UNIVERSIDADE FEDERAL DO ESPÍRITO SANTO

CENTRO DE CIÊNCIAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

MARLONNI MAURASTONI ARAUJO

Dynamics of the papaya meleira virus complex during the development of papaya (*Carica papaya* L.)

VITÓRIA, ES

2021

MARLONNI MAURASTONI ARAUJO

Dynamics of the papaya meleira virus complex during the development of papaya (*Carica papaya* L.)

Tese apresentada ao Programa de Pós-Graduação em Biotecnologia do Centro de Ciências da Saúde da Universidade Federal do Espírito Santo, como requisito parcial para obtenção do título de Doutor em Biotecnologia.

Orientador: Prof. Dr. Patricia Machado Bueno Fernandes

VITÓRIA, ES

2021

Ficha catalográfica disponibilizada pelo Sistema Integrado de Bibliotecas - SIBI/UFES e elaborada pelo autor

---

ARAUJO, MARLONNI MAURASTONI, 1996-
A658d Dynamics of the papaya meleira virus complex during the development of papaya (Carica papaya L.) / MARLONNI MAURASTONI ARAUJO. - 2021.
173 f. : il.

Orientadora: PATRICIA MACHADO BUENO FERNANDES.
Tese (Doutorado em Biotecnologia) - Universidade Federal do Espírito Santo, Centro de Ciências da Saúde.

1. Totiviruses. 2. Proteína capsidial. 3. Interação proteína proteína. 4. Interação vírus-hospedeiro. 5. Meleira do mamoeiro. 6. Vetores de vírus. I. FERNANDES, PATRICIA MACHADO BUENO. II. Universidade Federal do Espírito Santo. Centro de Ciências da Saúde. III. Título.

CDU: 61

MARLONNI MAURASTONI ARAUJO

Dynamics of the papaya meleira virus complex during the development of papaya (*Carica papaya* L.)

Tese apresentada ao Programa de Pós-Graduação em Biotecnologia do Centro de Ciências da Saúde da Universidade Federal do Espírito Santo, como requisito parcial para obtenção do título de Doutor em Biotecnologia.

Apresentada em 11 de novembro de 2021.

Prof. Dr. Patricia Machado Bueno Fernandes
Universidade Federal do Espírito Santo
Orientadora

Prof. Dr. Antônio Alberto Ribeiro Fernandes
Universidade Federal do Espírito Santo

Prof. Dr. Anna Elizabeth Whitfield
North Carolina State University

Prof. Dr. Francisco Murilo Zerbini Junior
Universidade Federal de Viçosa

Prof. Dr. Francisco José Lima Aragão
Empresa Brasileira de Pesquisa Agropecuária – Recursos Genéticos e Biotecnologia

Prof. Dr. Silas Pessini Rodrigues
Universidade Federal do Rio de Janeiro

VITÓRIA, ES

2021

DEDICATION

Dedicated to everyone fighting against the COVID-19 pandemic using Science as a weapon against the SARS-CoV.

ACKNOWLEDGEMENTS

I would like to thank the Federal University of Espírito Santo (UFES) and the UFES Biotechnology postgraduation program for the resources and infrastructure provided to develop my work.

I would also like to thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for my scholarship and Fundação de Amparo à Pesquisa do Espírito Santo (FAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for funding.

I am grateful to all professors and professionals for their guidance and their institutions they belong.

In particular faculty, staff and students of UFES: P. M. B. Fernandes, A. A. R. Fernandes, J. A. Ventura, A. M. C. Santos, T. S. F. Antunes, O. F. Quadros, A. B. Vaz, A. C. Tosi, R. D. Moura, L. A. M. Castro, T. Carneiro, M. L. Silva, R. C. Pereira and F. Torres.

Federal University of Viçosa (UFV): F. M. Zerbini Jr. and A. F. Orílio;

Federal University of Rio de Janeiro (UFRJ): S. P. Rodrigues

EMBRAPA Genetic Resources and Biotechnology (CENARGEN): E. F. M. Abreu, F. J. L. Aragão, S. G. Ribeiro, D. M. A. Dusi. C. C. Lacorte, M. M. Sanches, A. Mehta, A. H. Vidal, H. M. Farias and Y. Santos.

University of Brasília: W. Fontes and N. M. F. S. Domingues

North Carolina State University: A. E. Whitfield, D. Rotenberg, C. A. D. Xavier, S. Kanakala, S. P. Rajarapu, J. Han, K. Lahre, Y. Chen, H. W. Teh, K. A. Shaw and E. Smith.

THESIS STRUCTURE

This thesis is presented in Scientific Article format. The lists of figures and tables contain the illustrations and tables presented in the papers in preparation for publication, as per ABNT. The item References contains the bibliographical references presented in the introduction of this thesis.

DINÂMICA DO COMPLEXO PAPAYA MELEIRA VIRUS DURANTE O DESENVOLVIMENTO DO MAMOEIRO (*Carica papaya* L.)

RESUMO

MAURASTONI, M. A. **Dinâmica do complexo papaya meleira virus durante o desenvolvimento do mamoeiro (*Carica papaya* L.)**. 2021. 173f. Tese de doutorado em Biotecnologia – Programa de Pós-graduação em Biotecnologia, UFES, Espírito Santo, Brasil.

A meleira do mamoeiro (PSD - do inglês, *papaya sticky disease*) está entre as doenças mais graves causadas por vírus e que afetam a produção de mamão. Essa doença foi relatada pela primeira vez no Brasil em 1993, associada a um vírus de RNA fita dupla, denominado papaya meleira virus (PMeV). Desde então, avançou-se no conhecimento da dispersão da doença no campo, na caracterização do agente etiológico e suas interações com o mamoeiro. Porém, em 2016, o papaya meleira virus 2 (PMeV2), com genoma de RNA fita simples de senso positivo, também foi identificado em plantas doentes, impondo um repensar no patossistema. Neste trabalho, avaliamos criticamente os achados dos últimos 30 anos para entender a dispersão da doença em campo. Mostramos que espécies de cigarrinhas e moscas-branca precisam ser melhor estudadas como potenciais vetores no Brasil uma vez que técnicas de diagnóstico molecular mais sensíveis estão disponíveis. Não obstante, desenvolvemos uma técnica de RT-PCR *multiplex* (mPCR) capaz de detectar ambos os vírus em uma única reação a partir de amostras de plantas em pré-florescimento, que é um método alternativo para o diagnóstico precoce de PSD. Mostramos também que os laticíferos da nervura central de folhas do mamoeiro doente são os locais preferenciais de infecção do PMeV e PMeV2. O capsídeo do PMeV é composto de dois polipeptídios principais com sequências sobrepostas, sendo que um fragmento central desses polipeptídios (aa 321-670) interage com a proteína ribossomal 50S L17 (RPL17), que especulamos como importante no acúmulo de ambos os vírus. Assim, esta tese discute a PSD em três esferas principais: biologia do agente etiológico e sua interação com o hospedeiro, a disseminação da doença no campo e o desenvolvimento de tecnologias para seu manejo.

Palavras-chave: Totiviruses. Proteína capsidial. Interação proteína-proteína. Interação vírus-hospedeiro. Meleira do mamoeiro. Vetores de vírus.

DYNAMICS OF THE PAPAYA MELEIRA VIRUS COMPLEX DURING THE DEVELOPMENT OF PAPAYA (Carica papaya L.)

ABSTRACT

MAURASTONI, M. A. **Dynamics of the papaya meleira virus complex during the development of papaya (*Carica papaya* L.)**. 2021. 173p. Thesis for the Degree of Ph.D. in Biotechnology – Postgraduation Biotechnology Programme, UFES, Espirito Santo. Brazil.

Among the most serious virus-incited diseases in papaya production is papaya sticky disease (PSD). This disease was first reported in Brazil in 1993, associated with a double-stranded RNA virus, called papaya meleira virus (PMeV). Since then, progress has been made in the knowledge of the disease dispersion in the field, the etiological agent characterization, and its interactions with papaya. However, in 2016, the papaya meleira virus 2 (PMeV2), with a positive single-stranded RNA genome, was also identified in diseased plants, imposing a rethinking of the pathosystem. Therefore, in this work, we critically evaluate the latest findings on PSD and the last 30 years of research done to understand its dispersion in the field. We show that leafhopper and whitefly species need to be better studied as potential vectors of the PSD-associated viruses in Brazil now that more sensitive molecular diagnostic techniques are available. Nevertheless, we developed a multiplex RT-PCR (mPCR) technique capable of detecting both viruses in a single reaction from pre-flowering plant samples, which is a useful tool for the early diagnosis of PSD. Here we show that laticifers of the main vein of papaya sticky diseased leaves are the preferential infection site of PMeV and PMeV2. We also show that the PMeV capsid is composed of two major polypeptides with overlapping sequences. A central fragment of these polypeptides (aa 321-670) interacts with the 50S ribosomal protein L17 (RPL17), which we speculate as an important player in virus accumulation. Overall, this thesis discusses PSD in three main spheres: the biology of the etiological agent and its interaction with the host, the spread of the disease in the field, and the development of technologies for its management.

Keywords: Totiviruses. Capsid protein. Protein-protein interaction. Virus-host interaction. Papaya sticky disease. Virus vectors.

LIST OF ABBREVIATIONS

3-AT	3-Amino-1,2,4-triazole
AAP	Acquisition access period
AD	Activation domain
BD	Binding domain
BiFC	Bimolecular fluorescence complementation
bp	Base pairs
CMV	Cucumber mosaic vírus
CP	Capsid protein
DAPI	4,6-diamino-2-fenil-indol
DDO	Double dropout
DDO/X/A	Double dropout plus X-alpha-gal and Aureobasidin A
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
FAO	Food and agriculture organization of the United Nations
GFP	Green fluorescent protein
MATV	maize-associated totivirus
MP	Movement protein

nm	Nanometer
nt	Nucleotide
ORF	Open reading frame)
PCR	Polymerase chain reaction
PMeV	papaya meleira virus
PMeV2	papaya meleira virus 2
PMeV-ES	papaya meleira virus Espírito Santo isolate
PMeV-Mx	papaya meleira virus Mexico isolate
PMeV-RN	papaya meleira virus Rio Grande do Norte isolate
PnVA	panax notoginseng virus A
PPI	Protein-protein interaction
PpVQ	papaya virus Q
PVX	Potato virus X
QDO	Quadruple dropout
QDO/X	Quadruple dropout plus X-alpha-gal
QDO/X/A	Quadruple dropout plus X-alpha-gal and Aureobasidin A
RdRp	RNA-dependent RNA polimerase
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RPL17	50S ribosomal protein L17
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction

SD	Synthetical defined
SDO	Single dropout
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
ssRNA	Single-stranded RNA
TMV	Tobacco mosaic virus
Y2H	Yeast two-hybrid
YFP	Yellow fluorescent protein

INDEX

GENERAL INTRODUCTION

Papaya (*Carica papaya* L.) has been widely cultivated in tropical and subtropical regions. In 2019, the world production of papaya reached approximately 13.7 million tons. In 2018 the gross value of production reached $4.9 billion, the highest yield since the 1990s (FAO, 2019). In 2019, Brazil was placed as the third-largest producer, after the Dominican Republic and India. In the same year, more than 44 thousand tons of papaya fruit were exported from Brazil, which placed the country as the third-largest exporter of fruit in the world, only after Guatemala and Mexico (FAO, 2019).

The main importers of Brazilian papaya, the United States and European countries, demand high-quality fruits, but the papaya crop is susceptible to pathogens which affect exportation-quality fruit yields. From 2007 to 2015 more than 5.4 million diseased plants were eliminated in Brazilian fields (IDAF, 2015). Part of the losses can be attributed to viral diseases, including papaya sticky disease (PSD), known to affect the yield and quality of fruit in Brazil (VENTURA *et al*., 2004), Mexico (PEREZ-BRITO *et al.*, 2012), and Australia (PATHANIA *et al.*, 2019). So far, the only method for disease control consists of rouging symptomatic plants (VENTURA *et al.*, 2004).

To discuss and advance on what we know about the disease, this thesis produced three research manuscripts and two review manuscripts, and a patent deposited at the *Instituto Nacional da Propriedade Intelectual* (INPI). The first manuscript summarized recent papers published regarding PSD, including its etiology, epidemiology, and its interaction with the *C. papaya* host at the molecular level, and is attached to the introduction section. The second manuscript localizes PMeV complex RNA in papaya leaf tissues and uses somatic embryogenesis as a non-laticifer tissue system to show that the PMeV complex preferentially accumulates in laticifer cells. The third manuscript characterizes the PMeV ORF1 by adding a new non-structural function of totiviruses coat protein which we speculate to be relevant during virus-host interaction. The fourth article was originated from the need to develop a technique for early diagnosis of PSD that would include the

recently discovered class 1 umbravirus-like associated RNA (ulaRNA) virus associated with diseased plants. The last manuscript is a synthesis of the work carried out over the last 30 years in an attempt to identify the PSD vector in Brazil, and the main findings in other countries.

REFERENCES

FAO. Food Outlook - Biannual Report on Global Food Markets. Rome, p. 164. 2019.

IDAF, I. D. D. A. E. F. D. E. S. Controle do mamão no Espírito Santo é apresentado em workshop sobre defesa vegetal. DOI: https://idaf.es.gov.br/controle-do-mamao-no-espirito-santo-e-apresen.

PATHANIA, N.; VALERIANA JUSTO; PABLITO MAGDALITA; FE DE LA CUEVA; LORNA HERRADURA; AIRA WAJE; ADELFA LOBRES; ARCANGEL CUETO; NATALIE DILLON; LYNTON VAWDREY; LOUISE HUCKS; DONNA CHAMBERS; GRACE SUN; CHEESMAN, J. Integrated disease management strategies for the productive, profitable and sustainable production of high quality papaya fruit in the southern Philippines and Australia (FR2019-89). ACIAR. Australia: Australian Centre for International Agricultural Research (ACIAR) 2019: 64 p. 2019.

PEREZ-BRITO, D.; TAPIA-TUSSELL, R.; CORTES-VELAZQUEZ, A.; QUIJANO-RAMAYO, A.; NEXTICAPAN-GARCEZ, A.; MARTÍN-MEX, R. First report of papaya meleira virus (PMeV) in Mexico. African Journal of Biotechnology, 11, p. 13564-13570, 2012.

VENTURA, J. A.; COSTA, H.; TATAGIBA, J. D. S. Papaya Diseases and Integrated Control. In: NAQVI, S. A. M. H. (Ed.). Diseases of Fruits and Vegetables: Volume II: Diagnosis and Management. Dordrecht: Springer Netherlands, 2004. p. 201-268.

This work will be presented in the format of a Scientific Article in five chapters. Thus, the items: Methods, Conclusions, and References will be presented in the chapters, according to the methods and references used in each chapter.

MANUSCRIPT #1. BATTLE OF THREE: THE CURIOUS CASE OF PAPAYA STICKY DISEASE

Paper published in the *Plant Disease journal* (ISSN 0191-2917; IF 4.438, 2021; Qualis A2 Biotecnologia, 2013-2016). https://doi.org/10.1094/PDIS-12-19-2622-FE

Battle of three: The curious case of papaya sticky disease

Tathiana F. Sá-Antunes[1], **Marlonni Maurastoni**[1], L. Johana Madroñero[1,2], Gabriela Fuentes[3], Jorge M. Santamaria[3], José Aires Ventura[4], Emanuel F. Abreu[5], A. Alberto R. Fernandes[1] and Patricia M. B. Fernandes[1]*

[1]Nucleo de Biotecnologia Universidade Federal do Espírito Santo, Av. Marechal Campos 1468, Vitória, Espírito Santo 29040-090, Brazil;
[2]Universidad El Bosque, Vicerrectoría de Investigaciones, Bogota, Colombia
[3]Centro de Investigación Científica de Yucatán, Calle 43 No. 130, Col. Chuburná de Hidalgo, 97200 Mérida, Yucatán, Mexico;
[4]Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural, Vitória 29050790, Espírito Santo, Brazil
[5]Embrapa Recursos Genéticos e Biotecnologia, PqEB W5 Norte, Brasília, DF, 70770-900, Brazil.
*****Corresponding author: P. M. B. Fernandes; E-mail: patricia.fernandes@ufes.br

ABSTRACT

Among the most serious virus-incited diseases in papaya production is papaya sticky disease (PSD). PSD concerns producers worldwide because the disease is extremely aggressive. As no resistant cultivar is available, several management strategies have been used in affected countries, such as selecting healthy seeds for planting, excluding the pathogen, and roguing. In the 1990s, a dsRNA virus, papaya meleira virus (PMeV), was identified in Brazil as the causal agent of PSD. However, in 2016 a second virus, papaya meleira virus 2 (PMeV2), with an ssRNA genome, was also identified in PSD plants. PMeV has been detected in asymptomatic plants, whereas all symptomatic plants contain both viruses. Viral RNAs are packaged separately in particles formed by the PMeV capsid protein. PSD also affects papaya plants in Mexico, Ecuador, and Australia. PMeV2-like viruses have been identified in the affected plants, but the partner virus(es) in these countries are still unknown. In Brazil, PMeV and PMeV2 reside in laticifers, stimulating latex exudation that results in the affected papaya fruit's sticky appearance. Genes modulated in plants affected by PSD include those involved in reactive oxygen species and salicylic acid signaling, proteasomal degradation, and photosynthesis, which are key components of plant defenses against the PMeV complex. However, complete activation of the defense response is impaired by the expression of negative effectors modulated by the virus. This review presents a summary of the current knowledge of the *Carica papaya*-PMeV complex interaction and disease management strategies.

Keywords: papaya meleira virus, papaya meleira virus 2, virus-host interactions, pre-flowering tolerance.

INTRODUCTION

World papaya production is concentrated in five countries (India, Brazil, Mexico, Indonesia, and the Dominican Republic), with global estimates of over 13 million tons in 2018 (FAOSTAT 2018). Production in India, the leading producer, is mainly destined for internal consumption. Brazil is the second-largest papaya producer, accounting for 1 million tons of world production (FAOSTAT 2018). Mexico plays a pivotal role as a key supplier to the USA, which is the largest import market. Approximately 80% of papayas in the USA originate from Mexico (FAO 2019). Although most of the papaya produced worldwide is consumed in the domestic markets, high levels of fruit export provide a significant source of income and employment year-round (FAO 2017).

Papaya diseases have diverse biotic and abiotic etiologies that affect the plant and fruit quality, causing severe economic losses. In the world's leading papaya production regions, the major diseases are caused by viruses. Although more than ten different virus species have been reported in papaya worldwide (Table 1), only three present a threat to papaya cultivation in Americas: *Papaya ringspot virus* (PRSV-P), *Papaya mosaic virus* (PapMV), and the papaya meleira virus complex, comprised of papaya meleira virus (PMeV) and papaya meleira virus 2 (PMeV2).

PRSV-P causes severe damage in the main papaya production areas of Brazil and Mexico with crop losses of up to 85%. PRSV-P is mainly transmitted by aphid species in a non-persistent manner (Wu et al. 2018). PapMV was first reported in 1962 in Florida, USA, and has spread to Bolivia, Peru, Venezuela, and Mexico (Varun et al. 2017). PRSV-P and PapMV mixed infections present a synergistic interaction that leads to increased virus accumulation and symptoms (Chávez-Calvillo et al. 2016; García-Viera et al. 2018).

Papaya sticky disease (PSD), first reported as early as 1980 in Brazil, reached Mexico in 2008 (Perez-Brito et al. 2012) and Australia in 2019 (Pathania et al. 2019). In recent years, research on the etiology of the disease (Abreu et al. 2015b; Antunes et al. 2016), transmission (García-Cámara et al. 2018; García-Cámara et al. 2019; Tapia-Tussell et

al. 2015), and the plant-virus interaction (Abreu et al. 2014; Madroñero et al. 2018; Soares et al. 2016) has led to significant advances in the understanding of the PSD pathosystem. Here, we review the key developments in the literature, propose an interaction map for the *Carica papaya*-PMeV complex, and summarize the current management strategies for PSD.

PAPAYA STICKY DISEASE: TWO VIRUSES, ONE DISEASE

In the northeast region of Brazil, in the late 1980s, papaya began to exhibit an exudation of fluid and aqueous latex (Nakagawa et al. 1987). This was credited to boron and calcium deficiencies as the symptoms were similar to those observed on plants with these abiotic stresses (Nakagawa et al. 1987). In 1989, epidemiological studies identified a biotic pattern, which was confirmed by the appearance of disease in healthy plants inoculated with latex from diseased plants (Rodrigues et al. 1989). The sticky appearance of infected papaya fruits after oxidation of the latex by exposure to the air led to the name papaya sticky disease ("*meleira*" in Portuguese) (Figure 1). In Brazil, PSD is currently distributed in the northeastern states of Ceará, Rio Grande do Norte, Pernambuco, Bahia, and Espírito Santo (Meissner Filho et al. 2017).

Transmission electron microscopy images showed isometric particles of approximately 42 nm in the laticifers of diseased plants (Kitajima et al. 1993) (Supplementary Fig. S1). The purification of those particles from papaya latex and subsequent inoculation on healthy papaya seedlings that later developed typical symptoms of PSD confirmed the causal agent as a virus (Maciel-Zambolim et al. 2003).

Initially, experiments to identify the causal agents involved nucleic acid extraction of the latex tapped from papaya plants with typical symptoms of PSD (Maciel-Zambolim et al. 2003; Rodrigues et al. 2005). Total RNA was analyzed by gel electrophoresis leading to

the visualization of two bands: a double-stranded RNA (dsRNA) band estimated to be either ~10 kb (Kitajima et al. 1993) or ~12 kb (Maciel-Zambolim et al. 2003), designated PMeV, and another then-unnoticed band of approximately 4.5 kb (Antunes et al. 2016).

PMeV dsRNA sequences were obtained from isolates from Espírito Santo (Antunes et al. 2016) and Rio Grande do Norte (Abreu et al. 2015a). Sequence alignment showed a high similarity between the PMeV isolates and totiviruses. This family includes viruses with a single-component dsRNA genome that infects fungi and protozoa and forms virions (Fauquet and Fargette 2005). PMeV dsRNA contains two open reading frames (ORFs) coding for a capsid protein (CP) and an RNA-dependent RNA polymerase (RdRp) (Figure 2A) (Abreu et al. 2015a; Antunes et al. 2016).

Initially regarded as a PMeV subgenomic RNA, the 4.5 kb RNA band is now recognized as a genomic RNA from a second virus, PMeV2, associated with PMeV in infected plants from Brazil. Sequence alignment showed a high similarity between PMeV2 and umbraviruses (Antunes et al. 2016). Umbraviruses are single-stranded RNA (ssRNA) viruses that do not encode a CP gene and, consequently, do not form conventional virus particles. Genome encapsidation and transmission requires an auxiliary virus, typically a polerovirus or an enamovirus. The hybrid virus particles, formed of the umbraviral RNA and the helper virus CP, are transmitted by the helper virus vector (Taliansky and Robinson 2003).

Antunes et al. (2016) showed, using degenerate primers targeting conserved domains of the CP gene from members of the *Luteoviridae* family, that there were no recognized poleroviruses, enamoviruses or luteoviruses in symptomatic papaya plants. Peptides obtained by mass spectrometry from viral particles containing PMeV2 RNA matched with the predicted amino acid sequence of PMeV ORF1. This indicates that hybrid virus particles are formed from PMeV CP and PMeV2 ssRNA, supporting the idea that PMeV is an auxiliary virus for PMeV2 (Antunes et al. 2016).

To the best of our knowledge, this is the first known case of an umbra-like virus associated with a totivirus and an early example in plants of a viral CP encapsidating viral ssRNA and dsRNA genomes (Figure 2B). A similar relationship was shown by Zhang et al. (2016)

in which the capsidless ssRNA mycovirus, yado-kari virus 1 (YkV1), using the CP of the dsRNA mycovirus yado-nushi virus 1, forms hybrid particles encasing the YkV1 RdRp, allowing replication as a dsRNA virus.

PMeV, but not PMeV2, can be detected in asymptomatic papaya plants, suggesting that this virus alone cannot induce PSD symptoms (Antunes et al. 2016). This is similar to persistent viruses, which induce little or no overt effects on their hosts and do not encode a movement protein (Roossinck 2013). Although PMeV systemically infects papaya plants, no PMeV movement protein was reported (Abreu et al. 2015a). PMeV localization in laticifers and the increased latex exudation and fluidity during PSD (Kitajima et al. 1993) could be used by the virus to move systemically throughout the plant. Another possibility is that PMeV replicates in meristematic cells, allowing it to infect all plant tissues.

PMeV2 does not infect papaya on its own and all papaya plants displaying typical PSD symptoms have a double infection by PMeV and PMeV2. The apparent requirement of both PMeV and PMeV2 for PSD symptoms led to a reconsideration of the disease etiology in Brazil (Antunes et al. 2016).

In Mexico, similar symptoms to those of PSD were observed in papaya cv. Maradol. Gel electrophoresis of total RNA extracted from fruit latex also displayed two bands at approximately 10 and 4.5 kb. The disease could also be transmitted through the latex of infected papaya to healthy papaya plants (Perez-Brito et al. 2012). Together, these findings indicated the same viral etiology for the Brazilian and Mexican diseases.

A cDNA library obtained from symptomatic plants identified an 1154 bp sequence, partially covering the genome of the Mexican isolate (PMeV-Mx), showed high similarity to an umbravirus found in Ecuador (papaya virus Q - PpVQ) (Quito-Avila et al. 2015) and PMeV2, but no similarity to PMeV. Only one ORF with the characteristic domains of an RdRp has been predicted for PpVQ, while both PMeV2 and PMeV-Mx have a putative uncharacterized ORF upstream to their RdRp (Figure 2B). In addition, primers based on the Mexican isolate sequence amplified fragments from both Brazilian and Mexican symptomatic plants, and the amplicons had 100% nucleotide identity (Zamudio-Moreno et al. 2015).

In 2019, PSD was officially reported for the first time in Queensland, Australia (Pathania et al. 2019). Although virus particles were observed in Australian plants with PSD symptoms, next-generation sequencing revealed only an umbra-like virus (PMeV2-Au) (Campbell 2018). Currently, the auxiliary virus for the Mexican and Australian isolates is unknown.

PAPAYA AND PMeV COMPLEX INTERACTION

During the interaction between viruses and plants, the virus hijacks host factors to complete its infection cycle and the plant responds with a complex multilayered immune defense. In PSD, the outcome of this interaction depends on the papaya's development stage. Papaya is susceptible to the PMeV complex, but infected plants remain asymptomatic for 6–8 months (Ventura et al. 2004). This phenomenon supports the idea that a tolerance mechanism in pre-flowering plants allows the co-existence of plant and virus without causing significant loss of vigor or fitness to their hosts (Madroñero et al. 2018). For this reason, several physiological, biochemical, structural, and molecular aspects have been investigated to elucidate the interaction between the PMeV complex and pre- and post-flowering papaya. We present a schematic view of this interaction in Figure 3.

PMeV complex effect on the laticifers

Alteration in the physical and chemical properties, and spontaneous exudation of papaya latex during PSD, suggested that the PMeV complex viruses could be directly involved in

PSD symptoms (Rodrigues et al. 2009). Papaya proteases are usually activated during latex exudation and contribute to latex viscosity, the clotting process, and antiviral defense (Rodrigues et al. 2009). In PSD-affected plants, the reduction of protease levels and activity seems to have an inhibitory effect on latex coagulation, thus increasing its fluidity which could facilitate its flow through laticifers and allow virus spread within the plant (Rodrigues et al. 2012). Additionally, the accumulation of H_2O_2, a systemic response elicitor (Rodrigues et al. 2009), could play a negative regulatory role in cysteine-protease activity, possibly by oxidizing and inactivating the active site of the enzyme. The negative modulation of papaya latex cysteine proteases could also be a viral strategy to delay the progression of programmed cell death (PCD) in laticifers (Solomon et al. 1999) and minimize virus particle degradation.

PMeV complex effect on the proteasome

The PMeV complex also has a role in other papaya tissues, including necrotic lesions on the leaf tip (Ventura et al. 2004). The global protein expression profile of PSD leaf tissues showed an accumulation of calreticulin, proteasome-related proteins such as 20S proteasome b subunit, and stress-response proteins such as pathogenesis-related (PR) proteins, endochitinase and PR-4, while proteins related to metabolism are down-regulated (Rodrigues et al. 2012), demonstrating a major investment in plant defense (El Moussaoui et al. 2001).

The involvement of the ubiquitin/26S proteasome system (UPS) in the signaling and regulation of plant–pathogen interactions has been described in several studies (Sorel et al. 2018). The UPS machinery contributes to antiviral immunity by degrading viral effectors but viruses can usurp the UPS machinery to target proteins that inhibit viral infection to the degradation pathway (Verchot 2016). The accumulation of proteasome-related proteins in PSD symptomatic papaya plants supports the idea that defense host

proteins, which are essential for the plant response against viral infection, are targeted for degradation (Rodrigues et al. 2011).

During PMeV complex infection, the levels of several microRNAs (miRNAs) involved in the modulation of genes related to the UPS system are reduced. This indicates that PMeV coopts the UPS system for its benefit, promoting virus replication, movement, and a rapid turnover of viral proteins. For example, structural proteins, generally produced in large amount in a short time, are not able to fold correctly leading to the formation of misfolded proteins which are targeted for degradation (Alcaide-Loridan and Jupin 2012). Thus, we suggest that the rapid turnover of viral proteins can favor viral infection since it maintains an ideal cellular environment for plant and virus coexistence (Abreu et al. 2014; Verchot 2016).

In another study, the global protein profile of field-grown PMeV-infected pre-flowering *C. papaya* plants exhibited low levels of 26S proteasome-related proteins (Soares et al. 2016), an opposite pattern to that previously observed for symptomatic plants (Rodrigues et al. 2011). Lower activity of the 20S and 19S proteasome increases the levels of polyubiquitinated proteins that result in increased PCD, which could successfully contain a virus infection (Rodrigues et al. 2011). However, the full spectrum of anti-viral defenses involves the activity of caspase-like serine proteases (Citovsky et al. 2009; Rodrigues et al. 2011), such as subtilase 1.3, whose levels are reduced in PMeV-infected pre-flowering *C. papaya* tissues.

PMeV complex effect on redox balance and defense gene expression

Photosynthesis light-dependent reactions are important in plant responses against viruses and disturbances in this process favor viral accumulation (Soares et al. 2016). A higher electron flow ratio induced by the accumulation of photosynthesis-related proteins

in pre-flowering infected papaya (Soares et al. 2016) promotes a cascade of events in chloroplasts leading to reactive oxygen species (ROS) production. Low ROS levels induce antioxidant enzymes; however, when the ROS levels reach a certain threshold, a signal transduction pathway is activated that eventually leads to PCD (Solomon et al. 1999). ROS-related metabolic changes occur in *C. papaya*-PMeV complex interactions, including an increase in H_2O_2 production in the laticifers (Rodrigues et al. 2009). Moreover, pre-flowering *C. papaya* leaves treated with nitric oxide (NO) show an accumulation of compounds used for defense and an increased activity of detoxification enzymes (Buss et al. 2011). Supporting these data, several genes coding for ROS-detoxifying enzymes in PMeV complex-infected plants are up-regulated at pre-flowering (Madroñero et al. 2018). Although defense mechanisms such as ROS-signaling features are present at an early stage of infection, this is not enough to mediate resistance.

ROS have signaling effects in the chloroplast itself and other parts of the cell, often involving hormonal cross-talk that regulates the activation of defenses in plants, especially salicylic acid (SA) (Xia et al. 2015). At pre-flowering, *PR1, PR2, PR5*, and other genes involved in SA signaling are up-regulated. Moreover, the exogenous application of SA on pre-flowering plants before virus inoculation results in a trend of diminished viral load (Madroñero et al. 2018). These insights indicate the existence of defense mechanisms at pre-flowering, which could hamper the development of PSD symptoms. However, other genes known for their antagonistic roles in SA signaling, such as genes involved in ethylene metabolism and the NPR1-inhibitor, are also up-regulated, which could prevent full-scale and durable resistance. At post-flowering, the *PR1* gene is down-regulated and negative modulators of SA signaling are up-regulated (Madroñero et al. 2018). Together, the development of symptoms during post-flowering implies an incomplete activation of defense response mechanisms upon PMeV complex infection.

PMeV complex effect on cell wall structure

Spread of the PMeV complex could also be facilitated by changes in structure and composition of the papaya cell wall during the switch to the flowering stage. At pre-flowering, infected plants show modulation of transcripts coding for cell wall remodeling and structural proteins that may be part of the papaya response to hamper PMeV complex traffic (Madroñero et al. 2018; Soares et al. 2016). At post-flowering, in contrast, cell wall genes are induced in infected plants, which indicates that the PMeV complex could be inducing cell wall turnover at the plasmodesmata site to promote systemic viral infection. Analysis of the topography and mechanical properties of papaya leaves infected by PSD show that their midribs are fragile and susceptible to breakage (Magaña-Álvarez et al. 2016), which suggests a weakening in the cell walls of leaf tissues that could extend to laticifers. Cell rupture and latex exudation, the main symptoms in PSD plants, could be explained by cell wall turnover associated with increased water content and internal pressure.

EPIDEMIOLOGY

The epidemiological behavior of PSD has been extensively studied (Abreu et al. 2015a; Rodrigues et al. 1989; Tapia-Tussell et al. 2015; Ventura et al. 2003). Environmental factors and agricultural practices have been the main factors responsible for the disease progression and appearance of symptoms in the field, which may vary according to the source of virus inoculum—seed, alternative hosts, or vectors—and papaya variety.

Until 2012 most studies on PSD were based on virus detection through the visualization of the viral dsRNA band in agarose gel electrophoresis. The sequencing of both PMeV and PMeV2 (Antunes et al. 2016) allowed the development of more sensitive techniques such as RT-PCR (Abreu et al. 2012; Antunes et al. 2016; Maurastoni et al. 2020) and

qRT-PCR (Abreu et al. 2012), which have been applied to understand key aspects of PSD epidemiology.

Observations of PSD dispersal patterns in orchards pointed to insects as PMeV complex vectors. In a study conducted in Brazil, after exposure to whiteflies (*Bemisia tabaci* type B) that fed on infected plants, asymptomatic papaya plants developed PSD symptoms and PMeV dsRNA was detected. However, the virus was not detected in these whiteflies, which are not a papaya pest (Vidal et al. 2003). Several whiteflies can transmit viruses in a non-propagative manner in which viruses are not internalized inside the insect cells. Viral retention time in the insect's body depends on the virus half-life and viral load is often low (Whitfield et al. 2015). Therefore, the absence of PMeV dsRNA in *B. tabaci* type B does not exclude an ability to transmit the PMeV complex.

A whitefly commonly found in papaya orchards and considered a pest to Brazilian papaya is *Trialeurodes variabilis.* This whitefly appears unable to transmit viral dsRNA from inoculated plants to healthy plants (Rodrigues et al. 2009). The dsRNA was detected in adults and nymphs but not in latex collected from plants 20 days after being exposed to the viruliferous whiteflies. However, the ability of *T. variabilis* to vector the PMeV complex cannot be ruled out, as the time required for dsRNA visualization may be longer than that analyzed.

Recently, leafhoppers (Hemiptera,: Cicadellidae) were proposed as a potential PMeV complex insect vector in Brazil as their distribution in the crop is correlated with the distribution of the disease (Gouvea et al. 2018). In Mexico, *Empoasca papayae* Oman (Hemiptera: Cicadellidae) adults, but not nymphs, have been shown to transmit PMeV-Mx to *C. papaya* cv. Maradol. These leafhoppers can acquire the virus after 6 hours exposure to infected plants and viral titer increases with longer exposure time (up to 5 days). Little is known about the biology of *E. papayae* and research is now focused on understanding the behavior of this insect in the field (García-Cámara et al. 2019). In Brazil, transmission experiments to study leafhoppers' ability to vector PMeV complex are necessary to implement adequate management strategies.

Identification of alternative hosts for the PMeV complex is also essential for the development of control strategies. In Brazil, several plants were tested for their susceptibility to PMeV infection, but the dsRNA was detected only in *Brachiaria decumbens* (*Poaceae*) (Maciel-Zambolim et al. 2003), which is commonly found close to papaya plantations. In Mexico, intercropping between watermelon (*Citrullus lanatus* Thunb.) and papaya led to the hypothesis that watermelon could be an alternative host for PMeV-Mx. Indeed, PMeV-Mx can replicate in watermelon seedlings and, more surprisingly, induce necrotic lesions on the leaf tip, a typical PSD symptom (García-Cámara et al. 2018). The alternative hosts proposed by Maciel-Zambolim et al. (2003) need to be revisited using more sensitive techniques, with additional detection for PMeV2. *B. decumbens* and *C. lanatus* Thunb. also need to be assessed as viral reservoirs.

Understanding PSD etiology, dynamics of viral populations, and transmission are imperative for integrated management of papaya production. Since PSD symptoms appear only after flowering, an infected asymptomatic plant may remain an undetected virus source for months before being eradicated. Thus, developing an efficient strategy for virus control remains a challenge.

MANAGEMENT OF PAPAYA VIRUS DISEASES

The papaya crop has experienced significant improvements through the use of innovative technologies (Costa et al. 2019). However, there are significant challenges, necessitating quality- and genetically-certified seeds and cultivars, and most of all, resistance to major crop diseases (Ventura et al. 2019). Several strategies have been recommended for papaya virus control. The major control strategies applicable to all papaya viruses are the following:

1. Use of healthy seeds and exclusion of the pathogens by seedling/crop certification.

PSD has been reported as a seed-borne disease in Mexico and Australia, and the use of healthy seeds in new plantations has been recommended (Tapia-Tussell et al. 2015). The Australian government initiated a program to produce clean seeds (Campbell 2019b) using embryo rescue and tissue culture, which was able to produce 98% PMeV2-free plants (Campbell 2019a).

A seed transmission route has not yet been confirmed for the PMeV complex in Brazil. However, measures have been adopted by Brazilian farmers to mitigate the possible dispersion of PSD and other viral diseases: (i) the use of certified seedlings in the establishment of new orchards; (ii) the use of seeds from plants that have been grown under conditions that will prevent infection; (iii) the establishment of nurseries and orchards as far as possible from other orchards especially if viral diseases had been reported in the region; and (iv) exclusion of the pathogens by crop quarantine.

2. Control of pathogen by eradication (roguing) of infected plants.

Viruses can spread both within and between orchards. Growers and field workers must learn to identify the early symptoms of viral diseases. The best way to manage the virus is the prompt identification and immediate destruction of diseased trees, as delays enable

the virus to spread to additional trees (Ventura et al. 2003). Additionally, the possibility of virus spread from asymptomatic papaya implies a need for additional disease management strategies such as early detection of the PMeV complex (Maurastoni et al. 2020).

In Brazil, roguing of papaya is governed by Normative Instruction number 17, May 27th, 2010. Weekly inspections are performed throughout the crop, and plants with PSD symptoms are removed (Figure 4A) (Ventura et al. 2004). From 2011 to 2014 more than 4.9 million plants were eradicated in the largest papaya exporting state in Brazil, preventing the spread of various diseases to healthy orchards (Figure 4) (Fernandes et al. 2018).

3. Control of pathogen vectors.

Several viral pathogens of papaya have insect vectors and their control plays an important role in the management of papaya diseases. Efficacy of insecticide application is determined by the manner of transmission, vector population dynamics, and vector host range (Perring et al. 1999). In non-circulative transmitted viruses such as PRSV-P, the use of insecticides results only in reduction of the populations of potential vectors, without preventing the transmission. While the insect which vectors PSD-associated viruses is still unknown in Brazil, Mexico, and Australia, measures used to control the PRSV-P vectors have also been adopted for PSD control. An insecticide control strategy can be enhanced by using additional control measures, such as the elimination of weeds before the major peak of aphid flights, which could prevent the vector from acquiring virus from reservoir plants, disrupting the virus cycling.

4. Selection of tolerant and/or resistant cultivars.

The development of resistant papaya cultivars has been recognized as the most effective strategy for virus control. Since *C. papaya* cultivars are susceptible to several viruses, breeders have been seeking sources of resistance in other species within the family *Caricaceae*. Resistance to PRSV-P has been found in the genus *Vasconcellea* but its use in conventional breeding has been hampered by sexual incompatibility between species (Haireen and Drew 2014; Lin et al. 2019). No resistance to the PMeV complex has yet been found in thirty *C. papaya* genotypes or in non-cultivated plants (Meissner Filho et al. 2017).

Expression of pathogen-derived genes can interfere with the virus cycle in the host plants, inhibiting viral infection. So far, PRSV-resistant transgenic papaya have been developed based on a sequence homology-dependent strategy (Azad et al. 2014; Jia et al. 2017) which requires knowledge of virus diversity for its success. Transgenic resistance to PRSV-P has already been broken due to the emergence of divergent virus strains (Jia et al. 2017). To date, two PMeV isolates have been identified in Brazil (Abreu et al. 2015a; Antunes et al. 2016). Although their CPs share 75% similarity, studies with more isolates are required to understand PMeV diversity so that unlike PRSV, the PMeV complex will not overcome transgenic resistance.

5. Control of alternative hosts.

The possibility of papaya virus spread from alternative hosts necessitates management strategies for weed control (Alcalá-Briseño et al. 2020). Pathogen emergence results from interactions between susceptible hosts and pathogenic viruses in conducive

environments, causing disease outbreaks in new geographic regions or hosts (Alcalá-Briseño et al. 2020). In addition to the removal of weeds that grow close to papaya plants, special attention should be given to plants that are confirmed as a reservoir for the PMeV complex in Brazil and Mexico.

6. Cleaning harvesting tools.

Spatial distribution of the PMeV complex was studied in experimental plots over a year, and a high percentage of infected plants (~78%) per row during and after the harvest pointed to mechanical transmission (Abreu et al. 2015b; Ventura et al. 2003). This implies that agricultural practices, including fruit thinning, may also be responsible for the spread of PSD within orchards.

In orchards where appropriate agricultural practices were not carried out, the disease spread to the whole crop and led to total yield loss. In contrast, in orchards that strictly followed these principles including a weekly plant eradication based on the presence of initial PSD symptoms, the incidence of eradicated plants was less than 5% during the crop cycle (Ventura et al. 2004; Ventura et al. 2019).

CLOSING REMARKS AND FUTURE PROSPECTS

Over the last decade, much has been learned about PSD including details of the etiology, epidemiology, defense mechanisms of papaya against the PMeV complex, and disease

management strategies. In the 1990s, the causal agent of PSD was identified as a dsRNA virus, PMeV, but in 2016 a second virus, PMeV2, with an ssRNA genome, was also discovered in PSD plants. All PSD symptomatic papaya plants are infected by both viruses; however, asymptomatic plants analyzed in Brazil were positive only for PMeV. Thus, evidence suggests that contrary to what was originally believed, PMeV is not the etiological agent of PSD. Interestingly, in countries other than Brazil where PSD has been reported, only the umbra-like virus has been identified. Thus, it is possible that a different helper virus may be identified in these countries.

An intriguing question has been why infected plants remain asymptomatic until flowering. Proteomic and transcriptomic data have increased our understanding of the interaction between papaya and the PMeV complex. The infected papaya plant mounts an anti-viral defense mechanism during pre-flowering, when several genes related to the SA pathway and other defense pathways are highly expressed. After flowering, however, increased expression of genes that negatively regulate SA production leads to depression in defense responses.

The susceptibility of papaya plants to viruses results in economic and environmental impact as it increases the use of agrochemical products and water resources, without achieving the predicted crop yield. PSD may affect 20% of the plants during the economic cycle of the crop in orchards where roguing is performed, but it affects up to 100% of the plants where phytosanitary protocols are not implemented, causing a total yield loss (Abreu et al. 2015a).

The development of papaya plants resistant to viruses is urgently needed. Plants challenged by viral RNA initiate defense responses based upon RNA silencing, and this strategy was used to develop virus-resistant crops (Lindbo and Falk 2017). Resistance to infection against a range of individual viruses has been engineered into several plant species. Because of the durability and success of transgenic papaya cultivars in Hawaii (USA), similar transgenic papaya plants have been engineered in other countries. Resistance, however, was inconsistent for many reasons, such as the emergence of

recombinant strains, increased strength of viral silencing suppressors, and unfavorable environmental conditions.

Brazilian researchers have been working on the development of resistant papaya to combat PSD. Until 2016, PSD was associated only with PMeV, and all efforts to obtain a resistant plant considered only this virus. Now, groups working towards this goal must consider both viruses, as well as the defense mechanisms activated in papaya-PMeV/PMeV2 interactions reviewed in the present paper, if a breeding program to develop a PSD resistant papaya plant is to succeed.

ACKNOWLEDGEMENTS

Author Contributions: All the authors have equal contribution. All authors read and approved the manuscript.

Acknowledgments: Tathiana F. Sá Antunes and L. Johana Madronero would like to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES (Ministry of Education of Brazil), and Marlonni Maurastoni thanks the Fundação de Amparo à Pesquisa do Espírito Santo, FAPES for their fellowship. P.M.B. Fernandes and J.A. Ventura acknowledge the Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq for their research productivity award (grants # 303432/2018-7 and # 308631/2016-1). This work was supported by the Fundação de Amparo à Pesquisa do Estado do Espírito Santo (FAPES) grants # 76437906/16 and # 80598609/17. Thanks are due to Dr. Raul Tapia for providing the papaya Maradol picture presented in Fig.1.

Conflicts of Interest: The authors declare that they have no conflict of interest.

REFERENCES

Abreu, E. F. M., Daltro, C. B., Nogueira, E. O., Andrade, E. C., and Aragão, F. J. 2015a. Sequence and genome organization of papaya meleira virus infecting papaya in Brazil. Arch. Virol. 160:3143-3147.

Abreu, P., Antunes, T., Magaña-Álvarez, A., Pérez-Brito, D., Tapia-Tussell, R., Ventura, J., Fernandes, A., and Fernandes, P. 2015b. A current overview of the Papaya meleira virus, an unusual plant virus. Viruses 7:1853-1870.

Abreu, P. M. V., Piccin, J. G., Rodrigues, S. P., Buss, D. S., Ventura, J. A., and Fernandes, P. M. B. 2012. Molecular diagnosis of Papaya meleira virus (PMeV) from leaf samples of *Carica papaya* L. using conventional and real-time RT-PCR. J. Virol. Methods 180:11-17.

Abreu, P. M. V., Gaspar, C. G., Buss, D. S., Ventura, J. A., Ferreira, P. C. G., and Fernandes, P. M. B. 2014. *Carica papaya* microRNAs are responsive to Papaya meleira virus infection. PLoS One 9.

Alcaide-Loridan, C., and Jupin, I. 2012. Ubiquitin and Plant Viruses, Let's Play Together! Plant Physiol. 160:72-82.

Alcalá-Briseño, R. I., Casarrubias-Castillo, K., López-Ley, D., Garrett, K. A., and Silva-Rosales, L. 2020. Network Analysis of the Papaya Orchard Virome from Two Agroecological Regions of Chiapas, Mexico. mSystems 5:e00423-00419.

Antunes, T. F. S., Amaral, R. J. V., Ventura, J. A., Godinho, M. T., Amaral, J. G., Souza, F. O., Zerbini, P. A., Zerbini, F. M., and Fernandes, P. M. B. 2016. The dsRNA Virus Papaya Meleira Virus and an ssRNA Virus are Associated with Papaya Sticky Disease. PloS One 11:e0155240.

Azad, M. A. K., Amin, L., and Sidik, N. M. 2014. Gene Technology for Papaya Ringspot Virus Disease Management. Sci. World. J. 2014:768038.

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.

Buss, D. S., Dias, G. B., Santos, M. P., Ventura, J. A., and Fernandes, P. M. B. 2011. Oxidative Stress Defence Response of *Carica papaya* Challenged by Nitric Oxide, Papaya meleira virus and *Saccharomyces cerevisiae*. Open Nitric Oxide J. 3:55-64.

Campbell, P. 2018. Papaya Press. Page 1 in: New test to offer early detection of Papaya Sticky Disease G. Kath, ed., Australia.

Campbell, P. 2019a. Papaya Press. Page 1 in: Papaya clean seed update G. Kath, ed., Australia.

Campbell, P. 2019b. Papaya Press. Page 1 in: Papaya Clean Seed Program Underway, G. Kath, ed., Australia.

Chávez-Calvillo, G., Contreras-Paredes, C. A., Mora-Macias, J., Noa-Carrazana, J. C., Serrano-Rubio, A. A., Dinkova, T. D., Carrillo-Tripp, M., and Silva-Rosales, L. 2016. Antagonism or synergism between papaya ringspot virus and papaya mosaic virus in *Carica papaya* is determined by their order of infection. Virology 489:179-191.

Citovsky, V., Zaltsman, A., Kozlovsky, S. V., Gafni, Y., and Krichevsky, A. 2009. Proteasomal degradation in plant-pathogen interactions. Semin. Cell Dev. Biol. 20:1048-1054.

Costa, A. d. F. S. d., Abreu, E. F. M., Schmildt, E. R., Costa, A. N. d., and Schmildt, O. 2019. Advances observed in papaya tree propagation. Rev. Bras. Frutic. 41.

El Moussaoui, A., Nijs, M., Paul, C., Wintjens, R., Vincentelli, J., Azarkan, M., and Looze, Y. 2001. Revisiting the enzymes stored in the laticifers of *Carica papaya* in the context of their possible participation in the plant defence mechanism. Cell. Mol. Life Sci. 58:556-570.

FAO. 2017. Food Outlook - Biannual Report on Global Food Markets. Rome.

FAO. 2019. Food Outlook - Biannual Report on Global Food Markets. Page 164, Rome.

FAOSTAT. 2014. Food and Agriculture Organization of the United Nations. FAO, Rome, Italy.

Fauquet, C., and Fargette, D. 2005. International Committee on Taxonomy of Viruses and the 3,142 unassigned species. Virol. J. 2:64.

Fernandes, P. M. B., Fernandes, A. A. R., Ventura, J. A., Antunes, T. F. S., Quadros, O. F., Madroñero, J., Carminati, L. S., and Maurastoni, M. 2018. Um novo complexo viral: o caso da meleira do mamoeiro. in: VII Simpósio do Papaya Brasileiro. Produção e Sustentabilidade, Vitória-Espírito Santo, Brazil.

García-Cámara, I., Pérez-Brito, D., Moreno-Valenzuela, O., Magaña-Álvarez, A., Fernandes, P. M. B., and Tapia-Tussell, R. 2018. Molecular and experimental evidence of Watermelon (*Citrullus lanatus*) as host of the Mexican variant of Papaya meleira virus. Eur. J. Plant Pathol. 151:117-123.

García-Cámara, I., Tapia-Tussell, R., Magaña-Álvarez, A., Cortés Velázquez, A., Martín-Mex, R., Moreno-Valenzuela, O., and Pérez-Brito, D. 2019. *Empoasca papayae* (Hemiptera: Cicadellidae)-Mediated Transmission of Papaya Meleira Virus-Mexican Variant in Mexico. Plant Dis. 103:2015-2023.

García-Viera, M., Sánchez-Segura, L., Chavez-Calvillo, G., Jarquín-Rosales, D., and Silva-Rosales, L. 2018. Changes in leaf tissue of *Carica papaya* during single and mixed infections with *Papaya ringspot virus* and *Papaya mosaic virus*. Biol. Plant. 62:173-180.

Gouvea, R., da Vitória, R., Rosa, R., Alves, W. d. S., Giuriatto, N., Calatroni, D., Fanton, C., Martins, D. d. S., and Queiroz, R. 2018. Flutuação populacional de cigarrinhas (Hemiptera: cicadellidae) e ocorrência do vírus da meleira do mamoeiro. in: VII Simpósio do Papaya Brasileiro. Produção e Sustentabilidade, Vitória-Espírito Santo, Brazil.

Haireen, M. R. R., and Drew, R. A. 2014. Isolation and Characterisation of PRSV-P Resistance Genes in *Carica* and *Vasconcellea*. Int. J. Genomics 2014:145403-145403.

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. Sci. Rep. 7:12636.

Kitajima, E. W., Rodrigues, C. H., Silveira, J. S., and Alves, F. 1993. Association of isometric viruslike particles, restricted to lacticifers, with "meleira" (sticky disease) of papaya (*Carica papaya*). Fitopatol. Bras. 18:118.

Lin, Q., Singh, R., and Yu, Q. 2019. Isolation and characterization of translation initiation factor 4E in *Carica papaya* and *Vasconcellea species*. Pages 39-44 International Society for Horticultural Science (ISHS), Leuven, Belgium.

Lindbo, J. A., and Falk, B. W. 2017. The Impact of "Coat Protein-Mediated Virus Resistance" in Applied Plant Pathology and Basic Research. Phytopathology 107:624-634.

Maciel-Zambolim, E., Kunieda-Alonso, S., Matsuoka, K., De Carvalho, M., and Zerbini, F. 2003. Purification and some properties of Papaya meleira virus, a novel virus infecting papayas in Brazil. Plant Pathol. 52:389-394.

Madroñero, J., Rodrigues, S. P., Antunes, T. F. S., Abreu, P. M. V., Ventura, J. A., Fernandes, A. A. R., and Fernandes, P. M. B. 2018. Transcriptome analysis provides insights into the delayed sticky disease symptoms in *Carica papaya*. Plant Cell Rep. 37:967-980.

Magaña-Álvarez, A., Vencioneck Dutra, J. C., Carneiro, T., Pérez-Brito, D., Tapia-Tussell, R., Ventura, J. A., Higuera-Ciapara, I., Fernandes, P. M. B., and Fernandes, A. A. R. 2016. Physical Characteristics of the Leaves and Latex of Papaya Plants Infected with the Papaya meleira virus. Int. J. Mol. Sci. 17:574.

Maurastoni, M., Sá-Antunes, T. F., Oliveira, S. A., Santos, A. M. C., Ventura, J. A., and Fernandes, P. M. B. 2020. A multiplex RT-PCR method to detect papaya meleira virus complex in adult pre-flowering plants. Arch. Virol. 165:1211-1214.

Meissner Filho, P., Lima Neto, F., de Oliveira, C., Santana, S., and Dantas, J. 2017. Avaliação da resistência de genótipos de mamoeiro ao vírus da meleira no Semiárido. Report No. 1809-5003. Page 21. Embrapa Mandioca e Fruticultura, Cruz das Almas, BA.

Nakagawa, J., Takayama, Y., and Suzukama, Y. 1987. Exudação de látex pelo mamoeiro. Estudo de ocorrência em Teixeira de Freitas, BA. Pages 555-559 in: Congresso Brasileiro de Fruticultura.

Pathania, N., Valeriana Justo, Pablito Magdalita, Fe de la Cueva, Lorna Herradura, Aira Waje, Adelfa Lobres, Arcangel Cueto, Natalie Dillon, Lynton Vawdrey, Louise Hucks, Donna Chambers, Grace Sun, and Cheesman, J. 2019. Integrated disease management strategies for the productive, profitable and sustainable production of high quality papaya fruit in the southern Philippines and Australia (FR2019-89). Page 64, ACIAR, ed. Australian Centre for International Agricultural Research (ACIAR) 2019, Australia.

Perez-Brito, D., Tapia-Tussell, R., Cortes-Velazquez, A., Quijano-Ramayo, A., Nexticapan-Garcez, A., and Martín-Mex, R. 2012. First report of papaya meleira virus (PMeV) in Mexico. Afr. J. Biotechnol. 11:13564-13570.

Perring, T. M., Gruenhagen, N. M., and Farrar, C. A. 1999. Management of plant viral diseases through chemical control of insect vectors. Annu. Rev. Entomol. 44:457-481.

Quito-Avila, D. F., Alvarez, R. A., Ibarra, M. A., and Martin, R. R. 2015. Detection and partial genome sequence of a new umbra-like virus of papaya discovered in Ecuador. Eur. J. Plant Pathol. 143:199-204.

Rodrigues, C., Ventura, J., and Maffia, L. 1989. Distribuição e transmissão da meleira em pomares de mamão no Espírito Santo. Fitopatol. Bras. 14:118.

Rodrigues, S., Andrade, J., Ventura, J., Lindsey, G., and Fernandes, P. 2009. Papaya meleira virus is neither transmitted by infection at wound sites nor by the whitefly *Trialeurodes variabilis*. J. Plant Pathol. 91:87-91.

Rodrigues, S. P., Galvao, O. P., Andrade, J. S., Ventura, J. A., and Fernandes, P. M. B. 2005. Simplified molecular method for the diagnosis of Papaya meleira virus in papaya latex and tissues. Summa Phytopathol 31:281-283.

Rodrigues, S. P., Ventura, J. A., Aguilar, C., Nakayasu, E. S., Almeida, I. C., Fernandes, P., and Zingali, R. B. 2011. Proteomic analysis of papaya (*Carica papaya* L.) displaying typical sticky disease symptoms. Proteomics 11:2592-2602.

Rodrigues, S. P., Ventura, J. A., Aguilar, C., Nakayasu, E. S., Choi, H., Sobreira, T. J. P., Nohara, L. L., Wermelinger, L. S., Almeida, I. C., Zingali, R. B., and Fernandes, P. M. B. 2012. Label-free quantitative proteomics reveals differentially regulated proteins in the latex of sticky diseased *Carica papaya* L. plants. J. Proteomics 75:3191-3198.

Roossinck, M. J. 2013. Plant virus ecology. PLoS Pathog 9:e1003304.

Soares, E., Werth, E. G., Madroñero, L. J., Ventura, J. A., Rodrigues, S. P., Hicks, L. M., and Fernandes, P. M. B. 2016. Label-free quantitative proteomic analysis of pre-flowering PMeV-infected *Carica papaya* L. J. Proteomics 151:275-283.

Solomon, M., Belenghi, B., Delledonne, M., Menachem, E., and Levine, A. 1999. The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. Plant Cell 11:431-444.

Sorel, M., Mooney, B., de Marchi, R., and Graciet, E. 2018. Ubiquitin/Proteasome System in Plant Pathogen Responses. Annu. Plant Rev. online 2:65-116.

Taliansky, M. E., and Robinson, D. J. 2003. Molecular biology of umbraviruses: phantom warriors. J. Gen. Virol. 84:1951-1960.

Tapia-Tussell, R., Magaña-Alvarez, A., Cortes-Velazquez, A., Itza-Kuk, G., Nexticapan-Garcez, A., Quijano-Ramayo, A., Martin-Mex, R., and Perez-Brito, D. 2015. Seed transmission of Papaya meleira virus in papaya (*Carica papaya*) cv. Maradol. Plant Pathol. 64:272-275.

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

Varun, P., Ranade, S. A., and Saxena, S. 2017. A molecular insight into papaya leaf curl—a severe viral disease. Protoplasma 254:2055-2070.

Ventura, J. A., Costa, H., and da Silva Tatagiba, J. 2004. Papaya diseases and integrated control. Pages 201-268 in: Diseases of Fruits and Vegetables: Volume II. Springer.

Ventura, J. A., Costa, H., Tatagiba, J. d. S., Andrade, J. d. S., and Martins, D. d. S. 2003. Meleira do mamoeiro: Etiologia, sintomas e epidemiologia. Pages 267-276 in: Papaya Brasil: Qualidade do mamão para o mercado interno, Vitória-Espírito Santo, Brazil.

Ventura, J. A., Lima, I. d. M., Martins, M. V. V., Culik, M. P., and Costa, H. 2019. Impact and management of diseases in the propagation of fruit plants. Rev. Bras. Frutic. 41:e-647.

Verchot, J. 2016. Plant Virus Infection and the Ubiquitin Proteasome Machinery: Arms Race along the Endoplasmic Reticulum. Viruses 8:314.

Vidal, C., Nascimento, A., and Habibe, T. 2003. Transmissão do vírus da meleira do mamoeiro por insetos. Pages 612-615 in: Papaya Brasil: Qualidade do Mamão Para o Mercado Interno, Vitória, Espírito Santo, Brazil.

Whitfield, A. E., Falk, B. W., and Rotenberg, D. 2015. Insect vector-mediated transmission of plant viruses. Virology 479-480:278-289.

Wu, Z., Mo, C., Zhang, S., and Li, H. 2018. Characterization of Papaya ringspot virus isolates infecting transgenic papaya ‘Huanong No. 1’in South China. Sci. Rep. 8:8206.

Xia, X.-J., Zhou, Y.-H., Shi, K., Zhou, J., Foyer, C. H., and Yu, J.-Q. 2015. Interplay between reactive oxygen species and hormones in the control of plant development and stress tolerance. J. Exp. Bot. 66:2839-2856.

Zamudio-Moreno, E., Ramirez-Prado, J., Moreno-Valenzuela, O., and Lopez-Ochoa, L. 2015. Early diagnosis of a Mexican variant of Papaya meleira virus (PMeV-Mx) by RT-PCR. Gen. Mol. Res. 14:1145-1154.

Zhang, R., Hisano, S., Tani, A., Kondo, H., Kanematsu, S., and Suzuki, N. 2016. A capsidless ssRNA virus hosted by an unrelated dsRNA virus. Nat. Microbiol. 1:15001.

TABLES

Table 1 –Viruses of papaya, by family and genus, in main growing regions worldwide.

Family	Genus	Virus species
Bunyaviridae	*Tospovirus*	*Tomato spotted wilt virus*, TSWV
	Tenuivirus	*Papaya mild yellow leaf virus*, PMYLV
Geminiviridae	*Begomovirus*	*Papaya leaf curl virus*, PaLCuV
		Papaya leaf crumple virus, PaLCrV
		Chilli leaf curl virus, ChiLCuV
		Tomato leaf curl New Delhi virus, ToLCuNDV
		Croton yellow vein mosaic virus, CYVMV
Potyviridae	*Potyvirus*	*Papaya ringspot virus*, PRSV-P
		Papaya leaf distortion mosaic virus, PLDMV
		Zucchini yellow mosaic virus, ZYMV
Rhabdoviridae	*Rhabdovirus*	*Papaya apical necrosis virus*, PANV
		Papaya droopy necrosis virus, PDNV
Tombusviridae[1]	*Carmovirus*[1]	*Papaya lethal yellowing virus*, PLYV
Alphaflexiviridae	*Potexvirus*	*Papaya mosaic virus*, PapMV
NE[2]	NE[2]	Papaya meleira virus[2] (PMeV[a] + PMeV-2[b])

[1-] Molecular research indicates homology with the family *Sobemoviridae* and genus *Sobemovirus*.

[2-] Not established. Molecular characterization of virus genome is in progress; a- tentatively classified in *Totivirus* genus; b- tentatively classified in *Umbravirus* genus.

FIGURES

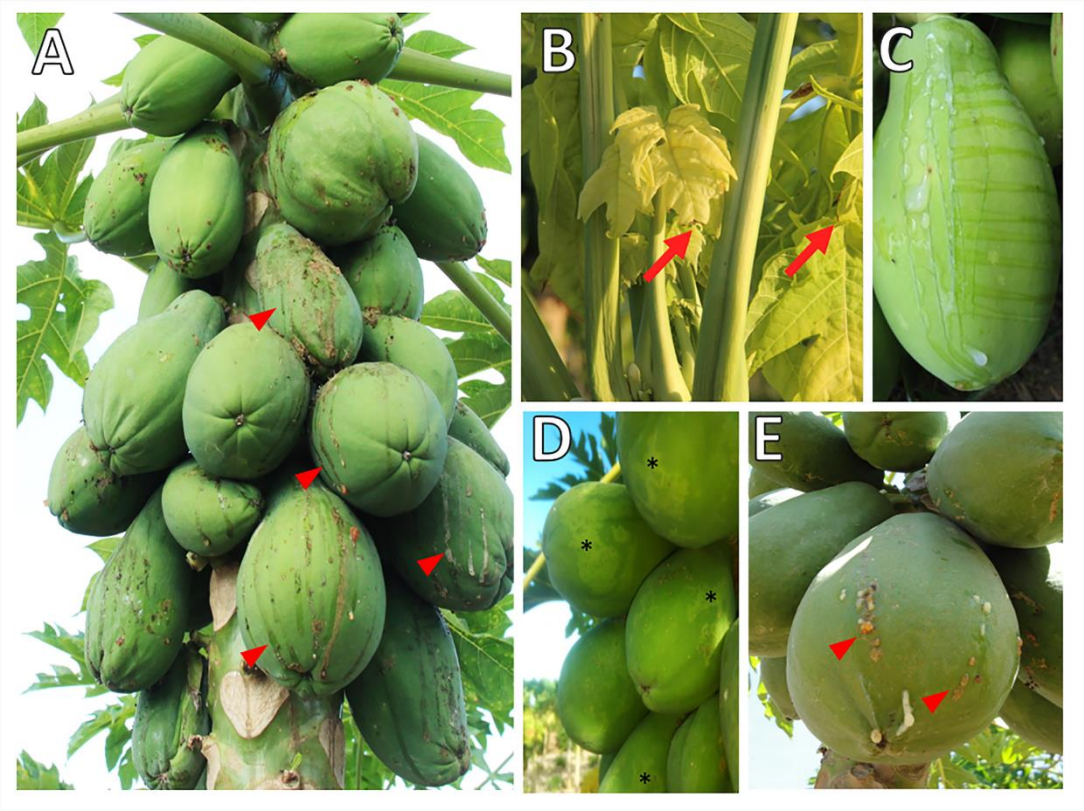
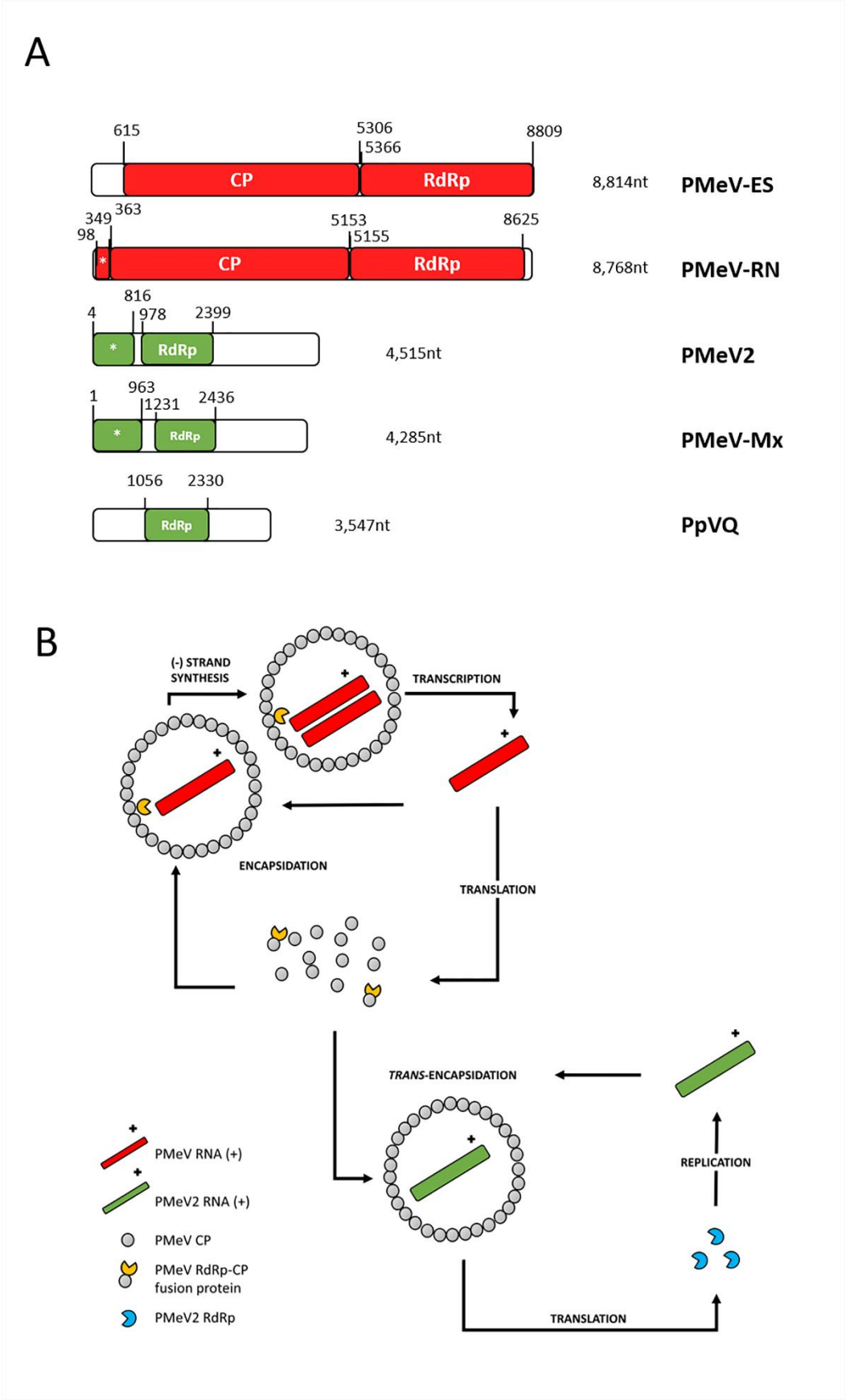


Figure 1. Papaya sticky disease (PSD) symptoms. (A) Papaya tree with green fruits presenting an exudation of fluid latex on their surface which darkens after oxidation by atmospheric exposure resulting in a sticky aspect (red arrowhead). (B) This exudation also results in the appearance of small necrotic lesions on the edges of young leaves (red arrow). (C) Watery, fluid, and translucent latex of an infected fruit after wound with a scalpel. (D) Irregular light-green and yellowish areas in green fruit (black asterisk). (E) Papaya fruit from symptomatic Mexican plant (cv. Maradol).


A
615
5306
5366
8809
CP
RdRp
8,814nt
PMeV-ES
349
363
98
5153
5155
8625
*
CP
RdRp
8,768nt
PMeV-RN
816
4
978
2399
*
RdRp
4,515nt
PMeV2
963
1
1231
2436
*
RdRp
4,285nt
PMeV-Mx
1056
2330
RdRp
3,547nt
PpVQ
B
(-) STRAND SYNTHESIS
TRANSCRIPTION
ENCAPSIDATION
TRANSLATION
TRANS-ENCAPSIDATION
REPLICATION
TRANSLATION
PMeV RNA (+)
PMeV2 RNA (+)
PMeV CP
PMeV RdRp-CP fusion protein
PMeV2 RdRp

816

Figure 2. Current isolates of PMeV and PMeV2 and proposed model for interactions between PMeV complex. (A) Genomic organization of PMeV (red) and PMeV2 (green) isolates showing their ORFs and their putative encoded proteins. NCBI accession numbers: PMeV-ES (KT921784); PMeV-RN (KT013296); PMeV2 (KT921785); PMeV-Mx (KF214786.1); PpVQ (KP165407). Hypothetical proteins are indicated with an asterisk. (B) PSD in Brazil occurs during a double infection, by PMeV, a toti-like virus, and PMeV2, an umbra-like virus. A possible scenario for PMeV and PMeV2 interplay is illustrated here. PMeV can complete its replication cycle in the host cell without PMeV2. Like an umbravirus, PMeV2 is a capsidless ssRNA virus and is not found alone and uses PMeV CP for encapsidation. A -1 ribosomal frameshifting produces the RdRp-CP fusion protein.

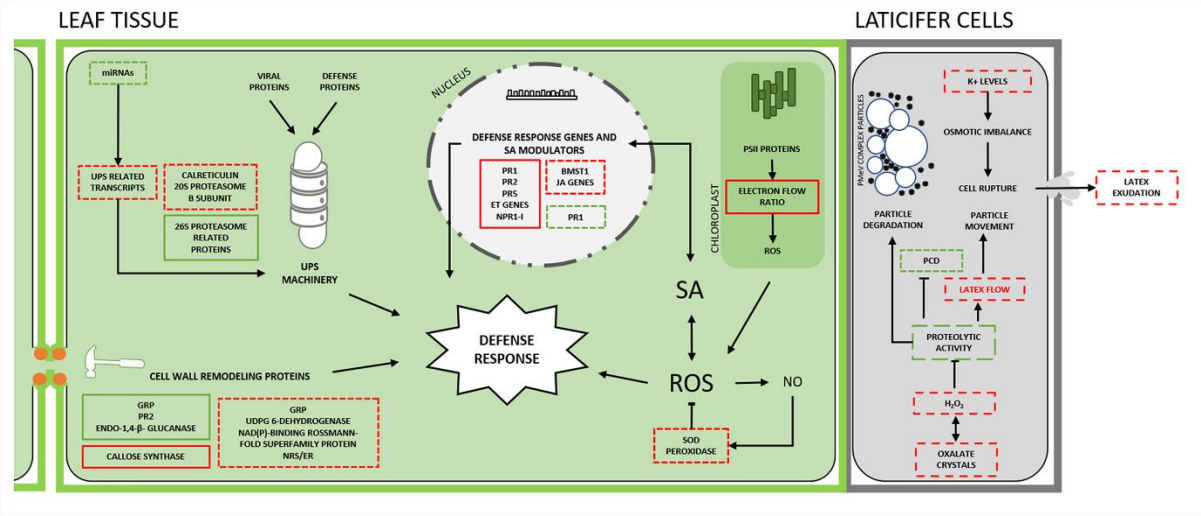


Figure 3. Papaya and PMeV complex interaction. In pre-flowering plants, a multilayer immune system triggered by PMeV complex infection results in a pre-flowering tolerance that is partially disabled in post-flowering. See the text (Papaya and PMeV complex interaction) for details and further references. SA, salicylic acid; *PR1*, *PR2* and *PR5* are pathogenesis-related protein genes; *BSMT1*, benzoic acid/SA carboxyl methyltransferase; NPR1-I, non-expressor of pathogenesis-related protein 1; ET, ethylene; JA, jasmonate; GRP, glycine-rich protein; NRS/ER, nucleotide-rhamnose synthase/epimerase-reductase; UPS, ubiquitin/26S proteasome system; PCD, programmed cell death. ROS, reactive oxygen species; NO, nitric oxide; SOD, superoxide dismutase; PSII, photosystem II. Dashed box: post-flowering events (PSD); solid box: pre-flowering events; red: induced or partially induced processes; green: repressed or partially repressed; black arrows: direction or order of the cellular event; blocked arrow: inhibition of the cellular event. Viral icosahedral particles and vesicles are represented in laticifer cells.

Figure 4. The roguing of infected plants. (A) An agricultural technician specialized in recognizing plants with papaya ringspot and papaya sticky disease symptoms, known in Portuguese as ‘mosaiqueiro’, performs the roguing using a machete. Until now, this is the only management applied to control the PSD. (B) If appropriate agricultural practices were not carried out, the disease spread the whole crop and brought about total yield losses.

SUPPLEMENTARY MATERIAL

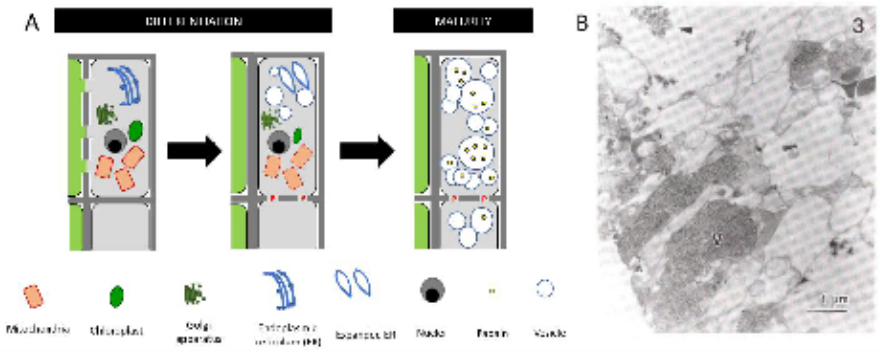


Figure S1. *Carica papaya* laticifers and PMeV complex viral particles. Xylem and phloem are complex tissues that form channels in the plant. Likewise, laticifers correspond to a system of additional channels, but comprised exclusively of living cells. Laticifers are specialized in synthesis and accumulation of latex which varies in biochemical composition depending on the plant species (Hagel et al. 2008). Stored under pressure, this latex is exuded on wounding being an important defense response against pathogens. In *C. papaya*, laticifers are articulated, anastomosed (Hagel et al. 2008) and found in all papaya organs. In the primary growing stem, laticifers localize in parenchyma region between xylem and phloem (Fisher 1980). They are also found in roots, but seem to septate their walls, becoming isolated cells at maturity (Rao et al. 2013). In the immature fruit, laticifers form an anastomosed network throughout the parenchyma, being more numerous and smaller in the fruit periphery. In the ripe fruit, they occur throughout the mesocarp tissue in a parallel arrangement to the vascular bundles (Fisher 1980). Differentiating laticifers have numerous well-developed and active mitochondria, ribosomes, and endoplasmic reticulum (ER). As development progresses, organelles are gradually degenerated, ER expands and splits into fragments, and the autophagy of the cytoplasm by the vacuole becomes evident. In their cell walls, perforations are produced in different places of contact with adjacent laticifers. Reaching maturity, laticifers become filled with vesicles containing proteases, organelles disappear, but the plasma membrane remains intact (Zeng et al. 1994). Some authors report that they rarely find nuclei (Evert 2006; Kitajima et al. 1993) while others report that *C. papaya* laticifers are multinucleated (Fisher 1980). ER and polyribosomes are involved in papain synthesis which is temporally stored in ER-derived vesicles and then in vesicles containing other latex components (Kitajima et al. 1993). A schematic diagram was organized summarizing those findings (Panel A). To date, there is no information on the cellular communication of *C. papaya* laticifers with surrounding cells. PMeV complex particles are visualized within laticifers from fruits and leaves but not in other papaya tissue. Figure B shows electron micrograph of thin sections of latex vessel from PSD fruit. Isometric particles appeared in two patterns: scattered randomly among latex vesicles (arrowheads) or forming aggregates of different sizes (V). Reprinted from Kitajima et al. (1993). P – perforation. Structures outlined with dashed lines are degenerating.

THESIS OBJECTIVE

GENERAL OBJECTIVE

To understand the papaya sticky disease pathosystem at host-pathogen interaction and pathogen dispersion level, and develop a diagnosis methodology to contribute to disease management.

SPECIFIC OBJECTIVES

- To localize papaya meleira virus (PMeV) and papaya meleira virus 2 (PMeV2) RNA in *Carica papaya* tissues assessing their preferential site of infection during somatic embryogenesis in papaya (Manuscript #2);

- To identify interactions between PMeV ORF1 and plant proteins, finding important players in the pre-flowering tolerance mechanism, mainly affecting PMeV complex replication (Manuscript #3);

- To validate a method to detect PMeV complex in samples collected from Brazilian orchards (Manuscript #4);

- To open an important discussion for directing new research to understand the vectors of PMeV complex and the use of new management practices in papaya orchards (Manuscript #5).

MANUSCRIPT #2. LATICIFERS OF PAPAYA STICKY DISEASED PLANTS ARE THE PREFERENTIAL INFECTION SITE OF PAPAYA MELEIRA VIRUS (PMeV), A TOTI-LIKE VIRUS AND UMBRAVIRUS-LIKE ASSOCIATED RNA, PAPAYA MELEIRA VIRUS 2 (PMeV2)

Manuscript in preparation for *Archives of Virology journal* (ISSN 0304-8608; IF 2.574, 2021; Qualis B1 Biotecnologia, 2013-2016).

Laticifers of papaya sticky diseased plants are the preferential infection site of papaya meleira virus (PMeV), a toti-like virus and umbravirus-like associated RNA, papaya meleira virus 2 (PMeV2)

Marlonni Maurastoni[1], Tathiana F. Sá-Antunes[1], Emanuel F. Abreu[2], Diva M. A. Dusi[2], Ana C. M. M. Gomes[2], Francisco J. L. Aragão[2], Simone G. Ribeiro[2], Francisco M. Zerbini[3], Anna E. Whitfield[4] and Patricia M. B. Fernandes[1]

[1]Núcleo de Biotecnologia, Universidade Federal do Espírito Santo, Vitória, Espírito Santo, Brazil;

[2]Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, 70770-900, Brazil

[3]Dep. de Fitopatologia/BIOAGRO, Universidade Federal de Viçosa, 36570-900, Viçosa, Minas Gerais, Brazil.

[4]Department of Entomology and Plant Pathology, North Carolina State University, 840 Main Campus Drive, Raleigh, NC 27606, USA

ABSTRACT

Papaya sticky disease (PSD) has affected production and caused the destruction of several orchards in Brazil, Mexico, and Australia. PSD is associated with a viral complex comprised of papaya meleira virus (PMeV), a totivirus-like, and papaya meleira virus 2 (PMeV2), a umbravirus-like associated RNA. In Brazil, both PMeV and PMeV2 are separately packaged by capsid protein coded by PMeV. Asymptomatic plants are detected with PMeV while symptoms can be visualized only after flowering and infection by PMeV2. Spontaneous exudation of aqueous latex from fruits and necrosis at the edges of young leaves are PSD symptoms caused by an osmotic imbalance in laticifer cells. Electron microscopy studies have shown that viral particles are localized in these cells. Here we aim to understand the distribution of both viral RNAs throughout the papaya tissues. *In situ* hybridization targeting both PMeV and PMeV2 RNA shows a preference of both viruses to laticifers in PSD plants. PMeV accumulates in laticifers cells of the main vein, while PMeV2 can infect both the main vein and mesophyll laticifers. To confirm the preference for these cells, we took advantage of a non-laticifer cell producing stages of papaya development using somatic embryogenesis to further characterize PMeV tissue tropism. The results show the PMeV complex is detected in embryogenic calli and somatic-embryogenesis regenerated plants, but not in callus tissue. No plasmodesmata were visualized in *Carica papaya* laticifers, suggesting an uncommon strategy for PMeV entry in these cells.

Keywords**:** laticifers; tissue culture; plasmodesmata; virus particle

INTRODUCTION

Papaya sticky disease (PSD) has been a causing the destruction of papaya (*Carica papaya* L.) orchards in Brazil (NAKAGAWA *et al.*, 1987), Mexico (PEREZ-BRITO *et al.*, 2012) and Australia (PATHANIA *et al.*, 2019), countries in which it was officially reported. In Brazil, PSD is associated with a viral complex that comprises papaya meleira virus (PMeV) and papaya meleira virus 2 (PMeV2). PMeV has a dsRNA genome in a typical arrangement of the *Totiviridae* family, while PMeV2 has an ssRNA genome and it has been grouped in the class I umbravirus-like associated RNAs (ulaRNA) (LIU *et al.*, 2021). Both PMeV and PMeV2 are separately encapsidated in particles assembled by PMeV capsid protein (ANTUNES *et al.*, 2016).

In PSD plants, isometric viral particles of approximately 42 nm in diameter are visualized only in laticifers. Viral particles are not visualized in any other plant tissue e.g. epidermis, parenchyma, fibers, xylem, and phloem vessels of PSD or asymptomatic plants (KITAJIMA *et al.*, 1993; MAGAÑA-ÁLVAREZ *et al.*, 2016; RODRIGUES *et al.*, 2009). Also, leaf dip preparations made from leaves or fruits of diseased plants only rarely show similar particles (KITAJIMA *et al.*, 1993). The infection in these unusual cells leads to the main PSD symptoms, the spontaneous exudation of fluid latex from green fruits. In contact with air, the oozed latex oxidizes, darkens, and marks the fruit, reducing its commercial value (ABREU *et al.*, 2015; ANTUNES *et al.*, 2020; VENTURA *et al.*, 2004). These symptoms are visualized only after flowering possibly due to a depletion of tolerance mechanisms signalized by salicylic acid (MADROÑERO *et al.*, 2018). Although viral particles are not visualized in asymptomatic plants, sensitive diagnostic techniques e.g., RT-PCR, show that it is not uncommon to detect PMeV and PMeV2 in these plants (ANTUNES *et al.*, 2016; MAURASTONI *et al.*, 2020). Therefore, the non-visualization of viral particles in infected asymptomatic plants but its visualization in symptomatic plants support a correlation of virus accumulation and symptom onset.

In *C. papaya*, laticifers are distributed as an articulated and anastomosed channel through all organs of the plant. In the primary growing stem, they are found among parenchyma cells located between the primary xylem and phloem, probably derived from the fascicular cambium (FISHER, 1980). During differentiation, laticifer cells undergo autophagy that results in the complete elimination of their organelles, including the nucleus. The active endoplasmic reticulum swells and fragments producing several vesicles containing proteases. Upon reaching maturity, the tonoplast disappears remaining only the plasma membrane which surrounds a lumen filled with vesicles (ZENG *et al.*, 1994).

Plasmodesmata are important cellular communications that allow the transport of molecules through the symplast. In latex-bearing plants such as *Papaver somniferum* L., plasmodesmata connect laticifers cells to the phloem cells, where there is an exchange of enzymes and mRNAs (FACCHINI; DE LUCA, 2008). On the other hand, plasmodesmata of *Hevea brasiliensis* laticifers are active only during cell differentiation. At maturity, these cells are symplastically isolated from the surrounding cells exchanging molecules with the apoplast through membrane proteins (DE FAY *et al.*, 1989). There is still a lack of information on the cellular communication of *C. papaya* laticifers with other cells. Therefore, the mechanism of import and export of molecules, mRNAs, and viral RNAs to these tissues remains unknown. More importantly, is still unknown if mature papaya laticifers are metabolic active or rely on adjacent cells to support its metabolism,

PMeV complex movement within the plant is still unknown, but a hypothesis has been raised based on the biochemical and physiological changes presented by laticifers of diseased plants. The latex of PSD plants is more fluid due to an imbalance of potassium ions and an increase in water content which possibly leads to cell disruption (RODRIGUES *et al.*, 2009). There is a lack of information regarding long-distance transport, cytoplasmic movements, or vesicle traffic in laticifers (PICKARD, 2008). Thus, the rupture and subsequent latex drainage could be used by the PMeV complex as an unusual transport mechanism through the plant (ANTUNES *et al.*, 2020; RODRIGUES *et al.*, 2009).

To date, electron microscopy studies have only detected viral particles in laticifers cells. However, there is no information regarding RNA localization in papaya tissues. Given that some viruses are known to be limited by a cell type, e.g. phloem tissue, we hypothesize that the PMeV complex is limited to laticifers. Here we show using in situ hybridization that PMeV and PMeV2 RNA accumulates preferentially in the laticifers of infected leaves in PSD plants. Using somatic embryogenesis (SE) as a non-vascular cell culture system, we show that PMeV complex infection is reestablished in cells where laticifers are differentiated. As no plasmodesma are visualized in laticifers of papaya, we speculate an alternative route for the PMeV complex to reach laticifer cells.

METHODS

PCR amplification and cloning

To obtain the probe for in situ hybridization experiments, cDNA was synthesized from the total RNA extracted from plants with symptoms using the High-Capacity cDNA Reverse Transcription kit (Invitrogen, Carlsbad, USA). DNA fragments were amplified from cDNA samples using the Platinum ® Taq DNA Polymerase High Fidelity enzyme (Invitrogen, Carlsbad, USA) and PMeV primer pair (F: 5’ CTTGGTTAGGCATAACTGTAGGT 3’; R: 5’ CACGGACTCTTAGAAACGTCTATC 3’) or PMeV2 primer pair (F: 5’ CGCCAAGTGGGATAAGTTTAGA 3’; R: 5’ CGATTTGAGCACAAGGGTTAATG 3’). PCR fragments were cloned into pGEM®-T (Promega, Madison, USA) generating plasmids containing fragments of PMeV (pGEM®-T-PMeV-ES-2446-2816) and PMeV2 (pGEM®-T-PMeV2-1430-2244). Numbers in the plasmid names represent the region in the genome of each virus target by the probe (NCBI accession number: KT921784 KT921785).

***In situ* Hybridization**

The plant material was selected from papayas trees growing in farms in the north of Espirito Santo state, Brazil. Samples were grouped into two conditions: symptomatic and asymptomatic. Each condition consisted of 6 plants from which the most expanded green leaf (second pair) was collected. From these leaves, three 1cm² segments from 3 different regions of the central rib (close to the petiole, middle of the leaf, and close to the leaf tip) were removed and immediately fixed in 4% (w/v) paraformaldehyde 0.01M phosphate buffer pH 7.2, dehydrated in an ethanol series and embedded in Paraplast® plus (Sigma, San Luis, MO). Specimens were cut into 10 µm sections and placed onto slides treated with 100 µg/mL of poly-L-lysine (Sigma, San Luis, MO). The Paraplast® was removed with HistoChoice® (Sigma, San Luis, MO) series.

The plasmids pGEM®-T-PMeV-ES-2446-2816 and pGEM®-T-PMeV2-1430-2244 were linearized using restriction enzymes NcoI or SalI (Promega, Madison, WI). Sense and anti-sense probes were labeled with the Roche® Dig RNA Labeling kit (SP6/T7), following the manufacturers' instructions, and hydrolyzed to 150–200 bp fragments. Prehybridization was carried out in 0.05 M Tris–HCl pH 7.5 buffer containing 1 µg/mL proteinase K in a humid chamber at 37°C for 10 min. Hybridization was carried out overnight in a humid chamber at 42°C, in 10 mM Tris–HCl pH 7.5 buffer containing 300 mM NaCl, 50% formamide (deionized), 1 mM EDTA pH 8, 1 X Denhardt's solution, 10% dextran sulfate, 600 ng/mL tRNA and 600 ng/mL of the RNA probe.. Detection was performed following the instructions of the Roche® Dig Detection kit, using anti-DIG conjugated alkaline phosphatase and NBT/BCIP as substrates. Sections were mounted in glycerol 50% (v/v) and regions of the main vein and mesophyll were observed under Leica DMRX or Zeiss-Axiophot light microscopes. Treatment with 10 mg/ml of RNAse A was performed after visualization to ensure the hybridization signals were genuine. This experiment was performed three times and at least 10 regions of the main vein and

mesophyll of each section in a total of 18 sections were analyzed for each plant group (see results section).

PMeV complex monitoring during somatic embryogenesis

Somatic embryogenesis tissue culture media and culture conditions were performed according to KOEHLER *et al.* (2013). Four 2-month-old plants growing in a greenhouse were inoculated with latex tapped from infected plants (ABREU *et al.*, 2012). The diagnosis was made on young leaves 30 days after inoculation. New emerged leaves were removed and used as explants for somatic embryogenesis. The experiment was set up on 15 Petri dishes per plant, each dish containing 4 leaf disks (1cm^2 each).

Leaf disks were inoculated in an induction medium. Monthly for three months, friable embryogenic calli (FEC) were transferred to a fresh tissue culture medium containing half of the 2,4-D concentration of the previous medium. FEC exhibiting somatic embryo clusters were transferred to a maturation medium with conditions described by Koheler et al., 2013. During 3 months, somatic embryos in the mature cotyledonary stage were isolated and placed on germination medium (Koheler et al., 2013). After germination, seedlings were transferred to the regeneration medium (Koheler et al., 2013) in glass flasks (14cm x 8 cm) containing 50 ml of culture medium. Each month the seedlings were transferred to a new fresh medium until they reach an approximate 8-10 cm height.
The monitoring of the PMeV complex was performed in 3 different stages of somatic embryogenesis: (i) three-month-old calli, (ii) calli exhibiting somatic embryo in mature cotyledonary stage, and (iii) regenerated seedlings. The diagnosis of PMeV complex was performed according to ANTUNES *et al.* (2016).

Plasmodesmata identification

Plasmodesmata were identified by staining papaya tissue with an aniline blue reagent. Semi-thin sections of the leaf main vein (12 µm) obtained from fixed material and included in paraffin were immersed in a solution of aniline blue 0.1% (w/v) in 1M glycine (pH 9.5). Sections were incubated for 5 min in the dark under gentle agitation and then washed in deionized water for 5 min. Slides were mounted in deionized water and visualized under a fluorescence microscope (NIKON ® Ti-Eclipse) using an excitation filter: bandpass (BP) 365/12 nm; emission: long pass (LP) 397 nm. The images were photographed and analyzed with the aid of the Nis-Elements AR 4.20.00 software. At least 10 regions of the main vein of 18 sections were observed.

RESULTS

PMeV and PMeV2 RNA accumulates preferentially in the laticifers of infected leaves in PSD plants

After diagnosis, symptomatic and asymptomatic plants were grouped in (i) asymptomatic and positive for PMeV only (ii) asymptomatic and positive for PMeV complex, and (iii) symptomatic and positive for PMeV complex. *In situ* hybridization assays using semi-thin sections of *C. papaya* leaves were performed to localize the PMeV and PMeV2 RNA in papaya tissues. The main vein and mesophyll regions of the second pair of leaves were analyzed (Figure 1 and 2). The results show that in group iii PMeV RNA is restricted to the laticifers of the main vein. Both sense and antisense strands were detected in this cell. These results corroborate those discovered by Rodrigues (2006) who detected the PMeV dsRNA in the layers with the parenchyma, xylem, vascular cambium, and phloem.

On the other hand, the sense RNA of PMeV2 is restricted to the laticifers of the main vein as well as mesophyll. The PMeV2 antisense RNA was not detected in the analyzed material. RNA of both viruses was not detected in any other tissue of diseased plants or the laticifers of plants in the groups i and ii. Curiously, in all groups, a hybridization signal is visualized in non-identified structures (Figure S1). These structures are present inside cells surrounded by parenchyma. In transversal sections of the main vein, they are found between phloem bundles and forming a ring near to collenchyma cells. In longitudinal sections, this ring is found as parenchymatic rays. These structures are also visualized in the transitioning parenchyma between the main vein and mesophyll (Figure S1).

PMeV complex is detected in embryogenic calli and somatic embryogenesis-regenerated plants

To monitor the presence of the PMeV complex in a system comprised of partially undifferentiated cells (i.e., devoid of laticifers), we induced somatic embryogenesis using the leaf of PMeV complex-infected plants as explants (Figure 3.A). PMeV complex diagnosis was conducted by RT-PCR in three different SE stages: (i) 3-month-old calli, (ii) calli exhibiting somatic embryo in mature cotyledonary stage, and (iii) regenerated seedlings.

The callogenic response was observed in the explants after 30 days in the culture medium. In the next two months, the callus tissue covered the entire leaf explant (Figure 3.B). During the following months, successive reductions of 2,4-D were made to half the molarity per month. At this stage, only embryogenic calluses were transferred to the maturation medium (Figure 3.C). At this time, somatic embryos can be visualized at different stages of development. Embryos in the mature cotyledonary stage were removed and transferred to the germination medium. After 2 weeks, embryos germinated in normal plants (Figure 3.F). Then, seedlings were transferred to flasks where their

growth was monitored for 3 months (Figure 3. G, H, and I). After 3 months of cultivation, seedlings were 8-10 cm long and their leaves were used for molecular diagnosis.

RT-PCR diagnosis shows that all inoculated explant-donor plants were infected with the PMeV complex (Table 1). After inducing somatic embryogenesis from inoculated plants, PMeV and PMeV2 were monitored in 3-month-old calluses and plants regenerated by SE. PMeV was detected in eight of sixteen calii while none of the analyzed calluses showed detection for PMeV2. Interestingly, all samples obtained from embryogenic callus and regenerated plants tested positive for the viral complex.

No plasmodesmata are visualized in *C. papaya* laticifers

To identify cellular communications between laticifers and adjacent tissues we used aniline blue as a fluorescent dye to detect the presence of callose, a polysaccharide well known to be found in plasmodesmata. It was possible to identify plasmodesmata associated with parenchymal tissues (Figure 4) and located between sieve tube elements and between sieve tube/companion cell complex in the phloem (Figure 4.A and B - arrowheads). No staining was visualized between laticifer cells and adjacent tissues (Figure 4.B - arrows).

DISCUSSION

During the virus cycle, the production of new particles is directly associated with the replication of their viral RNA (HULL, 2014). In PSD plants viral particles of the PMeV complex are only seen in laticifers, not in any other cell type (epidermis, parenchyma, fibers, xylem, and phloem vessels), while no particles have been found in asymptomatic plants (KITAJIMA *et al.*, 1993; RODRIGUES *et al.*, 1989). Since the discovery of the viral nature of PSD etiology, the visualization in agarose gel of the viral RNAs extracted from latex has been used as the simplest procedure to identify infected plants (TAVARES et al., 2004). However, the viral RNA is not always detected by the method above when total RNA is extracted from latex of asymptomatic plants neither from leaf tissues of symptomatic plants (data not shown). This suggests that the PMeV complex can accumulate in the latex of diseased plants, more so than in other green tissues. The sequencing of the PMeV complex allowed the use of more sensitive techniques for detection (ABREU *et al.*, 2012; ANTUNES *et al.*, 2016; MAURASTONI *et al.*, 2020) and changed the diagnosis of false-negative plants as PMeV and PMeV2 could now be detected in asymptomatic plants (ANTUNES *et al.*, 2016; MAURASTONI *et al.*, 2020). Taken together, this supports a correlation between increasing virus titer, the accumulation of virions in laticifer, and the symptom's onset. Supporting this idea, only diseased plants showed detection by the PMeV complex through *in situ* hybridization. Both asymptomatic plants with single infection (PMeV) and plants infected with PMeV complex do not show signs of infection through *in situ* hybridization, suggesting that additional factors, besides PMeV2 infection, (e.g the development stage when the plants are infected) lead to virus accumulation in these cells. The depletion of the tolerance mechanism in post-flowering plants and the biochemical and physiological modifications directly associated with laticifers could be important for this process (MADROÑERO *et al.*, 2018; RODRIGUES *et al.*, 2009; RODRIGUES *et al.*, 2012; SOARES *et al.*, 2016).

The vascular system is the main route used by viruses to systemically infect a plant. The phloem, for example, is the most advantageous conduit as it leads to almost all cells and organs (SEO; KIM, 2016). Although some viruses can reach phloem cells, several mechanisms limit their ability to escape from it, as seen during potato leafroll virus (PLRV) and citrus tristeza virus (CTV) infection (BENDIX; LEWIS, 2018). Similar to phloem cells, papaya laticifers are distributed among all tissues and organs of the papaya plant

(FISHER, 1980), but their role in exchanging molecules with surrounding cells still needs to be addressed. Here, we observed a preferential infection of both viruses in laticifers which suggests that PMeV complex accumulation is limited to laticifers. PMeV and PMeV2 also present a differential distribution throughout the leaf of diseased plants. Both positive and negative strands of PMeV RNA are detected only in laticifers of the main vein, while PMeV2 positive strand is detected in the main vein and by itself in mesophyll laticifers. PMeV2 has been grouped in class I of umbravirus-like associated RNAs, which are coat protein-dependent subviral RNA replicons related to umbraviruses (LIU *et al.*, 2021). As they do not encode a capsid protein, umbraviruses need an auxiliary virus responsible for packing their RNA to be transmitted by a vector, however when mechanically inoculated they can establish systemic infection (TALIANSKY; ROBINSON, 2003). The fact that PMeV2 is found alone in mesophyll laticifers suggests that it is capable of infecting tissue independent of its auxiliary virus. The reason that PMeV is not detected in other mesophyll cells or how PMeV2 reached these cells remains inconclusive. In *Hevea brasiliensis*, laticifers that originated from primary meristematic zones have a different morphology and transcriptome profile from the ones that originated from the cambium, with the former associated with defense against biotic stresses and the latter to abiotic stresses (TAN *et al.*, 2017). This supports the idea that laticifers from mesophyll and main vein could have different physiology which in turn will affect the distribution of the PMeV complex through the leaf.

The preference of PMeV complex for laticifer cells was also evaluated using somatic embryogenesis as a non-laticifer cell system. Ultrastructural analysis showed that *C. papaya* callus consists of partially differentiated cells containing numerous lipid bodies. Laticifers are observed in somatic embryos regenerated from the callus and no laticifers are observed in all the callogenic tissue (YAMAMOTO; TABATA, 1989). In this work, we used infected leaves as explants and followed the same material until the regeneration of in vitro plants. Monitoring of the PMeV complex during somatic embryogenesis showed that embryogenic calluses and SE-regenerated plants were infected with the viral complex. However, in 3-month old callus only few samples were detected with PMeV, while none were detected with PMeV2. The non-detection in this material could be attributed to the low viral titer in these cells supporting the idea that the system could not

support virus replication at the same extent as laticifers. The ability of the PMeV complex to infect plants regenerated from SE raises questions about how PMeV can move from the infected callogenic tissue to the differentiating embryo. In indirect somatic embryogenesis, embryos originate from pro-embryogenic complexes. Pro-embryogenic complexes are compact structures, formed by an aggregation of cells with high similarity to meristematic cells (FEHÉR, 2015). Histochemical analyzes of the indirect SE process in *C. papaya* show that pro-embryogenic complexes are delimited by cell walls with high deposition of callose (β-1,3-glucan) (KOEHLER, 2004). This thickening collapses plasmodesmata keeping them isolated from the other callus cells (FERNANDO *et al*., 2001). As a result, there is a restriction to symplastic transport, preventing the physiological influence of external cells on the embryogenic pathway of the complex's cells. In this work, somatic embryos are produced through indirect SE, as can be observed by the formation of embryogenic calluses, where there is a production of pro-embryogenic complexes. In addition to the level of cell organization, the somatic embryo has laticifers that are not visualized in the rest of the callus (YAMAMOTO; TABATA, 1989). Therefore, it is likely that, in embryogenic callus, the PMeV complex infects somatic embryos and accumulates in laticifers. The visualization of particles in embryogenic calluses of *C. papaya* could confirm this hypothesis.

Plasmodesmata are fundamental nanochannels connecting the plant cell symplast which permit the passage of several small molecules, including ions, hormones, nucleic acids, and photosynthates. Viruses exploit these structures for their intercellular movement mainly in the form of ribonucleoprotein complexes and interacting with several proteins present in the plasmodesmata site (HULL, 2014; KUMAR *et al.*, 2015). It has been pointed out that the lacking or the dynamics of the plasmodesmata network in some cells is detrimental for the symplastic exclusion and cell differentiation, which is the case of the apical meristem cells. However, the mechanisms that exclude some viruses or viroids in this structure are not fully understood (BRADAMANTE *et al.*, 2021). Here, plasmodesmata were identified mainly between sieve tube elements and between sieve tube/companion cell complexes present in the phloem, but not between papaya laticifers and adjacent tissues. Staining with aniline blue showed that in *Hevea brasiliensis* there are no plasmodesmata between the laticifers and adjacent parenchyma cells. Similarly,

laticifers of poinsettia lack cellular communication at maturity (FINERAN, 1983). Interestingly, in these plants, laticifers are visualized containing occluded plasmodesmata resulted from cell differentiation (DE FAY *et al.*, 1989) which supports the hypothesis that this cell type is independently programmed (JOHNSON *et al.*, 2021). In the absence of plasmodesmata, the most favorable moment for the PMeV complex to reach laticifer cells would occur in young organs containing initial cells from laticifers that initiated their cell differentiation. Supporting this idea, it has been pointed out that PMeV has a persistent lifestyle (ANTUNES *et al.*, 2016). Despite the PMeV complex infecting papaya systemically, there are no ORFs capable of encoding typical viral movement proteins. Most persistent plant viruses have a dsRNA genome and encode only one RdRp and one CP, without the MP essential for systemic infection (ROOSSINCK, 2010; ROOSSINCK, 2013). Therefore, there is no cell-to-cell movement or virus transport within the plant, except when the cell divides (BOCCARDO *et al.*, 1987), an event characteristic of plant meristematic cells. Papaya laticifers are articulated, a class of laticifers originated from multiple initials that later in their development become a multinucleate structure through cell fusion (JOHNSON *et al.*, 2021). Therefore, it is reasonable to imagine that PMeV infects laticifers initials earlier in its differentiation, being able to reach all organs through the further cytoplasm fusion of adjacent laticifers.

Taken together, we show that laticifers of the main vein are the preferential site of PMeV complex accumulation and PMeV2 alone can infect laticifers of the mesophyll. No plasmodesmata were visualized in mature laticifers of the main vein leading to the assumption that the PMeV complex reaches mature laticifers early in its differential through infection of laticifer initials. Supporting this idea, we show using somatic embryogenesis as a non-laticifer tissue system that PMeV complex can infect somatic embryos as well as regenerated plants.

REFERENCES

ABREU, P. M. V.; ANTUNES, T. F. S.; MAGAÑA-ÁLVAREZ, A.; PÉREZ-BRITO, D.; TAPIA-TUSSELL, R.; VENTURA, J. A.; FERNANDES, A. A. R.; FERNANDES, P. M. B. A current overview of the Papaya meleira virus, an unusual plant virus. **Viruses**, 7, n. 4, p. 1853-1870, 2015.

ABREU, P. M. V.; PICCIN, J. G.; RODRIGUES, S. P.; BUSS, D. S.; VENTURA, J. A.; FERNANDES, P. M. B. Molecular diagnosis of Papaya meleira virus (PMeV) from leaf samples of *Carica papaya* L. using conventional and real-time RT-PCR. **Journal of Virological Methods**, 180, n. 1, p. 11-17, 2012/03/01/ 2012.

ANTUNES, T. F. S.; AMARAL, R. J. V.; VENTURA, J. A.; GODINHO, M. T.; AMARAL, J. G.; SOUZA, F. O.; ZERBINI, P. A.; ZERBINI, F. M.; FERNANDES, P. M. B. The dsRNA Virus Papaya Meleira Virus and an ssRNA Virus are Associated with Papaya Sticky Disease. **PloS One**, 11, p. e0155240, 2016.

ANTUNES, T. F. S.; MAURASTONI, M.; MADROÑERO, L. J.; FUENTES, G.; SANTAMARÍA, J. M.; VENTURA, J. A.; ABREU, E. F.; FERNANDES, A. A. R.; FERNANDES, P. M. B. Battle of Three: The Curious Case of Papaya Sticky Disease. **Plant Disease**, 104, n. 11, p. 2754-2763, 2020.

BENDIX, C.; LEWIS, J. D. The enemy within: phloem-limited pathogens. **Molecular Plant Pathology**, 19, n. 1, p. 238-254, 2018.

BOCCARDO, G.; LISA, V.; LUISONI, E.; MILNE, R. G. Cryptic plant viruses. **Advances in Virus Research**, 32, p. 171-214, 1987.

BRADAMANTE, G.; MITTELSTEN SCHEID, O.; INCARBONE, M. Under siege: virus control in plant meristems and progeny. **The Plant Cell**, 33, n. 8, p. 2523-2537, 2021.

DE FAY, E.; SANIER, C.; HEBANT, C. The distribution of plasmodesmata in the phloem ofHevea brasiliensis in relation to laticifer loading. **Protoplasma**, 149, n. 2, p. 155-162, 1989/06/01 1989.

FACCHINI, P. J.; DE LUCA, V. Opium poppy and Madagascar periwinkle: model non-model systems to investigate alkaloid biosynthesis in plants. **The Plant Journal**, 54, n. 4, p. 763-784, 2008.

FERNANDO, J. A.; MELO, M.; SOARES, M. K.; APPEZZATO-DA-GLÓRIA, B. Anatomy of somatic embryogenesis in Carica papaya L. **Brazilian Archives of Biology and Technology**, 44, p. 247-255, 2001.

FEHÉR, A. Somatic embryogenesis - stress-induced remodeling of plant cell fate. **Biochimica et Biophysica Acta - Gene Regulatory Mechanisms**, 2015.

FINERAN, B. A. Differentiation of Non-articulated Laticifers in Poinsettia (*Euphorbia pulcherrima* Willd.). **Annals of Botany**, 52, n. 3, p. 279-293, 1983.

FISHER, J. B. The vegetative and reproductive structure of papaya (*Carica papaya*). **Lyonia**, 1, n. 4, p. 191-208, 1980.

GAMBINO, G.; DI MATTEO, D.; GRIBAUDO, I. Elimination of Grapevine fanleaf virus from three Vitis vinifera cultivars by somatic embryogenesis. **European Journal of Plant Pathology**, 123, n. 1, p. 57-60, 2009/01/01 2009.

GAMBINO, G.; VALLANIA, R.; GRIBAUDO, I. In situ localization of Grapevine fanleaf virus and phloem-restricted viruses in embryogenic callus of Vitis vinifera. **European journal of plant pathology**, 127, n. 4, p. 557-570, 2010.

HULL, R. Chapter 10 - Movement of Viruses Within Plants. *In*: HULL, R. (Ed.). **Plant Virology (Fifth Edition)**. Boston: Academic Press, 2014. p. 531-603.

JOHNSON, A. R.; MOGHE, G. D.; FRANK, M. H. Growing a glue factory: Open questions in laticifer development. **Current Opinion in Plant Biology**, 64, p. 102096, 2021/12/01/ 2021.

KITAJIMA, E. W.; RODRIGUES, C. H.; SILVEIRA, J. S.; ALVES, F.; VENTURA, J. A.; ARAGÃO, F. J. L.; OLIVEIRA, L. H. R. Association of isometric viruslike particles, restricted to lacticifers, with "meleira" (sticky disease) of papaya (*Carica papaya*). **Fitopatologia Brasileira**, 18, p. 118, 1993.

KOEHLER, A. D. EMBRIOGÊNESE SOMÁTICA EM MAMOEIRO (*Carica papaya* L.): ANATOMIA, HISTOQUÍMICA E INFLUÊNCIA DE ACC, AVG E STS E DE PULSOS DE 2,4-D. 2004. Universidade Federal de Viçosa, 2004.

KOEHLER, A. D.; CARVALHO, C. R.; ABREU, I. S.; CLARINDO, W. R. Somatic embryogenesis from leaf explants of hermaphrodite *Carica papaya*: a new approach for clonal propagation. **African Journal of Biotechnology**, 12, n. 18, 2013.

KUMAR, D.; KUMAR, R.; HYUN, T. K.; KIM, J.-Y. Cell-to-cell movement of viruses via plasmodesmata. **Journal of Plant Research**, 128, n. 1, p. 37-47, 2015/01/01 2015.

LIU, J.; CARINO, E.; BERA, S.; GAO, F.; MAY, J. P.; SIMON, A. E. Structural Analysis and Whole Genome Mapping of a New Type of Plant Virus Subviral RNA: Umbravirus-Like Associated RNAs. **Viruses**, 13, n. 4, p. 646, 2021.

MADROÑERO, J.; RODRIGUES, S. P.; ANTUNES, T. F. S.; ABREU, P. M. V.; VENTURA, J. A.; FERNANDES, A. A. R.; FERNANDES, P. M. B. Transcriptome analysis provides insights into the delayed sticky disease symptoms in *Carica papaya*. **Plant Cell Reports**, 37, n. 7, p. 967-980, 2018/07/01 2018.

MAGAÑA-ÁLVAREZ, A.; VENCIONECK DUTRA, J. C.; CARNEIRO, T.; PÉREZ-BRITO, D.; TAPIA-TUSSELL, R.; VENTURA, J. A.; HIGUERA-CIAPARA, I.; FERNANDES, P. M. B.; FERNANDES, A. A. R. Physical Characteristics of the Leaves and Latex of Papaya

Plants Infected with the Papaya meleira virus. **International Journal of Molecular Sciences**, 17, p. 574, 2016.

MAURASTONI, M.; ANTUNES, T. F. S.; OLIVEIRA, S. A.; SANTOS, A. M. C.; VENTURA, J. A.; FERNANDES, P. M. B. A multiplex RT-PCR method to detect papaya meleira virus complex in adult pre-flowering plants. **Archives of Virology**, 165, n. 5, p. 1211-1214, 2020/05/01 2020.

NAKAGAWA, J.; TAKAYAMA, Y.; SUZUKAMA, Y. Exudação de látex pelo mamoeiro. Estudo de ocorrência em Teixeira de Freitas, BA. *In*: Congresso Brasileiro de Fruticultura, 1987, **9**. p. 555-559.

PATHANIA, N.; VALERIANA JUSTO; PABLITO MAGDALITA; FE DE LA CUEVA; LORNA HERRADURA; AIRA WAJE; ADELFA LOBRES; ARCANGEL CUETO; NATALIE DILLON; LYNTON VAWDREY; LOUISE HUCKS; DONNA CHAMBERS; GRACE SUN; CHEESMAN, J. Integrated disease management strategies for the productive, profitable and sustainable production of high quality papaya fruit in the southern Philippines and Australia (FR2019-89). ACIAR. Australia: Australian Centre for International Agricultural Research (ACIAR) 2019**:** 64 p. 2019.

PELAH, D.; ALTMAN, A.; CZOSNEK, H. Tomato yellow leaf curl virus DNA in callus cultures derived from infected tomato leaves. **Plant Cell, Tissue and Organ Culture**, 39, n. 1, p. 37-42, 1994/10/01 1994.

PEREZ-BRITO, D.; TAPIA-TUSSELL, R.; CORTES-VELAZQUEZ, A.; QUIJANO-RAMAYO, A.; NEXTICAPAN-GARCEZ, A.; MARTÍN-MEX, R. First report of papaya meleira virus (PMeV) in Mexico. **African Journal of Biotechnology**, 11, p. 13564-13570, 2012.

PICKARD, W. F. Laticifers and secretory ducts: two other tube systems in plants. **New Phytologist**, 177, n. 4, p. 877-888, 2008.

RODRIGUES, C.; VENTURA, J.; MAFFIA, L. Distribuição e transmissão da meleira em pomares de mamão no Espírito Santo. **Fitopatologia Brasileira**, 14, p. 118, 1989.

RODRIGUES, S. P.; DA CUNHA, M.; VENTURA, J. A.; FERNANDES, P. M. B. Effects of the Papaya meleira virus on papaya latex structure and composition. **Plant Cell Reports**, 28, p. 861-871, 2009.

RODRIGUES, S. P.; VENTURA, J. A.; AGUILAR, C.; NAKAYASU, E. S.; CHOI, H.; SOBREIRA, T. J. P.; NOHARA, L. L.; WERMELINGER, L. S.; ALMEIDA, I. C.; ZINGALI, R. B.; FERNANDES, P. M. B. Label-free quantitative proteomics reveals differentially regulated proteins in the latex of sticky diseased *Carica papaya* L. plants. **Journal of Proteomics**, 75, p. 3191-3198, 2012.

ROOSSINCK, M. J. Lifestyles of plant viruses. **Philosophical transactions of the Royal Society of London. Series B, Biological sciences**, 365, n. 1548, p. 1899-1905, 2010.

ROOSSINCK, M. J. Plant virus ecology. **PLoS Pathog**, 9, n. 5, p. e1003304, 2013.

SEO, J.-K.; KIM, K.-H. Long-Distance Movement of Viruses in Plants. *In*: WANG, A. e ZHOU, X. (Ed.). **Current Research Topics in Plant Virology**. Cham: Springer International Publishing, 2016. p. 153-172.

SOARES, E.; WERTH, E. G.; MADROÑERO, L. J.; VENTURA, J. A.; RODRIGUES, S. P.; HICKS, L. M.; FERNANDES, P. M. B. Label-free quantitative proteomic analysis of pre-flowering PMeV-infected *Carica papaya* L. **Journal of Proteomics**, 151, p. 275-283, 2016.

TALIANSKY, M. E.; ROBINSON, D. J. Molecular biology of umbraviruses: phantom warriors. **Journal of General Virology**, 84, n. 8, p. 1951-1960, 2003.

TAN, D.; HU, X.; FU, L.; KUMPEANGKEAW, A.; DING, Z.; SUN, X.; ZHANG, J. Comparative morphology and transcriptome analysis reveals distinct functions of the primary and secondary laticifer cells in the rubber tree. **Scientific Reports**, 7, n. 1, p. 3126, 2017/06/09 2017.

TAVARES, E. T.; TATAGIBA, J. S.; VENTURA, J. A.; SOUZA JR, M. T. Dois novos sistemas de diagnose precoce da meleira do mamoeiro. **Fitopatologia Brasileira**, 29, n. 5, p. 563-566, 2004.

VENTURA, J. A.; COSTA, H.; TATAGIBA, J. D. S. Papaya Diseases and Integrated Control. *In*: NAQVI, S. A. M. H. (Ed.). **Diseases of Fruits and Vegetables: Volume II: Diagnosis and Management**. Dordrecht: Springer Netherlands, 2004. p. 201-268.

YAMAMOTO, H.; TABATA, M. Correlation of papain-like enzyme production with laticifer formation in somatic embryos of papaya. **Plant Cell Rep**, 8, n. 4, p. 251-254, Apr 1989.

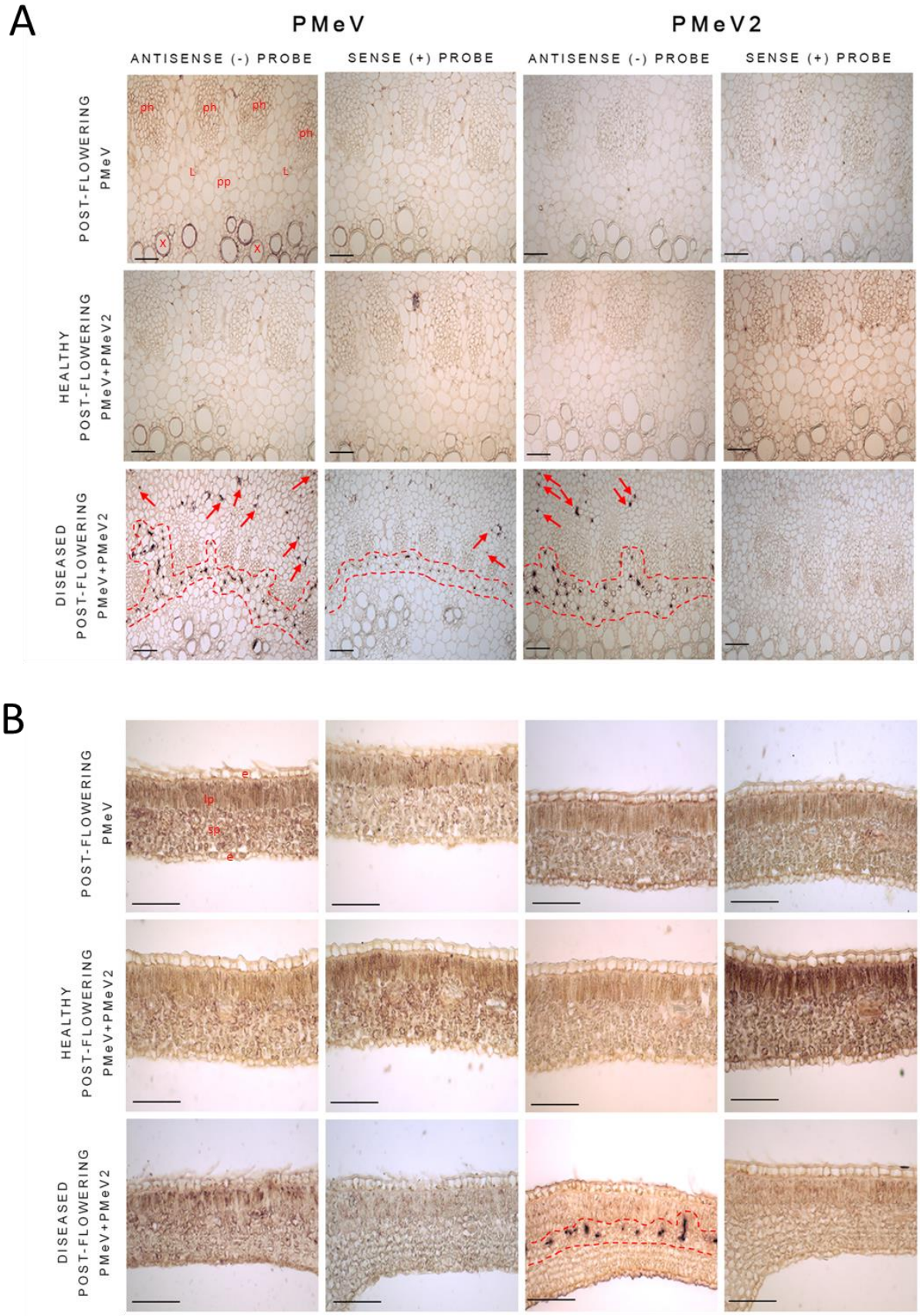
ZENG, Y.; JI, B.; YU, B. Laticifer ultrastructural and immunocytochemical studies of papain in *Carica papaya*. **Acta Botanica Sinica**, 36, n. 7, p. 497-501, 1994.

TABLES

Table 1. Results of RT-PCR diagnosis monitoring PMeV complex during somatic embryogenesis in *Carica papaya*

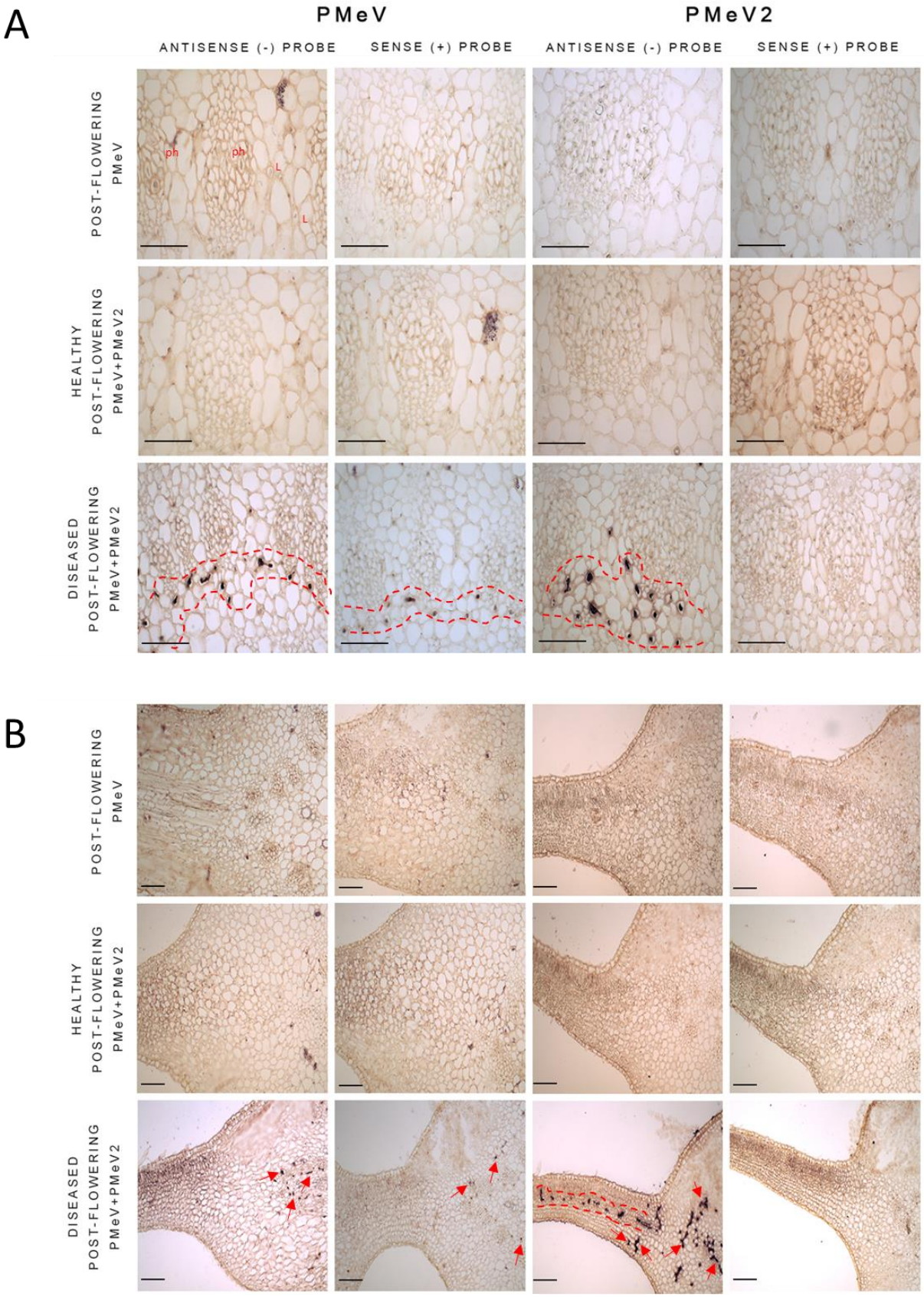
	No. infected/total	
Plant material	**PMeV**	**PMeV2**
Donor-plant	4/4	4/4
3-month-old calli	8/16	0/16
Embryogenic calli	6/6	6/6
SE-regenerated plants	16/16	16/16

FIGURES


A
PMeV
PMeV2
ANTISENSE (-) PROBE
SENSE (+) PROBE
ANTISENSE (-) PROBE
SENSE (+) PROBE
POST-FLOWERING PMeV
HEALTHY POST-FLOWERING PMeV+PMeV2
DISEASED POST-FLOWERING PMeV+PMeV2
ph
L
pp
X
B
POST-FLOWERING PMeV
HEALTHY POST-FLOWERING PMeV+PMeV2
DISEASED POST-FLOWERING PMeV+PMeV2
e
lp
sp

1425

Figure 1. PMeV complex localization in the main vein and mesophyll laticifers cells of *C. papaya* by *in situ* hybridization. Cross sections of main vein (**A**) and mesophyll (**B**) obtained from infected leaves were hybridized with a specific probe for PMeV and PMeV2 sense and antisense strand. Red arrows and dashed lines representing laticifers where the signal was visualized. ph: phloem; L: laticifer; x: xylem; pp: parenchyma; e: epidermis; lp: lacunar parenchyma; sp: spongy parechyma.


A
PMeV
PMeV2
ANTISENSE (-) PROBE
SENSE (+) PROBE
ANTISENSE (-) PROBE
SENSE (+) PROBE
POST-FLOWERING PMeV
HEALTHY POST-FLOWERING PMeV+PMeV2
DISEASED POST-FLOWERING PMeV+PMeV2
ph
ph
L
L
B
POST-FLOWERING PMeV
HEALTHY POST-FLOWERING PMeV+PMeV2
DISEASED POST-FLOWERING PMeV+PMeV2

1434

Figure 2. PMeV complex localization in the main vein and mesophyll laticifers cells of *C. papaya* by *in situ* hybridization. Cross sections obtained from infected leaves were hybridized with a specific probe for PMeV and PMeV2 sense and antisense strand. **A.** Detail of phloem bundles of the main vein. **B.** Transitioning region of main vein to mesophyll. Red arrows and dashed lines representing laticifers where the signal was visualized.ph: phloem; L: laticifer.

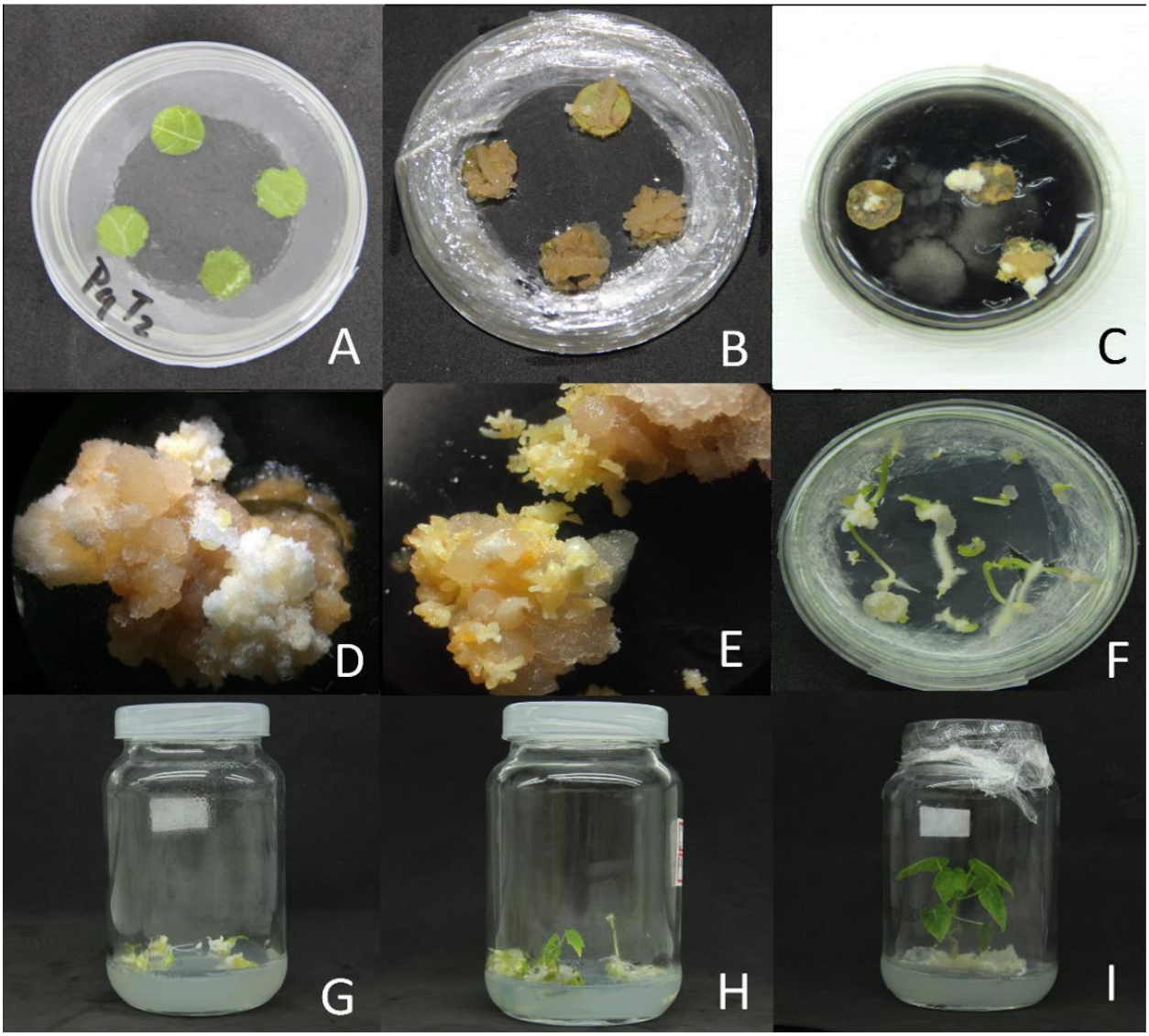


Figure 3. Development stages of somatic embryogenesis in *Carica papaya.* **A.** Establishment of ES, from infected leaf discs grown in semi-solid medium supplemented with 2,4-D- at 1st day of cultivation. **B.** Callus proliferation in 45 days of cultivation. **C.** Proliferation of embryogenic calluses in semi-solid medium supplemented with activated carbon. **D.** Non-embryogenic callus. **E.** Embryogenic callus. **F.** Somatic embryos germinating in germination medium after 2 weeks of cultivation. **G-I**. The sequential growth process of papaya seedlings obtained by ES in vitro. **I**. Seedlings regenerated in basal MS medium, without the addition of growth regulators. 3-month-old callus (not shown), somatic embryogenic callus (E), and regenerated plants (I) were used for monitoring PMeV complex.

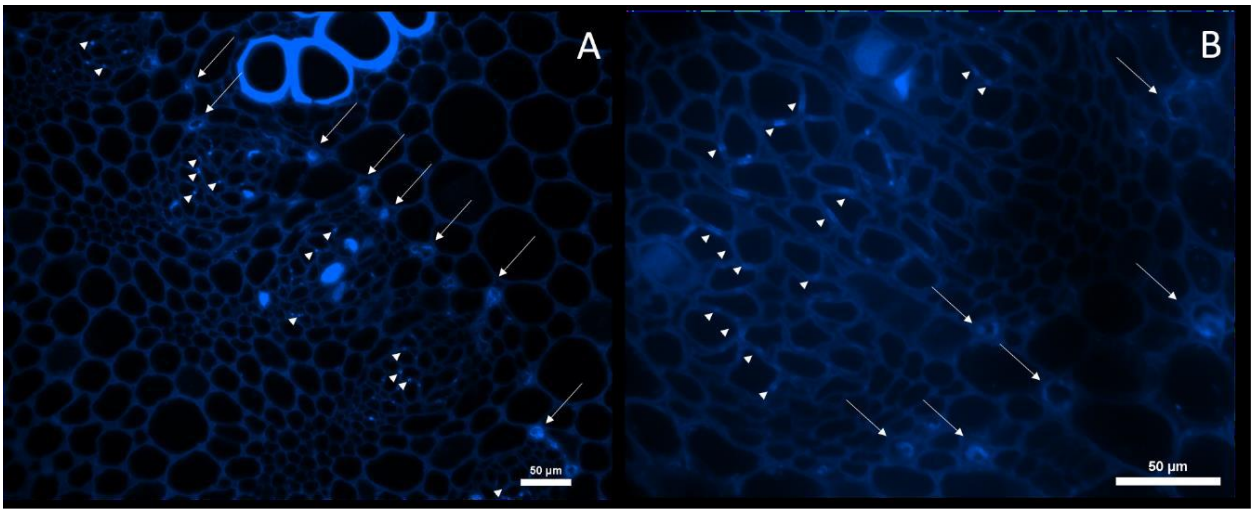
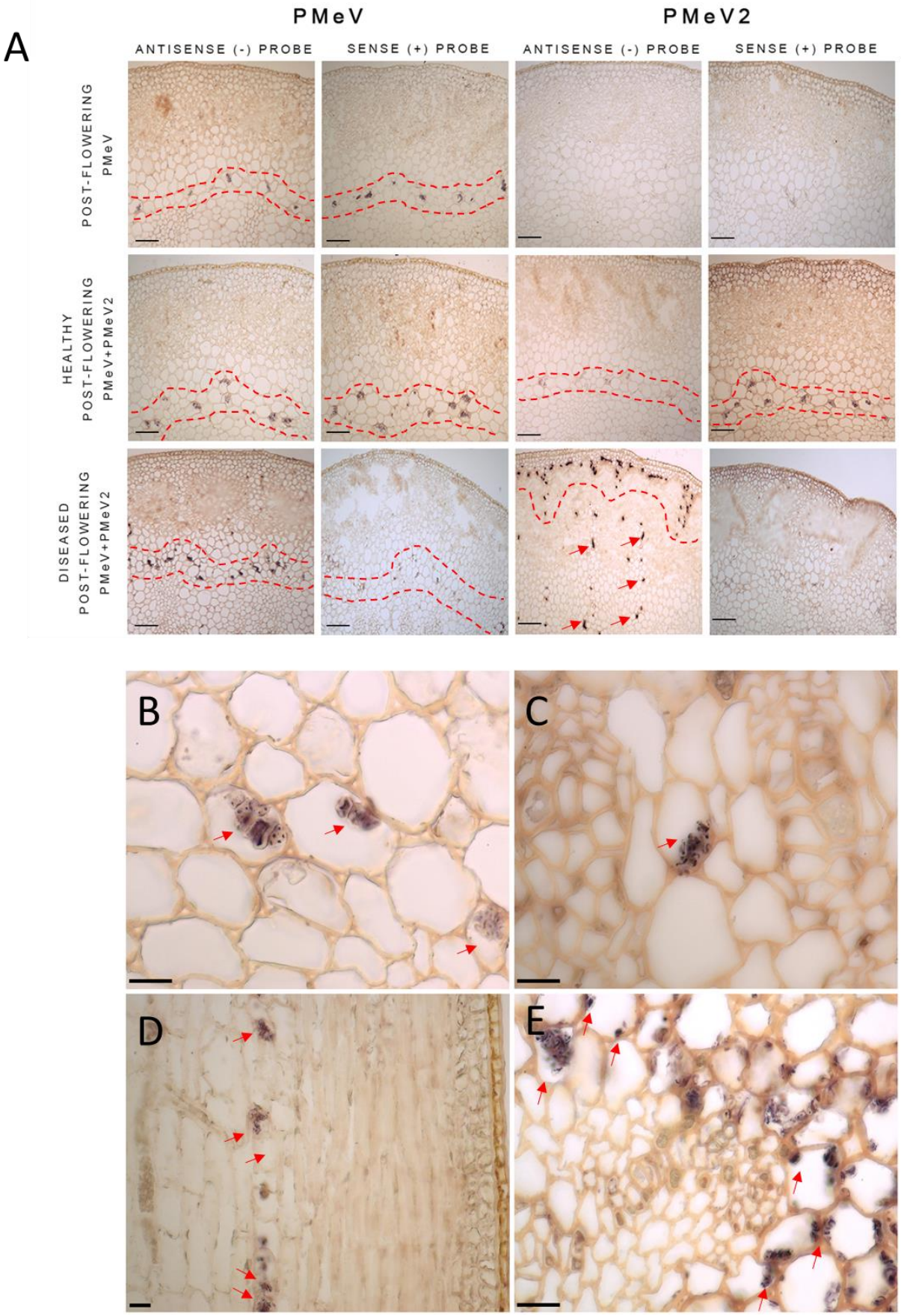


Figure 4. Identification of plasmodesmata in vascular tissue cells of *C. papaya* by aniline blue staining. Cross-sections obtained from leaves were observed under a fluorescence microscope. **A** and **B**. Visualization of plasmodesmata between phloem cells. No plasmodesmata are visualized between laticifers and adjacent cells. White arrowheads: plasmodesmata stained with aniline blue; white arrows: laticifers.

SUPPLEMENTARY MATERIAL

Figure S1. Hybridization signal observed in structures that resemble plastids. **A.** Signal is distributed as a ring near to the epidermis in cross sections of the main vein. **B.** Detail of parenchyma cells near to the epidermis. **C.** Detail in parenchyma cells between two phloem bundles. **D.** Longitudinal section of the parenchyma near to the main vein collenchyma. **E.** Transversal section of the transition between main vein to mesophyll.


A
PMeV
PMeV2
ANTISENSE (-) PROBE
SENSE (+) PROBE
ANTISENSE (-) PROBE
SENSE (+) PROBE
POST-FLOWERING
PMeV
HEALTHY
POST-FLOWERING
PMeV+PMeV2
DISEASED
POST-FLOWERING
PMeV+PMeV2
B
C
D
E

1468

MANUSCRIPT #3. A CAPSID PROTEIN FRAGMENT OF PAPAYA MELEIRA VIRUS (PMeV), A TOTI-LIKE VIRUS FOUND IN PAPAYA LATEX INTERACTS WITH THE 50S RIBOSOMAL PROTEIN L17

Manuscript in preparation for *Molecular Plant Pathology* (ISSN 1464-6722; IF 5.663, 2021; Qualis A1 Biotecnologia 2013-2016)

A capsid protein fragment of papaya meleira virus (PMeV), a toti-like virus found in papaya latex interacts with the 50S ribosomal protein L17

Marlonni Maurastoni[1], Tathiana F. Sá-Antunes[1], Emanuel F. Abreu[2], Angela Mehta[2], Marcio M. Sanches[2], W. Fontes[3], Fabiano T. Cruz[1], Alexandre M. C. Santos[1], Francisco J. L. Aragão[2], Elliot W. Kitajima[4], Simone G. Ribeiro[2], Francisco M. Zerbini[5], Anna E. Whitfield[6] and Patricia M. B. Fernandes[1]

[1]Núcleo de Biotecnologia, Universidade Federal do Espírito Santo, Vitória, Espírito Santo, Brazil;

[2]Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, 70770-900, Brazil

[3]Toxinology Laboratory, Department of Physiological Sciences, University of Brasilia, Brasilia/DF, Brazil, 70.910-900

[4]Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, CEP, Piracicaba, SP, 13418-400, Brazil

[5]Dep. de Fitopatologia/BIOAGRO, Universidade Federal de Viçosa, 36570-900, Viçosa, Minas Gerais, Brazil.

[6]Department of Entomology and Plant Pathology, North Carolina State University, 840 Main Campus Drive, Raleigh, NC 27606, USA

ABSTRACT

An unusual trans-encapsidation phenomenon is observed in the PMeV complex, the association of a totivirus-like and a umbravirus-like associated RNA (ulaRNA), papaya meleira virus (PMeV), and papaya meleira virus 2 (PMeV2), respectively. Both viruses are encapsidated by particles produced by PMeV coat protein (CP) which is a translation product of the putative PMeV ORF1, predicted to encode a polypeptide of 177kDa. In previous work, purification from papaya latex by sucrose gradient followed by mass spectrometry analysis identified nine peptide fragments accounting for 8% of the PMeV ORF1 predicted amino acid sequence (125 of 1563 aa) and encompassing the central region of the putative protein (from aa 356 to 785). However, the structural proteins of PMeV capsid remain unknown. In this work, an additional purification step, a cesium chloride gradient, resulted in obtaining high- and low-density fractions that both enriched for viral particles with similar morphology as visualized by transmission electron microscopy. The separation of these fractions by SDS-PAGE revealed the presence of two major polypeptides with a molecular mass of ~40kDa and ~55kDa. Peptide mass fingerprint analysis of both bands revealed overlapping peptides that match with the PMeV ORF1 increasing its coverage to the N-terminal side. To identify interactions between PMeV capsid protein and plant proteins, a yeast two-hybrid assay identified several *Arabidopsis* proteins potentially interacting with a fragment of these polypeptides. A PPI network using differentially accumulated proteins of PMeV complex host (*Carica papaya*) and PMeV CP fragment-interacting protein shows the 50S ribosomal protein L17 family protein (RPL17) as an important player potentially associated with modulated translation-related proteins. The *At*RPL17 co-localizes with PMeV CP fragment when transiently expressed and shows interaction with PMeV CP fragment in vivo by BiFC and yeast two-hybrid. We speculate that the interaction of capsid protein with RPL17 could be an important player in the totivirus-like virus-plant interactions.

Keywords: totivirus; protein-protein interaction; coat protein.

INTRODUCTION

Additional functions have been reported for coat proteins (CP) of plant viruses in addition to protection of the viral genome. The multifunctional nature of a CP is observed in its roles as participating in the virus cell-to-cell and systemic movement, component of genome transcription and replication complex, modulating host defense pathways, and processing host mRNA (A. CALLAWAY *et al*., 2001; BOL, 2008; HERRANZ *et al.*, 2017; VAIRA *et al.*, 2018). Dimers of a single CP are the building blocks of icosahedral capsids, mainly found among dsRNA viruses which usually need the enclosed structure to avoid host cell defense mechanisms. This T = 1 capsid, comprised of 120 subunits of 60 asymmetrical polypeptides, are found in members of the families *Reoviridae*, *Picobirnaviridae*, and *Cystoviridae*, and in the mycoviruses of the families *Totiviridae*, *Partitiviridae*, and *Megabirnaviridae* (LUQUE *et al.*, 2018).

Most of our knowledge regarding non-structural functions of totiviruses capsids relies on *Saccharomyces cerevisiae L-A virus* (ScV-L-A), the type species of the genus *Totivirus* (family *Totiviridae*). A remarkable function is performed by this virus and its host cell. To avoid viral RNA degradation by host exoribonuclease, ScV-L-A decaps the 7-methylguanosine 5'-monophosphate (m7GMP) from host mRNA hijacking it for its RNAs. This enzymatic activity is performed by the segment Gln139-Ser182 of ScV-LA capsid (FUJIMURA; ESTEBAN, 2011). To date, only a few totiviruses have been found infecting plants (AKINYEMI *et al.*, 2018; GUO *et al.*, 2016; ZHANG *et al.*, 2021). Our main understanding of how plants respond to totiviruses comes from the papaya sticky disease (PSD) pathosystem which has been studied at the biochemical and molecular level (ABREU *et al.*, 2015; MADROÑERO *et al.*, 2018; RODRIGUES *et al.*, 2011; RODRIGUES *et al.*, 2012; SÁ ANTUNES; *et al.*, 2020; SOARES *et al.*, 2016). Proteomic and transcriptomic analysis of infected *Carica papaya* plants shows a tolerance mechanism to symptoms before flowering, mainly related to changes in hormone-responsive genes, protein turnover, and chloroplast-related proteins. Although we know the effect of infection

on the accumulation of proteins and transcripts, there is still a lack of information about the key aspects of the plant-virus interaction and data on the viral protein-plant protein interactions, which hinders the development of more effective strategies to control PSD.

The PSD is associated with the PMeV complex which is comprised of papaya meleira virus (PMeV), a toti-like dsRNA virus, and papaya meleira virus 2 (PMeV2), a umbravirus-like associated RNA (ulaRNA) ssRNA virus (LIU *et al.*, 2021). Similar to the virus-virus interaction between the dsRNA yadokari virus 1 (YkV1) and the positive-sense single-stranded [(+) ssRNA] yadonushi virus 1 (YnV1) (DAS *et al.*, 2021), a trans-encapsidation phenomenon is observed between the PMeV complex as they are both encapsidated in particles formed by PMeV CP. The full-length PMeV ORF1 is predicted to encode a polypeptide of 1563 aa which is 20-26% identical to analogous proteins found in mycoviruses, but these putative proteins have no significant matches in protein databases. The PMeV CP is known to be part of the predicted PMeV ORF1 polypeptide (ANTUNES et al., 2016). Although the precise PMeV CP sequence remains unknown, virus purification from papaya latex by sucrose gradient followed by mass spectrometry analysis identified nine peptide fragments accounting for 8% of the PMeV ORF1 predicted amino acid sequence (125 of 1563 aa) encompassing the central region of the putative protein (from aa 356 to 785) (ANTUNES *et al.*, 2016).

We report herein that PMeV capsid, coded by PMeV ORF1, contains two major polypeptides with overlapping sequences. We sought to identify *Arabidopsis* proteins that interact with two fragments of PMeV ORF1 by using a yeast two-hybrid (Y2H) assay which identified 28 interacting proteins mostly targeted to the chloroplast. We also built a protein-protein interaction (PPI) network showing that PMeV capsid can be indirectly responsible for the modulation of several proteins during pre- and post-flowering stages of infected papaya plants. We speculate that one of these proteins, a 50S ribosomal protein L17 family protein (RPL17) can be an important target to modulate virus infection.

METHODS

Virus purification and polypeptide composition

Sucrose gradient purification was performed according to (ANTUNES *et al.*, 2016). Additional purification was performed by layering all the three fractions collected from the sucrose gradient onto a 50% (w/w) CsCl gradient and centrifuged for 18 h at 145,000 g. Fractions were collected and centrifuged again for 3.5 h at 35,000 g. The final pellet was resuspended in 0.01M borate buffer pH 9.0. To visualize viral particles, viral preparations were negatively stained in 2% (w/v) potassium phosphotungstate, pH 6.8, and observed in a JEOL JEM-1011 transmission electron microscope (TEM).

To determine the composition of structural proteins, preparations of CsCl purified virions were applied to a 15% (w/v) polyacrylamide gel after boiling in Laemmli buffer (LAEMMLI, 1970). Following electrophoresis, the gel was stained overnight with 0.1% (w/v) Coomassie blue and destained for 1 h to visualize the separated proteins. The molecular mass of the proteins was estimated from the measurement of electrophoretic mobilities (WEBER; OSBORN, 1969). Polypeptides p40 and p55 obtained in the gel from M fraction were subjected to in situ digestion according to Shevchenko *et al*. (2006) and subsequent mass spectrometry analysis using a Bruker Autoflex II TOF/TOF instrument (Bruker Daltonics, Bremen, Germany). Samples were resuspended in acetonitrile/water (1:1, v/v) containing TFA 0.1% (v/v), and 1 µL of sample was applied to a stainless-steel plate with 1 µL of HCCA (10 mg/mL). The data were analyzed using MASCOT software (Matrix Sciences, Chicago, IL, USA). The search parameters used were: type of search as peptide mass fingerprint, enzyme as trypsin, fixed modification as carbomidomethyl (C), variable modification as oxidation (M), mass values as monoisotopic, protein mass as unrestricted, peptide mass tolerance as ± 200 ppm, peptide charge state as 1+ and max missed cleavages as 1. Search results with significant MASCOT scores (>90) were taken into consideration to identify the peptides matching with the predicted PMeV ORF1. To avoid missing any proteins during viral preparation, crude virion preparations of M fraction

obtained after the sucrose gradient were subjected to protein extraction according to Carmo *et al*. (2013) and its peptides were identified as described above.

Secondary structure prediction

The secondary structure prediction was done with five different prediction tools Frag1D, Porter 5.0, PsiPred, RaptorX, and SOMPA and a consensus structure was generated using the combined results.

Cloning of PMeV ORF1 and *At*RPL17

To generate a PMeV ORF1 clone, total RNA was extracted from 100 µl of a pool of latex obtained from PSD plants using TRIzol® reagent (Thermo Fisher Scientific, Walthan, MA, USA), according to the manufacturer's protocol. One microgram of total RNA was treated with DNAse I (Thermo Fisher Scientific, Walthan, MA, USA) and used for cDNA synthesis using Superscript III Reverse Transcriptase and the sequence-specific reverse primer according to the manufacturer’s instructions. PCR was performed using Platinum High Fidelity DNA Polymerase (Invitrogen, Walthan, MA, USA) and specific primers following the manufacturer’s instructions in a Mastercycler Thermocycler (Eppendorf, Hamburg, Germany). PCR amplicon was visualized on 1% agarose gel, excised, and purified using PureLink™ Quick Gel Extraction Kit (Thermo Fisher Scientific, Walthan, MA, USA). The amplicon was cloned into Gateway™ pDONR™221 Vector and the whole clone was submitted to Sanger sequencing. The clone was named pDONR™221-PMeVORF1. To generate PMeV ORF1 fragments, a total of 10 primers pairs were designed based on the pDONR™221-PMeVORF1 sequence. *At*RPL17 full-length gene was amplified from a

pGADT7 plasmid recovered from an *Arabidopsis* cDNA library in yeast. The plasmid was sequenced and compared against the NCBI database. Amplicons from both PMeV ORF1 fragments and *At*RPL17 were cloned into pENTR-D/TOPO then recombined to yeast and plant expression vectors using Gateway cloning techniques. Primers used in this work are listed in the supplementary material (Supplementary materials Table S1).

Binary interactions and library screening using yeast two-hybrid assay

A yeast two-hybrid system (Y2H) was used to test binary interactions between PMeV ORF1 fragments. We recombined a pENTR-D/TOPO clone containing a PMeV ORF1 fragment with the pDESTGADT7 and pDESTGBKT7 destination vectors to generate a protein fused to the GAL4 activating domain (AD) and -binding domain (BD). Briefly, the yeast reporter strain Y2HGold (Takara Bio Inc., Kusatsu, Shiga, Japan) was co-transformed with both AD- and BD- plasmids according to the manufacturer's instruction. Co-transformants were selected by culture on double dropout media (DDO), i.e. synthetic defined minimal media (SD) lacking leucine and tryptophan (SD/-L/-W). Positive interactions were selected by culture on quadruple dropout media, i.e. SD lacking leucine, tryptophan, adenine, and histidine, (SD/-L/-W/-H/-A/-). We co-transformed plasmids containing GAL4 DNA-BD fused with murine p53 (pGBKT7-p53) plus GAL4 AD fused with SV40 large T-antigen (pGADT7-T) as positive control and GAL4 BD fused with lamin (pGBKT7-Lam) plus pGADT7-T as a negative control. We also tested the autoactivation of each fragment by co-transforming BD and AD plasmids with empty plasmids. An overnight culture of all co-transformants was normalized to OD_{600} 2, spotted in DDO and QDO/X/A (QDO media supplemented with Aureobasidin A and the chromogenic substrate X-alpha-gal) plates, and kept at 30°C for 4 days. The entire experiment was performed three times.

Yeast two-hybrid library screening was performed using the Matchmaker® Mate & Plate Two-Hybrid System (Takara Bio Inc., Kusatsu, Shiga, Japan). To choose the bait, we performed an expression assay in yeast following the manufacturer's instructions. Plasmids containing the fragments fused to GAL4 BD were transformed in the yeast strain Y2HGold according to the manufacturer's protocol and spread on single dropout media (SDO), i. e. SD lacking tryptophan (SD/-W). Protein expression was induced by growing the positive transformants on YPD media. Yeast proteins were extracted according to Kushnirov (2000) and normalized to the equivalent of 2-3 OD_{600} units. The expression of fused proteins was verified by SDS-PAGE and Western blotting assay using mouse anti-myc monoclonal antibody (Invitrogen, Waltham, Massachusetts) at 2: 10,000 dilution followed by incubation with a goat anti-mouse IgG (H+L)-HRP Conjugated (Biorad, Hercules, California) at 1: 10,000 dilution according to standard protocols.

A normalized cDNA *Arabidopsis* library fused to GAL4 AD in the yeast strain Y187 (Mate & Plate™ Library - Takara Bio Inc., Kusatsu, Shiga, Japan) was used to mate with Y2HGold containing the ORF1 fragment 4 fused to GAL4 BD. Cells were initially screened on 60 150mm plates containing DDO/X/A media. Then, 306 blue colonies were patched on QDO/X/A media and QDO/X/A media supplement with 2.5mM and 5mM of 3-Amino-1,2,4-triazole (3-AT). Plasmids from colonies picked from all media were extracted from yeast, recovered in *Escherichia coli*, sequenced, and identified.

A total of 28 unique plasmids were transformed in yeast with an empty GAL4 BD plasmid and fragment 4 AD-containing plasmid to test autoactivation and interaction, respectively. Given that most of our peptides obtained in the mass spectrometry analysis covered the fragment 2, we took advantage of the interaction between fragment 4 and fragment 2 (see results section) and also tested the 28 unique plasmids interaction with fragment 2. Positive interactors were spotted in DDO, QDO/X/A, and QDO/X/A supplemented with 0mM, 1mM, 2.5mM, and 5mM of 3–AT media. The interaction of pGBKT7-p53 and pGADT7-T was used as positive control and pGBKT7-Lam and pGADT7-T as a negative control. The experiment was performed three times.

Protein-protein interaction network

A protein-protein interaction network was constructed with differentially modulated proteins of PMeV complex-infected papaya at 4- and 7-months post-germination (SOARES et al., 2016), and PMeV ORF1 fragment 2 and 4-interacting proteins. Biomart (https://phytozome.jgi.doe.gov/biomart/) was used to obtain *Arabidopsis* orthologues from the Phytozome database (http://phytozome.jgi.doe.gov/) in April 2021. Sequences were uploaded on String (SZKLARCZYK *et al*., 2018) and an interaction network of high confidence level was exported to Cytoscape (SHANNON *et al*., 2003).

Transient expression and detection of protein interaction in *Nicotiana benthamiana*

To identify the interaction of PMeV capsid protein and RPL17 in vivo, we first aimed to localize RPL17 and PMeV fragment 2 in plant cells fusing proteins to red or green fluorescent proteins. For EGFP fusions, we used the binary plasmids pSITE-2CA and pSITE-2NB and for RFP fusions we used pSITE-4CA and pSITE-4NB. “-CA” or “-NB” indicates that the plasmid allows cloning of fluorescent protein fused to the N-terminus and C-terminus of the desired protein, respectively (CHAKRABARTY *et al*., 2007). For the bimolecular fluorescence complementation (BiFC) assay, we recombined *At*RPL17 and ORF1 fragment 2 into the BiFC vectors (pSITE-nen, pSITE-cen, pSITE-nec, pSITE-cec) as fusions either the C- or N-terminal sequence of both halves of the eYFP gene (MARTIN *et al*., 2009). As a negative control, we challenged fragment 2 with Glutathione S-transferase (GST) fusions to YFP halves. A positive interaction was considered if the fluorescence was above the observed as for the negative control and if at least 50 cells with similar localization signal for the interaction were visualized. For Agrobacterium-mediated expression, an overnight culture of *A. tumefaciens* cells containing the plasmids

was inoculated in a fresh media containing the appropriated antibiotics and brought to standard concentration (OD_{600}=0.5-1.0) in 10mM MES buffer, pH 5.6, containing 10mM $MgCl_2$. The culture was incubated for at least 2 h at 28°C with no agitation in the presence of 200µM acetosyringone (Sigma-Aldrich, St. Louis, MO, USA).

Agroinfiltration was performed using 1 ml syringes without a needle on the abaxial side of *N. benthamiana* leaves wild type or transgenic expressing the histone 2B protein fused to RFP (MARTIN *et al.*, 2009). Leaves were analyzed for five days after agroinfiltration. Transient expression and localization of fluorescent fusion proteins, EGFP, RFP, or YFP in leaf cells of *N. benthamiana* were visualized using BioTek ® Cytation 5 image reader. Images were analyzed in Gen5 version 3.04 Microplate reader and imager software. This experiment was performed three times, with a least two leaves per infiltration treatment.

RESULTS

PMeV capsid is composed of two major polypeptides with overlapping sequences

To determine the composition of PMeV capsid, virus particles were purified using sucrose gradient centrifugation followed by a CsCl isopycnic gradient purification. Three opalescent zones, as reported by Antunes *et al*. (2016), were visible in the sucrose gradient. The additional purification in the CsCl gradient yielded bands for the top and bottom fraction, while the middle fraction appeared as a zone (Figure 1.A). The presence of nucleic acid in these bands was confirmed through RNA extraction from each fraction (Figure 1.B). Fraction B contained a single band of the approximate size of PMeV. RNAs extracted from fraction M present a band of the approximated size of PMeV2 and a smear above this band. The presence of PMeV and PMeV2 was also confirmed by visualization of viral particles by transmission electron microscopy (Figure 1.C). Fraction M contained

a higher number of particles compared to fraction B, which shows a fibrillary material associated with them. No nucleic acid or viral particle was obtained for the top fraction. Purified virions from both B and M fractions showed two major polypeptides after SDS-PAGE with a molecular mass of approximately 55kDa and 40kDa, named p55 and p40 (Figure 1.D). Mass spectrometry analysis from both p40 and p50 shows an accumulation of overlapping peptides extending from aa 287 to 731 (Figure 3.E). Three additional minor bands of higher molecular mass, named p68, p85, and p100, and several minor bands below p40 were also visualized, but no matches with PMeV or PMeV2 sequences were obtained for these polypeptides.

PMeV ORF1 predicted protein is mostly composed of alpha-helix

A consensus sequence based on the secondary structure prediction of five prediction tools was built for PMeV ORF1 (Figure 2). PMeV ORF1 has 34 alpha helixes, 39 beta-sheets, and 11 non-consensus structures, which account for 22.3 %, 11.3 %, and 3.3 % of the whole sequence, respectively. Three low complexity regions extending from aa 105 to 125, 214 to 227, and 1260 to 1270 are also found in the sequence. No other domains are found in protein database.

A central region of the PMeV complex capsid protein interacts with a PMeV ORF1 fragment (aa 961-1200)

To determine dimerization regions on PMeV capsid protein, we tested binary interactions of the full length and five non-overlapping fragments of PMeV ORF1 using a yeast two-hybrid system (Takara®) (Figure 3.A). ORF1 fragments for expression in yeast were selected based on secondary structure (no disruption of predicted alpha-helix or beta strands, i.e., the fragments start and end at random coil regions), and peptide coverage of the polypeptide obtained from purified particles. The five non-overlapping fragments tested were named CP1 (aa 1 to 320), CP2 (aa 321 to 670), CP3 (aa 671-960), CP4 (aa 961-1200), and CP5 (aa 1201-1563). Binary interactions were identified between CP2 and CP4, CP3 and CP4, and a self-interaction for CP4. No interaction was identified for the full-length protein, CP1 or CP5.

Arabidopsis library screening using 961-1200 ORF1 fragment identified 28 plant interacting proteins

A commercial *Arabidopsis* cDNA library (Mate & Plate^{TM} Library - Takara Bio Inc., Kusatsu, Shiga, Japan) was used to identify host proteins interacting with PMeV ORF1 fragments. The best candidate to use as bait was determined after testing the expression of each fragment fused to the GAL4-binding domain (Figure 4.B). Although most of the peptides identified from the mass spectrometry assay obtained from the major polypeptides match with CP2, this fragment showed a lower level of expression when compared to CP4. Moreover, given that CP4 interacts with CP2 in our binary yeast two-hybrid assay, we decided to use CP4 as bait and validate the interactors with both fragments. Using a GAL4-based yeast two-hybrid system, independent yeast transformants were screened using CP4 as bait. Three hundred six colonies, 36 and 48 were screened from QDO/X/A supplement with 0mm, 2.5mM, and 5mM of 3-AT, respectively. From 48 colonies sequenced, 28 represented unique sequences that also interact with CP2 (Table 1; Supplementary Materials Figure S1). A functional categorization analysis shows that most proteins are targeted to the chloroplast and have protein binding and catalytic activity (data not shown).

CP2 putatively associates with translation-related proteins differentially modulated in pre-flowering papaya

Arabidopsis orthologues were obtained from a list of differentially accumulated proteins of pre-and post-flowering PMeV complex-infected *C. papaya* and submitted to protein-protein interaction (PPI) analysis. From 130 differential accumulated proteins at pre-flowering, it was possible to retrieve 101 orthologues in *Arabidopsis* (Supplementary Materials Table S2), while at post-flowering 123 *Arabidopsis* proteins were obtained from 160 differential accumulated proteins (Supplementary Materials Table S3). Two PPI networks were built including the 28 CP2 and CP4-interacting proteins (Figure 4 and Supplementary Materials Table S4 and S5). In both networks, *At*RPL17 appears consistently predicted to interact with several modulated proteins, mainly translation-related proteins.

CP2 and *At*RPL17 co-localize and interact in *Nicotiana benthamiana* cells

To identify the interaction of PMeV capsid and *At*RPL17 we first determine the localization of CP2 and *At*RPL17 in plant tissue fusing GFP to the protein C- and N-terminus. CP2 is detected at the nucleus and cytoplasm; no frequent fluorescent signals were detected when GFP was fused to the N-terminal end of CP2. In the cytoplasm, punctate fluorescent signals are observed in epidermal cells (Figure 5.A). GFP::*At*RPL17 was observed at nucleus and chloroplasts (Figure 5.A). Co-localization of RFP::*At*RPL17 and CP2::GFP was observed as punctate signals (Figure 5.B). No changes in the localization of *At*RPL17 or CP2 were observed when proteins were co-infiltrated. BiFC assays showed that both

proteins do not interact in the nucleus as observed by the agroinfiltration with the transgenic *N. benthamiana* expressing RFP fused to the histone protein 2B (Figure 5.C and D).

DISCUSSION

Viruses tentatively classified in the *Totiviridae* family are found infecting a plethora of organisms: filamentous fungi, yeast, parasitic protozoa, mollusks, arthropods (including mosquitoes, ants, shrimps, and planthoppers), and plants (DE LIMA *et al.*, 2019). Our knowledge of interactions between totiviruses and plants is still very scarce, as the best-studied totiviruses are found infecting fungi. However, the papaya sticky disease pathosystem has been studied at the transcriptome and proteome level which revealed that papaya plants present a delay in the appearance of the symptoms until flowering due to a multilayered tolerance mechanism (ANTUNES *et al*., 2020, MADRONERO et al., 2018). Even though, key aspects of the viral protein-plant protein interactions in this pathosystem are still unknown, which hinders the development of more effective strategies to control PSD. In this scenario, capsid proteins are an important target of study as they possess a multifunctional nature (A. CALLAWAY *et al*., 2001; BOL, 2008; HERRANZ *et al.*, 2017; VAIRA *et al.*, 2018).

Here we show that the capsid of PMeV, a totivirus-like virus, is mainly composed of two major polypeptides with overlapping sequences matching with PMeV ORF1. A central region of these polypeptides, named here as CP2, interacts with 28 proteins mostly targeted to the chloroplast. One of these proteins, the 50S ribosomal protein L17 family protein (RPL17), co-localizes and interact with CP2 in *Nicotiana benthamiana* cells and putatively associates with translation-related proteins differentially modulated in pre-flowering papaya.

It has been pointed out that PMeV complex viral structural proteins could be originated from self-cleavage or cleavage by papaya latex proteases of the PMeV ORF1 translated product (ANTUNES et al., 2016; ANTUNES et al., 2020). The first analysis to identify the nature of PMeV coat protein applied trypsin digestion to the crude sucrose-purified virions followed by mass spectrometry analysis identifying peptides matching with the predicted PMeV ORF1 sequence (ANTUNES *et al.*, 2016). The

polypeptide coded by PMeV ORF1 is predicted to be 177kDa, which is unusual because most totiviruses coat proteins range in molecular mass from 70-100kDa (DE LIMA *et al.*, 2019). The additional CsCl purification followed by SDS-PAGE led us to identify the polypeptides of PMeV complex viral preparations. Both particles from M and B fractions presented the same banding profile on SDS-PAGE giving another support to the transcapsidation between these viruses. Interestingly peptide mass fingerprinting of the major polypeptides identified in CsCl preparations, p40 and p55, shows overlapping peptides supporting the idea that the capsid is composed of two proteins with slightly different compositions. The presence of two major components in virus purification is also visualized in the prototype of the genus *Victorvirus* (family *Totiviridae*), Helminthosporium victoriae virus 190S (HvV190S). Only one ORF1 is predicted for HvV190S but SDS-PAGE of purified virions shows three forms of the CP p88, p83, and p78, named after their relative molecular weights (GHABRIAL; HAVENS, 1992). Interestingly, p83 and p78 are products of the proteolytic processing of p88 although no protease-like protein is coded by HvV190S (HUANG *et al.*, 1997). The presence of two carboxy-terminal proteins making a viral capsid is also found in the Yado-nushi virus 1 (YkV1) a virus tentatively classified in the Totiviridae family (ZHANG et al., 2016). Proteins of 177kDa were not observed in our CsCl virus preparations but in our sucrose purification, even polypeptides with higher molecular mass are visualized (data not shown). This could indicate that a polyprotein is coded by PMeV but is not present in the assembled capsid. In this case, the ORF1 translation product may be separated into different polypeptides, which is not an uncommon phenomenon among viruses in the family *Totiviridae*. Totiviruses in the proposed Artivirus clade, possess 2A-like sequences in their genomes which mediates a skipping effect of the ribosome resulting in an apparent co-translation cleavage of polyproteins, therefore lacking the need of a protease. Pseudo 2A-sites are found in Giardivirus clade but their amino acid composition makes them unlikely to produce a skipping effect (DE LIMA *et al.*, 2019). No 2A-like sequences or pseudo 2A-sites are found in the PMeV ORF1, which suggests another strategy for ORF1 polyprotein processing. Another possibility is that the bands from both p40 and p50 contain more than one protein. Peptides obtained from p40 and p50 matching with PMeV ORF1 span a region with predicted molecular mass higher than their correspondent band. More sensible separation

methods as liquid chromatography or two-dimensional gel electrophoresis could give additional information.

It is noteworthy that PMeV complex is, hitherto, the only viruses described to inhabit papaya laticifer cells, a physical and chemical defense barrier against pathogens. In previous work, we showed that laticifers are the preferred site for PMeV complex accumulation (Manuscript #2). It is during laticifer differentiation that papaya proteases are accumulated. Early laticifer cells undergo autophagy of their well-developed organelles, but later in differentiation, their endoplasmic reticulum split in fragments and initiates the production and accumulation of proteases which are stored within vesicles of the mature laticifer (ANTUNES *et al.*, 2020; ZENG *et al.*, 1994). Diseased plants, however, present a reduction in the accumulation and activity of proteases, mainly cysteine proteases, which is likely to be caused by oxidation of the enzyme active site by hydrogen peroxide, a reactive oxygen species present in high levels in disease latex. This assumption is credited to promote (i) a reduction in latex coagulation facilitating virus flow through the laticifers and (ii) a delay in programmed cell death which has been attributed to PMeV infection (RODRIGUES *et al.*, 2009). Several viruses encode their own proteases which are necessary for post-translational modifications of their polyproteins to ensure that proteins can travel together to the viral assembly site, to ensure the proper time for the initiation of folding and assembly, and to control the concentration of key viral proteins (BABÉ; CRAIK, 1997). However, the ones that do not encode a protease rely on their host counterparts for protein processing, as seen with two members of the *Totiviridae* family. A specific host cysteine protease act as the protein responsible for separate capsid and replicase polyprotein of Giardiavirus (GLV) and Leishmania RNA virus (LRV) (LAGUNAS-RANGEL *et al.*, 2021; YU *et al.*, 1995). Here, we identified 28 Arabidopsis proteins interacting with two fragments of PMeV ORF1. One of these proteins, a cysteine protease, has homologs identified in papaya green tissues and latex (RODRIGUES *et al.*, 2011; RODRIGUES *et al.*, 2012; SOARES *et al.*, 2016), which supports the idea that cysteine proteases are important players during PMeV complex infection of papaya. Using Y2H, no dimerization or expression was detected for the full-length ORF1. Besides yeast, the full-length ORF1 protein is undetectable when expressed in *N. benthamiana*, sf9 insect, and *E. coli* cells (data not shown), indicating that a highly specific environment

is necessary for its correct expression and processing. Indeed, using transmission electron microscopy, Kitajima et al. (1993) analyzed different tissues and organs of asymptomatic and symptomatic papaya tissues looking for PMeV complex capsids that were not observed in any other symptomatic papaya tissue, but laticifers. Papaya latex is mainly composed of lipids, phenols, alkaloids, sugars, and oxalate crystals, polyisoprenes, and mostly proteins (RODRIGUES *et al.*, 2009). In papaya latex, cysteine proteases are only activated upon latex exudation (MOUTIM *et al.*, 1999), which is a spontaneous event in diseased plants. Taken together, PMeV could take advantage of the young active laticifer cells for translation of its ORF1 polypeptide which could be processed upon interaction with papaya cysteine proteases during the spontaneous exudation. Although proteins identified in latex (RODRIGUES *et al.*, 2011; RODRIGUES *et al.*, 2012), are also found in other papaya green tissues (SOARES *et al.*, 2016), their accumulation level associated with physiological changes in laticifer cells might be important for PMeV ORF1 correct expression, processing, and interaction with cellular factors.

The dimerization assay using yeast two-hybrid revealed that CP4 dimerizes and interacts with the other two fragments of PMeV ORF1, CP2 and CP3. Peptides corresponding to CP4 were not obtained in our CsCl purification, but in protein extracts of sucrose purified virions (data not shown), which supports the idea that CP4 is not part of the capsid but it could assist the virus in different moments of the infection through the interaction with plant factors. The yeast two-hybrid screening with *Arabidopsis* library identified 28 proteins interacting with both CP2 and CP4. To choose a protein that could play an important role in the PMeV complex-papaya pathosystem, we built a PPI network using *Arabidopsis* orthologs of *C. papaya* proteins modulated during PMeV infection at 4- and 7 months post-germination (mpg). The 50S ribosomal protein L17 (RPL17) appeared in both scenarios as putatively associated with 4 up-accumulated proteins and 1 down-accumulated protein at 4mpg and 3 up-accumulated proteins and 1 down-accumulated protein at 7 mpg. It is interesting to note that at 4 mpg, RPL17 is associated with several down-accumulated proteins related to the regulation of protein synthesis and ribosome biogenesis, which includes ribosomal protein S13A (RPS13A, AT4G00100), eukaryotic translation initiation factor 3E (EIF3E, AT3G57290), poly(A) binding protein 2 (PAB8, AT1G49760), fibrillarin 2 (FIB2,

AT4G25630) and NOP56-like pre-RNA processing ribonucleoprotein (NOP56-like, AT3G05060). Ribosomal proteins have been reported to directly affect several processes during virus infections, either with a pro- or antiviral activity (LI, 2019; MILLER *et al*., 2021). A proviral function is observed in the translational transactivation of *Cauliflower mosaic virus* (CaMV) in which RPL18 of *Arabidopsis thaliana* interacts with P6 of CaMV in a complex comprised of RPs including L18, L24, and eIF3 (BUREAU *et al*., 2004). On the other hand, it has been shown that RPL10 is an important player in the antiviral defense pathway in plants. The phosphorylation of RPL10 by its specific partner, the geminiviral nuclear shuttle protein-interacting kinase, redirects it to the nucleus to modulate viral infection (CARVALHO *et al*., 2008). The fact that translation-regulating proteins are down-accumulated at 4 mpg gives support to the tolerance mechanism presented by papaya plants at pre-flowering and the interaction of RPL17 with PMeV complex capsid protein could be detrimental to this process.

Despite modulation of translation could be an important mechanism used by PMeV complex or hosts to regulate virus levels, several other cellular mechanisms could be altered due to the interaction of plant proteins with the PMeV capsid protein. Our results point to other processes including polyprotein and RNA processing, cell wall modification, gene expression regulation, and reactive oxygen species detoxification that have been identified as modulated in our previous transcriptome and proteome analysis of PMeV complex-infected papaya. Thus, the identification of binding partners of PMeV CP provided a framework for a better understanding of the response of plants against totiviruses and potentially identified new targets for the development of more effective strategies to control PSD.

REFERENCES

A. CALLAWAY; D. GIESMAN-COOKMEYER; E. T. GILLOCK; T. L. SIT, A.; LOMMEL, S. A. THE MULTIFUNCTIONAL CAPSID PROTEINS OF PLANT RNA VIRUSES. **Annual Review of Phytopathology**, 39, n. 1, p. 419-460, 2001.

ABREU, P. M. V.; ANTUNES, T. F. S.; MAGAÑA-ÁLVAREZ, A.; PÉREZ-BRITO, D.; TAPIA-TUSSELL, R.; VENTURA, J. A.; FERNANDES, A. A. R.; FERNANDES, P. M. B. A current overview of the Papaya meleira virus, an unusual plant virus. **Viruses**, 7, n. 4, p. 1853-1870, 2015.

AKINYEMI, I. A.; WANG, F.; CHANG, Z.-X.; WU, Q. Genome characterization of the newly identified maize-associated totivirus Anhui. **Archives of Virology**, 163, n. 10, p. 2929-2931, 2018/10/01 2018.

ANTUNES, T. F. S.; AMARAL, R. J. V.; VENTURA, J. A.; GODINHO, M. T.; AMARAL, J. G.; SOUZA, F. O.; ZERBINI, P. A.; ZERBINI, F. M.; FERNANDES, P. M. B. The dsRNA Virus Papaya Meleira Virus and an ssRNA Virus are Associated with Papaya Sticky Disease. **PloS One**, 11, p. e0155240, 2016.

ANTUNES, T. F. S.; MAURASTONI, M.; MADROÑERO, L. J.; FUENTES, G.; SANTAMARÍA, J. M.; VENTURA, J. A.; ABREU, E. F.; FERNANDES, A. A. R.; FERNANDES, P. M. B. Battle of Three: The Curious Case of Papaya Sticky Disease. **Plant Disease**, 104, n. 11, p. 2754-2763, 2020.

BABÉ, L. M.; CRAIK, C. S. Viral Proteases: Evolution of Diverse Structural Motifs to Optimize Function. **Cell**, 91, n. 4, p. 427-430, 1997.

BOL, J. F. Role of Capsid Proteins. *In*: FOSTER, G. D.;JOHANSEN, I. E.*, et al* (Ed.). **Plant Virology Protocols: From Viral Sequence to Protein Function**. Totowa, NJ: Humana Press, 2008. p. 21-31.

BUREAU, M.; LEH, V.; HAAS, M.; GELDREICH, A.; RYABOVA, L.; YOT, P.; KELLER, M. P6 protein of Cauliflower mosaic virus, a translation reinitiator, interacts with ribosomal protein L13 from *Arabidopsis thaliana*. **Journal of General Virology**, 85, n. 12, p. 3765-3775, 2004.

CARMO, L. S. T.; RESENDE, R. O.; SILVA, L. P.; RIBEIRO, S. G.; MEHTA, A. Identification of host proteins modulated by the virulence factor AC2 of Tomato chlorotic mottle virus in Nicotiana benthamiana. **Proteomics**, 13, n. 12-13, p. 1947-1960, 2013.

CARVALHO, C. M.; SANTOS, A. A.; PIRES, S. R.; ROCHA, C. S.; SARAIVA, D. I.; MACHADO, J. P. B.; MATTOS, E. C.; FIETTO, L. G.; FONTES, E. P. B. Regulated nuclear trafficking of rpL10A mediated by NIK1 tepresents a defense strategy of plant cells against virus. **PLOS Pathogens**, 4, n. 12, p. e1000247, 2008.

CHAKRABARTY, R.; BANERJEE, R.; CHUNG, S.-M.; FARMAN, M.; CITOVSKY, V.; HOGENHOUT, S. A.; TZFIRA, T.; GOODIN, M. pSITE vectors for stable integration or transient expression of autofluorescent protein fusions in plants: probing *Nicotiana benthamiana*-virus interactions. **Molecular Plant-Microbe Interactions**, 20, n. 7, p. 740-750, 2007.

DE LIMA, J. G. S.; TEIXEIRA, D. G.; FREITAS, T. T.; LIMA, J. P. M. S.; LANZA, D. C. F. Evolutionary origin of 2A-like sequences in *Totiviridae* genomes. **Virus Research**, 259, p. 1-9, 2019/01/02/ 2019.

FUJIMURA, T.; ESTEBAN, R. Cap-snatching mechanism in yeast L-A double-stranded RNA virus. **Proceedings of the National Academy of Sciences**, 108, n. 43, p. 17667-17671, 2011.

GHABRIAL, S. A.; HAVENS, W. M. The Helminthosporium victoriae 190S mycovirus has two forms distinguishable by capsid protein composition and phosphorylation state. **Virology**, 188, n. 2, p. 657-665, 1992/06/01/ 1992.

GHOSH, S.; KANAKALA, S.; LEBEDEV, G.; KONTSEDALOV, S.; SILVERMAN, D.; ALON, T.; MOR, N.; SELA, N.; LURIA, N.; DOMBROVSKY, A.; MAWASSI, M.; HAVIV, S.; CZOSNEK, H.; GHANIM, M.; SIMON, A. E. Transmission of a new polerovirus infecting pepper by the whitefly *Bemisia tabaci*. **Journal of Virology**, 93, n. 15, p. e00488-00419, 2019.

GUO, L.; YANG, X.; WU, W.; TAN, G.; FANG, S.; ZHANG, S.; LI, F. Identification and molecular characterization of Panax notoginseng virus A, which may represent an undescribed novel species of the genus Totivirus, family *Totiviridae*. **Archives of Virology**, 161, n. 3, p. 731-734, 2016/03/01 2016.

HERRANZ, M. C.; PALLAS, V.; APARICIO, F. Multifunctional Roles for the N-Terminal Basic Motif of Alfalfa mosaic virus Coat Protein: Nucleolar/Cytoplasmic Shuttling, Modulation of RNA-Binding Activity, and Virion Formation. Molecular Plant-Microbe Interactions®, 25, n. 8, p. 1093-1103, 2012.

HUANG, S.; SOLDEVILA, A. I.; WEBB, B. A.; GHABRIAL, S. A. Expression, Assembly, and Proteolytic Processing of Helminthosporium victoriae 190S Totivirus Capsid Protein in Insect Cells. **Virology**, 234, n. 1, p. 130-137, 1997/07/21/ 1997.

KITAJIMA, E. W.; RODRIGUES, C. H.; SILVEIRA, J. S.; ALVES, F.; VENTURA, J. A.; ARAGÃO, F. J. L.; OLIVEIRA, L. H. R. Association of isometric viruslike particles, restricted to lacticifers, with "meleira" (sticky disease) of papaya (*Carica papaya*). **Fitopatologia Brasileira**, 18, p. 118, 1993.

KUSHNIROV, V. V. Rapid and reliable protein extraction from yeast. Yeast, 16, n. 9, p. 857-860, 2000.

LAGUNAS-RANGEL, F. A.; KAMEYAMA-KAWABE, L. Y.; BERMÚDEZ-CRUZ, R. M. Giardiavirus: an update. **Parasitology Research**, 120, n. 6, p. 1943-1948, 2021/06/01 2021.

LAEMMLI, U. K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. **Nature**, 227, n. 5259, p. 680-685, 1970/08/01 1970.

LI, S. Regulation of Ribosomal Proteins on Viral Infection. **Cells**, 8, n. 5, p. 508, 2019.

LUQUE, D.; MATA, C. P.; SUZUKI, N.; GHABRIAL, S. A.; CASTÓN, J. R. Capsid structure of dsRNA fungal viruses. **Viruses**, 10, n. 9, p. 481, 2018.

MADROÑERO, J.; RODRIGUES, S. P.; ANTUNES, T. F. S.; ABREU, P. M. V.; VENTURA, J. A.; FERNANDES, A. A. R.; FERNANDES, P. M. B. Transcriptome analysis provides insights into the delayed sticky disease symptoms in *Carica papaya*. **Plant Cell Reports**, 37, n. 7, p. 967-980, 2018/07/01 2018.

MARTIN, K.; KOPPERUD, K.; CHAKRABARTY, R.; BANERJEE, R.; BROOKS, R.; GOODIN, M. M. Transient expression in *Nicotiana benthamiana* fluorescent marker lines provides enhanced definition of protein localization, movement and interactions in planta. **The Plant Journal**, 59, n. 1, p. 150-162, 2009.

MATA, C. P.; LUQUE, D.; GÓMEZ-BLANCO, J.; RODRÍGUEZ, J. M.; GONZÁLEZ, J. M.; SUZUKI, N.; GHABRIAL, S. A.; CARRASCOSA, J. L.; TRUS, B. L.; CASTÓN, J. R. Acquisition of functions on the outer capsid surface during evolution of double-stranded RNA fungal viruses. **PLOS Pathogens**, 13, n. 12, p. e1006755, 2017.

MILLER, C. M.; SELVAM, S.; FUCHS, G. Fatal attraction: The roles of ribosomal proteins in the viral life cycle. **WIREs RNA**, 12, n. 2, p. e1613, 2021.

MOUTIM, V.; SILVA, L. G.; LOPES, M. T. P.; FERNANDES, G. W.; SALAS, C. E. Spontaneous processing of peptides during coagulation of latex from *Carica papaya*. **Plant Science**, 142, n. 2, p. 115-121, 1999/03/29/ 1999.

RODRIGUES, S. P.; DA CUNHA, M.; VENTURA, J. A.; FERNANDES, P. M. B. Effects of the Papaya meleira virus on papaya latex structure and composition. **Plant Cell Reports**, 28, p. 861-871, 2009.

RODRIGUES, S. P.; VENTURA, J. A.; AGUILAR, C.; NAKAYASU, E. S.; ALMEIDA, I. C.; FERNANDES, P.; ZINGALI, R. B. Proteomic analysis of papaya (*Carica papaya* L.) displaying typical sticky disease symptoms. **Proteomics**, 11, n. 13, p. 2592-2602, 2011.

RODRIGUES, S. P.; VENTURA, J. A.; AGUILAR, C.; NAKAYASU, E. S.; CHOI, H.; SOBREIRA, T. J. P.; NOHARA, L. L.; WERMELINGER, L. S.; ALMEIDA, I. C.; ZINGALI, R. B.; FERNANDES, P. M. B. Label-free quantitative proteomics reveals differentially regulated proteins in the latex of sticky diseased *Carica papaya* L. plants. **Journal of Proteomics**, 75, p. 3191-3198, 2012.

SHANNON, P.; MARKIEL, A.; OZIER, O.; BALIGA, N. S.; WANG, J. T.; RAMAGE, D.; AMIN, N.; SCHWIKOWSKI, B.; IDEKER, T. Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. **Genome Research**, 13, n. 11, p. 2498-2504, November 1, 2003 2003.

SHEVCHENKO, A.; TOMAS, H.; HAVLI, J.; OLSEN, J. V.; MANN, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. **Nature Protocols**, 1, n. 6, p. 2856-2860, 2006/12/01 2006.

SOARES, E.; WERTH, E. G.; MADROÑERO, L. J.; VENTURA, J. A.; RODRIGUES, S. P.; HICKS, L. M.; FERNANDES, P. M. B. Label-free quantitative proteomic analysis of pre-flowering PMeV-infected *Carica papaya* L. **Journal of Proteomics**, 151, p. 275-283, 2016.

SZKLARCZYK, D.; GABLE, A. L.; LYON, D.; JUNGE, A.; WYDER, S.; HUERTA-CEPAS, J.; SIMONOVIC, M.; DONCHEVA, N. T.; MORRIS, J. H.; BORK, P.; JENSEN, L. J.; MERING, CHRISTIAN V. STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. **Nucleic Acids Research**, 47, n. D1, p. D607-D613, 2018.

VAIRA, A. M.; LIM, H. S.; BAUCHAN, G.; GULBRONSON, C. J.; MIOZZI, L.; VINALS, N.; NATILLA, A.; HAMMOND, J. The interaction of Lolium latent virus major coat protein with ankyrin repeat protein NbANKr redirects it to chloroplasts and modulates virus infection. **Journal of General Virology**, 99, n. 5, p. 730-742, 2018.

WEBER, K.; OSBORN, M. The Reliability of Molecular Weight Determinations by Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. **Journal of Biological Chemistry**, 244, n. 16, p. 4406-4412, 1969.

YU, D.; WANG, C. C.; WANG, A. L. Maturation of giardiavirus capsid protein involves posttranslational proteolytic processing by a cysteine protease. **Journal of Virology**, 69, n. 5, p. 2825-2830, 1995.

ZENG, Y.; JI, B.; YU, B. Laticifer ultrastructural and immunocytochemical studies of papain in *Carica papaya*. **Acta Botanica Sinica**, 36, n. 7, p. 497-501, 1994.

ZHANG, Z. Y.; HUANG, H.; HAN, X. X.; LI, R.; WU, L. P.; WU, L. Identification and molecular characterization of tea-oil camellia-associated totivirus 1. **Archives of Virology**, 2021/04/17 2021.

ZHANG, R.; HISANO, S.; TANI, A.; KONDO, H.; KANEMATSU, S.; SUZUKI, N. A capsidless ssRNA virus hosted by an unrelated dsRNA virus. **Nature Microbiology**, 1, p. 15001, Jan 11 2016

TABLES

Table 1. Yeast-two-hybrid-derived clones obtained from a screening using PMeV ORF1 fragment 4 as bait

Clone No.	Growth media	NCBI or TAIR Description	TAIR accession	Gene ontology information
1	QXA	Beta-1,4-N-acetylglucosaminyltransferase family protein (AT3G01620), mRNA	AT3G01620	Located in Golgi apparatus
2	QXA	Zinc finger protein 2 (ZFP2), mRNA	AT5G57520	Located in nucleus
3	QXA	DHHC-type zinc finger family protein (AT2G40990), partial mRNA	AT2G40990	Is active in Golgi apparatus and endoplasmic reticulum
4	QXA	Polynucleotide adenylyltransferase family protein (AT5G23690), mRNA	AT5G23690	Involved in RNA processing
5	QXA	Transmembrane protein (AT2G35750)	AT2G35750	Located in mitochondrion
6	QXA	Inorganic carbon transport protein-like protein (NdhL), mRNA	AT1G70760.1	Located in chloroplast, chloroplast thylakoid membrane, thylakoid membrane

7	QXA 2.5mM	Cytochrome c oxidase assembly protein CtaG / Cox11 family (AT1G02410), mRNA	AT1G02410	Is located in chloroplast, integral component of mitochondrial membrane, mitochondrion
8	QXA 2.5mM	Pectin lyase-like superfamily protein (AT1G04680), mRNA	AT1G04680	Located in extracellular region
9	QXA 2.5mM	Plastid developmental protein DAG (MORF9), mRNA	AT1G11430	Located in chloroplast, chloroplast envelope, chloroplast stroma
10	QXA 2.5mM	Plant/protein (AT1G13990), mRNA	AT1G13990	Located in chloroplast
11	QXA 2.5mM	Peroxidase CB (PRXCB), mRNA	AT3G49120	Located in Golgi apparatus, apoplast, cell wall, cytosol, extracellular region, plant-type cell wall, plant-type vacuole, secretory vesicle
12	QXA 2.5mM	RmlC-like cupins superfamily protein (AT1G03890), mRNA	AT1G03890	Located in extracellular region
13	QXA 2.5mM	RNA polymerase transcriptional regulation mediator-like protein (MED6), mRNA	AT3G21350	Located in nucleus
14	QXA 2.5 mM	Chloroplast ribosomal protein S3	ATCG00800.1	Located in chloroplast, chloroplast envelope, chloroplast nucleoid, chloroplast stroma, plastid

15	QXA 2.5 mM	Chloroplast GRX 12, GRXS12	AT2G20270	Located in chloroplast, chloroplast stroma, mitochondrion
16	QXA 5mM	DNAJ heat shock family protein (AT2G22360), mRNA	AT2G22360	Located in chloroplast, chloroplast envelope, chloroplast thylakoid membrane, cytoplasm, vacuole
17	QXA 5mM	Ribosomal protein L17 family protein (AT3G54210), mRNA	AT3G54210	Located in chloroplast, chloroplast envelope, chloroplast stroma, cytosol
18	QXA 5mM	Sec14p-like phosphatidylinositol transfer family protein (AT1G72160), mRNA	AT1G72160	Located in plasma membrane
19	QXA 5mM	GDSL-like Lipase/Acylhydrolase superfamily protein (AT5G45670), mRNA	AT5G45670	Located in extracellular region
20	QXA 5mM	Chaperone protein dnaJ-like protein (AT5G06130), mRNA	AT5G06130	Located in chloroplast membrane, mitochondrion
21	QXA 5mM	GPI-anchored protein (AT3G18050), mRNA	AT3G18050	Located in anchored component of membrane, chloroplast
22	QXA 5mM	Pyrimidin 4 (PYR4), mRNA	AT4G22930	Located in chloroplast, cytosol, mitochondrion

23	QXA 5mM	Pectinacetylesterase family protein (AT4G19420), mRNA	AT4G19420	Located in extracellular region
24	QXA 5mM	Double Clp-N motif protein (AT4G12060), mRNA	AT4G12060	Located in chloroplast, chloroplast envelope, chloroplast stroma, cytosol, plastid stroma
25	QXA 5mM	PEBP (phosphatidylethanolamine-binding protein) family protein (FT), mRNA	AT1G65480	Located in cytoplasm nucleus
26	QXA 5mM	Clone RAFL09-89-G08 (R19778) putative cellulose synthase catalytic subunit (RSW1) (At4g32410), mRNA	AT4G32410.1	Located in Golgi apparatus, endosome, plasma membrane, trans-Golgi network
27	QXA 5mM	mRNA for plastid protein, complete cds, clone: RAFL15-06-D14	AT1G32580.1	Located in chloroplast, mitochondrion, nucleus
28	QXA 5mM	Papain family cysteine protease (AT4G16190), mRNA	AT4G16190	Located in extracellular region, lytic vacuole, plant-type vacuole, vacuole

2163

FIGURES

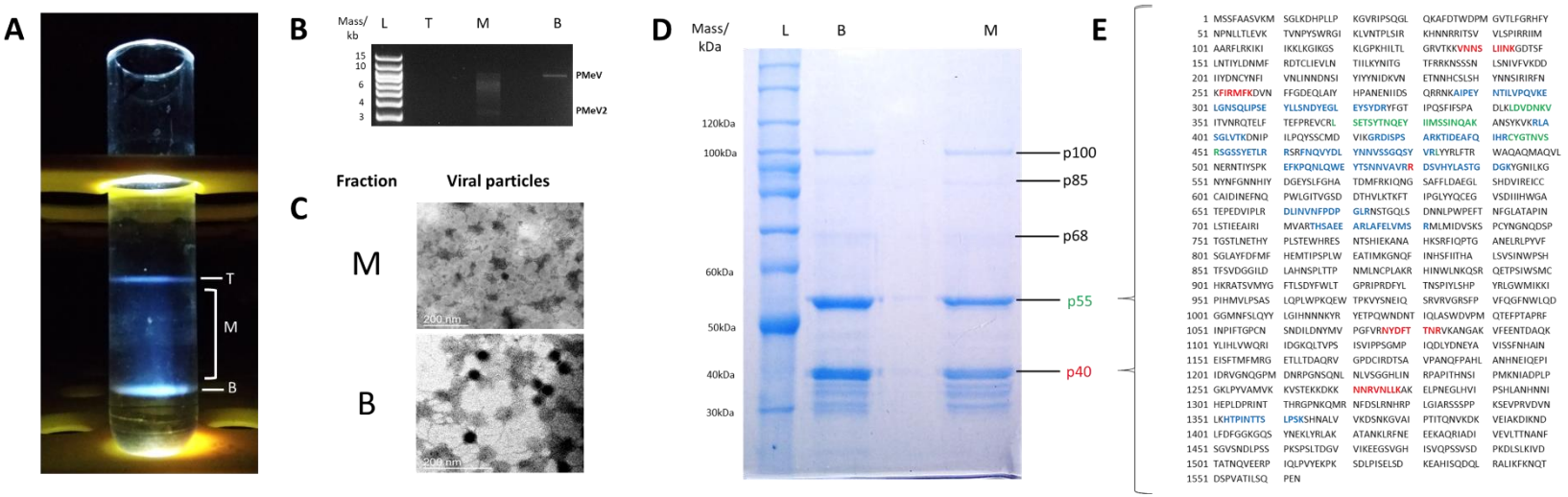


Figure 1. Characterization of PMeV complex capsid protein polypeptide composition. **a** The three opalescent fractions obtained after ultracentrifugation at 145,000 g for 18 h at 4ºC in a 50 % (w/v) CsCl isopycnic gradient. T- Top fraction. M- middle fraction. B- Bottom fraction. **b** Agarose gel electrophoresis of total RNA extracted from the fractions. Twenty micrograms of each fraction were submitted for phenol: chloroform (1:1) extraction and RNA was loaded in gel. L- 1kb plus DNA ladder Invitrogen®. **c** Transmission electron microscope images of viral particles from the M and B fractions, as shown in figure a. No particles were visualized for fraction T. **d** Coomassie blue-stained SDS-PAGE of fractions collected from M and B. Viral particles were boiled for 3 min in loading buffer and 40µg of protein was loaded in the gel. L- Benchmark^{TM} protein ladder Invitrogen®. **e.** Deduced amino acid sequence of ORF1 highlighting the positions of the peptides identified from the p40 and p55 bands

2175 extracted from the M fraction lane. Peptides identified from p40 and p55 bands are highlighted in red and green, respectively. Overlapping peptides

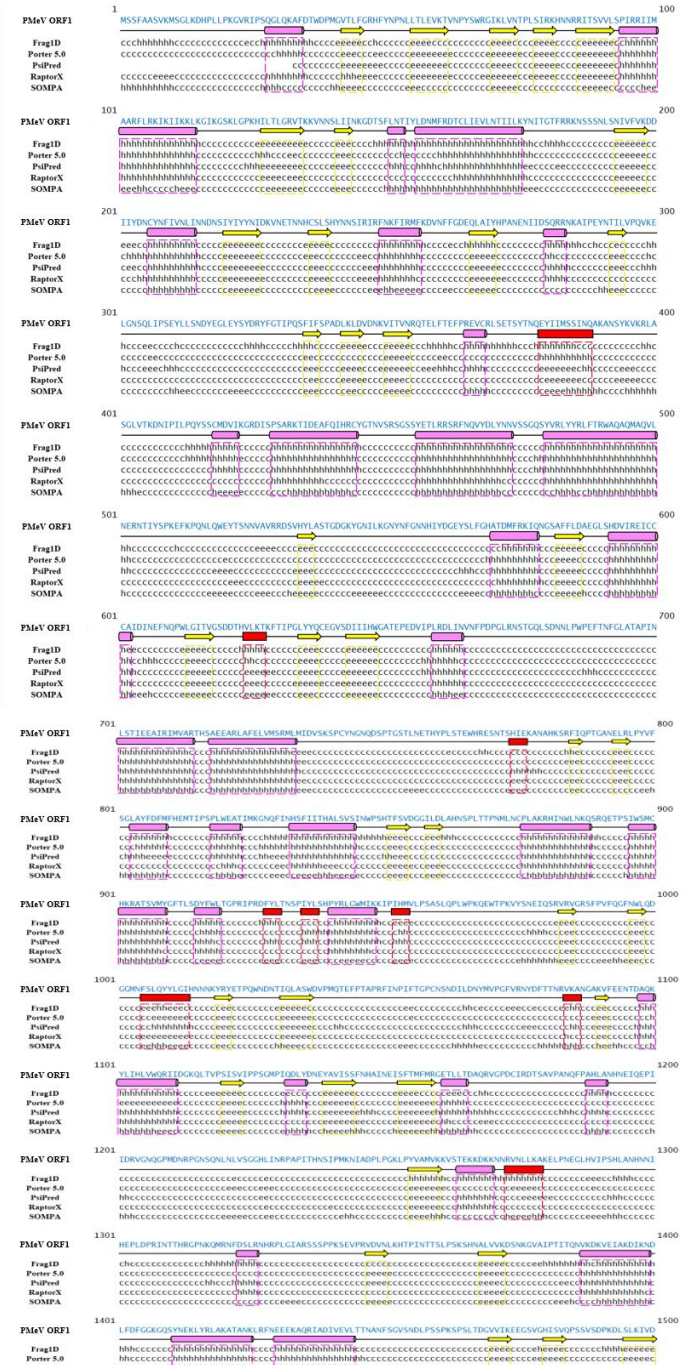

PMeV ORF1
Frag1D
Porter 5.0
PsiPred
RaptorX
SOMPA

Figure 2. A consensus secondary structure of the PMeV ORF1. The alpha-helix, beta-strand, and random coil segments are represented schematically as rectangles, arrows, and lines, respectively. All secondary-structure predictions were made with five different software (see Methods). GenBank accession number for PMeV ORF1: AMU19319.1.

A

ORF1 fragment	Range (aa)	No. amino acids	Approximated size (kDa)	BD	Yeast Growth in QDO/X/A — AD 1	2	3	4	5	6
CP1	1-320	320	35.2	**1**						
CP2	321-670	350	38.5	**2**						
CP3	671-960	290	31.9	**3**						
CP4	961-1200	240	26.4	**4**						
CP5	1201-1563	363	39.9	**5**						
Full length	1-1563	1563	177	**6**						

B

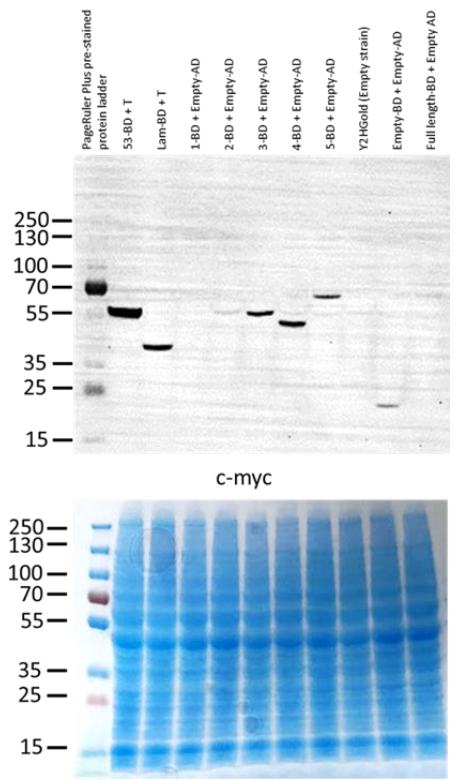
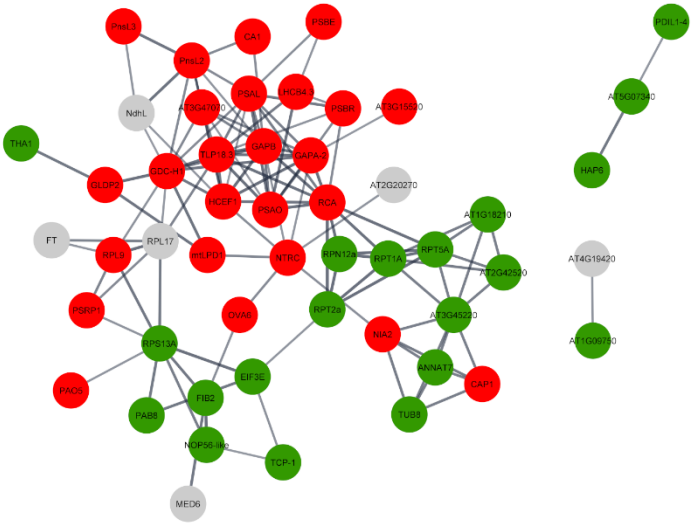


Figure 3. Summary of yeast two-hybrid assays to map dimerization fragments in PMeV ORF1 and detection of 5 fragments of PMeV ORF1. **a** Each fragment was fused either to GAL4 Binding domain (BD) or GAL4 Activation domain (AD) in pDESTGBKT7 and pDESTGADT7, respectively, and transformed in yeast strain Y2HGold (Takara®). Positive interactors were selected in QDO/X/A media. **b** Expression was verified by SDS-PAGE of yeast crude protein extracts and western blotting using an anti-c-myc antibody. The c-myc tag is fused to the expressed protein. 1 to 5-BD represents each fragment of PMeV ORF1 fused to GAL4 BD. GAL4 DNA-BD fused with murine p53, GAL4 BD fused with Lamin were used as a positive control. Untransformed yeast and yeast transformed with pDEST-BGKT7 plasmids were used as a negative control.

A



B

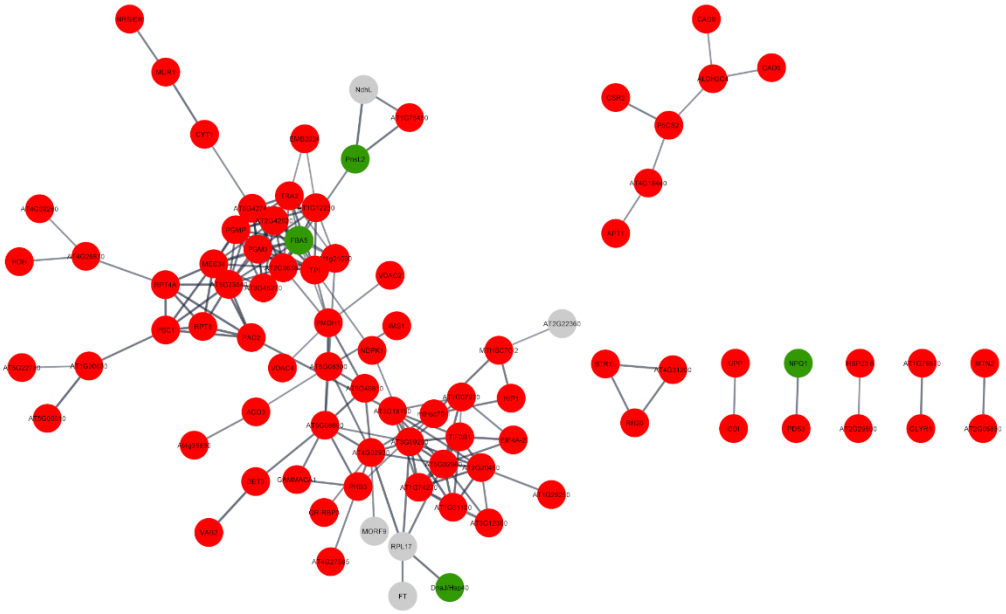
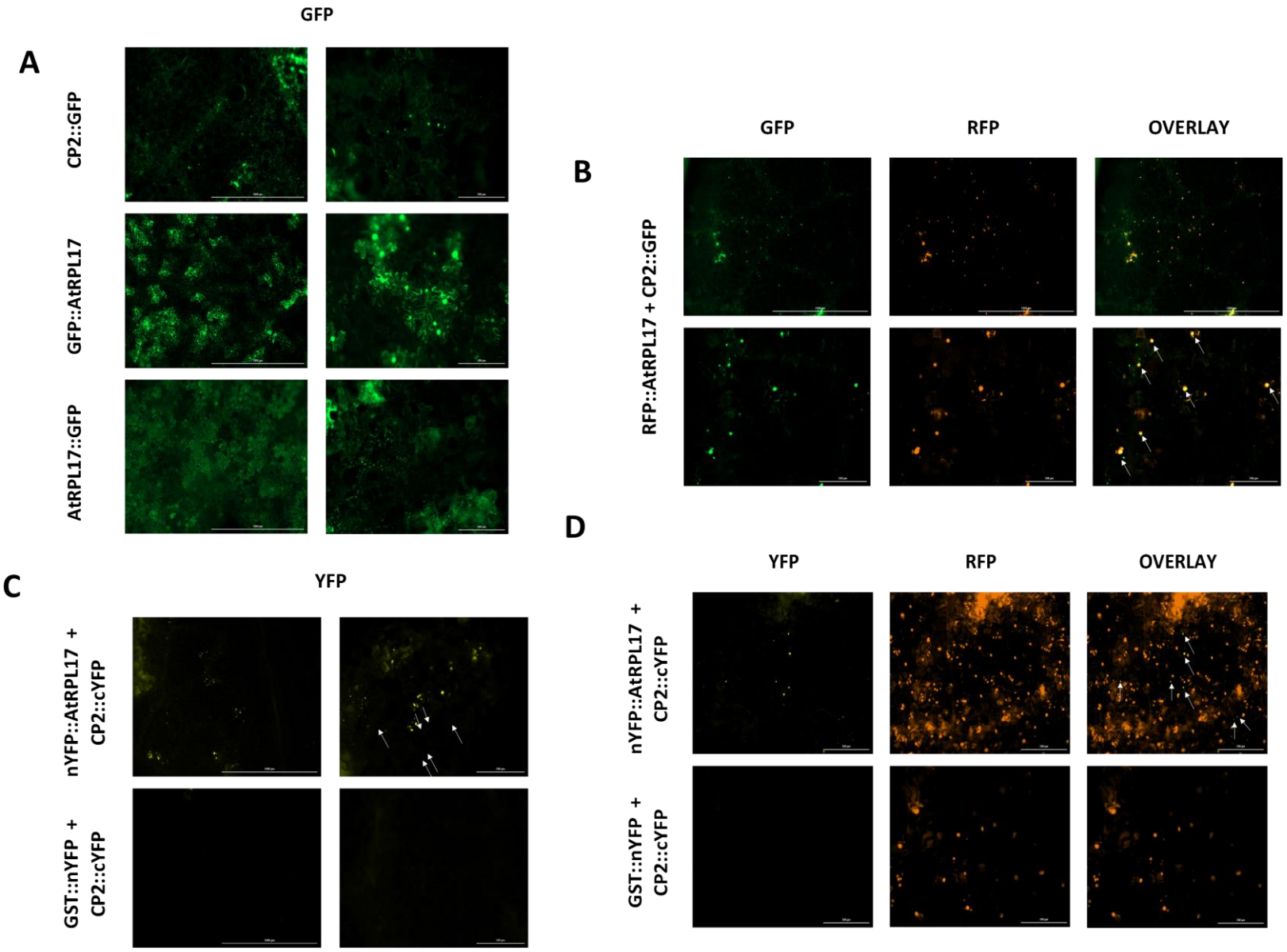


Figure 4. Protein-protein interaction network of differentially modulated proteins of PMeV complex-infected *Carica papaya* and PMeV CP2 and CP4-interacting proteins. PPI network of

an infected plant at (**a**) pre-flowering stage (4 months post-germination – 4MPG) and (**b**) post-flowering stage (7 months post-germination – 7MPG). Red nodes are up-accumulated proteins; green nodes are down-accumulated proteins; gray nodes are PMeV CP2 and CP4-interacting proteins.


A
GFP
CP2::GFP
GFP::AtRPL17
AtRPL17::GFP
B
GFP
RFP
OVERLAY
RFP::AtRPL17 + CP2::GFP
C
YFP
nYFP::AtRPL17 + CP2::cYFP
GST::nYFP + CP2::cYFP
D
YFP
RFP
OVERLAY
nYFP::AtRPL17 + CP2::cYFP
GST::nYFP + CP2::cYFP

2195

Figure 5. Transient expression of *At*RPL17 and CP2, and their interaction in *Nicotiana benthamiana*. **a** Localization of *At*RPL17 and CP2 proteins expressed as fusions to a green fluorescent protein (GFP) in leaf epidermal cells of *N. benthamiana.* The fusion proteins CP2::GFP, *At*RPL17::GFP, and GFP::*At*RPL17 were expressed and visualized at 2 days post infiltration. The right column represents an image in higher magnification. **b** Co-localization of *At*RPL17 and CP2 expressed as fusions to (GFP) or red fluorescent protein (RFP) in *N. benthamiana* epidermal leaf cells. First column, GFP channel; Second column, RFP channel; Third column, Overlay of GFP and RFP channels. The fusion proteins RFP::*At*RPL17 were expressed along with GFP::CP2. Lower row represents an image in higher magnification. **c and d** PMeV CP2-*At*RPL17 interaction in vivo by bimolecular fluorescence complementation (BiFC) in wild type (**c**) and transgenic *N. benthamiana* expressing RFP::H2B as a nuclear marker (**d**). Fusion proteins nYFP::*At*RPL17 or GST::nYFP were expressed along with CP2::cYFP by agroinfiltrating the encoding plasmids into leaves of *N. benthamiana*. The reconstitution of the yellow fluorescence was visualized 2 days post infiltration. Fusion protein combinations expressed in each sample are indicated at the left of the corresponding row of images. Scale bars are represented in the figures. White arrows: co-localization or interaction signals.

SUPPLEMENTARY MATERIAL

Supplementary Material 1

Table S1. List of primers used to generate the clones in this study.

Table S2. Arabidopsis orthologs obtained from proteins differentially accumulated in infected papaya leaf at 4 months post-germination.

Table S3. Arabidopsis orthologs obtained from proteins differentially accumulated in infected papaya leaf at 7 months post-germination.

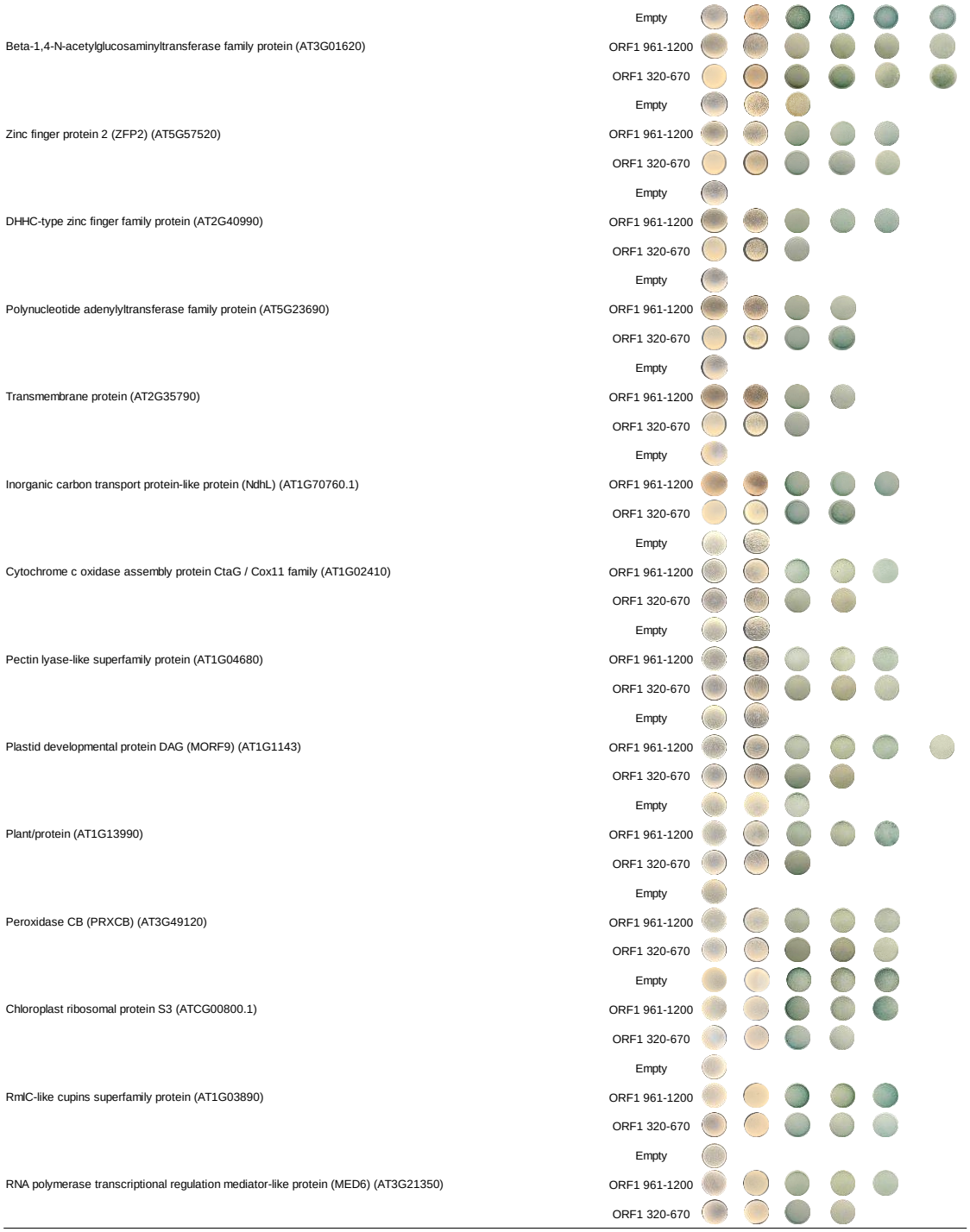
Table S4. Arabidopsis orthologs obtained from proteins differentially accumulated in infected papaya leaf (at 4 months post-germination) used retrieved the protein-protein interaction network of CP2 and CP4-interacting proteins.

Table S5. Arabidopsis orthologs obtained from proteins differentially accumulated in infected papaya leaf (at 7 months post-germination) used retrieved the protein-protein interaction network of CP2 and CP4-interacting proteins.

Link to access supplementary tables
https://1drv.ms/x/s!ApYzPMWzGjw8gdlc6kRIlIdNDYfjzQ?e=oLaeJL

Figure S1. Spot plating showing the validation of genuine positives interacting CP2 and CP4. After overnight growth, cultures were normalized to OD_{600}=2, and 20uL was spotted in nine different selective media. To test the autoactivation of baits, yeast transformed only with the BD-fused protein were spotted in SDO, SDO/X, and SDO/X/A media. Yeast co-transformed with bait and empty-AD plasmids were spotted in DDO, DDO/X, DDO/X/A, QDO, QDO/X, and QDO/X/A. None of the tested baits, except the positive control, shows growth in QDO/X and QDO/X/A media. The growth in these media depends on the activation of reporter genes.

p-AD	p-BD	Yeast Growth					
		DDO	QDO	QDOXA + 3-AT			
				0 mM	1 mM	2.5 mM	5 mM
T	53						
T	Lam						
	Empty						
DNAJ heat shock family protein (AT2G22360)	ORF1 961-1200						
	ORF1 320-670						
	Empty						
Ribosomal protein L17 family protein (AT3G54210)	ORF1 961-1200						
	ORF1 320-670						
	Empty						
Sec14p-like phosphatidylinositol transfer family protein (AT1G72160)	ORF1 961-1200						
	ORF1 320-670						
	Empty						
GDSL-like Lipase/Acylhydrolase superfamily protein (AT5G45670)	ORF1 961-1200						
	ORF1 320-670						
	Empty						
Chloroplast GRX 12, GRXS12 (AT2G20270)	ORF1 961-1200						
	ORF1 320-670						
	Empty						
Chaperone protein dnaJ-like protein (AT5G06130)	ORF1 961-1200						
	ORF1 320-670						
	Empty						
GPI-anchored protein (AT3G18050)	ORF1 961-1200						
	ORF1 320-670						
	Empty						
Pyrimidin 4 (PYR4) (AT4G22930)	ORF1 961-1200						
	ORF1 320-670						
	Empty						
Pectinacetylesterase family protein (AT4G19420)	ORF1 961-1200						
	ORF1 320-670						
	Empty						
Double Clp-N motif protein (AT4G12060)	ORF1 961-1200						
	ORF1 320-670						
	Empty						
PEBP (phosphatidylethanolamine-binding protein) family protein (FT) (AT1G6548)	ORF1 961-1200						
	ORF1 320-670						
	Empty						
Clone RAFL09-89-G08 (R19778) putative cellulose synthase catalytic subunit (RSW1) (At4g32410)	ORF1 961-1200						
	ORF1 320-670						
	Empty						
mRNA for plastid protein, complete cds, clone: RAFL15-06-D14 (AT1G32580.1)	ORF1 961-1200						
	ORF1 320-670						
	Empty						
Papain family cysteine protease (AT4G16190)	ORF1 961-1200						
	ORF1 320-670						


Empty
Beta-1,4-N-acetylglucosaminyltransferase family protein (AT3G01620)
ORF1 961-1200
ORF1 320-670
Empty
Zinc finger protein 2 (ZFP2) (AT5G57520)
ORF1 961-1200
ORF1 320-670
Empty
DHHC-type zinc finger family protein (AT2G40990)
ORF1 961-1200
ORF1 320-670
Empty
Polynucleotide adenylyltransferase family protein (AT5G23690)
ORF1 961-1200
ORF1 320-670
Empty
Transmembrane protein (AT2G35790)
ORF1 961-1200
ORF1 320-670
Empty
Inorganic carbon transport protein-like protein (NdhL) (AT1G70760.1)
ORF1 961-1200
ORF1 320-670
Empty
Cytochrome c oxidase assembly protein CtaG / Cox11 family (AT1G02410)
ORF1 961-1200
ORF1 320-670
Empty
Pectin lyase-like superfamily protein (AT1G04680)
ORF1 961-1200
ORF1 320-670
Empty
Plastid developmental protein DAG (MORF9) (AT1G1143)
ORF1 961-1200
ORF1 320-670
Empty
Plant/protein (AT1G13990)
ORF1 961-1200
ORF1 320-670
Empty
Peroxidase CB (PRXCB) (AT3G49120)
ORF1 961-1200
ORF1 320-670
Empty
Chloroplast ribosomal protein S3 (ATCG00800.1)
ORF1 961-1200
ORF1 320-670
Empty
RmlC-like cupins superfamily protein (AT1G03890)
ORF1 961-1200
ORF1 320-670
Empty
RNA polymerase transcriptional regulation mediator-like protein (MED6) (AT3G21350)
ORF1 961-1200
ORF1 320-670

MANUSCRIPT #4. A MULTIPLEX RT-PCR METHOD TO DETECT PAPAYA MELEIRA VIRUS COMPLEX IN ADULT PRE-FLOWERING PLANTS

Paper published in the *Archives of Virology journal* (ISSN 0304-8608; IF 2.574, 2021; Qualis B1 Biotecnologia, 2013-2016). https://doi.org/10.1007/s00705-020-04588-5

A multiplex RT-PCR method to detect papaya meleira virus complex in adult pre-flowering plants

Marlonni Maurastoni[a], Tathiana F. Sá-Antunes[a], Scarlett A. Oliveira[a], Alexandre M. C. Santos[a], José A. Ventura[a,b] and Patricia M. B. Fernandes[a*]

[a]Núcleo de Biotecnologia, Universidade Federal do Espírito Santo, Vitória, Espírito Santo, Brazil

[b]Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural, Vitória, Epírito Santo, Brazil

*Corresponding author: patricia.fernandes@ufes.br

ABSTRACT

Papaya sticky disease (PSD), which can destroy orchards, was first attributed to papaya meleira virus (PMeV). However, the discovery of papaya meleira virus 2 (PMeV2) associated with PSD plants impose the need to detect this viral complex. We developed a multiplex RT-PCR (mPCR) technique capable of detecting two viruses in a single assay from pre-flowering plant samples, which is a useful tool for early diagnosis of PSD. We also determined the limit of detection (LOD) using asymmetric plasmid dilutions of both PMeV and PMeV2, which revealed that a higher titer of one virus prevents detection of the other. Thus, this technique is an alternative method for detecting PMeV and PMeV2 in a single reaction.

Keywords**:** papaya sticky disease; Carica papaya; virus diagnosis

Officially reported in Brazil and Mexico, papaya sticky disease (PSD) is a severe disease that can devastate papaya orchards. Initially, the causal agent of PSD was identified as papaya meleira virus (PMeV), a virus with a double-stranded RNA genome similar to those of members of the family *Totiviridae* enclosed in a 42-nm-diameter isometric particle [1, 2]. Later, papaya meleira virus 2 (PMeV2), a single-stranded RNA virus closely related to members of the genus *Umbravirus*, was also discovered in association with PSD plants. These viruses have an interesting relationship in mixed infections, because the PMeV and PMeV2 genomes are separately encapsidated in particles formed by the PMeV capsid protein [3].

No papaya cultivars have been found that are resistant to PMeV and PMeV2 (PMeV complex) [4]. Visual identification of diseased plants and their eradication (roguing) is the only available control method [5]. However, symptoms of PSD appear only after flowering. Thus, an infected symptomless plant in a field may remain unnoticed for an extended period, acting as a virus inoculum source [5, 6]. Therefore, development of diagnostic procedures for early detection is imperative.

Previous reports have described alternative diagnostic methods for PMeV: (i) viewing in an agarose gel the viral dsRNA band purified from latex [7], (ii) conventional reverse transcription PCR (RT-PCR) from nucleic acids obtained from latex diluted in ammonium or sodium citrate [8], and (iii) conventional RT-PCR and quantitative RT-PCR (qRT-PCR) from small quantities of leaf-purified RNA [9]. Despite these advances, the discovery of PMeV2 associated with PSD plants [3] requires new diagnostic methodologies. A method modified from conventional RT-PCR was described by Antunes et al. [3], who used primers based on sequenced genomes. However, the methodology requires synthesis of two cDNAs and two PCR reactions, one for each virus, making it laborious and time-consuming, especially when screening a large number of samples.

In contrast, the multiplex PCR (mPCR) method is based on a single PCR that can simultaneously detect different viruses [10]. The method has been used to simultaneously detect papaya ringspot virus (PRSV-P), papaya leaf distortion mosaic virus (PLDMV), and papaya mosaic virus (PapMV). These viruses are difficult to distinguish visually since they cause similar symptoms [11].

The sensitivity or limit of detection (LOD) of a PCR method is an important parameter used to evaluate the minimum amount of amplicon DNA that can be detected and quantified [12, 13]. It is commonly determined using total nucleic acids [14, 15], nucleic acids extracted from viral particles purified from infected plants [16], or plasmids containing the target [17,18,19]. These templates are quantified, mixed in equimolar amounts, serially diluted and used as a template for mPCR. However, an equimolar mix may not be a proper template to determine the LOD. This can lead to misleading results, as the different viruses in mixed infection do not usually have the same titer in a host [20,21,22]. Here, we report a mPCR method for simultaneous identification of PMeV and PMeV2 in pre-flowering papaya plants. Moreover, we propose that an asymmetric mixture of PMeV and PMeV2 templates is the most appropriate target for determining the sensitivity of the mPCR method.

A survey was conducted on four groups of plants at different stages on several papaya production farms in the north of Espírito Santo state, Brazil. For the first group, (i) papaya seedlings (n = 10) were kept under greenhouse conditions for two months before leaves were collected. For the other groups, the papaya leaves in the field were collected from trees (ii) that were in the adult pre-flowering stage (n = 10), (iii) that were asymptomatic in the post-flowering stage (n = 16), and (iv) that were symptomatic in the post-flowering stage (n = 6). Leaf samples were taken on the same day from different papaya plants.

Total RNA was extracted from 100 mg of papaya leaves using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). RNA purity (A_{260}/A_{280}) was assessed using a NanoDrop® ND2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The templates used for RT-PCR reactions were obtained from 1 µg of purified RNA that had been treated with DNAse I (Invitrogen, Carlsbad, CA, USA). For the uniplex PCR reaction, the RNA was incubated at 96 °C for 3 min and 70 °C for 10 min to denature the dsRNA (PMeV) and ssRNA (PMeV2). For the mPCR reaction, the RNA was denatured at 96 °C for 3 min. First-strand cDNA synthesis was performed using random hexamers and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

Two primer pairs were utilized for both uniplex PCR and mPCR diagnosis. The PMeV-specific primer pair targets the predicted PMeV ORF1 at nucleotide position 2446-2816 (PMeVC1F, 5´CTTGGTTAGGCATAACTGTAGGT3´; PMeVC1R, 5´CACGGACTCTTAGAAACGTCTATC3´) [3]. The PMeV2-specific primer pair targets ORF2 at nucleotide position 1430-2244 (PMeV2F, 5´CGCCAAGTGGGATAAGTTTAGA3´; PMeV2R, 5´CGATTTGAGCACAAGGGTTAATG3´) based on an available genomic sequence (NCBI GenBank no. KT921785). The primers were designed using the PrimerQuest Tool (https://www.idtdna.com/PrimerQuest/Home/Index), and their specificity was verified using BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers for PMeV amplify a 370-bp fragment, and those for PMeV2 amplify an 814-bp fragment.

Uniplex and mPCR reactions were performed in a Mastercycler Thermocycler (Eppendorf, Hamburg, Germany) using Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA). To determine the optimal PCR conditions, different annealing temperatures (52 °C, 54 °C, 56 °C, 58 °C, 60 °C, 62 °C) and concentrations of each specific primer set (0.5:0.5 µM or 1.5:0.5 µM) were tested.

Following optimization, PCR amplification was performed in a 10-µl volume containing 1.54 µl of PCR mix (1 µl of 10X PCR Buffer -Mg^{2+}, 0.3 µl of 50 mM MgCl2, 0.2 µl of 10 mM dNTP mixture and 0.04 µl of recombinant Taq DNA polymerase [5 U/ µl]), and deionized water. The uniplex PCR reaction for detection of PMeV or PMeV2 contained 1 µl of PMeV or PMeV2 primers (10 µM), while multiplex PCR reactions were performed with half the amount of both primers. Both uniplex and mPCR reaction were performed with 55 ng of cDNA. The PCR mix and primers were manufactured by Invitrogen, Carlsbad, CA, USA.

The PCR protocol consisted of the following: 94 °C for 3 min followed by 35 cycles of amplification (94 °C for 45 s, 58 °C for 30 s, 72 °C for 1 min) and a final extension at 72 °C for 10 min. PMeV2 conditions were the same as for PMeV, but the extension time during the cycles was increased to 1.2 min. The mPCR program was the same as for the PMeV2 uniplex reaction. PCR amplicons were analyzed by electrophoresis on 1% (w/v) agarose gels stained with ethidium bromide and visualized under UV light.

To assess the LOD of the uniplex and mPCR assays, we generated recombinant plasmids by ligating the RT-PCR products into the plasmid pGEM®-T Easy Vector (Promega, Fitchburg, WI, USA). The specificity of PCR was validated by Sanger sequencing. The plasmid copy number was determined [18], and serial tenfold dilutions (10^8-10^1 copies/µl) were used as a template in 10-µL uniplex PCR mixtures. To determine the sensitivity of the mPCR, two different assays were performed. In the first one, equal volumes of each plasmid dilution were used as a template in different PCR reactions. In the second, different ratios of the PMeV and PMeV2 plasmids were used (10^8:10^3, 10^8:10^2, and 10^8:10^1) to mimic situations in which different viral titers are present in field samples. All reactions were performed according to the program described above for mPCR.

To determine the optimal annealing temperature for the PCR reactions, a gradient test was performed in uniplex and mPCR reactions. No differences in the efficiency of the reaction were found when different temperatures were tested; therefore, the annealing temperature was chosen to be 58 °C. This temperature was also used to test different PMeV and PMeV2 primer ratios in mPCR using cDNA from symptomatic post-flowering plants. Based on the intensity of amplicons, the 0.5:0.5 µM primer ratio was used in further reactions. Moreover, reliable diagnosis of the PMeV complex using current techniques requires synthesis of a cDNA with two different denaturation temperatures (one for each virus) [3], and this consumes double the materials and reagents for PCR detection. We tested these two cDNA samples in the mPCR assay, but only the PMeV dsRNA denaturation protocol (96 °C for 3 min) gave results that were consistent with those obtained with the uniplex RT-PCR (data not shown).

The sensitivity test showed that the uniplex RT-PCR assay could detect 10 copies of PMeV, whereas the LOD for PMeV2 was 100 copies (Online Resource 1). The sensitivity of the uniplex PCR was compared with that of the mPCR, and they were found to have equal sensitivity, although the band intensity was weaker at all dilutions in the mPCR.

To validate the mPCR assay for use in field surveys, samples collected from papaya plants at stages i, ii, iii, and iv were tested. Forty-two papaya plants were tested, and the results are summarized in Table 1. In seedlings, all samples were positive for PMeV

but negative for PMeV2. All adult pre-flowering plants tested positive for both PMeV and PMeV2, although some differences were found between the post-flowering groups. PMeV2 was detected in 12 out of 16 samples from group iii and in all samples of group iv, while fewer samples (three in the asymptomatic group and two in the symptomatic group) tested negative for PMeV. To rule out a false-negative diagnosis, we perform the uniplex RT-PCR assay with samples that tested negative for one of the viruses (data not shown). The results for the four samples from group iii that tested negative for PMeV2 and all seedling samples agreed with the mPCR results. On the other hand, uniplex RT-PCR confirmed the infection in the remaining samples, revealing a discrepancy between the results of this experiment and those of the LOD experiment.

The sensitivity test using an equimolar plasmid ratio demonstrated that PMeV and PMeV2 detection in the mPCR was not altered when compared to the uniplex PCR (Online Resource 1). Therefore, it was not clear why some field samples were positive for PMeV in the uniplex PCR but not in the mPCR assay. One possible reason could be related to differences in viral titer. The use of equal amounts of PMeV and PMeV2 recombinant plasmids in sensitivity assay may not reflect the actual amounts of these viruses in the papaya plants. To test this hypothesis, we performed assays with different PMeV:PMeV2 plasmid copy number ratios (10^8:10^3, 10^8:10^2, and 10^8:10^1) in the mPCR. The results showed that when one virus was present at a high titer, the band intensity for the other virus in the agarose gel was lower (Online Resource 1).

The discovery of a second virus associated with sticky disease in plants indicated the need for the development of a new diagnostic tool. In this study, an already available primer pair [3] and a new one were used to develop an mPCR assay to detect PMeV and PMeV2 in a single reaction and its sensitivity and applicability for use in field surveys were evaluated.

It is a common practice to use equimolar amounts of PCR templates to determine the detection limit of an mPCR assay [14, 17,18,19]. Here, we determined the detection limit when different ratios of templates were used and found that altering the relative amount of the templates indeed affected the results (Online Resource 1). Although PMeV was detected more frequently than PMeV2 in pre-flowering papaya plants [23],

this difference was more pronounced at later stages of infection (post-flowering symptomatic), as both viruses were successfully detected by mPCR in all adult pre-flowering plants. Amplicons produced by mPCR in the sensitivity test showed lower band intensity in an agarose gel. The presence of two primer pairs forces competition between the amplicons by the PCR reagents and thus reduces the yield of either the amplicons [24]. When templates were used in equal amounts, the reduced yield did not affect the LOD for both PMeV or PMeV2 amplicon.

In this study, we developed a multiplex PCR method for simultaneous detection of PMeV and PMeV2 in papaya pre-flowering plants. This method is very useful for early diagnosis because it can be used to screen simultaneously for both viruses in a large number of samples. Therefore, this procedure will contribute to a better understanding of PSD epidemiology and to the development of disease management strategies.

Acknowledgments: T.F. Sá Antunes and S. A. Oliveira would like to thank the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior*, CAPES (Ministry of Education of Brazil) and M. Maurastoni the*Fundação de Amparo à Pesquisa do Espírito Santo*, FAPES for their fellowship. P.M.B. Fernandes and José A. Ventura were granted with the research productivity award from the *Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq),* Grant #304719/2014-5 and #308631/2016-1, respectively.

Conflicts of Interest: The authors declare that they have no conflict of interest.

REFERENCES

1. Maciel-Zambolim E, Kunieda-Alonso S, Matsuoka K, De Carvalho M, Zerbini F (2003) Purification and some properties of Papaya meleira virus, a novel virus infecting papayas in Brazil. Plant Pathol 52:389-394.

2. Kitajima EW, Rodrigues CH, Silveira JS, Alves F, Ventura JA, Aragão FJL, Oliveira LHR (1993) Association of isometric viruslike particles, restricted to lacticifers, with" meleira"(" sticky disease") of papaya (*Carica papaya*). Fitopatol Bras 18:118-122.

3. Antunes TFS, Amaral RJV, Ventura JA, Godinho MT, Amaral JG, Souza FO, Zerbini PA, Zerbini FM, Fernandes PMB (2016) The dsRNA Virus Papaya Meleira Virus and an ssRNA Virus Are Associated with Papaya Sticky Disease. PloS One 11:e0155240.

4. Meissner Filho P, Lima Neto F, de Oliveira C, Santana S, Dantas J (2017) Avaliação da resistência de genótipos de mamoeiro ao vírus da meleira no Semiárido. Embrapa Mandioca e Fruticultura-Boletim de Pesquisa e Desenvolvimento (INFOTECA-E). Embrapa Mandioca e Fruticultura, Cruz das Almas, BA.

5. Ventura JA, Costa H, Tatagiba JdS (2004) Papaya Diseases and Integrated Control. In: Naqvi SAMH (ed) Diseases of Fruits and Vegetables: Volume II: Diagnosis and Management. Springer Netherlands, Dordrecht, pp 201-268.

6. Abreu PMV, Antunes TFS, Magaña-Álvarez A, Pérez-Brito D, Tapia-Tussell R, Ventura JA, Fernandes AAR, Fernandes PMB (2015) A current overview of the Papaya meleira virus, an unusual plant virus. Viruses 7:1853-1870.

7. Rodrigues SP, Galvao OP, Andrade JS, Ventura JA, Fernandes PMB (2005) Simplified molecular method for the diagnosis of Papaya meleira virus in papaya latex and tissues. Summa Phytopathol 31:281-283.

8. Araújo MMMd, Tavares ÉT, Silva FRd, Marinho VLdA, Júnior MTS (2007) Molecular detection of Papaya meleira virus in the latex of *Carica papaya* by RT-PCR. J Virol Methods 146:305-310.

9. Abreu PMV, Piccin JG, Rodrigues SP, Buss DS, Ventura JA, Fernandes PMB (2012) Molecular diagnosis of Papaya meleira virus (PMeV) from leaf samples of *Carica papaya* L. using conventional and real-time RT-PCR. J Virol Methods 180:11-17.

10. Elnifro EM, Ashshi AM, Cooper RJ, Klapper PE (2000) Multiplex PCR: optimization and application in diagnostic virology. Clin Microbiol Rev 13:559-570.

11. Tuo D, Shen W, Yang Y, Yan P, Li X, Zhou P (2014) Development and validation of a multiplex reverse transcription PCR assay for simultaneous detection of three papaya viruses. Viruses 6:3893-3906.

12. Forootan A, Sjöback R, Björkman J, Sjögreen B, Linz L, Kubista M (2017) Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). Biomol Detect Quantif 12:1-6.

13. Broeders S, Huber I, Grohmann L, Berben G, Taverniers I, Mazzara M, Roosens N, Morisset D (2014) Guidelines for validation of qualitative real-time PCR methods. Trends Food Sci Tech 37:115-126.

14. Zhao X, Liu X, Ge B, Li M, Hong B (2015) A multiplex RT-PCR for simultaneous detection and identification of five viruses and two viroids infecting chrysanthemum. Arch Virol 160:1145-1152.

15. Hyun JW, Hwang RY, Jung KE (2017) Development of multiplex PCR for simultaneous detection of citrus viruses and the incidence of citrus viral diseases in late-maturity citrus trees in Jeju Island. Plant Pathol J 33:307.

16. Kwon JY, Hong JS, Kim MJ, Choi SH, Min BE, Song EG, Kim HH, Ryu KH (2014) Simultaneous multiplex PCR detection of seven cucurbit-infecting viruses. J Virol Methods 206:133-139.

17. Tao Y, Man J, Wu Y (2012) Development of a multiplex polymerase chain reaction for simultaneous detection of wheat viruses and a phytoplasma in China. Arch Virol 157:1261-1267.

18. Deng X, Zhang J, Su J, Liu H, Cong Y, Zhang L, Zhang K, Shi N, Lu R, Yan X (2018) A multiplex PCR method for the simultaneous detection of three viruses associated with canine viral enteric infections. Arch Virol 163:2133-2138.

19. Zhang X, Peng Y, Wang Y, Zhang Z, Li D, Yu J, Han C (2016) Simultaneous detection and differentiation of three genotypes of Brassica yellows virus by multiplex reverse transcription-polymerase chain reaction. Virology J 13:189.

20. Syller J (2012) Facilitative and antagonistic interactions between plant viruses in mixed infections. Mol Plant Pathol 13:204-216.

21. Mascia T, Gallitelli D (2016) Synergies and antagonisms in virus interactions. Plant Sci 252:176-192.

22. Chávez-Calvillo G, Contreras-Paredes CA, Mora-Macias J, Noa-Carrazana JC, Serrano-Rubio AA, Dinkova TD, Carrillo-Tripp M, Silva-Rosales L (2016) Antagonism or synergism between papaya ringspot virus and papaya mosaic virus in *Carica papaya* is determined by their order of infection. Virology 489:179-191.

23. Madronero J, Rodrigues SP, Antunes TFS, Abreu PMV, Ventura JA, Fernandes AAR, Fernandes PMB (2018) Transcriptome analysis provides insights into the delayed sticky disease symptoms in *Carica papaya*. Plant Cell Rep 37:967-980.

24. Anitha S, Monyo E, Okori P (2014) Simultaneous detection of groundnut rosette assistor virus (GRAV), groundnut rosette virus (GRV) and satellite RNA (satRNA) in groundnuts using multiplex RT-PCR. Arch Virol 159:3059-30

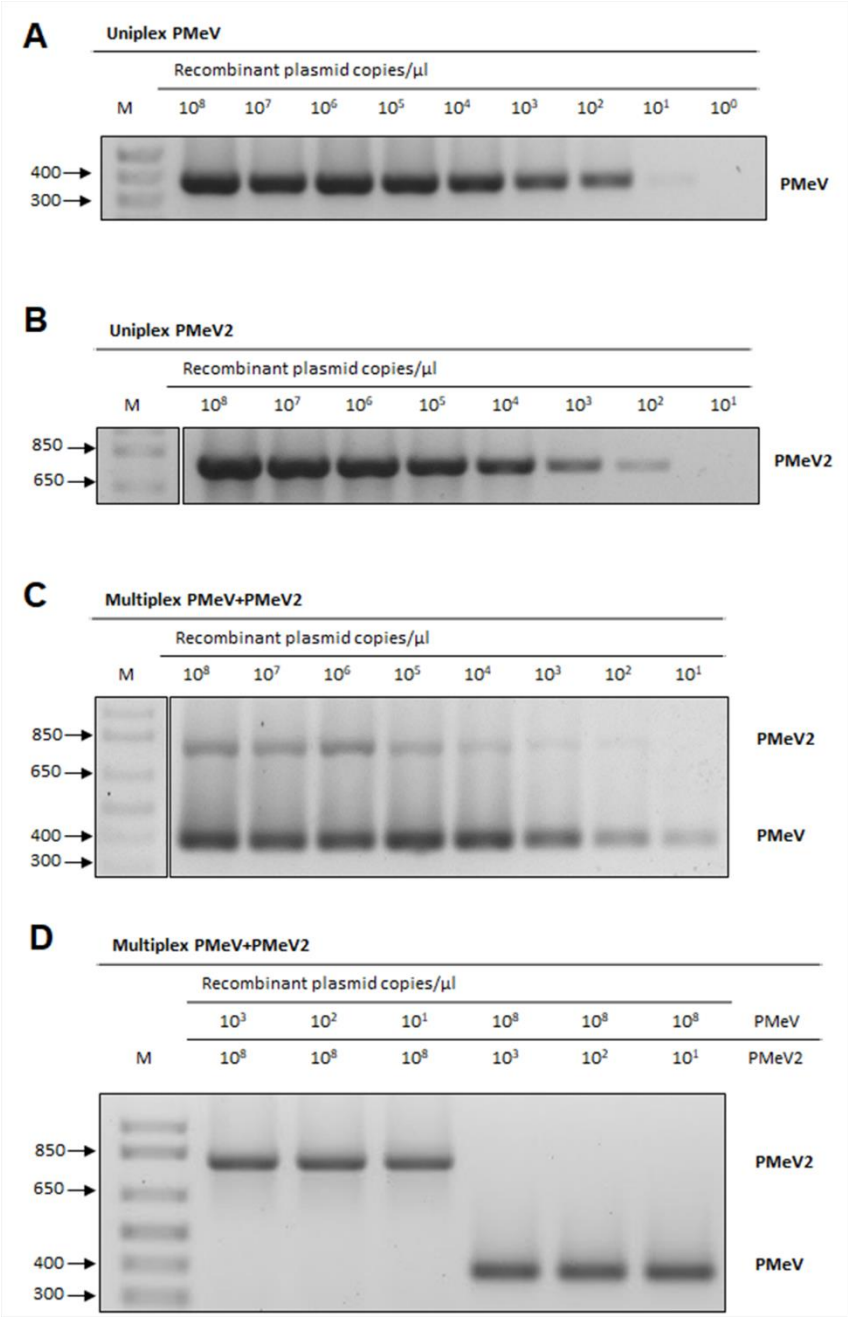
TABLES

Table 1. Results of uniplex and multiplex PCR survey testing 42 plants from greenhouse and papaya orchards in Espírito Santo, Brazil

Development stage	Uniplex*		Multiplex*	
	PMeV	PMeV2	PMeV	PMeV2
Seedlings	10/10	10/0	10/10	10/0
Adult Pre-flowering	10/10	10/10	10/10	10/10
Post-flowering asymptomatic	16/16	16/12	16/12	16/12
Post-flowering symptomatic	6/6	6/6	6/4	6/6

* No. of total plants/No. positives

SUPPLEMENTARY MATERIAL



MANUSCRIPT #5. EFFORTS TO UNDERSTAND TRANSMISSION OF THE PAPAYA MELEIRA VIRUS COMPLEX BY INSECTS

Manuscript in preparation for *Annals of Applied Biology* (ISSN 0003-4746; IF 2.75, 2020; Qualis Biotecnologia B1)

Efforts to understand transmission of the papaya meleira virus complex by insects

Marlonni Maurastoni[a], Tathiana F. Sá-Antunes[a], José A. Ventura[a,b] , Anna E. Whitfield[c*] and Patricia M. B. Fernandes[a*]

[a]Núcleo de Biotecnologia, Universidade Federal do Espírito Santo, Vitória, Espírito Santo, Brazil

[b]Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural, Vitória, Epírito Santo, Brazil

[c]Department of Entomology and Plant Pathology, North Carolina State University, Raleigh, North Carolina, USA

*Corresponding authors: awhitfi@ncsu.edu; patricia.fernandes@ufes.br

ABSTRACT

Papaya sticky disease (PSD) is an emerging disease causing significant crop losses in some of the major papaya-growing regions of the world. The vectors of the PSD-associated viruses in Brazil are still unknown. Publications on transmission and epidemiology of PSD have increased recently with the spread of the disease to additional papaya-growing countries. In this review, we present an overview of the vector biology studies of PSD transmission. Epidemiological analyses attributed fruit thinning as a mechanism spreading the disease, but an aerial vector was not ruled out. Hemipteran insects have been implicated as vectors but a definitive conclusion on the biologically relevant vector has not been reached. Leafhoppers have a population peak a month before the PSD incidence peak in the field and their ability to vector the PMeV Mexican isolate has been demonstrated. Whiteflies (*Bemisia tabaci* Middle East-Asia Minor 1) have been reported to occur in plants close to papaya trees in Brazil and to transmit an Ecuadorian virus similar to PMeV2. In Brazil, *Trialeurodes variabilis* which colonizes papaya trees, can acquire but not transmit the PMeV complex. The conflicting reports of insect vectors for PSD and related viruses highlights the need for additional research on this important pathosystem. Elucidation of the PMeV complex vector would contribute to the efficient management of papaya sticky disease and increase understanding of the transmission mechanisms of plant-infecting toti-like viruses.

Keywords**:** sticky disease, vector, Leafhoppers, Trialeurodes*,* transmission.

INTRODUCTION TO PAPAYA STICKY DISEASE

Papaya sticky disease (PSD) is one of the major diseases affecting papaya orchards in Brazil (RODRIGUES *et al.*, 1989), Mexico (PEREZ-BRITO *et al.*, 2012), and Australia (PATHANIA *et al.*, 2019), and capable of causing complete crop loss. The distribution of PSD under field conditions points to possible mechanical transmission and an aerial vector but the PSD insect vector is still unknown in Brazil (ABREU, *et al.*, 2015; ANTUNES *et al.*, 2020) even though, several transmission assays and epidemiology analyses have been done in the last 30 years. In Mexico, the dispersion occurs by the leafhopper *Empoasca papayae* Oman (Hemiptera: Cicadellidae) (GARCÍA-CÁMARA *et al.*, 2019), and also through the infected seeds (TAPIA-TUSSELL *et al.*, 2015). The presence of a vector in Australian orchards has not yet been confirmed, but seeds play an important role in the spread of the disease (CAMPBELL, 2019a; b).

The presence of a biotic agent responsible for PSD is dated in a 1989 publication showing that inoculation of infected latex leads to symptom development in healthy plants (RODRIGUES *et al.*, 1989). The disease's etiology was initially confirmed in 2003 as being caused by a virus (MACIEL-ZAMBOLIM *et al.*, 2003) and only in 2016 a second virus was identified associated with diseased plants (ANTUNES *et al.*, 2016).

Since the confirmation of its etiology, Brazilian researchers have tried to identify the insect vector. Initially, experiments have shown that healthy plants can develop symptoms when inoculated with macerated whiteflies (*Bemisia tabaci* Middle East-Asia Minor 1) collected near diseased papaya plants (HABIBE *et al.*, 2001). Although *B. tabaci* MEAM1 did not colonize papaya trees, this result paved the way to investigate the correlation of another species of whitefly associated with papaya: *Trialeurodes variabilis*. From 2002 to 2003 Brazilian papaya orchards were intensively analyzed in search of a positive correlation between a population of an insect species and plants with PSD symptoms. *Tiraleurodes variabilis* does not show this correlation in the field and experiments conducted in the greenhouse have shown that despite acquiring the infectious agent, this species is not able to transmit it (ANDRADE *et al.*, 2003; LIMA *et al.*, 2003; RODRIGUES, S. *et al.*, 2009). Surprisingly, field analysis showed a positive

correlation with leafhoppers (*Solanasca bordia*) which was confirmed in a second analysis conducted from 2017 to 2018.

During these last 30 years, publications on transmission and epidemiology, written only in Portuguese, have been presented and discussed at Brazilian meetings and are available across different libraries and journals (Table 1). Recently, the number of publications and experiments carried out to identify PSD vectors has intensified since the disease has reached other countries. Thus, in this review, we have compiled these works and discussed their main findings given the molecular diagnostic techniques developed over the years and the new proposed etiology. We open an important discussion for directing new research to understand the vectors of this virus complex and the use of new management practices in papaya orchards.

COULD PMEV COMPLEX BE TRANSMITTED BY FUNGI?

Spontaneous latex exudation from green fruits and necrosis in the edge of young leaves are the main PSD symptoms (VENTURA *et al.*, 2004) which are associated with infection by an unclassified viral complex (papaya meleira virus - PMeV and papaya meleira virus 2 - PMeV2, PMeV complex) (Figure 1) (ANTUNES *et al.*, 2016).

The viral structural proteins protect the viral genome and play a role in several biological processes such as virus movement within the host, replication, translation, and specificity of transmission by a vector (BOL, 2008). In mixed infections, capsid proteins (CPs) produced by PMeV are used for the package of PMeV2 (trans-encapsidation) resulting in virions with the same morphology but containing different RNAs (ANTUNES *et al.*, 2016) which supports the idea that PMeV and PMeV2 could be transmitted by the same vectors. The trans-encapsidation phenomenon is also found between members of the *Umbravirus* genus and poleroviruses or enamoviruses (family *Solemoviridae)*. Umbraviruses lack the CP gene and, as a result, do not form conventional virus particles, even though they can systematically infect a plant when mechanically inoculated (TALIANSKY; ROBINSON, 2003). Umbraviruses transmission between plants with the aid of insect vectors is only possible when the umbraviral genome is packaged by the luteovirids capsid protein, which results in the same host range (TALIANSKY *et al.*, 2000). In members of the *Totiviridae* family, the CP is typically encoded by the 5’ ORF (ORF1) which generally have sizes between 70–100 kDa (DE LIMA *et al.*, 2019) and are predominantly α-helical (LUQUE *et al.*, 2018). Members of the *Totiviridae* family generally associated with fungi, yeast, and parasitic protozoa, have been also found infecting mollusks, arthropods, including mosquitos, ants, shrimps, and planthoppers, and plants (DE LIMA *et al.*, 2019). Besides PMeV, maize-associated totivirus (MATV), panax notoginseng virus A (PnVA) and tea-oil camellia-associated totivirus 1 (TOCaTV1) are unclassified viruses that also

infect plants (AKINYEMI *et al.*, 2018; GUO *et al.*, 2016; ZHANG *et al*., 2021). Although there are no reports of a CP coded by these viruses (except for PMeV), the PnVA, MATV and TOCaTV1 ORF1 have a conserved region which includes the LA virus coat domain (pfam09220), present in all CPs of totiviruses infecting fungi (AKINYEMI *et al.*, 2018). The fact that the CP of these plant viruses is more similar to totiviruses that infect fungi than totiviruses that infect insects supports the idea that fungi may act as vectors of the PMeV complex. Given the opportunities for transfer during fungal colonization, it is possible that PMeV, MATV, PnVA and TOCaTV1 can be transmitted to plants via a fungal host species (ANDIKA *et al.*, 2017; ROOSSINCK, 2019). Under controlled conditions, *Rizoctania solani* can acquire and transmit a plant virus, cucumber mosaic virus (CMV), during plant infection (ANDIKA *et al.*, 2017). Several fungi are found infecting papaya leaves and they are included in the genus *Asperisporium*, *Stagonosporopsis* (Syn.: *Phoma*), *Colletotrichum*, and *Corynespora*, and recently an (+) ssRNA virus was found in *Phoma matteuccicola*, the causal agent of leaf blight disease in *Curcuma wenyujin* (ZHOU *et al.*, 2020). If fungi also play a role in PMeV complex transmission, important uncharacterized structural domains may be present in PMeV capsid.

HOW DOES AN INSECT ACQUIRE PMEV COMPLEX VIRIONS FROM A DISEASED PLANT?

A longstanding question in virus acquisition by an insect vector is how can they acquire PMeV virions from papaya laticifers, the only documented site of virus particle accumulation in papaya plants (KITAJIMA *et al.*, 1993). PMeV virions were visualized, using transmission electron microscopy (TEM), in laticifers cells, a structure well known for its defense role against pathogens. In *C. papaya*, laticifers are articulated, anastomosed (HAGEL *et al.*, 2008), and found in all papaya organs (FISHER, 1980; RAO *et al.*, 2013). Mature papaya laticifers are living cells that store, under high pressure, vesicles containing, carbohydrates, lipid salts, and proteins, mainly cysteine

proteases (EL MOUSSAOUI *et al.*, 2001). Upon tissue wounding, latex starts to exudate and cysteine proteases are activated resulting in the clotting of the wound (SILVA *et al.*, 1997). Whereas several studies report the different strategies adopted by mandibulate herbivores, little information is available on how sap-sucking insects can feed on latex-bearing plants. Any damage to laticifers could cause an overflow of harmful compounds (e.g proteolytic enzymes such as cysteine and serine proteases, organic acids, alkaloids, and terpenes) leading to the clogging or destruction of the insect's mouthparts. However, it has been shown that when feeding in two different latex-bearing plants, *Aphis nerii* can use its stylet to reach phloem cells avoiding the laticifers or completely circumscribing them during the probing (BOTHA *et al.*, 1975a; b). It is not yet clear how the PMeV viral particles are acquired by an insect. It is possible that insects can acquire viral particles present in other cells, which due to not accumulate are not observed by TEM but could be detected with immunocytochemical techniques, hitherto unavailable. In another scenario, the physiological and biochemical changes present in laticifers of PSD plants could help viral particles to be acquired by an insect. Laticifers of PSD plants present a reduction of protease levels and activity, and an increase in its fluidity (RODRIGUES, *et al.*, 2009) which could minimize the damage and the clogging to an insect mouthpart when probing a laticifer.

STUDIES OF PSD TRANSMISSION BY VECTORS

Insects are the most common vectors of plant viruses and are associated with more than 61% of virus species, and approximately 83% of insect-borne viruses are transmitted by hemipterans, e.g. aphids, whiteflies, leafhoppers, and planthoppers (reviewed in (COSTA, 2005) and (HOGENHOUT *et al.*, 2008)). Because the natural spread of viruses often depends on vectors, knowledge of the interrelationship between the virus and the vector is essential for establishing control strategies and mitigating the damage that the disease causes in plants.

The possible involvement of insects as sticky disease vectors has been suggested based upon early studies on the field spread pattern of this disease, especially with evidence of the existence of an aerial vector associated with the disease (MAFFIA *et al.*, 1993; RODRIGUES *et al.*, 1989). Although epidemiological studies of PSD implicate the involvement of vectors in the transmission of PMeV and PMeV2 viruses, the identity of the vector has not been determined in Brazil and in the other regions where the disease is present. Insects of the order Hemiptera, suborder Homoptera, have a large number of species that are reported as vectors of approximately 90% of the viruses transmitted by insects (COSTA, 2005). In addition to aphids (Family: *Aphididae*) reported as vectors of *Papaya ringspot virus* (PRSV-P), other homopterans, such as leafhoppers (Family: *Cicadellidae*) and whiteflies (Family: *Aleyrodidae*), are also reported as vectors of other diseases in papaya (LIMA *et al.*, 2003).

It is important to clarify that until 2007 most studies on PSD were based on virus detection through the visualization of the viral PMeV dsRNA. However, this technique requires that samples display a large amount of both viruses. The sequencing of both PMeV and PMeV2 (ABREU *et al.*, 2015; ANTUNES *et al.*, 2016; ARAÚJO *et al.*, 2007) allowed the development of more sensitive techniques such as RT-PCR (ABREU *et al.*, 2012; ANTUNES *et al.*, 2016; MAURASTONI *et al.*, 2020) and qRT-PCR (ABREU *et al.*, 2012) which have been applied to understand critical aspects of the PSD epidemiology. Through RT-PCR it was able to show that papaya plants infected by PMeV can remain asymptomatic in the field acting as a viral source for uninfected plants (ANTUNES *et al.*, 2016).

DO LEAFHOPPERS TRANSMIT PSD-ASSOCIATED VIRUSES?

Leafhoppers (Hemiptera: *Cicadellidae*) are threatening papaya pests because they cause significant damage and are potential vectors of viruses, phytoplasmas, and rickettsia. Leafhoppers emerged as potential vectors of the PMeV complex since their

distribution in the field is related to the spread of the PSD (LIMA *et al.*, 2003; VENTURA *et al.*, 2003). Surveys of leafhopper populations in papaya orchards in Brazil were conducted using sticky traps and circular sweep nets. During the one-year sampling period, most leafhoppers collected were identified as *Solanasca bordia* (Hemiptera: Cicadellidae: Typhlocybinae), accounting for 80% of the total, followed by species of the genus *Empoasca,* accounting for 5% of the total (GOUVEA *et al.*, 2018). Studies on the involvement of leafhoppers, especially those of the genus *Solanasca*, as vectors of PMeV have shown a high correlation between the insect population and the incidence of diseased plants (Figure 2). The population peak of leafhoppers precedes the highest peak of PSD incidence, which occurs about one month later (GOUVEA *et al.*, 2018). A delay of 45 days for symptom onset was also shown when papaya plants are mechanically inoculated with disease latex (VENTURA *et al.*, 2001). These results indicate that leafhoppers can be potential vectors of the PSD in Brazil and must be considered in further transmission assays (GOUVEA *et al.*, 2018; LIMA *et al.*, 2003; VENTURA *et al.*, 2003).

The population fluctuation of leafhoppers is compatible with the analysis of temporal evolution of papaya sticky disease and provides subsidies to verify the dispersion and generate information about the influence of biological and environmental factors on the population dynamics of the pathogen/disease. The most favorable period of the year for the disease development were colder and dry months, while the warmest and wettest months favored the mitigation of symptoms, and the model that best fitted the disease epidemics is the Gompertz (COSMI *et al.*, 2017).

In Mexico, the ability of the leafhopper *E. papayae* adults, but not nymphs, to transmit PMeV-Mx to *C. papaya* 'Maradol' has been proven. PMeV-Mx is an umbravirus-like associated RNA (ulaRNA) found infecting papaya plants in Mexico. It is 71% and 79% identical at nucleotide level to PMeV2 and the Ecuadorian virus, papaya virus Q (PpVQ) respectively. Under controlled conditions, *E. papayae* can acquire PMeV-Mx six hours after exposure to infected plants, and viral titer increases if the exposure time is longer up to 5 days (Figure 3). Little is known about the biology of *E. papayae,* and research is now focused on understanding the behavior of this insect in the field (GARCÍA-CÁMARA *et al.*, 2019). Despite the lower abundance among the collected

species, insects from the family *Cicadelidae* (*Agallia constricta, Agalliopsis novella*, *E. papayae, Draeculacephala. soluta*, *Hortensia* sp., and *Xyphon* sp.) and *Aphididae* (*Aphis* sp. and *Uroleucon taraxaci)* were also identified containing the PMeV-Mx but their potential as vectors has yet to be studied.

Research teams in Brazil are currently conducting experiments to identify the virus vector and elucidate the transmission mechanism. Under field conditions, research on the papaya-producing region of the north of Espírito Santo state found the most frequent leafhoppers in papaya plants belonging to *Cercopydae*, *Cicadellidae*, *Membracidae,* and *Delphacidae* families (VENTURA, J.A.; unpublished data).

DO WHITEFLIES TRANSMIT PSD-ASSOCIATED VIRUSES?

Whiteflies are considered secondary pests of papayas worldwide because they do not cause important damage to plants or fruits in field orchards. Among the whitefly species reported worldwide two species, *Bemisia tabaci* MEAM1 and *Trialeurodes variabilis,* have been reported occurring in different areas of Brazilian papaya orchards (MARTINS *et al.*, 2016) (Figure 4), and its ability to transmit the infectious agent of PSD in Brazil, was evaluated in three different works through the visualization of PMeV dsRNA (HABIBE *et al.*, 2001; RODRIGUES. *et al.*, 2009; VIDAL *et al.*, 2003).

B. tabaci MEAM1, despite being reported to cause damage to papaya in other biogeographic regions of the world, so far it has limited occurrence to protected cultivation environments and is not considered a papaya pest in Brazil under field conditions (VENTURA *et al.*, 2004). As a polyphagous insect, the whitefly colonizes and multiplies on numerous cultivated, wild, and invasive plants. The ability of *B. tabaci* MEAM1 to acquire and transmit the PSD infectious agent was assessed by two different experiments. Habibe et al., (2001) inoculated macerated bodies of whiteflies collected from areas with PSD into healthy papaya plants. Ninety days after inoculation, healthy plants presented viral dsRNA of similar size to that detected in PSD plants,

which suggested that *B. tabaci* MEAM1 is capable of acquiring the infectious form of the PMeV complex (HABIBE *et al.*, 2001). In another experiment, the ability of *B. tabaci* MEAM1 to transmit the PSD infectious agent was determined when the dsRNA of PMeV was detected in asymptomatic plants exposed for 24-72h to whiteflies that have previously been forced feeding for 48h and 30min on diseased papaya plants (Figure 5.A) (VIDAL *et al.*, 2003). In this experiment, the authors do not mention any diagnostic test in asymptomatic plants. After the development of sensitive techniques for PSD-associated virus diagnosis (e.g RT-PCR), it is not uncommon to detect viral RNA in asymptomatic plants (ANTUNES *et al*., 2016). This supports the idea that asymptomatic but infected plants were used for the experiment, instead of virus-free plants. Moreover, the fact that few plants were exposed to the whiteflies raises the necessity to include a higher number of plants in this experiment. This group also tested the virus transmission by aphid species, *Toxoptera citricidus,* and *Myzus persicae*, but they were unable to transmit the PMeV dsRNA to healthy plants. Under field conditions, Martins et al. (2016) when studying aphid population species and their host plants in commercial papaya orchards, found no evidence that these insects were involved in the transmission of PSD.

T. variabilis initially infest papaya leaves on the top of the canopies and then move to newly developed leaves. Eggs and nymphs are found in all parts of the canopy, but insects preferentially feed and lay their eggs on new leaves. Also, it is common to see oviposition concentrated in the basal region, and nymphs more frequently in the central part of older leaves (MARTINS *et al.*, 2016). The ability of *T. variabilis* to transmit the PMeV dsRNA was assessed under greenhouse conditions (Figure 5.B). Twenty-four plants were inoculated with papaya diseased-latex, and one month later, they were infested with a population of *T. variabilis* collected from fields with asymptomatic papaya plants. One month later, three healthy papaya plants of different cultivars each (cvs. Taiwan, Golden, and Sunrise Solo) were added inside the greenhouse to be infested by the whiteflies. Twenty days later, dsRNA was detected in plants used as initial inoculum, and in adults and nymphs exposed to latex-inoculated plants but not in healthy plants that were exposed to "viruliferous" whiteflies. The authors suggested that *T. variabilis* can acquire the virus from infected plants and it is not able to transmit it to healthy plants under controlled conditions (RODRIGUES *et al*., 2009). The amount

of virus inoculated through latex injection is higher than through vector transmission. This difference could result in a lower virus load in plants that were exposed to "viruliferous" whiteflies, undetectable for dsRNA visualization.

Epidemiological analysis revealed that PSD spread does not follow the same pattern as the fluctuation of the whitefly population (ANDRADE *et al.*, 2003; LIMA *et al.*, 2003). PSD occurs initially scattered and randomly in the orchard, later evolving to aggregation. Clouds of whiteflies are regularly observed in papaya crops during peak periods of the insect population and low incidence of plants with PSD, which suggests that whiteflies could not be the major insect involved in PSD spreading (MARTINS, D. *et al.*, 2016) (Figure 6). Whiteflies have a preference for certain hosts and even though they acquire viruses they only transmit a few, for example, viruses belonging to the genera *Begomovirus*, *Carlavirus, Crinivirus* and *Polerovirus* (GHOSH *et al.*, 2019).

In Ecuador, the latest transmission tests pointed to whiteflies (*B. tabaci*) as vectors of the ulaRNA PpVQ. Epidemiology data suggests an aerial vector for PpVQ which commonly occurs associated with *Papaya ringspot virus* (PRSV). However, efforts to transmit the virus from plants using aphids were only successful for PRSV but not for PpVQ (QUITO-AVILA *et al.*, 2015). To understand the vector of PpVQ in Ecuador, a field survey identified whiteflies, red mites, and mealybugs as the main arthropods present in papaya-infected plants and detected PpVQ in all three groups. These arthropods were collected and transferred to PpVQ virus-free papaya plants where they fed for 7 days. Ninety days after exposure to whiteflies, the virus was detected in three out of ten plants which whiteflies had fed, but not in field-collected whiteflies 7 days after feeding in PpVQ virus-free plants. None of the plants exposed to field-collected red mites and mealybugs tested positive for PpVQ (CORNEJO-FRANCO *et al.*, 2018).

Overall, the role of whiteflies as vectors of PSD needs to be carefully assessed since the experiments and analyzes carried out so far reach different conclusions. *T. variabilis* does not have a field distribution correlated with the incidence of diseased plants and is not able to transmit the infectious form of the viral complex to healthy plants. *B. tabaci* MEAM1 is not found colonizing papaya plants in the field which does

not support its role as a vector even though, they can acquire the PMeV dsRNA from papaya plants under greenhouse conditions.

CONCLUSIONS

For the past 30 years, research groups studying PSD have made efforts to understand the vector of PSD causative agents in the main papaya producers in the world. It is still challenging to assign a specific vector but epidemiological analysis shows insects as potential spreaders, among them leafhoppers and whiteflies. In Mexico and Ecuador, the question: "who are the vectors of the causal agents of the PSD?" is partially answered. Although leafhoppers and whiteflies were not found during survey analyzes conducted in Ecuador and Mexico, respectively, the sequence and genome organization similarity of PpVQ and PMeV-Mx supports the idea that both can be transmitted by the two insects. In Brazil, the results of the experiments conducted with whiteflies so far are contradictory but we cannot rule out that these insects may play a role in the dispersion of viruses in the field, among invasive plants as sources of inoculum. Leafhoppers need to be studied as potential vectors of the PMeV complex in Brazil, since these insects already play a role in the disease spread in Mexico and that their population fluctuation is related to the PSD occurrence in Brazil. Importantly, previous experiments need to be repeated and analyzed now that more sensitive molecular diagnostic techniques are available and with considerations of how the virus complex of a toti-like virus (PMeV) and the ulaRNA (PMeV2) may impact disease physiology and vector transmission. Knowledge about the diversity of viruses tentatively classified in the family *Totiviridae* infecting plants is very limited, as well as their modes of transmission. Therefore, studies that elucidate the PMeV complex vector and its transmission mechanisms could reveal uncharacterized relationships between viral structural proteins and insect vectors.

REFERENCES

ABREU, E. F. M.; DALTRO, C. B.; NOGUEIRA, E. O.; ANDRADE, E. C.; ARAGÃO, F. J. Sequence and genome organization of papaya meleira virus infecting papaya in Brazil. **Archives of virology**, 160, n. 12, p. 3143-3147, 2015.

ABREU, P. M. V.; ANTUNES, T. F. S.; MAGAÑA-ÁLVAREZ, A.; PÉREZ-BRITO, D.; TAPIA-TUSSELL, R.; VENTURA, J. A.; FERNANDES, A. A. R.; FERNANDES, P. M. B. A current overview of the Papaya meleira virus, an unusual plant virus. **Viruses**, 7, n. 4, p. 1853-1870, 2015.

ABREU, P. M. V.; PICCIN, J. G.; RODRIGUES, S. P.; BUSS, D. S.; VENTURA, J. A.; FERNANDES, P. M. B. Molecular diagnosis of Papaya meleira virus (PMeV) from leaf samples of *Carica papaya* L. using conventional and real-time RT-PCR. **Journal of Virological Methods**, 180, n. 1, p. 11-17, 2012/03/01/ 2012.

AKINYEMI, I. A.; WANG, F.; CHANG, Z.-X.; WU, Q. Genome characterization of the newly identified maize-associated totivirus Anhui. **Archives of Virology**, 163, n. 10, p. 2929-2931, 2018/10/01 2018.

ANDIKA, I. B.; WEI, S.; CAO, C.; SALAIPETH, L.; KONDO, H.; SUN, L. Phytopathogenic fungus hosts a plant virus: A naturally occurring cross-kingdom viral infection. **Proceedings of the National Academy of Sciences**, 114, n. 46, p. 12267-12272, 2017.

ANDRADE, J. D. S.; VENTURA, J. A.; RODRIGUES, S. P.; COUTO, A. D. O. F.; LIMA, R. D. C. A.; TATAGIBA, J. D. S.; FERNANDES, P. M. B.; MARTINS, D. D. S. Evidência da não transmissão do vírus da meleira por mosca-branca *Trialeurodes variabilis* (Quaintance, 1900). *In*: Papaya Brasil: qualidade do mamão para o mercado interno, 2003, Vitória-ES, Brazil. p. 605-608.

ANTUNES, T. F. S.; AMARAL, R. J. V.; VENTURA, J. A.; GODINHO, M. T.; AMARAL, J. G.; SOUZA, F. O.; ZERBINI, P. A.; ZERBINI, F. M.; FERNANDES, P. M. B. The dsRNA Virus Papaya Meleira Virus and an ssRNA Virus are Associated with Papaya Sticky Disease. **PloS One**, 11, p. e0155240, 2016.

ANTUNES, T. F. S.; MAURASTONI, M.; MADROÑERO, L. J.; FUENTES, G.; SANTAMARÍA, J. M.; VENTURA, J. A.; ABREU, E. F.; FERNANDES, A. A. R.; FERNANDES, P. M. B. Battle of Three: The Curious Case of Papaya Sticky Disease. **Plant Disease**, 104, n. 11, p. 2754-2763, 2020.

ARAÚJO, M. M. M. D.; TAVARES, É. T.; SILVA, F. R. D.; MARINHO, V. L. D. A.; JÚNIOR, M. T. S. Molecular detection of Papaya meleira virus in the latex of *Carica papaya* by RT-PCR. **Journal of Virological Methods**, 146, n. 1, p. 305-310, 2007/12/01/ 2007.

BOL, J. F. Role of Capsid Proteins. *In*: FOSTER, G. D.;JOHANSEN, I. E.*, et al* (Ed.). **Plant Virology Protocols: From Viral Sequence to Protein Function**. Totowa, NJ: Humana Press, 2008. p. 21-31.

BOTHA, C. E. J.; EVERT, R. F.; WALMSLEY, R. D. Observations of the penetration of the phloem in leaves of *Nerium oleander* (Linn.) by stylets of the aphid, *Aphis nerii* (B. de F.). **Protoplasma**, 86, n. 4, p. 309-319, 1975/12/01 1975a.

BOTHA, C. E. J.; EVERT, R. F.; WALMSLEY, R. D. Studies on *Gomphocarpus physocarpus*: Further evidence of preferential feeding by the aphid, *Aphis nerii* on the internal phloem. **Protoplasma**, 84, n. 3, p. 345-356, 1975/09/01 1975b.

CAMPBELL, P. Papaya Press. Papaya Clean Seed Program Underway. KATH, G. Australia**:** 1 p. 2019a.

CAMPBELL, P. Papaya Press. Papaya clean seed update KATH, G. Australia**:** 1 p. 2019b.

CORNEJO-FRANCO, J. F.; ALVAREZ-QUINTO, R. A.; QUITO-AVILA, D. F. Transmission of the umbra-like Papaya virus Q in Ecuador and its association with meleira-related viruses from Brazil. **Crop Protection**, 110, p. 99-102, 2018/08/01/ 2018.

COSMI, F. C.; ALVES, K. D. S.; MORAES, W. B.; VENTURA, J. A.; MORAES, S. D. P. C. B.; MORAES, W. B.; JESUS JÚNIOR, W. C. D. Análise epidemiológica da evolução temporal da meleira do mamoeiro. **Summa Phytopathologica**, 43, p. 303-309, 2017.

COSTA, C. L. As inter-relações vírus-afídeos vetores e o controle da mancha anelar do mamoeiro causada pelo Papaya ringspot virus-P. *In*: Papaya Brasil: mercado e inovações tecnológicas para o mamão, 2005, Vitória-ES, Brazil. p. 183-191.

DE LIMA, J. G. S.; TEIXEIRA, D. G.; FREITAS, T. T.; LIMA, J. P. M. S.; LANZA, D. C. F. Evolutionary origin of 2A-like sequences in *Totiviridae* genomes. **Virus Research**, 259, p. 1-9, 2019/01/02/ 2019.

EL MOUSSAOUI, A.; NIJS, M.; PAUL, C.; WINTJENS, R.; VINCENTELLI, J.; AZARKAN, M.; LOOZE, Y. Revisiting the enzymes stored in the laticifers of *Carica papaya* in the context of their possible participation in the plant defence mechanism. **Cellular and Molecular Life Sciences**, 58, p. 556-570, 2001.

FISHER, J. B. The vegetative and reproductive structure of papaya (*Carica papaya*). **Lyonia**, 1, n. 4, p. 191-208, 1980.

GARCÍA-CÁMARA, I.; TAPIA-TUSSELL, R.; MAGAÑA-ÁLVAREZ, A.; CORTÉS VELÁZQUEZ, A.; MARTÍN-MEX, R.; MORENO-VALENZUELA, O.; PÉREZ-BRITO, D. *Empoasca papayae* (Hemiptera: *Cicadellidae*)-mediated transmission of papaya meleira virus-mexican variant in Mexico. **Plant Disease**, 103, n. 8, p. 2015-2023, 2019.

GHOSH, S.; KANAKALA, S.; LEBEDEV, G.; KONTSEDALOV, S.; SILVERMAN, D.; ALON, T.; MOR, N.; SELA, N.; LURIA, N.; DOMBROVSKY, A.; MAWASSI, M.; HAVIV, S.; CZOSNEK, H.; GHANIM, M. Transmission of a new polerovirus infecting pepper by the whitefly *Bemisia tabaci*. **Journal of Virology**, 93, n. 15, p. e00488-00419, 2019.

GOUVEA, R.; DA VITÓRIA, R.; ROSA, R.; ALVES, W. D. S.; GIURIATTO, N.; CALATRONI, D.; FANTON, C.; MARTINS, D. D. S.; QUEIROZ, R. Flutuação populacional de cigarrinhas (Hemiptera: *Cicadellidae*) e ocorrência do vírus da meleira do mamoeiro. *In*: VII Simpósio do Papaya Brasileiro. Produção e Sustentabilidade, 2018, Vitória-Espírito Santo, Brazil. 22-25 August.

GUO, L.; YANG, X.; WU, W.; TAN, G.; FANG, S.; ZHANG, S.; LI, F. Identification and molecular characterization of Panax notoginseng virus A, which may represent an undescribed novel species of the genus Totivirus, family *Totiviridae*. **Archives of Virology**, 161, n. 3, p. 731-734, 2016/03/01 2016.

HABIBE, T. C.; VIDAL, C. A.; NASCIMENTO, A. S. Transmissão da meleira para mamoeiros inoculados com macerados de moscas-brancas *Bemisia tabaci* Genn. biótipo B. **Fitopatologia Brasileira**, 26 (supl.), p. 526-526, 2001.

HAGEL, J. M.; YEUNG, E. C.; FACCHINI, P. J. Got milk? The secret life of laticifers. **Trends in Plant Science**, 13, n. 12, p. 631-639, 2008.

HOGENHOUT, S. A.; AMMAR, E.-D.; WHITFIELD, A. E.; REDINBAUGH, M. G. Insect Vector Interactions with Persistently Transmitted Viruses. **Annual Review of Phytopathology**, 46, n. 1, p. 327-359, 2008.

KITAJIMA, E. W.; RODRIGUES, C. H.; SILVEIRA, J. S.; ALVES, F.; VENTURA, J. A.; ARAGÃO, F. J. L.; OLIVEIRA, L. H. R. Association of isometric viruslike particles, restricted to lacticifers, with "meleira" (sticky disease) of papaya (*Carica papaya*). **Fitopatologia Brasileira**, 18, p. 118, 1993.

LIMA, R. C. A.; COUTO, A. O. F.; ANDRADE, J. S.; MARTINS, D. D. S.; VENTURA, J.; TATAGIBA, J.; COSTA, H. Flutuação populacional de insetos vetores de doenças do mamoeiro e sua relação com a ocorrência de doenças viróticas. *In*: Papaya Brasil: qualidade do mamão para o mercado interno, 2003, Vitória-ES, Brazil. p. 539-541.

LUQUE, D.; MATA, C. P.; SUZUKI, N.; GHABRIAL, S. A.; CASTÓN, J. R. Capsid structure of dsRNA fungal viruses. **Viruses**, 10, n. 9, p. 481, 2018.

MACIEL-ZAMBOLIM, E.; KUNIEDA-ALONSO, S.; MATSUOKA, K.; DE CARVALHO, M. G. *et al.* Purification and some properties of Papaya meleira virus, a novel virus infecting papayas in Brazil. **Plant Pathology**, 52, n. 3, p. 389-394, 2003.

MAFFIA, L. A.; RODRIGUES, C. H.; VENTURA, J. A. Significância epidemiológica do conhecimento do arranjo espacial de plantas doentes em campo. I - Meleira do mamoeiro. **Fitopatologia Brasileira**, 18 (Supl.), p. 315, 1993.

MARTINS, D. D. S.; LIMA, A. F.; FORNAZIER, M. J.; BARCELLOS, B. D.; QUEIROZ, R. B.; FANTON, C. J.; JUNIOR, J. S. Z.; FORNAZIER, D. L. Whiteflies (Hemiptera:

Aleyrodidae) associated with papaya (*Carica papaya* L.). **Revista Científica Intelletto**, 2, n. 1, p. 78-86, 2016.

MARTINS, D. D. S.; VENTURA, J. A.; PAULA, R. D. C. A. L.; FORNAZIER, M. J.; REZENDE, J. A. M.; CULIK, M. P.; FERREIRA, P. S. F.; PERONTI, A. L. B. G.; ZONTA DE CARVALHO, R. C.; SOUSA-SILVA, C. R. Aphid vectors of Papaya ringspot virus and their weed hosts in orchards in the major papaya producing and exporting region of Brazil. **Crop Protection**, 90, p. 191-196, 2016/12/01/ 2016.

MARTINS, D.; FORNAZIER, M.; FANTON, C.; QUEIROZ, R.; ZANUNCIO JUNIOR, J. Pragas do mamoeiro (Pests of Papaya). **Informe Agropecuário**, 37, p. 30-43, 01/01 2016.

MAURASTONI, M.; ANTUNES, T. F. S.; OLIVEIRA, S. A.; SANTOS, A. M. C.; VENTURA, J. A.; FERNANDES, P. M. B. A multiplex RT-PCR method to detect papaya meleira virus complex in adult pre-flowering plants. **Archives of Virology**, 165, n. 5, p. 1211-1214, 2020/05/01 2020.

PATHANIA, N.; VALERIANA JUSTO; PABLITO MAGDALITA; FE DE LA CUEVA *et al.* Integrated disease management strategies for the productive, profitable and sustainable production of high quality papaya fruit in the southern Philippines and Australia (FR2019-89). ACIAR. Australia: Australian Centre for International Agricultural Research (ACIAR) 2019**:** 64 p. 2019.

PEREZ-BRITO, D.; TAPIA-TUSSELL, R.; CORTES-VELAZQUEZ, A.; QUIJANO-RAMAYO, A.; NEXTICAPAN-GARCEZ, A.; MARTÍN-MEX, R. First report of papaya meleira virus (PMeV) in Mexico. **African Journal of Biotechnology**, 11, p. 13564-13570, 2012.

QUITO-AVILA, D. F.; ALVAREZ, R. A.; IBARRA, M. A.; MARTIN, R. R. Detection and partial genome sequence of a new umbra-like virus of papaya discovered in Ecuador. **European Journal of Plant Pathology**, 143, n. 1, p. 199-204, 2015/09/01 2015.

RAO, K. S.; RAJPUT, K. S.; KIM, Y. S. Secondary growth and occurrence of laticifers in the root of papaya (*Carica papaya* L.). **Acta Botanica Gallica**, 160, n. 3-4, p. 255-260, 2013/12/01 2013.

RODRIGUES, C.; VENTURA, J.; MAFFIA, L. Distribuição e transmissão da meleira em pomares de mamão no Espírito Santo. **Fitopatologia Brasileira**, 14, p. 118, 1989.

RODRIGUES, S. P.; DA CUNHA, M.; VENTURA, J. A.; FERNANDES, P. M. B. Effects of the Papaya meleira virus on papaya latex structure and composition. **Plant Cell Reports**, 28, p. 861-871, 2009.

RODRIGUES, S.; ANDRADE, J.; VENTURA, J.; LINDSEY, G.; FERNANDES, P. Papaya meleira virus is neither transmitted by infection at wound sites nor by the whitefly *Trialeurodes variabilis*. **Journal of Plant Pathology**, 91, n. 1, p. 87-91, 2009.

ROOSSINCK, M. J. Evolutionary and ecological links between plant and fungal viruses. **New Phytologist**, 221, n. 1, p. 86-92, 2019.

SILVA, L.; GARCIA, O.; LOPES, M.; SALAS, C. Changes in protein profile during coagulation of latex from *Carica papaya*. **Brazilian Journal of Medical and Biological Research**, 30, n. 5, p. 615-616, 1997.

TALIANSKY, M. E.; ROBINSON, D. J. Molecular biology of umbraviruses: phantom warriors. **Journal of General Virology**, 84, n. 8, p. 1951-1960, 2003.

TALIANSKY, M. E.; ROBINSON, D.; MURANT, A. Groundnut rosette disease virus complex: biology and molecular biology. **Advances in virus research**, 55, p. 357-408, 2000.

TAPIA-TUSSELL, R.; MAGAÑA-ALVAREZ, A.; CORTES-VELAZQUEZ, A.; ITZA-KUK, G. *et al.* Seed transmission of Papaya meleira virus in papaya (*Carica papaya*) cv. Maradol. **Plant Pathology**, 64, n. 2, p. 272-275, 2015.

VENTURA, J. A.; COSTA, H.; TATAGIBA, J. D. S. Papaya Diseases and Integrated Control. *In*: NAQVI, S. A. M. H. (Ed.). **Diseases of Fruits and Vegetables: Volume II: Diagnosis and Management**. Dordrecht: Springer Netherlands, 2004. p. 201-268.

VENTURA, J. A.; COSTA, H.; TATAGIBA, J. D. S.; ANDRADE, J. D. S. Meleira do mamoeiro: Etiologia, sintomas e epidemiologia. *In*: Papaya Brasil: Qualidade do mamão para o mercado interno, 2003, Vitória-Espírito Santo, Brazil. p. 267-276.

VENTURA, J.; COSTA, H.; TATAGIBA, J. D. S. Sintomatologia da meleira do mamoeiro e sua importância para o “roguing”. **Fitopatologia Brasileira,** 26, n. 1, 2001.

VIDAL, C.; NASCIMENTO, A.; HABIBE, T. Transmissão do vírus da meleira do mamoeiro por insetos. *In*: Papaya Brasil: Qualidade do Mamão Para o Mercado Interno, 2003, Vitória, Espírito Santo, Brazil. p. 612-615.

ZHANG, Z. Y.; HUANG, H.; HAN, X. X.; LI, R.; WU, L. P.; WU, L. Identification and molecular characterization of tea-oil camellia-associated totivirus 1. **Archives of Virology**, 2021/04/17 2021.

ZHOU, J.; WANG, Y.; LIANG, X.; XIE, C.; LIU, W.; MIAO, W.; KANG, Z.; ZHENG, L. Molecular Characterization of a Novel Ourmia-Like Virus Infecting Phoma matteucciicola. **Viruses**, 12, n. 2, p. 231, 2020.

TABLES

Table 1. Summary of transmission assays conducted to date

		Experiment details				
Country	**Virus**	**Virus detection method**	**Vector implicated**	**Transmission assay**	**Main Conclusion**	**Citation**
Brazil	PMeV-ES	Visualization of viral dsRNA	*Bemisia tabaci* MEAM1*	A macerated of insects collected from diseased plants was inoculated in healthy plants which developed symptoms later	*B. tabaci* MEAM1 is capable of acquiring the infectious form of the PMeV complex	Habibe, 2001
Brazil	PMeV-ES	Visualization of symptoms	*Bemisia tabaci* MEAM1; *Toxoptera citricidus*; *Myzus persicae*	Asymptomatic plants were exposed to whiteflies and aphids that were forced to feed on diseased papaya plants	*B. tabaci* MEAM1 but not *Toxoptera citricidus* and *Myzus persicae* are capable to transmit the infectious form of the PMeV complex.	Vidal, 2003

Brazil	PMeV-ES	Visualization of viral dsRNA	*Trialeurodes variabilis*	Plants inoculated with papaya diseased-latex were infested with *T. variabilis* collected from fields with non-symptomatic papaya plants. Three healthy papaya plants of different cultivars were added into the greenhouse to be infested by the whiteflies.	*T. variabilis* can acquire the virus from infected plants and it is not able to transmit it to healthy plants under controlled conditions	Rodrigues et al., 2009
Ecuador	PpVQ	RT-PCR	*Bemisia tabaci*	Whiteflies, red mites, and mealybugs collected from infected plants were transferred to virus-free plants to feed for 7 days.	Papaya virus Q (PpVQ) is transmitted by the whitefly *Bemisia tabaci.*	Cornejo-Franco et al., 2018
Mexico	PMeV-Mx	qRT-PCR and visualization of symptoms	*Empoasca papayae*	After determination of optimal acquisition access period, "viruliferous" insects were allowed to fly from cages containing infected plants to healthy plants. Exposed	*E. papayae* can acquire the virus six hours after exposure to infected plants and transmit it to *C. papaya* 'Maradol'.	Garcia-camara et al., 2019

		plants were transferred to green-house.

3096

1* *Bemisia tabaci* is not considered a papaya pest in Brazil, but have been reported to occur in plants near papaya trees

3097

FIGURES

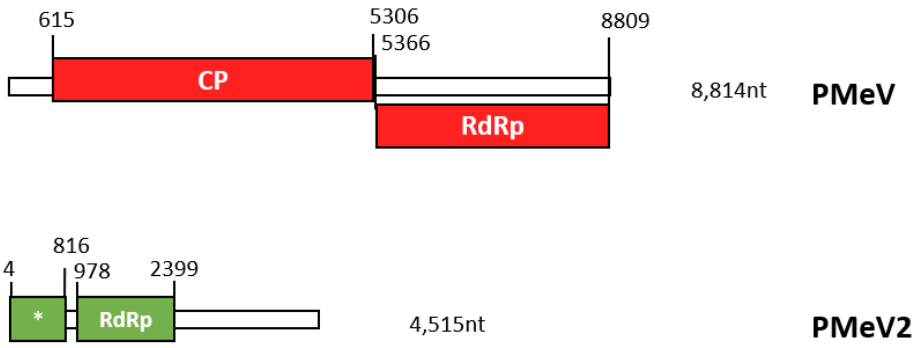


Figure 1. Genomic organization of PMeV-ES (red) and PMeV2 (green) isolates showing their open reading frames and their putative encoded proteins. PMeV is a double-stranded RNA (dsRNA) virus enclosed in a 42-nm-diameter icosahedral particle with its genome organized in two ORFs in different reading frames. ORF1 encodes a polypeptide predicted to be 1,563 amino acids long (177.6 kDa) in which a segment of the capsid from aa 356 to 785 was proved to be part of the virion. It is 75% identical to another Brazilian isolate of PMeV (PMeV-RN) and 20-26% identical to viruses infecting plant pathogenic fungi. Both PMeV-ES and PMeV-RN isolates are tentatively classified in the family *Totiviridae* (ANTUNES *et al*., 2016; ANTUNES *et al.,* 2020; ABREU *et al.*, 2015).

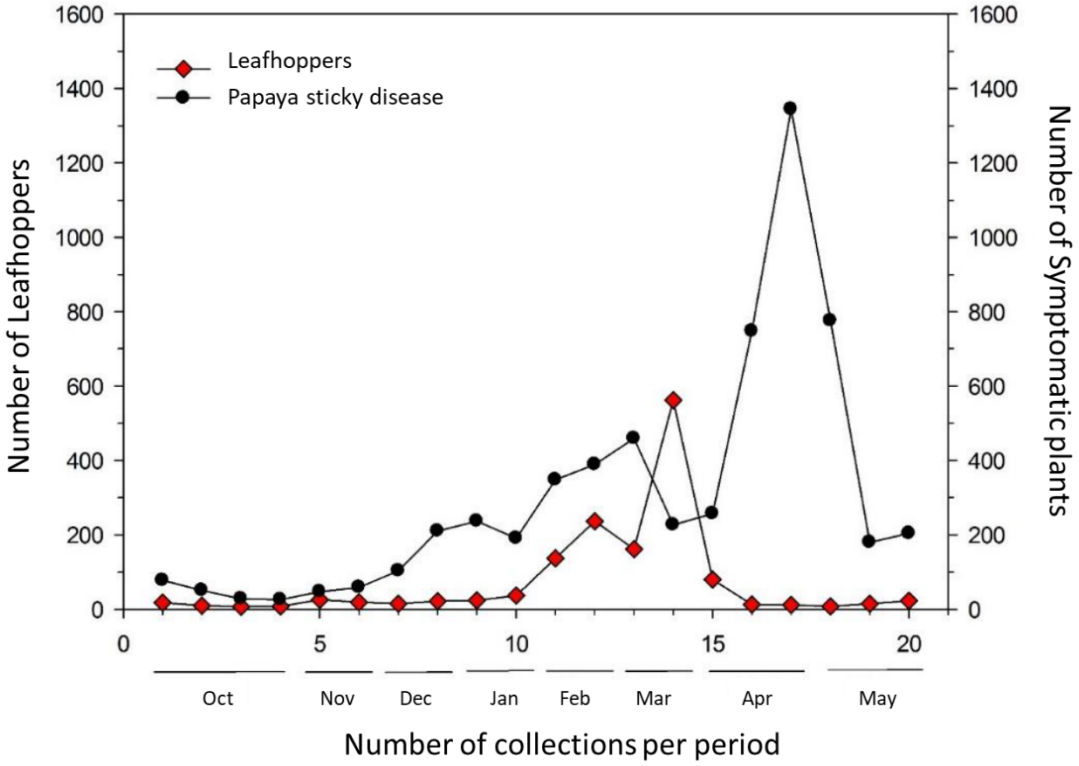


Figure 2. Population fluctuation of leafhoppers and incidence of plants with symptoms of papaya sticky disease in Northern Espírito Santo state, Brazil, with roguing management applied to control the PSD. Source: (GOUVEA et al., 2018).

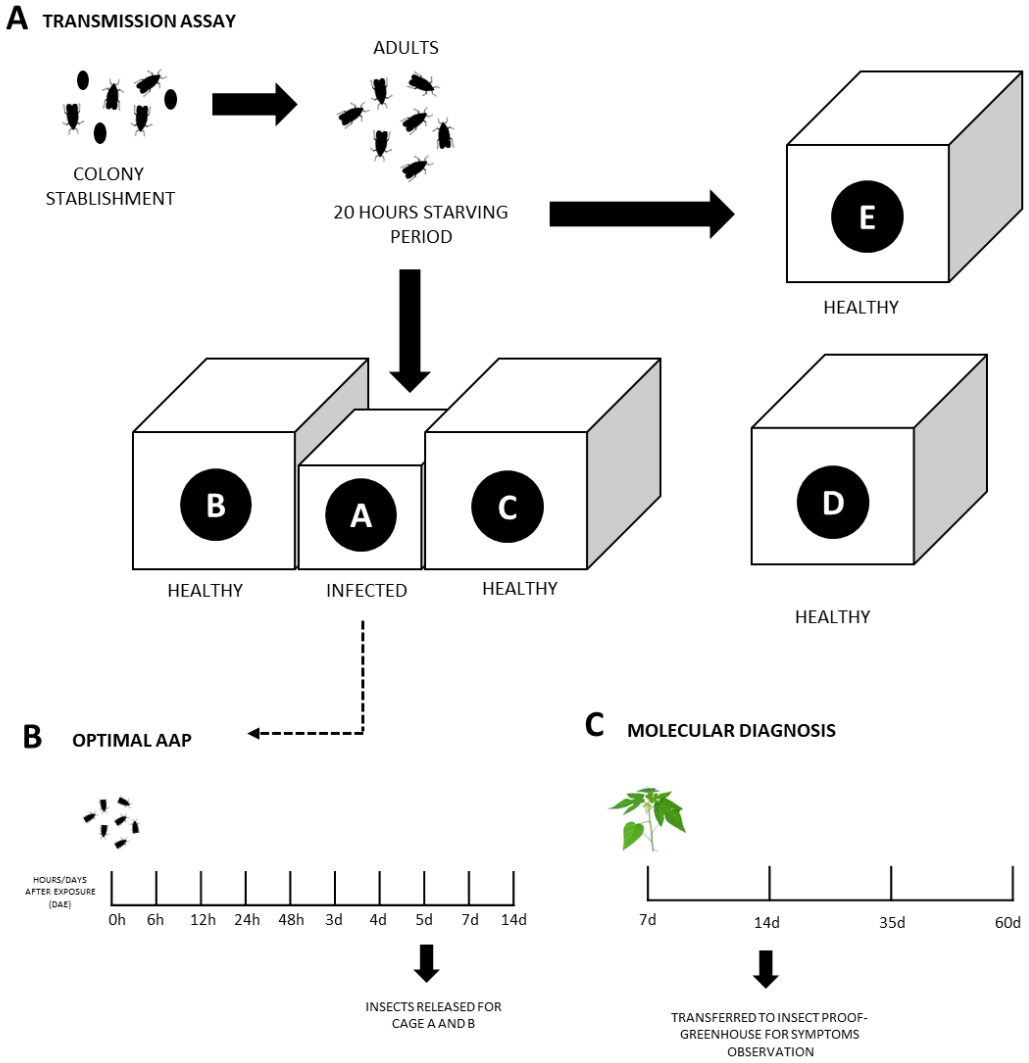


Figure 3. Schematic representation of the transmission assay of papaya meleira virus Mexican variant (PMeV-Mx) by *Empoasca papayae*.**a** A colony of *E. papayae* was established in the laboratory and periodically diagnosed for PMeV-Mx or phytoplasma infection. Two hundred adult insects were transferred to a cage containing PMeV-Mx infected plants after a 20-hour starvation period. **b** The optimal acquisition access period was determined by qRT-PCR at different time points after exposure to infected plants. Five days after exposure (optimal AAP) insect-proof meshes that separate cage A from two other cages, named B and C, were removed allowing insects to fly from infected plants to healthy plants. **c** The plants in cages B and C were diagnosed at 7, 14, 35, and 60 days after exposure to insects that had fed on infected plants. After 14 days the plants were transferred to a greenhouse. Symptoms were observed within 3 to 4 months after insect exposure. Two controls were included in this experiment represented on cages D and E (**a**). In cage D six plants were exposed to 100 adult insects that had not fed on infected plants. In cage E, six plants were not exposed to insects. Plants in both cages remained healthy throughout the experiment.

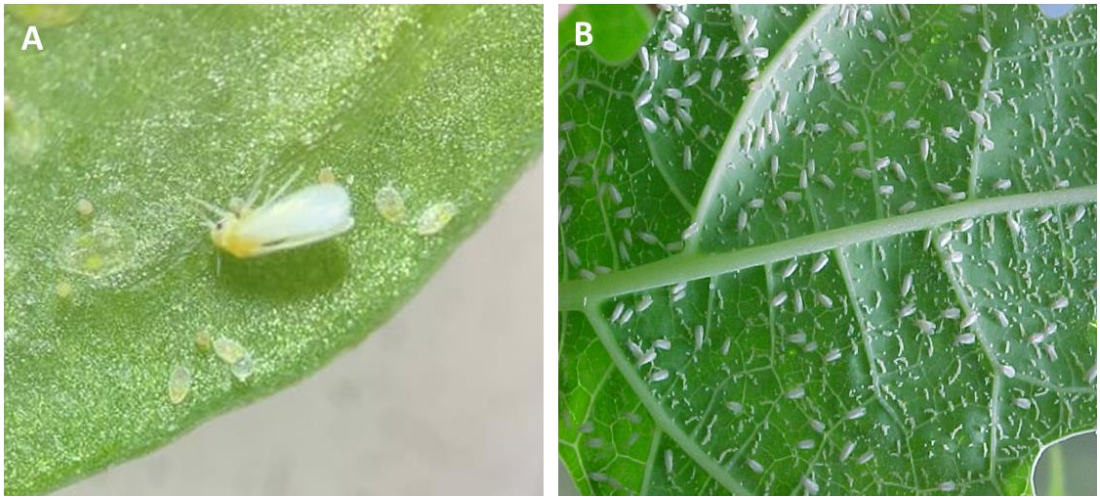


Figure 4. Whitefly species reported occurring in different areas of Brazilian papaya orchards.**a** *Bemisia tabaci* MEAM1 in a tomato leaf. The species is reported to occur in plants close to papaya trees Source: A. Nogueira-UFV. **b** High incidence of *Trialeurodes variabilis* population in the papaya leaf cv. Golden. Source: JA Ventura-Incaper

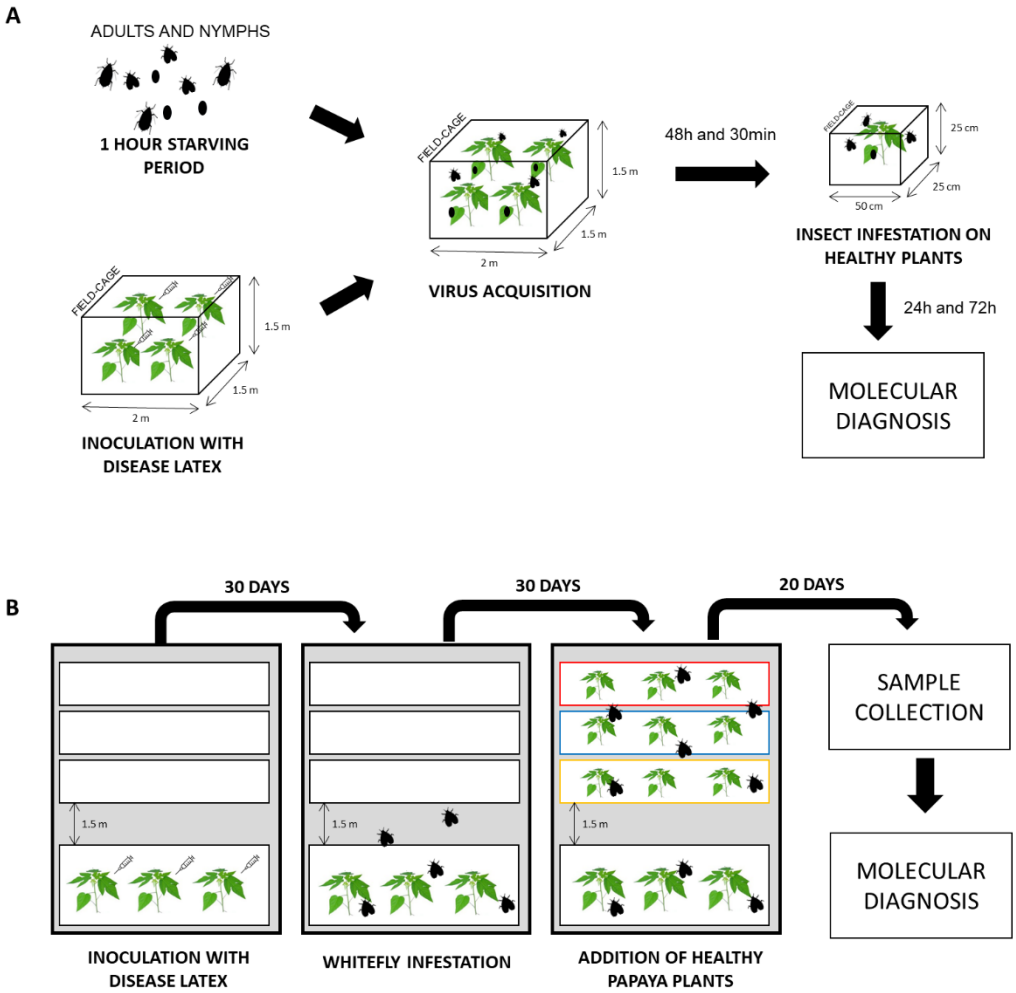


Figure 5. Transmission assays conducted to test the ability of whiteflies (A and B) to transmit the PMeV dsRNA.**a** Plants inoculated with latex collected from disease fruits were kept under cages until the experimental assay. Aphids (*Toxoptera citricidus* and *Myzus persicae*) and whiteflies (*Bemisia tabaci* MEAM1) were kept in separate cages containing asymptomatic papaya plants. 10-20 nymphs and adults were kept in a starving period of 1 hour. Then, insects were transferred to a diseased plant for 48h and 30min (virus acquisition). Then 10-20 insects of each species were transferred to a cage containing a 3 month-old asymptomatic papaya plant where they fed for 24-72h. Insects obtained from the same colonies but submitted to feeding in healthy plants were used as negative controls. Infested plants were kept in a greenhouse for 30 days and subsequently transferred to field cages (two plants per cage) for 9 months or until fructification. Three and eight months after the virus acquisition, new emerging leaves of all plants were collected and submitted for diagnosis by detection of the viral dsRNA. Plants were monitored monthly until the visualization of symptoms. **b** This experiment was conducted in greenhouse conditions. A total of 32 plants were analyzed: 24 were inoculated with diseased latex and 8 were kept non-inoculated. One month after latex inoculation, plants were infested with a population of *T. variabilis* collected from fields with asymptomatic papaya plants. 30 days after the infestation, three healthy plants of different cultivar each (cv. Taiwan, cv. Golden and cv. Sunrise – outlined red, blue, and yellow rectangle, respectively) were added inside the greenhouse. 20 days after the exposition, latex from all

plants was collected for detection of the viral dsRNA. Adults and nymphs exposed to healthy plants and inoculated plants were collected and submitted to molecular diagnosis by detection of the viral dsRNA.

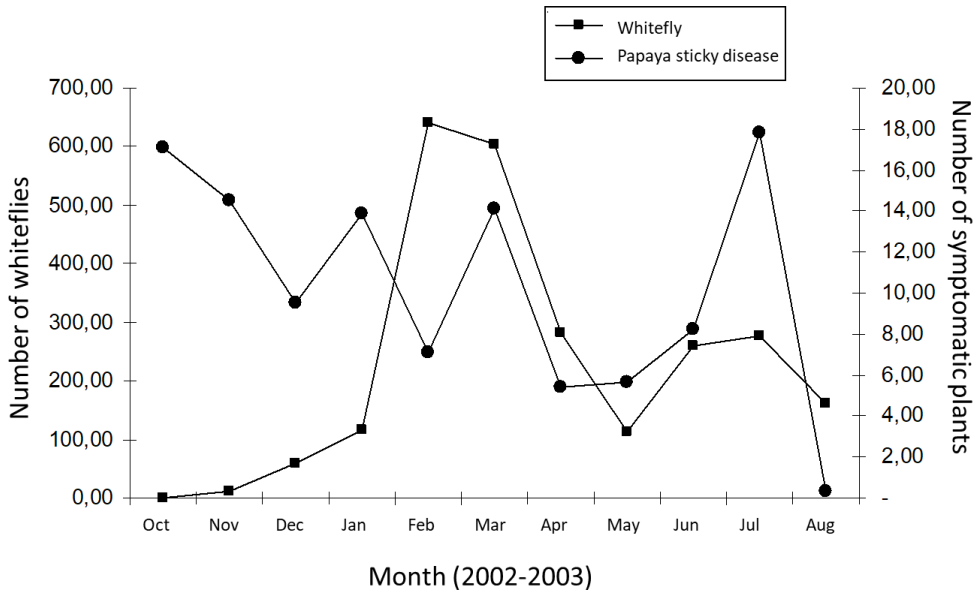


Figure 6. Population of whitefly (Trialeurodes variabilis) and incidence of plants with symptoms of PSD in Northern Espírito Santo, Brazil. Source: (Andrade et al., 2003).

THESIS CONCLUSIONS AND FUTURE PERSPECTIVES

Since the identification of a virus as an etiological agent, researchers have had to try to understand papaya sticky disease in three major spheres: the biology of the etiologic agent and its host, the spread of the disease in the field, and the development of technologies for its management. Here, we compiled and analyzed critically the latest publications on the disease. Through the first manuscript, we highlighted the points of the greatest progress in the last 30 years and those that still need further investigation. Among them, “what is the tolerance mechanism that plants present at the pre-flowering stage?”, “how is the virus able to infect laticifers?”, “who is the vector?”, “is the virus transmitted through the seed?”, “How can we diagnose the disease early?”.

In the sphere of virus-host interaction, we show that the PMeV complex accumulates preferentially in the laticifers of the main vein and that PMeV2 can infect alone laticifers in the mesophyll. Here, we proposed that the PMeV complex reaches mature laticifers early in its differentiation. Two pieces of evidence were presented here supporting this idea: the absence of plasmodesmata in mature laticifer cells and the detection of PMeV in non-laticifer tissue systems.

The virus-host interaction was also explored in the fourth manuscript, where we show that the PMeV capsid protein is mainly composed of two major polypeptides with overlapping sequences. We also show that the central fragment of these polypeptides can interact with an *Arabidopsis* ribosomal protein, RPL17, potentially modulating an important pathway for virus infection. This pathway is mainly composed of translation-associated proteins which are mostly down-regulated at pre-flowering. The meaning of this interaction is still unknown, but we can speculate that reducing the levels of these proteins could be important to avoid virus accumulation. This could be an important strategy presented by pre-flowering plants to tolerate virus effects. The effect of silencing or overexpression of RPL17 in virus replication could give additional thoughts in the RPL17 or another capsid protein-interacting proteins in the virus life cycle.

To contribute to disease management, a new diagnostic method is proposed in this thesis for the detection of the viral complex in adult-pre-flowering asymptomatic plants. This method takes into account the new proposed etiology and will contribute to a better understanding of PSD epidemiology. Several new diagnostic procedures are available across the literature and have been used in the diagnosis of plant viruses. With some adaptations, methods like loop-mediated isothermal amplification (LAMP) could be an alternative diagnosis of PMeV complex virus. Other approaches could also be explored for diagnosis focusing on the changes of metabolite composition of infected and healthy plants, including paper chromatography or detection of volatile organic compounds (VOCs). Quantitative Elisa and lateral flow tests targeting viral proteins could also be explored once established a threshold of protein accumulation when comparing infected and healthy plants.

The last manuscript focused on a major unresolved problem in Brazil, the vector of the PMeV complex. We can conclude that the role of whiteflies and leafhoppers as vectors needs to be addressed. Previous experiments need to be repeated using new more sensitive diagnostic methodologies, which were developed due to advances in the understanding of etiology. Also, the possibility of fungi as vectors of PMeV complex also needs to be assessed due to the similarities of PMeV capsid protein, a plant virus, with other viruses infecting fungi.

The PSD pathosystem does not follow several common topics in plant virology, starting with the cells PMeV complex are capable to infect. Besides the questions presented above, which are still open, the results in this thesis pave the way for new research to understand the papaya sticky disease pathosystem.

ANNEX

ANNEX #1

GOVERNO DO ESTADO
DO ESPÍRITO SANTO
Secretaria da Agricultura, Abastecimento, Aquicultura e Pesca

PERMISSION TO REUSE A MATERIAL PUBLISHED IN PAPAYA BRAZIL

The Editor of Papaya Brazil agrees that you use in **Archives of Virology**, the Figure 1 of the article: Evidência da não transmissão do vírus da meleira por mosca-branca *Trialeurodes variabilis* (Quaintance, 1900), in Papaya Brasil: qualidade do mamão para o mercado interno, D.d.S. MARTINS, Editor. 2003: Vitória-ES, Brazil. p.605-608, and Figure 1 of the article: Flutuação populacional de cigarrinhas (Hemiptera: cicadellidae) e ocorrência do vírus da meleira do mamoeiro, in VII Simpósio do Papaya Brasileiro. Produção e Sustentabilidade. 2018: Vitória-Espírito Santo, Brazil.
Proper credits to the authors and journal/publication should be provided.

David dos Santos Martins

Editor Papaya Brazil
Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural-Incaper
Rua Afonso Sarlo 160, (Bento Ferreira)
29052-010, Vitória-ES, Brasil.

ANNEX #2

PROTOCOLS

Preparation of competent yeast cells for co-transformation

Clontech Matchmaker Gold Yeast Two-Hybrid System [Cat#630489]

Yeast strains included in the kit: Y187 and 2YHGold

For co-transformation use 2YHGold. If single plasmids need to be transformed into yeast do as follows: pGADT7 in 2YHGold (resuspending the cells in -Leu plates at the last step) and pGBKT in Y187 (-Trp plates). This protocol is different from “Yeastmaker Yeast transformation system 2 User Manual” but solutions and media preparation are the same.

This procedure can make 12 transformation reactions and **the whole protocol takes 2 days**

1.1X TE/LiAc	**10mL**	**PEG/LiAc**	**10ml**
10X TE	1.1 mL	50 % PEG 4000 or 3350	8 mL
10X LiAc	1.1 mL	10X TE	1 mL
ddH2O (sterilized)	7.8 mL	10X LiAc	1 mL
These two solutions need to be prepared freshly before transformation and pH of all DROPOUT media should be adjusted to 5.8.			

YPDA = YPD + Adenine hemisulfate (15 mL 0.2 % for 1 L), no need to adjust the pH (autoclavable), but it is added to the media before pouring. The adenine hemisulfate is filter sterilized.

Methods:

1. From glycerol stock: streak a **YPDA** agar plate with Y2HGold and incubate at 30 ºC for ~2days. If it is from a streaked plate, just take one colony and streak it again (no more than 3 times).

NOTE: Use filter tips for all steps that need to pipette.

1st Day

2. Inoculate a 2-3mm colony into 5 mL YPDA in a falcon tube (it is better to use yeasts that were in the plate no more than 2 weeks, otherwise, it will take a longer time to reach the desired OD600). Incubate on a shaker at 250 rpm at 30 ºC for 8 h (start from 8 am to 4 pm)

3. Inoculate 5 µL to 50 mL YPDA in a 250 mL erlenmeyer flask. Incubate in a shaker at 250 rpm overnight (16-20 h) until the OD600 reaches 0.15-0.3 (check in the next morning).

2nd Day

4. Check at 8 am the OD600 from overnight culture from step 3.

5. Spin down cells at 700-1000 g for 7 min at RT. Discard the supernatant and gently resuspend the pellet in 100 mL of fresh YPDA in a 500 mL Erlenmeyer flask.

6. Incubate in a shaker at 250 rpm at 30 ºC for another 3-5 h until the OD600 reaches 0.4-0.6.

7. Spin down cells using 2x 50 mL falcon tubes at 700 g for 7 min at RT.

8. Discard the supernatant and resuspend each pellet using 30 mL of sterile ddH20. Spin down at 700 g for 7 min at RT. Total of 60 mL of ddH20. As soon as you add the water, pellets will resuspend.

9. Discard the supernatant and resuspend each pellet using 1.5 mL 1.1X TE/LiAc (total 3 mL 1.1X TE/LiAc solution). **Do not pour!** Use a serological pipette. Yeast pellets do not attach very well. Otherwise, you will lose some pellets.

10. Transfer cell suspension to 1.5 mL microtube (2 tubes).

11. Spin down 14 000 rpm 30 s RT in a centrifuge bench

12. Remove supernatant. **Do not pour!** Use a micropipette. Yeast pellets do not attach very well. Otherwise, you will lose some pellets.

13. Use a total of 1.2 mL of 1.1X TE/LiAc to resuspend all pellets (for example step 9 I used 2 tubes, so 600 µL of 1.1X TE/LiAc will be added into each tube. Given that every 100 µL competent cell will be used for one co-transformation reaction, this procedure can make 12 transformation reactions, if more reactions are needed, scale-up. **These competent cells cannot be frozen! They stay competent for several hours at room temperature.**

Yeast co-transformation protocol

Prepare all combinations of prey+bait+carrier DNA during step 6 of the previous protocol.

1. Perform in a hood: In each 1.5 mL sterile microtube add sequentially: 1500 ng prey plasmid (pGADT7) + 1500 ng of bait plasmid (pGBKT7) + 5 µL denatured carrier DNA [Cat#630440]. Close the tubes, vortex to mix, and do a short spin. Spray 70% ethanol on tubes before bringing to hood.
2. Add 100 µL of freshly made competent cells (Y2HGold) in each tube, mix by pipetting 2-3 times.
3. Add 500 µL PEG/LiAc to each tube, mix by inverting tubes or short vortex. The PEG/LiAc solution is viscous, after adding them to cells+DNA make sure that they are well mixed.
4. Incubate at 30 ºC in a water bath for 30 min (mix by inverting tubes every 15 min).
5. Spray 70% ethanol before returning the tubes to the hood. Add 20 µL of DMSO to each tube and mix by inverting tubes.
6. Incubate at 42 ºC for 20 min, mix every 5 min. **Do not shake! just mix gently.**
7. Spin down cells at 14 000 rpm (bench centrifuge) for 30 s.
8. Perform in a hood: Remove supernatant and resuspend cells using 800 µL - 1mL of YPDA. Because PEG was added, cells are very sticky, so pipette carefully.
9. Incubate at 30 ºC for 90 min in a shaker at 225 rpm.
10. Spin down cells at 14 000 rpm (bench centrifuge) for 30 s.
11. Perform in a hood: Remove YPDA and resuspend in 150 µL 0.9 % NaCl (sterile). This volume is recommended for 1 plate. Spread on DDO (SD/-Leu/-Trp) and QDO media (SD/-Ade/-Leu/-Trp/-His)

Positive control: pGADT7-T + pGBKT7-53 show colonies on both DDO + QDO plates

Negative control: pGADT7-T + pGBKT7-Lam show colonies only on DDO media

If show colonies in DDO = cotransformation work

If show colonies in QDO = interaction occur.

Protocol for protein-protein interaction map building on String using Arabidopsis orthologs.

Obtaining the orthologs

1. Open the Phytozome website, select “Tools” and then “Biomart”
2. In the Biomart software go to the dropdown menu “Choose database” and select “ V13 Genomes and Families”. Then in the dropdown menu “Choose dataset” select “Phytozome V13 Genomes”.
3. Now you’re going to add your input data, which means the list of the proteins from a certain species that you’re looking for in the orthologs in the other. In my case, I used the PAC transcript ID, which refers to the papaya transcript, available in the dataset from Soares, 2016.
    1. Go to “Filters - click to specify” on the left menu and select “Organism”
    2. Then, select on the list on your left “*Carica papaya* ASGPBv0.4:
    3. Then open the GENE tab and select “ID List Filter”
    4. In the dropdown menu “Gene name(s)”, select PAC transcript ID and add your list of ID in the box below.
4. Now you’re going to choose the species that you want the orthologs. In my case, Arabidopsis

a. Go in “Attributes - click to specify” on the left menu and select “Orthologs”

b. Click on the box “Select all”

c. Choose the species you want the orthologs, in my case “Arabidopsis TAIR 100”

5. Click on results
6. The results are displayed and downloadable in text format, you have to convert for a table format in excel.

It is important to note that one gene can retrieve more than one ortholog and some genes do not have orthologs identified in that species. As output, you will have the gene name (ex. evm.TU.supercontig_12.98), and the TAIR code corresponded to that papaya gene in Arabidopsis. You have to organize your data in the excel table to match the gene name with your PAC transcript ID and TAIR code. Now with the TAIR codes, we can proceed to the String analysis.

Creating the PPI network on String

1. Go to string website https://string-db.org/
2. Select “Search”
3. On the menu on your left, select “Multiple proteins”
4. In “List of names” Add the list of proteins that you obtained from the previous analysis. In my case, I included the TAIR codes of proteins that I found interacting with the PMeV ORF1 segment used to test the interaction.
5. Select “Organism”, which in my case would be Arabidopsis, and press search
6. A list of proteins based on the codes you provided will appear and sometimes more than one option for a protein will be present. Select the best one for your case and press “Continue”
7. Now your map is built. In my case, I went to “Settings” and selected high confidence in the dropdown menu “minimum required interaction score”.
8. You can now export your network as JPG or for a different software, including Cytoscape.

Purification of PMeV complex viral particles from papaya latex

This protocol was written based on a procedure performed at CENARGEN under the guidance of Dr. Márcio Sanches, Dr. Tathiana Antunes (UFES), Dr. Murilo Zerbini (UFV) and Dr. Simone Ribeiro. Therefore, for any questions regarding the procedure, contact these experts. The protocol was based on Zambolim et al., 2003. and Lane (1992).

The procedure is described for purification from the supernatant of papaya latex. However, some adaptations can be made if the procedure is performed from leaves. Considering that most particles are found in latex, the ideal is to use this plant material for the procedure.

Material collection:

1. One day before collection, prepare 0.1M sodium citrate buffer pH 5.0 containing protease inhibitor E64 at a final concentration of 10µM. We usually prepare enough for 4 falcon tubes (100ml). Distribute 25 mL of the buffer in each falcon and store in the refrigerator.

2. Collect latex from the fruits of diseased plants in the buffer. Maintain the 1:1 latex: buffer volume ratio. We usually do it from a pool of plants, because that's the only way we can get enough latex volume. Once collected, the material should be stored on ice. **Must not freeze**!! this can denature the proteins.

Separation of viral particles:

The entire procedure must be performed on ice.

3. Add 2 volumes (~20ml) of 0.1M ammonium citrate pH 6.5 containing 0.037M of iodoacetamide, 0.15M of NaDIECA and 100 µg/ml of PMSF (iodoacetamide, NaDIECA and PMSF have to be add freshly) to 10ml of the latex:sodium citrate solution. Vortex the solution to mix. If there are problems with the solubility of NaDIECA, omit it from the preparation. It is ideal to use a PMSF that sells already solubilized.

4. Centrifuge the mixture at 3800g for 10min at 4ºC (Falcon Centrifuge 5804R-Eppendorf). Collect supernatant (~30mL).

5. Transfer supernatant to a beaker on ice. Add Triton X-100 3% (v/v) dropwise to the supernatant (this step should be done in the hood). After the addition of the triton, clear clumps will form. Stir in a cold chamber for 3 h with the aid of a magnetic stirrer. Stirring should not be too fast or too slow, but gently. After 3h the solution turns slightly yellow and the clumps disappear.

6. Ultracentrifuge at 100,000 g for 90 min (add 10min to centrifuge reach the speed) at 4°C, in fixed angle rotor through a 20% (w/v) sucrose cushion. Use a proportion of 4 of viral preparation and 1 of 20% (w/v) sucrose.

    a. Prepare the 20% (w/v) sucrose solution with the buffer used in step 3;

    b. Transfer the viral preparation to the tubes of the fixed angle rotor. These tubes have a capacity of 30ml. So, I transferred 15ml of viral preparation into 2 different tubes. Add 3.75 ml of sucrose solution by placing the pipette at the bottom of the tube and gently releasing it.

    c. Weigh tubes and balance in pairs adding more buffer from step 3 if necessary. Place a beaker on the scale (previously tared) to keep the tube upright. As the process is carried out on the ice, it is necessary to dry the tubes well with paper before weighing them so that water does not interfere with the weight. Be careful when handling the tubes to not disturb the sucrose cushion.

7. Remove supernatant and refrigerate, in case something goes wrong you still have the supernatant stored.

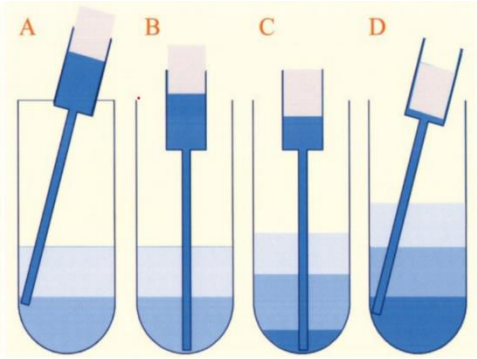
8. Add 500µL of ice-cold 0.01M borate buffer pH 9.0 to each pellet. Keep the tubes slanted in the refrigerator (so that the solution completely covers the pellet) overnight.

9. Prepare different 10, 20, 30 and 40% (w/v) sucrose solutions in 0.01M borate buffer pH 9.0.

    a. Mount the gradient in polycarbonate tubes for ultracentrifugation in a Swing Bucket rotor. **TUBES MUST BE POLYCARBONATE OR ANY VERY TRANSPARENT MATERIAL. OTHERWISE, IT WILL NOT BE POSSIBLE TO SEE THE BANDS BY REFRACTION OF THE LIGHT.** The tube has a capacity of 8ml. Therefore, it

fitted approximately 1.4ml of each sucrose fraction and 1-2ml of the viral preparation.

b. This process requires a lot of patience, so be calm and relaxed. Add 10% solution to each tube. With the aid of a Pasteur pipette (glass) coupled to an automatic pipettor (these instruments are important as the procedure requires great care and precision) add the 20% solution by touching the pipette to the bottom of the tube and gently releasing (always pipette 0.5 mL more volume than you want to pipette as part of the solution does not come out of the pipette. There must be no bubbles, this will disturb your gradient!! Always dry the Pasteur pipette before placing it in your gradient tube. When you remove the pipette, it is normal for part of the solution to come off creating a trail along with the sucrose layers). The lighter solution (10%) will float above the 20% solution. Do the same with the next denser solutions up to 40%. Avoid sudden shaking with the tubes!! this will destroy your gradient. You need to see the layers as they are added, if you don't see, something is wrong with your sucrose solution. Over time the layers become less defined, but if you pay attention it is possible to observe a subtle transparent line between each layer. Leave the tubes in the refrigerator overnight. This will cause the regions between two layers to slowly homogenize creating a continuous gradient. The figure below illustrates how to perform the gradient.



10. The next day, ultracentrifuge the resuspended pellets through the sucrose gradient.

11. Transfer the gradient tubes to the rotor adapters supported on a hack to avoid sudden movement and gradient dispersion.

a. Resuspend the pellets that stayed overnight in the refrigerator (ideally, the solution is very homogeneous, despite having some small clumps). Mix the resuspended pellet from 2 different tubes (1-2ml total, do not add more buffer!). Slowly load the

resuspended solution over the sucrose solution in the gradient tube. This solution is whiter than the transparent gradient.

b. Weigh tubes with their adapters by balancing in pairs. Use 0.01M borate buffer pH 9.0, if necessary. Also prepare a tube to balance if necessary.

c. Place the tubes in the rotor and ultracentrifuge at 114,000g for 90 min at 4ºC under vacuum. (The centrifuge time is 80 min; the extra 10 min is needed to reach speed). If the centrifuge loses the vacuum, the run is lost as it slows down.

12. Analyze the viral bands that scatter light. The environment must be as dark as possible (a small room with a closed door and no windows is ideal). Place a flashlight light (the light source must be powerful) under the tube. The ideal is to assemble a box in which only one hole of approx. 0.5 cm of light pass. Place the tube supported with claws or on support over this light string. Photograph and collect the bands. In the purification of latex from diseased plants usually, 3 opalescent layers appear, the heavier one being more separated from the others and tending to form a band.

13. The collection of bands is performed with a 3mL syringe and a hypodermic needle. The needle must be as thin as possible. With the tube positioned over the light, collect the lighter bands (the ones on top) by slowly pulling the syringe plunger and transfer to the polycarbonate tubes (3-5mL) for ultracentrifugation, previously identified as TOP, MIDDLE, AND BOTTOM.

14. Ultracentrifuge at 100,000g for 2 hours at 4 ºC. After ultracentrifugation, it is possible (sometimes not) to see a transparent and gelatinous pellet at the bottom of the tube. Remove the supernatant (refrigerate if necessary) and resuspend the pellet in 100µL of 0.01M borate buffer pH 9.0. Wash and scrub with the pipette tip the tube walls well where the pellet may be.

15. Quantify in nanodrop on A280 or UV.
    a. Commonly found values:

Sample	Protein	Unit	A 260/280
T	0.496	mg/mL	1.66
M	2.283	mg/mL	1.07
B	1.427	mg/mL	1.56

16. The purification using Cesium chloride is done similarly to the sucrose. All the care applied for sucrose purification is also applied here.

    a. Load 1mL of the three collected fractions over 4mL of a 50% (w/v) CsCl solution in 0.01M borate buffer pH 9.0.

    b. Centrifuge at 145,000 g for 18 h in the swing bucket rotor.

    c. Visualize and collect the opalescent bands in the same way for the sucrose solution

    d. Centrifuge at 35,000 g for 3.5 h to pellet the viral particles

17. Resuspend the pellet in 100 µL 0.01M borate buffer pH 9.0buffer

18. Quantify in nanodrop A280 or UV

    a. Commonly found values:

Sample	Protein	Unit	A 260/280
T	0.471	mg/mL	
M	0.994	mg/mL	
B	0.437	mg/mL	

ANNEX #3

INPI INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

31/01/2018 870180008444 13:19

29409161800979443

Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2018 002105 2

Dados do Depositante (71)

Depositante 1 de 1

Nome ou Razão Social: UNIVERSIDADE FEDERAL DO ESPÍRITO SANTO - UFES

Tipo de Pessoa: Pessoa Jurídica

CPF/CNPJ: 32479123000143

Nacionalidade: Brasileira

Qualificação Jurídica: Instituição de Ensino e Pesquisa

Endereço: RUA FERNANDO FERRARI,514

Cidade: Vitória

Estado: ES

CEP: 29075-910

País: Brasil

Telefone: 027-4009-7885

Fax:

Email: josecarlos.init@gmail.com

PETICIONAMENTO ELETRÔNICO Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 31/01/2018 às 13:19, Petição 870180008444