INFECTIVITY ANALYSIS OF *Ageratum yellow vein virus*-MALAYSIA ISOLATE AND ITS ASSOCIATED DNA BETASATELLITE COMPONENT IN *Solanum lycopersicum*

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INFECTIVITY ANALYSIS OF *Ageratum yellow vein virus*-MALAYSIA ISOLATE AND ITS ASSOCIATED DNA BETASATELLITE COMPONENT IN *Solanum lycopersicum*

ABSTRACT

The previous study has shown that the virus isolated from infected tomato leaves in peninsular Malaysia, showing leaf curl disease symptoms is a monopartite whitefly-transmitted *Begomovirus* from *Geminiviridae* family. The virus isolate was identified with a single-stranded DNA-A like genome and its associated DNA betasatellite. Despite the finding on these viral DNAs, no detail study has been down to investigate the effect of the betasatellite on the helper virus. Therefore, in this study, infectivity analysis was conducted using newly constructed agroinfectious clones harboring individual amplified viral DNAs, in the binary vector pCAMBIA1304. The susceptible tomato plants (*Solanum lycopersicum*) were either co-inoculated with both virus and betasatellite or single inoculated with only the virus. Our results showed both developed agroinfectious clones were infectious and could produce the leaf curl symptoms in the inoculated *S. lycopersicum*. However, the co-inoculation with betasatellite caused more severe leaf curl phenotypes, slower growth rate, shorter latent period and higher virus accumulation in the symptomatic co-inoculated plants as compared to single inoculated plants without betasatellite. This study marks significant findings on the fundamental knowledge of the begomovirus-betasatellite complex in *S. lycopersicum* as well as an important milestone towards future improvements on effective RNAi-mediated defense strategy against this virus.

Keywords: Geminivirus, *Begomovirus*, *Solanum lycopersicum*, *Ageratum yellow vein virus*, betasatellite

ANALISIS INFEKSI BAGI *Ageratum yellow vein virus*-MALAYSIA DAN KOMPONEN BETASATELLITE DNA YANG BERKAIT DENGANNYA DIDALAM *Solanum lycopersicum*

ABSTRAK

Kajian yang telah dijalankan sebelum ini menunjukkan asid deoksiribonukleik (DNA) yang diasingkan dari daun yang mempunyai simptom 'daun kerinting' di kebun tomato di Semenanjung Malaysia adalah daripada virus tumbuhan yang dikenali sebagai *Begomovirus*. *Begomovirus* tergolong dalam keluarga *Geminiviridae* yang mempunyai satu komponen DNA-A dan hanya boleh disebarkan melalui serangga lalat putih. Selain DNA-A, satu lagi komponen DNA yang dikenali sebagai 'betasatellite' telah ditemui. Tetapi, sehingga kini tiada kajian dijalankan untuk mengkaji kesan atau pengaruh betasatellite terhadap virus ini. Maka, didalam kajian ini, analisis infeksi telah dijalankan secara terperinci dengan menggunakan klon-klon yang boleh menghasilkan jangkitan dalam pokok tomato yang dikenali sebagai 'agroinfectious clones'. Setiap klon yang dihasilkan dalam vektor tumbuhan (pCAMBIA1304) membawa virus dan betasatellite dengan berasingan, yang kemudiannya disuntik dalam pokok tomato (*Solanum lycopersicum*) yang sihat melalui kaedah penyusupan atau 'inoculation assay'. Hasil kajian menunjukkan klon-klon yang telah dihasilkan adalah stabil dan mampu menghasilkan simptom daun keriniting dalam pokok tomato yang disuntik sama ada dengan campuran virus dan betasatellite atau hanya dengan virus sahaja. Apabila dibandingkan dengan pokok tomato yang hanya disuntik dengan virus, pokok yang disuntik bersama-sama dengan betasatellite menunjukkan infeksi yang lebih tinggi termasuklah simptom yang dihasilkan kelihatan lebih jelas dan dihasilkan dalam masa yang lebih singkat serta tahap pengumpulan virus menjadi lebih tinggi dalam pokok tersebut. Hasil kajian yang telah diperolehi turut dibincangkan dengan lebih lanjut didalam tesis ini serta diharapkan dapat meningkatkan pengetahuan sedia ada untuk

penambahbaikan strategi pertahanan dalam tumbuhan melalui kaedah ‘interferensi RNA’ (RNAi) yang lebih efektif dalam kajian yang seterusnya bagi mengelakkan penularan virus yang lebih serius pada masa hadapan.

Kata kunci: Geminivirus, *Begomovirus*, *Solanum lycopersicum*, *Ageratum yellow vein virus*, betasatellite

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Finally, I dedicated this works to my beloved parents; Omar and Nooraini, as well as my siblings; Taufik, Hidayah and Adib. In good, in bad, in parts or in whole, they are the ones who showed up, who stayed in there, regardless. Other things may change us, but I always know in my heart that we start and end with family.

"*He who travels in the search of knowledge, to him God shows the way of Paradise*".

(Muhammad S.A.W)

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LIST OF SYMBOLS AND ABBREVIATIONS

~	:	Approximately
%	:	Percentage
^{o}C	:	Degree celsius
$^{\Delta}$	:	Delta
µg	:	Micro gram
µl	:	Micro liter
n	:	Sample size
m	:	Mean
bp	:	Base pair
$CaCl_2$	:	Calcium chloride
cDNA	:	Complementary deoxyribonucleic acid
cm	:	Centimeter
CT	:	Cycle threshold
df	:	Degrees of freedom
DNA	:	Deoxyribonucleic acid
DNase	:	Deoxyribonuclease
dNTP	:	Deoxy ribonucleotides triphosphate
dpi	:	Day post inoculation
E.coli	:	Escherichia coli
g	:	Gram
h	:	Hour
ICTV	:	International committee on taxonomy of viruses
IR	:	Intergenic region
kb	:	Kilo bases

L	:	Liter
LB	:	Luria bertani
M	:	Molar
mg	:	Mili gram
$MgCl_2$	:	Magnesium chloride
min	:	Minute
ml	:	Milliliter
mM	:	Millimolar
ng	:	Nanogram
No	:	Number
ns	:	Not significant
nt	:	Nucleotide
ORF	:	Open reading frame
PCR	:	Polymerase chain reaction
RE	:	Restriction enzyme
RFLP	:	Restriction fragment length polymorphism
RNA	:	Ribonucleic acid
RNAi	:	RNA interference
RPM	:	Revolutions per minute
s	:	Second
ssDNA	:	Single stranded deoxyribonucleic acid
TBE	:	Tris borate ethylenediaminetetraacetic acid
UBI	:	Ubiquitin

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CHAPTER 1: INTRODUCTION

1.1 Research Background

Geminiviridae comprises a group of plant viruses with a circular, single-stranded DNA (ssDNA) genome with a geminate particle morphology (Hanley-Bowdoin et al., 2013). The *Geminiviridae* family was established only in 1978 and since then it has become an emerging viral pathogen that poses a serious threat to the food security around the world. According to the International Committee on Taxonomy of Viruses (ICTV), there are seven genera in *Geminiviridae* including *Begomovirus*, *Mastrevirus*, *Curtovirus*, *Becurtovirus, Topocuvirus, Eragrovirus* and *Turncurtovirus* (Harrison & Robinson, 1999; Varsani et al., 2014). As a result of recent advances in molecular tools and approaches, two more genera have been added to the *Geminiviridae* family members which are *Capulavirus* and *Grablovirus* (Varsani et al., 2017). Each genus is different based on host range, insect vector, genome organization and genome-wide pairwise sequence identities (Fauquet et al., 2008). Collectively, these *Geminiviridae* family members can cause a variety of major crop diseases such as upward curling of leaf margins, mottling and yellowing of the young leaves and experience stunted growth.

In addition, *Begomoviruses*, which constitute the largest genus are exclusively transmitted by whiteflies and consists of either monopartite or bipartite genomes designated as DNA-A and DNA-B of similar size (2.5-2.8 kb). For bipartite *begomoviruses* such as *African cassava mosaic virus* (ACMV) and *Tomato golden mosaic virus* (TGMV), both DNA-A and DNA-B components are required for virus infection and symptoms induction (Hartitz et al., 1999; Mgbechi-Ezeri et al., 2008). Meanwhile, monopartite *begomoviruses* like *Tomato leaf curl virus* (ToLCV) and *Tomato yellow leaf curl virus* (TYLCV) only need its' single genome, a homolog of DNA-A to induce the disease symptoms in the infected plants (Kil et al., 2016; Pandey et al., 2009). Early studies have revealed that the monopartite *begomovirus* with a single genome such

as *Ageratum yellow vein virus* (AYVV) was unable to produce symptomatic infection in the host plants from which they were isolated (Briddon et al., 2001; Tan et al., 1995). These findings implied that there may be an additional factor required to produce systemic infection and later a novel betasatellite molecule (previously known as DNA-β) was isolated and characterized from these infected plants (Saunders et al., 2004; Stanley et al., 1997).

1.2 Purpose and the Significance of the Study

Over the past three decades, monopartite *begomoviruses* have emerged in many tropical and subtropical regions of the world including Southeast and East Asia. The most well-established studies of this plant virus in these regions are on TYLCV isolated from the infected local S*olanaceous* crops, particularly tomato (*Solanum lycopersicum*) and peppers (*Capsicum spp*.). In Malaysia, only two monopartite *begomoviruses* have been reported across the country since the first outbreak in 1997 for *Tomato leaf curl Malaysia virus* [Malaysia-Klang-1997] (ToLCMYV-MY[MY-Kla-97]) (GenBank Accession No. AF327436) then followed by isolation of *Pepper leaf curl virus* [Malaysia-Klang-1997] (PepLCV-MY[MY-Kla-97]) (GenBank Accession No. AF414287) in 2001 (Koravieh et al., 2008; Shih et al., 2000). In 2016, a preliminary study was conducted on tomato leaves affected by leaf curl disease that associated with the presence of whiteflies. This incidence was observed in the tomato fields located at Peninsular Malaysia. Subsequently, a monopartite *begomovirus* strain was isolated and identified from a selected field, along with its associated DNA betasatellite from the same tomato leaves. The purified PCR products were sequenced and submitted in the GenBank database, defined by *Ageratum yellow vein virus*-[Malaysia-Tomato Leaf curl-2011] (AYVV-MY [MY-Leaf Curl-11]) (GenBank Accession No. KM051527) and *Tomato leaf curl Malaysia betasatellite* (GenBank Accession No. KM051528) (Koravieh, 2016).

Despite the deposition of the DNA sequences of this virus and its associated betasatellite in GenBank database, to the best of our knowledge no report has been published yet and not much is known about the virus pathogenicity and infectivity, particularly on the association between the virus and its cognate betasatellite. Yet, the concerns about the potential of this plant virus to become a devastating causal agent for the loss of tomato in Peninsular Malaysia should not be neglected. Therefore, understanding the relationship of the begomovirus-betasatellite complex can greatly enhance our knowledge on how these two viral DNAs interact to initiate the virus infections in the host plants and what factors influence this process. The knowledge on this aspects is crucial in developing the sustainable strategies for the control of viral infections and its spread in the future.

Like most members of the *Geminiviridae* family, both begomovirus and betasatellite cannot be transmitted to the host plants by mechanical inoculation and the only experimental transmission method available is the use of insect vectors of whiteflies. This constraint could cause researchers to struggle in conducting biological studies, determining the host range and importantly in searching for potential sources of genetic resistance. However, with the recent availability of technologies and tools to develop infectious clones of whole virus genomes, this constraint could be overcome, thus providing an excellent strategy for the researches on plant-virus interactions. Hence, to achieve our objectives in this study, we had described the construction of agroinfectious clones harboring individual monopartite *begomovirus* and its betasatellite in the plant binary vector of pCAMBIA1304, prior to the analysis on the virus infectivity by inoculating the wild-type tomato plants with the virus and betasatellite (co-inoculation assay) or without the betasatellite (single inoculation assay).

1.3 Hypotheses

i. The developed agroinfectious clones harboring individual viral inserts (the virus and its betasatellite) are stable and infectious (can establish the leaf curl disease symptoms) in the inoculated wild-type tomatoes.

ii. Leaf curl symptoms induced by the virus are enhanced by its cognate betasatellite in the co-inoculated tomato plants.

iii. Viral loads in the infected tomato plants co-inoculated with both virus and the betasatellite are higher than those tomatoes inoculated without the betasatellite.

1.4 Objectives

i. To develop a stable inoculation system of agroinfectious clones harboring individual virus DNA and its betasatellite in the plant binary vector backbone of pCAMBIA1304.

ii. To perform infectivity assay on the wild-type tomato plants based on agroinoculation methods using the developed agroinfectious clones.

iii. To compare the infectivity levels in terms of the disease severity and viral loads between inoculated tomato plants with or without betasatellite.

CHAPTER 2: LITERATURE REVIEW

2.1 Geminivirus: An Important Plant Virus

Plant viruses are economically important plant pathogens for many host plants. Among them, *Geminiviridae* is a well-studied plant virus family by researchers worldwide and also the only family categorized as a circular, single-stranded DNA (ssDNA) virus (Hanley-Bowdoin et al., 2013). Plant diseases caused by these viruses can result in global agriculture crisis thus leading to poor crop production. The name *Geminiviridae* was derived from Gemini, the Zodiac sign representing the twin icosahedral (geminate) capsid structure of its member as shown in Figure 2.1 (Abhary et al., 2007). Each member of *Geminiviridae* has a circular ssDNA genome with different sizes between 2.5 kb to 3.0 kb, depending on the virus type (Fauquet et al., 1995). There are nine genera associated with *Geminiviridae* family including *Begomovirus* (288 species), *Mastrevirus* (29 species), *Curtovirus* (3 species), *Becurtovirus* (2 species), *Topocuvirus* (1 species), *Eragrovirus* (1 species) and *Turncurtovirus* (1 species), as well as the latest discovery of *Capulavirus* (4 species) and *Grablovirus* (1 species) (Fauquet et al., 2008; Varsani et al., 2017). Different geminiviruses can infect and transmit the viral particles into different host plants, either monocotyledonous or dicotyledonous plants depending on its genome organization of bipartite or monopartite (Hanley-Bowdoin et al., 2013). For instance, *Masterviruses* have a single genome composition and being transmitted by leafhoppers to infect either monocotyledonous or dicotyledonous plants. Even though *Curtoviruses* share similar biological properties as *Masterviruses* including monopartite genome and vector of leafhoppers, *Curtoviruses* can only infect dicotyledonous plants (Hernandez & Brown, 2010).

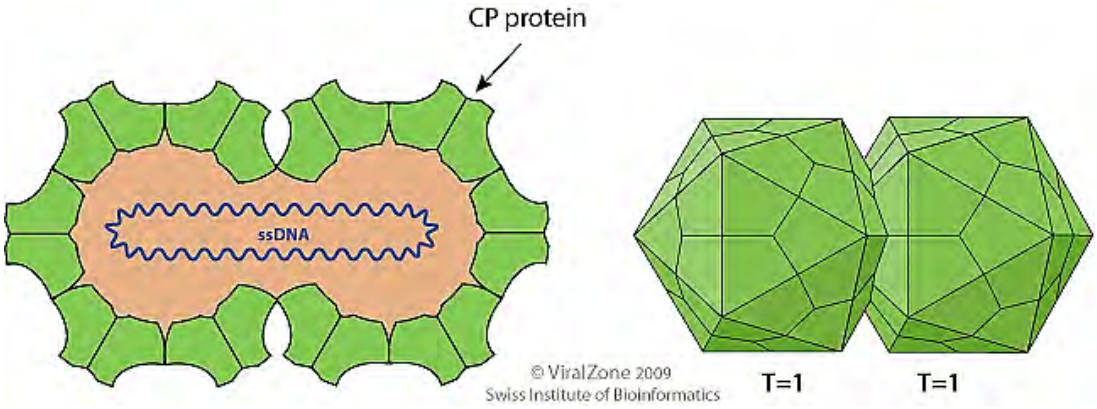

Figure 2.1: Virion of *Geminiviridae*. *Geminiviridae* is a non-enveloped virion with an approximate length of 38 nm and 22 nm in diameter. It is twinned (geminate) incomplete T=1 icosahedral symmetry capsid and made of capsid proteins (CP). Each geminate particle has only a single circular ssDNA. Image adapted from ViralZone (2009), with permission from SIB Swiss Institute of Bioinformatics.

2.2 Genus *Begomovirus*: A Whitefly-transmitted Geminivirus

Prior to the discovery and classification of the biggest and well-characterized member of the *Geminiviridae* family; *Begomovirus* was referred to as 'rugaceous' whitefly-transmitted viruses (Abhary et al., 2007). Begomovirus has a wide host range, infecting only dicotyledonous plants and exclusively transmitted by whitefly (*Bemisia tabaci*) in a persistent, circulative manner. The general disease symptoms caused by this genus including leaf curling, mosaic and bright yellow or yellow-green on the curled leaves of endemic host and cultivated crop species (Fauquet et al., 2003).

2.2.1 Genome Organization of *Begomovirus*

Begomoviruses have been categorized into two groups of either a bipartite or monopartite genome (Figure 2.2). The first well-studied bipartite *begomoviruses* were *African Cassava Mosaic Virus* (ACMV) from the Old World (OW) and *Tomato Golden Mosaic Virus* (TGMV) from the New World in 1981 (Nawaz-ul-Rehman et al., 2009). These bipartite *begomoviruses* consisting two DNA genomes designated as DNA-A and

DNA-B. Meanwhile, the first monopartite *begomovirus* with single DNA-A (a homolog of DNA-A of bipartite *begomoviruses*) was discovered in Israel and then was subsequently classified as *Tomato yellow leaf curl Sardinia virus* (TYLCSV) (GenBank Accession No. X15656) (Navot et al., 1991). Since then, many more monopartite *begomoviruses* were discovered and mostly were originated from the OW including *Tomato Yellow Leaf Curl Virus* (TYLCV) and *Tomato Leaf Curl Virus* (ToLCV) isolated from the infected tomatoes.

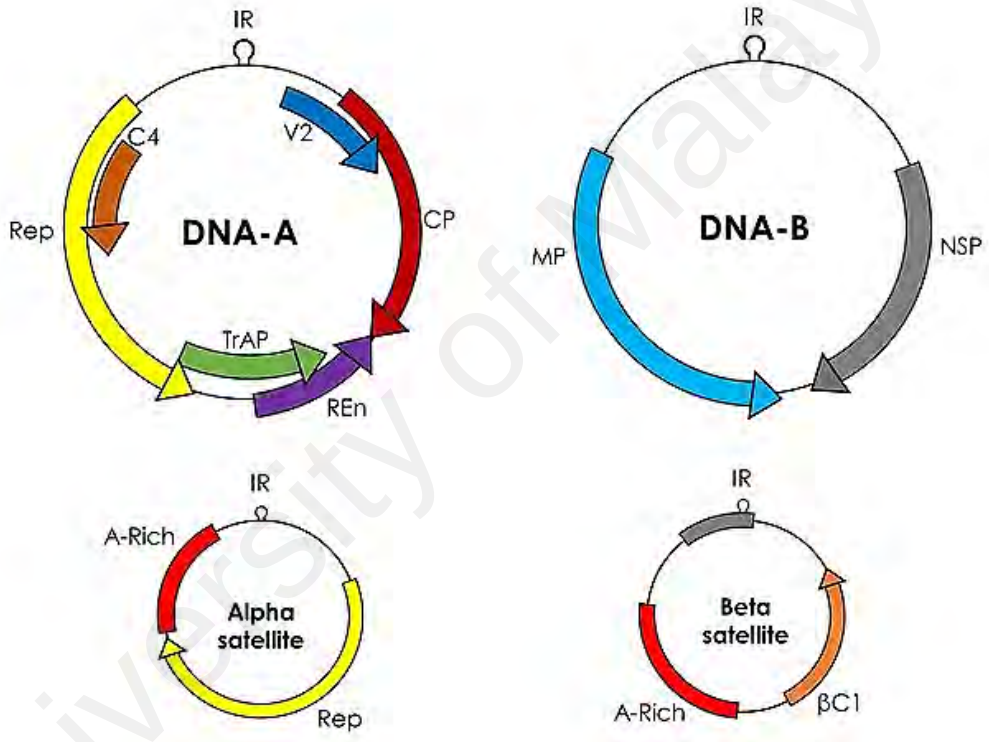

Figure 2.2: Genome organization of *begomoviruses* and their associated DNA satellites. *Begomoviruses* have an either bipartite or monopartite genome. Bipartite *begomoviruses* have both DNA-A and DNA-B components, whereas monopartite *begomoviruses* only have a single genome homolog to DNA-A of bipartite *begomoviruses*. Most *begomoviruses* are associated with DNA satellites that are half the size of the viruses termed alphasatellites and betasatellites. Alphasatellites have Rep gene in virion sense orientation, whereas betasatellites encode C1 gene in the complementary sense orientation. Both DNA and betasatellite molecules contain a stem-loop structure within the intergenic region (IR) that responsible for the replication of viral DNA in the virus-infected plants. Image reproduced from Zaidi et al. (2016), with permission from Frontiers in Plant Science.

2.2.2 Mode of *Begomovirus* DNA Replication

According to the current reviews, the replication of *begomovirus* is predominantly control by a rolling circle replication (RCR) mode with the help of other viral and host factors as represented in Figure 2.3 (Hanley-Bowdoin et al., 2000). It is started with the injection of the virus into a plant cell by the insect vector. Then, the circular ssDNA is released into the nucleoplasm following a coat protein-based nuclear localization signal of the targeted viral particle to the nucleus. Subsequently, this circular ssDNA is converted to circular dsDNA by the host DNA polymerase.

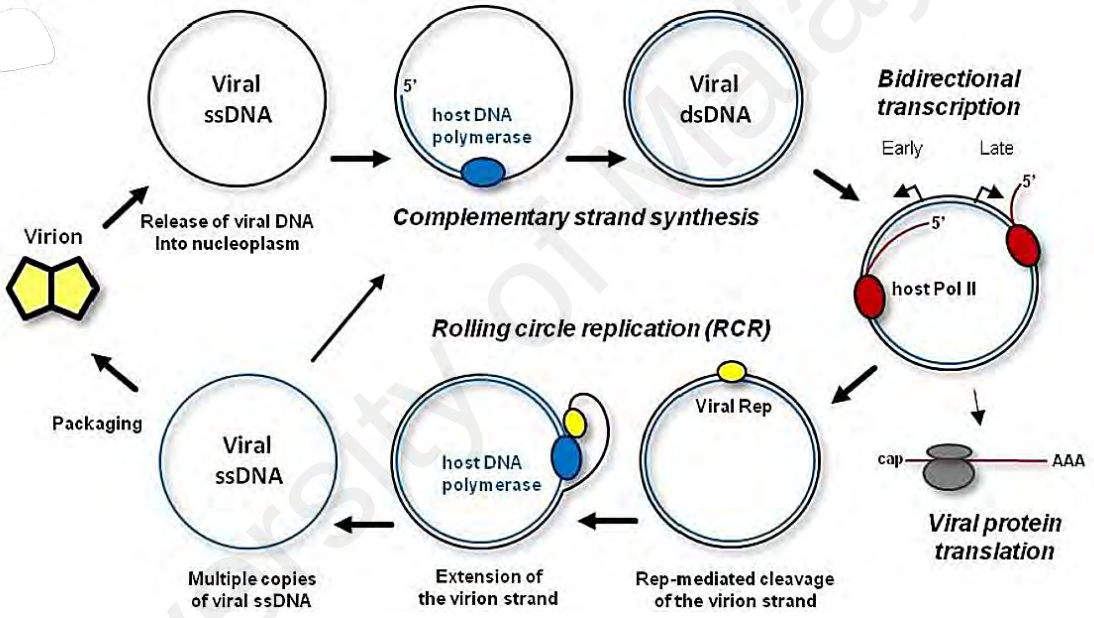

Figure 2.3: Models for RCR mode of begomovirus DNA replication. The RCR process started when the viral circular ssDNA is released from the virion (yellow) into the host plant nucleus, in which later was converted to the circular dsDNA by the host DNA polymerase through complementary strand synthesis. Then, this dsRNA went through bidirectional transcription of the early leftward (Rep) and the late rightward (coat protein) genes, resulting in viral mRNAs that were transported to the cytoplasm. The replication of the viral dsDNA was initiated by the viral Rep (resulted from protein translation) in the nucleus through a rolling circle replication (RCR) mechanism. The process continued until the newly synthesized one or more circular ssDNA re-enter the replication cycle or get packaged into virions. Image reproduced from Poogin (2013), with permission from International Journal of Molecular Sciences.

2.3 Monopartite *Begomoviruses*: A Widespread Plant Viruses

Monopartite *begomoviruses* are important pathogens to the cultivated crops and increasingly spreading throughout the world. To date, extensive researches have been done focusing on these viruses that mostly isolated from infected tomato. One of them is *Ageratum yellow vein virus* (AYVV), which is the focus of this research study. AYVV was first identified in a common weed, *A. conyzoides* which is mostly grown in moist and shady areas, particularly in many regions of South East Asia (Tan et al., 1995). AYVV-infected plants displayed similar symptoms of a whitefly-borne virus disease as other host plants and later was classified as a geminivirus species based on its host range, vector type, virion morphology and cytopathology.

Monopartite *begomovirus* consist of a circular ssDNA genome consisting of six overlapping open reading frames (ORFs) with bidirectional organization, in which two of them, VI and V2 are expressed from the viral sense orientation while the rest of C1, C2, C3 and C4 are transcribed to RNA in the complementary strand (Figure 2.2). V1 encodes the Capsid Protein (CP) that essential in many processes during the life cycle of the virus including the encapsidation of ssDNA and the formation of virus particles for protection during transmission by the insect vector (Zrachya et al., 2007). Besides, the CP is well-studied viral protein in which was shown to involve in a long-distance viral movement in the infected host plants. V2 is a pathogenicity gene that encodes a distinct motion related protein responsible for cell-to-cell viral movement and systemic spread (Padidam et al., 1996). Meanwhile, the ORFs in the complementary sense orientation, C1 encodes a replication associated protein (Rep) required for viral DNA replication, while C2 encodes a transcription activator protein (TrAP) that acts as a suppressor of post-transcriptional gene silencing (PTGS) (Hartitz et al., 1999). C3 encodes a replication enhancer protein (REn) which is crucial for the effective viral DNA replication, thus leads

to viral DNA accumulation. Finally, C4 protein interacts well with C3 protein in the infection process through PTGS suppression in the host plant cells.

The ORFs of monopartite *begomoviruses* are structured bi-directionally in its genome and specifically separated by an intergenic region (IR) that comprise of main elements for the replication and transcription of the viral genome (Figure 2.2). The size of IR is about 300 nucleotides and contains conserved TATA box promoter elements (TAATATT↓AC) in almost all monopartite *begomoviruses* (Varma & Ramachandran, 2013). In order to increase the viral infectivity, many studies had shown that most infectious clones of Geminivirus need more than one mer (unit) of the viral genome, thus the clone will have at least two replication origins (V-ori) in its tandem repeat sequences. Studies had showed that the only viral protein needed for viral replication is viral replication associated protein (Rep) encoded by the C1 ORF (Blawid et al., 2008).

2.4 Monopartite *Begomovirus*-betasatellite Complex

2.4.1 Origin and Evolution of *Begomovirus*-betasatellite Complex

A study reported that DNA-A of *Ageratum yellow vein virus* (AYVV) in which originally resulted in typical symptomatic infection in *S. lycopersicum* and *Nicotiana tabacum* was later found unable to give the same result when reintroduced into *A. conyzoides* (Saunders et al., 2000). Besides, they could not verify that the DNA-B is responsible for this infections after many unsuccessful attempts to isolate it. Therefore, they came to the conclusion that there was an additional factor contributed to this disease. Later, this unknown factor was isolated, analyzed and termed as betasatellite (previously known as DNA-β) (Singh et al., 2012). Unlike bipartite *begomoviruses*, few evidences showed that monopartite *begomoviruses* only need a single DNA-A component to infect the plants and could cooperate well with its DNA satellite molecule in compensating for

the lack of a DNA-B component (Briddon et al., 2003; Saeed et al., 2007). Plus, betasatellite associated with AYVV was reported to be the potential substitute for the DNA-B of *Sri Lanka Cassava mosaic virus* (SLCMV) that encoded for viral movement genes to cause systemic infection in the infected plants (Saunders et al., 2002).

2.4.2 Genome Composition of Betasatellite

Similar to the single DNA-A genome of monopartite *begomovirus*, betasatellite also a circular single-stranded DNA (ssDNA) molecule of approximately half the size of their helper *begomoviruses* (1350 nt) with a potential stem-loop structure containing nona-nucleotide sequence (TAATATTAC) (Varma & Ramachandran, 2013; Pandey et al., 2010). This conserved inverted repeat sequence was known as the intergenic region (IR) or satellite conserved region (SCR) for *begomovirus* and its betasatellite, respectively. The IR/SCR region is essential for the replication of the viral DNA genome in the infected host plant. Plus, the betasatellites comprise an adenine-rich region (A-rich) and a single ORF of the βC1 gene in the complementary orientation of the molecule (Figure 2.2). Betasatellites are helper *begomoviruses*-dependent for their replication, movement in plants and transmission between plants, through trans-encapsidation in the helper virus coat protein (CP). So far, betasatellites have been reported to encode essential component(s) for the establishment of the viral systemic infection pathogeny determinant, a potential suppressor of posttranscriptional gene silencing (PTGS) as well as its involvements in virus movement in the infected plants by AYVV, TYLCV and *Cotton leaf curl Gezira virus* (Andou et al., 2010; Idris et al., 2005). Besides, many reports have demonstrated that in order to have symptomatic infection, most infectious clones of Geminivirus need more than one mer (unit) of its tandem repeat sequences of IR (Bang et al., 2014; Jin et al., 2012).

2.5 Monopartite *Begomoviruses* Cause Significant Yield Losses to Tomato Crops

The cultivated tomato, *Solanum Lycopersicum* is one of 3000 species in the diverse family *Solanaceae* and it is grown worldwide mostly for its edible fruits. *S. lycopersicum* is an herbaceous perennial plant with slender hairs, a weekly woody stem that normally scrambles over other plants and can grow up to three meters tall (Kenyon et al., 2014). The fruit's varieties including fresh or beefsteak types, plum, cherry, grapes or paste and others, making *S. lycopersicum* commercially importance vegetables globally. It is native to South America and yet growing in temperate climates worldwide including Malaysia. Based on Malaysia agricultural statistical analysis, the production of tomatoes, mostly in Cameron Highlands, Pahang (1000 to 1500 meters above sea level) has increased from 513 ha in 1997 to 2,200 ha in 2008 with the annual rate growth of 13.8% (Islam et al., 2012). Furthermore, with the improvements in rain shelter system as the alternative to the conventional system in cultivating the tomatoes, the average yearly net income for one hectare of tomato reported by The Malaysian Agricultural Research and Development Institute (MARDI) was significantly increased, thus remark the importance of local tomato industry.

However, like other vegetable cultivation, the tomato farmers all around the world also suffer from the yield loss due to the pathogen of *begomoviruses*. According to Kenyon et al. (2014), there are more than 60 species of tomato-infecting *begomoviruses* are identified worldwide due to outbreaks of whiteflies, mostly affected by tomato yellow leaf curl disease (TYLCD) and tomato leaf curl disease (ToLCD) (Figure 2.4). Likewise, the emerging plant virus diseases pose a continued threat to profitable agriculture including tomato productions in Malaysia. So far, there are six types of plant viruses that have been discovered in Malaysia including *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV), *Potato virus Y* (PVY), *Tomato mosaic virus* (ToMV), *Tomato*

spotted wilt tospovirus (TSWV) and *Leaf curl virus* (LCV) (Roff et al., 2005). Among them, LCV, a whitefly-transmitted begomovirus contributes to the major crop losses in the 1990s and later in 2001, *Pepper leaf curl virus* [Malaysia-Klang-1997] (PepLCV-MY[MY-Kla-97]) (GenBank Accession No. AF414287) has been reported with a complete sequence of DNA-A. Therefore, it is crucial to find alternative in managing the leaf curl diseases, concentrating in finding the resistance to these pathogens based on genetic engineering approaches.

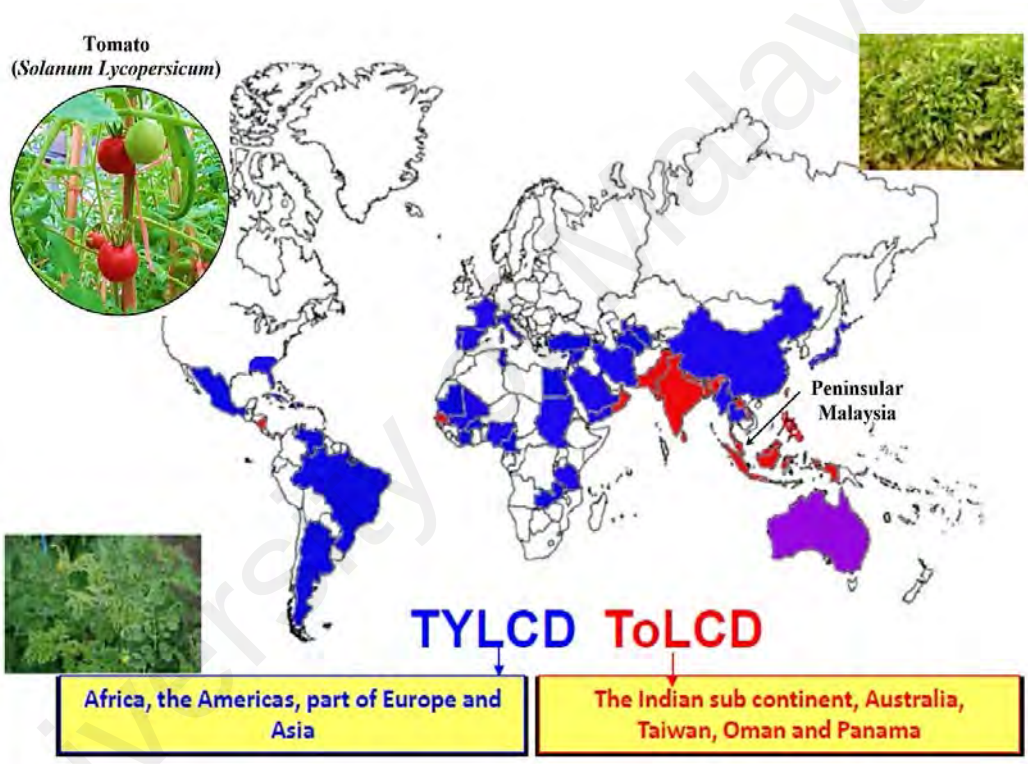

Figure 2.4: Distribution map of *begomovirus* species in tomato fields. Most part in Africa, Americas, Europe and Asia were infected with tomato yellow leaf curl disease (TYLCD), while the regions in Indian sub-continent, Australia, Taiwan, Panama and Peninsular Malaysia were mostly infected by tomato leaf curl disease (ToLCD). Image reproduced from King et al. (2011), with permission from Elsevier Science.

2.6 Inoculation Techniques for Virus Transmission into Host Plants.

To have simple and effective strategies involve in developing resistant varieties in the crop plants to combat virus infection, it is first important to establish the improved inoculation method. Most monopartite *begomoviruses* rely on whiteflies, *Bemisia tabaci* as the only natural inoculation method to successfully transmit the virus into the host plants.

2.6.1 Several Transmission Methods Used for Infectivity Studies

Alternatively, there are other transmission methods that have been extensively used to test the infectivity of monopartite *begomoviruses* as summarize in Table 2.1. However, a few listed methods have proven to be laborious and pose a potential threat to the environment. For instance, the variability of whitefly populations was unpredictable over the years, therefore, the infectivity results of conducted research studies at different time point will be varied depending on the whitefly populations (Polston & Capobianco, 2013). Plus, methods using whitefly inoculation in cages and sap-transmission using either rubbing using abrasive materials or by immersion (dip) are laborious and inefficient in some host plants (Chauhan et al., 2018; Picó et al., 1998). Besides, the used of biolistic inoculations (particle bombardment) also showed inconsistency in the results within different plant species (Lapidot et al., 2007; Morilla et al., 2005).

Table 2.1: Transmission methods for infectivity analysis of *begomoviruses.*

Method of virus transmission	*Begomoviruses*	Inoculated plants	References
Insect vector (*Bemisia tabaci*)	*Cucurbit leaf curl virus*	Pumpkin (*Cucurbita L.)*	Brown et al. (2000)
Mechanical sap-transmission	*Chilli leaf curl virus*	Chilli (*Capsicum annuum L.*)	Chauhan et al. (2018)
Biolistic delivery / Particle bombardment	*Tomato yellow leaf curl virus*	Tomato (*S. lycopersicum*)	Lapidot et al. (2007)
	Bipartite: a)*Bean golden yellow mosaic virus,* b)*Squash leaf curl virus* Monopartite: *Tomato yellow leaf curl virus*	Bean (*Phaseolus vulgaris*) Squash (*Cucurbita pepo*) Tomato (*S. lycopersicum*)	Guenoune-Gelbart et al. (2010)
Agrobacterium tumefaciens-mediated inoculation / Agroinoculation	a)*Tomato yellow leaf curl virus* b)*Tomato yellow leaf curl Málaga virus*	Bean (*Phaseolus vulgaris*)	Monci et al. (2005)

2.6.2 Highly Efficient Agroinoculation Method for Tomato Plants

Hence, it is crucial to use a simple and controlled biological inoculation method to have efficient virus transmission into the host and to prevent the unfavorable spread of the viruses to the environment. In this study, we used *Agrobacterium*-mediated inoculation (agroinoculation) to achieve our objectives. Most geminiviruses can be infectious when they are delivered to the host plants as a full or partial-length repeat of DNA sequences within the T-DNA of a binary vector via *Agrobacterium tumefaciens* (Komari et al., 2004). This agroinoculation technique is ultimately different to those whitefly-mediated inoculation (Figure 2.5) and was anciently used for many plant viruses.

It was first reported in 1986, in which the cloned DNA of *Cauliflower mosaic virus* (CaMV) was released from the T-DNA of *A. tumefaciens* to the plant cells, resulting in disease symptoms upon agroinoculation (Grimsley et al., 1986). This inserted cloned DNA was later termed as an infectious DNA clone. It is a double-stranded DNA copy of the viral genome inserted in a bacterial plasmid to be introduced into the target cells, thus producing infectious virus (Wieczorek et al., 2015). The discovery of this infectious clone was made in the late 1970s, and since then many other infectious DNAs of nearly every virus family have been reported (Jin et al., 2012; Wu et al., 2008). The infectious viral DNA clone is preferable by many plant virologists since it enables manipulation of the viral genome at will as well as permitting unprecedented genetic analysis. Therefore, the combination of infectious viral DNA clone with the agroinoculation/agroinfection techniques is a double-edged sword. It allows many researchers to do the isolating, genotyping and phenotyping genetic variants of most geminiviruses in the experimental studies, particularly nowadays for transient RNAi and the virus-induced gene silencing (VIGS) studies.

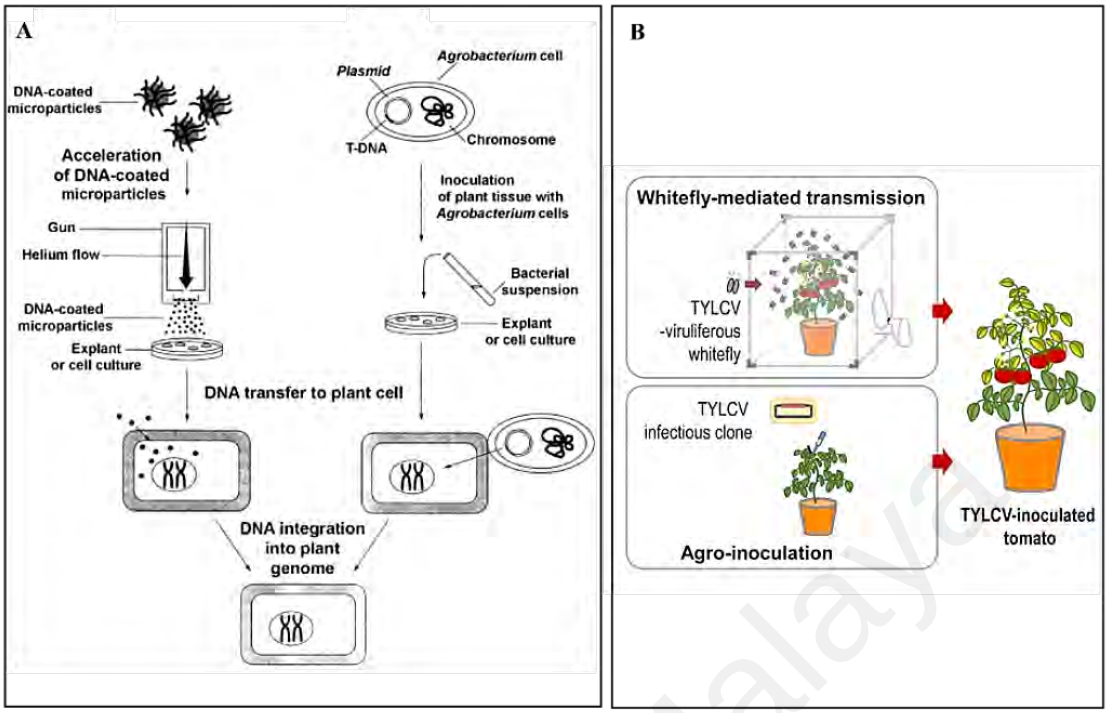

Figure 2.5: Schematic representations of virus inoculation methods in plant tissues. The virus particles can be transferred to the plants cell by few methods. (A) It can be transferred through particle bombardment or inoculation using *Agrobacterium.* Image reproduced from Mourgues et al. (1998), with permission from Trends in Biotechnology. (B) Another known method is using whitefly-mediated transmission or by molecular agroinoculation technique using the infectious clone. Image reproduced from Kil et al. (2016), with permission from Scientific Reports.

CHAPTER 3: METHODOLOGY

3.1 Plant Materials, Vector and Virus Sources

Seeds of wild-type tomato, *Solanum lycopersicum* (Variety: MT1) obtained from MARDI Station Jalan Kebun, Klang, Selangor were sown and grown to four to five true leaves for inoculation assay. All tested tomato plants were maintained in the greenhouse (Biosafety level 2) with a constant temperature at 24 ± 2°C, (16/8 h light/dark cycle) and humidity (65% - 75%).

Plant binary vector of pCAMBIA1304 harboring the 35S CaMV promoter (35S), kanamycin resistant gene and GFP-GUS marker genes was used for the construction of each agroinfectious clone. The vector size is approximately 12.3 kb. The empty pCAMBIA1304 plasmid was obtained from bacterial glycerol stocks, stored stably at -80°C freezer in our laboratory.

For the virus source, total genomic DNA from the virus-infected leaves was kindly provided by Dr. Mohtaram from our virus group (Koravieh, 2016). It was extracted from leaf tissue of symptomatic whitefly-infected tomato plants collected in selected tomato field located at Selangor, Malaysia in 2009 (2.98305°N, 101.7006°E). The genomic DNA was stored in -20°C freezer till further use for the construction of the agroinfectious clones.

3.2 General Methods

3.2.1 Seeds Sterilization

Seeds were surface sterilized for 15 minutes in sterilization solution containing 5.25% sodium hypochlorite (1:5 dilution) and a drop of Tween-20 per 50 µl solution. Then the seeds were rinsed twice (20 min per rinse) with sterile distilled water and placed onto the filter paper to remove the excess water.

3.2.2 Greenhouse Care of the Tomato Plants

Sterilized tomato seeds of WT-plants were sown into autoclaved universal organic soil mix (with perlite) in the standard open flat cell with drainage holes (three seeds per square cell). After two weeks, the tomato plantlets were individually transferred to the properly labeled small pots with an approximate size of 10 inches containing the soil mix and grown until the inoculation steps. The grown tomato plants with four to five true leaves were used for inoculation experiments. The plants were maintained at 24 ± 2°C, (16/8 h light/dark cycle) and humidity (65% - 75%) under a natural photoperiod in the greenhouse in accordance with biosafety requirements (Biosafety level 2).

3.2.3 Symptoms Development and Leaf Sampling

Disease symptoms were monitored daily and the plant heights were measured weekly after the agroinoculation assay. The symptoms of leaf curl disease (plants exhibiting leaf curl and yellowing) were recorded on the basis of no symptom, a mild or severe symptom in comparison to control (mock) plants.

For DNA quantification, leaf samples were collected at different time points after inoculation; 7, 14, 21 and 28 days of post inoculation (dpi) from each tested tomato plant, either inoculated with only the virus or co-inoculated with both virus and the betasatellite. Tomato plants inoculated with *Agrobacterium tumefaciens* containing empty pCAMBIA1304 were served as control plants (mock). The collected leaves were properly labeled and stored in -80°C freezer until further use for the virus quantification.

3.2.4 The Primers Design

The primers used in this study were specifically self-designed mainly for normal polymerase chain reaction assay (PCR) which was used for cloning, detection of viral DNAs and analysis of viral load via quantitative real-time PCR (qPCR) in each tested tomato plant. All of these primers were custom synthesized using the service provided by Integrated DNA Technologies, IDT (Singapore) as listed in Table 3.1. To have successful PCR amplification with specific and high yields, few parameters were taken into consideration when designing these primers. These general parameters for PCR primer design including: (a) Primer length is around 18 bp to 22 bp, (b) Primer melting temperatures in the range of 55°C to 60°C, (c) The GC content is approximately 40% to 60%, (d) Primer has no self-dimer to avoid the reduction of product yield.

For the amplification of full-length DNA sequence of individual virus and betasatellite, the primer pairs were designed in such a way that the forward and reverse sequences overlap at the similar single restriction (RE) site of the sequences (Figure 3.1A: Table 3.1). Another specific primer pair; pGA101/pGA201 and pGβ301/ pGβ401 were designed for the selection of positive bacterial colonies (containing the corresponding recombinant plasmid) in colony PCR (Figure 3.1B: Table 3.1). Since each constructed agroinfectious clones containing monomeric repeats of viral DNA sequences, these primer pairs were designed in such a way to target the short gene sequences in between those monomeric repeats. Therefore, using these primers, we can be ensured that the whole tandem dimers of corresponding individual viral genome within the *A. tumefaciens* T-DNA borders was properly released into the plant cells.

Table 3.1: The list of primers used in this study.

Primer Names	Primer sequences (5' to 3')	Target size	Remarks
pFSA01	CCC GGG *CTGCAG*ATTTTAATATA	2.8 kb	Amplification of full length virus
pFSA02	AGA*CTGCAG* AGA GCT TCA CGA		
pFLβ03	CCCA*GAATTC*TAAATAGCATAG	1.3 kb	Amplification of full length betasatellite
pFLβ04	GCAT*GAATTC*GTAAATAGCAGT		
pGA101	TTTCTTTGCCAGTCCCTTTGG	516 bp	Virus sequence integrity verification, pCAY-1.7mer
pGA201	GGACGAATGGGGAGAATTTCA		
pGβ301	GCCATCTAAATAGCATAGAGGT	502 bp	Betasatellite sequence integrity verification, pCMYβ-1.9mer
pGβ401	GCCCTACAGTGCTAATTATTAAAGG		
pwA-1F	GGACCCGGGCTGCAGATTTTAA	787 bp	DNA sequence analyses for pGEM-28A
pwA-1R	GCCTGGGCACATCAGGGCTTCTG		
pwA-2F	GGTTGTGCCCAAAGGTTGTGAAG	783 bp	
pwA-2R	CCCAGCTTCCAGATCAGGACTCA		
pwA-3F	GGAGAAGCTCAGAAAACACTGG	702 bp	
pwA-3R	CACCCAAGAGTATAGTTGTAGAG		
pwA-4F	GGATCTTGGGTCTCCATGGCCGC	512 bp	
pwA-4R	TCGAGACTGCAGAGAGCTTCACG		
pwβ-1F	GCCGAATTCTAAATAGCATAGA	519 bp	DNA sequence analyses for pGEM-13β
pwβ-1R	GGGAGTGTTTCTCCTGTATGCAT		
pwβ-2F	CCGACACTTATTTATACAATGA	445 bp	
pwβ-2R	CCCATAATTCATGTCCACATA		
pwβ-3F	GGGATTATATACGCGTTCAATTGT	394 bp	
pwβ-3R	CCGGAATTCGTAAATAGCAGTTA		
qpCA501	CTTTGCCAGTCCCTTTGGG	125 bp	Relative viral DNA quantification
qpCA601	GGTCACCTCGACCTGAGTC		
qpCβ711	GGTAACTTTGTCCCCAATAGGT	105 bp	Relative betasatellite quantification
qpCβ811	CACATTTTTAAGGGTATTTTTGTCTG		
qUbi01	TCGTAAGGAGTGCCCTAATGCTGA	120 bp	Ubiquitin gene housekeeping gene
qUbi02	CAATCGCCTCCAGCCTTGTTGTAA		

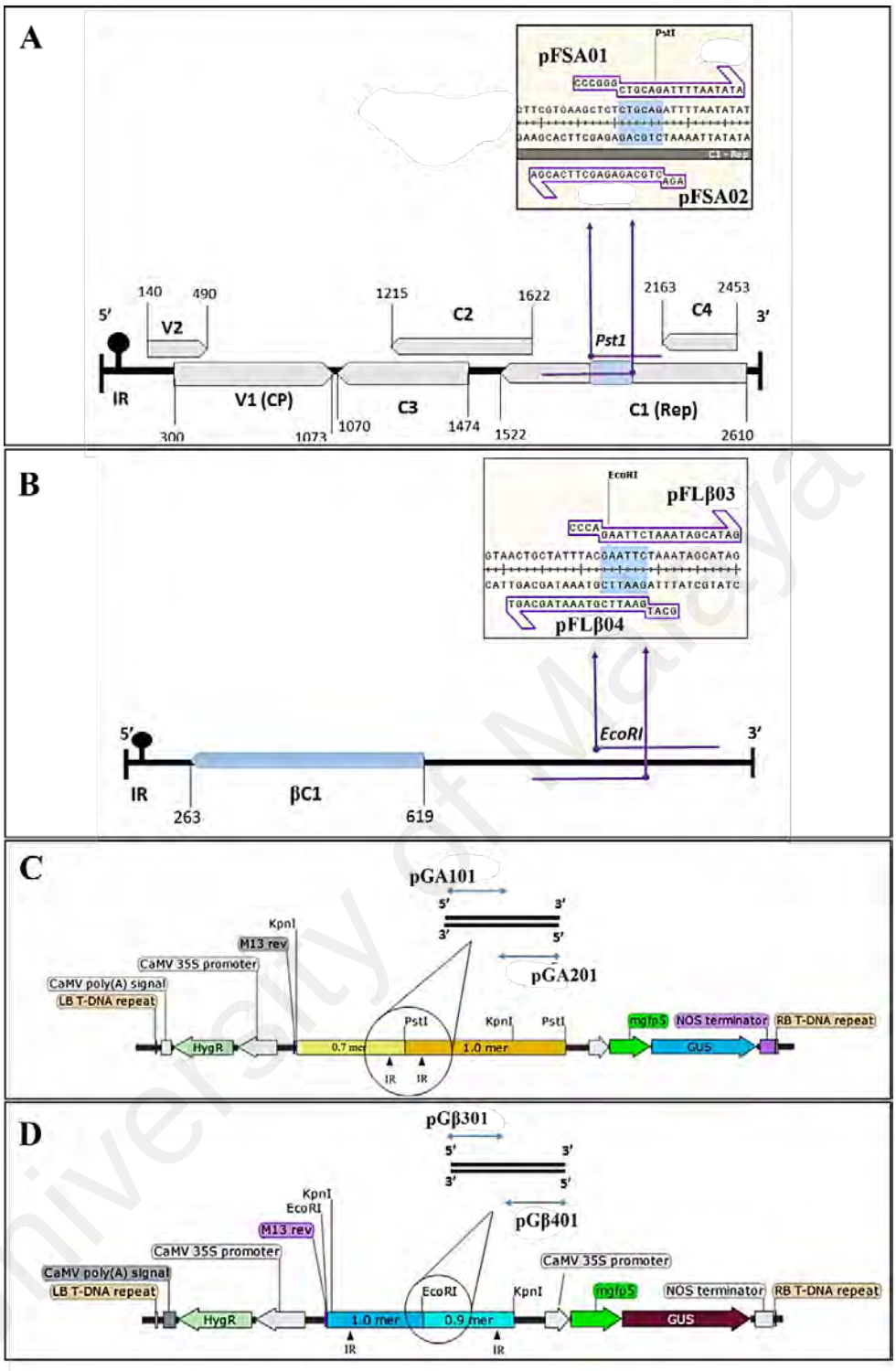

Figure 3.1: The strategies to design specific primer pairs for the amplification and cloning purposes of both viral DNAs. For the amplification of the full-length virus (A) and betasatellite (B) DNA sequences, the forward and reverse primer sequences were designed in such a way they are overlapped at single RE site. The primer pairs of pFSA01 and pFSA02 were overlapped at *Pst*1, while pFLβ03 and pFLβ04 were overlapped at *Eco*R1 to amplify the virus and betasatellite DNAs, respectively. The selected RE sites were chosen to enable the 1.0 mer viral DNAs to ligate into the pCAMBIA1304 that has the similar RE sites in the cloning process. To quantify the relative DNA of each viral DNA in the inoculated plants, another primer pairs were specifically designed to target the short DNA sequence within the tandem repeats of individual plasmid harboring the virus (C) and betasatellite (D), separately.

3.2.5 Cloning Reactions

3.2.5.1 Ligation of PCR amplified products with sticky ends

The ligation of PCR amplified products with 3'-ends (sticky ends) was performed using pGEM-T Easy Vector System (Promega) based on the manufacturer's protocol. The reaction set up as follows:

Purified PCR product	:	20-50 ng
pGEM-T Easy vector	:	1 µl
10x T4 DNA ligation buffer	:	2 µl
T4 DNA ligase	:	1 µl
Final volume with autoclaved water	:	**20 µl**

The reactions were mixed by pipetting and incubated at 4°C overnight (12-16 h) prior to the transformation process.

3.2.5.2 Ligation of PCR amplified products with blunt ends

The ligation of pfu-amplified PCR products with blunt ends was performed using pEASY-Blunt Zero cloning Kit (Transgen Biotech) according to the manufacturer's protocol. The reaction set up as follows:

Purified PCR product	:	4 µl (~ 50ng)
pEASY-Blunt Zero cloning vector	:	1 µl
Final volume with autoclaved water	:	**5 µl**

The reaction mixture was mixed well and incubated at 37°C for 15 min and then was placed on the ice to be used directly for transformation.

3.2.5.3 Ligation of both viral insert(s) and plasmid vector

To insert the viral DNAs into the plasmid vector, individually, the ligation was set up in the 0.5 ml PCR tube using T4 DNA Ligase kit (Promega) as follows:

Purified vector DNA	:	20-50 ng
Purified insert DNA	:	50-200 ng
10x T4 DNA ligation buffer	:	2 µl
T4 DNA ligase	:	1 µl
Final volume with autoclaved water	:	**20 µl**

The reaction mixture was incubated at 16°C for 12 h and hold at 12°C in the thermocycler machine. The ligation product was stored in -20°C fridge until used for the transformation step.

3.2.6 Transformation of Competent Cells

Ligated product was transformed into the chemically competent cell (Trans1-T1 Phage, Transgen Biotech) by heat shock based on the manufacturer's protocol. Basically, the cells were given heat shock at 42°C for 90 s and immediately transferred to the ice for at least 6 min. 700 µl of prepared LB broth was added to this mixture and incubated at 37°C for 1 h in the incubator shaker set at 200 rpm. The cells were pelleted down in the centrifuge at 4000 rpm for 4 min and then re-suspended in 250 µl of LB broth. Transformed cells (100-180 µl) were spread uniformly on the LB agar supplemented with appropriate antibiotics before incubated the LB plates in the oven at 37°C, for 12 h to 16 h until the bacterial colonies grew big enough to select. For selection of the recombinant plasmids by blue-white selection (ligation with pGEMT-Easy), ampicillin (50 ng/ml) and X-gal (50 ng/ml) were used in appropriate amounts. Meanwhile, both kanamycin (50 ng/ml) and streptomycin (50 ng/ml) were used for the selection of recombinant plasmids-pCAMBIA1304 on the LB agar.

3.2.7 Restriction Enzyme Digestion for Cloning Purposes

The restriction enzymes (REs) used in this study are high-fidelity (HF®) REs from New England Biolabs (NEB). The procedures for single or double digestion of the DNA inserts and vectors are based on the manufacturer's protocol. General restriction reactions for single digestion were set up as follows:

DNA insert / vector	:	200-500 ng
10x CutSmart® Buffer	:	2 µl
Restriction enzyme- HF®	:	0.7-1 µl
Final volume with autoclaved water	:	**20 µl**

Meanwhile, for double digestion, another corresponding RE was added in this reaction mixture of single digestion with a similar amount. The reaction mixtures were incubated in 37°C incubator for at least 4 h and the reaction was stopped by adding an appropriate amount of 6X blue/orange loading dye (Promega) before directly electrophoresed in 1.0% agarose gel. The gel was excides and purified for the next step of ligation.

3.2.8 Sequencing Analysis

The amplified fragments were purified using Wizard® SV Gel and PCR Clean-Up Start-Up Kit (Promega), then were cloned into either pGEM-T Easy vector (Promega) or pEASY-Blunt Zero vector (Transgene Biotech) for sequencing. The clones in this study were sequenced by 1st BASE DNA Sequencing Services, Singapore. Sequencing data were processed and analyzed using the Molecular Evolutionary Genetics Analysis (MEGA) software, version 6.0 (Tamura et al., 2013) or BioEdit version 7.2 software (Hall, 1999). Sequence similarity analyses were performed using the Basic Local Alignment Search Tool (BLAST) available online at http://blast.ncbi.nlm.nih.gov. The analyzed viral sequences for each construct were assembled into the corresponding vector

sequences using the molecular biology SnapGene Viewer software (from GSL Biotech and available online at snapgene.com).

3.2.9 Purification Process and Agarose Gel Electrophoresis

The products either from PCR amplification or digestion reaction were purified using FavorPrep™ GEL/PCR Purification Kit (Favorgen Biotech Corp) based on the manufacturer's protocol. Briefly, the excised agarose gel was completely dissolved in the FADF buffer by incubating the mixture at 55°C for 10 min. Then the DNA was eluted by a series of columns washing and centrifugations using the provided wash (ethanol added) and elution buffers.

Gel electrophoresis process started with the boiling of an appropriate amount of agarose powder (1-2% as per requirement) in 1x TAE buffer until completely dissolved. The mixture was cooled and gel staining was added before poured into the appropriate gel casting tray with the clot-forming comb. When the agarose was solidified, the comb was removed and the gel was placed carefully in the gel tank for electrophoresis to take place. Any PCR products or digested REs products were properly mixed with gel loading dye and loaded onto the wells of the gel. DNA ladder (either 100 bp or 1 kb ladder, Promega) was also loaded to compare the size of the PCR /digested REs products. Electrophoresis was run at constant voltages of 60 V, 100 V or 120 V, based on the size of the target templates (the bigger the size the lower the voltage). When the electrophoresis was ended, the gel was visualized using ChemiDoc MP System, gel documentation system from Bio-Rad.

3.2.10 DNA Isolation

In this study, the isolation of DNA was achieved by using TissueLyser LT (Qiagen) with its adapter. Leaf tissue (approximately 100 mg) was disrupted to a fine powder using this machine in which the adapter can efficiently disrupt 12 leaf samples per run. Disruption and homogenization are achieved through the beating and grinding effect of beads on the sample material as they are shaken together in 2 ml sample tubes. The disruption process begin when the precooled insert with leaf sample tubes (flash-frozen in liquid nitrogen) were placed into the base of the precooled adapter (at least 2 h in -80°C freezer). Then, after fastened the lid of the TissueLyser LT adapter, the machine was immediately operated at 50 Hz for 2 min. Once the operation is done, the disrupted leaf samples were removed from the insert and then were kept in -80°C freezer until used. From the beginning till the end of the process, the leaf samples (before and after disruption) were kept in the transportable Dewar flask filled with liquid nitrogen to avoid the leaf tissues from thaw (potential DNA degradation).

3.3 Amplification of Individual Viral DNA

3.3.1 Full-length Sequence of the Virus

Total purified virus DNA was used for the amplification of full-length or one unit (1 mer; 2750 bp) using normal PCR with the specific overlapping primer pair, pFSA01 and pFSA02 (Figure 3.1A: Table 3.1). The reactions were conducted in a reaction volume of 150 µl comprising of 40 µl Q5® High-Fidelity 2X Master Mix buffer (NEB), 7.5 µl of each primer (final concentration of 0.5 µM), 100 ng of template DNA (diluted in 1:20 in water) in a thermal cycler (T100™, Bio-Rad) with the following parameters: initial denaturation at 98°C for 30 s ; subsequent 32 cycles of PCR cycles at 98°C for 10 s, 58°C for 30 s, 72°C for 90 s; and final extension step of 6 min at 72°C. The amplified DNA fragments were determined by 1% agarose gel electrophoresis prior to gel purification and then were individually cloned into pGEM-T-Easy (Promega). Selection for positive

plasmid was performed on Luria-Bertani (LB) plate supplemented with ampicillin (50 μg/ml) and colony PCR using the corresponding primer pairs were carried out to detect the presence of 1.0 mer full-length DNA sequence. The positive plasmid was denoted as pGEM-28A and stored in either -20°C (purified plasmid) or in -80°C (glycerol stock) for further use.

3.3.2 Full-length Sequence of Betasatellite

Since there is no conserved RE site on the betasatellite sequence that available to those multiple cloning sites on the pCAMBIA1304 for ligation to take place, a new RE site of *Eco*RI was introduced into the betasatellite genome sequence by replacing the conserved *Sty*I with *Eco*RI without protein changes. Using the designed overlapping primers in which overlapped at introduced *Eco*RI site, a full length of betasatellite (1 mer: 1356 bp) was amplified by PCR. These primers were termed as pFLβ03 pFLβ04 and they were designed with a similar concept as designing the pFSA01/ pFSA02 (Figure 3.1A:Table 3.1), except the primer sequences were overlapped at *Eco*RI site of the virus from genomic DNA extracted from the infected tomato leaves. The PCR reaction and conditions were as follows; initial denaturation at 98°C for 30 s and subsequent 32 cycles of PCR cycles at 98°C for 10 s, 55°C for 45 s, 72°C for 90 s; and a final extension step of 6 min at 72°C. The DNA fragment amplified with this PCR reaction was then purified, cloned and sequenced. The positive plasmid was denoted as pGEM-13β and stored in either -20°C (purified plasmid) or in -80°C (glycerol stock) for further use.

3.4 Construction Process of Agroinfectious Clones

3.4.1 Infectious Clone Harboring Virus DNA

The 0.7 mer (~1.9 kb) fragment was released from pGEM-28A plasmid by double digestion with *Pst*I and *Kpn*I, and then was subsequently ligated into a linearized binary vector, pCAMBIA1304 that had been digested with the same REs (Figure 3.2). The

ligated plasmids were screened using REs digest reaction with the same REs and the selected positive plasmids were denoted as pCAY-0.7mer. Another 1.0 mer (~2.8 kb) viral fragment released from pGEM-28A by single digestion with *Pst*I was subsequently ligated into pCAY-0.7mer, digested with the same RE. The generated plasmid was pCAY-1.7mer a binary vector containing two IR sequences of the virus.

3.4.2 Infectious Clone of Virus Associated Betasatellite

The 0.9 mer (~1.3 kb) fragment of betasatellite was released from pGEM-13β by double digestion with *Eco*RI and *Kpn*I. The released fragment was subsequently ligated into the linearized binary vector, pCAMBIA1304 that had been digested with the same REs (Figure 3.2). The positive plasmids were denoted as pCMYβ-0.9mer. The remaining steps were similar as those steps described for the construction of pCAY-1.7mer. The final generated vector was termed pCMYβ-1.9mer.

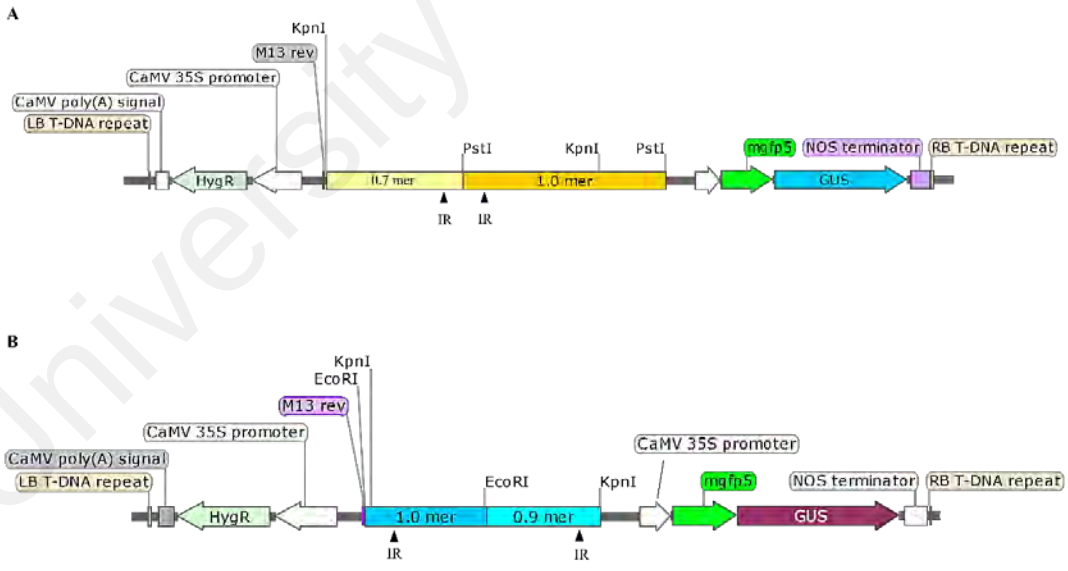

Figure 3.2: Linear map of constructed agroinfectious clones. Each agroinfectious clone carrying the tandem repeats of individual viral DNAs which are (A) pCAY-1.7mer harboring virus DNA and (B) pCMYβ-1.9mer harboring betasatellite DNA. The viral DNAs were inserted individually between the respective RE sites in the pCAMBIA1304 to enable them to be released and replicated in the inoculated leaf tissues.

3.5 Infectivity Assay

3.5.1 Transformation of *Agrobacterium* Competent Cells

Agroinoculation method was used to test the infectivity of infectious clones constructed in this study. The T-DNA vectors carrying tandem repeats of the virus and betasatellite clone constructs were transformed individually into *A. tumefaciens* strain LBA4404 competent cells via heat shock transformation as described by Bang et al. (2014). Briefly, the plasmid DNA (1µg) was mixed with the competent cells and then were immediately frozen in the liquid nitrogen for 5 min. Then, the mixture was immediately incubated in 37°C incubator for 15 min. After the incubation, 1 ml of LB broth (without antibiotic) was added into the mixture and the cells were grown in 28°C incubator for 3 h with continuous shaking at 200 rpm. Once the incubation was ended, the cells were pelleted down by centrifugation at 12,000 rpm for 2 min. When the supernatant was discarded, 100 µl LB broth was added and the cells were dispensed in it. Then, the cells were spread on LB agar plates supplemented with streptomycin (100µg/ml), rifampicin (25µg/ml) and kanamycin (50µg/ml). Lastly, the plates were incubated in the incubator at 28°C for 3 to 4 days.

3.5.2 Selection of Transformed *Agrobacterium* by Colony PCR

Verification of the positive transformants of *A. tumefaciens* was carried out by performing colony PCR using specific primer pairs for every different fragment. A single *A. tumefaciens* colony was picked using a toothpick in the sterile area and was placed in 50 µl sterile water in PCR tube. Then the mixture was boiled at 98°C for 2 min and then immediately put on ice. The PCR reaction using GoTaq® Green Master Mix (Promega) was set up as follows:

2X GoTaq® Green Master Mix	:	25 µl
Forward primer	:	1 µl
Reverse primer	:	1 µl
Boiled mixture (*A.tumefaciens* colony)	:	1 µl
Final volume with autoclaved water	:	**50 µl**

The PCR reaction was conducted at 95°C for 2 min, followed by annealing temperature based on target fragments and the final extension at 72°C for 5 min. After completion, the reaction was subjected to electrophoresis in 1% agarose gel to detect the presence of desired amplification.

3.5.3 Agroinoculation of Infectious Clones into the Tomato Plants

A. tumefaciens culture was grown individually in a conical flask containing LB media supplemented with kanamycin (50 µg/ml) and rifampicin (50 µg/ml) for 36 to 48 h at 28°C in the incubator with agitation at 200 rpm. The bacterial cells were then harvested by centrifugation at 4000 rpm for 10 min and resuspended the pellets in 0.5 ml infiltration medium supplemented with 10 mM MgCl, 10 mM MES and 100 µM acetosyringone to final concentration of OD600 = 1.0, then stabilized in the room temperature (4 h incubation time) prior the used for agroinoculation. Agroinoculation was performed based on methods described by Leuzinger et al. (2013) and Ma et al. (2012). The four-week-old tomato plants were agroinoculated using a needless 1.0 ml syringe (Terumo®), into the lower side of both third and fourth leaves per plant (Figure 3.3). The piston was pushed slowly down to force the entry of the agroinoculation media into the leaf tissue until a dark-green sector is visible, indicating the bacterial culture is filled as apoplastic spaces. To visualize specific area on the leave triggered by the inoculated virus and to distinguish the inoculated area for future reference during the leaf sampling, the inoculated area was marked gently using a fine black marker.

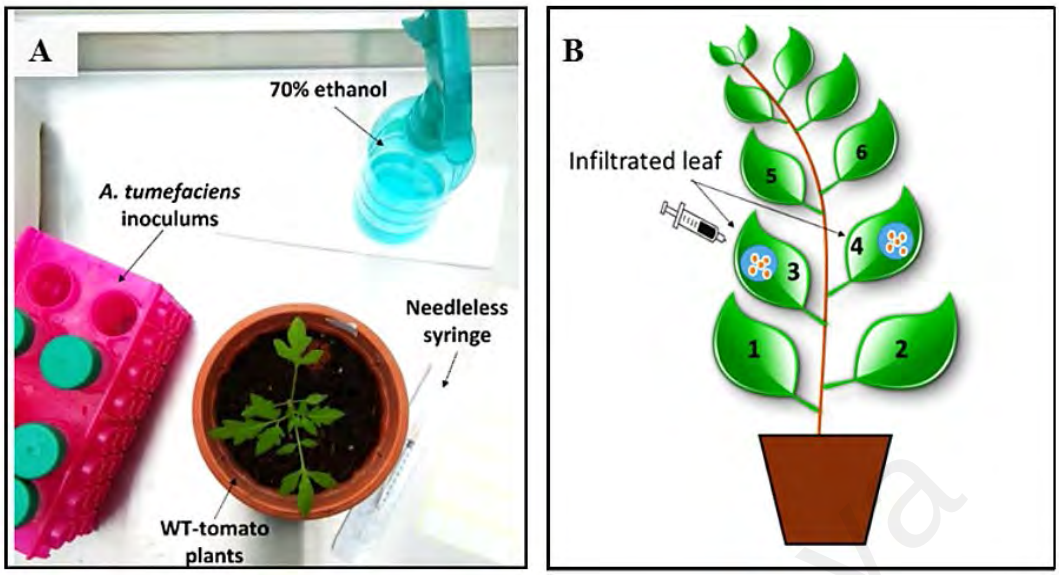

Figure 3.3: Plant set up for the infectivity assay. (A) The experimental setup prior to agroinoculation assay including materials used such as 70% ethanol for disinfection, needless syringe to inoculate the WT-tomato plants with *A. tumefaciens* inoculums containing the virus or the betasatellite, separately. (B) A schematic diagram to illustrate the techniques used to inoculate the leaves of the individual tomato plant with the *A. tumefaciens* inoculums. The leaves were numbered accordingly and the inoculum was inoculated on the leaf no. 3 and 4. For the infectivity analysis including the symptom appearances, detection of viral DNAs, and measurement of viral loads, all of these parameters were done on both inoculated leaves (leaf no. 3 and 4) and the non-inoculated upper leaves (leaf no. 5 and 6).

For all leaf inoculation assay conducted in this study, three independent experiments (three biological replicates) were performed with 10 plants (n=10) inoculated per treatment (single and co-infections) as well as the control (mock) to account for variation. The control plants inoculated with *A. tumefaciens* inoculum containing the empty binary vector of pCAMBIA1304 were used as the negative control in this study. The infectivity of PCR-derived clones was analyzed as follows:

$$\text{Infectivity level} = \frac{\text{Number of virus-infected plants}}{\text{Number of inoculated plants}} \qquad (3, 1)$$

All the agroinoculated plants were grown in the greenhouse (Biosafety Level 2) for four weeks post inoculation, a timing selected to allow the full symptom expression in tomato plants (Refer to Section 3.2.3). Along the course time, the symptoms appearances and plant heights were observed and measured, periodically. All the single / co-inoculated leaves (Leaf no. 3 and 4) and non-inoculated upper leaves (Leaf no. 5 and 6) of the individual treated tomato plants were harvested at 7, 14, 21 and 28 (dpi) to quantify the relative amount of viral DNAs. The harvested leaf samples were immediately frozen and ground in the liquid nitrogen and stored at -80°C until processing. Step-by-step procedures of agroinoculation assay conducted in this study are illustrated in Figure 3.4.

3.6 Detection of Replicon DNA in the Virus-infected Plants

3.6.1 Extraction of Total Genomic DNAs

Total genomic DNA was extracted from the frozen leaf material of symptomatic infected leaves of the tomato plants and was subjected for PCR assay to detect the presence of viral DNAs. The inoculated leaves of leaf three and four (local infection), as well as non-inoculated upper leaves of leaf number five and six (systemic infection), were harvested at several different time point after the inoculation assay: 7, 14, 21 and 28 dpi. To detect the viral components in the systemically infected leaves per inoculated plant, the total genomic DNA was extracted using FavorPrep™ plant genomic DNA extraction kit (FavorGen Biotech) following the manufacturer steps. Briefly, the plant sample (~100 mg) was ground to make a fine powder using TissueLyser LT as described in Section 3.2.10. Then, a series of lysis, bind, wash and elute was performed with corresponding buffers as described in the manufacturer's protocol. Prior to the final step of elution to release the pure DNA, an extra centrifugation step was performed after washing to dry the column of residual ethanol. The concentration and purity of the genomic DNA were assessed using NanoDrop™ 2000/2000c Spectrophotometers (Thermo Fisher Scientific).

Finally, the eluted DNA was stored at 4°C fridge for frequent use or -20°C fridge for long-term storage.

3.6.2 PCR Amplification for the Detection of Viral DNAs

The presence of viral DNA in the inoculated plants was detected using PCR assays performed with GoTaq® Green Master Mix (Promega) with primer pairs of pGA101/pGA201 and pGβ301/ pGβ401 for the DNA virus and betasatellite, respectively (Section 3.2.4; Table 3.1). The PCR reaction was carried out with a denaturation step at 95°C for 2 min followed by 30 cycles of 30 s at 9°C, 40 s at 58°C, 1 min at 72°C and end with a final extension at 72°C for 6 min. The band detection was analyzed on 1% agarose gel. The positive PCR products were used as the template for sequencing to confirm the presence of viral DNA sequences of the corresponding virus.

3.7 Quantitative Measurements of Relative Viral DNA Loads

Quantitative PCR (qPCR) was used to determine the relative viral DNA accumulation in five virus-infected plant samples (biological replicates) of each treatment with three technical replicates (Péréfarres et al., 2011). Prior to that, total DNA was isolated from 100 mg of symptomatic frozen leaf at regular intervals of 7, 14, 21 and 28 dpi (Refer to Section 3.2.10). The qPCR reaction consisted of a total volume of 20 µL with 1x HOT FIREPOL® EvaGreen® qPCR Supermix (Solis BioDyne), 100-200 nM of each primer, and 20 ng of DNA. The qPCR cycling conditions consisted of 95°C for 12 min, followed by 31 cycles of 95°C for 15s, 60°C for 30s and 72°C for 30s. Reactions were performed using QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems). The tomato ubiquitin (UBI) gene was used as a reference gene for normalization and plants inoculated with empty pCAMBIA1304 were used as the calibrator. The primer pair of qpCA501 and qpCA601 was used to quantify virus DNA, while primer pair of qpCβ711 and qpCβ811 was used for quantification of the betasatellite DNA (Table 3.1). Relative

viral DNA was calculated as fold-change in normalized virus DNA relative to the calibrator using the $2^{-\Delta\Delta CT}$ method. The calibrator DNA was set to 1 for all treatments at all time points as no virus accumulation in the inoculated plants (Livak and Schmittgen, 2001).

3.8 Data Analysis

Related nucleotide sequences in GenBank database were identified using the NCBI-BLASTn algorithm (www.ncbi.nlm.nih.gov). We used an AlignX (Vector NTI Suite V 5.5, InforMax) and ClustalW algorithm to do multiple sequence alignments and identification of ORFs. The Species Demarcation Tool (SDT) was used to calculate the percent nucleotide sequence identity values from pairwise sequence alignments as described by Muhire et al. (2014).

The data of viral DNA accumulation were analyzed using the Student's t-test. The calculated relative viral DNA in the treated plants was compared between the control and treatments, and among the treatments. Each treatment and the control had sample sizes of five plants (n=5) with three biological replicates. The threshold significance level was set as listed in Table 3.2 to find the conclusion for each analyzed result. Bar graphs with standard errors were prepared based on the pooled results from three independent experiments at each dpi.

Table 3.2: The P-values levels of statistical significance.

P-value	Wording	Summary
< 0.001	Very significant	***
0.001 to 0.01	Very significant	**
0.01 to 0.05	Significant	*
≥ 0.05	Not significant	ns

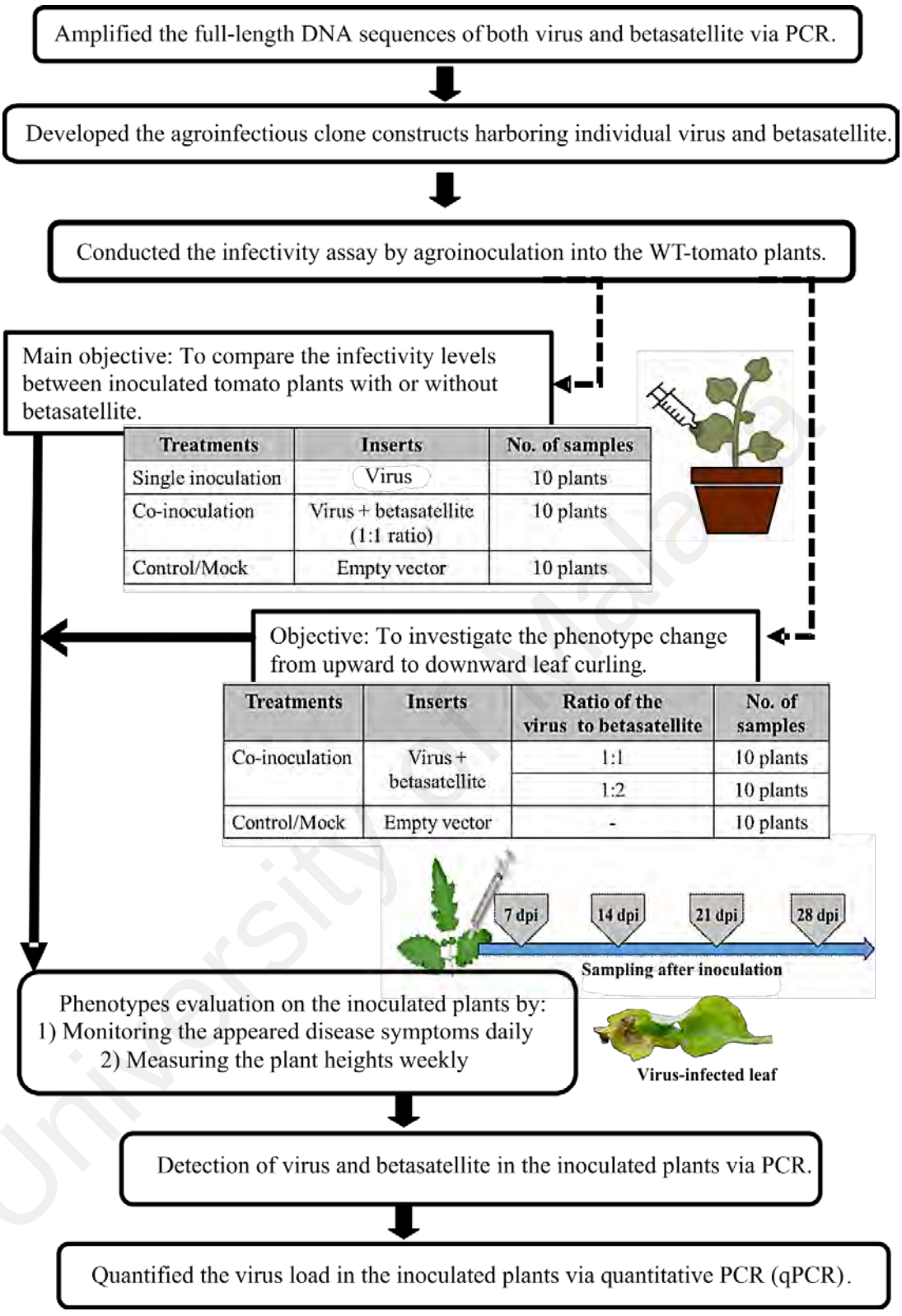

Figure 3.4: Flowchart diagram summarizing the experimental designs adapted in this study. The infectivity analysis was conducted to achieve the objective in this study which is to investigate the effects of betasatellite on the virus in the inoculated plants. Thus, the analysis was done by treating the plants with or without the betasatellite. Meanwhile, an additional work was performed to investigate the probability that the level of betasatellite (two different *A. tumefaciens* inoculum ratios) has an influence to the virus infectivity too. For each treatments, three biological replicates were performed to test the variability between plant samples (n=10).

CHAPTER 4: RESULTS

4.1 Analysis of Viral DNAs by PCR and DNA Sequencing

Total genomic DNA extracted from the leaves showing leaf curl disease symptoms was first subjected to polymerase chain reaction (PCR) with specific overlapping primer sets; pFSA01/ pFSA02 and pFLβ03/ pFLβ04 to amplify the full sequences of both DNA virus and its betasatellite, respectively. Approximately, 2765 nt (~2.8kb) of the virus and 1356 nt (~1.3 kb) of betasatellite were amplified and the presence for both amplified target DNA genes were detected on the 1% agarose gel (Figure 4.1). No band was detected for PCR reactions with the absence of the primer pair or target DNA (negative controls), suggesting that the produced products from the PCR are specific to those designed primer pairs. These amplified full-length DNA sequences were then ligated individually, into pGEM-T Easy vector for the construction of agroinfectious clones.

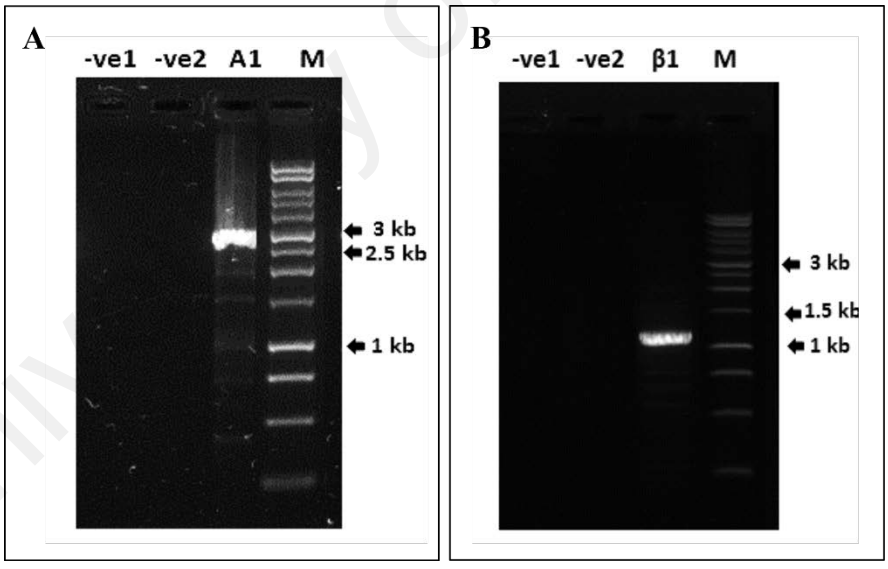

Figure 4.1: Agarose gel electrophoresis of PCR amplification products. (A) Detection of ~2.8 kb DNA virus (lane A1) using a primer set; pFSA01 and pFSA02. (B) The presence of ~1.3 kb betasatellite (lane β1) was detected on the agarose gel using a primer set; pFLβ03 and pFLβ04. No band was detected for healthy wild-type tomato plant (lane –ve1) and PCR template without primer pair (lane –ve2). 1 kb ladder was used as a molecular size marker (lane M).

4.1.1 The Sequence Similarity of DNA Virus

Sequencing results of pGEM-28A (harboring full-length virus DNA) showed that the single genome of the virus (homolog to bipartite DNA-A) contained six conserved ORFs with a bidirectional organization, which are V1 and V2 in the virion-sense strand and C1, C2, C3 and C4 in the complementary strand. Results of the sequence similarity comparison using BLAST analysis showed the virus sequence obtained here to have high levels of sequence identity with isolates of *Ageratum yellow vein virus* (AYVV) (Table 4.1). Sequence analysis revealed that our *begomovirus* isolate was closely related to four AYVV isolated from Indonesia with the highest sequence identity of 99% which are; AYVV-BA_D1-2, AYVV-BA_E2-2, AYVV-BA_C1-2 and AYVV-BA_A6-2 (Koeda et al., 2016). Besides, our isolate also shared 94% sequence identity with another AYVV isolate which is AYVV-JP[JP-Soy] (Samretwanich et al., 2001). Following the current taxonomic criteria for begomovirus species demarcation (91% for species and 94% for strain) the virus characterized here is an isolate of AYVV for which the name Indonesia 2 (ID2) is proposed, with the isolate descriptor [Malaysia:tomato:2011] (AYVV-ID2[MY: tom:11]) (Brown et al., 2015). The name is abbreviated to AYVV-ID2 (MY:MY11A:tom:11). The virus sequence of 2765 nt was submitted to the GenBank database with the accession number of MH745157.

4.1.2 The Sequence Similarity of DNA Betasatellite

Meanwhile, the sequence of pGEM-13β (harboring full-length betasatellite DNA) encodes a single conserved gene in the complementary sense (βC1). The sequence has a low sequence identity with previously characterized betasatellites available in the GenBank database (Table 4.1), with the exception of *Pepper yellow leaf curl betasatellite* (PYLCB) isolated by Jamsari and Pedri (2013) at Tanah Datar, Indonesia (GenBank Accession No. GU382667), at 88.5% nucleotide sequence identity (calculation was conducted using CLUSTAL V; Briddon et al., 2008). Based on the suggested minimum

threshold cut-off value for the proposed nomenclature of betasatellites (78% sequence identity; Briddon et al., 2008), the betasatellite identified here is an isolate of PYLCB. The isolate descriptor [Malaysia:tomato:2011] (PYLCB-[MY:tom:11]) is proposed. The betasatellite sequence of 1356 nt was mitted to the GenBank database with the accession number of MH745158.

Table 4.1: Nucleotide sequence identity between viral DNAs under study and other closely related *begomoviruses*.

Viral DNA	***Begomovirus* isolates**	**Sequence/ nucleotide identity**	**GenBank Accession No.**	**References**
DNA virus (homolog to bipartite DNA-A)	*Ageratum yellow vein virus* (AYVV) Indonesia isolates: AYVV-BA_D1-2 AYVV-BA_E2-2 AYVV-BA_C1-2 AYVV-BA_A6-2	99.0%	 LC051119 LC051120 LC051118 LC051117	Koeda et al. (2016)
	Ageratum yellow vein virus - [Japan-Soybean crinkle leaf]	94.0%	AB050781	Samretwanich et al. (2001)
DNA betasatellite	*Pepper yellow leaf curl betasatellite*-isolate Tanah Datar (PepYLCB)	88.5%	GU382667	Jamsari & Pedri (2013)

4.2 Successful Production of Agroinfectious Clones

Since monopartite *begomovirus* under study is exclusively whitefly-transmitted virus and cannot be mechanically transmitted into the tomato plants, agroinfectious clones were constructed to prove Koch's postulates. The overall workflows for both amplification and construction of these agroinfectious clones are represented in the schematic diagrams in Figure 4.2. Using the amplified and sequenced viral DNAs, two complete agroinfectious clones for both virus (1.7mer) and betasatellite (1.9mer) were constructed individually in the pCAMBIA1304 binary vector. The restriction fragment length polymorphism (RFLP) analyses confirmed the presence of the full-length *Pst*I monomeric virus DNA (1.0 mer) and a direct repeat of *Pst*I / *Kpn*I DNA fragment (0.7 mer) in pCAY-1.7mer. Similar result was also found for pCMYβ-1.9mer that harboring the full length *Eco*RI monomeric betasatellite DNA (1.0 mer) and a direct repeat of *Eco*R1/ *Kpn*I betasatellite fragment (0.9 mer). The complete result of RFLP analysis for the developed agroinfectious clones was summarized in Table 4.2. Representative pictures for the detection of the presence or absence of DNA inserts in each plasmid vectors through digestion reaction with appropriate REs were shown in Figure 4.3. Meanwhile, the DNA sequencing analysis confirmed both positive agroinfectious clones have no random mutation or deletion suggesting the DNA sequences are conserved without protein changes. The complete DNA sequences of both virus and betasatellite can be found in Appendix A. The vector maps of both agroinfectious clones harboring the tandem repeats of individual viral DNA is shown in Figure 4.4.

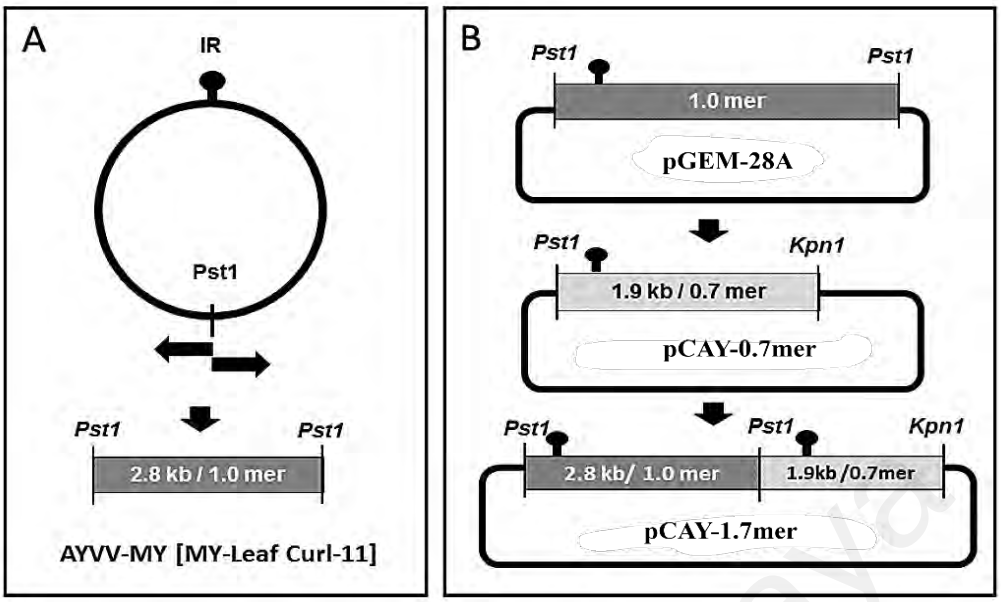

Figure 4.2: Diagrams of the workflow and construction of virus-infectious clones. (A) The full-length DNA virus was amplified via PCR-based method using specifically designed primer pair that overlapped at *Pst*1 site. (B) The amplified full-length DNA virus (~2.8 kb/1.0 mer) was then ligated into pGEM-T Easy vector, resulting to pGEM-28A. The plasmid of pGEM-28A was first digested with *Pst*I and *Kpn*I in which produced 0.7 mer fragment (~1.9 kb). This 0.7 mer fragment was then ligated into the pCAMBIA1304 digested with the same REs that produced pCAY-0.7mer with a total size of ~14.2 kb. The construction of this infectious clone was proceeded with the ligation of another 1.0 mer of the full-length virus into the pCAY-0.7mer plasmid vector in which both insert and vector were single digested with the same RE of *Pst*I. The product of this ligation reaction was denoted as pCAY-1.7mer with an approximate size of 16.9 kb. The processes described here are similar for the construction of agroinfectious clones containing tandem dimer repeats of the betasatellite (pCMYβ-1.9mer).

Table 4.2: RFLP patterns of recombinant plasmids after digestion with specific REs.

Plasmids	**Respective RE(s) for plasmid digestion**	**Digested fragment (kb)**	**Total size (insert+vector) (kb)**
pCAY-0.7mer	*Pst*I and *Kpn*I	1.9	14.2
pCAY-1.7mer	*Pst*I	2.8	**15.1**
pCMYβ-0.9mer	*Eco*RI and *Kpn*I	1.3	13.6
pCMYβ-1.9mer	*Eco*RI	1.4	**13.7**

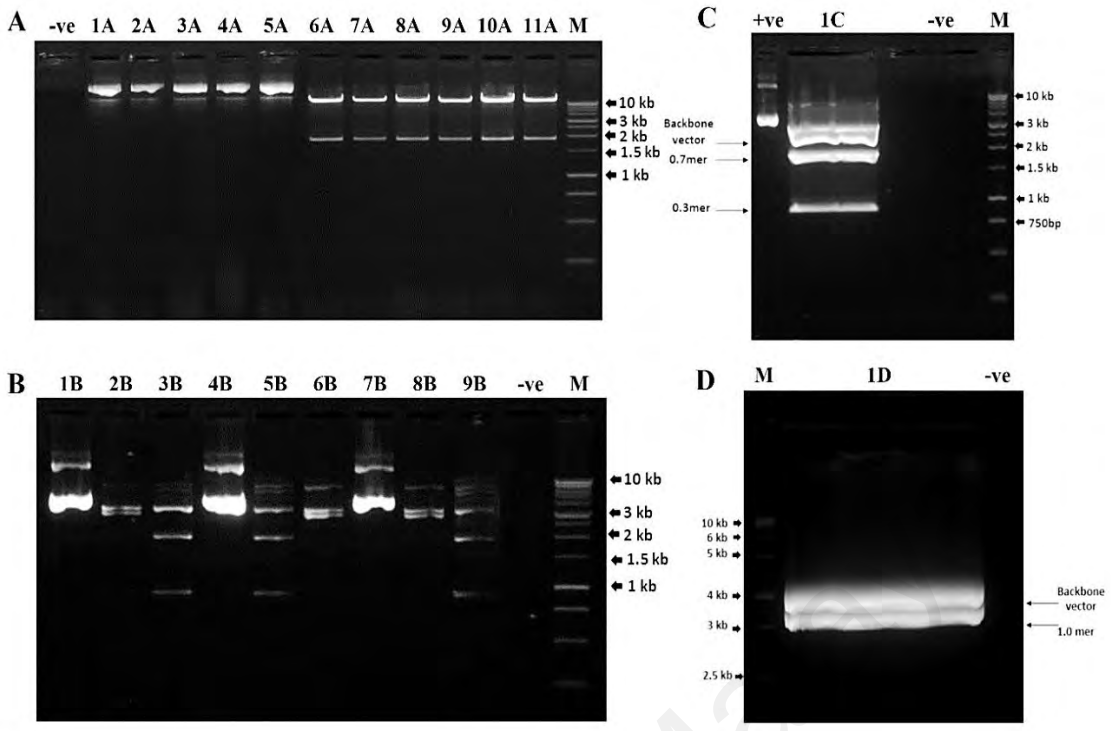

Figure 4.3: RFLP patterns of digested plasmids on the agarose gel. (A) Few selected pCAY-0.7mer plasmids digested with *Pst*I and *Kpn*I (lanes 6A-11A) showed the expected band of 1.9 kb, while the respective undigested plasmids, serve as the positive controls (lanes 1A-5A) showed no digested insert. (B) Single digestion of pCAY-1.7mer plasmids with *Pst*I (lanes 2B, 6B, 8B) produced 2.8 kb bands on the agarose gel, while the double digestion of the same plasmids with *Pst*I and *Kpn*I showed the expected bands of 1.9 kb (lanes 3B, 5B, 9B). No insert was produced from the undigested pCAY-1.7mer plasmids (lanes 1B, 4B, 7B). (C) Focus view of single RFLP pattern of digested pCAY0.7mer on the agarose gel when double digested with *Pst*I and *Kpn*I (1C). Two bands were detected with the approximate sizes of 1.9 kb and 0.8 kb. (D) RFLP pattern of digested pGEM-28A in the single digestion with *Pst*I to release the full-length insert (2.8 kb / 1.0 mer) from the pGEM-T backbone vector (lane 1D). No band was detected on negative control sample (-ve). 1 kb ladder was used as a molecular size marker (lane M).

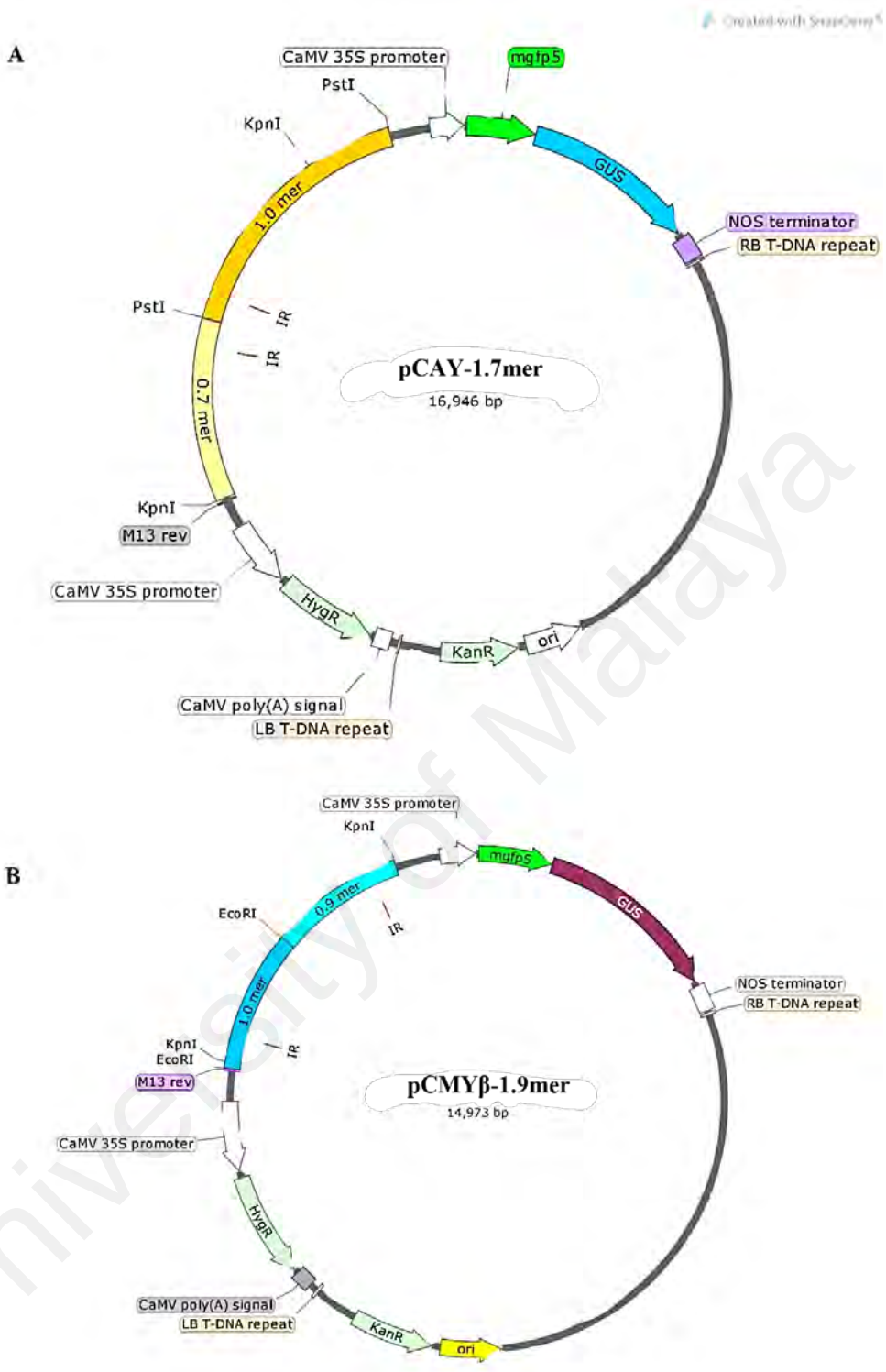

Figure 4.4: Map of constructed agroinfectious clones. (A) pCAY-1.7mer plasmid harboring two tandem repeats virus DNA, which are the full-length sequences (1.0 mer) and another partial DNA sequences of 0.7 mer. *Pst*I and *Kpn*I are chosen as the restriction sites of interest. Two intergenic regions (IR) can be found in pCAY-1.7mer. (B) pCMYβ-1.9mer plasmid harboring two tandem repeats of betasatellite DNA with a total size of 1.9 mer, ligated between *Eco*R1 and *Kpn*1 sites. This plasmid also has two IRs to initiate the replication process of betasatellite molecule in the inoculated plants.

4.3 Phenotypic Evaluation of the Virus-infected Tomato Plants

4.3.1 Leaf Curl Symptoms were Observed On the Virus-inoculated Plants

The typical leaf curl symptoms including leaf curling, upward cupping, yellowing and vein darkening were observed on the inoculated plants, similar to those observed in the field condition, suggesting the successful virus transmission into the leaf tissues. The agroinfectious constructs were highly infectious in the tomato plants when co-inoculated with betasatellite in the tomato leaves, resulting in chlorotic lesions on the co-inoculated leaves (leaf no.3 and 4) as early as 7 to 8 dpi, followed by severe leaf curl symptoms at 14 to 21 dpi. The symptoms on upper non-inoculated leaves (leaf no.5 and 6) of the same inoculated plants became pronounced mild yellowing to distortion at 21 to 24 dpi with moderate upward curling in the later stages (Figure 4.5). The symptoms appearance on these upper mom-inoculated leaves demonstrating that the virus did replicated in the inoculated lower leaves and subsequently moved to the upper leaves of the same inoculated plants.

Single inoculation with virus alone induced mild leaf curl symptoms consisting of upward curling, yellowing and vein swelling on both inoculated and non-inoculated leaves. Besides, the recorded period between the inoculation assay and the first appearance of the disease symptoms (latent period) observed on the infected plants became longer when inoculated without betasatellite. The first leaf curl symptoms in the single inoculated leaves were recorded at 14 to 16 dpi, and were delayed by four to six days in comparison to leaves co-inoculated with betasatellite (Table 4.3). In addition, only 56.7% infection rates were recorded for the single inoculated leaves at 21 dpi, whereas all the co-inoculated leaves displayed complete disease symptoms with 100% infection rates. Plants inoculated with the empty binary vector (mock-inoculated) did not develop any symptoms, suggesting that the observed leaf curl symptoms on the inoculated plants did caused by the presence of this virus under study (Figure 4.5 and Table 4.3).

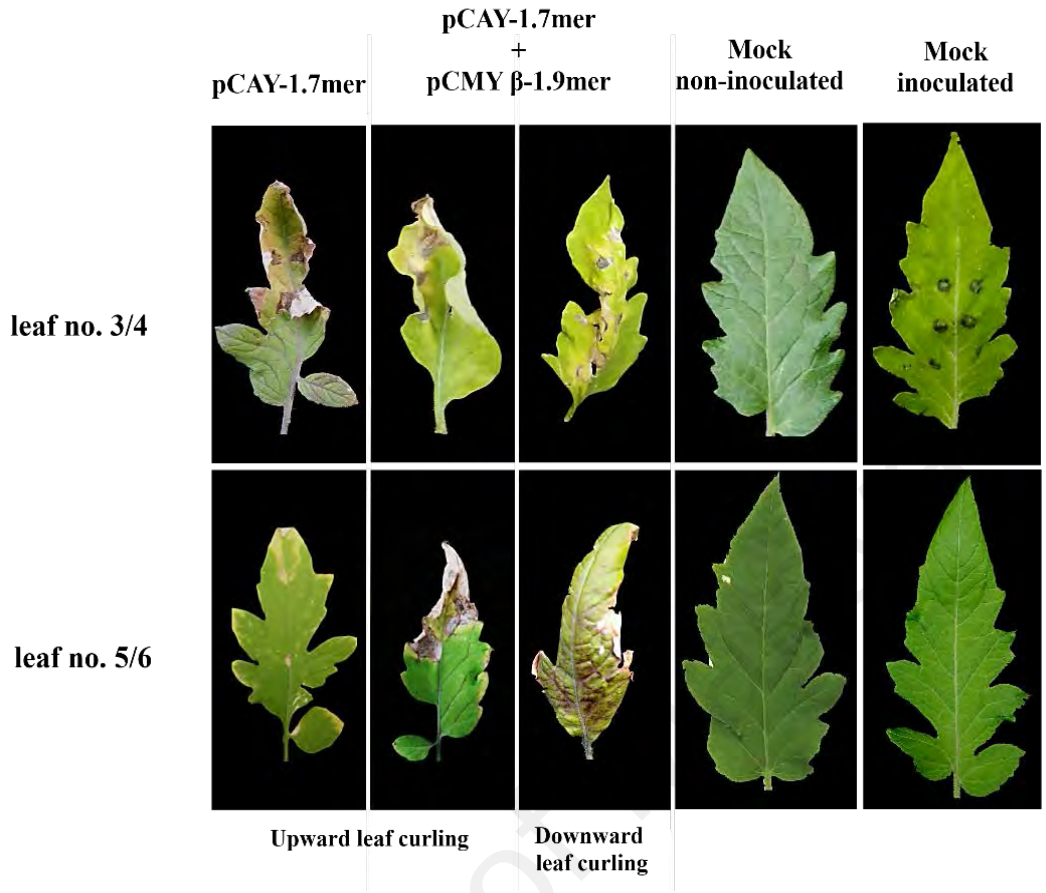

Figure 4.5: Plant phenotypes following inoculation with virus (pCAY-1.7mer) alone or with its associated betasatellite (pCMY β-1.9mer). Typical leaf curl phenotypes including upward leaf curling and yellowing could be observed on both inoculated leaves (leaf no. 3 or 4) and non-inoculated upper leaves (leaf no. 5 or 6) with or without betasatellite. However, symptoms induced by the infectious constructs of both viral DNAs in the co-inoculated leaves are more severe than the inoculated leaves with pCAY-1.7mer only. Some co-infected leaves with the presence of betasatellite displayed downward leaf curling accompanied with bright yellow chlorosis along the veins. No symptom detected on mock inoculated and mock non-inoculated leaves. The leaf pictures were taken at 21 dpi.

4.3.2 Effect of Virus Infections on the Plant's Growth

The virus infectivity was also recorded on the basis of the height of the inoculated plants in single or co-inoculation assays. The inoculated plants under treatments were compared to mock plants inoculated with empty pCAMBIA1304. As expected, all inoculated plants with or without the betasatellite showed gradual growth but at a slower rate as compared to the mock plants (Figure 4.6). When compared the results between the treatments, the virus-infected plants inoculated with betasatellite did have a slower growth than those inoculated plants without the betasatellite. Statistical analysis on the infected plants at each dpi revealed significant differences in growth rate not only among treatments but also between the virus-inoculated and control plants (Student's t-test at $P < 0.05$) (Appendix F). This result suggesting that the virus particle might have formed a specific interaction with the betasatellite molecule to disrupt the development of the infected plants at a severe rate. The measured height for each individual inoculated plants in the single and co-inoculation treatments at each time points can be found in Appendix C.

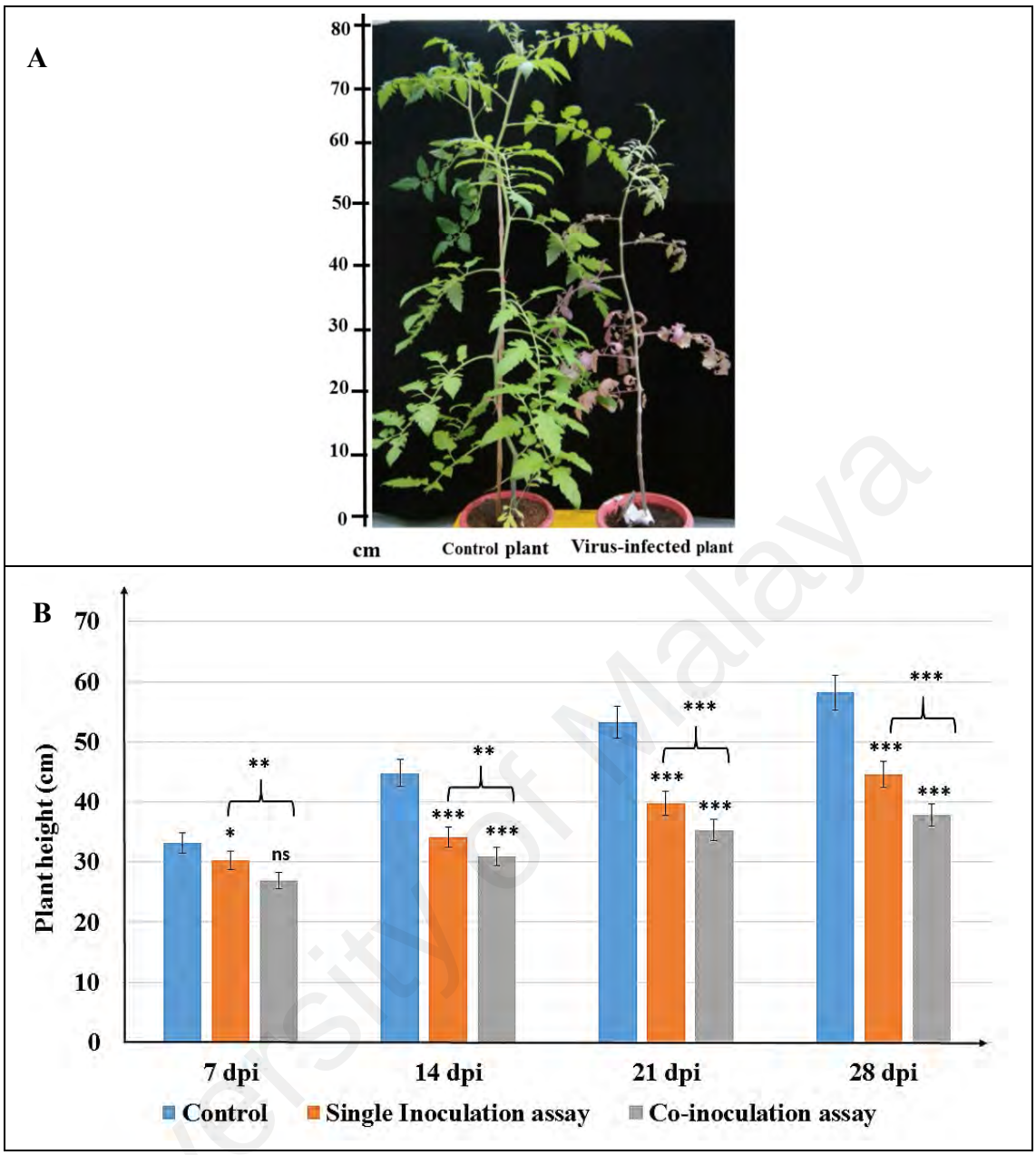

Figure 4.6: Effect of virus infection on the plant's growth. (A) The virus-infected plant is shorter with 20 cm different in height and unhealthier than the mock-inoculated plant at 28 dpi. (B) Graph showing the average of measured height at 28 dpi for the inoculated tomato plants in the inoculation assays, either with or without betasatellite. The co-inoculated plants recorded slowest growing rates as compared to the inoculated plants with the virus alone, and also to the control plants. The control was inoculated plant with empty pCAMBIA1304 and is set to 1 for no virus accumulation. Error bars indicate the standard error for three biological replicates (n: 5 plants). P-values: ***<0.001, **0.001-0.01, *0.01-0.05, ns ≥0.05.

Table 4.3: Summary of the infectivity assays in tomato plants inoculated with and without betasatellite for three independent experiments.

Inoculum	Time point intervals	No. of plants infected / No. of plants inoculated			Infectivity (%)	Symptom description	[a] PCR-based detection of virus (No. of plants showed replication)	[b] PCR-based detection of betasatellite (No. of plants showed replication)
		Exp. 1	Exp. 2	Exp. 3				
pCAY-1.7mer	**7 dpi**	0/10	0/10	0/10	-	No symptom	No	No
	14 dpi	3/10	2/10	2/10	23.3	Mild upward leaf curl and mild leaf yellowing	Yes (7)	No
	21 dpi	6/10	5/10	6/10	56.7		Yes (17)	No
	28 dpi	10/10	10/10	10/10	100		Yes (30)	No
pCAY-1.7mer and pCMYβ-1.9mer	**7 dpi**	2/10	2/10	3/10	23.3	Severe upward leaf curl, severe leaf yellowing, stunting. Some plants showed mild downward leaf curling.	Yes (7)	Yes (7)
	14 dpi	5/10 (*2)	4/10 (*1)	5/10 (*2)	46.7		Yes (14)	Yes (14)
	21 dpi	10/10 (*3)	10/10 (*2)	10/10 (*2)	100		Yes (30)	Yes (30)
	28 dpi	10/10 (*4)	10/10 (*3)	10/10 (*3)	100		Yes (30)	Yes (30)
pCAMBIA1304 (Control)	**7, 14, 21, 28 dpi**	0/10	0/10	0/10	-	No symptom	No	No

[a,b] The presence of virus and betasatellite DNAs in the inoculated plants was confirmed by PCR amplification using the specific primer pairs.

(*) Number of co-inoculated plants with downward-curling leaves.

4.4 PCR Screening of Virus-infected Tomato Plants

The presence of the viral genomic DNAs in all the inoculated leaves was analyzed by PCR amplification with specific primer pairs of GapA-F/R and Gapβ-F/R for the virus and its betasatellite, respectively (Table 4.4). The amplification results showed the presence of both DNA molecules in the co-inoculated leaves (leaf 3 and 4) at 7 dpi and 14 dpi, that yielded the expected fragments of 516 bp (virus) and 502 bp (betasatellite) using these specific primer pairs (Figures 4.8A and 4.8B).

Subsequently, viral DNAs could also be detected in the non-inoculated leaves (leaf 5 and 6) of individual symptomatic plants subjected to the co-inoculation assay at 21 dpi and 28 dpi, thus indicating that replication and systemic movement of viral DNA had occurred with or without the betasatellite (Figures 4.8C and 4.8D). Betasatellite amplicon could neither be detected in inoculated nor non-inoculated leaves of single inoculation plants (Figure 4.8E and 4.8F). Plus, no viral DNAs from the healthy mock inoculated plants that were used as a negative control was detected with either primer sets. These results confirmed the presence of the T-DNA virus construct carrying the viral DNA in the inoculated plants. Sequencing analyses on the DNA molecules, confirmed these as the fragments from the virus and the betasatellite in the infected plants.

Table 4.4: PCR results for the inoculated plants with or without betasatellite.

Plant No.	PCR Result (Target gene: virus)					Plant No.	PCR Result (Target gene: betasatellite)				
	A[1]	A+β[2]		A[1]	A+β[2]		A[1]	A+β[2]		A[1]	A+β[2]
7 dpi			**21 dpi**			**7 dpi**			**21 dpi**		
1	+	+	1	+	+	1	-	+	1	-	+
2	+	+	2	+	+	2	-	+	2	-	+
3	+	+	3	+	+	3	-	+	3	-	+
4	+	+	4	+	+	4	-	+	4	-	+
5	+	+	5	+	+	5	-	+	5	-	+
C	-	-	C	-	-	C	-	-	C	-	-
14 dpi			**28 dpi**			**14 dpi**			**28 dpi**		
1	+	+	1	+	+	1	-	+	1	-	+
2	+	+	2	+	+	2	-	+	2	-	+
3	+	+	3	+	+	3	-	+	3	-	+
4	+	+	4	+	+	4	-	+	4	-	+
5	+	+	5	+	+	5	-	+	5	-	+
C	-	-	C	-	-	C	-	-	C	-	-

[1]Inoculated plants subjected to single inoculation assay with virus only

[2]Co-inoculated plants subjected to co-inoculation assay with both virus and betasatellite

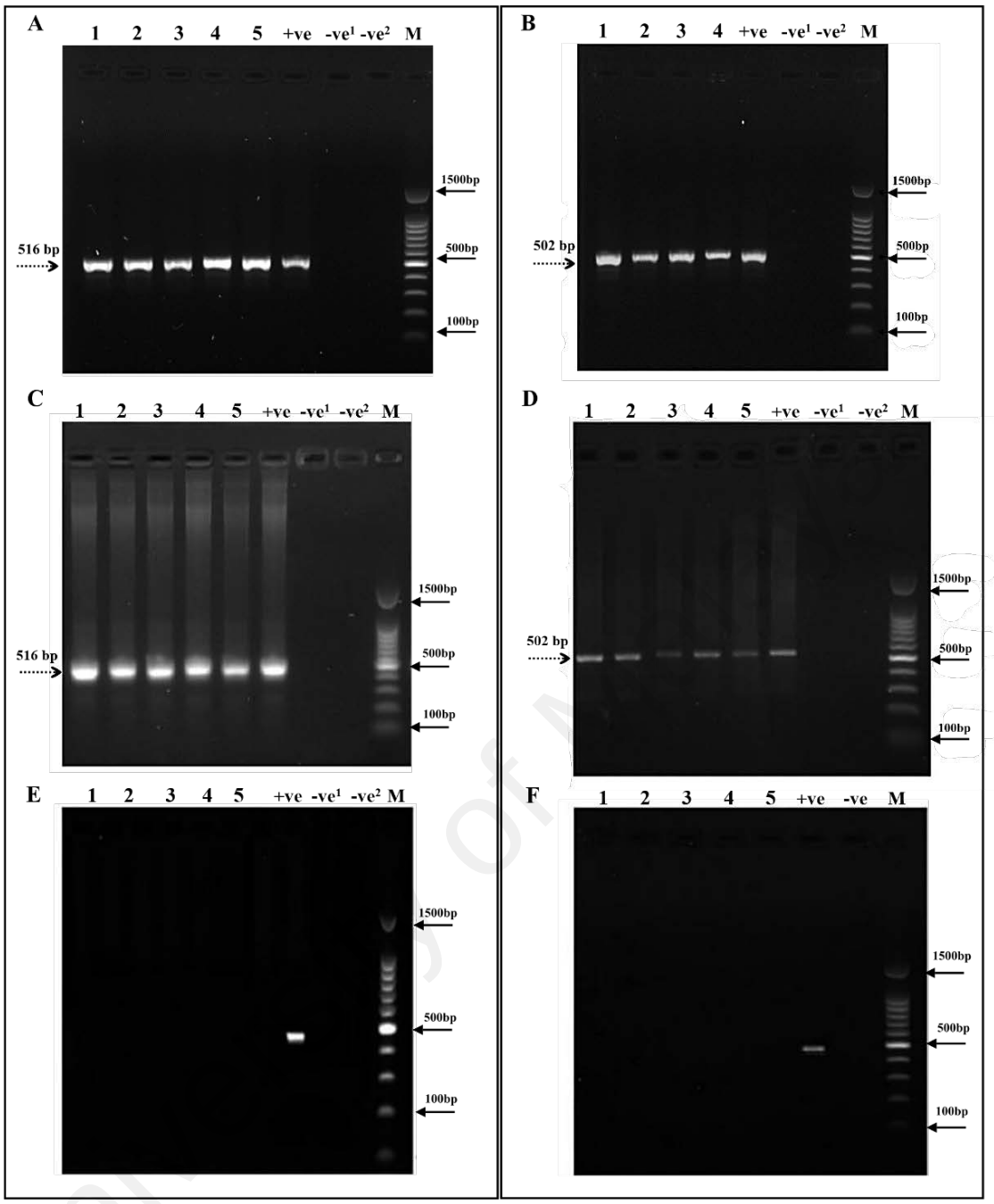

Figure 4.7: PCR analyses of inoculated tomato plants. The presence of virus (A) and betasatellite (B) molecules, extracted from co-inoculated leaves (Lanes 1A-5A; Lanes 1B-4B) and non-inoculated leaves (Lanes 1C-5C; Lanes 1D-5D) could be detected on the 1% agarose gel with the target sizes of 516 bp and 502 bp, respectively. No betasatellite amplicon can be detected on the agarose gel for the inoculated (Lanes 1E -5E) and non-inoculated leaves (Lanes 1F-5F) of the same virus-infected plants in the single inoculation assay. All the leaf samples used in this PCR assay were collected at 14 dpi. No band was detected for the PCR samples that serve as negative control (-ve). The positive controls (+ve) used in this PCR assay were plasmid DNAs pCAY-1.7mer for virus and pCMYβ-1.9mer for betasatellite. 100 bp ladder is a molecular marker (M).

4.5 Quantification of Viral DNA Levels In the Virus-infected Tomato Plants

The accumulation of the virus and betasatellite in the inoculated plants were quantified separately at several time points; 7, 14, 21 and 28 dpi using quantitative real-time PCR (qPCR) with the specific primer pairs, qpCA501/qpCA601 and qpCβ711/qpCβ811, respectively. Using the $2^{-\Delta\Delta CT}$ method, the data here is presented as the virus accumulation that was normalized to an endogenous control of Ubiquitin (UBI) gene and relative to the calibrator; extracted DNA from the leaves of control (mock) plant which was inoculated with empty vector pCAMBIA1304 under the same conditions as other inoculated plants with agroinfectious clones. The calibrator had viral DNA level equal to 1 for all treatments (either single inoculated with the virus only or co-inoculated with both virus and the betasatellite at equal inoculum ratio) at each dpi because ΔΔCT equals to zero, thus 2^0 equals to one (means no virus accumulation) (Livak & Schmittgen, 2001). For each treatment, five virus-infected plants from the single and co-inoculation assays were analyzed. The melting curves for the amplification of both target genes and UBI can be referred to Appendix D. All the data analysis of qPCR assays conducted in this study can be referred to Appendix E. These melting curves confirms the specificity of the product as only one amplification peak was monitored.

Our results showed that the co-inoculated leaf tissues had overall 1.9x higher virus accumulation rate as compared to the inoculated leaves (leaf no. 3 and 4) without the betasatellite at each dpi (Figure 4.9A). Similar increasing pattern of the virus levels were also observed in the non-inoculated upper leaf tissues (leaf no. 5 and 6) in the co-inoculation assay (Figure 4.9B). The highest accumulation rate was recorded at 28 dpi with the difference of 5.1x higher virus DNA level than the non-inoculated leaves subjected to single inoculation. This result suggests that the accumulation of virus was increased in response to the presence of its betasatellite. Moreover, it is important to note that the non-inoculated upper leaves in the co-inoculation treatment also had an overall

lower relative virus DNA than the inoculated leaves of the same infected individual plants. This result indicates that the attenuated symptoms observed on the non-inoculated upper leaves are likely associated with lower virus DNA level. Plus, it is noticeable that the virus titter in the co-inoculated leaves of virus-infected plants treated in both single and co-inoculation assays were gradually decreasing from 14 dpi to 28 dpi (Figure 4.9A). However, both co-inoculated and non-inoculated upper leaves of the virus-infected plants showed gradual decrease of betasatellite titter from 14 dpi to 28 dpi (Figure 4.9C). These results might be due to the potential occurrence of host recovery mechanisms against the virus-infection in the treated plants.

We also quantified the average level of betasatellite in the co-inoculated and non-inoculated leaf tissues of the symptomatic plants subjected to the co-inoculation assay with both virus and the betasatellite (Figure 4.9C). As expected, a high accumulation of betasatellite is observed in the co-inoculated plants with the presence of betasatellite. The co-inoculated leaves recorded an average of 2x higher accumulations of betasatellite when compared to the non-inoculated upper leaves of the same infected plants at each dpi.

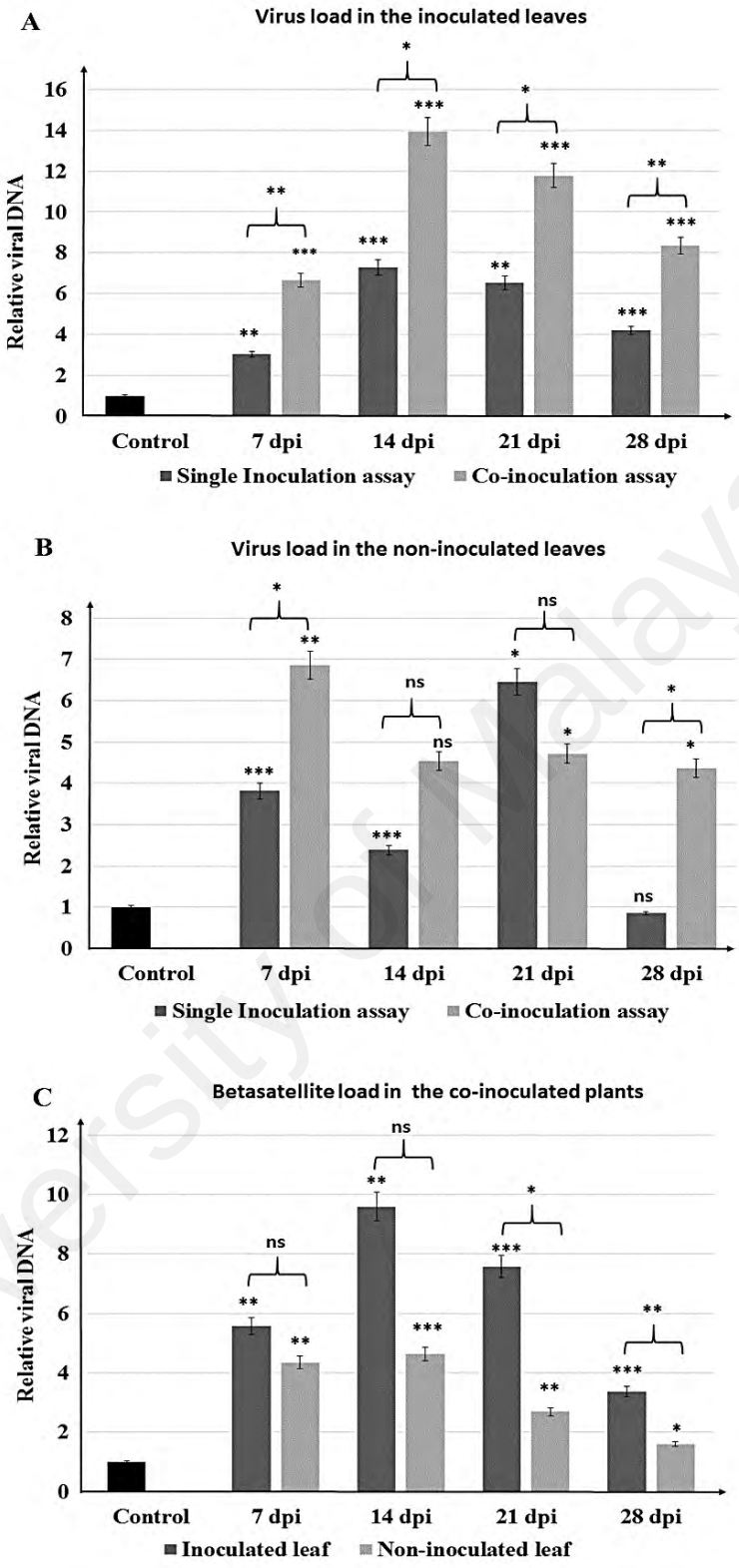

Figure 4.8: Accumulation of viral DNAs in the inoculated and non-inoculated tomato leaves. Virus DNA accumulation was measured quantitatively in the inoculated leaves (A) and non-inoculated upper leaves (B) of the same inoculated plants subjected to the single and co-inoculation assay. Betasatellite titers were also measured in the co-inoculated and non-inoculated upper leaves of individual plants subjected to the co-inoculation assay (C). The control was inoculated plant with empty pCAMBIA1304 and is set to 1 for no virus accumulation. Error bars indicate the standard error for three biological replicates (n: 5 plants). P-values: ***<0.001, **0.001-0.01, *0.01-0.05, ns ≥0.05

4.6 Betasatellite Could Possibly be a Factor for Variation in Symptom Expression.

Interestingly, few plants co-inoculated with both virus and betasatellite from each triplicate experiment showed downward leaf curl instead of typical upward leaf curl phenotypes that associated with AYVV and TYLCV-like diseases (Figure 4.5). No downward leaf curl was observed on the infected plants in the single infection without the betasatellite. The number of plants recorded for such symptom appearance of downward curling was presented in Table 4.3. This suggests that the phenotype change could be due to the presence of betasatellite in the infected plants.

Therefore, we conducted an additional qPCR experiment using specific primer pair of qpCβ711 and qpCβ811 for five leaf samples from co-infected plants that showed the downward-curling leaf at 14 dpi to quantify the relative amounts of betasatellite in comparison to the leaf samples showing upward-curling leaf. The co-infected plants that displayed downward-curling leaf indeed have high levels of betasatellite with 2.7x higher than the co-infected plants that displayed the upward-curling leaf (Figure 4.9). These results indicate that the level of betasatellite in the co-inoculated leaves could be the factor of a symptom change. To test this hypothesis, other batches of tomato plants were co-inoculated with two different inoculum mixtures consisted of either 1:1 or 1:2 ratio of *A. tumefaciens* cultures harboring 1.7-mer viral DNA and 1.9-mer betasatellite.

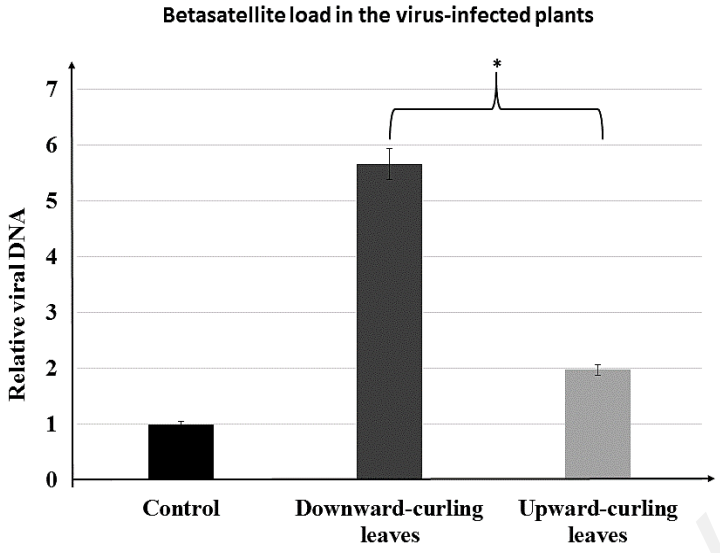

Figure 4.9: The level of betasatellite in the downward and upward curling leaves. An extra qPCR assay was conducted for the co-inoculated plants with upward and downward-curling leaves at 14 dpi. The control was inoculated plant with empty pCAMBIA1304 and is set to 1 for no virus accumulation. Error bars indicate the standard error for three biological replicates (n: 5 plants). P-values: ***<0.001, **0.001-0.01, *0.01-0.05, ns ≥0.05.

4.6.1 Symptoms Evaluation on the Co-inoculated Plants at Different Inoculums Ratios

All co-inoculated plants (regardless the inoculum ratios of virus to betasatellite) showed typical leaf curl disease symptoms including chlorotic margins on the leaflets and leaf curling and yellowing at 21 dpi similar to symptoms showed in Figure 4.10. Out of 10 virus-infected plants co-inoculated with a 1:1 inoculum ratio, only one to two co-infected plants exhibited downward-curling leaves and the rest showed the typical upward-curling leaves (Table 4.5). However, virus-infected plants co-inoculated at 1:2 inoculum ratio developed more leaves with downward curling symptom (7 to 8 virus-infected plants).

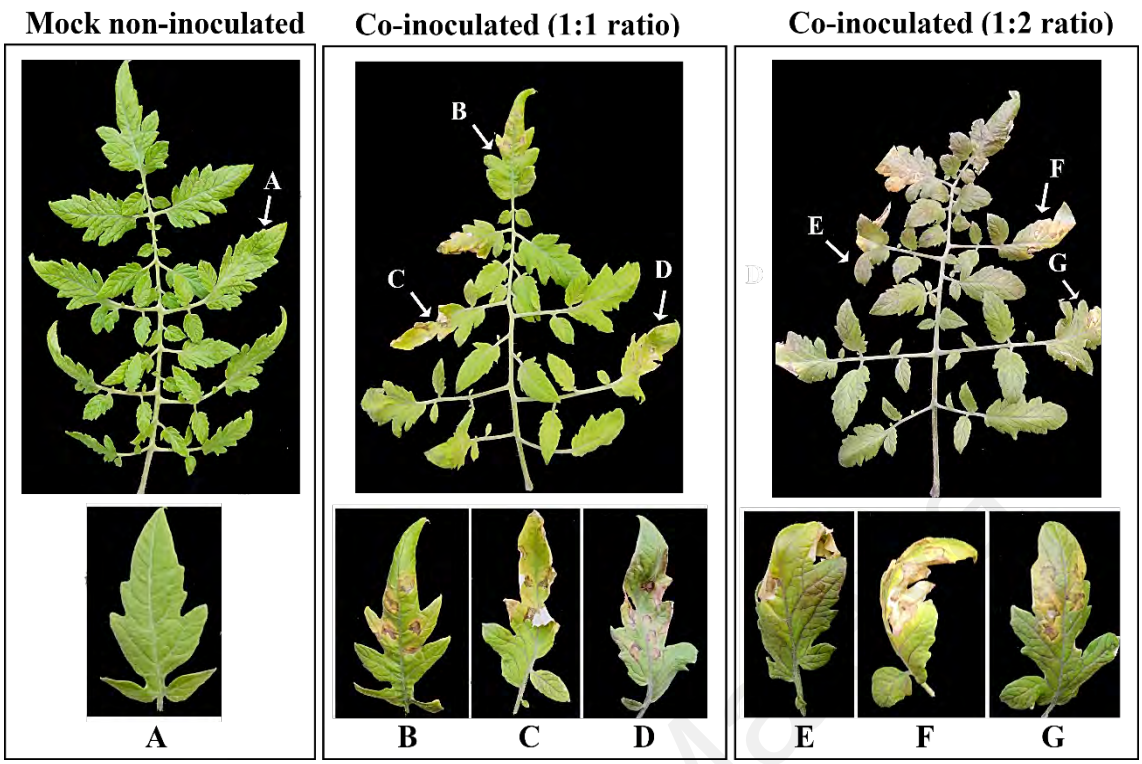

Figure 4.10: Virus-infected leaves showing disease symptoms of upward and downward leaf curl. The tomato plants were subjected to two different treatments; either co-inoculated with an equal ratio of virus to betasatellite (1:1 ratio) or with the double betasatellite inoculum (1:2 ratio). At 21 dpi, all the co-inoculated plants in both treatments displayed disease symptoms of leaf yellowing and curling but it was noticeable that the co-inoculated plants at 1:2 ratio had downward leaf curl (E, F, G) instead of typical upward leaf curl as those infected leaves in the 1:1 ratio (B, C, D). The mock non-inoculated plants remained symptomless (A).

4.6.2 Analysis of the Viral DNA Loads in the Co-inoculated Plants with Downward Leaf Curl Symptoms

The qPCR analysis confirmed a relatively high accumulation of both viral DNAs in the co-inoculated leaves at 1:2 inoculum ratio (virus: betasatellite) as compared to the co-inoculated leaves with 1:1 inoculum ratio at 21 dpi. The virus-infected plants subjected to co-inoculation assay with 1:2 inoculum ratio recorded 4.6x and 2.6x higher virus DNA levels in the co-inoculated and non-inoculated leaves, respectively, as compared to those plants co-inoculated at 1:1 inoculum ratio (Figure 4.11A). The same high viral load pattern was also observed for the betasatellite level in the co-inoculated plants. The betasatellite titters in the co-inoculated and non-inoculated leaves of the individual co-

inoculated plants at 1:2 inoculum ratio were recorded at 3.4x and 3x higher, respectively than those plants subjected to 1:1 inoculum ratio (Figure 4.11B). These results suggest there is a specific feature of betasatellite that responsible for the induction of the downward-curling leaf in the virus-infected plants.

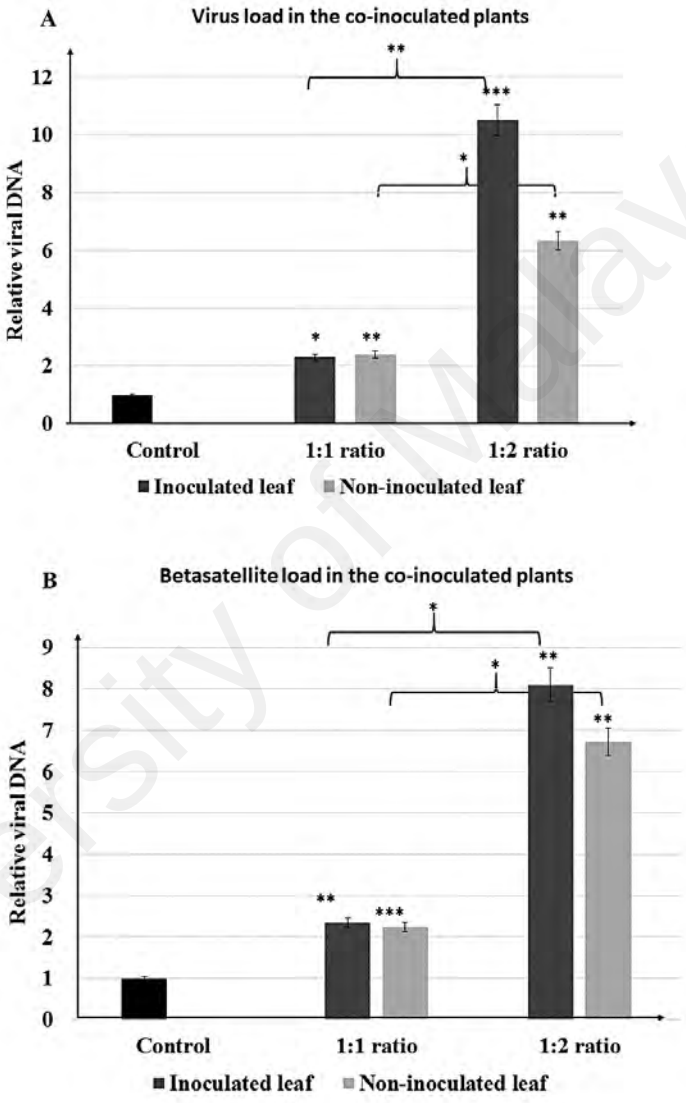

Figure 4.11: Viral DNAs accumulation in the inoculated plants subjected to two different inoculum ratios. The graphs showing the virus (A) and betasatellite (B) DNA loads between the inoculated and non-inoculated leaves of virus-infected plants. The control was inoculated plant with empty pCAMBIA1304 and is set to 1 for no virus accumulation. Error bars indicate the standard error for three biological replicates (n: 5 plants). P-values: ***<0.001, **0.001-0.01, *0.01-0.05, ns ≥0.05.

Table 4.5: Summary of the infectivity assay in the tomato plants co-inoculated with viral DNA and the betasatellite at two different inoculum ratios

Inoculum / Ratio	Experiment	No. of infected plants/ no. of inoculated plants	No of co-inoculated plants with upward or downward curling leaf		Infectivity	Symptoms
			Upward	Downward		
pCAY-1.7mer and pCMY β-1.9mer/ 1:1 ratio	1	10/ 10	8	2	100%	Severe upward leaf curl, severe leaf yellowing, stunting.
	2	10/10	8	2	100%	
	3	10/10	9	1	100%	
pCAY-1.7mer and pCMY β-1.9mer/ 1:2 ratio	1	10/10	2	8	100%	Severe downward leaf curl, severe leaf yellowing, stunting.
	2	10/10	3	7	100%	
	3	10/10	2	8	100%	
pCAMBIA1304 (Control)	1, 2, 3	0 / 30	-	-	0%	No symptoms

CHAPTER 5: DISCUSSION

In Malaysia, leaf curl disease caused by monopartite *begomoviruses* has not been intensively studied, particularly in tomato plants. The first etiology study reported that the transmission of *Leaf curl virus* (LCV) by the whitefly in the tomato plants exhibited leaf curl phenotypes together with yellowing and mosaic pattern (Shih et al., 1998). Then, another study demonstrated that LCV transmitted by whitefly epidemic was polycyclic by analyzing the temporal and spatial patterns of the disease in the tomato plants (Roff et al., 2005). Nevertheless, more intensive researches would be needed to investigate this plant disease in tomatoes for developing an efficient control management system in the future.

5.1 New Names for the Virus and Betasatellite Under Study Based on Current Taxonomy

In this study, we had described the cloning, sequencing and infectivity of a new strain of a monopartite *begomovirus* isolate designated as *Ageratum yellow vein virus*-Indonesia 2 [Malaysia:MY11A:tomato:2011] (AYVV-ID2 (MY:MY11A:tom:11)) (previously known as AYVV-MY [MY-Leaf Curl-11]; KM051527) and its associated betasatellite from infected tomato showing leaf curl disease in Peninsular Malaysia. The betasatellite is an isolate of *Pepper yellow leaf curl betasatellite* (PYLCB) with a proposed descriptor of [Malaysia:tomato:2011] (PYLCB-[MY:tom:11]). The naming of these two viral DNAs was proposed in accordance to the current taxonomic criteria for begomovirus species demarcation as explained in Section 4.1.1 and Section 4.1.2. This is the first report for a monopartite begomovirus-betasatellite complex from Malaysia. Koch's postulates were satisfied for AYVV-ID2 (the virus) and its associated PYLCB (the betasatellite) as the causal agents of the disease observed in tomato plants (*Solanum lycopersicum*).

The isolation of AYVV-ID2 could suggest few lines of evidences that can explain the nature of local spread of AYVV in Malaysia. Firstly, the increased of planted area in different regions of the country for the cultivation of tomato provided a highly susceptible host for the virus. It was reported that the cultivation area is expanding every year since 2008 in which the tomato production are mostly concentrated in Peninsular Malaysia such as Cameron Highlands (627 ha) and Kelantan Lodging (368 ha) (Rahim et al., 2017). Secondly, the spread observed on the tomato field could be due to the presence of local progenitor of other monopartite *begomovirus* existed in native or cultivated plants. Lastly, the abundance of polyphagous insect vector, whitefly (*B. tabaci*) could facilitated the introduction of this local progenitor *begomovirus* into tomato crop plants (Seal et al., 2006). The incidence of whitefly was first recorded in Malaysia in 1935, observed on chilli (*C. annuum*), soybean (*Glycine max*) and okra (*A. esculentus*) (Corbett, 1935). Over the years, the whitefly infestation is widespread to other crops such as angled loofah (*Luffa acutangula*), brinjal (*S. melongena*), cucumber (*Cucumis sativus*) and tomato (Syed et al., 2000). The distribution of this whitefly population on many different crops in Malaysia, showed the unique behavioral response of adult whiteflies upon the preferred crops, in which they reduce the crop yields by direct feeding and transmitting the geminiviruses from one crop to another. Nevertheless, future studies on the geography and distribution of AYVV-ID2 in Malaysia will increase the understanding on the *begomovirus* disease. Plus, it is useful to direct the further studies on estimating the relative contributions of recombination or mutation to the diversification and evolution of AYVV-ID2 and its betasatellite.

5.2 The Developed Agroinfectious Clones are Stable and Functional in the Inoculated Plants

Using the constructed agroinfectious clones containing the individual full-length DNA of AYVV-ID2 and PYLCB (pCAY-1.7mer and pCMYβ-1.9mer, respectively), we successfully demonstrated transfections of these dimeric constructs into tomato leaf tissues resulting in the initiation of typical leaf curl symptoms including leaf curling and yellowing appearances (Figure 4.5). Further detection of the amplified DNA forms through PCR analyses indicate that the full biological cycle can be reproduced experimentally in the inoculated plants using these developed agroinfectious clones (Figure 4.7: Table 4.4). We thus show that our constructed clones are stable and infectious to the tomato host plants. Agroinfectious clones of viral DNAs have been reported for other begomoviruses such as for AYVV and TYLCV (Saunders & Stanley 1999; Navot et al., 1991). The use of infectious clones as a reverse genetic tool can overcome several limitations that cannot be experimentally regulated in vivo, thus providing greater insights into the begomovirus-satellite complex. Based on our experience, the developed agroinfectious clones have redeeming features as an alternative for bioassays requiring virus transmission by insect vector (the whitefly) to the target host plants which is difficult and inconvenient to replicate requiring expertise and special equipment such as polypropylene cages with ventilation and aspiration devices.

5.3 The Effects of PYLCB on the AYVV-ID2 in the Virus-infected Plants

5.3.1 Symptom Severity in the Infected Plants Could be Correlated with PYLCB

Variation in the severity of disease symptoms found in inoculated plants with and without PYLCB indicates that it is not necessary for infection but intensify the disease symptoms caused by AYVV-ID2 (Table 4.3). The results presented here are consistent with findings by previous studies that showed accompanying betasatellites are involved in modulating the host defenses and, therefore, enhances helper begomoviral

pathogenicity resulting in an increased intensity of the typical symptoms in the hosts (Briddon et al., 2003; Zhou et al., 2003; Cui et al., 2004). It is well-established that βC1 encoded by betasatellite could act as a suppressor of post-transcriptional gene silencing (PTGS). Plant viruses encode silencing suppressors (VSRs) to counteract host defense of PTGS that provides an adaptive immune system for recognizing and inactivating the viruses (Rodríguez et al., 2009; Burgyan & Havelda, 2011). Besides, βC1 protein is a dominant symptom determinant. We speculate that βC1 of PYLCB could be a reason that such variations in phenotypic expression were observed in the co-inoculated tomato plants. For instance, studies showed that the expression of βC1 encoded by *Chili leaf curl betasatellite* (ChLCB) in the agroinoculated *Nicotiana benthamania* plants had developed severe leaf curl symptoms in which also resembles phenotypes induced by βC1 of *Cotton leaf curl Multan betasatellite* (CLCuMB) (Qazi et al., 2007; Tahir & Mansoor, 2011).

5.3.2 PYLCB Increased the Accumulation of Viral DNAs

Our results also demonstrated the increased of viral DNA levels with the presence of PYLCB in both co-inoculated leaves and non-inoculated upper leaves (Figure 4.8). One hypothesis is that the viral DNA of AYVV-ID2 cooperatively interacts with PYLCB, thus contributing to the increased in the virus DNA in the co-inoculated leaves. According To Saeed et al. (2007) such interactions possibly due to several factors including rapid cell division, enhanced movement or suppression of RNA silencing (host defense mechanism). These possibilities were further supported by earlier findings on virus helper-encoded replication associated protein that is essential for many roles including the initiation of rolling-circle DNA replication (Blawid et al., 2008; Zhang et al., 2015). Previous studies also showed that V2 and C4 encoded proteins by *Cotton leaf curl Multan virus* (CLCuMV) and *Bhendi yellow vein mosaic virus* (BYVMV), respectively resulting in strong suppression activities against gene silencing with the presence of its associated βC1 (Gopal et al., 2007: Amin et al., 2011). It is possible in this study, βC1 of PYLCB

could have VSR properties and interacting with the virus to target different effector molecules in the RNA silencing pathway and disable the host-defenses, thus contributes to severe disease symptoms and increased in the viral DNA loads in the co-inoculated plants. The precise mechanism of action of the viral proteins encoded by AYVV-ID2 and PYLCB will be the focus of our future research.

5.3.3 Co-inoculation with PYLCB Induced Systemic Virus Spread

The phenotypic appearances and detections of viral DNAs in the non-inoculated upper leaves of the same plants inoculated with or without PYLCB as shown in our results, demonstrated the successful initiation of systemic infection. It is therefore likely that DNA-A like genome of AYVV-ID2 might have been delivered and replicated in the epidermal tissues of the inoculated leaves, spread to the neighboring cells (local spread) and move systemically throughout the plants. It is also worth noting that the virus transmission from the inoculated leaves to non-inoculated leaves of the same infected plants is at the highest rate in the presence of PYLCB (Table 4.3). Our theory is that the accumulation of PYLCB may be responsible for a cell to cell movement function that led to the dissemination of AYVV-ID2 throughout the plants. This was supported by our result of qPCR analysis, where noticeably higher levels of virus DNA found in the co-inoculated leaves with PYLCB (Figure 4.8). Our findings are in accordance with the previous studies conducted by Saeed et al. (2007) in which considered the high accumulation of virus DNA at the sites of inoculation as dependent on cell-to-cell movements. Besides, the betasatellite has been shown to play an important role in the replication and movement of the monopartite begomovirus in plants through the multifunctional pathogenicity of βC1 (Briddon et al., 2003). Saeed et al. (2007) demonstrated that βC1 can, at least in part, mimic DNA-B functions, particularly in virus movement in the co-inoculated plants with *Cotton leaf curl Multan betasatellite* (CLCuMB) and *Tomato leaf curl virus* (ToLCV) DNA-A. A better understanding of the

viral movement is achievable if future studies concentrate on the association of βC1 and virus DNA. To achieve this, a series of recombinant plasmids for fluorescently tagged viral movement genes can be created either by replacing the GFP/GUS reporter genes with the viral coat protein (CP) sequences or by fusions to movement protein (MP) sequences under promoter element of intergenic region (IR). Such developed construct can then be used to analyze RNAi-mediated host–virus-interactions in the transgenic plants (perhaps in T3 lines) expressing siRNA mediated resistance mechanism to the virus infection.

5.3.4 PYLCB Caused Phenotypic Changes of Leaf Curling

Some of the virus-inoculated plants showed the downward-curling leaves instead of the typical upward-curling leaves. Since the level of PYLCB was higher for those symptomatic downward-curling leaves compared to the symptomatic upward-curling leaves at 14 dpi (Figure 4.9), this result suggest that the level of betasatellite may have contributed to this phenotype change. Therefore, an additional infectivity study was conducted on the co-inoculated plants with both viral DNAs in two different *A. tumefaciens* inoculum ratios. The results showed that the plants co-inoculated with higher inoculum ratio of virus to PYLCB (1:2 inoculum ratio) resulted in a greater number of leaves with downward leaf curling, rather than the typical upward-curling leaf (Table 4.5). These results suggested that the level of PYLCB may contribute to symptoms by changing the spread of infection in the plant's vascular system, thus leading to downward-curling leaf. A possible explanation to this observation is the high accumulation of betasatellite in the co-inoculated plants (1:2 inoculum ratio) directly increased the replication of betasatellite by Rep protein encoded by viral DNA. We also thought that the high accumulation of PYLCB, specifically at the early infection yields to high expression of its βC1, therefore more potential interactions occur between βC1 and host proteins to contribute to the phenotype change. Earlier studies showed that high levels of

betasatellite associated with cotton leaf curl disease in infected plants displayed severe vein yellowing and downward leaf curling (Briddon et al., 2003; Mansoor et al., 2003; Qazi et al., 2007). They suggested that the formation of secondary vascular elements caused by abnormal cambium activity in phloem parenchyma could be responsible for this change to downward leaf curling. Nevertheless, future study will be conducted to develop an understanding of which DNA components of AYVV-ID2 that interact specifically with βC1 of PYLCB, that contribute to the distinctive phenotype of leaf curl disease.

5.4 Possible Occurrence of Host Recovery in the Virus-infected Plants

Additionally, the gradual decreased of AYVV-ID2 and PYLCB levels recorded for the virus-infected plants in single and co-inoculation assays are noticeable (Figures 4.9A and 4.9C). This could suggest that the high expression of both viral DNAs in the infected plants results in the stress response and the plants respond to these stresses by increasing their host proteins, most likely their evolutionarily conserved WRKY transcription factors to downregulate these viral DNAs as demonstrated by previous researchers in their recent studies (Huang et al., 2016; Liu et al., 2014). We further speculate that there is a potential occurrence of host recovery in the tomato plants which restricts virus replication and movement therefore prevents disease onset. Several lines of evidence support the claim of such host recovery occurrence in the begomoviruses-infected plants particularly in the studies focusing on RNA silencing as a natural antiviral host defense (Carrillo-Tripp et al., 2007; Melgarejo et al., 2013). Future work will be directed toward exploring this possibility.

CHAPTER 6: CONCLUSION

In summary, our findings provide few substantial pieces of evidence that firstly, the tentatively new isolate monopartite *begomovirus* in Malaysia which is AYVV-ID2 and its associated betasatellite, PYLCB can be amplified and developed into stable and functional agroinfectious clones for the inoculation assay to susceptible WT-tomato plants (*S. lycopersicum*) in the control environment. Secondly, using these developed agroinfectious clones, we had demonstrated an experimental infectivity analysis of AYVV-ID2 as expressed by; (i) Severity of the disease symptoms, (ii) Percentage of the infected plants, (iii) Detection of viral DNAs on the agarose gel and (iv) The accumulation of viral DNAs in the virus-infected plants, inoculated with or without PYLCB. Our results showed that the mentioned parameters became more severe and increased significantly with the presence of PYLCB in the co-inoculated plants as compared to the single inoculated plants with only AYVV-ID2. It is safe to conclude that PYLCB is not necessary for the infection but it is essential to intensify the leaf curl symptoms as demonstrated in our findings. Therefore, we suggest that the variations in the infectivity demonstrated here could be due to the dynamic yet specific interactions between AYVV-ID2 and PYLCB, as well as between the combination of both viral DNAs and the host factors. This might be due to the faster and better synthesized of mRNA and protein in the inoculated plants with higher viral DNAs accumulation, therefore efficiently establishing systemic infection possibly by evading the plant host defense system. We further demonstrated the importance of PYLCB level in eliciting the virus infectivity in the inoculated plants, therefore the identification of its host-interacting proteins should become our focus in future studies. For now, we can only speculate that βC1 protein of PYLCB perhaps is the best candidate for pathogenicity factor that specifically interacts with AYVV-ID2 in the infected plants. Future study is directed towards this hypothesis. The results described here may be particularly useful for us to devise efficient novel

strategies in controlling the virus spread in Malaysia, particularly in developing the resistant transgenic tomato lines by expression of hairpin RNAi (hp-RNAi) based on post-transcriptional gene silencing (PTGS) system.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

1. Omar, M. & Othman, R. Y. (2017). *A betasatellite DNA component of tomato Ageratum yellow vein-virus (Malaysia) is an important pathogenic determinant of the virus in tomato plants.* Paper presented at the International Union of Microbiological Societies Congresses (IUMS), 7th-21st July 2017, Sands Expo and Convention Center, Singapore.

2. Omar, M. & Othman, R. Y. (2016). *Betasatellite DNA component of Ageratum yellow vein-virus (Malaysia) increases the infectivity of the virus in tomato plants.* Paper presented at the 21st Biological Science Graduate Congress (BSGC), 15-17th December 2016, University of Malaya, Kuala Lumpur, Malaysia.

3. Omar, M. & Othman, R. Y. (2015). *A simplified method for amplification of Ageratum yellow vein virus-Malaysia isolates using bacteriophage phi-29 DNA polymerase*. Paper presented at the 20th Biological Sciences Graduated Congress (BSGC), 9-11th December, Chulalongkorn University 254 Pathumwan, Bangkok, Thailand.