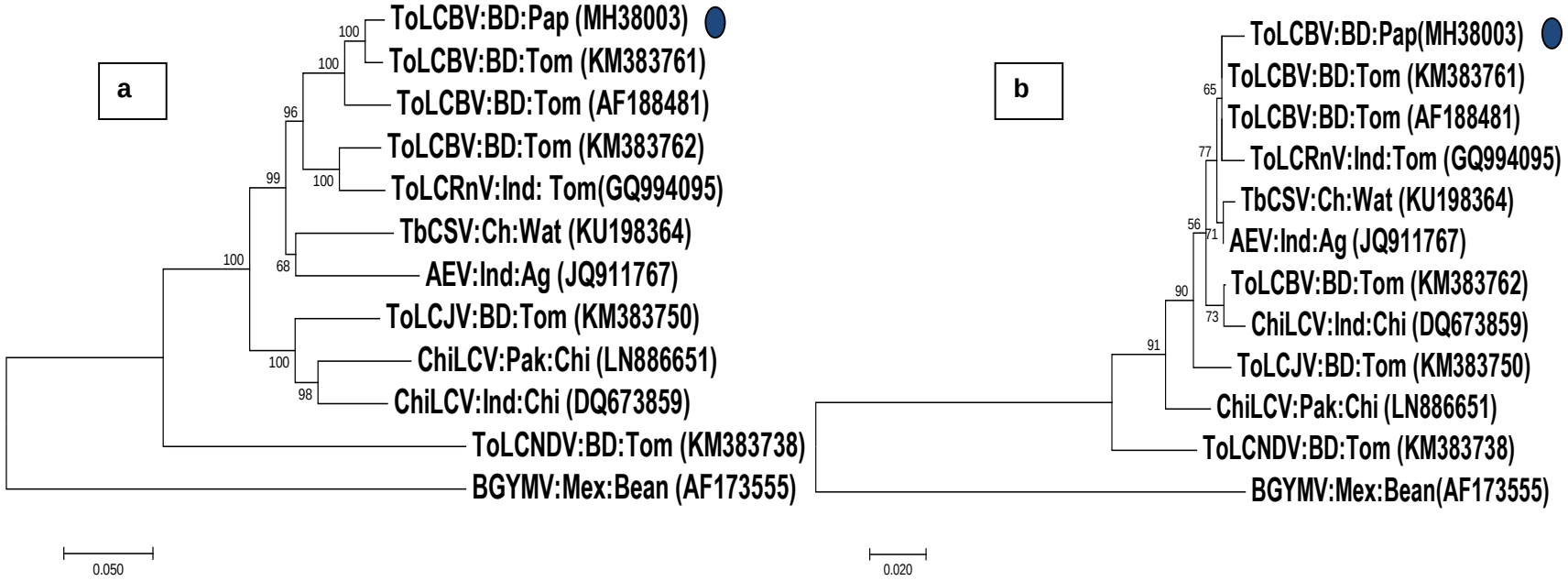


Figure .4.3. Phylograms showing the (a) phylogenetic relationship of ToLCBV-[BD-Pap-17] (ToLCBV: BD:Pap) (highlighted by blue ovals) to other begomoviruses based on complete nucleotide genome sequences and (b) the phylogenetic relationship among ToLCBV-[BD-Pap-17] (ToLCBV:BD:Pap) and other begomoviruses based on their CP amino acid sequences (highlighted by blue ovals), respectively. ToLCBV=tomato leaf curl Bangladesh virus, BD=Bangladesh, Tom=tomato, ToLCRnV=tomato leaf curl Ranchi virus, Ind=India, TbCSV=tobacco curly shoot virus, Wat=watermelon, Ch=China, AEV=ageratum enation virus, Ag=*Ageratum* sp., ChiLCV=chili leaf curl virus, Chi=Chili, Pak=Pakistan, ToLCJV=tomato leaf curl Joydebpur virus, ToLCNDV=tomato leaf curl New Delhi virus, Pap=papaya

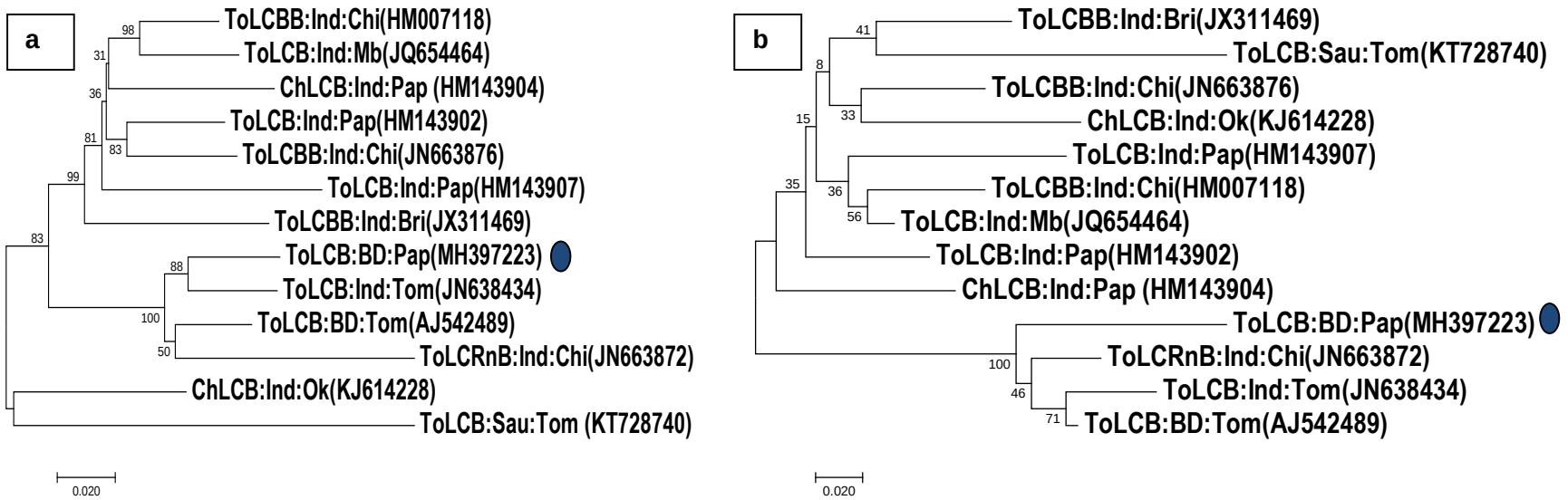
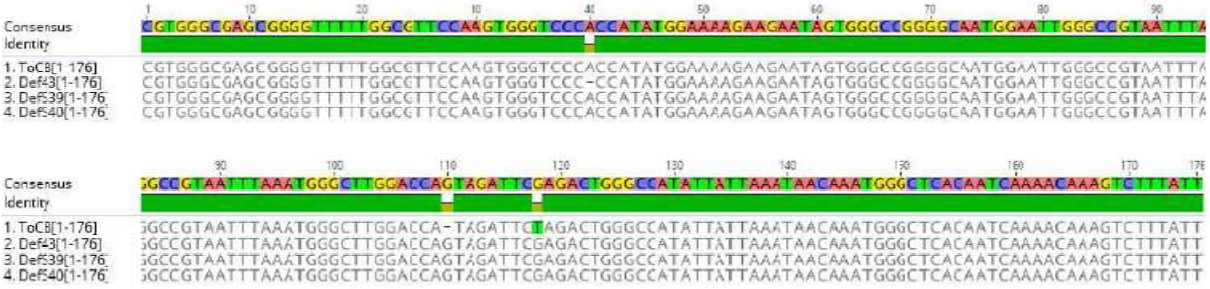


Figure 4.4. Phylograms showing the (a) phylogenetic relationship of ToLCB-[BD-Pap-17] (ToLCB:BD:Pap) (highlighted by blue ovals) to other betasatellites associated with begomoviruses based on complete nucleotide genome sequences and (b) the phylogenetic relationship among ToLCB-[BD-pap-17] (ToLCB:BD:Pap) (highlighted by blue ovals) and other betasatellites associated with begomoviruses based on their beta C1 amino acid sequences, respectively. ToLCB=tomato leaf curl beta-satellite, Ind=India, Tom=tomato, BD=Bangladesh, ToLCRnV=tomato leaf curl Ranchi betasatellite, Ind=India, ToLCBB=tomato leaf curl Bangladesh beta satellite, Chi=chili, Pap=papaya, Mb=mung bean, Ok=okra, Bri=brinjal, Sau=Saudi Arabia

SCR upstream of nonanucleotide (a)



Region derived from helper virus(b)

Conserved region with stem-loop structure (c)

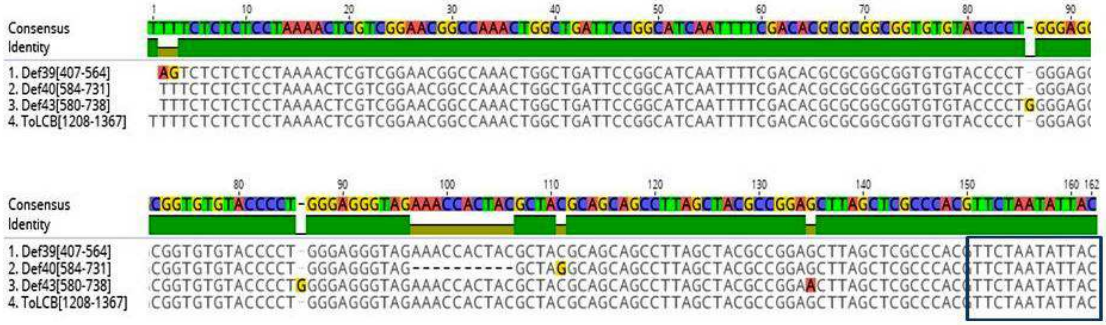


Figure 4.5 Alignement of ToLCBV-associated defective betasatellites with tomato leaf curl beta-satellite (ToLCB) and tomato leaf curl Bangladesh virus (ToLCBV) (a) Alignment of the sequences immediately upstream of the nonanucleotide (nona)-containing hairpin (TCTAATATTAC) of Defs39, Defs40, Defs43 and the SCR of ToLCB (MH397223). (b) Alignment of the helper-virus derived regions of the satellites Defs39, Defs40 and Defs43 with the iteron-containing sequence of ToLCBV (MH380003). The TATA box of the Rep promoter is highlighted within the red box. The predicted iteron sequences of the virus are in the black boxes. (c) Comparisons of the sequences encompassing the nonanucleotide (TAATATTAC) containing hairpin structures of the small DNA molecules Defs39, Defs40, Defs43 and tomato leaf curl betasatellite (MH397223).

CHAPTER V

MOLECULAR CHARACTERIZATION OF TOMATO LEAF CURL JOYDEBPUR VIRUS AND TOMATO LEAF CURL NEW DELHI VIRUS IN PAPAYA IN BANGLADESH

Introduction

Papaya (*Carica papaya* L., Caricaceae) is an extensively grown, economically important fruit crop in Bangladesh. Abundant in minerals and nutrients, this appetizing berry-type fruit is consumed not only as a dessert but also as a green vegetable, in salads, and used to tenderize meat. Rich in papain, papaya is used in food processing, the leather industry, and medicine (Milkovich 2017). Viral diseases are important limitations to papaya production worldwide, and cause symptoms of ringspot, leaf curl, mosaic, and distortion, all of which have been reported from different papaya growing regions in Bangladesh (Milkovich et al. 2017). These diseases can be caused by either single or mixed infections of various RNA or DNA viruses from diverse genera, including *Begomovirus*, *Cucumovirus*, *Ilarvirus*, *Potyvirus*, *Potexvirus*, *Nepovirus*, *Tospovirus*, *Tobravirus* and *Crinivirus* (Hamim et al. 2018a).

Leaf curl of papaya is an emerging disease in Asia and there are several reports of the association of begomoviruses with leaf curl diseases of papaya in different countries. Plants infected by begomoviruses suffer serious production losses and can act as sources of virus inoculum for whitefly (*Bemisia tabaci)* vectors. Begomovirus also can cause leaf curl disease in tomato (*Solanum lycopersicum*), chili (*Capsicum spp*.) and in papaya (Singh-Pant et al. 2013).

Begomoviruses, in the family *Geminiviridae,* are characterized by circular single-stranded DNA (ssDNA) genomes. They are grouped into monopartite and bi-partite viruses

(Brown et al. 2012). The genomes of monopartite begomoviruses are homologous to the DNA-A components of bipartite begomoviruses, including the V1, C1, C2, C3, and C4 genes that encode the coat protein (CP), replication-associated protein (Rep), transactivator protein (TrAP), replication enhancer protein (REn), and C4 proteins, respectively. Monopartite begomoviruses are usually associated with betasatellites or defective betasatellites and these satellites depend on a begomovirus for their replication, encapsidation, transmission via insects, and movement within or between plants (Briddon and Stanley 2006). DNA-A components occur in bipartite begomoviruses, along with a second component of similar genomic size that encodes a movement protein (BV1 or NSP) and a nuclear shuttle protein (BC1 or MP). DNA-A encodes two genes on the virus strand (+), the coat protein (AV1 or CP) and AV2, and four genes on the complementary strand. DNA-A also encodes the replication-associated protein (AC1 or Rep), transcriptional activator protein (AC2 or TrAP), replication enhancer protein (AC3 or REn), and AC4. A common region (CR) of near nucleotide identity is shared between DNA-A and DNA-B. The CR contains promoters and iteron sequences required in virus replication (Raj et al 2008).

In many regions of Bangladesh, begomoviruses and associated betasatellite molecules are emerging as serious pathogens infecting vegetables and weeds. Previously, tomato leaf curl New Delhi virus (ToLCNDV) was the only begomovirus reported from papaya in Bangladesh (Maruthi et al. 2007). However, begomoviruses other than papaya leaf curl virus (PLCV) also have been reported on papaya from India, Nepal, and the USA. Studies from India have shown that papaya is also a host of tomato leaf curl Joydebpur virus (ToLCJoV), ToLCNDV, and betasatellites (Shahid et al. 2013).

Severe whitefly infestations and leaf curl symptoms of papaya were observed during surveys of seven districts in Bangladesh in 2016 and 2017. The incidence of plants afflicted by

leaf curl disease was low compared to that of papayaringspot disease, but the symptoms of leaf curl on plants were severe (Hamim et al. 2018d). It may be that changes in agricultural practices have led to a higher incidence of this more severe disease in papaya. It is also possible that changes in *B.tabaci* species have allowed the virus to spread from vegetable crops to papaya.

In this paper, we report the molecular characterization of tomato leaf curl Joydebpur virus (ToLCJoV) and tomato leaf curl New Delhi virus (ToLCNDV) in papaya from Bangladesh. This is the first time these viruses have been molecularly characterized based on their genome components from papaya in Bangladesh. Findings from this study will be important in developing strategies to sustainably manage papaya leaf curl disease and its associated virus complexes in Bangladesh.

Materials and Methods

Papaya leaf curl sample collection and DNA extraction

Leaf samples were collected from five papaya plants growing in the Pabna district of Bangladesh. Two of these plants displayed typical leaf curl symptoms, the other three were asymptomatic. All papaya leaf samples were immediately stored in RNA*later*® (Qiagen, Inc., USA) and shipped to the plant virology laboratory at the University of Hawaii at Manoa, Oahu, USA, under USDA PPQ 526 permits (P526-160413-009 and P526P-16-03662). Total genomic DNA was extracted from the symptomatic and asymptomatic leaves using a DNeasy®Plant Mini Extraction Kit (Qiagen, Inc., USA) and quantified with a NanoDrop® spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Determination of the full-length genomes of two begomoviruses

We amplified the full-length genome sequence of ToLCJoV by PCR using primer pairs described in Table 5.1 from the J1-Pap isolate obtained from the Pabna district in Bangladesh. The same isolate was used to amplify the ToLCJoV cognate betasatellite using primer sets in Table 5.1. PCR assays were performed under the following cycling conditions: initial denaturation at 94°C for 5 min; 35 cycles, each consisting of denaturation at 94°C for 1 min, annealing for 1 min at the temperatures listed in Table 5.1 and Table 5.2 for the primer pair used, and extension at 72°C for 1.5 min; followed by a final extension at 72°C for 7 min. We used primer pairs mentioned in Table 5.2 and Table 5.3 using the same PCR cycling conditions and respective annealing temperatures to amplify the DNA-A and DNA-B from ToLCNDV, respectively. The amplified PCR products were cloned and sequenced as described above. Sequences were assembled using the CAP3 genome assembly program (Huang et al. 1999).

Pairwise identity, phylogenetic and recombination analysis

All sequences obtained by PCR were compared to sequences available in GenBank using BLASTn and BLASTx analysis. The open reading frames (ORFs) and their predicted amino acid sequences were analyzed with the ORF finder (www.ncbi.nlm.nih.gov/projects/gorf/). The sequence demarcation tool (SDT) was used with default parameters to obtain the specific nucleotide sequence identity of the isolates of begomovirus and betasatellite identified here with respective sequences of isolates of begomovirus and betasatellite from different countries and hosts (Muhire et al. 2017).

Table 5.1 Primers used to detect begomoviruses in papaya and characterize the complete genomes of tomato leaf curl Joydebpur virus (ToLCJoV) and its associated betasatellites

Primers	**Sequences**	**Annealing temperatures (℃)**	**Target**
ToLCJoBEGAF4	CTATGTACTGGGCAAGATTTGGATG	55	647bp to 1756bp
ToLCJoBEGAR4	CAAAGGGACTGGCAATCAAATACG		
ToLCJVPA	CTAATATTACCGGATGGCCGCGAT	55	2756bp to 1441bp
ToLJoVJ1R2	CAGGCCGAGAATGGCGTGTTTATC		
ToLCJoVDNA2F	AGCTGCAGTGATGGGTTCC	55 ℃	1442bp to 543bp
ToLCJVPB	TGGACCTTACATGGGCCTTCACA		
ToLCJoVJ1DNAF1	ACCGGATGGCCGCGATTT	57	1bp to 1441bp
ToLCJoVJ1R2	CAGGCCGAGAATGGCGTGTTTATC		
ToLCJoV DNAF2	AGCTGCAGTGATGGGTTCC	55	1422bp to 2762bp
ToLCJoVJ1R1	AATATTAGTGCGGATGGCCGCTTT		
Beta 01	GGTACCACTACGCTACGCAGCAGCC	60	1301bp to 1325bp
Beta 02	GGTACCTACCCTCCCAGGGGTACAC		1306bp to 1282bp
ToLJoBbetaPaF	ACCGTGGGCGAGCGGGGTCTTTGGC	65	1bp to 25bp
ToLJoBbetaPaR	AATATTAGAACGTGGGCGAGCTAGG		1365 to 1341

Table 5.2 Primers used to determine the complete genomes of tomato leaf curl NewDelhi virus (ToLCNDV) DNA A component

Primers	**Sequences**	**Annealing Temperature** (℃)	**Target**
ToLCNDVBEGAF4	CTATGTGCTGGGGAAGATATGGATGGA	56	628bp-1929bp
ToLCNDVBEGAR5	GGATAGTAGGACAGGCAAAACAATGTG		
ToLCNDVPA	TAATATTACCGAATGGCCGC	52	2734bp-1415bp
ToLNDV71R2	CGAGCAGAGAGTGGCGTATATACC		
ToLCNDVDNA2F	ATCCGCAGTGATGTATTCC	55	1416bp-501bp
ToLCNDVPB	CACCTTGCAAGGGCCTTCACA		
ToLCNDVDNA A1F	ACCGAATGGCCGCGCAAAT	58	1bp-1415bp
ToLCNDVJ1R2	CGAGCAGAGAGTGGCGTATATACC		
ToLCNDV DNA2F	ATCCGCAGTGATGTATTCC	51	1416bp-2740bp
ToLCNDV71R1	AATATTATACGAATGGCCGCTTT		

Table 5.3 Primers used to determine the complete genomes of tomato leaf curl New Delhi virus (ToLCNDV) DNA B component

Primers	**Sequences**	**Annealing temperature (℃)**	**Targets (bp)**
ToLCNDVB1F1	ACCCGTAACGATCTTGAACTTTGTCC	58	1544-1569
ToLCNDVB1R1	GGTACCATATTTGGCAATAGGTCCGAA		1520-1546
ToLCNDVDNA-BF	CAATATACGCGTAAGGAAATATGTG	52	623-1517
ToLCNDVDNABR	AATCATGGGCTAGCAGATCG		
ToLCNDV DNA B 1F)	ACCGAAAGGCCGCGAAAAT	55	1-1458
ToLCNDV DNA B 2R	AAC CTG AGC GGA CTG GAC GAT T		
ToLCNDV DNA B 1R	AATATTATACGAAAGGCCGCTT	52	1437-2688
ToLCNDV DNA B 2F	AATCGTCCAGTCCGCTCAGGTT		
TLCN7102FP	TGCAATCTCTTCCCTTGTGATA	51	1375-767
TLCND7102RP	TTAGCTTGATGTACGAACGAA		

Multiple sequence alignment was done with Clustal W (www.ebi.ac.uk) followed by phylogenetic analysis using MEGA 7.0 (www.megasoftware.net), and phylogenetic trees were constructed by the neighbor joining method (NJ), bootstrapped with 1000 replicates (Kumar et al. 2016). Recombination analyses were conducted to detect potential recombinants, major and minor parental sequences and recombination break points in the genomes using the RDP4 software (version 4.72). This software includes RDP, GENECOV, Bootscan, Max Chi, Chimara, Siscan, and 3Seq methods (Martin et al. 2010). A multiple comparison corrected P-value cutoff (0.05) system with default settings was used. We considered only recombination events confirmed by at least four independent methods (Martin et al. 2010).

Results

Molecular characterization of ToLCJoV and associated betasatellite in papaya

The complete 2,762-nucleotide sequence of the ToLCJoV-[BD-Pap-J1] isolate was determined for both orientations. The sequence contained features typical of the genome of ToLCJV: two ORFs, V1-coat protein (CP); V2-pre-coat protein on the virion-sense strand; and five ORFs, C1- replication associated protein (Rep), C2- the transcriptional-activator protein (TrAP), C3- the replication-enhancer protein (REn), separated by an intergenic region (IR) (Table 5.4). The genome sequence of ToLCJoV-[BD-Pap-J1] had identities of 90 to 95% at the nucleotide level to the sequence of ToLCJoV isolates in GenBank (Table 5.4). It shared the highest nucleotide identity (95%) with ToLCJoV (KM383750) infecting tomato in Bangladesh and the lowest nucleotide identity (90%) to ToLCJoV (JX311468) infecting brinjal in India.

Table 5.4. Pairwise percentages of nucleotide identities between the genomic components and amino acid sequence identities of encoded genes from ToLCJoV [BD:Pap:J1] and ToLCNDV-[BD:Pap:ND71], with the components and genes of ToLCJoV and ToLCNDV available in GenBank

ToLCJV-[BD:Pap:J1]			**ToLCJoB -[BD-Pap-J1]**		
DNA components/ genes	Nt	aa		Nt	aa
ssDNA	90-95	-	ssDNA	82-95	-
			A-rich region	86-91	-
V1 (CP)	92-99	97-99	SCR regions	93-98	-
V2	92-99	90-94	Beta C1	86-98	77-98
C1(Rep)	90-94	90-94			
C2 (TrAP)	93-99	90-99			
C3(REn)	92-99	88-100			
ToLCNDV-[BD:Pap:ND71]			**ToLCNDV-DNA B-[BD-Pap-ND71]**		
	Nt	aa		nt	aa
DNA A	93-98	-	DNA B	83-93	
AV1(CP)	96-99	98-100	BC1	87-97	88-99
AV2	97-99	93-100	BV1	89-95	92-99
AC1(Rep)	93-98	94-98			
AC2 (TrAP)	95-99	87-99			
AC3 (Ren)	92-99	85-98			

Genes are indicated as coat protein (CP), replication-associated protein (Rep), transcriptional activator protein (TrAP), and replication enhancer (REn). nt = nucleotide, aa = amino acid

This result was well supported by phylogenetic analyses based on alignment of the entire ssDNA genome of ToLCJoV-[BD-Pap-J1] with the complete genome sequences of other related begomoviruses (Fig. 5.1A). The phylogenetic tree was based on nucleotide sequences of the newly identified ToLCJoV-[BD-Pap-J1] and other ToLCJV isolates worldwide. It revealed that ToLCJoV-[BD-Pap-J1] was closely related to ToLCJV isolates infecting tomato in Bangladesh (KM38750, AJ875159, KM383749 and KM383751), India (KF551591), and a ToLCJoV isolate infecting chili in Kolkata, India (HM007109), all of which shared 94 to 95% nucleotide identity with ToLCJoV-[BD-Pap-J1]. The comparison of individually encoded proteins revealed the C3 (Rep) gene of ToLCJoV-[BD-Pap-J1] had the lowest sequence identitiesof 90 to 94%, at both the nucleotide and amino acid levels to the C3 gene of other ToLCJV isolates. A second phylogenetic tree (Figure 5.1B) generated from amino acid sequence comparisons of the C3 gene of the ToLCJoV-[BD-Pap-J1] and ToLCJoV isolates was constructed that also supported the phylogenetic relationship of ToLCJoV-[BD-Pap-J1]and ToLCJoV isolates (Fig.5.1B). A comprehensive recombination analysis using the software program RDP4 was conducted to detect anypossible recombination events within the genome of ToLCJoV isolates (Table 5.4 and Fig. 5.3). We identified two recombinant isolates: ToLCJoV-[BD-Pap-J1] and ToLCJoV (JQ654461) infecting mungbean (*Phaseolus aureus*) in India. ToLCJoV-[BD-Pap-J1] was the recombinant of a minor parent, ToLCJV (AJ875159), and a major parent, ToLCJV (KM383751). ToLCJV (KM383751) contributed the larger region of the sequence to ToLCJoV-[BD-Pap-J1], whereas ToLCJV (AJ875159) contributed the smaller region. ToLCJV (JQ654461) was also a recombinant, to which ToLCJoV-[BD-Pap-J1] donated the major portion and ToLCJV (AJ875159) donated the minor portion. These results suggested that the ToLCJoV-[BD-Pap-J1] isolate from papaya was a recombinant variant of ToLCJoV isolates infecting tomato in

Bangladesh, based on the current species and strain demarcation criteria for begomoviruses (89% and 93% nucleotide sequence identity cut-off for species and strains, respectively) (Fauquet et al. 2008).

The complete nucleotide sequence of betasatellite ToLCJoB-[BD-Pap-J1] was 1,365bp in length and had similar structural features to other betasatellites: an (A)-rich region, a single gene in the complementary sense (βC1) with the capacity to encode 126 amino acids, and a satellite conserved region (SCR) at its 3'-end, and a predicted hairpin structure containing a loop with the sequence TAATATTAC having similarity to the origin of replication of geminiviruses (Briddon et al. 2003; Venkataravanappa et al. 2011). Pairwise sequence comparisons of full-length betasatellites determined that the genome sequence of ToLCJoB -[BD-Pap-J1] was 82 to 95% identical at the nucleotide level to ToLCJoV-associated betasatellites in GenBank (Table 5. 3). At the nucleic acid level, ToLCJoB-[BD-Pap-J1] exhibited the lowest identity (82%) to the ToLCJoB isolate from Kalyani, West Bengal, India (LN614513), and the highest identity (95%) to the ToLCJoB-BD[Gaz:Tom:05] (AJ966244) isolate infecting tomato in Gazipur, Bangladesh. The βC1 gene showed 77 to 98% nucleotide identities to the beta C1 of other ToLCJB isolates. The βC1 gene of ToLCJoB-[BD-Pap-J1] had the highest sequence identity, 98% at the amino acid level, to the βC1 of ToLCJoB-BD [Gaz:Tom:05]. A phylogenetic tree was prepared based on the entire nucleotide sequences of the newly identified ToLCJoB-[BD-Pap-J1] isolate and other ToLCJB isolates worldwide. It revealed that the ToLCJB isolates could be divided into two major phylogroups. ToLCJoB-[BD-Pap-J1] was closely related to the ToLCJoB-BD[Gaz:Tom:05] (AJ966244) isolate infecting tomato in Bangladesh. Another phylogenetic tree (Figure 5.2D) generated from amino acid sequence comparisons of the βC1 gene of ToLCJoB-[BD-Pap-J1] and other ToLCJoB isolates was constructed. Results of this analysis showed the

βC1 gene of ToLCJoB-[BD-Pap-J1] formed a cluster with the βC1 gene of ToLCJoB (CAI84641) (Fig.5.2D). We used the recombination analysis program RDP4 to screen for possible recombination events within the genome sequences of betasatellies associated with ToLCJoV (Table 5.4 and Figure 5.3). We found that ToLCJoB-[BD-Pap-J1] and ToLCJoB (HM007110) were recombinants. ToLCJoB-[BD-Pap-J1] was the recombinant of a minor parent, a strain of ToLCJB (JN176566) known to infect tomato in Kolkata, India, and a major parent, another strain of ToLCJB (KJ605117) also known to infect tomato in Kolkata, India. ToLCJoB (HM007110) infecting chili in Kolkata was a recombinant of a minor parent, a strain of ToLCJoB (JN176566) known to infect tomato in the area and a major parent, another strain of ToLCJoB (KJ605116) also known to infect tomato in Kolkata. These results suggested that ToLCJoB-[BD-Pap-J1] from papaya was a recombinant variant of several ToLCJoB isolates infecting tomato.

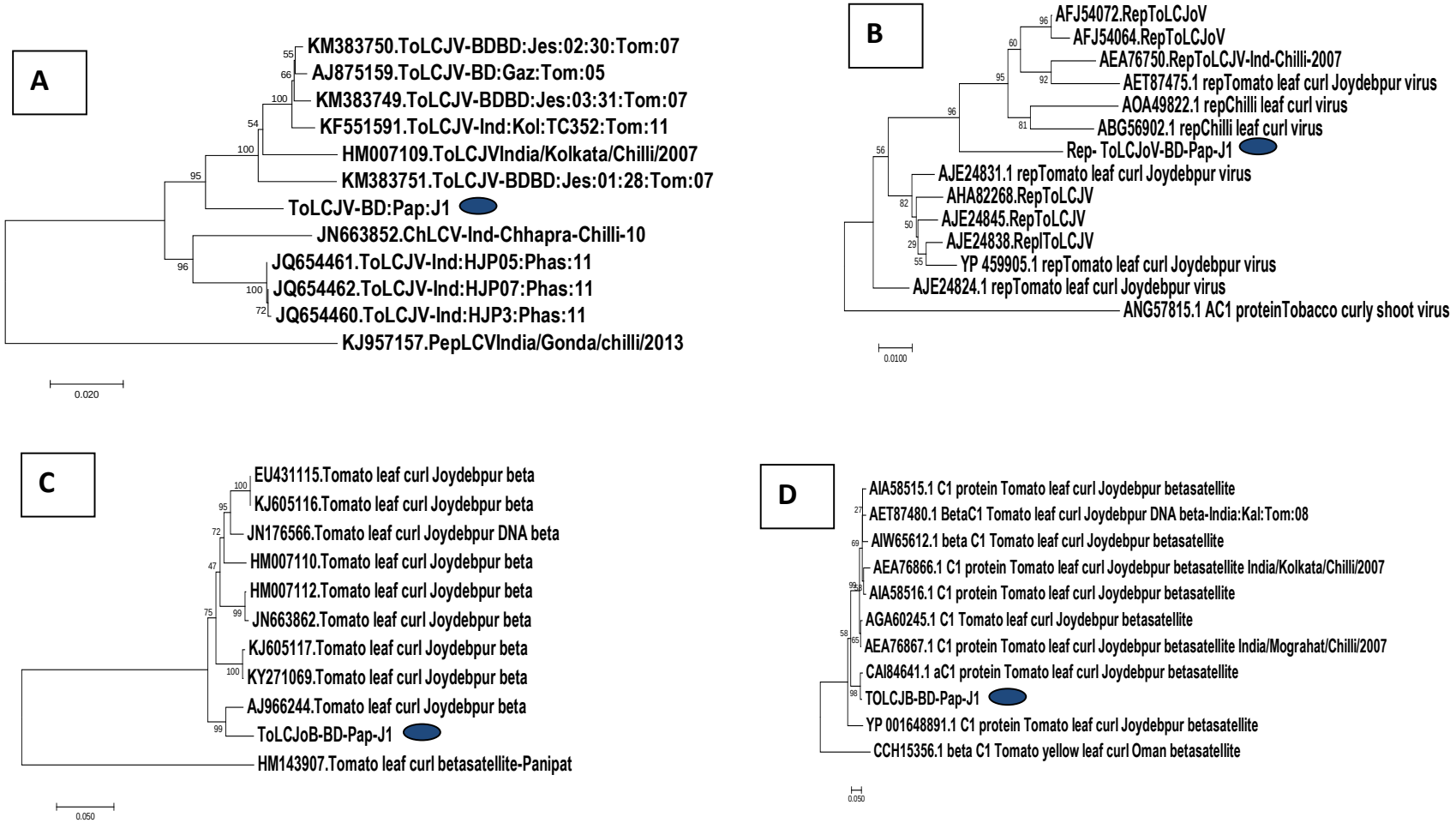

A
KM383750.ToLCJV-BDBD:Jes:02:30:Tom:07
AJ875159.ToLCJV-BD:Gaz:Tom:05
KM383749.ToLCJV-BDBD:Jes:03:31:Tom:07
KF551591.ToLCJV-Ind:Kol:TC352:Tom:11
HM007109.ToLCJVIndia/Kolkata/Chilli/2007
KM383751.ToLCJV-BDBD:Jes:01:28:Tom:07
ToLCJV-BD:Pap:J1
JN663852.ChLCV-Ind-Chhapra-Chilli-10
JQ654461.ToLCJV-Ind:HJP05:Phas:11
JQ654462.ToLCJV-Ind:HJP07:Phas:11
JQ654460.ToLCJV-Ind:HJP3:Phas:11
KJ957157.PepLCVIndia/Gonda/chilli/2013
0.020
B
AFJ54072.RepToLCJoV
AFJ54064.RepToLCJoV
AEA76750.RepToLCJV-Ind-Chilli-2007
AET87475.1 repTomato leaf curl Joydebpur virus
AOA49822.1 repChilli leaf curl virus
ABG56902.1 repChilli leaf curl virus
Rep- ToLCJoV-BD-Pap-J1
AJE24831.1 repTomato leaf curl Joydebpur virus
AHA82268.RepToLCJV
AJE24845.RepToLCJV
AJE24838.ReplToLCJV
YP 459905.1 repTomato leaf curl Joydebpur virus
AJE24824.1 repTomato leaf curl Joydebpur virus
ANG57815.1 AC1 proteinTobacco curly shoot virus
0.0100
C
EU431115.Tomato leaf curl Joydebpur beta
KJ605116.Tomato leaf curl Joydebpur beta
JN176566.Tomato leaf curl Joydebpur DNA beta
HM007110.Tomato leaf curl Joydebpur beta
HM007112.Tomato leaf curl Joydebpur beta
JN663862.Tomato leaf curl Joydebpur beta
KJ605117.Tomato leaf curl Joydebpur beta
KY271069.Tomato leaf curl Joydebpur beta
AJ966244.Tomato leaf curl Joydebpur beta
ToLCJoB-BD-Pap-J1
HM143907.Tomato leaf curl betasatellite-Panipat
0.050
D
AIA58515.1 C1 protein Tomato leaf curl Joydebpur betasatellite
AET87480.1 BetaC1 Tomato leaf curl Joydebpur DNA beta-India:Kal:Tom:08
AIW65612.1 beta C1 Tomato leaf curl Joydebpur betasatellite
AEA76866.1 C1 protein Tomato leaf curl Joydebpur betasatellite India/Kolkata/Chilli/2007
AIA58516.1 C1 protein Tomato leaf curl Joydebpur betasatellite
AGA60245.1 C1 Tomato leaf curl Joydebpur betasatellite
AEA76867.1 C1 protein Tomato leaf curl Joydebpur betasatellite India/Mograhat/Chilli/2007
CAI84641.1 aC1 protein Tomato leaf curl Joydebpur betasatellite
TOLCJB-BD-Pap-J1
YP 001648891.1 C1 protein Tomato leaf curl Joydebpur betasatellite
CCH15356.1 beta C1 Tomato yellow leaf curl Oman betasatellite
0.050

Figure 5.1.Phylogenetic analysis of sequences of ToLCJoV isolates (A and B) and their associated betasatellites(C and D). A.Neighbor-joining phylogram obtained from the alignment of nucleotide (nt) sequences of ToLCJoV-[BD-Pap-J1] with other ToLCJoV isolates obtained from GenBank. The tree was rooted with PepLCV (KJ957157). The symbol shows a newly sequenced variant of ToLCJoV from Bangladesh, ToLCJoV-[BD-Pap-J1] (marked by a blue oval).B.Neighbor-joining phylogram obtained from the alignment of amino acid (aa) sequences of AC1 (Rep) gene of ToLCJoV-[BD-Pap-J1] with AC1 gene of other ToLCJoV isolates obtained from GenBank. The tree was rooted with the AC1 gene (ANG57815) of tobacco curly shoot virus (TbCSV). The symbol shows the aa sequence of the AC1 gene of ToLCJoV-[BD-Pap-J1] from Bangladesh (marked by a blue oval). C. Neighbor-joining phylogram obtained from the alignment of nt sequences of ToLCJoB-[BD-Pap-J1] with other ToLCJoB isolates obtained from GenBank. The tree was rooted with ToLCB (HM143907). The symbol shows a newly sequenced variant of ToLCJoB from Bangladesh, ToLCJoB-[BD-Pap-J1] (marked by a blue oval). D. Neighbor-joining phylogram obtained from the alignment of aa sequences of the βC1 gene of ToLCJoB-[BD-Pap-J1] with βC1 gene of other ToLCJoV isolates obtained from GenBank. The tree was rooted with the βC1 gene (ANG57815) of tomato yellow leaf curl betasatellite (TbCSV). The symbol shows the aa sequence of the βC1gene of ToLCJoB-[BD-Pap-J1] from Bangladesh (marked by a blue oval). All trees were constructed in MEGA 7.0.14 using Clustal W with 1,000 replicates. Boot strap values are percentages with only values over 75% shown at the nodes.

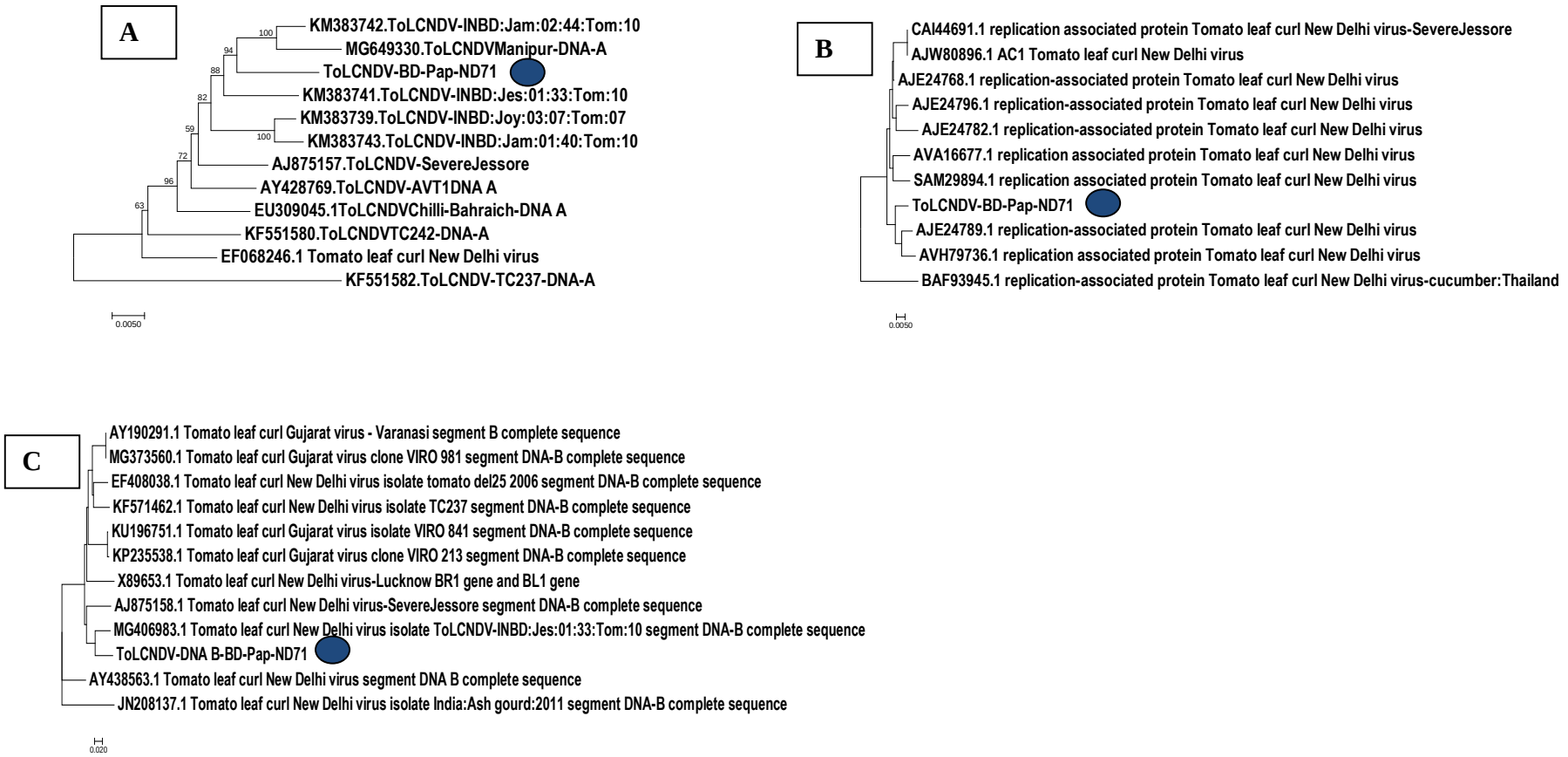


Figure 5.2. Phylogenetic analysis of sequences DNA A (A and B) and DNA B (C) components of ToLCNDV isolates A. Neighbor-joining phylogram obtained from the alignment of nucleotide (nt) sequences of DNA A of ToLCNDV-[BD-Pap-ND71] with other ToLCNDV isolates obtained from GenBank. The tree was rooted with ToLCNDV (KF551582). The symbol shows newly sequenced

variant of ToLCNDV from Bangladesh, ToLCNDV-[BD-Pap-ND71] (marked by a blue oval). B. Neighbor-joining phylogram obtained from the alignment of nt sequences of AC1 (Rep) gene of ToLCNDV-[BD-Pap-ND71] with AC1 gene of other ToLCNDV-[BD-Pap-ND71] isolates obtained from GenBank. The tree was rooted with AC1 gene (BAF93945) of ToLCNDV. The symbol shows the aa sequence of the AC1 gene of ToLCNDV-[BD-Pap-ND71] from Bangladesh (marked by a blue oval). C. Neighbor-joining phylogram obtained from the alignment of nt sequences of DNA –B of ToLCNDV-[BD-Pap-ND71] with other ToLCNDV isolates obtained from GenBank. The tree was rooted with DNA B of ToLCNDV (JN208137). The symbol show newly sequenced variant of ToLCNDV from Bangladesh, ToLCNDV-[BD-Pap-ND71] (marked by a blue oval). All the trees were constructed in MEGA 7.0.14 using Clustal W with 1,000 replicates. Bootstrap values are percentages with only values over 75% shown at the nodes.

Molecular characterization of ToLCNDV-[BD-Pap-ND71] in papaya

The DNA-A and DNA-B components of ToLCNDV-[BD-Pap-ND71] were comprised of 2,740nt and 2,688nt respectively, and their genome organizations were identical to those of previously characterized bipartite ToLCNDV isolates. DNA-A contained two ORFs: AV1 and AV2 were in the virion sense, and the other four ORFs, AC1, AC2 and AC3 were in the complementary sense. These ORFs were separated by a 276-nt-long intergenic region (IR) located between ORFs AC1 and AV2. The DNA-B component contained two ORFs designated as BVI on the sense strand and BC1 on the complementary strand. The DNA-A and DNA-B genomes did not exhibit any sequence similarity to one another, except for the approximately 160 nt common region.

The nucleotide sequence comparison of the completesequence of DNA-A of ToLCNDV-[BD-Pap-ND71] isolate had identities of 93 to 98% at the nucleotide level to the sequences of ToLCNDV isolates in GenBank (Table 5.1). At the nucleic acid level, ToLCNDV-[BD-Pap-ND71] exhibited the lowest identity (93%) to the tomato leaf curl New Delhi virus isolate TC237 segment DNA-A (HM007098) infecting soybean and the highest identity (98%) to the tomato leaf curl New Delhi virus isolate ToLCNDV-IN[BD:Jam:02:44:Tom:10] segment DNA-A (KM383742) infecting tomato. The AC1 protein of DNA-A of ToLCNDV-[BD-Pap-ND71] had the lowest identity to AC1 genes of other reported ToLCNDV-[BD-Pap-ND71] isolates. Phylogenetic analysis based on the nucleotide sequence of the DNA-A genome of the newly identified ToLCNDV-[BD-Pap-ND71] with the DNA-A sequences of other ToLCNDV isolates worldwide revealed that the ToLCNDV-[BD-Pap-ND71] isolate has a close relationship tothe isolates ToLCNDV (KM383742) infecing tomato in Jamalpur, Bangladesh, ToLCNDV infecing tomato in Jessor, Bangladesh (KM383741), and ToLCNDV infecing tomato in Manipur,

India (MG649330), sharing a 97 to 98% nucleotide identity among them. We constructed a second phylogenetic analysis (Figure 5.2a) generated from amino acid sequence comparisons of the AC1 gene of ToLCNDV-[BD-Pap-ND71] and other ToLCNDV isolates. Results of this analysis revealed that the AC1 gene of ToLCNDV-[BD-Pap-ND71] clustered with the AC1 genes of ToLCNDV (KM383742) and ToLCNDV (MG649330), supporting the phylogenetic relationship of ToLNDV-[BD-Pap-ND71] and the ToLCNDV isolates (Figure 5. 2B).

We used the recombination analysis program RDP4 to screen for possible recombination events within the genome sequences of the DNA-A component of ToLCNDV isolates (Table 5.5 and Figure 5.4) and found that ToLCNDV-DNA-A(AJ875157) infecting tomato in Jessore, Bangladesh, ToLCNDV-DNA-A(KF551580) infecting tomato in Punjab, India,and ToLCNDV-DNA-A (EF068246) infecting tomato in India were recombinants. ToLCNDV-[BD-Pap-ND71] was the minor parent of ToLCNDV-DNA-A (AJ875157) infecting tomato in Jessore, Bangladesh. These results suggested that ToLCBV-[BD-Pap-17] from papaya was a variant of the ToLCNDV isolates infecting tomato, according to the latest International Committee on Taxonomy of Viruses guidelines for species demarcation in the genus (Fauquet et al. 2008).

The nucleotide sequence analysis of DNA-B of the ToLCNDV-[BD-Pap-ND71] isolate with DNA-Bsequences of other ToLCNDV isolates in the NCBI database showed that it had the highest sequence identity (93%) with ToLCNDV-DNA-B (MG406983) infecting tomato in Jessore, Bangladesh, and the lowest with ToLCNDV-DNA-B (JN208137). ORFs BC1 and BV1 were 87 to 97% and 89 to 95% identical to corresponding ORFs of DNA-Bs of other ToLCNDV isolates. Phylogenetic analysis based on nucleotide sequences showed that ToLCNDV-[BD-Pap-ND71] was nearly identical to the newly identified ToLCNDV-[BD-Pap-ND71] and other

ToLCNDV-associated isolates worldwide. These results suggested that ToLCBV-[BD-Pap-17] from papaya was a variant of the ToLCNDV-IN [BD:Jam:02:44:Tom:10] segment of DNA-A (KM383742). We determined that ToLCNDV-DNA-B (AY438563) infecting tomato in India was a recombinant isolate. These results suggested that ToLCBV-[BD-Pap-17] from papaya was a variant of ToLCNDV isolates infecting tomato in Bangladesh (Table 5.5 and Figure 5.4).

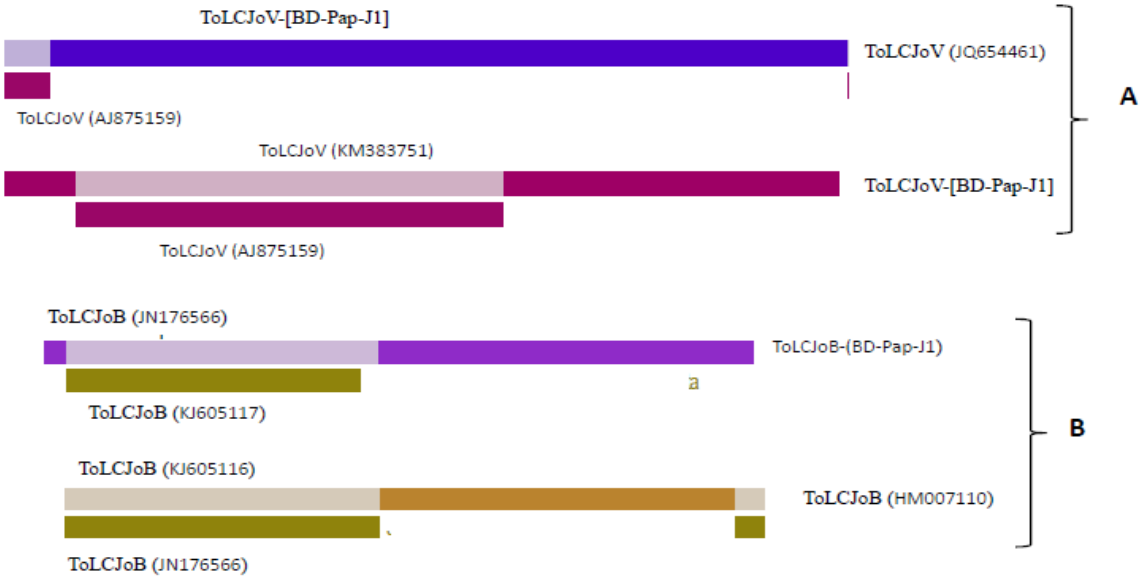


Figure 5.3. Recombination analysis of ToLCJoV isolates and associated betasatellites A. Two recombinant isolates: ToLCJoV-[BD-Pap-J1] and ToLCJoV (JQ654461). ToLCJoV-[BD-Pap-J1] was the recombinant of a minor parent, ToLCJoV (AJ875159), and a major parent, ToLCJoV (KM383751). B. The recombination analysis program RDP4 was used to screen for possible recombination events within the genome sequences of betasatellites associated with ToLCJoV. Two recombinant betasatellite isolates: ToLCJoB-[BD-Pap-J1] and ToLCJoB (HM007110) were recombinants. ToLCJoB-[BD-Pap-J1] was the recombinant of a minor parent, ToLCJoB (JN176566) infecting tomato in Kolkata, India and a major parent, ToLCJB (KJ605117) infecting tomato Kolkata in India. ToLCJoB (HM007110) infecting chili was the recombinant of a minor parent, ToLCJB (JN176566) ToLCJB (JN176566) infecting tomato in Kolkata, India and a major parent, ToLCJB (KJ605116) infecting tomato in Kolkata in India.

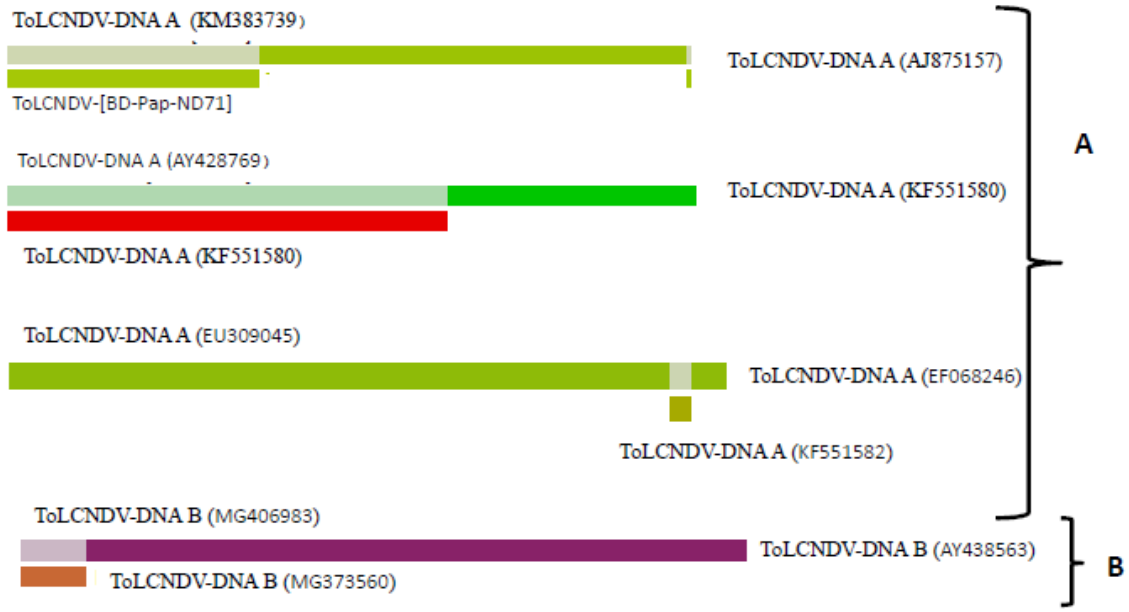


Figure 5.4. Recombination analysis of ToLCNDV isolates. A. ToLCNDV-DNA A (AJ875157) infecting tomato in Jessore, Bangladesh, ToLCNDV-DNA-A (KF551580) and ToLCNDV-DNA-A(EF068246) were recombinants. ToLCNDV-[BD-Pap-ND71] was the minor parent of ToLCNDV-DNA A (AJ875157) infecting tomato in Jessore, Bangladesh. B. ToLCNDV-DNA B (AY438563) is a recombinant isolate.

Table 5.5. Information about recombinant events identified in the ToLCJoV isolates and associated betasatellites as analyzed by RDP4

Recombination event	Breakpoint positions		Recombinant sequence(s)	Minor parental strain	Major parental strain	Detection methods (P value)				
						RDP	Maxchi	Chimaera	3Seq	GENECONV
6	2752	148	JQ654461	AJ875159	ToLCJoV-[BD-Pap-J1]	$2.47x10^{-17}$	$1.71x10^{-05}$	$2.78x10^{-02}$	$3.28x10^{-07}$	$1.12x10^{-06}$
7	236	1650	ToLCJoV-[BD:Pap:J1]	AJ875159	KM383751	$3.41x10^{-10}$	$1.91x10^{-50}$	$2.36x10^{-11}$	$5.67x10^{-04}$	$4.40x10^{-10}$
6	41	641	ToLCJoB-BD-Pap-J1	JN176566.Tomato leaf curl Joydebpur DNA beta	KJ605117.Tomato leaf curl Joydebpur beta	$1.367x10^{-07}$	$3.71x10^{-12}$	$4.245x10^{-07}$	$1.26x10^{-11}$	$1.71x10^{-06}$
7	1308	615	HM007110.Tomato leaf curl Joydebpur beta	JN176566.Tomato leaf curl Joydebpur DNA beta	KJ605116.Tomato leaf curl Joydebpur beta	$3.64x10^{-04}$	$5.54x10^{-18}$	$1.74x10^{-09}$	$5.72x10^{-07}$	$6.10x10^{-10}$

Minor parental strain: this strain contributes a small sequence fraction in the recombinant strain
Major parental strain: this strain contributes a large sequence fraction in the recombinant strain

Table 5. 6. Information about recombinant events identified in the ToLCNDV isolates as analyzed by RDP4

Recombination event	Breakpoint Positions		Recombinant Sequence(s)	Minor parental strain	Major parental strain	Detection methods (P value)				
						RDP	Maxchi	Chimaera	3Seq	GENE CONV
2	2724	1012	AJ875157. ToLCNDV-Severe[Jessore]	ToLCNDV-[BD-Pap-ND71]	KM383739. ToLCNDV-IN[BD:Joy:03:07:Tom:07]	$4.86x10^{-02}$	$3.49x10^{-04}$	$9.60x10^{-04}$	$7.74x10^{-03}$	NS
3	2738	1750	KF551580. ToLCNDV[TC242-DNA-A]	MG649330. ToLCNDV [Manipur-DNA-A]	AY428769. ToLCNDV-AVT1[DNA A]	$2.64x10^{-57}$	$5.67x10^{-17}$	$7.52x10^{-18}$	$1.58x10^{-46}$	$1.74x10^{-54}$
4	2520	2606	EF068246.1 Tomato leaf curl New Delhi virus	KF551582.ToLCNDV-[TC237-DNA-A]	EU309045.1ToLCNDV[Chilli-Bahraich-DNA A]	$8.22x10^{-04}$	$1.01x10^{-03}$	$8.59x10^{-04}$	$4.72x10^{-04}$	$3.04x10^{-03}$
1	2688	228	AY438563	MG373560	MG406983	$5.51x10^{-36}$	$1.74x10^{-10}$	$5.14x10^{-09}$	$5.50x10^{-14}$	NS

Minor parental strain: this strain contributes a small sequence fraction in the recombinant strain
Major parental strain: this strain contributes a large sequence fraction in the recombinant strain

Discussion

Papaya cultivation in Bangladesh is hindered economically by the extensive occurrence of PRSV infections. However, leaf curl disease and the occurrence of begomoviruses in papaya are increasing, possibly due to the broad host range of begomoviruses, their whitefly vectors, and the practice of mixed cropping (Alabi et al. 2017; Maruthi et al. 2007). Leaf curl diseases of papaya causes yield losses that pose an economic threat to the papaya industry in Bangladesh. Leaf curl symptoms in papaya have been reported from other countries, including India, Pakistan, Nepal, Iran, and China, where monopartite begomoviruses are known to infect papaya (Singh-Pant et al. 2012; Bananej et al. 2016). Singh-Pant and colleagues (2012) reported the association of monopartite begomoviruses, such as chili leaf curl virus (ChiLCuV) and papaya leaf crumple virus (PaLCrV) in papaya. The bipartite begomovirus ToLCNDV is known to infect papaya in Bangladesh and India (Singh-Pant et al. 2012; Maruthi et al. 2007). Other begomoviruses found in papaya include papaya leaf curl Guandong virus, papaya leaf curl China virus, malvastrum leaf curl virus, okra enation leaf curl virus, cotton leaf curl Multan virus, and ageratum yellow vein virus (Shahid et al. 2013; Bananej et al. 2016, Sinha et al. 2013; Cai et al. 2005; Wang et al. 2004).

The ToLCJoV and ToLCNDV isolates with severe symptoms of leaf curl were detected and molecularly characterized in our study from papaya samples J1-pap and ND-71. The genome organization of ToLCJoV and ToLCNDV infecting papaya are typical of ToLCJV and ToLCNDV infecting tomato. Based on the current criteria for demarcating species and strains of begomoviruses (Fauquet et al. 2008; Padidam et al. 1995), ToLCJoV and ToLCNDV infecting papaya are identified as new variants of ToLCJoV and ToLCNDV infecting tomato in Bangladesh (Green et al. 2003; Ha et al. 2008). ToLCJoV was reported earlier from the

Joydebpur district of Bangladesh (Maruthi et al. 2005). The genomic characteristics of ToLCJoV-[BD:Pap:J1] at the nucleotide and amino acid levels are typical of the monopartite begomoviuses and indicate that ToLCJoV-[BD:Pap:J1] is closely related to ToLCJoV infecting tomato in Bangladesh. This result suggests that ToLCJoV-[BD:Pap:J1] is a variant of the previously reported tomato-infecting ToLCJoV. It further suggests that the virus may have been introduced into papaya from tomato infected with ToLCJoV in Bangladesh. In support of this hypothesis, others have reported the host range expansion of ToLCJoV to chili (Shih et al. 2007) and kenaf (Paul et al. 2009) in India. The ToLJoCB satellite associated with ToLCJoV-[BD:Pap:J1] had the highest identity (95%) to ToLCJoB-BD (AJ966244) infecting tomato in Gazipur, Bangladesh. Paul et al. (2008) reported that the ToLJoB satellite of the related virus ToLCJV infects kenaf plants in India. Synergistic interactions between a particular virus and its related satellite may alter the host range or increase the pathogenicity of the virus. The emergence of ToLCJoV infecting papaya in Bangladesh, where the environmental conditions support large *B. tabaci* populations, poses a new challenge for the production of papaya.

In this study, recombination events were detected among the ToLCJoV isolates (Figure 5.3). Similar reports of recombination within the AV1 gene, specifically the CR/IR (ori) and Rep regions of ToLCJoV, have been published (Kirthi et al 2002; Tiwari et al. 2013). The high genetic diversity of tomato-infecting begomoviruses and their recombination has been documented in the neighboring country of India. The high rates of mutation and recombination in begomoviruses may stimulate the evolution of new species and different groups of associated satellites in papaya (Singh-Pant et al. 2012).

We discovered that ToLCJoV infecting papaya in Bangladesh was associated with a betasatellite, ToLCJoB. The ToLJoB associated with papaya identified in our study was

associated with tobacco leaf curl disease in India and Bangladesh (Bull et al. 2004; Singh et al. 2011). Phylogenetic analysis of betasatellites revealed their close relationship with the diseases of many solanaceous crops, indicating the possible spread of betasatellites across geographic regions. Available evidence indicates that betasatellites do not contain iteron sequences of their helper viruses, yet still depend on a helper virus for their replication (Briddon et al. 2001; Kon et al. 2006, 2007; Saunders et al. 2000). In the last decade, a large number of alphasatellites and betasatellites associated with begomoviruses were found causing severe diseases of many crop plants. Interactions of helper begomoviruses with their cognate betasatellites range from an absolute requirement for typical symptom induction in primary hosts to enhanced symptom severity due to a synergism between PTGS suppressors. ToLCJoV does not require its betasatelliteto infect plants and produce disease symptoms (Tiwari et al. 2013).

ToLCJoV-[BD-Pap-J1] was closely related to ToLCJoV (KM383750) infecting tomato in Bangladesh and ToLCJoV-[BD-Pap-J1] is associated with severe leaf curl symptoms of papaya. Maruthi et al. (2005) reported that ToLCJoV infection produced foliar yellowing, mild leaf curling, and slightly stunted growth symptoms on tomato. However, Tiwari et al. (2013) described a different disease reaction to the ToLCJoV isolate on tomato, including severe leaf curl, deformation, and stunting. In this case, symptoms were severe, even when DNA-A alone was used for inoculation. This isolate is more closely related to one that infects chili. It may be that a change in the host produced more severe symptoms in the new host. Recombination is a major driving force in the evolution of geminiviruses (Seal et al. 2006) and the evidence suggests that recombination played a part in the origin of ToLCJoV infecting papaya.

ToLCNDV-[BD-Pap-ND71] had the highest identity to tomato leaf curl New Delhi virus (ToLCNDV) isolates infecting tomato. ToLCNDV is present in various crop plants in India,

Pakistan, Thailand, and Bangladesh (Fauquet et al. 2008). ToLCNDV was reported for the first time in India from tomato; it requires both DNA-A and DNA-B for symptom development in this host (Padidam et al. 1995). It has also been reported from various crops and weeds such as chili (Hussain et al. 2004), cucurbits (Ito et al. 2008), potato (Usharani et al. 2004), papaya (Raj et al. 2008), bittergourd (Tahir and Haider, 2005), cotton (EF063145) and black nightshade (*Solanum nigrum*) (AJ620187).

ToLCJoV and ToLCNDV infect tomato and other vegetables in Bangladesh and India. Intercropping, layer cropping, and mixed cropping systems are common in and around papaya orchards in Bangladesh. It is possible that ToLCJoV and ToLCNDV were introduced from tomato or other hosts to papaya in Bangladesh. Further studies are needed to determine the diversity of ToLCJV and ToLCNDV in papaya and other crops, including weeds in papaya orchards in Bangladesh.

Serious leaf curl disease of papaya is caused by at least three tomato begomoviruses: tomato leaf curl Bangladesh virus (ToLCBV), ToLCJoV, and ToLCNDV. Infection of other crops by tomato begomoviruses might be due to high inoculum pressure by tomato begomoviruses and their strains, intensive year-round intercropping of brinjal, tomato, chili, and cucurbits, and a variety of whitefly biotypes in Bangladesh. Increased overlapping cultivation of closely related solanaceous and cucurbitaceous crops may have resulted in an expanded host range of existing begomoviruses and led to the emergence of new strains and recombinants. This is also relevant for papaya, which grows in diverse agroclimatic zones throughout the year, perpetuating the virus inoculum and vector population.

The possibility of an epidemic of this destructive disease is of great concern to papaya cultivation in Bangladesh. The need to monitor the prevalence of ToLCJoV and ToLCNDV is

crucial to the management of this emerging threat. Plants with symptoms of leaf curl should be removed and planting alternate hosts of begomovirusesin or around papaya orchards avoided. Strict quarantine measures to prevent the movement of infected vegetable hosts among farms are needed. The role of the whitefly vector in the transmission of ToLCJoV and ToLCNDV needs clarification. Presently, there are no known papaya cultivars with resistance to begomoviruses in Bangladesh (Hamim, *unpublished*). Efforts towards developing resistance against begomoviruses in papaya would have substantial beneficial effects for the papaya industry in Bangladesh.

CHAPTER VI

CONCLUSION, RECOMMENDATIONS AND FUTURE RESEARCH DIRECTIONS

One of the crucial horticultural crops in Bangladesh is papaya. It is cultivated at the commercial level and by private subsistence farmers. The major antagonists to papaya production are plant viruses, which can eliminate entire crops and affect the food security system of Bangladesh. Papaya ringspot virus (PRSV) currently affects 90 to 100% of existing papaya plants, causing the notorious papaya ringspot disease. Two main challenges in managing this disease are non-persistent aphid-mediated transmission, and the high genetic diversity of PRSV strains within a country or among geographical regions.

Papaya cultivars with transgenic resistance to specific viruses have reduced the impact of PRSV and it is the only solution in places where disease pressure is extreme. Transgenic papayas saved the papaya industry in Hawaii and have maintained their resistance to PRSV for decades. This success story encouraged researchers from other countries to develop PRSV-resistant transgenic papaya cultivars. In most cases, these attempts produced only a transitory resistance that was later lost. In othercases the resistance was inconsistent, with some transgenic lines showing resistance while others failed. Different factors can trigger the loss of PRSV-resistance in transgenic papaya.

A specific, ultrasensitive assay is needed for epidemiological studies and quarantine protocols. It must provide early detection of PRSV at very low titers in the field and in imported plant samples. To address this demand, we developed a single-tube nested PCR (STNP) assay with two sets of nested PCR primers designed to target the P3 gene of PRSV. The annealing temperatures and concentrations of these primer pairs were optimized to avoid potential

competition between them during the assay. STNP had a more sensitive reaction than conventional RT-PCR and ELISA. It also detected PRSV from asymptomatic plants with very low virus titers.

The new full-length genome sequences of Bangladeshi PRSV strains and their distribution provide important information on the dynamics of papaya ringspot disease in Bangladesh. The complete genome sequences of two distinct PRSV strains, PRSV BD-1 (10,300 bp) and PRSV BD-2 (10,325 bp) from severely infected papaya plants were determined using the Illumina NextSeq 500 platform, RT-PCR, and Sanger DNA sequencing. PRSV BD-2 is a recombinant strain and most closely related to PRSV-Meghalaya (MF356497) from papaya in India. PRSV BD-1 formed a distinct branch from other PRSV sequences by nucleotide as well as amino acid sequence comparisons. The occurrence of PRSV BD-1 and PRSV BD-2 in the sampled areas of Bangladesh was approximately 19% and 69%, respectively. Plants infected with both strains (11%) exhibited more severe symptoms than plants infected with either strain alone.

During our surveys, we identified 43 papaya plants positive for begomoviruses and 29 positive for tomato leaf curl Bangladesh virus (ToLCBV). The latter had severe leaf curl symptoms and large infestations of its whitefly vector. The complete genome sequence of ToLCBV (accession no. MH380003) infecting papaya was most closely related to ToCLBV (accession no. KM383761) causing leaf curl disease of tomato reported from Bangladesh. ToLCBV had the highest homologies at the nucleotide and amino acid levels to the betasatellite associated with tomato leaf curl virus (ToLCV) from tomato in India (JN638434). Non-coding DNA satellites with notable sequence homology to the conserved region of betasatellites, and a stem-loop structure containing the nonanucleotide sequence motif TAATATTAC, were found in

ToLCBV-infected papaya plants. Based on a phylogenetic tree analysis, these small non-coding satellites produced a distinct cluster. This study confirmed papaya as a host of ToLCBV, ToLCV-associated betasatellites and small non-coding DNA satellites in Bangladesh.

The complete genome sequence of the tomato leaf curl Joydebpur virus (ToLCJoV) isolate infecting papaya was most closely related to ToCJoV reported from Gazipur, Bangladesh, where it causes tomato leaf curl disease. ToLCB from papaya had the highest homologies at the nucleotide and amino acid levels to ToLCB from tomato in India. The complete genome sequence of the DNA A component of the tomato leaf curl New Delhi virus (ToLCNDV) infecting papaya was most closely related to ToCNDV reported from Gazipur, Bangladesh, where it causes tomato leaf curl disease. The DNA B component of ToLNDV from papaya had the highest homologies at nucleotide and amino acid levels to DNA B ToLCNDV from tomato in India. Asymptomatic leaf samples and samples with PRSV-like symptom were negative for both ToLCJoV and ToLCNDV.

In Bangladesh, diseases caused by whitefly-transmitted begomoviruses are increasing. The severe leaf curl disease symptoms of papaya are caused by at least three tomato begomoviruses: tomato leaf curl Bangladesh virus (ToLCBV), tomato leaf curl Joydebpur virus (ToLCJoV), and ToLCNDV. These begomoviruses have wide host ranges that include different vegetables, ornamentals, and weeds in Bangladesh and India. Increased overlapping cultivation of closely related solanaceous and cucurbitaceous crops has resulted in an expanded host range of existing begomoviruses and may have contributed to the emergence of new strains and recombinants. This is especially true for papaya, which grows in diverse climatic zones throughout the year, perpetuating the vector population and virus inoculum. Intercropping, layer cropping, and mixed cropping systems are practiced in and around papaya orchards in

Bangladesh. The infection of other crops by tomato begomoviruses in Bangladesh might be due to the high inoculum pressure of these viruses and their strains; year-round growing of brinjal, tomato, chili, and cucurbits side by side; and existing whitefly biotype complexes. Therefore, tomato-infecting begomoviruses could be introduced into papaya from tomato or other hosts in Bangladesh or neighboring India. Further studies are needed to determine the diversity of ToLBV, ToLCJoV, and ToLCNDV in papaya, other agronomic crops, and weeds in and around the papaya orchards of Bangladesh.

The need to monitor the prevalence of viruses infecting papaya is crucial to the management of this emerging threat. Plants with symptoms of ringspot or leaf curl should be removed and planting of alternate hosts within or around papaya orchards avoided. Therefore, strict quarantine measures to prevent the movement of infected vegetable hosts among farms, and internationally are also needed. The role of insect vectors in the transmission of papaya viruses cannot be ignored. Presently, there are no known papaya cultivars with resistance to PRSV or begomoviruses in Bangladesh (Hamim, *personal observation*). Efforts towards developing resistance against PRSV or begomoviruses in papaya could have important beneficial effects for the country's papaya industry.

Transgene-derived resistance is currently the only technology of managing PRSV successfully in papaya (Gonsalves 1998, Hamim et al. 2018a). PRSV-resistant transgenic papaya cultivars are highly specific to local virus strains (Hamim et al. 2018a). My studies indicated that PRSV strains in Bangladesh were diverse compared to those from other geographical locations. Therefore, characterization of local PRSV populations and identification of conserved regions in the Bangladesh strains is needed to develop transgene-derived, PRSV-resistant papaya for Bangladesh. Newly emerged virulent PRSV strains, or the increased

virulence of existing strains arising from synergistic interactions with other viruses, have caused severe disease outbreaks among transgenic cultivars in many papaya-growing areas. PRSV-resistant transgenic plants are still the most effective means of controlling papaya ringspot disease. Several biotic and abiotic factors may cause partial or total loss of virus resistance in transgenic papaya lines. CRISPR/Cas9 and other new technologies may lead to the development of durable, broad-spectrum, PRSV-resistant transgenic cultivars. Transgenic resistance should be combined with appropriate elements of an IPM program. This combination will maximize the effect of transgenic virus resistance and extend its viability. Local, national, and international quarantine efforts can help reduce the risk of losing virus resistance by avoiding the introduction of new viruses or strains from other regions.

CHAPTER VII

REFERENCES

Adams, M.J., Antoniw, J.F., and Beaudoin, F. 2005. Review: overview and analysis of the polyprotein cleavage sites in the family Potyviridae. Molecular Plant Pathology 6:471–487.doi:10.1111/j.1364-3703.2005.00296.x.

Agrawal, N., Dasaradhi, P.V.N., Mohmmed, A., Malhotra, P., Bhatnagar, R.K., and Mukherjee, S.K. 2003. RNA interference: biology, mechanism, and applications. Microbiology and Molecular Biology Reviews.67(4):657–685. doi: 10.1128/MMBR.67.4.657-685.2003

Akhter, M.S., Basavaraj, Y.B., Akanda, Mandal, A.M. B. and Jain, R. K. 2013. Genetic diversity based on coat protein of *Papaya ringspot virus* (Pathotype P) isolates from Bangladesh. Indian J. Virology 24(1):70-3. doi: 10.1007/s13337-013-0127-2.

Alabi O. J., Rwahnih, M. Al, Brown, J. K., Idris, A. M.Gregg, L., Kmieciak, E., Sétamou,M., and Jifon. J.L. 2016. First report of papaya (*Carica papaya*) naturally infected with the introduced *Tomato yellow leaf curl virus-Israel*. Plant Disease 100(9):1959. https://doi.org/10.1094/PDIS-04-16-0469-PDN

Alabi, O.J., Rwahnih, M.A., Jifon, J.L., Sétamou, M., Brown, J.K., Gregg, L., Park, J.W. 2017. Mixed infection of *Lettuce chlorosis virus*, *Papaya ringspot virus*, and *Tomato yellow leaf curl virus-IL* detected in a Texas papaya orchard affected by a virus-like disease outbreak. Plant Disease 101 (7): 1094-1102.https://doi.org/10.1094/PDIS-01-17-0118-RE

Ali, Z., Abul-faraj, A., Idris, A., Ali, S., Tashkandi, M., and Mahfouz, M.M. 2015. CRISPR/Cas9-mediated viral interference in plants. Genome Biology, 16, 1–11. doi: 10.1186/s13059-015-0799-6 ·

Al Rwahnih, M., Rowhani, A., Westrick, N., Stevens, K., Diaz-Lara, A., Trouillas, F., Preece, J., Kallsen, C.E., Farrar, K. and Golino, D.A., 2018. Discovery of viruses and virus-like pathogens in pistachio using high-throughput sequencing. Plant Disease. 102:1419–1425.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. 1990. Basic local alignment search tool. J Mol Biol. 215:403-410.

Anandalakshmi, R., Pruss, G.J., Ge, X., Marathe, R., Mallor, A.C., Smith, T.H., & Vance, V.B. 1998. A viral suppressor of gene silencing in plants. Proceedings of the *National* Academy of Sciences,95 (22).13079-13084. doi: 10.1073/pnas.95.22.13079

Anderson, T.P., Beynon, K.A., Murdoch, D.R. 2003. Comparison of real-time PCR and conventional hemi-nested PCR for the detection of *Bordetella pertussis* in nasopharyngeal samples. Clinical Microbiology Infection 9 : 746–749.

Azad, M.A.K., Amin, L., and Sidik, N.M. 2014. Gene technology for *Papaya ringspot virus* disease management. The Scientific World Journal. doi:10.1155/2014/768038.

Badillo, V. 1993. Caricaceae, segundo esquema. *Revista de la Facultad de Agronornia* (Maracay) 43,:111.

Baltes, N.J., Hummel, A.W., Konecna, E., Cegan, R., Bruns, A.N., Bisaro, D.M, Voytas, D.F. 2015. Conferring resistance to geminiviruses with the CRISPR-Cas prokaryotic immune system. Nature Plants. 1*:* 15145. https://doi.org/10.1038/nplants.2015.145

Bananej, K., Kraberger, S., Varsani, A. 2016. Okra enation leaf curl virus in papaya from Iran displaying severe leaf curl symptoms. J Plant Pathol. 98(3): 637-639. https://doi.org/10.4454/JPP.V98I3.043

Bau, H.J., Cheng, Y.H., Yu, T.A., Yang, J.S., and Yeh, S.D. 2003. Broad-spectrum resistance to different geographic strains of *Papaya ringspot virus* in coat protein gene transgenic papaya. Phytopathology,93:112–120. https: //doi.org /10.1094 /PHYTO. 2003.93.1.112

Bau, H.J., Kung, Y.J., Raja, J., Chan, S.J., Chen, K.C., Chen, Y.K., Wu, H.W., and Yeh, S.D. 2008. Potential threat of a new pathotype of *Papaya leaf distortion mosaic virus* infecting transgenic papaya resistant to *Papaya ringspot virus*. Phytopathology 98, 848–856. doi: 10.1094/PHYTO-98-7-0848.

Baulcombe, D., English, J., Mueller, E., and Davenport, G. 1996. Gene silencing and virus resistance in transgenic plants, in *Mechanisms and applicationsof gene silencing* (Grierson, G. W., Lycett, G. W., and Tucker, G. A., eds.) Nottingham University Press, Nottingham, pp. 127–138

Bayot, R. G., Villegas, V. N., Magdalita, P. M., Jovellana, M. D., Espino, T. M., and Exconde, S. B. 1990. Seed transmissibility of *Papaya ringspot virus* . Philippines J. Crop Sci. 15:107-111

Beachy, R. N. 1997. Mechanisms and applications of pathogen-derived resistance in transgenic plants. Current Opinion Biotechnoogy. 8,215–220.

Bonfim, K., Faria, J.C., Nogueira, E.O., Mendes, E.A., and Aragão, F.J.L. 2007. RNAi-Mediated Resistance to *Bean golden mosaic virus* in genetically engineered common bean

(*Phaseolus vulgaris*). Molecular Plant Microbe Interaction. 20(6), 717–726. doi:10.1094 / MPMI -20-6-0717.

Briddon, R.W., Stanley, J. 2006. Subviral agents associated with plant single-stranded DNA viruses. Virology 344: 198–210. doi:10.1016/j.virol.2005. 09.042

Briddon, R.W., Bull, S.E.., Mansoor, S., Amin, I., Markham, P.G. 2002. Universal primers for the PCR-mediated amplification of DNA β; a molecule associated with some monopartite begomoviruses. Mol Biotechnol. 20: 315-318.

Brigneti, G., Voinnet, O., Li, W.X., Ji, L.H., Ding, S.W., and Baulcombe, D.C. 1998. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. The EMBO Journal. 17:6739–6746.doi:10. 15252 / embj. 201570030

Brown, J.K., Zerbini, F.M., Navas-Castillo, J., Moriones E, Ramos-Sobrinho R, Silva JF, Fiallo-Olivé E, Briddon RW, Hernández-Zepeda C, Idris A, Malathi, VG, Martin DP, Rivera-Bustamante, R., Ueda, S., Varsani, A. 2015. Revision of Begomovirus taxonomy based on pairwise sequence comparisons. Arch Virol. 160: 1593-1619.

Brown, J.K., Fauquet, C.M., Briddon, R.W., Zerbini, M., Moriones, E., Navas-Castillo, J. 2012. "Geminiviridae,"in *Virus Taxonomy-Ninth Report of the International Committeeon Taxonomy of Viruses*, eds A.M.Q. King, E. Lefkowitz, M. J. Adams, and E. B. Carstens (New York City, NY: Associated Press), 351–373.

Burgyan, J. and Havelda, Z. 2011. Viral suppressors of RNA silencing. Trends in Plant Sciences, 16, 265–272. https: //doi.org/10.1016/ j.tplants.2011.02.010

Cai, J.H., Wang, X.Y., Li, G.X., Qin, B.X., Zhou, X.P. 2005. Genomic organization of Papaya leaf curl China virus isolated from Nanning. Acta Phytopathol Sin. 35:446-450.

Carvalho, F.D., Gheysen, G., Kushnir, S.M., Inze, D. and Castresana, C. 1992. Suppression of beta-1,3-glucanase transgene expression in homozygous plants. European Molecular Biology Organization Journal. 11: 2595–2602

Chan Jr, H. T., and Tang, C.-S. 1979. The chemistry and biochemistry of papaya. In G. E. Inglett, & C. G. (Eds.), Tropical Foods: Chemistry and Nutrition (Vol. 1, pp. 33-53). New York: Academic press.

Chandrasekaran, J.,Brumin, M., Wolf, D., Leibman, D., Klap, C., Pearlsman, M., Sherman, A, Arazi, T, and Gal-On, A. 2016. Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. Molecular Plant Pathology, 17(7) :1140-53. doi: 10.1111/mpp.12375

Chang, L.S., Lee, Y.S., Su, H.J., Hung, T.H. 2003. First report of papaya leaf curl virus infecting papaya plants in Taiwan. Plant Disease 87**:** 204.

Chaves-Bedoya, G., and Ortiz-Rojas, L. Y. 2015. Genetic variability of papaya ringspot virus isolates in Norte de Santander - Colombia. Agronomia Colombiana, 33(2) :84–193.

Cheng, Y.H., Yang, J.S., and Yeh, S.D. 1996. Efficient transformation of papaya by coat protein gene of *Papaya ringspot virus* mediated by Agrobacterium following liquid-phase wounding of embryogenic tissues with carborundum. Plant Cell Report 16: 127–132. doi: 10.1007/BF01890852.

Chen, K.C., Chiang, C.H., Raja, J.A., Liu, F.L., Tai, C.H., Yeh, S.D. 2008. A single amino acid of NIaPro of Papaya ringspot virus determines host specificity for infectionof papaya. Molecular Plant Microbe Interaction. 21:1046–1057.

Chiang, C.H., Wang, J.J., Jan, F.J., Yeh, S.D., and Gonsalves, D. 2001. Comparative reactions of recombinant papaya ringspot viruses with chimeric coat protein (CP) genes and wild-type viruses on CP-transgenic papaya. Journal of General Virology ,82:2827–2836. doi:10.1099/0022-1317-82-11-2827

Chung, B. Y.W., Miller, W. A., Atkins, J. F., and Firth, A. E. 2008. An overlapping essential gene in the Potyviridae. Proceedings of the National Academy of Sciences of the United States of America 105:5897–5902.

Coutts, B. A., Kehoe, M. A., Webster, C. G., Wylie, S. J., and Jones, R. A. C. 2011. Zucchini yellow mosaic virus: biological properties, detection procedures and comparison of coat protein gene sequences. Arch. Virol. 156:2119-2131.

Cruz, F.C.S., Tanada, J.M., Elvira, P.R.V., Dolores, L.M., Magdalita, P.M., Hautea, D.M., and Hautea, R.A. 2009. Detection of mixed virus infection with *Papaya ringspot virus* (PRSV) in papaya (*Carica papaya L.)* grown in Luzon, Philippines. Philipp. Journal of Crop Science 34: 62–74.

Datta, S., Budhauliya, R., Das, B., Chatterjee, S., Bora, A., Vairale, M.G., Gogoi, H.K., Veer, V. 2015. First report of tomato leaf curl Bangladesh virus (ToLCBV) infecting *Gomphostemma niveum* plants in Assam, India. New Dis Rep 31:19. http://dx.doi.org/10.5197/j.2044-0588.2015.031.019.

Davis, M.J., and Ying, Z. 2004. Development of papaya breeding lines with transgenic resistance to *Papaya ringspot virus*. Plant Disease, 88, 352-358. https:// doi.org/ 10.1094/ PDIS.2004.88.4.352.

De, Barro P.J., Liu, S.S., Boykin L.M, Dinsdale, A.B. 2011. Bemisia tabaci: a statement of species status. Annual Review of Entomology 56:1–19

Dey, K.K., Hong, L., Borth, W.B., Melzer, M.J., and Hu, J.S. 2012. A highly sensitive single-tube nested PCR assay for the detection of Pineapple mealybug wilt associated virus-2 (PMWaV-2). Journal of virological methods 183**:** 215– 218.

Dıaz-Pendon, J.A., and Ding, S.W. 2008. Direct and indirect roles of viral suppressors of RNA silencing in pathogenesis. Annual Review of Phytopathology 46: 303– 26. doi: 10.1146/annurev.phyto.46.081407.104746.

Ding, S.W., and Voinnet, O. 2007. Antiviral immunity directed by small RNAs. Cell, 130, 413–26.doi: 10.1016 /j.cell.2007.07.039

Dinant, S., Maisonneuve, B., Albouy, J., Chupeau, Y., Chupeau, M., Bellec, Y., Gaudefroy, F., Kusiak, C., Souche, S., Robaglia, C., and Lot, H. 1997. Coat protein gene mediated protection in *Lactucasativa* against lettuce mosaic potyvirus strains. Molecular Breeding, 3: 75–86.

Dry, I.B., Rigden, J.E., Krake, L.R., Mullineaux, P.M. and Rezaian, M.A. 1993. Nucleotide sequence and genome organization of tomato leaf curl geminivirus. Journal of General Virology. 74:147–151.

Duprat, A., Caranta, C., Revers, F., Menand, B., Browning, K.S., and Robaglia, C. 2002. The Arabidopsis eukaryotic initiation factor (iso) 4E is dispensable for plant growth but required for susceptibility to potyviruses. Plant Journal. 32:927–934.

Fagard, M. and Vaucheret, H. 2000. (Trans) gene silencing in plants: how many mechanisms? Annual Review of Plant Physiology and Plant Molecular Biology , 51:167–194

FAOSTAT. 2014. Production. http://faostat.fao.org/site/339/default.aspx. Accessed 19 December 2014.

Fauquet, C.M., Briddon, R.W., Brown, J.K., Moriones, E., Stanley, J., Zerbini, M., Zhou, X. 2008. Geminivirus strain demarcation and nomenclature. Archives of Virology 2, 153:783-821.

Fermin, G. and Gonsalves, D. 2001. Towards the development of short synthetic genes for multiple virus resistance. Phytopathology. 91**:**28.

Fermin-Munoz, G. A. 2002. Use, application, and technology transfer of native and synthetic genes for engineering single and multiple transgenic viral resistances. Department of Plant Pathology, Cornell University, Ithaca, NY, p. 293.

Fermin, G.A., Inglesses, V., Garbozo, C., Rangel, S., Dagert, M. and Gonsalves, D. 2004. Engineered resistance against PRSV in Venezuelan transgenic papayas. Plant Disease, 88,:516–522.

Fermin, G., and Gonsalves, D. 2004. Control of viral diseases of papaya: Native, chimeric and synthetic transgenes to engineer resistance against Papaya ringspot virus. In G. Loebenstein, & G. Thottappilly (Eds.), Virus and virus-like diseases of major crops in

developing countries (pp. 497-518). The Netherlands: Kluwer Academic Press Publishers.

Fermin, G. A., Castro, L. T., and Tennant, P. F. 2010. CP-transgenic and non-transgenic approaches for the control of papaya ringspot: current situation and challenges. Transgenic Plant Journal 4: 1-15.

Fermin, G. A., and Randle, M. 2015. Papaya ringspot. In P. Tennant and G. A. Fermin (Eds.), Virus diseases of tropical and subtropical crops. London: Cabi.

Fermin, G.A.and Randle, M. 2015. Papaya ringspot . Virus Diseases of tropical and subtropical crops edited by Paula Tennant, Gustavo A Fermin. Papaya ringspot. Cabi.

Ferreira, S., Mau, R., Manshardt, R., Pitz, K., and Gonsalves, D. 1992. Field evaluation of papaya ringspot virus cross protection. In Proc. 28th Annual Hawaii Papaya Industry Association Conference, (pp. 29-30).

Ferreira, S.A., Pitz, K.Y., Manshardt, R., Fitch, M., and Gonsalves, D. 2002. Virus coat protein transgenic papaya provides practical control of Papaya ringspot virus in Hawaii. Plant Disease. 86 (2): 101–105.

Fitch, M.M. and Manshardt, R. M. 1990. Somatic embryogenesis and plant regeneration from immature zygotic embryos of papaya (*Carica papaya* L.). Plant Cell Reports 9:320- 324. doi: 10.1007/BF00232860.

Fitch, M.M., Manshardt, R.M., Gonsalves, D., Slightom, J.L., and Sanford, J.C. 1992. Virus resistant papaya derived from tissues bombarded with the coat protein gene of papaya ringspot virus. Bio/Technology 10: 1466-1472. doi:10.1038/nbt1192-1466

Fuchs, M., and Gonsalves, D. 2007. Safety of virus-resistant transgenic plants two decades after their introduction: lessons from realistic field risk assessment studies. Annual Review of Phytopathology,*45*, 173–202. DOI:10. 1146/ annurev. phyto. 45.062806.094434

Green, J. C., and Hu, S.J. 2017. Editing plants for virus resistance using CRISPR-Cas. Acta virologica 61**:**,138 – 142. doi: 10.4149/av_2017_02_02

Gómez, P., Rodríguez-Hernández, A.M., Moury, B. and Aranda, M.A. 2009. Genetic resistance for the sustainable control of plant virus diseases: breeding, mechanisms and durability. European Journal of Plant Pathology 125:1–22.

Gonsalves D. 1998. Control of papaya ringspot virus in papaya: a case study. Annual Review Phytopathology,36, 415–437. doi:10.1146/annurev.phyto.36.1.415.

Gonsalves, D. 2002. Coat protein transgenic papaya: ‘‘acquired’’ immunity for controlling papaya ringspot virus. Current Topics in Microbiology and Immunology 266:73–83. doi:10.1007/BF00232860

Gonsalves, D., Gonsalves, C., Ferreira, S., Pitz, K., Fitch, M., Manshardt, R., and Slightom, J. 2004. Transgenic virus resistant papaya: from hope to reality for controlling papaya ringspot virus in Hawaii. APSnetFeatures, (http://www.apsnet.org/online/feature/ringspot/) doi:10.1094/APSnetFeature-2004-0704

Gonsalves, D. 2004. Tansgenic papaya in Hawaii and beyond. AgBioForum, 7(1*and* 2), 36-40.

Gonsalves, D., Suzuki, J.Y., Tripathi, S., Ferreira, S.A. 2008. Papaya ringspot virus (Potyviridae). In: Mahy, B.W.J., van Regenmortel, M.H.V. (Eds.), Encyclopedia of Virology. Elsevier Ltd., Oxford. pp. 1–8.

Gonsalves, D.Tripathi, S., Carr, J.B. and Suzuki, J.Y. 2010. Papaya ringspot virus. The Plant Health Instructor. doi: 10.1094/PHI-I-2010-1004-01

Guo, J., Yang, L., Liu, X., Guan, X., Jiang, L., and Zhang, D. 2009. Characterization of the exogenous insert and development of event-specific PCR detection methods for genetically modified Huanong No. 1 papaya. Journal of Agricultural and Food Chemistry 57:7205–7212. doi:10.1021/jf901198x.

Guo, T., Guo, Q., Cui, X., Liu, Y., Hu, J., Liu, S. 2015. Comparison of transmission of papaya leaf curl China virus among four cryptic species of the whitefly *Bemisia tabaci* complex. Sci Rep. 5:15432 doi: 10.1038/srep15432.

Hamim, I., Alam, M.Z., Ali, M.A., and Ashrafuzzaman, M. 2014. Incidence of post-harvest fungal diseases of ripe papaya in Mymensingh. Journal of Bangladesh Agricultural University 12(1): 25–28

Hamim, I., Borth, W.B., Marquez, J., Green, J.C., Melzer, M. J., and Hu, J.S. 2018a. Transgene-mediated resistance to papaya ringspot virus: challenges and solutions. Phytoparasitica 46:1 https://doi.org/10.1007/s12600-017-0636-4. https://doi.org/10.1007/s12600-017-0636-4

Hamim, I., Borth, W.B, Melzer, M.J., Hu, J.S. 2018b. Ultra-sensitive detection of papaya ringspot virus using single-tube nested PCR. Acta Virol. 62(4):379-385. doi: 10.4149/av_2018_405.

Hamim, I., Rwahnih, M.A., Borth, W.B., Melzer, M.J., Gonsalves, D., Suzuki, J.Y., Wall, M.M., Hu, J.S. 2018c. Deep sequencing of total RNAs in papaya for genome characterization of Papaya ringspot virus Bangladesh strain. Phytopathology 108(10):120

Hamim, I., Borth, W.B., Melzer, M.J.,Gonsalves, D., Suzuki, J.Y., Wall, M.M., Hu, J.S. 2018d. Molecular analysis indicates that papaya in Bangladesh is a host of multiple

begomoviruses. Phytopathology 108(10):120. (Abstr.). https://doi.org/10.1094/PHYTO-108-10-S1.120.

Hamim, I., Borth, W.B., Melzer, M.J., Suzuki, J.Y., Wall, M.M. and Hu, J.S., 2019. Occurrence of tomato leaf curl Bangladesh virus and associated subviral DNA molecules in papaya in Bangladesh: molecular detection and characterization. Archives of Virology pp.1-5. https://link.springer.com/article/10.1007/s00705-019-04235-8

Hanley-Bowdoin, L., Bejarano, E.R., Robertson, D., and Mansoor, S. 2013. Geminiviruses: masters at redirecting and reprogramming plant processes. Nature Reviews Microbiology, 11, 777–788. https://doi.org/10.1038/ nrmicro3117

Harrison, B.D., Robinson, D.J. 1999. Natural genomic and antigenic variation in whitefly-transmitted geminiviruses (begomoviruses). Annu Rev Phytopathol . 37:369–398.

Huo-gen, X., Hu, J.S., Fan, H.Z. 2000. Detection of papaya ringspot virus by immuno-capture PCR，nested-PCR and ELISA·PCR Assays. Virologia Sincia l 5**:**367-372.

Huang, X., Madan, A. 1999. CAP3: A DNA sequence assembly program. Genome Res. 9: 868-877. doi:10.1101/gr.9.9.868.

Huang, C., Xie, Y, Zhao, L., Ren, H., Li, Z. 2013. A naturally occurring defective DNA satellite associated with a monopartite begomovirus: evidence for recombination between alphasatellite and betasatellite. Viruses 5(9):2116-28. doi:10.3390/v5092116.

Hussain, M., Mansoor, S., Iram, S., Zafar, Y., Briddon, R.W. 2004. First report of tomato leaf curl New Delhi virus affecting chilli pepper in Pakistan. Plant pathology, 53:794-794.

Hsu, P. D., Lander, E. S. and Zhang, F. 2014. Development and applications of CRISPRCas9 for genome engineering. Cell 157:1262–1278.

Ingham, D.J., Pascal, E., Lazarowitz, S.G. 1995. Both bipartite geminivirus movement proteins define viral host range, but only BL1 determines viral pathogenicity. Virology 207:191-204.

Ito, T., Sharma, P., Kittipakorn, K., Ikegami, M. 2008. Complete nucleotide sequence of a new isolate of tomato leaf curl New Delhi virus infecting cucumber, bottle gourd and muskmelon in Thailand. Archives of Virology. 153:611-613.

Jain, R.K., Nasiruddin, K.M., Sharma, J., Pant, R.P., and Varma, A. 2004. First report of occurrence of papaya ring spot virus infecting papaya in Bangladesh. Plant Dis. 88:221.

Jensen, D.D. 1949. Papaya virus diseases with special reference to papaya ringspot. Phytopathology 39:191–211.

Jeong, J.J., Ju, H.J., Noh, J. 2014 . A review of detection methods for plant viruses. Research in Plant Disease, 20 (3) : 173-181.

Ji, X., Zhang, H., Zhang, Y., Wang, Y., and Gao, C. 2015. Establishing a CRISPR-Cas-like immune system conferring DNA virus resistance in plants. Nature Plants 1:15144. https://doi. org/10.1038/nplants.2015.144.

Jia, R., Zhao, H., Huang, J., Kong, H., Zhang, Y., Guo, J., Huang, Q., Guo, Y., Wei, Q., Zuo, J., Zhu, Y.J., Peng, M., and Guo, A. 2017. Use of RNAi technology to develop a PRSV-resistant transgenic papaya. Scientific report. 7:12636. doi:10.1038/s41598-017-13049-0

Jiang, J. and Laliberté, J.F. 2011. The genome-linked protein VPg of plant viruses-a protein with many partners. Current opinion virology. 1: 347–354.

Kalantidis, K.., Psaradakis, S., Tabler, M., and Tsagris, M. 2002. The occurrence of CMV specific short RNAs in transgenic tobacco expressing virus-derived doubles tranded RNA is indicative of resistance to the virus. Molecular Plant Microbe Interactions 15:826–33. doi:10.1094/MPMI.2002.15.8.826

Kasschau, K.D., and Carrington, J.C. 1998. A counter defensive strategy of plant viruses: suppression of post transcriptional gene silencing. Cell 95, 461–470. https://doi.org/10.1016/S0092-8674(00)81614-1.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Mentjies, P., Drummond, A. 2012. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649

Kheyr-Pour, A., Bendahmane, M., Matzeit V., Accotto, G.P., Crespi, S., Groenenborn, B. 1991. Tomato yellow leaf curl virus from Sardinia is a whitefly-transmitted monopartite geminivirus. Nucleic Acids Res. 19:6763–6769

Kirthi, N., Maiya, S. P., Murthy, M. R. N., and Savithri, H. S. 2002. Evidence of recombination among the tomato leaf curl virus strains/ species from Bangalore, India. Arch. Virol. 147:255-272.

Kon, T., and Gilbertson, R.L. 2012. Two genetically related begomoviruses causing tomato leaf curl disease in Togo and Nigeria differ in virulence and host range but do not require a betasatellite for induction of disease symptoms. Arch Virol. 157:107–120

Kos, M., Loon, J.J.V., Dicke, M., and Vet, L.E.M. 2009. Transgenic plants as vital components of integrated pest management.Trends in Biotechnology, 27(11) : 621-627.

Kung, Y.J., Bau, H.J., Wu, Y.L., Huang, C.H., Chen, T.M., and Yeh, S.D. 2009. Phytopathology 99: 1312–1320. doi: 10.1094/PHYTO-99-11-1312.

Kung, Y., You, B., Raja, J.A.J., Chen, K., Huang, C., Bau, H., Yang, C., Huang, C., Chang, C. and Yeh, S. 2015. Nucleotide sequence-homology-independent breakdown of transgenic resistance by more virulent virus strains and a potential solution. Science Reports 5:9804. http://dx.doi.org/10.1038/srep09804.

Kumar, S., Glen, S., and Tamura, K.. 2015. MEGA7: Molecular evolutionary genetics analysis version 7.0. Molecular Biology and Evolution, Volume 33, Issue 7, 1 July 2016, Pages 1870–1874 https://doi.org/10.1093/molbev/msw054

Kumar, S., Stecher, G., Tamura, K. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33:1870-1874.

Lakatos, L., Csorba, T., Pantaleo, V., Chapman, E. J., Carrington, J. C., and Liu, Y. P., 2006. Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. EMBO Journal 25,:2768–2780

Lellis, A.D., Kasschau, K.D., Whitham, S.A. and Carrington, J.C. 2002. Loss of susceptibility mutants of *Arabidopsis thaliana* reveal an essential role for *eIF(iso)4E* during Potyvirus Infection. Current Biology 12,:1046–1051.

Léonard, S., Plante, D., Wittmann, S., Daigneault, N., Fortin, M.G. and Laliberté, J.F. 2000. Complex formation between Potyvirus VPg and translation eukaryotic initiation factor 4E correlates with virus infectivity.Journal of Virology. 74:7730–7737

Li, Z., Xie, Y., Zhou, X. 2005. Tobacco curly shoot virus DNAβ is not necessary for infection but intensifies symptoms in a host-dependent manner. Phytopathology. 95:902-908.

Ling, K., Namba, S., Gonsalves, C., Slightom, J.L., and Gonsalves, D. 1991. Protection against detrimental effects of potyvirus infection in transgenic tobacco plants expressing the papaya ringspot virus coat protein gene. Biotechnology, 9**:** 752–758.

Lines, R.E., Persley, D., Dale, J.L., Drew, R., and Bateson, M.F. 2002. Genetically engineered immunity to papaya ringspot virus in Australian papaya cultivars. Molecular Breeding 10:119-129. doi: 10.1023 / A:1020381110181

Lin, H., Chen, C., Doddapaneni, H., Duan, Y., Civerolo, E.L., Bai, X., Zhao, X. 2010. A new diagnostic system for ultra-sensitive and specific detection and quantification of *Candidatus Liberibacter asiaticus*, the bacterium associated with citrus Huanglongbing. Journal of Microbiological Methods. 81:17- 25.

Ling, K., Namba, S., Gonsalves, C., and Slightom, J.L., Gonsalves, D. 1991. Protection against detrimental effects of potyvirus infection in transgenic tobacco plants expressing the papaya ringspot virus coat protein gene. Biotechnology, 9752–758.

Lius, S., Manshardt, R.M., Fitch, M.M., Slightom, J.L., Sanford, J.C. and Gonsalves, D. 1997. Pathogen-derived resistance provides papaya with effective protection against papaya ring spot virus. Molecular Breeding, 3:161-168.

Lu, Y.W., Shen, W.T., Zhou, P., Tang, Q.J., Niu, Y.M., Peng, M., and Xiong, Z. 2008. Complete genomic sequence of a papaya ringspot virus isolate from Hainan Island, China. Archives of Virology 153: 991–993. doi:10.1007/s00705-008-0056-3.

Llop, P., Bonaterra, A., Penalver, J., Lopez, M.M. 2000. Development of a highly sensitive nested PCR procedure using a single closed tube for detection of *Erwinia amylovora* in asymptomatic plant material. Applied Environmental Microbiology. 66 :2071–2078.

Lu, Y.W., Shen, W.T., Zho, P., Tang, J.Q., Niu, Y.M., Peng, M., Xiong, Z. 2008. Complete genomic sequence of a papaya ringspot virus isolate from Hainan Island, China. Archives of Virology153: 991–993.

Mahatma, L., and Mahatma, M.K. 2012. First report of a variant of tomato leaf curl Bangladesh virus infecting Gaillardia. New Dis Rep. 26:4. http://dx.doi.org/10.5197/j.2044-0588.2012.026.004.

Maoka, T., Kashiwazaki, S., Tsuda, S., Usugi, T., Hibino, H. 1996. Nucleotide sequence of the capsid protein gene of papaya leaf-distortion mosaic potyvirus. Archives of Virology 141 197–204.

Manshardt, R.M. 1992. Papaya. In Biotechnology of perennial fruit crops (Biotechnology in Agraculture No. 8); Hammerschlag, F.A., Litz, R.E., Eds.; CIBA: Wallingford, UK. 489–511.

Martin, D.P., Lerney, P., Lott, M., Moulton, V., Posada, D., Lefeuvre, P. 2010. RDP3: a flexible and fast computer program for analyzing recombination. Bioinformatics 26:2462–2463.

Maruthi, M.N., Alam, S.N., Kader, K.A., Rekha, A.R., Cork, A., Collvin, J. 2005. Nucleotide sequencing, whitefly transmission and screening tomato for resistance against two newly described begomoviruses in Bangladesh. Virology 95:1472–1481

Mallory, A.C., Reinhart, B.J., Bartel, D., Vance, V.B., and Bowman, L.H. 2002. A viral suppressor of RNA silencing differentially regulates the accumulation of short interfering RNAs and micro-RNAs in tobacco. Procedings of National Academic of Science. USA 99:15228–15233. doi: 10.1073/pnas.232434999

Mangrauthia, S. K.., Shakya, V.P.S., Jain, R.K., and Praveen, S. 2009. Ambient temperature perception in papaya for papaya ringspot virus interaction. Virus Genes 38: 429–434. doi: 10.1007/s11262-009-0336-3.

Mangrauthia, S.K., Priyanka, S.E., and Praveen, E.S. 2010. Genomics of helper component proteinase reveals effective strategy for papaya ringspot virus resistance. Molecular Biotechnology. 44:22–29 DOI 10.1007/s12033-009-9205-5

Mangrauthia, S. K., Parameswari, B., Jain, R. K., and Praveen, S. 2008. Role of genetic recombination in the molecular architecture of papaya ringspot virus. Biochemical Genetics 46, 11–12, 835–846. doi:10.1007/s10528-008-9198-y.

Mansoor, S., Briddon, R.W., Zafar, Y., and Stanley, J..2003. Geminivirus disease complexes: an emerging threat. Trends in Plant Sciences 8: 128–134.

Manshardt, R.M. 1992. Papaya. InF. A. Hammerschlag&R. E. Litz (Eds.), Biotechnology of perennial fruit crops (Biotechnology in Agraculture No. 8) (pp. 489–511). Wallingford, UK:CIBA.

Manshardt, R.M. 1999. 'UH Rainbow' papaya a high-quality hybrid with genetically engineered disease resistance. University of Hawaii College of Tropical Agriculture and Human Resources(CTAHR), New Plants for Hawaii (NPH)-1, revised. Available at: http://www.ctahr.hawaii.edu/ctahr200l/P]O/FreePubs/FreePubsO7.asp#NewPlantsFor Hawaii.

Maruthi, M.N., Rekha, A.R., Mirza, S.H., Alam, S.N., Colvin, J. 2007. PCR-based detection and partial genome sequencing indicate high genetic diversity in Bangladeshi begomoviruses and their whitefly vector, *Bemisia tabaci*. Virus Genes 34: 373–385.

Matzke, M.A. and Matzke, A.J.M. 1998. Epigenetic silencing of plant transgenes as a consequence of diverse cellular defence responses. Cellular and Molecular Life Sciences,54, 94–103.

Mazier, M., Flamain, F., Nicolaï, M., Sarnette, V. and Caranta, C. 2011. Knock-down of both eIF4E1 and eIF4E2 genes confers broad-spectrum resistance against potyviruses in Tomato. PLoS ONE 6:e29595.

McDonald, J., Brandle, F., Gleddie, S., Hermans, J. and Kermali, I. 1997. Resistance to homologous and heterologous strains of potato virus Y in transgenic tobacco carrying the PVY (N) coat protein gene. Canadian Journal of Plant Science,77:167–171.

Mendoza, T.E.M., Laurena, A., and Botella, J.R. 2008. Recent advances in the development of transgenic papaya technology. Biotechnology Annual Review. 14:423–462. http://dx.doi.org/10.1016/S1387-2656(08)00019-7.

Merai, Z., Kerenyi, Z., Kertesz, S., Magna, M., Lakatos, L., and Silhavy, D. 2006. Double-stranded RNA binding may be a general plant viral strategy to suppress RNA silencing. Journal of Virology. 80,:5747–5756.

Milkovich, M. 2017. BHEARD scholar sheds light on papaya viruses in Bangladesh. Borlaug Higher Education for Agricultural Research and Development (BHEARD). Michigan State University. 2017. https:// www canr.msu.edu /news/bheard_scholar_ sheds _light_on _papaya _viruses _in _ bangladesh.

Mishra, R. and, Basavaprabhu, L.P. 2018. First complete genome sequence of a distinct papaya ringspot virus isolate from the Northeastern Region of India. Genome announcements vol. 6,22 e00437-18. 31 doi:10.1128/genomeA.00437-18

Moffat, A. S. 1999. Geminiviruses emerge as serious crop threat. Science 286: 1835.

Muhire, B.M., Varsani, A., Martin, D.P. 2014. SDT: A virus classification tool based on pairwise sequence alignment and identity calculation. PLoS ONE 9(9): e108277. https://doi.org/10.1371/journal.pone.0108277

Murphy, J. F., and Bowen, K. L. 2006. Synergistic disease in pepper caused by the mixed infection of cucumber mosaic virus and pepper mottle virus. Phytopathology, 96,:240-247.

Namba, R., and Higa, S. Y. 1981. Papaya mosaic transmission as affected by the duration of the acquisition probe of the green peach aphid – *Myzus persicae* (Sulzer). P. Hawaii Entomol. Soc. 23:431-443. 12.

Nault, L. R. 1997. Arthropod transmission of plant viruses – A new synthesis. Ann. Entomol. Soc. Am. 90:521-541.

Nawaz-Ul-Rehman, M.S. and Fauquet, C.M. 2009. Evolution of geminiviruses and their satellites. FEBS Lett. 583:1825–1832

Nawaz-ul-Rehman, M.S., Mansoor, S., Briddon, R.W., Fauquet, C.M. 2009. Maintenance of an Old World betasatellite by a New World helper begomovirus and possible rapid adaptation of the betasatellite. J Virol. 83:9347–9355

Nicaise, V., German-Retana, S., Sanjuán, R., Dubrana, M.P., Mazier, M., Maisonneuve, B., Candresse, T., Caranta, C. and LeGall, O. 2003. The eukaryotic translation initiation factor 4E controls Lettuce susceptibility to the potyvirus lettuce mosaic virus. Plant Physiology 132: 1272–1282.

Noa-Carrazana, J., González-de-León, D., Ruiz-Castro, B., Piñero, D., and Silva-Rosales, L. 2006. Distribution of papaya ringspot virus and papaya mosaic virus in papaya plants (*Carica papaya*) in Mexico. Plant Disease,90:1004–1011. https://doi.org/10.1094/PD-90-1004

Ohshima, K.., Tomitaka, Y., Wood, J.T., Minematsu, Y., Kajiyama, H., Tomimura, K., and Gibbs, A.J. 2007. Pattern of recombination in turnip mosaic virus genomic sequences indicates hotspots of recombination. Journal of General Virology. 88:298–315

Ortiz-Rojas, L. and Chaves-Bedoya, G. 2017. Molecular characterization of two papaya ringspot virus isolates that cause devastating symptoms in Norte de Santander, Colombia. Eur J Plant Pathol. 148: 883. https://doi.org/10.1007/s10658-016-1143-z

Padidam, M., Beachy, R.N., Fauquet, C.M. 1995. Tomato leaf curl geminivirus from India has a bipartite genome and coat protein is not essential for infectivity. J Gen Virol. 1995, 76(Pt 1):25-35.

Paolla, M.V.A., Tathiana, F.S.A., Anuar, M.Á., Daisy, P.B., Raúl, T.T., José, A.V, Antonio, A.R.F., and Patricia, M.B.F. 2015. A current overview of the papaya meleira virus, an unusual plant virus. Viruses 7:1853-1870. doi:10.3390/v7041853

Paul, S., Ghosh, R., Das, S., Palit, P., Acharyya, S., Das, A. and Roy, A. 2009. First report of tomato leaf curl Joydebpur virus and associated betasatellite in kenaf (*Hibiscus cannabinus*) plants showing leaf curl symptoms from southern India. Plant Pathology, 58(2).

Phironrit, N., Chowpongpang, S., Warin, N., Bhunchoth, A., and Attathom, S. 2005. Small scale field testing of PRSV resistance in transgenic papaya line KN116/5. In I International Symposium on Papaya 740:169-176.

Powell-Abel, P., Nelson, R.S., De, B., Hoffmann, N., and Rogers, S.G. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science, 232, 738-743. doi: 10.1126/science.232.4751.738.

Prasad, S. M. and Sarkar, D. P. 1989. Some ecological studies on papaya ringspot virus in Ranchi. Indian Journal Virolog ,5:118-122.

Pruss, G., Ge, X., Shi, X. M., Carrington, J.C., and Vance, V.B. 1997. Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. The Plant Cell 9:859-868.doi: https://doi.org/10.1105/tpc.9.6.859

Purcifull, D, Edwardson, J., Hiebert, F. and Gonsalves, D. 1984. Papaya ringspot virus. CM1/AAB Descriptions of Plant Viruses.No. 292. (No. 64 Revised, July 1984), pp. 8.

Pyott, D.E., Sheehan, E., and Molnar, A .2016. Engineering of CRISPR/Cas9-mediated potyvirus resistance in transgene-free Arabidopsis plants. Molecular Plant Pathology 17, 1276–1288. https://doi.org/10.1111/mpp.12417. doi: 10.1111/mpp.12417. Epub 2016 Jun 27.

Raj, S.K., Snehi, S.K., Khan, M.S., Singh, R, Khan, A.A. 2008. Molecular evidence for association of tomato leaf curl New Delhi virus with leaf curl disease of papaya (*Carica papaya* L.) in India. Aust Plant Dis Notes. 3:152–155.

Rodríguez-Hernández, A.M., Gosalvez, B., Sempere, R.N., Burgos, L., Aranda, M.A. and Truniger, V. 2012. Melon RNA interference (RNAi) lines silenced for Cm-eIF4E show broad virus resistance. Molecular Plant Pathology. 13:755–763.

Rodríguez, D., Alonso, M., Tornet, Y., Valero, L., Raimildes Lorenzetti, E., and Pérez, R. 2013. Evaluación de accesiones cubanas de papaya (*Carica papaya* L.) ante lamancha anular. Summa Phytopathologica. 39: 25.

Ruffel, S., Gallois, J.L., Moury, B., Robaglia, C., Palloix, A. and Caranta, C. 2006. Simultaneous mutations in translation initiation factors eIF4E andeIF(iso)4E are required to prevent pepper veinal mottle virus infection of pepper. Journal of General. Virology. 87, 2089–2098.

Quemada, H., L Hostis, B., and Gonsalves, D. 1990. The nucleotide sequences of the 3'-terminal regions of papaya ringspot virus strains w and p. Journal of General Virology 71**:**203–210.

Ruanjan, P., Kertbundit, S., Juricek, M. 2007. Post-transcriptional gene silencing is involved in resistance of transgenic papayas to papaya ringspot virus. Biologia Plantarum 51 (3) : 517-520.

Sakuanrungsirikul, S., Sarindu, N., Prasartsee, V., Chaikiatiyos, S., Siriyan, R., Sriwatanakul, M., Lekananon, P., Kitprasert, C., Boonsong, P., Kosiyachinda, P., Fermin, G. and Gonsalves, D. 2005. Update on the development of virus-resistant papaya: virus-resistant transgenic papaya for people in rural communities of Thailand. Food Nutrition Bulletin 26: 422–426.

Sanfaçon, H. 2015. Plant translation factors and virus resistance. Viruses 7: 3392–3419.

Sanford, J. C. and Johnston, S. A. 1985. The concept of parasite-derived resistance - Deriving resistance genes from the parasite's own genome. Journal of Theoretical Biology 113, 395–405.

Sanford, J.C., Klein, T.M., Wolf, E.D., and Allen, N. 1987. Delivery of substances into cells and tissues using a particle bombardment process. Journal of Pharmaceutical Science and Technology, 5:27–37.

Shahid, M.S., Yoshida, S., Khatri-Chhetri, G.B., Briddon, R.W., Natsuaki, K.T. 2013. Complete nucleotide sequence of a monopartiteBegomovirus and associated satellites infecting Carica papaya in Nepal. Virus Genes 46(3):581-4. doi 10.1007/s11262-013-0888-0.

Sato, M., Nakahara, K., Yoshii, M., Ishikawa, M. and Uyeda, I. 2005. Selective involvement of members of the eukaryotic initiation factor 4E family in the infection of *Arabidopsis thaliana* by potyviruses. FEBS Letter 579: 1167–1171.

Saeed, M., Zafar, Y., Randles, J.W., Rezaian, M.A. 2007. A monopartite begomovirus associated DNA beta satellite substitutes for the DNA B of a bipartite begomovirus to permit systemic infection. J Gen Virol. 88:2881-2889.

Saunders, K., Stanley, J. 1999. A nanovirus-like DNA component associated with yellow vein disease of *Ageratum conyzoides*: evidence for interfamilial recombination between plant DNA viruses. Virology 264:142–152.

Saunders, K., Bedford, I.D., Stanley, J. 2001. Pathogenicity of a natural recombinant associated with Ageratum yellow vein disease: implications for geminivirus evolution and disease aetiology. Virology 282: 38-47.

Saunders, K., Bedford, I.D., Briddon, R.W., Markham, P.G., Wong, S.M., Stanley, J. 2000. A unique virus complex causes Ageratum yellow vein disease. Proc Natl Acad Sci USA. 97:6890-6895.

Saunders, K., Briddon, R.W., Stanley, J. 2008. Replication promiscuity of DNA-β satellites associated with monopartite begomoviruses; deletion mutagenesis of the ageratum yellow vein virus DNA-β satellite localises sequences involved in replication. J Gen Virol. 89: 3165-3172.

Schumann, U., Smith, N.A., Kazan, K., Michael, A. M., and Wang, M.B. 2013. Analysis of hairpin RNA transgene-induced gene silencing in *Fusarium oxysporum*. Silence,4, 3. http://www.silencejournal.com/content/4/1/3. doi: 10.1186/1758-907X-4-3.

Shen, W., Tuo, D., Yan, P., Li, X., Zhou, P. 2014a. Detection of papaya leaf distortion mosaic virus by reverse-transcription loop-mediated isothermal amplification. Journal of Virological Methods 195: 174–179.

Shen, W., Tuo, P., Yan, X., Li, P., Zhou, P. 2014b. Reverse transcription loop-mediated isothermal amplification assay for rapid detection of papaya ringspot virus. Journal of Virological Methods 204**:**93–100. http://dx.doi.org/10.1016/j.jviromet.2014.04.012

Shih, S. L., Tsai, W. S., Green, S. K., and Singh, D. 2007. First report of tomato leaf curl Joydebpur virus infecting chilli in India. Plant pathology 56(2).

Singh-Pant, P., Pant, P., Mukherjee, S.K., Mazumdar, S. 2012. Spatial and temporal diversity of begomoviral complexes in papayas with leaf curl disease. Archives of Virology 157: 1217–1232. DOI 10.1007/s00705-012-1287-x

Sinha, V., Kumar, A., Bhatnagar, D., Khan, J.A. 2013. Association of cotton leaf curl Multan virus and its satellite molecules with leaf curl disease of papaya in India. New Dis Rep 27: 9. http://dx.doi.org/10.5197/j.2044-0588.2013.027.009

Souza, M.T., Nickel, O. and Gonsalves, D. 2005. Development of virus resistant transgenic papayas expressing the coat protein gene from a Brazilian isolate of papaya ringspot virus. Fitopatol Bras. 30:357–365.

Souza, J.M.T., Tennant, P.F., and Gonsalves, D. 2005. Influence of coat protein transgene copy number on resistance in transgenic line 63–1 against papaya ringspot virus isolates. Hort-Science, 40, 2083–2087

Sreenivasulu, M. and Sai Gopal, D.V.R. 2010. Development of recombinant coat protein antibody based IC-RT-PCR and comparison of its sensitivity with other immunoassays for the detection of papaya ringspot virus isolates from India. Plant Pathology Journal 26(1):25-31.

Stanley, J., Saunders, K., Pinner, M.P., Wong, S.M. 1997. Novel defective interfering DNAs associated with ageratum yellow vein geminivirus infection of *Ageratum conyzoides.* Viroloogy 239: 87-96.

Sturrock, D. 1940. Tropical fruits for Southern Florida and Cuba and their uses. The Arnold Arboretum of Harvard University. Jamaica Plain, MA.

Stokstad, E. 2008. Papaya takes on ringspot virus and wins. Science, 320:472. doi:10.1126/science.320.5875.472.

Swain, S., and Powell, D. 2001. Papaya ringspot virus resistant papaya: A case study. Online. University of Guelph. Available at http://www. Food safetynetwork. ca/gmo/papayarep. Htm

Tahir, M., Haider, M.S. 2005. First report of tomato leaf curl New Delhi virus infecting bitter gourd in Pakistan. Plant pathology. 54:807-807.

Tennant, P.F., Gonsalves, C., Ling, K.S., Fitch, M.M., Manshardt, R., Slightom, L.J. Gonsalves, D. 1994. Differential protection against papaya ringspot virus isolates in coat protein gene transgenic papaya and classically cross-protected papaya. Phytopathology,84(11) :, 1359-1366.

Tennant, P., Fermin, G., Fitch, M.M., Manshardt, R.M., Slightom, J.L., and Gonsalves, D. 2001. Papaya ringspot virus resistance of transgenic Rainbow and SunUp is affected by gene dosage, plant development, and coat protein homology. European Journal of Plant Pathology,107(6), 645-653. doi:10.1023/A:1017936226557

Tennant, P.F., Ahmad, M.H., and Gonsalves, D. 2002. Transformation of *Carica papaya L.* with virus coat protein genes for studies on resistance to papaya ringspot virus from Jamaica. Tropical Agriculture (Trinidad), 79(2), 105-113.

Tennant, P., Ahmad, M.H., and Gonsalves, D. 2005. Field resistance of coat protein transgenic papaya to papaya ringspot virus in Jamaica. Plant Disease 89:841–847. *apsjournals.apsnet.org/doi/pdf/10.1094/PD-89-0841*

Thompson, J.D., Higgins, D.G., and Gibson, T.J. 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting,

positions-specific gap penalties and weight matrix choice. Nucleic Acid Res. 22:4673–4680 .

Titanji, V.P.K., Zofou, D., and Ngemeneya, M.N. 2008. The Antimalarial potential of medicinal plants used for the treatment of malaria in Cameroonian Folk Medicine. The African Journal of Traditional, Complementary and Alternative medicines 5(3): 302-321.

Tiwari, N., Singh, V.B., Sharma, P.K., Malathi, V.G. 2013. Tomato leaf curl Joydebpur virus: a monopartite begomovirus causing severe leaf curl in tomato in West Bengal . Arch Virol 158:1–10 DOI 10.1007/s00705-012-1440-6

Torres-Barcelo, C., Daros, J. A., and Elena, S.F. 2010. HC-Pro hypo- and hypersuppressor mutants: differences in viral siRNA accumulation in vivo and siRNA binding activity in vitro. Archives of Virology, 155, 251–254. DOI:10.1007/s00705-009-0563-x

Tripathi, S., Bau, H.J., Chen, L.F. and Yeh, S.D. 2004. The ability of papaya ringspot virus strains overcoming the transgenic resistance of papaya conferred by the coat protein gene is not correlated with higher degrees of sequence divergence from the transgene. European Journal of Plant Pathology 110:871–882. doi:10.1007/s10658-004-0607-8

Tripathi, S., Suzuki, J.Y., Ferreira, S.A., and Gonsalves, D. 2008. Papaya ringspot virus - P: characteristics, pathogenicity, sequence variability and control. Molecular Plant Pathology, 9, 269-280.doi: 10.1111/j.1364-3703.2008.00467.x.

Tuo, D., Shen, W., Yan, P., Li, C., Gao, L., Li, X., Li, H., Zhou, P. 2013. Complete genome sequence of an isolate of papaya leaf distortion mosaic virus from commercialized PRSV-resistant transgenic papaya in China. Acta Virologica 57:452–455.

USDA/ARS. 2001. USDA nutrient data base for standard reference. Release 13.Nutrient Data Laboratory Home Page [online]. United States Department of Agriculture/Agricultural Research Service, 1999 [cited February 7, 2001]. Available from http:www.nal.usda.gov/fnic/foodcomp; accessed March 17, 2006.

Usharani, T.R., Laxmi, V., Jalali, S., Krishnareddy, M. 2013. Duplex PCR to detect both papaya ring spot virus and papaya leaf curl virus simultaneously from naturally infected papaya (*Carica papaya* L.). Indian Journal of Biotechnology. 12: 269–272

Valli, A., Lopez-Moya, J. J., and Garcia, J. A. 2007. Recombination and gene duplication in the evolutionary diversification of P1 proteins in the family Potyviridae. The Journal of General Virology 88:1016–1028. doi:10.1099/vir.0.82402-0.

Varma, A., Mandal, B., Singh, M.K. 2011. Global emergence and spread of whitefly (*Bemisia tabaci*) transmitted Geminiviruses.In: Thompson WMO (ed) The whitefly, *Bemisia tabaci* (Homoptera: Aleyrodidae) interaction with geminivirus-infected host plants. Springer, Netherlands, pp 205–292

Vela´zqueza, K., Renovella, A., Comellasa, M., Serraa, P., Garcı´ab, M.L., Pinaa, J.A., Navarroa, L., Morenoa, P. and Guerria, J. 2010. Effect of temperature on RNA silencing of a negative stranded RNA plant virus*:* Citrus psorosis virus. Plant Pathology. 59, 982–990.

Ventura, J.A., Costa, H., Tatagiba, J.S., Andrade, J.S., and Martins, D.S. 2003. Meleira do mamoeiro: Etiologia, sintomas e epidemiologia. InMartins DS (Ed.) Papaya Brasil: Qualidade do Mamão Para o Mercado Interno.; Incaper: Vitória, Brazil, pp 267–276.

Ventura, J.A., Costa, H., and da Silva Tatagiba, J. 2004. Papaya diseases and integrated control. In,Diseases of Fruits and Vegetables (pp. 201–268).Berlin/Heidelberg, Germany:Springer

Villegas, V.N., Magdalita, P.M., Valencia, L.D. and Ocampo, T.D. 2001. Development of transgenic papaya resistance to ringspot virus. Country progress report at the papaya Biotechnology Network of Southeast Asia held in Hanoi, Vietnam, 22–23 October 2001.

Wang, H. L. 1981. Aphid transmission of papaya ringspot virus in Taiwan. Plant Protection Bulletin, 23:229-233.

Wang, X.Y., Xie, Y., Zhou, X.P. 2004. Molecular characterization of two distinct begomoviruses from papaya in China. Virus Genes 29:303-309.

Wang, Y.N., Wu, B., Borth, W. B., Hamim, I., Green, J. C., Melzer, M.J., Hu, J. S. 2017. Molecular characterization and distribution of two strains of dasheen mosaic virus on Taro in Hawaii. Plant Dis. 101(12), 1980–1989. . https://doi.org/10.1094/PDIS-04-17-0516-RE.

Wang, Y.N, Borth, W.B, Green, J.C., Hamim, I, Cao, K., Hu, J.S. and Melzer, M.J. 2017. Genome characterization and distribution of taro bacilliform CH virus on taro in Hawaii, USA. European Journal of Plant Pathology 387:1-5.

Wang, Y., Shen, W., Wang, S., Tuo, D., Yan, P., Li, X., Zhou, P. 2013. Complete genomic sequence of papaya mosaic virus isolate from Hainan Island. China. Chinese Journal of Tropical Crops 34:297–300.

Wylie, S.J., Adams, M., Chalam, C., Kreuze, J., López-Moya, J.J., Ohshima, K., Praveen, S., Rabenstein, F., Stenger, D., Wang, A., and Zerbini, F.M. 2017. ICTV Report consortium ICTV virus taxonomy profile: potyviridae. Journal of General Virology 98:352–354.

Yeh, S.D. and Gonsalves, D. 1984. Evaluation of induced mutants of papaya ringspot virus for control by cross protection. Phytopathology 74(9) :1086-1091

Yeh, S.D., Gonsalves, D., Provvidenti, R. 1984. Comparative studies on host range and serology of papaya ringspot virus and watermelon mosaic virus 1. Phytopathology, 74:1081–1085.

Yeh, S. D., Jan, F. J., Chiang, C. H., Doong, T. J., Chen, M. C., Chung, P. H., et al. 1992. Complete nucleotide sequence and genetic organization of papaya ringspot virus RNA. The Journal of General Virology, 73: 2531–254. doi:10.1099/0022-1317-73-10-2531.

Yeh, S.D. and Gonsalves, D. 1994. Practices and perspectives of control of papaya ringspot virus by cross protection. Advance Disease Vector Research 10:237-257.

Yeh, S.D., Kung, Y.J., Bau, H.J., Yu, T.A., and Raja, J.A.J. 2010. Generation of a papaya hybrid variety with broad-spectrum transgenic resistance to papaya ringspot virus and papaya leaf-distortion mosaic virus. Transgenic Plant Journal.4 37–44.

Yeh, S.D., Raja, J., Kung, Y.-J., and Kositratana, W. 2014. Agbiotechnology: costs and benefits of genetically modified papaya. In N. K. Van Alfen (Ed.), Encyclopedia of agriculture and food systems. London, UK: Elsevier. doi: 10.1016/B978-0-444-52512-3.00252-7

Ye, C. and Li, H. 2010. 20 years of transgenic research in China for resistance papaya ringspot virus. Transgenic plant journal. Global science book. 4(1). 58-63.

Yue, F., Cui, S., Zhang, C., Yoon, K.J. 2009. A multiplex PCR for rapid and simultaneous detection of porcine circovirus type 2, porcine parvovirus, porcine pseudorabies virus, and porcine reproductive and respiratory syndrome virus in clinical specimens. Virus Genes 38:392–397.

Zhao, G., Yan, P., Shen, W., Tuo, D., Li, X., and Zhou, P. 2015. Complete genome sequence of papaya ringspot virus isolated from genetically modified papaya in Hainan island, China.Genome Announcements 3(5): e01056-15.doi: 10.1128 /genomeA. 01056- 15.

Zhang, Y, Yu, N., Huang, Q., Yin, G., Guo, A., Wang, X., Xiong, Z., and Liu, Z. 2014. Complete genome of Hainan papaya ringspot virus using small RNA deep sequencing. Virus Genes. 48, 502–508. doi:10.1007/s11262-014-1042-3.

Zimmerman, T. W., Joseph, L., St. Brice, N., and Kowalski, J. A. 2005. Development and selection for homozygous transgenic papaya seedling. In International Symposium on Papaya 740: 177-182.

Zhou, X., Xie, Y., Tao, X., Zhang, Z., Li, Z., and Fauquet, C.M. 2003. Characterization of DNAbeta associated with begomoviruses in China and evidence for coevolution with their cognate viral DNA-A. J Gen Virol. 84:237-247.