A study of C2 and V2 pathogenicity genes of Tomato curly stunt virus variants in tomato and *Nicotiana benthamiana*

by

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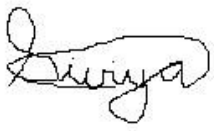
Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



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Abstract

Tomatoes are the second most important agricultural crop in the world. Due to selection and inbreeding, genetic variation in tomato is restricted, rendering the crop susceptible to disease and pest outbreaks. Tomatoes have been shown to be infected by a number of viruses. Tomato production is largely affected by begomovirus infections, with tomato yellow leaf curl virus (TYLCV) being the most destructive virus. Tomato yellow leaf curl disease (TYLCD) can have a massive impact on fruit yield, reducing the value of commercial tomato products. In 1997 a disease emerged in tomatoes that were planted in the Onderberg region of South Africa. Plants showed symptoms that were similar to those caused by tomato yellow leaf curl virus (TYLCV), namely leaf curling, yellowing and plant stunting. A new begomovirus species was found to cause these symptoms and the virus was *named* tomato curly stunt virus (ToCSV) Infection of tomato plants with ToCSV can lead to yield loses of up to 100% thus making ToCSV a major concern to the South African economy. A later study described two variants of ToCSV in South Africa, ToCSV_V30 (severe) and ToCSV_V22 (mild). Both variants have a genomic sequence identical to the Old World monopartite begomovirus with six Open Reading Frames(ORFs): V1 (CP), V2 (pre-coat protein), C1 (Rep), C2 (TrAP), C3 (REn), and C4 (REn). The aim of this study was to determine the importance of pathogenicity determinates C2 and V2 encoded by either ToCSV_V30 or ToCSV_V22 variants and their contribution to symptom phenotype or severity in tomato and *Nicotiana benthamiana*.

To investigate if C2 and/or V2 are crucial for viral replication and viral DNA accumulation in tomato cv. Rooikhaki, mutational studies were carried out to determine the impact of mutations in the C2 and V2 ORFs of ToCSV variants. A chimeric infectious clone was produced by substituting the entire V2 region of ToCSV_V30 with that of the ToSCV_V22. Tomato cv. Rooikhaki was subsequently agroinfiltrated with the infectious clone, symptom scores (according to the disease severity index (DSI)) and viral load (using qPCR) were assessed. Plants inoculated with the chimeric infectious clone containing the V2 swap showed no significant differences in symptom severity when compared with symptoms induced by ToCSV_V30. However, when compared to wild-type ToCSV V30 and ToCSV V22, plants infected with the chimeric infectious clone, ToCSV_V30(V22V2$_{aa1-116}$) (V2 swap), had higher levels of viral DNA. Relative viral load analyses indicated that the viral load of the swap was 5-fold and 3-fold higher at 21 dpi when compared to ToCSV_V30 and ToCSV_V22, respectively. These results suggest that disease severity is coupled to viral DNA levels in tomato plants infected with the V2 swap. The results indicated that the V2 swap did affect the levels of DNA replicated when compared to ToCSV_V22 and ToCSV_V30. In order to determine the function of C2 in pathogenicity, three C2 mutants were produced by substituting the amino acids (aa) in the severe ToCSV_V30 with the corresponding ones from the mild V22 variant; Mut 10 (ToCSV_V30(V22C2$_{aa46}$)), Mut 9 (ToCSV_V30(V22C2$_{aa48}$)), and Mut 7 (ToCSV_V30(V22C2$_{aa109}$)). Plants infected with the C2 mutants developed symptoms that were similar to those produced by ToCSV_V30. Relative viral load analyses showed that the C2 mutants accumulated higher levels of DNA at 21 dpi when compared to the wild-type virus variants. Relative viral load of mut 7, mut 9 and mut 10 was 2.3-, 2.7-, and 2-fold, respectively, higher than ToCSV_V22 at 21 dpi, whereas the relative viral load change for the

mutants were 4.38, 4,4 and 3.7-fold higher than ToCSV_V30. This suggests that the specific amino acids in the C2 region studied did affect viral DNA replication.

By overexpressing C2 using a PVX expression vector, pGR107, the putative function of C2-encoded TrAP in pathogenicity by both mild and severe variants was studied in *N. benthamiana*. Plants inoculated with PXV expressing C2_V22 enhanced viral symptoms in inoculated leaves at 7dpi, however, plants inoculated with PVX expressing C2_V30 did not alter the symptoms induced by PVX. These results indicated that C2_V22 is a pathogenicity determinant in *N. benthamiana*. Intriguingly, plants inoculated with C2 expressed in PVX from both V30 and V22 showed symptom recovery in systemic leaves at 14 dpi and 28 dpi. RNA silencing has been found to be involved in the recovery of geminiviral symptoms. Therefore, to investigate if indeed ToCSV C2 was a suppressor of RNA silencing, the C2 of both severe and mild variants were cloned into pTRV VIGS vector and co-inoculated with GFP into transgenic *N. benthamiana* expressing GFP. Results indicated that ToCSV C2 weakly suppresses local RNA silencing in inoculated leaves at 4 dpi, however, C2 was unable to suppress systemic silencing in leaves at 14 and 28 dpi. These results have given an insight into the putative roles of C2 and V2 genes encoded by ToCSV variants showing different symptom severity and phenotype in *N. benthamiana* and tomato plants.

Dedication

Dedicated to my beautiful daughter in heaven,
Sijabuliseni Tshegofatso Siviya

-My guiding star, my shining light, my perfect Angel is who you are.

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I would like to acknowledge and express my deepest appreciation to the following individuals:

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Abbreviations

µl	microlitre
ACMV	African cassava mosaic virus
AGO	Argonaute
AGV	Apple geminivirus
ANOVA	Analysis of variance
BCTV	Beet curly top virus
CLCuMuV	Cotton Leaf Curl Multan virus
CP	Coat protein
cv	cultivar
DCL	Dicer-like
DNA β	Betasatellite
dpi	Days post inoculation
DSI	Disease severity index
g	gram
IPM	Integrated pest managements
L	Litre
MCS	Multiple clonig site
ml	millilitre
MMDaV	Mulberry mosaic dwarf-associated virus
MP	Movement protein
nt	Nucleotide
ORFs	Open reading frames
PTGS	Posttranscriptional gene silencing
PVX	Potato virus X
qPCR	Quantitative polymerase chain reaction
RCR	Rolling circle replication
REn	Replication enhancer protein
Rep	Replication-associated protein
SAMS	S-adenosyl methionine synthetase
siRNA	small interfering RNA
SLCMV-LK	Sri Lankan cassava mosaic virus
ss	single stranded
TGMV	Tomato golden mosaic virus
TGS	Transcriptional gene silencing
TMV	Tobacco mosaic virus
ToBYMV	Tomato bright yellow mosaic virus
ToCMoV	Tomato chlorotic mottle virus
ToCSD	Tomato curly stunt disease
ToCSV	Tomato curly stunt virus
ToLCKuV	Tomato leaf curl Kumasi virus
ToLCTGB	Tomato leaf curl Togo betasatellite
ToLDV	Tomato leaf distortion virus
ToSRV	Tomato severe rugose virus

ToYVSV	Tomato yellow vein streak virus
TrAP	Transcription activation protein
TRV	Tobacco rattle virus
TYLCCNV	Tomato yellow leaf curl China virus
TYLCD	Tomato yellow leaf curl disease
TYLCSV	Tomato yellow leaf curl Sardinia virus
TYLCV	tomato yellow leaf curl virus
VIGS	Virus-induced gene silencing
VSR	Viral suppressors

Chapter 1

Literature Review

1.1. Introduction

Tomatoes (*Solanum lycopersicum* L.) are economically important and are grown worldwide (Gerszberg et al., 2015). They belong to the family *Solanaceae*. Tomatoes originate from the Andean Mountains and were later exported to Europe in the 16th century (Gerszberg et al., 2015). Genetic analyses have shown that tomato domestication may have occurred in Central America (Kimura & Sinha, 2008). Tomatoes have high nutritive value and are abundant in Vitamin A, C, lycopene and beta-carotene (Spaldon & Kumar, 2017) . Despite being classified as vegetables due to nutritional content, tomatoes are botanically classified as fruits (Malherbe & Marais, 2015)

The annual global production of tomatoes amounted to 376 004 hectogram per hectare (hg/ha) (or 82 metric tons/ ha) (Figure 1.1) thus making them one of the most vital crops worldwide (FAO, 2018). Tomatoes are cultivated on every continent except Antarctica. The worldwide ranking of the top three countries for 2015 to 2017 in terms of tomato productivity include United States of America (107 metric tons/ha, or 47.7 short tons per acre), Australia (99 metric tons/ha) and Canada (more than 93 metric tons/ha) (WPTC, 2018). Production of tomatoes in the Southern African Development Corporation (SADC) region has also shown the same growth between 2001 and 2011 period, with approximately 20% increase in production (Malherbe & Marais, 2015). South Africa is the leading producer in the SADC region, producing 54% of tomatoes (Malherbe & Marais, 2015). Tomato production in South Africa increased by 8.3%, from 563 188 tons in 2015/2016 (July to June) to 610 008 tons in 2016/2017(Department of Agriculture Forestry & Fisheries, 2019). The tomato sector is the second most essential vegetable division in the agricultural economy of South Africa. Tomato production occurs in all provinces of South Africa, with Limpopo producing the most at 3590 ha per year. The Onderberg area of Mpumalanga Province and Eastern Cape Province are the next major producing areas, with 770 ha and 450 ha, respectively. The utilization of tomatoes in South Africa is measured as 12 kg per annum, compared to 32 kg per annum in Europe. South African households consume 5 to 10 tomatoes per week on average (Department of Agriculture Forestry & Fisheries, 2019). Advantages of tomatoes are that they are short duration crops, can be grown in protected cultivation and they have a high economic value (van Dam et al., 2005) .

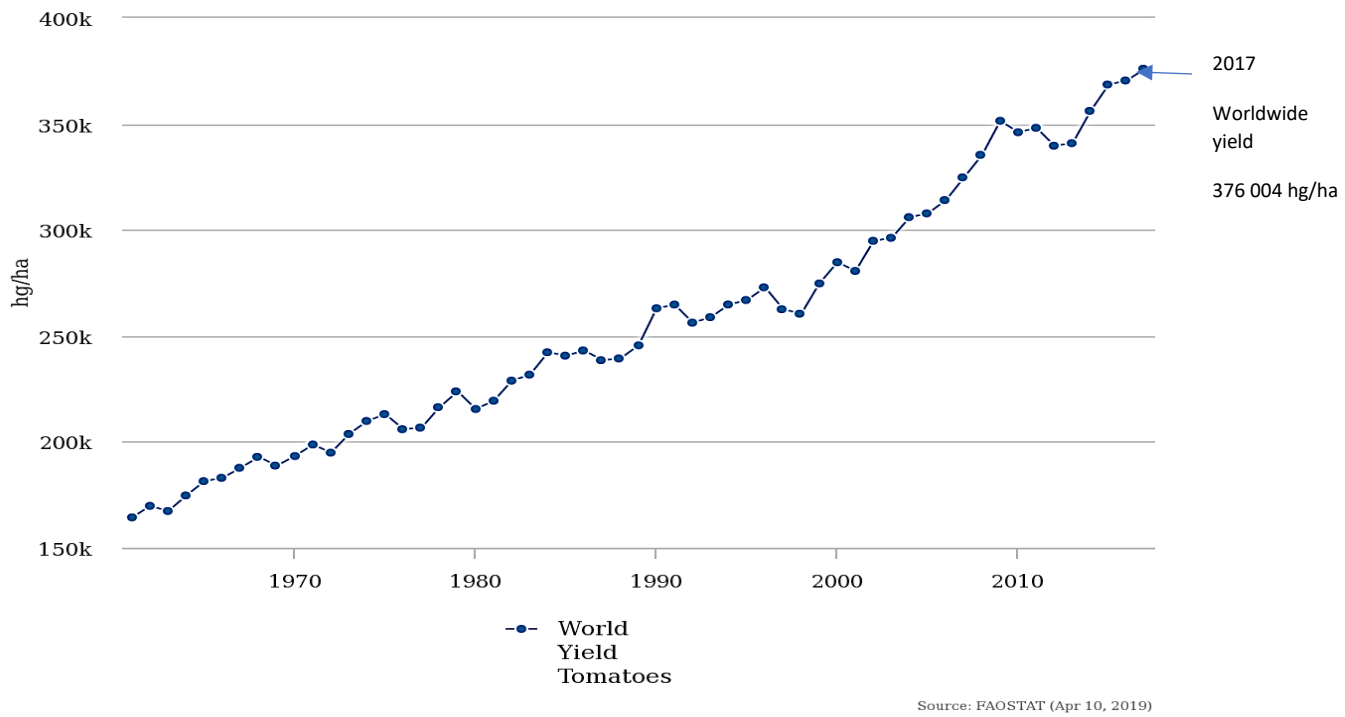

Figure 1.1: The worldwide production of tomatoes from 1961 to 2017 (FAO, 2018).

One of the most most limiting factors to tomato production is their infection by begomoviruses which cause diseases that impact agriculturally important crops (Prasanna & Rai, 2007). These viruses are transmitted by the whitefly *Bemisia tabaci* Genn. (Legg, 1996). A survey conducted between 2002 and 2009 revealed that *B. tabaci* whitefly haplotype diversity in eight provinces in SA on cassava and tomato hosts (Esterhuizen et al., 2013). Tomato yellow leaf curl virus (TYLCV) is the most widespread monopartite begomovirus (Tripathi et al., 2018). Tomato yellow leaf curl Sardinia virus (TYLCSV) is one of the agents responsible for tomato yellow leaf curl disease (TYLCD) and can cause yield losses of up to 100% in tomato fields (Moriones & Navas-Castillo, 2000). Symptoms include severe leaf yellowing, leaf apex cupping, and severe size reduction in normal plant height. These symptoms can have a major impact on the production of the fruits. Plants infected with the viruses can lead to the reduction of fruit size, that often do not meet the required commercial size, thus cause a decrease in quality of produce (Pico' et al., 1996). In South Africa, an undiscovered virus emerged over a decade ago that infected tomato plants. These tomato plants showed symptoms that were similar to those caused by tomato yellow leaf curl virus (TYLCV) (Pietersen et al., 2008). Disease spread was initially controlled using pesticides by integrated pest managements (IPM). This has however been unsuccessful due to difficulties in the vector such as development of resistance (Azizi et al., 2008; Lapidot et al., 1997). Breeding programs have been developed to screen for resistance genes from wild solanaceous plants. Tolerant plants that express resistance genes have shown a delay in symptom appearance and low viral accumulation (Lapidot et al., 1997). However, these bred

cultivars are not immune to the virus but to develop milder symptoms when infected by TYLCV (Legarrea et al., 2015).

1.2. *Geminiviridae*

Geminiviruses belong to the family *Geminiviridae*. The family is made of 9 genera, namely, *Becurtovirus, Begomovirus, Capulavirus, Curtovirus, Eragrovirus, Grablovirus, Mastrevirus, Topocuvirus and Turncurtovirus* (Zerbini *et al.,*2017). Classification of geminiviruses into genera is in accordance with the genome, host and insect vector (Fauquet & Stanley, 2003). These viruses have circular DNA that is single stranded (ss) and is encapsulated in twinned particles (Figure 1.2) Geminiviruses occur as monopartite (one ssDNA molecule) or bipartite (two ssDNA molecules). Viruses that belong to the genera *Becurtovirus, Capulavirus, Curtovirus, Eragrovirus, Grablovirus, Mastrevirus, Topocuvirus* and *Turncurtovirus* have monopartite genomes, whereas viruses that belong to the genera *Begomovirus* have monopartite and bipartite genomes (Zerbini et al., 2017).

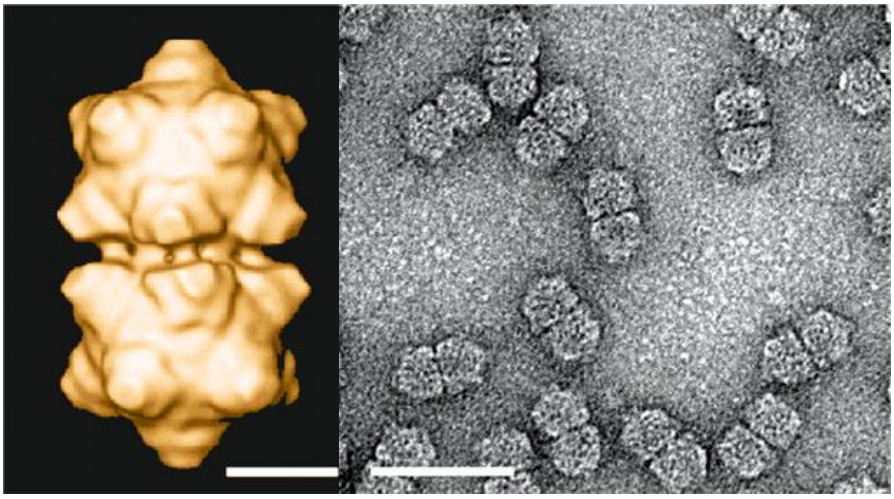

Figure 1.2: Cryo-electron microscopic reconstruction of geminivirus twinned capsid (F. Murilo Zerbini et al., 2017)

Based on the composition of the genome, variation of the genes and geographical allocation, these viruses have been separated into Old World (Europe, Africa, Asia, and Australia) and New World (America). All geminiviruses share a conserved nonanucleotide (TAATATT/AC), which is indicated as the origin of replication (Nawaz-ul-Rehman and Fauquet, 2009). The origin of geminiviruses is believed to be in South East Asia, and eight centers of origin have been described, (Figure 1.3 :Nawaz-ul-Rehman & Fauquet, 2009).

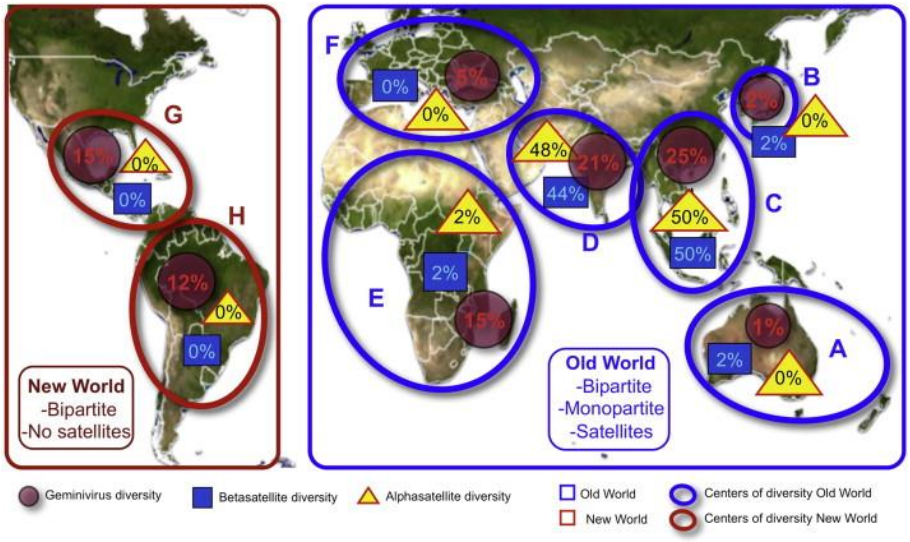

Figure 1.3: Ecological spreading of geminiviruses. There are eight distinct regions of variation that have been found (A–H) according to the percentage of species of each molecule. Percentages circled in red show the geminivirus species percentage, percentages circled in blue show the β-satellite percentage (see section 1.3. and 1.5), and percentages in yellow triangles show the alphasatellite species percentages. Regions are marked with alphabets: A – Australia, B – Japan, C – China, D – Indian subcontinent, E – Africa, F – Mediterranean-European region, G – Central America, H – South America. (Adapted from Nawaz-ul-Rehman & Fauquet, 2009).

Geminiviruses are one of the most economically important plant viruses. They are usually transmitted by phloem-feeding insects (whitefly vector), which belong to the genus *Besmia* (Harrison & Robinson, 2003; Moriones & Navas-Castillo, 2000). Once they are transmitted into the host, they rely on the host's DNA polymerase to enable their replication (Bass *et al.,*2000). Geminiviruses multiply in the nucleus by a rolling cycle mechanism (Stanley, 1993), and encode multifunctional proteins that are encoded by various open reading frames (ORFs) (Brown et al., 2015). Coded proteins include those involved in symptom development, cell-to-cell movement, transcriptional activation and host plant defense suppression (Brown et al., 2015). Depending on the functions, these proteins have been named as pre-coat protein (V2 or AV2), coat protein (CP, V1 or AV1), replication enhancer protein (REn, C3 or AC3), transcription activation protein (TrAP, C2 or AC2), replication-associated protein (Rep, C1/AC1) and C4 or AC4 protein (Stanley et al., 2005).

1.3. Begomovirus classification and structure

Begomovirus species belonging to the genus *Begomovirus* have become one of the most economically important plant virus genera, affecting fruit and vegetables, crops, cereal grains, spices, and legumes. (Rey et al., 2012), and are transmitted by the whitefly *Bemisia tabaci* Genn. (Legg, 1996). Begomoviruses can be monopartite or bipartite (DNA-A and DNA-B components) and the components for bipartite begomoviruses share a common region with an origin of replication (*ori*) (Brown et al., 2015). There have been more than 50 species of begomoviruses identified as natural pathogens infecting tomato. Tomato yellow leaf curl

virus (TYLCV) is the most widespread monopartite begomovirus, Figure 1.4 (Tripathi et al., 2018). Most of the Old World begomoviruses are monopartite with a few that are bipartite and New World begomoviruses most exclusively bipartite (Melgarejo et al., 2013).

Differentiation of species for the genus *Begomovirus* requires the genome sequence to be less than 91% in nucleotide sequence identity for DNA A component (Brown et al., 2015). A new species' name should contain the host it infects and the symptom it causes. The inclusion of names of countries, cities, towns, villages, or provinces in the naming of new viruses is discouraged. It has also been proposed that if a sequence share <94 % identity to all isolates described for that species, a strain name should be provided (Brown et al., 2015).

Bipartite begomoviruses genome components, DNA A and DNA B, are required for systematic infection and the induction of symptoms (John Stanley & Gay, 1983). Monopartite viruses consist of only one genome component and some monopartite begomoviruses like TYLCV and tomato leaf curl virus (TLCV)) can cause sufficient disease symptoms with a single DNA component (Dry et al., 1993; Navot et al., 1991; Tao & Zhou, 2008). However most monopartite begomoviruses, for example tomato yellow leaf curl China virus (TYLCCNV), require a novel DNA molecule known as DNA β (betasatellite) to induce symptoms (Cui et al., 2005). Phylogenetic analysis of DNA β has suggested that they have co-evolved with their helper viruses (Tao & Zhou, 2008). These betasatellites are important in pathogenicity of the virus and are approximately 1.4 kb in size (Ueda et al., 2012).

The bipartite begomoviruses DNA-A have six ORFs, two that are located on the virion sense strand (AV1 and AV2) and four in the complementary sense strand (AC1, AC2 and AC3 and AC4). The DNA-B component is composed of two ORFs, with the virion strand coding the BV1 ORF and the complementary strand coding the BC1 ORF. The AV2 ORF is found in Old World bipartite begomoviruses, but not in New World begomoviruses (Fondong, 2013). Monopartite begomovirus ORFs are made up of the virion-sense, V1 and V2 genes, as well as the complementary-sense, C1, C2, C3, and C4 genes (Fondong, 2013). The AC1 of bipartite or C1 of monopartite encodes the replication associated protein (Rep). Expression of the Rep gene is under the control of a bidirectional core promoter in the IR region (Hanley-Bowdoin *et al.*, 1999). Rep is important for rolling circle replication (RCR) and regulation of gene expression (Haley et al., 1992). AC2 or C2 encodes the transcriptional activator protein (TrAP). TrAP is involved in expression of viral genes, pathogenicity of the virus and suppression of gene silencing (Fondong, 2013; Sharma & Ikegami, 2010a). AC3 or C3 encodes the replication enhancer protein (REn). REn causes an upregulation of viral DNA in infected plants and development of symptom (Sunter et al., 1990). AC4 or C4 is the least conserved protein found in all geminiviruses, with varying functions in monopartite and bipartite geminiviruses. C4 is known to be a movement protein in monopartite geminiviruses and is important in symptom development (Fondong, 2013). AC4 of TGMV has been shown to be involved in the movement of the virus (Pooma & Petty, 1996), however, it is not particularly important in the infection by the virus (Elmer et al., 1988). AV1 or V1 ORFs encode the coat protein (CP) (Zhang et al., 2001). The CP is the only structural protein necessary for insect transmission, viral DNA movement into and out of the nucleus, cell-to-cell movement, and virus systemic spread (John

Stanley & Gay, 1983). AV2 or V2 encodes the pre coat protein (Stanley et al., 2005) and is known to be a pathogenicity determinant (Fondong, 2013). It is also suggested that V2 encodes a movement protein (MP) which is necessary for cell-to-cell transmission and long-distance mobility (Noueiry et al., 1994).

Begomovirus genomic sequence also includes an intergenic region (IR). Bipartite begomoviruses genome components, DNA-A and DNA-B, are different from each other except for an approximately 200 bp IR (Padidam et al., 1996). The IR contains two TATA motifs, one for AV1 andAV2 and another for AC1/AC4 and the bidirectional promoter of these ORFS are present on the IR (Snehi et al., 2017). The IR also contains inverted repeats, whereby binding of the Rep protein occurs (Argüello-Astorga et al., 1994). The IR has a conserved nonanucleotide (TAATATT/AC), which is indicated as the origin of replication. The nonanucleotide is present in a stem loop which varies in composition and length between different viruses (Harrison & Robinson, 2003).

1.4. Begomoviruses infecting tomatoes

1.4.1. Tomato begomoviruses worldwide

Begomoviruses infecting tomato are distributed worldwide and cause diseases that impact agriculturally important crops (Prasanna and Rai, 2007). Tomato yellow leaf curl disease (TYLCD) caused by TYLCV is one of the most damaging tomato diseases in the world (Lefeuvre et al., 2010). TYLCV is an Old World monopartite begomovirus and its symptoms were first described in tomato crops in Israel in the Middle East in 1939, and is transmitted by the whitefly *Bemisia tabaci* Genn. (Czosnek & Laterrot, 1997; Pico' et al., 1996). This viral agent was isolated and sequenced in 1988 (Czosnek & Laterrot, 1997; Pico' et al., 1996). The most widespread TYLCV strains are TYLCV-Israel (TYLCV-IL) and TYLCV-Mild (TYLCV-Mld) (Lefeuvre et al., 2010). TYLCSV is also another begomovirus that causes tomato yellow leaf curl disease (TYLCD), resulting in up to 100% yield losses in tomato fields (Moriones and Navas-Castillo ,2000) . Several more tomato-infecting begomoviruses have been identified. Tomato golden mosaic virus (TGMV) is a bipartite begomovirus that was first discovered in Brazil and was the first tomato-infecting begomovirus to be molecularly defined (Costa, 1976; Wyant et al., 2012). Following this first report, the occurrence of TGMV did not grow in line with the increase in *B. tabaci* populations (Zerbini et al., 2005).

Brazil is one of the world's most diverse tomato-infecting begomovirus hotspots (Duarte et al., 2021). Tomato severe rugose virus (ToSRV) is the most prevalent begomovirus, found in all of Brazil's major tomato-producing areas (Mituti et al., 2019). Vein thinning, chlorosis, mosaic, foliar creasing, and stunted growth are all symptoms of ToSRV infection in tomato plants (Mituti et al., 2019). ToSRV has been found infecting a variety of cultivated crops, including pepper, potato, eggplant, and a variety of weeds, in addition to tomatoes (Barbosa et al., 2009; Mituti et al., 2019; Moura et al., 2018; Souza-Dias et al., 2008). Tomato yellow vein streak virus (ToYVSV) has been shown to infect tomato plants in Sao Paulo, the most important tomato growing region in Brazil (Colariccio et al., 2007). Infection with ToYVSV has been shown to cause up to 80% yield loss and induces symptoms that include yellowing leaf

reduction and stunted growth (Colariccio et al., 2007). Several other tomato-infecting begomoviruses have been discovered in Brazil, including tomato bright yellow mosaic virus (ToBYMV), tomato leaf distortion virus (ToLDV) and tomato chlorotic mottle virus (ToCMoV) (Duarte et al., 2021)

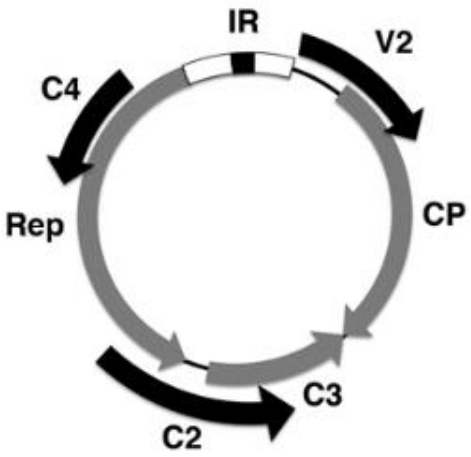

Figure 1.4: Tomato yellow leaf curl virus genome organization illustrating the ORFs encoded by the virus sense; V2 and CP and complementary sense ; Rep, C4, C2, and C3 (adapted from Luna et al., 2012)

1.4.2. Tomato begomoviruses in South Africa

A new disease emerged in 1997 in tomatoes in the Onderberg region of South Africa (Pietersen et al., 2000)and in Mozambique (Pietersen et al., 2008). These tomato plants showed symptoms that were similar to the TYLCV (Pietersen et al., 2000). Symptoms included leaf chlorosis, leaf curling, stunting and fruit set reduction (Figure 1.5). The new disease was named tomato curly stunt disease (ToCSD). An investigation was done in South Africa to determine the identity of the virus that caused the disease, and this led to the discovery of a new begomovirus species, *Tomato curly stunt virus* (Figure 1.6). The nucleotide sequence of the core coat protein (AV1), which shared just under 86% nucleotide identity with strongly related begomoviruses (Pietersen et al., 2007, was used to classify the virus(Brown et al., 2015; F. Murilo Zerbini et al., 2017)). The AV1 sequence comparison also showed that there are other begomovirus species closely related to the members of the TYLCV cluster, including south African cassava mosaic virus (SACMV,77.4%), east African cassava mosaic virus (EACMV, 77.3%) and TYLCV-Israel (76.2%) (Pietersen *et al.*, 2000). A later study (Esterhuizen, 2012) described two variants of ToCSV in South Africa, ToCSV_V30 and ToCSV_V22. PCR-RFLP was used to differentiate the two variants based on symptom phenotype. ToCSV__V30 variants have a wide geographical distribution and cause severe symptom phenotypes including severe stunting, chlorosis, and leaf curling. Tomato plants inoculated with ToCSV_V22

demonstrate a mild symptom phenotype such as slight chlorosis and leaf curling. ToCSV V30 isolates were found in Limpopo, Mpumalanga, and Kwazulu-Natal provinces, with only a few samples found in the Western Cape. ToCSV_V22 was also identified in the northern regions, and it was the only strain responsible for the outbreak in East London.

Tomato curly stunt virus (ToCSV) has been found to be transmitted by *B. tabaci* type B in South Africa (Esterhuizen et al., 2013). A survey was conducted by Dr. Esterhuizen between 2002 and 2009, which revealed *B. tabaci* whitefly haplotype diversity in eight provinces in SA on cassava and tomato hosts. The *B. tabaci* whitefly haplotypes were distinguished using mtCOI sequences and RFLP-PCR. The methods could tell the difference between the B, Q, and SSAF subclades (SSAF-1 and SSAF-5). The B-type was found to be the most common haplotype, with 99% nucleotide identity with the mtCOI sequences of other Middle East-Asian minor 1 members. The study was the first to report the Q type haplotype in South Africa. This haplotype was first identified in East London in 2009. The Q type is able to transmit ToCSV in tomatoes and has since been reported in vegetable production areas of Mozambique (Esterhuizen et al., 2013).

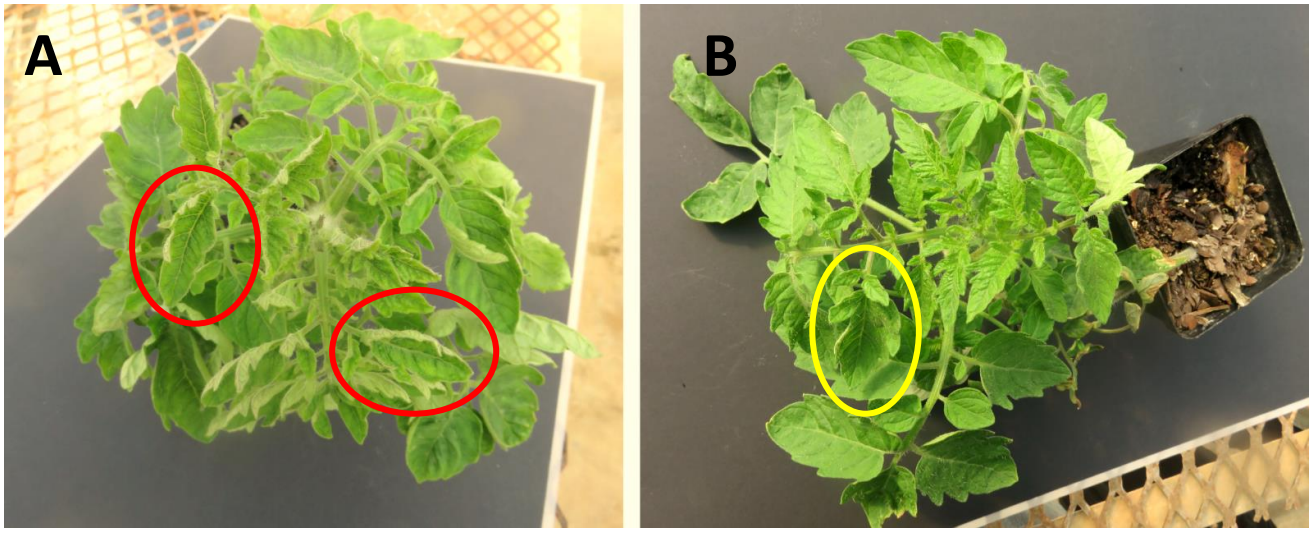

Figure 1.5: Symptom phenotype of ToCSV strains. A) ToCSV_V30 which causes severe symptoms including severe stunting, chlorosis, and leaf curling (red circles) and B) ToCSV_V22 which cause mild symptoms including slight chlorosis and leaf curling (yellow circles) in tomato Moneymaker.

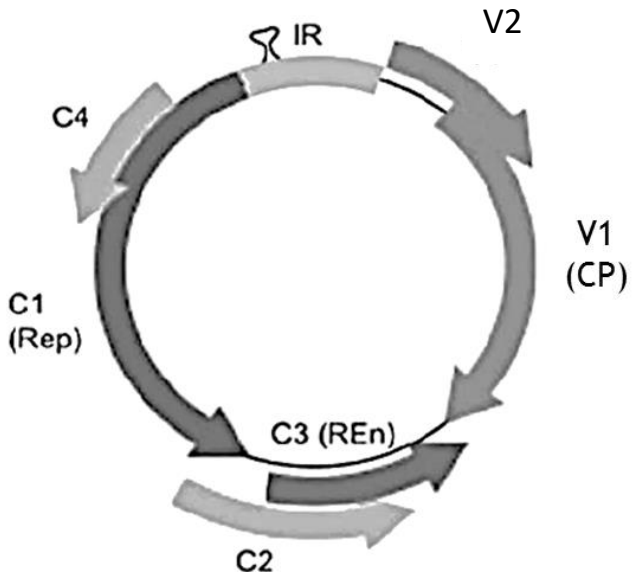

Figure 1.6: Genome organization of ToCSV showing ORFs (Esterhuizen, 2012). V1 encodes a coat protein (CP) and V2 encodes a movement protein, C1 encodes the replication initiator protein (Rep), C2 encodes a transcriptional activator protein (TrAP), C3 encodes a replication enhancer protein (REn) and C4 a symptom determinant.

1.5. Pathogenicity of begomoviruses

1.5.1. Role of viral ORFs in pathogenicity

In monopartite begomoviruses, CP is important for systemic spread throughout the plant (Briddon et al., 1989), however, as shown for the bipartite tomato golden mosaic virus (TGMV) , the CP is not necessary for infectivity (Gardiner et al., 1988). C2 has been shown to interact with V2 which is important in systematic infection and cell-to-cell movement (Priyadarshini et al., 2011). The C1 ORF is conserved in sequence and function and is expressed under the control of a bidirectional core promoter in the IR. C1 encodes the replication associated protein (Hanley-Bowdoin et al., 2000). The pathogenicity of C1 has been reported in many geminiviruses. Zhan *et al.,* (2018) reported that C1 from apple geminivirus (AGV), expressed in PVX, enhanced the pathogenicity of the virus. The C1 protein induced creasing and necrosis symptoms in *N. benthamiana.* The monopartite begomoviruses coat protein is important in systemic infection, however, the coat protein is not required by bipartite begomoviruses for systemic infection (Rojas et al., 2005).

RNA silencing is an important antiviral mechanism in plants. It involves gene silencing directed by small RNAs that target viral RNA/mRNA. During the infection of the host by the virus, the plant can cleave the viral RNA into siRNAs and use them to initiate antiviral silencing activity (Luna et al., 2012). Geminiviruses are able to suppress host defenses, such as transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS), thus leading to symptoms and disease (Rodríguez-Negrete et al., 2009). The C4 protein is a pathogenicity determinant involved in suppression of the host gene silencing defense response, and localizes to the plasma membrane in monopartite begomoviruses as it is important in cell-to-cell movement via the plasmodesmata (Rojas et al., 2005). Ismayil et al. (2018a), showed that C4 of cotton Leaf Curl Multan virus (CLCuMuV) suppresses TGS and PTGS. The study also showed that C4 binds to S-adenosyl methionine synthetase (SAMS), an important enzyme in the methyl cycle, and inhibits SAMS enzymatic activity.

The AC4 of Sri Lankan cassava mosaic virus (SLCMV-LK) , a bipartite begomovirus, was also shown to suppress PTGS in *N. benthamiana* leaf assays (Vanitharani et al., 2004). AC4 protein of African cassava mosaic virus Cameroon strain (EACMV-CM) has been shown to associate with single stranded siRNAs and miRNAs *in vitro and in vivo* (Chellappan et al., 2005). AC4 is also a pathogenicity determinant in bipartite Old-World viruses, such as cassava geminiviruses, but not in various New World bipartite begomoviruses. In TGMV for example, the AC4 homolog is not required for pathogenicity of the virus or can only cause a disease when infected with one or more of the other ORF proteins in *N. benthamiana* (Pooma and Petty, 1996). Bipartite begomoviruses are able to infect phloem and non-phloem tissues, stimulate leaf curling, crumping and mosaic/mottling symptoms and are sap-transmissible (Melgarejo et al., 2013).

The V2 protein, unique to the Old World begomoviruses, also serves as an inhibitor of PTGS and induces hypersensitive response-like cell death including severe systemic necrosis. It has been suggested that V2 suppresses post-transcriptional gene silencing by delaying siRNA secondary amplification (Luna et al., 2012; Sharma & Ikegami, 2010b). Zhao et al. (2018)

suggest that TYLCV V2 could be involved in a counter-defense response by self-interaction. The V2 protein has also been shown to suppress PTGS by interacting with SGS3 thus disrupting the spread of RNA silencing signal (Zrachya et al., 2007). Serine 71 of V2 could be important for pathogenicity of V2 (Zhao et al., 2018). For TYLCV, V2 has been shown to be involved in viral movement (Hanley-Bowdoin et al., 2013).

A study by Gong et al. (2021) showed that geminivirus genome contains additional ORFs. These ORFs are expressed during viral infection and exhibit certain subcellular localization. An additional, conserved ORF, V3, of TYLCV was shown to localize in the Golgi apparatus and is essential for infection and suppresses RNA silencing. Additional ORF have also been discovered in mulberry mosaic dwarf-associated virus (MMDaV). Although these putative ORF (V3, V4 and V5) of MMDaV virion-sense strand did not share sequence homology with other proteins, coiled-coil and transmembrane (TM) domains were identified in V4 and V3, respectively. Coiled-coil domains were previously reported in movement of viral proteins, viral transmission by insects or suppression RNA silencing of DNA or RNA viruses (Ma et al., 2015)

Viral replisomes are complexes that contain viral proteins and host factors that are crucial in reprogramming of the plant cell cycle (Hanley-Bowdoin et al., 2013). Geminiviruses usually infect leaf cells that have completed the cell cycle and no longer express DNA polymerase. To overcome this , geminiviruses modify the host cell cycle (Hanley-Bowdoin et al., 2013). It has been shown that geminivirus infection activates a host DNA synthesis protein known as proliferating cell nuclear antigen (PCNA) (Nagar et al., 1995). PCNA is highly conserved in eukaryotes and is able to interact with varies proteins that are required in cell cycle regulation, DNA replication and DNA repair (Hanley-Bowdoin et al., 2013). Nagar et al. (1995), also showed that *Rep* proteins of geminiviruses stimulate the accumulation of PCNA in terminally differentiated cells. A transcriptome profile of Arabidopsis infected with cabbage leaf curl virus (CaLCuV) revealed that infection by the virus inhibits expression of genes that are expressed in the G1 and M phase of the cell cycle, while infection activates genes that are expressed in the G2 and S phase (Ascencio-Ibáñez et al., 2008). Retinoblastoma- related protein (RBR) is an important modulator of the cell cycle. It binds to E2F transcription factors and is a suppressor of host replication processes. Geminiviruses disrupt the RBR-E2F interaction by binding of Rep and REn to RBR (Hanley-Bowdoin et al., 2013).

1.5.2. Role of betasatellites in pathogenicity

DNA satellites (known as betasatellites or alphasatellites) are widely associated with monopartite begomoviruses, that mostly regulate disease symptoms or disease symptom severity Briddon et al., 2010). Betasatellites are important for the induction of disease symptoms and rely on their helper viruses for replication, systemic transmission, encapsidation, and transmission to new host through insert vectors (Zhou, 2013). In the absence of betasatellites many monopartite begomoviruses can infect and induce symptoms in *Nicotiana benthamiana*, an experimental host (Melgarejo et al., 2013). Bipartite begomoviruses do not require DNA satellites for development of typical symptoms however

bipartite Begomoviruses and betasatellites can be found co-infecting plants (Melgarejo et al., 2013).

Tomato-infecting begomoviruses can cause mild disease symptoms in the natural host without betasatellites (Melgarejo et al., 2013). This has been shown in tomato infected with tomato leaf curl Kumasi virus (ToLCKuV) which induced stunted growth and leaf curl symptoms in the absence of a betasatelite. However, the association of a betasatelite, tomato leaf curl Togo betasatellite (ToLCTGB), had no effect on disease symptoms in tomatoes (Kon & Gilbertson, 2012) . Betasatellites have also been reported in tomato. The C1 protein of tomato yellow leaf curl China virus (TYLCCNV) is associated with a betasatellite (TYLCCNB-βC1), which encodes a single pathogenicity protein, βC1, and functions by suppressing the host RNA silencing defense mechanism, as well as functioning as a symptom determinant (enhances symptoms). Over expression of TYLCCNB-βC1 in *Nicotiana* (host) and *Arabidopsis* (non-host), resulted in virus-like symptom, such as leaf curling, vein swelling and blistering of leaves. TYLCCNB-βC1 also leads to an upregulation of an endogenous RNA silencing suppressor, *Nicotiana benthamiana calmodulin-like* gene (*Nbrgs-CaM)*. The Nbrgs-CaM protein is responsible for the regulation of RNA silencing and promotes geminivirus infection by suppressing the expression of *RDR6* and thus suppressing gene silencing via the autophagy pathway (Zhong et al., 2017).

1.5.3. Role of host factors in pathogenicity

Geminiviruses have recently been at the frontier of studying host factors involved in host-virus interactions. These studies have revealed host genes that are expressed differentially either at infection or at expression of a viral protein (Lozano-Durán et al., 2011). The ability of a plant virus to infect a host is determined by the interaction of host defenses and the virus life cycle, which includes replication and movement (Luna et al., 2012). All geminivirus proteins interact with host proteins enabling the life cycle of the virus in the host (Hanley-Bowdoin et al., 2000). Viruses encode proteins, several of which act as host defense suppressors (Csorba et al., 2015). Geminiviruses also interact with plant hormones like salicylic acid, ethylene and jasmonic acid pathways (Hanley-Bowdoin et al., 2013). C4 of beet curly top virus (BCTV) has been shown to interact with SHAGGY-like protein kinase (AtSK) family (AtSK11, AtSK12, AtSK13, AtSK21, AtSK22, AtSK23 and AtSK32) of *Arabidopsis thaliana* (Piroux et al., 2007). A study performed by Bi et al. (2017), revealed that the C4 protein of the sweet potato leaf curl virus-Jiangsu (SPLCV-JS) altered the Brassinosteroid (BR) Signaling Pathway. This suggests that the SPLCV-JS C4 protein interacts with the BR-signaling pathway to cause viral infection. Brassinosteroids are plant hormones that induce elongation of the stem, pollen tube development, leaf bending and downward leaf curving, inhibition of root development, stimulation of ethylene synthesis, proton-pump activation, differentiation of xylem tissue, and modulation of gene expression (Li & Chory, 1999). Geminiviruses also activate salicylic acid and ethylene pathways, as shown by infection of Arabidopsis with CaLCuV, which increases the resistance of the plant to the infection (Ascencio-Ibáñez et al., 2008). The jasmonic acid pathway was suppressed during infection, however, a study done by Soitamo et al. (2012) showed that AC2 from african cassava mosaic virus (ACMV), expressed in tobacco plants up-regulated jasmonate biosynthesis. The study also showed that

AC2 caused a decrease in photosynthesis-related transcripts. Heat shock proteins 70 (HSP70s) have been shown to be associated with plant viral infections. HSP70s are cellular chaperones that belong to a conserved family (Park & Seo, 2015). They are involved in various cellular processes such as synthesis of new polypeptides, refolding of proteins that have been misfolded or aggregated, translocation of proteins and degradation of proteins. HSP70 is targeted by a variety of plant viruses to facilitate the synthesis, folding, and distribution of viral proteins, as well as to regulate virus replication and alter the host's antiviral response. Evidence has shown the involvement of HSP70 in the life cycle of geminiviruses. The silencing of HSP70 and HSP70-1 resulted in reduced TYLCSV infections in tomato (Gorovits et al., 2013). It has also been suggested that interaction of HSP70 and CP is important in enabling CP translocation into the host's nucleus, thereby stimulating replication (Gorovits et al., 2013).

In a study performed by Sahu et al. (2010), tolerant and susceptible tomato cultivars were infected with ToLCNDV. It was shown that a low abundance of viral replicative intermediates correlated with a high accumulation of siRNA. Infection of the cultivars with ToLCNDV also induced the accumulation of other host transcripts. A transcript that was characterized as bHLH transcription factor was upregulated in tolerant cultivars. bHLH acts as a transcriptional activator in abscisic acid (ABA) signaling in *Arabidopsis* (Abe et al., 2003). ABA is known to regulate abiotic and biotic stress tolerance related processes and disease resistance (Finkelstein et al., 2002). Another transcript that was characterized from the study was serine/threonine protein kinase PSB1, similar to that found in *Ricinus communis.* Protein kinases play an important role in pathogen recognition signaling and the activation of plant defense systems (Romeis, 2001). PSB1 was upregulated in tolerant cultivars and downregulated in susceptible cultivars. This suggested that the protein kinases play a crucial role in ToLCNDV tolerance. Tolerant cultivars also showed an upregulation of gibberellin regulatory protein 1 a regulator of gibberellic acid (GA), which is a growth hormone that upregulates plant development and may act as a defense gene (Swain & Singh, 2005).

1.6. Developing begomovirus resistance in tomatoes

For more than 20 years, efforts have been put into developing TYLCV-resistant tomato cultivars. Initially, control measures for disease development included limitation of the vector population by the use of pesticides in integrated pest managements (IPM) strategies and mesh nets (Azizi et al., 2008; Lapidot et al., 1997). Since tomato cultivars are so susceptible to TYLCV, wild Solanum plant species have been screened for their virus resistance. Breeding programs have thus been developed to transfer the resistance genes from the wild solanacea plants to the susceptible domesticated plants. TY20 was the first commercial tolerant cultivar with resistance genes derived from *Solanum peruvianum*. This tolerant cultivar showed a delay in symptoms and accumulation of TYLCV DNA. A TYLCV-resistance genes, *TY-1*, was mapped on chromosome 6 of *S. chilense* (Lapidot et al., 1997). Another TYLCV resistance locus was mapped on *S. habrochaites.* This resistance QTL or locus was localized on chromosome 11. This locus was delimited and designated as *Ty-2*. Many other wild tomato species have served as the source of resistance to TYLCV. These species include *S. pimpinellifolium* and *S. cheesmaniae* (Ji et al., 2007) . Ji et al. (2007), mapped a resistance gene, designated *Ty-3*, on

the long arm of chromosome 6. However, these bred cultivars are not completely immune to the virus but rather develop milder symptoms when infected by TYLCV (Legarrea et al., 2015).

1.7. Virus induced gene silencing

RNA silencing was first described in 1928 in a paper published by Wingard. The initially infected leaves of tobacco plants were necrotic and showed diseases symptoms relating to tobacco ringspot virus (TRSV), however, no symptoms appeared on the upper leaves (Baulcombe, 2004; Wingard, 1928). No explanation could be given for the recovery and resistances to secondary infection. The recovery was later attributed to the upper leaves containing lower concentrations of the viral RNA (Ratcliff et al., 1997). Plants have developed mechanisms of defence against pathogen attack, thus preventing disease development. One mechanism used by plants is RNA silencing. The RNA silencing mechanism includes three distinct pathways: cytoplasmic short interfering (siRNA) silencing, microRNAs (miRNAs) silencing of endogenous mRNAs via DNA methylation, and transcription suppression (Baulcombe, 2004). The first mechanism, siRNA silencing, is triggered by viral double stranded RNA (dsRNA) transcribed from internal base-paired stem loop structures of DNA viruses or from dsRNA that could be a replication intermediates (Baulcombe, 2004). The viral dsRNA are processed by a Dicer-like (DCL) enzyme into small interfering RNA (siRNA), approximately 21–24 nt in length (Baulcombe, 2004). These siRNAs direct the silencing process in a sequence-specific manner. The RNA-induced silencing complex (RISC) is formed when small viral RNAs bind to an Argonaute (AGO) protein and other proteins. AGO directs the siRNAs to homologous regions of the virus mRNA initiating cleavage and inhibition of viral transcription and preventing infection by the virus and the development of disease symptoms, Figure 1.7 (Liu et al., 2017). This process is known as posttranscriptional gene silencing (PTGS) and takes place in the cytoplasm. Transcriptional gene silencing occurs when 24nt siRNAs are transported to the nucleus and mediate methylation of viral DNA or DNA-associated histones as is the case with begomoviruses (Pooggin, 2013).

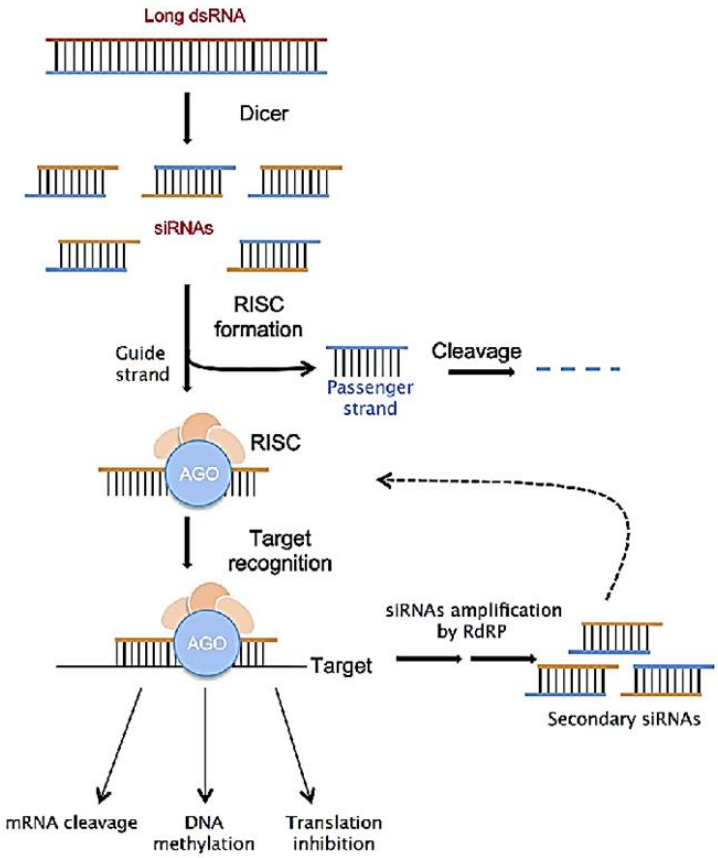

Figure 1.7: Schematic diagram of RNA silencing (Limera et al., 2017)

Like many viruses, geminiviruses induce and target RNA silencing (Yang et al., 2011). mRNA transcribed bi-directional from promoters in the IR regions of DNA A leads to overlapping ORFs which can be filled in by RDRP6 to form dsRNA, or the transcribed RNA folds back to form a dsRNA-like structure which acts as a template for dicer (DCL1) (Vanitharani et al., 2005). Akbergenov et al. 2006) characterized three major size-classes of ACMV siRNAs in infected *N. benthamiana* and cassava plants. These siRNA were derived from the sense and antisense strands of viral DNA A and B, and many are derived from AC2 ORF. Accumulation of these siRNA suggested that that more than one DCL might be involved in the synthesis of these RNAs (Rodríguez-Negrete et al., 2009).

Virus-induced gene silencing (VIGS) is a plant RNA-silencing method that employs viral vectors containing a fragment of a gene of interest to generate double-stranded RNA, which induces PTGS silencing of the target gene (Vaucheret et al., 2001). VIGS can be used to produce a transient loss of function of host or virus gene which enables analysis of gene function (Baulcombe, 1999). VIGS in plants occurs when there is a similarity in the cloned host gene or gene fragment and the plant endogenous or transgene (Teresa Ruiz et al., 1998). Tobacco mosaic virus (TMV), potato virus X (PVX) and tomato golden mosaic virus (TGMV) were the first viruses to be used for VIGS (Watson et al., 2005), however, to date many more virus-based vectors have been used effectively to silence genes in plants and these include barley stripe mosaic virus, bean pod mottle virus (BPMV), brome mosaic virus, cabbage leaf curl

virus, cotton leaf crumple virus, grapevine leafroll-associated virus-2, pea early browning virus and TGMV (Wang et al., 2014). The efficiency of the vector firstly relies on the viral DNA replication and accumulation of viral DNA to adequate levels in the plant to produce dsRNA that will induce silencing. Secondly, the virus vector should lack a strong PTGS suppressor, which protects the virus against any RNA silencing mechanism. TMV, PVX and TGMV have been optimized in *Nicotiana benthamiana* (Faivre-Rampant et al., 2004). RNA viruses have been greatly used as expression vectors. PVX can be used as a virus-based vector as it is easily transmitted by inoculating the sap (phloem and xylem tissues) and it can infect a wide variety of host plants (Roggero et al., 2001). PVX is a member of the potexvirus group and is composed of a single stranded RNA of 6430 nt. It is capped at the 5' end with an m^7GpppG structure and is polyadenylated at the 3' end. The viral-sense strand contains five ORFs. The ORF closer to the 5' end encodes a protein that is 166kDa and is involved in viral replication (encodes an RNA-dependent RNA polymerase). This ORF is followed by the triple gene block (TGB), which consists of three partially overlapping ORFs. TGB encodes proteins of 25 kDa (TGBp1), 12 kDa (TGBp2), and 8 kDa (TGBp3). The last ORF encodes the 25 kDa CP and it is transcribed by a sub-genomic promoter (SGP) (Chapman et al., 1992; Wang et al., 2014). The limitation with using PVX as a VIGS vector is that it induces symptoms that are associated with the virus that include chlorosis, leaf distortion and localized cell death, which make PTGS phenotype analysis difficult. Tobacco rattle virus (TRV) vector is a widely used VIGS vector as it can infect a larger range of host plants, can cause systematic infection as well as spreading to the meristematic tissues and does not cause severe symptoms in infected host (Ramegowda et al., 2014; Valentine et al., 2004). RNA1 encodes genes that are important for viral replication and these include 134 kDa and 194 kDa replicase proteins, a 29-kDa movement protein and a 16 kDa cysteine-rich protein (which has been shown to be important in allows entry of TRV into meristems and to infect the reproductive organs(Martin-Hernandez & Baulcombe, 2008)) (Liu et al., 2002)

Even though plants have RNA mediated mechanisms to combat viral infection, viruses can counteract the silencing mechanism using viral suppressors (VSR). VSR enhance virus replication in infected cells, and inhibit local or long-distance spread of RNA silencing by the plant (Raja et al., 2010). VSR act by affecting with the production of small RNA, by binding and removing small RNA, or by directly or indirectly inhibiting the activity of proteins required for silencing (Raja et al., 2010). Many begomoviruses encode VSR proteins for RNA silencing (Sharma & Ikegami, 2008). The C2 protein of (TYLCCNV) has been shown to be VSR of PTGS. The C2 protein was expressed with a PVX vector tagged with a GFP molecule. The C2 protein would have to be localized to the nucleus and bind zinc to determine its correct biological function in PTGS suppression (Dong et al., 2003; Van Wezel et al., 2002). However, C2 protein of tomato leaf curl Java virus (ToLCJAV) expressed with a GFP protein showed to be a weak suppressor of PTGS. The C4 protein has been shown to be involved in suppressing gene-silencing defense mechanism of the plant CLCuMuV (Ismayil et al., 2018). The AC4 of sri Lankan cassava mosaic virus (SLCMV), a bipartite begomovirus, was also shown to suppress PTGS (Vanitharani et al., 2004) (see section 1.5.1). Kon et al. (2007), showed that ToLCJAV C4 protein does not suppress PTGS. Cui et al, (2005), showed that βC1 of TYLCCNV Y10 isolate

functions as a PTGS suppressor. *N. benthamiana* inoculated with C4 showed a suppressed GFP. In a recent study it has been shown that CLCuMV V2, Trap (C2), C4 and βC1 proteins exhibit suppressor activity (Amin et al., 2011). V2 binds long RNA which suggests that it interferes with silencing by removing long dsRNA, thus preventing cleavage by Dicer and production of siRNA (Amin, 2010).

1.8. Rationale for Study

Tomato is the second most important crop commodity in South Africa after potatoes. It contributes about 24% of the total vegetable production in South Africa. About 6000 hectares of tomatoes are planted. Ripe tomatoes are mostly consumed fresh in salads, cooked, fried or sundried. Processed tomatoes are used in jams, chutney, tomato sauce and as canned tomatoes. The consumption of tomatoes in South Africa is measured at 12kg per annum. South African households consume 5 to 10 tomatoes per week on average. They provide a good source of vitamins A and C as well as essential minerals (Arc, 2013; DAFF, 2017)

Plant viruses classified under the family *Geminiviridae* are able to infect food crops, leading to major crop failure and economic losses worldwide. Along with potyviruses, geminiviruses are the most widely spread and devastating. These phytopathogens mostly affect diverse crop plants in tropical and subtropical areas of many countries. They can evolve into new strains, and also new species due to recombination, and thus are able to broaden their host range (Tripathi et al., 2018). Tomato begomoviruses can causes great quantitative and qualitative yield decrease in tomato crops which can lead to 100% losses (Pico' et al., 1996). TYLC disease was first reported in South Africa in 1997 and in 1998 140 hectares of tomato fields in Onderberg were tested for the presence of TYLCV. The symptoms observed were similar to those caused by the Israel isolate of TYLCV. Yield losses caused by this disease ranged to from negligible to 100%. Analysis of the AV1 nucleotide of the viruses led to the designation of a new tomato begomovirus species, ToCSV. The disease has spread to other tomato growing regions (Trichardtsdal, Pongola and Nkwalini) South Africa, Swaziland and Mozambique (Pietersen et al., 2000, 2008) . Determining the factors responsible for the pathogenicity of the virus can provide information for the development of tolerant or resistant crops.

Since the identification of ToCSV in tomatoes in the Onderberg region of South Africa Pietersen et al., 2008) and Mozambique, it has been reported in seven of South Africa's nine provinces (Esterhuizen, 2012). Two variants of the virus were then identified using PCR_RFLP, ToCSV_V30 (severe) and ToCSV_V22 (mild). The variants were differentiated depending on the presence or absence of recombination fragments in the V2 region. Recombination between two species produces new begomoviruses (Marwal et al., 2011). Variants of ToCSV have acquired an additional fragment of the V2 region from a virus that appears to be related to ToLCUV-[Ug:Iga:05] (Esterhuizen, 2012). ToCSV_V30 showed no recombination in the V2 region, while ToCSV_V22 (mild) showed recombination in the V2 region (Esterhuizen, 2012). The geographical distribution ToCSV V30 isolates were discovered in northern South African production lines such as Limpopo, Mpumalanga, and Kwazulu-Natal, with a minor number of samples discovered in the Western Cape. Distribution of ToCSV_V22 was also shown in the northern regions, and it was the only strain responsible for the outbreak in East London,

Eastern Cape. Infectious clones of both strains were constructed by modification of a pCambia 2300 binary vector. Infectious clones were agroinfected into tomato cv. Rooi Khaki. ToCSV_V30 inoculated plants showed severe symptoms, including yellowing, and curled leaflet margins and reduced leaf surface whereas plants inoculated with ToCSV_V22 showed mild symptoms, which include slight yellowing and curling of leaves, with no decrease in leaf surface area. An infectious clone with the V2 ORF mutant swap was constructed by deleting ToCSV_V30 V2 ORF and ToCSV_V22 V2 ORF. Plants inoculated with the swap showed, through southern blot analysis, that the V2 region does not affect the viral infectivity and the ability of the virus to replicate. It was also demonstrated that changes in the V2 area are not involved in the altered symptom phenotype. (Esterhuizen, 2012). Thirteen site directed mutants were synthesized for C1, C2, C3 and V1 ORF in ToCSV_V22 to determine whether the difference between the mild and severe strain symptoms are due to single amino acid sequence differences (Esterhuizen, 2012). Ten of the thirteen mutants were inoculated into tomato cv. moneymaker and *N. benthamiana*. Only a single trial was performed and no significant change to the symptom phenotype was observed.

To gain an overall understanding of molecular mechanisms involved in pathogenesis, host-virus interaction studies have become a standard practice. This has become possible by obtaining nucleotide sequences of several viral genomes thus contributing to the identification and understanding of the expression strategies of different plant viruses in different plant (natural and/or experimental hosts). The general aim of this study was to determine what role pathogenicity determinates C2 (transcriptional activator protein) and V2 (pre-coat protein) encoded by either V30 or V22 variants contribute to symptom phenotype or severity in tomato and *N. benthamiana*.

1. The specific aims and objectives were: To investigate the effect of mutations in the C2 and V2 ORFs of ToCSV variants in order to determine whether C2 and/or V2 are important for viral replication and viral DNA accumulation in tomato cv. Rooikhaki.
    - Symptom scoring of plants inoculated with chimeric ToCSV_V30 infectious clone containing entire V2 swap and ToCSV_V30 containing C2 mutants.
    - Quantitative PCR analysis of extracted DNA to determine accumulation of viral DNA.
2. To determine the possible role of C2-encoded TrAP by both mild and severe variants in pathogenicity by overexpressing C2 using a PVX expression vector, pGR107, in Nicotiana benthamiana.
    - Cloning the full length of C2 gene from both ToCSV_V22 and ToCSV_V30 into the multiple cloning site of PVX vector.
    - Symptom evaluation of inoculated *N. benthamiana.*
    - RT-qPCR analysis of extracted RNA to determine relative mRNA expression levels of C2 and PVX coat protein.
3. To determine whether C2 of ToCSV mild (ToCSV_V22) and severe (ToCSV_V30) variants are a suppressors of host posttranscriptional gene silencing (PTGS) activity.

- Cloning the full length of C2 ORF from both ToCSV_V22 and ToCSV_V30 were cloned into the multiple cloning site of TRV2.
- Observation of GFP expression in N. benthamiana 16 C line plants.
- RT-qPCR analysis of extracted RNA to determine relative GFP mRNA expression levels.

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Chapter 2

Pathogenicity assays with ToCSV nucleotide single-site C2 and V2 swap mutants in tomato cv. Rooikhaki

Abstract

Tomatoes are one of the world's most vital crops, and geminiviruses have a significant effect on them. Tomato curl stunt virus (ToCSV), a monopartite begomovirus, has been shown to be a major tomato-infecting virus in South Africa. The V2 protein encoded by begomoviruses is important in viral infection, cell-to-cell movement and suppression of host defense response. The C2 proteins have been shown to be important for viral assembly and transmission. In this study, we analyzed the effects of V2 swap and C2 mutations of ToCSV variants to determine the importance of V2 and C2 genes in viral infection and DNA accumulation in tomato plants. When compared to symptoms caused by ToCSV V30, plants inoculated with the chimeric infectious clone containing the V2 swap showed no significant differences in symptom severity. Plants infected with the chimeric infectious clone, ToCSV V30(V22V2aa1-116) (V2 swap), had higher levels of viral DNA than wild-type ToCSV V30 and ToCSV V22. Relative viral load analyses revealed that the swap had a 5-fold and 3-fold higher viral load at 21 dpi when compared to ToCSV V30 and ToCSV V22, respectively. The results indicated that the V2 swap did affect the levels of DNA replicated when compared to ToCSV_V22 and ToCSV_V30. In order to determine the function of C2 in pathogenicity, three C2 mutants were produced by substituting the amino acids (aa) in the severe ToCSV_V30 with the corresponding ones from the mild V22 variant; Mut 10 (ToCSV_V30(V22C2$_{aa46}$)), Mut 9 (ToCSV_V30(V22C2$_{aa48}$)), and Mut 7 (ToCSV_V30(V22C2$_{aa109}$)). Plants infected with the C2 mutants developed symptoms that were similar to those produced by ToCSV_V30. Relative viral load analyses showed that the C2 mutants accumulated higher levels of DNA at 21 dpi when compared to the wild-type virus variants. Relative viral load of mut 7, mut 9 and mut 10 was 2.3-, 2.7-, and 2-fold, respectively, higher than ToCSV_V22 at 21 dpi, whereas the relative viral load change for the mutants were 4.38, 4,4 and 3.7-fold higher than ToCSV_V30. This suggests that the specific amino acids in the C2 region studied did affect viral DNA replication.

2.1. Introduction

Geminiviruses belong to the family *Geminiviridae*. The family is made of 9 genera, namely, *Becurtovirus, Begomovirus, Capulavirus, Curtovirus, Eragrovirus, Grablovirus, Mastrevirus, Topocuvirus* and *Turncurtovirus* (Zerbini et al., 2017). The genus *Begomovirus* consist of

viruses that have monopartite and bipartite genomes (Zerbini et al., 2017). Depending on the structure of the genome, diversity of the genes and geographical location, begomoviruses have been separated into Old World (OW) and New World (NW). All geminiviruses have in common a conserved nonanucleotide (TAATATT/AC), which is indicated as the origin of replication and is located in the long intergenic region (Nawaz-ul-Rehman & Fauquet, 2009; Zerbini et al., 2017) . The bipartite genome consists of a DNA-A and DNA-B component. The DNA-A component encode for proteins that are important in initiating DNA replication (replication initiator protein/Rep, AC1; and replication enhancer protein/REn, AC3), regulating host genes and suppressing gene silencing (transcription activation protein/ TrAP, AC2; AC4; and the pre-coat protein, AC2) and proteins involved in viral assembly and transmission (coat protein/CP, AV1). The DNA-B component encodes for proteins that are required for viral movement (nuclear shuttle protein/NSP, BC1; and movement protein/MP, BV1) (Yadava et al., 2010). The begomovirus monopartite genome resembles the DNA-A component (V1, V2, C1,C2,C3,C4) of the bipartite genome (Rojas et al., 2005). The V2 protein and CP of monopartite begomoviruses have functions similar to those of the NSP and MP of the DNA-B component of the bipartite genome (Rojas et al., 2005). The V2 protein is conserved between begomoviruses from the OW, however, NW begomoviruses lack AV2 (Mubin et al., 2010; Zerbini et al., 2017).

The V2 protein of tomato begomoviruses has been regarded as important for viral movement. This important function has been determined due to mutations introduced into the V2 gene that have resulted in the reduction of viral load as well as symptoms (Padidam et al., 1996; Wartig et al., 1997). The V2 gene has also been shown to be a pathogenicity determinant and is also important in inducing severe necrosis in plants due to systemic infection thus demonstrating that it is a target of host defense responses (Mubin et al., 2010; Sharma & Ikegami, 2010). This severe necrosis has been associated with hypersensitive response (HR)-like lesions. Mutational studies have shown that the C terminal domain of V2 is involved in inducing the HR, a plant defense response (Sharma & Ikegami, 2010). The expression of V2 also causes the production of H_2O_2, which is characteristic of HR (Sharma & Ikegami, 2010).

The V2 protein has been reported to form aggregates in the cell. These aggregates usually contain viral and host proteins and can differ in position, size , function and content (Zhao et al., 2018). The formation of these aggregates and their degradation by 26S proteasome is dependent on actin and microtubule cytoskeleton integrity and thus suggests that the formation of the V2 aggregates play a crucial role in viral movement during infection (Moshe et al., 2015; Zhao et al., 2018). The expression of V2 has been shown to function with C4 in localization of viral DNA from nuclear membrane to the cell membrane and to adjacent cells (Rojas et al., 2001). It has also been shown that V2 is a suppressor of posttranscriptional gene silencing (PTGS), however, mutations of V2 ORF that disrupt PTGS activity indicate that there is a reduction in viral movement, confirming that V2 plays a vital role in movement. A double mutation (C84S/C86S) in the V2 region of tomato yellow leaf curl virus-Israel (TYLCV-Is) has been carried out to eliminate the silencing suppression activity of V2 (Hak et al., 2015). Hak et al (2015) indicated that a majority of the plants infected with the mutant were positive for viral DNA showing that the V2 protein is also important for suppressing RNA silencing.

Transcriptional activator protein (TrAP) is a product of the C2 gene. The AC2/C2 gene is conserved between all begomoviruses (Guerrero et al., 2020). TrAP of tomato leaf curl New Delhi virus (ToLCNDV) has been shown to inhibit cell death caused by the NSP, which can also act as an avirulence determinant (Garrido-Ramirez et al., 2000; Hussain et al., 2007). In monopartite viruses, the expression of C2 has been shown to inhibit cell death elicited by V2 (Mubin et al., 2010). TrAP is important for the transcription of the coat protein (CP or V1 gene) and has shown to activate, in mesophyll cells, the CP promoter, however depressing it in phloem cells (Sunter & Bisaro, 1997). This transcription activity is due to the properties of the protein. TrAP consist of a basic domain in the N-terminal region and an acidic domain in the C-terminal region. It has been shown that the basic domain of transcription factors is required for the binding of DNA and/or movement of protein to the nucleus, whereas the acidic domains are important for some transcriptional activation domains. The central domain of TrAP contains a sequence of cysteine and histidine residues that are conserved which form a binding site for zinc. Zinger fingers are also used in DNA binding (Hartitz et al., 1999). Mutational studies of cysteine residues within the zinc finger, and arginine-rich nuclear localization signal within the basic domain, have shown that the N-terminal and the zinc finger domains are important in suppressing host RNA silencing activity (van Wezel et al., 2002; Dong et al., 2003). Two of the mutants (Mut10 and Mut9) that will be tested in our study contain a mutation within the zinc finger domain. It has also been suggested that AC2 could be involved in inducing the activation of specific host genes which are required to suppress RNA silencing. These endogenous suppressors could be involved in regulating silenced genes required for host development or preventing local or systemic spread of silencing (Trinks et al., 2005).

2.2 Aim

The aim of this study was to investigate the effect of the mutations in the C2 and V2 genes of ToCSV variants to determine if C2 and/or V2 are important for viral replication, viral DNA accumulation and systemic infection of tomato plants.

2.3. Methodology

2.3.2. Construction of C2 single-site mutants and V2 swap

A comparison of each Open Reading Frame (ORF) from ToCSV V30 and ToCSV V22 revealed that both variants contained 41 nucleotide variations or insertions, 22 amino acid substitutions, and substitutions in the V2 region. These nucleotide difference contributed to seven amino acid differences in the C2 region between both variants (Esterhuizen, 2012). The mutants generated from this study were selected from the mentioned amino acid difference. The difference in amino acids between the variants could affect DNA binding function. To determine the function of certain amino acids in C2, three different single site- directed ToCSV_V30 mutants have been generated for C2. The mutants were generated for three

different amino acids (aa); aa 46 Asp was mutated to Asn (gac->aac) [Mut 10, ToCSV_V30(V22C2$_{aa46}$)], aa 48 Ile was mutated to Ser (atc->agc) [Mut 9, ToCSV_V30(V22C2$_{aa48}$)], aa 109 was mutated to Ile (act->att) Thr (Mut 7, ToCSV_V30(V22C2$_{aa109}$)). These mutants were generated by substituting the aa in the severe ToCSV V30 with the corresponding aa difference from the mild V22 variant (Figure 2.1-A). To investigate whether the recombinant V2 protein is important for infectivity, a chimeric infectious clone ToCSV_V30(V22V2$_{aa1-116}$ was constructed by swapping out the V2 ORF between the V30 and V22 (Figure 2.1-B). The TOCSV_V30 and ToCSV_V22 V2 ORFs were digested with *BamHI* and *XhoI* to release the V2 fragment, which was then cloned into the corresponding region within the TOCSV V30 (Esterhuizen, 2012). All mutants were cloned into pCambia 2300 and transformed into *Agrobacterium tumefaciens* C58C1.

A)

ToCSV_V30_C2 atg cgg tct tca tca ccc tca acc agc cac tgt act caa gtc cca atc aag gtc cag cat cga ata
M R S S S P S T S H C T Q V P I K V Q H R I
ToCSV_V22_C2 atg cga tct tca tca ccc tca acc agc cac tgt act caa gtc cca atc aag gtc cag cat cga ata
M R S S S P S T S H C T Q V P I K V Q H R I

ToCSV_V30_C2 gcc aag aag aga gta gtc agg cgt agg cga gtt gat ctt gat tgc ggg tgt aca tat tac ctc cac
A K K R V V R R R R V D L D C G C T Y Y L H
ToCSV_V22_C2 gcc aag aag aga gta gtc agg cgt agg cga gtt gat ctt gat tgc ggg tgt aca tat tat ctc cat
A K K R V V R R R R V D L D C G C T Y Y L H

ToCSV_V30_C2 atc gac tgc atc aac cat gga ttt acg cac cgg gga act cat cac tgc tca tca ggc cca gaa tgg
I D C I N H G F T H R G T H H C S S G P E W
ToCSV_V22_C2 atc aac tgc agc aac cat gga ttt acg cac cgg gga act cat cac tgc tca tca ggc cca gaa tgg
I N C S N H G F T H R G T H H C S S G P E W

ToCSV_V30_C2 cgt cta tat ttg gga gat aac aaa tcc cct cta ttt caa gat tat caa cca cga cag cag acc gtt
R L Y L G D N K S P L F Q D Y Q P R Q Q T V
ToCSV_V22_C2 cgt cta tat ttg gga gat aac aaa tcc cct ata ttt caa gat tat caa cca cga gaa acg gcc att
R L Y L G D N K S P I F Q D Y Q P R E T A I

ToCSV_V30_C2 cca cac gaa cca cga cat cat atc gat cca gat acg gtt caa cca caa cct ccg gaa gga act
P H E P R H H I D P D T V Q P Q P P E G T
ToCSV_V22_C2 cca cac gaa cca cga cat cat atc gat cca gat acg gtt caa cca caa cct ccg gaa gga att
P H E P R H H I D P D T V Q P Q P P E G I

ToCSV_V30_C2 ggg gat tca caa atg ttt tct caa ctt cca agt ctg gac gac ctt aca gcc tca gac tgg tcg
G D S Q M F S Q L P S L D D L T A S D W S
ToCSV_V22_C2 ggg gat tca caa atg ttt tct caa ctt cca agt ctg gac gac ctt aca gcc tca gac tgg tcg
G D S Q M F S Q L P S L D D L T A S D W S

ToCSV_V30_C2 ttt ctt aag cgt att taa
F L K R I -
ToCSV_V22_C2 ttt ctt aag cgt att tag
F L K R I -

B)

ToCSV_V30_V2 atg tgg gat cca ctg tta aac gaa ttc cca gac tcc gtc cac ggt ttt cgt tgt atg ctt

M W D P L L N E F P D S V H G F R C M L

ToCSV_V22_V2 atg tgg gat cca ctg tta aac gag ttc cca gac tct gtt cat ggg ttt cgt tgt atg ctt

M W D P L L N E F P D S V H G F R C M L

ToCSV_V30_V2 gct gtt aaa tat ctg cag tcc gtt gaa gcc aca tac gag ccc aac aca ttg ggc cac gat

A V K Y L Q S V E A T Y E P N T L G H D

ToCSV_V22_V2 gct ata aaa tac ttg cag gtt att gag tcc act tat gag ccc aat act ttg ggc cac gat

A I K Y L Q V I E S T Y E P N T L G H D

ToCSV_V30_V2 ctt ata cgg gat ctg att ctg gtt att agg gca aaa gat tat gtc gaa gcg tcc cgc cga

L I R D L I L V I R A K D Y V E A S R R

ToCSV_V22_V2 tta att cga gat ctt att tct gtt att aga gcc cgg gat tat gtc gaa gcg acc cgc cga

L I R D L I S V I R A R D Y V E A T R R

ToCSV_V30_V2 tat aat cat ttc cac gcc cgc ctc gaa ggt gcg gag aag gct gaa ctt cga cag ccc gtt

Y N H F H A R L E G A E K A E L R Q P V

ToCSV_V22_V2 tat aat cat ttc cac gcc cgc ctc gaa ggt gcg gag aag gct gaa ctt cga cag ccc gtt

Y N H F H A R L E G A E K A E L R Q P V

ToCSV_V30_V2 cac cag ccg tgc tgt tgt ccc cat tgc ccc agg cac aaa caa gcg acg atc atg gac gta

H Q P C C C P H C P R H K Q A T I M D V

ToCSV_V22_V2 cac cag ccg tgc tgt tgt ccc cat tgt ccc agg cac aaa caa gcg acg atc atg gac gta

H Q P C C C P H C P R H K Q A T I M D V

ToCSV_V30_V2 cag gcc cat gta tcg aaa gcc caa gat gta cag aat gta tag

Q A H V S K A Q D V Q N V -

ToCSV_V22_V2 cag gcc cat gta tcg aaa gcc cag gat gta cag aat gta cag aag ccc tga

Q A H V S K A Q D V Q N V Q K P -

Figure 2.1: C2 and V2 swap mutations. A) Mutations were generated by substituting the amino acid in the severe ToCSV V30 (green) with the corresponding amino acid difference from the mild ToCSV_V22 (red). Three mutants were generated in C2 region: Mut 10 (circled in yellow), mut 9 (circled in blue) and mut 7 (circled in purple). **B)** V2 swap was generated by swapping out the V2 ORF from ToCSV_V30 with that of ToCSV_V22 (highlighted in green)

2.3.3. Transformation into *Agrobacterium tumefaciens*

For transformation of mutants into *Agrobacterium tumefaciens* C58C1, competent C58C1 cells were allowed to thaw on ice. Once cells were thawed, 100 ng of plasmid C2 and V2 swap mutants were introduced to 100 µl of C58C1 competent cells. The solution was placed on ice for 10 minutes before being placed in liquid nitrogen for 5 minutes to snap freeze. The cells were allowed to thaw at 37°C and placed back on ice for 2 min. The cells were used to inoculate 500 µl LB and incubated at 28°C shaking at 220 rpm for 2 hours. After 2 hours, the cells were spun at maximum speed for 1 minute to collect the cells into a pellet. About 400 µl of the supernatant was removed from the tube and the remaining supernatant was used to resuspend the cells. The resuspended pellet was spread on an LB agar plate with 100 g/ml rifampicin and 100 g/ml kanamycin for 48 hours at 28 °C. Colonies were grown to saturation in 10ml LB media containing 100 µg/ml rifampicin and 100 µg/ml kanamycin. The cultures were used to make glycerol stocks and stored at -80°C.

2.3.4. Growing conditions of tomato cv. Rooikhaki

Tomato cv. Rooikhaki seeds obtained from Sakata Seed Southern Africa (Pty) Ltd. were sown in cocopeat and allowed to germinate at 25 °C with 16 hours light and 8 hours dark photoperiodism. When seedlings reached the two-week stage, they were transplanted into pots containing germination mix composed of Culterra cocopeat soil and vermiculite (3:1). The pots were placed into deep trays, covered with cling wrap, and acclimatized for a week. The fertilizer is composed of .65 g/L Calcium nitrate, 0.09 g/ L Mono-ammonium phosphate technical grade, 0.09 g/ L Mono-Potassium Phosphate, 0.15 g/L Potassium Sulphate, 0.4 g/L Potassium nitrate, 0.3 g/L Magnesium Sulphate, 0.02 g/L Microplex- Micromix EDTA and 0.04 X of Kelp P Max Bio Power seed weed extract.

2.3.5. Agroinoculation and symptom scoring

Agrobacterium transformed with the C2 mutants and V2 swap were cultured in LB agar plates that contained 100 µg/ml rifampicin and 100 µg/ml kanamycin for 48 hours at 28 °C. Six tomato cv. Rooikhaki plants were inoculated using the agro-prick method outlined by Urbino *et al.*, 2008) at the two leaf stage. Plants were inoculated after 18 days post germination. The plants were pricked three times from the shoot apical meristem going down towards the roots with a sterile needle. Six plants were inoculated, and three biological replicates were performed for each construct. As a positive control, plants were inoculated with *A. tumefaciens* the wild-type infectious clones of ToCSV_V30 and ToCSV_V22 and plants were inoculated with *A. tumefaciens* with an empty pCambia 2300 were used as negative controls. The disease severity index (DSI) described by Friedmann *et al.*, (1998) was used to measure symptom severity in inoculated plants on a daily basis. The scale used contained the following score: 0 -no visible symptoms, inoculated plants show same growth and development as non-inoculated plants; 1- slight yellowing of leaflet margins mainly on apical leaves; 2- some yellowing and minor leaf curling at the edges and margins of the leaves; 3- wide range of leaf yellowing, curling and cupping, plant size reduction; 4-very severe plant stunting and yellowing, pronounced leaf cupping and curling, and plant ceased to grow. Symptoms scoring and plant height was measured for all plants at 14 days post inoculation (dpi), 21 dpi and 28

dpi. Two leaves (below the apical meristem the shoot) were also collected at the times mentioned above.

2.3.6. DNA extraction

DNA from collected plant material was extracted for each time point using the CTAB method as described by Doyle & Doyle, 1987. Liquid nitrogen was used to grind approximately 50 mg of leaf material. The ground up samples were resuspended in 2% CTAB and 0.2% v/v β-mercaptoethanol and incubating the mixture at 65°C for 30 min. The top aqueous layer containing total nucleic acid (TNA) was extracted with chloroform: isoamyl alcohol (24:1), followed by inverted mixing and centrifugation at maximum speed at 4°C for 10 min. The top aqueous layer was placed into a new tube and resuspended in chloroform: isoamyl alcohol and the centrifugation step was repeated. Isopropanol was added to the newly extracted TNA to precipitate the nucleic acids and tube was gently inverted until DNA was precipitated. This was followed by centrifuging at maximum speed at 4°C for 10 min and the supernatant was discarded. The pellet was treated with 70% ice cold molecular grade ethanol before being centrifuged at maximum speed for 10 minutes at 4°C, and the supernatant was disposed of. The wash step was repeated and was followed by air drying the pellet then resuspension in 50 µl TE buffer containing 20 µg/ml RNase A. The DNA was incubated at 4°C overnight to allow for DNA resuspension. DNA integrity was checked by running 1 µg of the extracted DNA on a 1% agarose gel.

2.3.7. Determining viral load using quantitative polymerase chain reaction (qPCR)

To determine the viral load, DNA extracted from each sample was amplified using qPCR. The V1 gene (coat protein) was amplified using qPCR_V1 primers (**Table 2.1**). The reactions were performed in the LightCycler ® 1.5 Realtime system using Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix to a total volume of 20 µl. To the reaction, 10 µl Maxima SYBR green master mix, final concentration of 0.3 µM V1 primers (qPCR_V1), 2 µl of the extracted DNA and nuclease free water were added. The qPCR was conducted for 40 cycles. Initial denaturation occurred at 95 °C for 10 min, followed by denaturation at 95 °C for 15 s, annealing at 60 °C for 30 seconds and extension at 72 °C for 30 s. The qPCR reactions were normalized using *actin 7* primers (Bokhale et al., 2020).

Table 2-1: Primers used to amplify V1 gene to determine viral load

Primer name	Primer sequence	Fragment size
qPCR_V1F	5'- ACTTCGACAGCCCGTTCACCAG- 3'	140 bp
qPCR_V1R	5'-AGCCCTGATGTCCCTCGAGGTT-3'	

For the standard curves, the ToCSV_V30 and ToCSV_V22 infectious clones were digested using HindIII and SmaI to linearize the DNA using Thermo Scientific™ FastDigest protocol, respectively. The linear plasmid was run on a 1% agarose gel then gel extracted using Thermo Scientific GeneJET Gel Extraction Kit. The extracted DNA was diluted 10-fold from a starting concentration of 10 ng to 1 pg. The standard curve was constructed using qPCR_V1 primers

and conditions and the primer efficiency was calculated using the slope. Relative fold change was calculated using the crossing point method (Pfaffl, 2001) and the following equation:

Equation 2.3.7-1

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta \text{CP}_{\text{target}}(control-sample)}}{(E_{\text{ref}})^{\Delta \text{CP}_{\text{ref}}(control-sample)}}$$

Where E= efficiency of amplified gene

Target= V1

Ref= *actin 7* gene

ΔCp= difference in Cp values of control (healthy) and Cp of treatments (mut 7, mut 9, mut 10, V2 swap, ToCSV_V22 and ToCSV_V30)

2.3.8. Statistical analysis

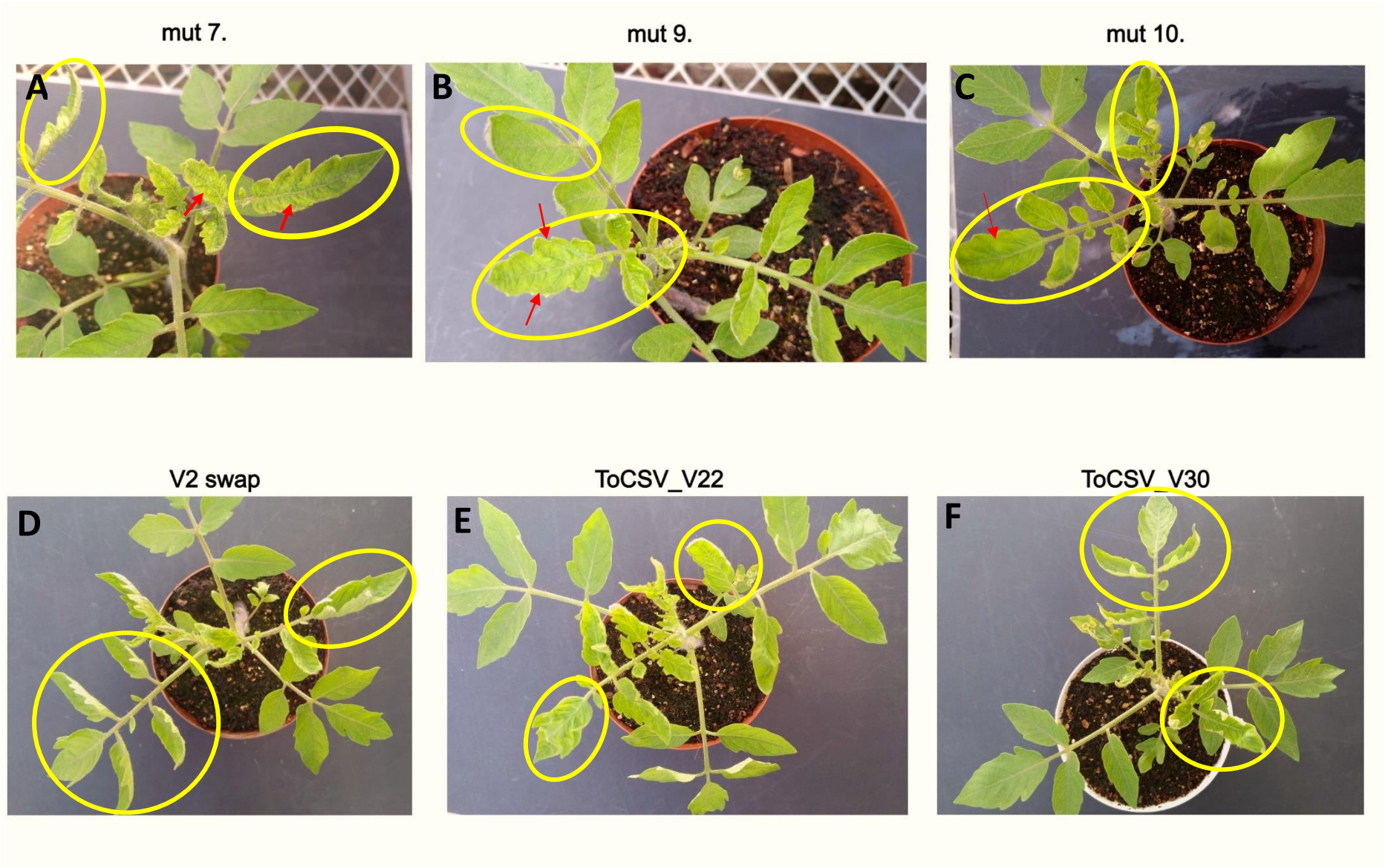
A two-way analysis of variance (ANOVA) was performed using the relative expression ratios from qPCR analysis to determine statistical significance between the treatments, and between treatments and controls. Significant differences between infections were calculated with $\alpha = 0.05$ (*).

2.4. Results

2.4.1. Symptoms scoring and height evaluation

The putative roles of C2 and V2 proteins were investigated through mutations and a ORF swap. The C2 mutants were constructed by substitution of amino acids in the severe ToCSV variant V30 with the corresponding aa from the mild ToCSV_V22 (Figure 2.1-A). The V2 swap was constructed by swapping out the V2 ORF between the ToCSV_V30 and V22 (Figure 2.1-B). Plants inoculated with the mutants and V2 ORF swap showed severe symptoms similar to plants inoculated with ToCSV_V30 infectious clone. At 14 dpi, most plants did not show any symptom development, however, from 17 dpi (results not recorded) plants began to develop symptoms, mainly slight leaf yellowing at the edges of leaves (DSI 1). By 21 dpi, there was yellowing and minor leaf curling at the edges and margins of the leaves of plants inoculated with mutants (DSI – Mut 7= 2.6, mut 9= 2.0 and mut 10= 2.4) and V2 swap (DSI= 2.5) as shown in Figure 2.2**.** The same symptoms were observed on plants inoculated with ToCSV_V30 (Figure 2.2-F). Plants inoculated with ToCSV_V22 showed minor leaf curling (Figure 2.2-E)**.** At 28 dpi, infections showed a wide range of leaf yellowing, curling, and cupping symptoms on plants inoculated with mut 7, mut 9, mut 10, V2 swap and ToCSV_V30, while plants inoculated with ToCSV_V22 showed mild leaf yellowing and mild lead curling (Figure 2.3**).** Infection with both ToCSV_V30 and ToCSV_V22 caused stunting, with ToCSV_V30 infections causing more

severe stunting (Figure 2.**4**). Mutants (mut 7= 175.6 mm; mut 9= 151.7 mm; mut 10=173.9 mm) and V2 swap (178,9 mm) gave similar height measurements to ToCSV_V30.Symptom scoring of infected plants showed that C2 mutants (DSI – Mut 7= 3.1, mut 9= 2.6 and mut 10= 3.1) and V2 swap (DSI= 3.1) was similar to the scoring of symptoms of plants infected with ToCSV_V30 (DSI=3.2) as shown in Figure 2.5. ToCSV_V22 showed lower symptom scoring (DSI=2.2) when compared to ToCSV_V30 (DSI=3.2) at 28 dpi (Figure 2.5). Infection of plants with ToCSV_V30 did cause slight stunting. Plants infected with ToCSV_V30 had an average height of 167.8 mm by 28 dpi, whereas plants infected with ToCSV_V22 had an average height of 189.4 mm. This average plant height was compared to non-infected plants which had an average plant height of 211.2 mm. ANOVA analysis using *p*-value of 0.05 indicated that symptom scoring, and height were not significantly different between the mutants and wild type infections, respectively.


mut 7.
mut 9.
mut 10.
A
B
C
V2 swap
ToCSV_V22
ToCSV_V30
D
E
F

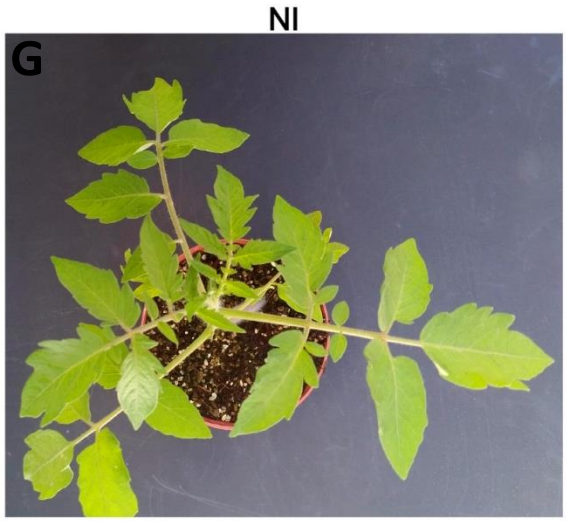
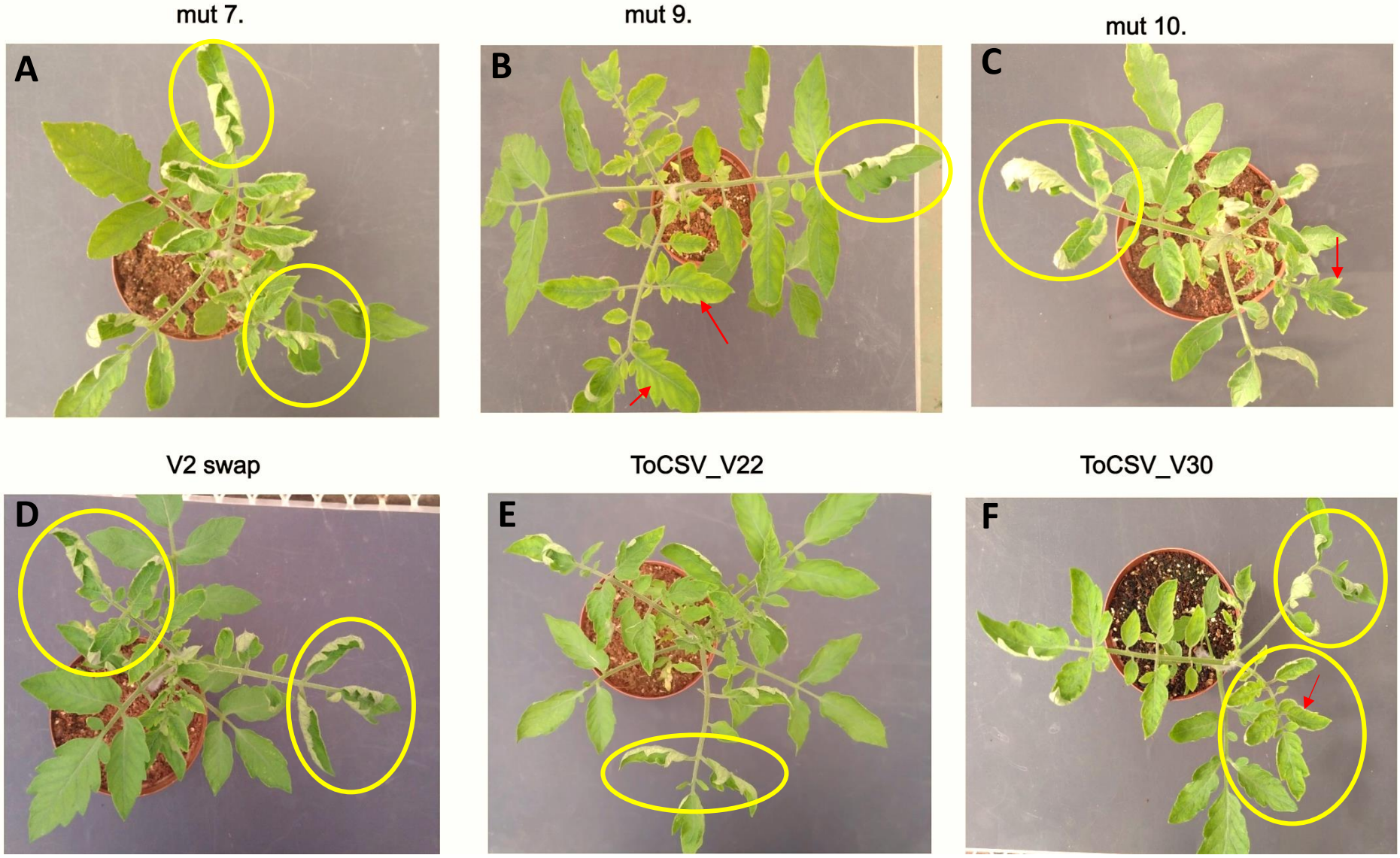


Figure 2.2: Symptoms induced in tomato cv. Rooikhaki infected with C2 mutants and V2 swap at 21 dpi. Leaf yellowing (red arrows) and minor leaf curling (yellow circles) symptoms were induced by **A)** mut 7, **B)** mut 9, **C)** mut 10 and **D)** V2 swap. These symptoms were similar to plants inoculated with **F)** ToCSV_V30**.** Yellowing can be seen mostly around the edges and margins of the leaves. Plants inoculated with **E)** ToCSV_V22 showed minor leaf curling. Plants infected with ToCSV_V22 and ToCSV_V30 were used as positive controls.


mut 7.
A
mut 9.
B
mut 10.
C
V2 swap
D
ToCSV_V22
E
ToCSV_V30
F

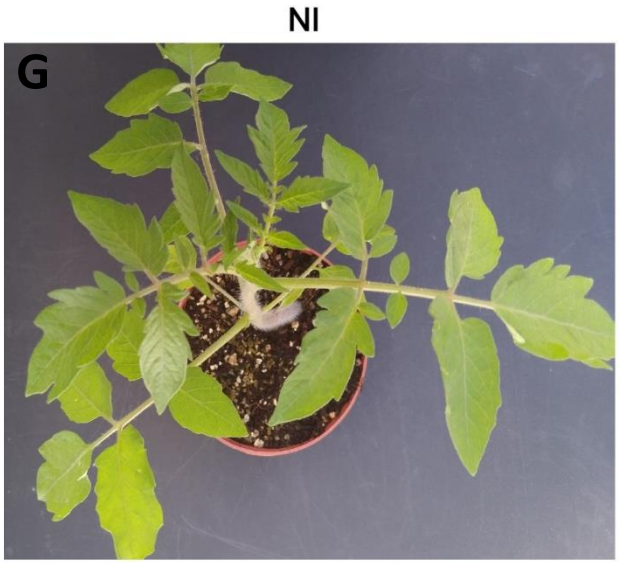


Figure 2.3: Infection of tomato cv. Rooikhaki with C2 mutants, V2 swap and infectious clones at 28 dpi. Plants inoculated with A) mut 7, B) mut 9, C) mut 10, D) V2 swap and F) ToCSV_V30 at 28 dpi showed a wide range of leaf yellowing (red arrows), curling, and cupping (yellow circles). Plants inoculated with E) ToCSV_V22 showed mild leaf yellowing and mild lead curling (yellow circles).

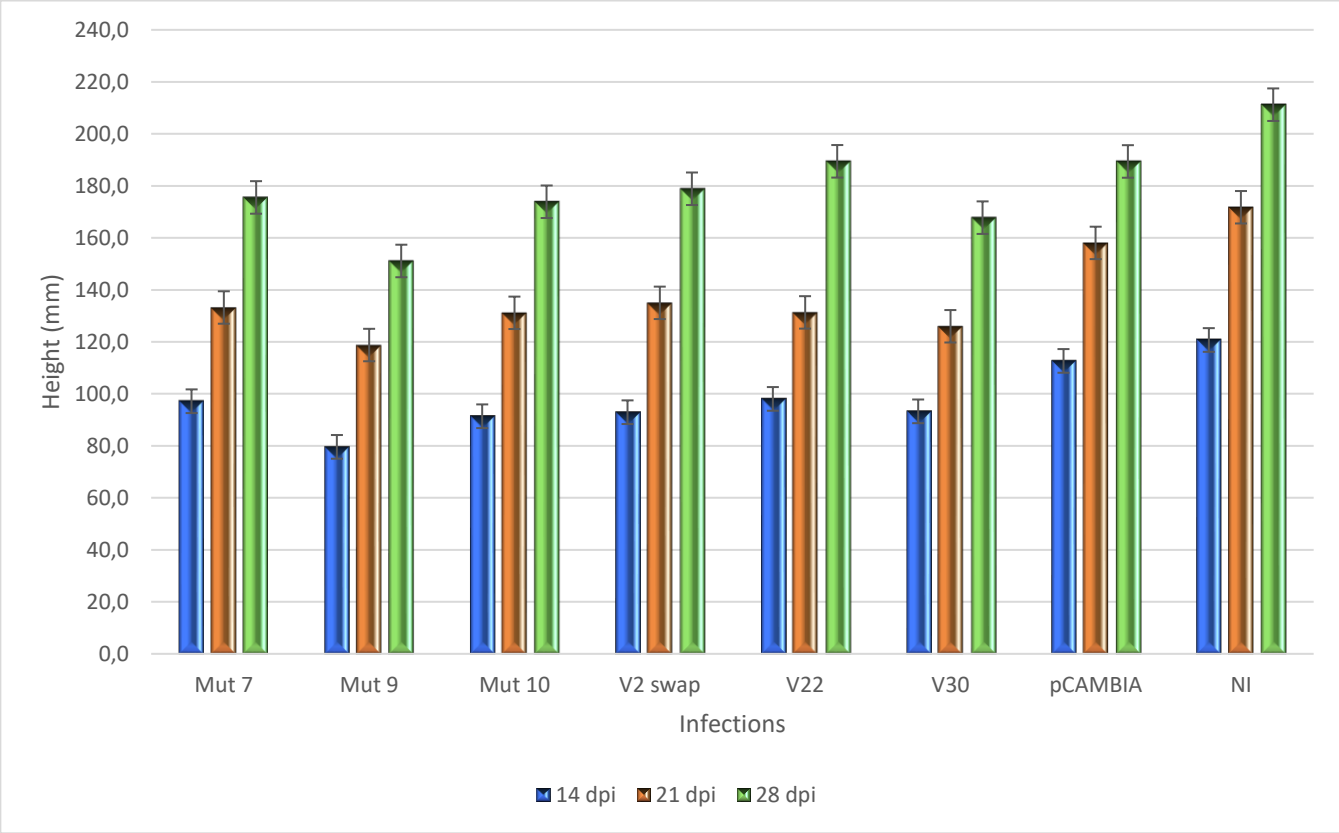


Figure 2.4*:* Height of tomato plants record at each time interval. Heights were measured at each time point for each treatment to determine the effect of the infections of plant growth. Plant height was measured to indicate stunting caused by infection of plants with C2 mutants, V2 swap and wild type infectious clones when compared to non-inoculated plants**.**

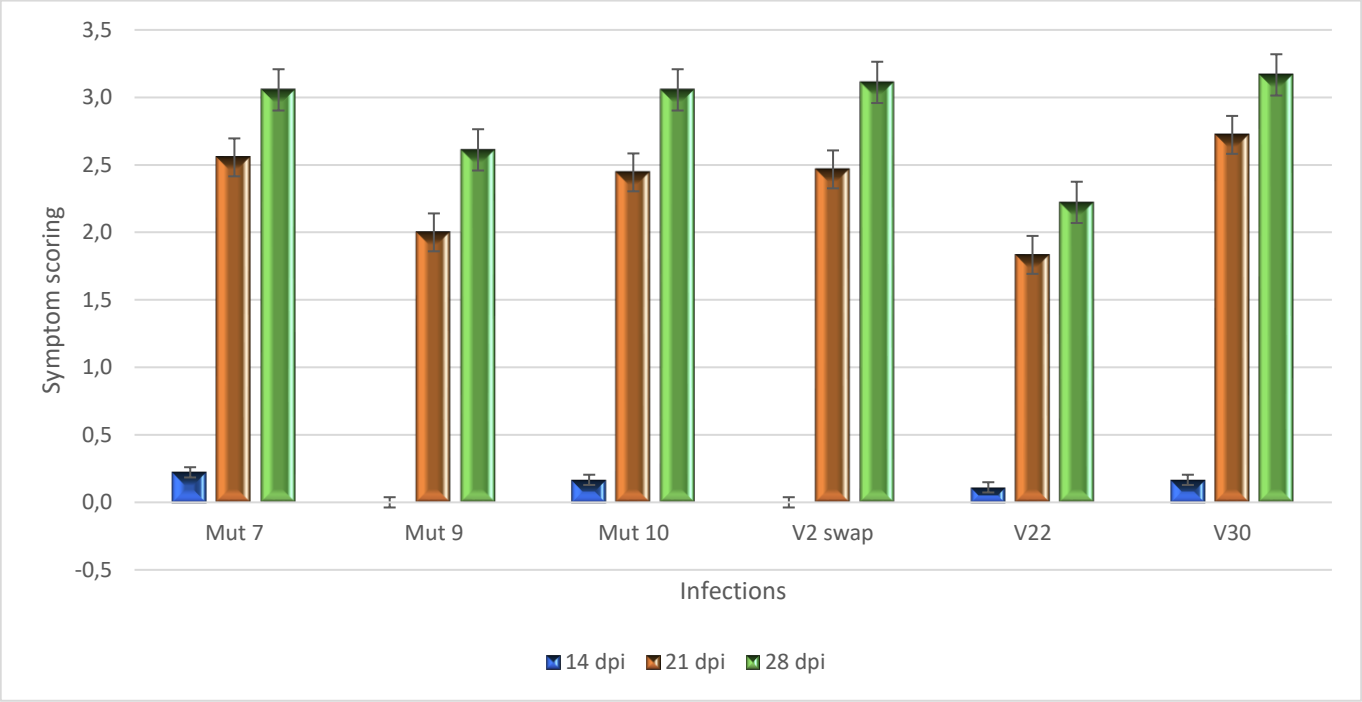


Figure 2.5: Symptom scoring of infected plants. Symptoms observed on infected plants were scored using disease severity index (DSI) published by Friedmann et al., 1998) which range from a score of 0 (no symptoms observed) to 4 (severe plant stunting and yellowing, prominent leaf cupping and curling, and plant ceased to grow). The average symptoms scores of C2 mutants and V2 swap at each time point was compared to the average symptom score of wild type infectious clones. Symptoms were scored at 14, 21 and 28 dpi.

2.4.3. Relative viral load

The relative viral load change was determined at each time point using qPCR and normalized against *actin 7* gene. A standard curve was initially constructed using linearized ToCSV_V30 and ToCSV_V22 infectious clones at different concentrations (Figure 2.6). One of the standard concentrations was always included in each run to determine the viral load.

The viral load change was obtained using the Pfaffl method based on the efficiencies and delta C_p values of the target gene in comparison to the reference gene, (Pfaffl, 2001). Efficiencies obtained from standard curves for gene specific primers and reference gene primers were used (Figure 2.6**).** The viral load change was graphed as shown in Figure 2.7. At 14 dpi, viral DNA accumulation showed a 21.3% decrease for mut 7 and a 95.2% decrease for V2 swap when compared to ToCSV_V30. However, there was a 58% and 138% (1.38-fold) increase in viral DNA for mut 9 and mut 10, respectively. At 21 dpi, the viral load spiked up for the mutants and V2 swap. Viral DNA increased by 438% (4.38-fold) for mut 7, 444% (4.44-fold) for mut 9, 368% (3.68-fold) and 508% (5.08-fold) for V2 swap when compared to ToCSV_V30. This significant increase was due to a decrease in relative fold change of viral DNA accumulation in ToCSV_V30 at 21 dpi. At 28 dpi, there was a 60% (0.601-fold), 24% (0.24-fold), 58% (0.58-fold) and 26 % (0.26-fold) increase in viral load of plants infected with mut 7, mut 9, mut 10 and V2 swap, respectively, when compared with plants infected with the wild type ToCSV_V30. These results show a higher viral load in mutants and V2 swap when compared to wild-type variants. However, the viral load between ToCSV_V30 and ToCSV_V22 were similar, with ToCSV_V30 showing a 14% decrease in viral load when compared to ToCSV_V22.

An ANOVA analysis was performed to determine whether there was a stastical significance in viral load between each mutant (C2 mutant and V2 swap) and the wild-type variants (ToCSV_V30 and ToCSV_V22). The ANOVA results indicated that viral load change was not significant between any of the mutants and wild-type variants. When comparing the viral load of mut 7, mut 9, mut 10 and V2 swap to the viral load of ToCSV_V30, there is a 95%, 71%, 92% and 91% probability, respectively, of seeing a difference in viral load in this study. When comparing the viral load of mut 7, mut 9, mut 10 and V2 swap to the viral load of ToCSV_V22, there was a 63%, 89%, 67% and 70% chance, respectively, of observing a change in viral load.

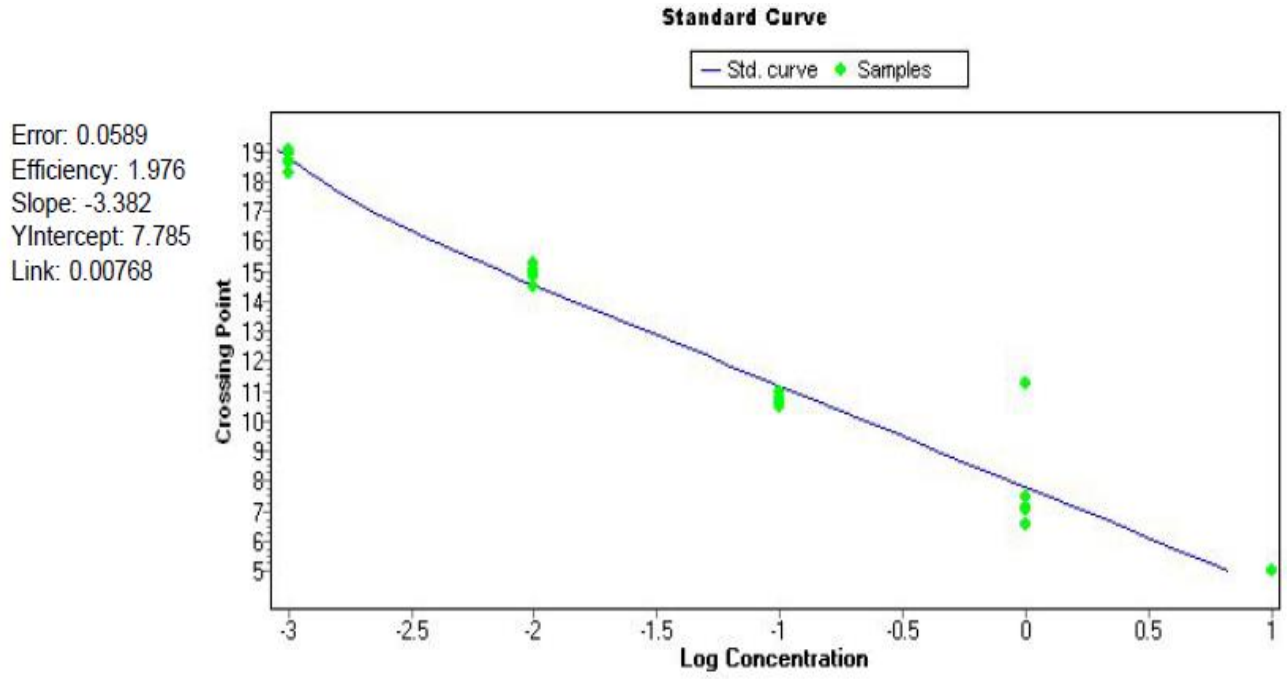


Figure 2.6: Standard curve of ToCSV_V30 and ToCSV_V22 plasmid using V1 primers. ToCSV_V30 and ToCSV_V22 infectious clones were digested and serially diluted 10-fold from 10ng to 1pg. Diluted plasmids were used to construct a standard curve using the LightCycler II 480 software. Primer efficiency was determined using the slope of the curve.

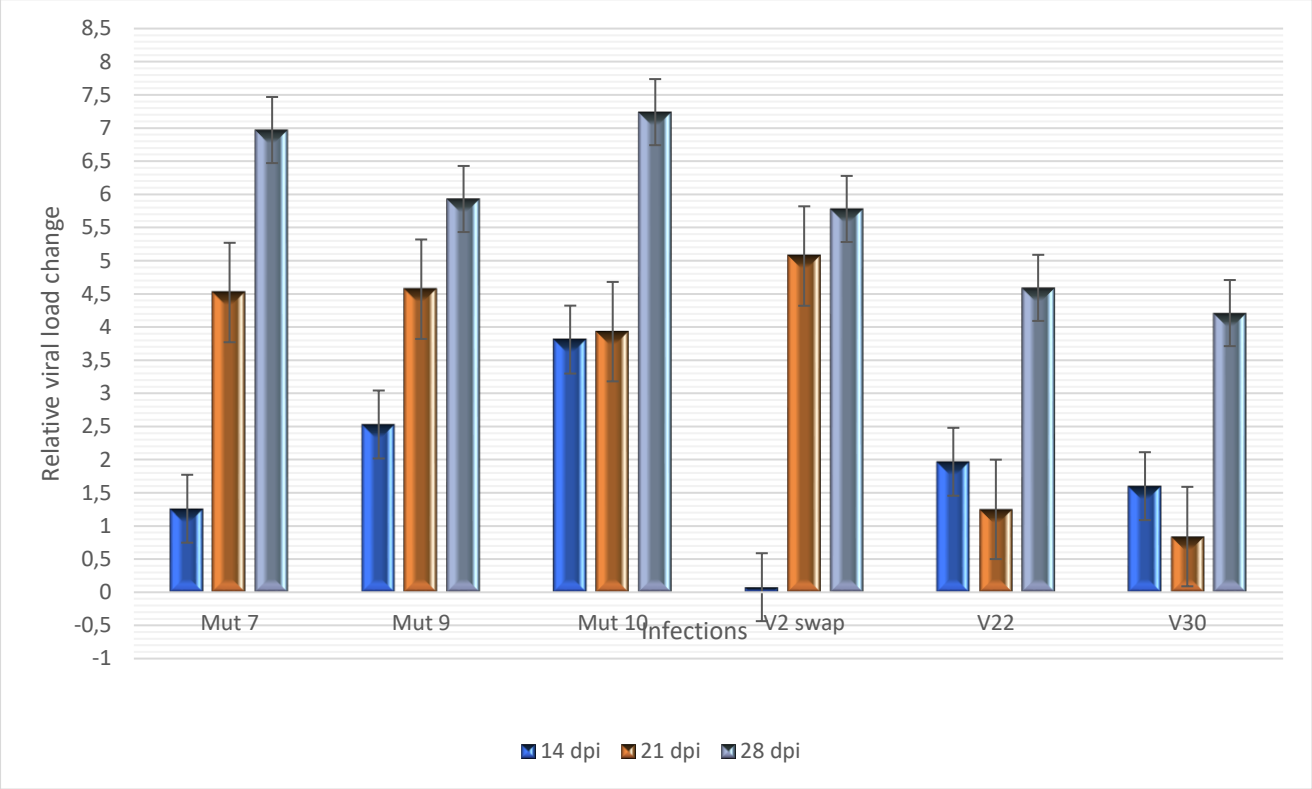


Figure 2.7: Relative viral load of C2 mutants and V2 swap compared to wild-type V22 and ToCSV_V30.The viral load change was calculated using the Pfaffl method based on the efficiencies and delta Cp values of the target gene (V1) in comparison to the reference gene (actin 7). Expression values are represented on a log 10 scale. Significant differences calculated with $\alpha = 0.05$. Error bars show standard error.

2.5. Discussion

Genetic variation in viruses is mostly caused by mutations that occur during replication (Yang et al., 2017). Site-directed mutagenesis has become an important tool in the analysis of gene structure and function (Kunkel, 1985; Liu & Lomonossoff, 2006). In this study, single-site mutations have been produced in regions of the C2 ORF sequences and a chimeric infectious clone was produced by swapping the V2 region of ToCSV_V30 with that of the ToSCV_V22. Tomato plants infected with these constructs were used to determine the effects of the infectivity on symptoms induced when compared to infectivity of wild type ToCSV_V30 and ToCSV_V22.

Mutated V2 ORF of ToLCV- India has shown the importance of V2 in accumulation of viral DNA and the development of symptoms in infected plants (Padidam et al., 1996). This was shown by low levels of viral DNA which was associated with very mild or no symptoms. In our study,

there was no significant difference in symptoms induced by the chimeric infectious clone (V2 swap), ToCSV_V30(V22V2$_{aa1-116}$), when compared with symptoms induced by ToCSV_V30. However, the swap induced more severe symptoms in comparison to ToCSV_V22. Relative viral load indicated that the viral load of the swap was 5-fold and 3-fold higher at 21 dpi when compared to ToCSV_V30 and ToCSV_V22, respectively. Viral load for the swap was 0.37-fold and 0,25-fold higher at 28 dpi compared to ToCSV_V30 and ToCSV_V22, respectively. ToCSV_V30 and ToCSV_V22 accumulated similar levels of viral DNA, and these results were similar to those reported by Esterhuizen (2012). The results from this experiment suggested that the entire V2 region exchanged between ToCSV_V30 and ToCSV_V22 did affect the replication of viral DNA and that it is associated with symptom severity, however, a specific domain within the V2 protein may be responsible for symptom development and viral DNA accumulation. A mutational study performed on TYLCV, which had a Cysteine85 (C85S) mutation integrated into the TYLCV infectious clone caused a delay in symptom development and showed a reduction in viral DNA accumulation in both tomato and *N.benthamiana* (Zhao et al., 2020). These findings suggested that the C85 domain of the V2 protein is critical in viral systemic infection. *Nicotiana benthamiana* plants inoculated with a mutated V2 of tomato leaf curl Java virus-A (ToLCJV-A[ID]) cloned into a PVX vector developed very mild chlorotic lesions and mosaic symptoms when compared to plants infected with a non-mutated V2, which resulted in mild symptoms(Sharma & Ikegami, 2010). These results suggested that V2 is a symptom determinant (Sharma & Ikegami, 2010). Deletion mutants were generated in the N-terminal and C-terminal of V2 of ToLCJV-A. The N-terminal mutants induced systemic symptoms and hypersensitive response (HR) however, the C-terminal mutant did not induce any HR symptoms. This demonstrated that a domain with 58 amino acids of the C-terminal of V2 is important in inducing HR (Sharma & Ikegami, 2010). Further deletion studies of V2 showed that specific domains of the C-terminal are important for inducing HR while others are required for severity of HR (Mubin et al., 2010). This suggests that symptoms induced by V2 may be dependent on the virus and the host plant. Further mutant studies of the V2 ORF of ToCSV should indicate which domains within V2 are important in plant-virus interactions contributing to symptom severity between both variants.

The C2 protein is conserved between all monopartite begomoviruses. The expression of late genes, CP and NSP, during viral replication is up-regulated by C2, which is required for infections by production of virions and symptom development (Guerrero et al., 2020; Mubin et al., 2010). The C2 protein, TrAP, has been shown to function as transcription factor necessary for the expression of the CP by up-regulating the CP promoter in mesophyll cells and down-regulating it in vascular tissue (Sunter & Bisaro, 1997). DNA binding assays have shown that C2 of tomato golden mosaic virus (TGMV) and TYLCV is required for nonspecific binding of ssDNA and binds weakly to dsDNA (Hartitz et al., 1999; Noris et al., 1996). However, C2 mutants of TYLCV have been shown to accumulate higher levels of dsDNA and lower levels of ssDNA than infections with the wild-type virus. The increased accumulation of TYLCV dsDNA was similar to that produced by V2, suggesting that C2 is important in the activation of virion- sense gene expression (Dry et al., 1997; Dry et al., 2000). Mutations of the C2 gene of cotton leaf curl Kokhran virus (CLCuKoV) induced no symptoms in *N. benthamiana*, which

also resulted in low levels of viral DNA (Iqbal et al., 2012). In our study, infection with C2 mutants developed symptoms that were equivalent to ToCSV_V30. The C2 mutants accumulated higher levels of DNA at 21 dpi when compared to the wild-type virus variants. Relative viral load of mut 7, mut 9 and mut 10 was 2.3-, 2.7-, and 2-fold, respectively, higher than ToCSV_V22 at 21 dpi, whereas fold change for the mutants were 4.38, 4,4 and 3.7-fold higher than ToCSV_V30. At 28 dpi a slight change difference was observed for mutants and wild-type virus variants. The relative viral load of mut 7, mut 9 and mut 10 was 0.6-, 0.2-, and 0.58-fold, respectively, higher than ToCSV_V30 at 28 dpi. This suggests that the specific amino acids in the C2 region studied did affect viral DNA replication and are required for symptom severity in context of the severe ToCSV_V30 background. It can be noticed that C2 mutants accumulated slightly more viral DNA than the V2 swap (mut 7-20,5%, mut 9-3,5% and mut 10-25% more viral DNA than V2 swap), indicating that C2 may be involved in expression of the V2 gene.

Viral load is not always necessarily associated with symptom severity. Pathogenicity of plant viruses may be caused by different interactions between the virus and specific components and pathways of the host. Replication and movement of the virus within the different host cells are important for the development of symptoms associated with the disease. The rate and extent of viral replication and interactions could be the basic determinants for the development of symptoms (Pallas & García, 2011), and the outcome is often host-virus specific. This could be seen in our studies, as viral load was not generally altered by symptom severity. Symptoms that developed from infection with the C2 mutants were similar to those produced by the severe ToCSV_V30. Although, ToCSV_V30 produced more severe symptoms when compared to symptoms with ToCSV_V22 infection, there was no significant fold change in the viral load. It would be interesting to determine how the interaction of C2 and V2 genes with host genes may be important in the role of disease development. Symptoms caused by C2 and V2 could also be affected by their interaction with other viral gene. Co-infiltration of TYLCV C2 gene with V2 and C1 in *N. benthamiana* showed that they can prevent local necrosis caused by C2(Matić et al., 2016). Therefore, V2 and C2 may interact with other viral genes which may affect their expression and these interactions could also affect the symptom severity.

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Chapter 3

The effect on pathogenicity of C2 by PVX-mediated expression in *Nicotiana benthamiana*

Abstract

The expression of viral proteins using viral vectors such as PVX allows for the identification of pathogenicity determinants and to describe additional functions of these viral proteins. The PVX vector (pGR107) causes very mild symptoms in *Nicotiana benthamiana*When a gene is inserted into the vector, controlled by the coat protein promoter, and expressed alongside the other PVX proteins during infection, this may induce a phenotype that differs from that which the PVX vector produces. In this study, the role of ToCSV C2 in pathogenicity was determined by overexpressing C2 gene of ToCSV variants using PVX expression system. The function of C2 in pathogenicity of mild and severe variants was investigated in *N. benthamiana*. Plants inoculated with PXV vector expressing C2_V22 enhanced viral symptoms in inoculated leaves at 7 dpi, however, plants inoculated with PVX vector expressing C2_V30 did not alter the symptoms, suggesting that C2_V22 is a pathogenicity determinant at early infection in *N. benthamiana*. Intriguingly, plants inoculated with C2 expressed in PVX vector from both V30 and V22 showed symptom recovery in systemic leaves at 14 dpi and 28 dpi. This suggests that C2 of ToCSV_V22 variant could be responsible for induction of severe symptoms elicited in *N. benthamiana*.

3.1. Introduction

Plant virus-based vectors are used to express genes from viruses or other sources in hosts. These virus-based vectors are mainly used for transient expression because they can multiply to high levels and thus lead to a high expression level of the genes under study (Lico et al., 2008; Porta et al., 1996). The development of plant-virus based vectors have been shown to be important in the production of biopharmaceutical proteins and in studying the function of plant genes by including virus induced gene silencing to negatively regulate specific host genes (Hefferon, 2014). The use of viral vectors also entails phenotypic evaluation of plant responses to transient gene expression (Porta et al., 1996).

Geminivirus transcriptional activation protein (TrAP) encoded by the C2 ORF is important for an effective interaction between the virus and host plants (Tu et al., 2017). TrAP is a transcription factor that is essential for the transcription of late genes CP and V2 for bipartite and some monopartite viruses, respectively (Briddon et al., 2014). TrAP of bipartite tomato golden mosaic virus (TGMV) is important in the expression of the coat protein (CP) promoter by activating the CP promoter in mesophyll cells and drepressing in vascular tissue (Sunter & Bisaro, 1997). Co-expression of monopartite V2 papaya leaf curl virus (PaLCuV) and cotton leaf curl Kokhran virus (CLCuKoV) in Nicotiana benthamiana with C2 revealed that C2 protein suppressed the hypersensitive response (HR) induced by V2, indicating the significance of C2

protein in transcriptional activation of TLCV virion-sense gene expression (Mubin et al., 2010). TrAP also affects the expression of host genes such as the upregulation of heat shock cognate 70 kDa protein (cpHSC70-1) by C2 protein, which is important in protein transportation into chloroplasts (Liu et al., 2014); induces plant genes required for cell-to-cell and long-distance movement of viruses (Liu et al., 2014). Two biological proteins, serine/threonine kinase (SNF1) and adenosine kinase (ADK), have been shown to interact with begomovirus tomato golden mosaic virus (TGMV), and curtovirus spinach curly top virus (SCTV) AC2 protein (Baliji et al., 2010). The AC2 Zn-finger domain mediates the initial contact, which inactivates SNF1 kinase, a crucial modulator of cellular metabolism involved in intrinsic antiviral defense, and hence increases vulnerability to DNA and RNA virus infection (Baliji et al., 2010; Sunter et al., 2001). The second interaction inactivates ADK, which may operate as an upstream activator of SNF1, implying that geminiviruses have developed a counter-defensive approach to deal with the intrinsic antiviral response (Baliji et al., 2010; Wang et al., 2005). It's also possible that AC2 inactivation of ADK suppresses silence indirectly by interfering with a general methylation mechanism that needs ADK (Jackel et al., 2015; Wang et al., 2005) and by degrading S-adenosyl-methionine decarboxylase 1 (SAMDC1) (Zhang et al., 2011). This was shown by the loss of suppression activity of Zn finger and acidic activation domain mutants, which are important in transcription activation of host genes. This shows that suppressor activity is linked to transcription factor activity (Trinks et al., 2005). TrAP was also shown to regulate the expression of miRNAs which are also important in the regulation of plant gene expression (Briddon et al., 2014). The TrAP protein of african cassava mosaic virus (ACMV), tomato yellow leaf curl virus (TYLCV) and cabbage leaf curl virus (CbLCuV) has been shown to interact with miR164 (Amin et al., 2011). miR164 suppresses the expression of several genes that encode NAC-like transcription factors. CUP-SHAPED COTYLEDON 1 (CUC1) and CUC2, which are expressed in meristems and developing organs, and are required for their development, are among these genes. If organ borders are not established, significant developmental effects result (Amin et al., 2011).

TrAP is also required for the suppression of host defense systems such as the suppression of jasmonate-mediated defense in Arabidopsis by tomato leaf curl Sardinia virus (TYLCSV) C2 protein (Lozano-Durán et al., 2011). More recently, infection with either TYLCV or papaya leaf curl China virus (PaLCuCNV) C2 directly was shown to interact with plant ubiquitin and disrupted the activation of terpene synthase genes, decreasing plant resistance to whiteflies (Li et al., 2019). C2 proteins have been shown to suppress PTGS i.e. AC2 proteins of ACMV and mungbean yellow mosaic Indian virus (MYMIV) have been shown to suppress PTGS (Kumar et al., 2015; Voinnet et al., 1999). While both AC2 of bipartite and C2 of monopartite can prevent PTGS from occurring, only AC2 can suppress systemic silencing by inhibiting the spread of silencing signals throughout the host (Guerrero et al., 2020). C2 of monopartite viruses, tomato leaf curl Java virus (ToLCJAV) and bhendi yellow mosaic virus (BYVMV) were shown to only suppress local silencing but could not inhibit systemic silencing (Gopal et al., 2007; Kon et al., 2007).

The transient expression of begomovirus genes in various viral vectors has been studied in plant hosts. The expression of TLCV *C2* gene using TMV-30B vector in *N. benthamiana* induces

necrotic lesions on the inoculated leaves. The expression of the gene can also spread systemically causing severe veinal necrosis (Selth et al., 2004). This leaf necrosis has also been shown in the expression of ACMV AC2 using the PVX expression vector (Hong et al., 1997). A similar reaction occurred with the infection of plants with the C2 of tomato yellow leaf curl virus-China (TYLCV-C) leading to cell death (Van Wezel et al., 2001), thus demonstrating that this protein has a pathogenic role. A study performed showed that the polyglutamine stretch of the TYLCSV C2 protein could play a role in cell death and plant defense (Matić et al., 2016).

3.2. Aim

The aim of this study was to determine the possible role of C2-encoded TrAP by both mild and severe variants of ToCSV in pathogenicity by overexpressing *C2* using a PVX expression vector, pGR107, in *Nicotiana benthamiana*.

3.3. Methodology

3.3.1. Construction of PVX constructs

The PVX expression vector (Jones et al., 1999) was used to design the constructs. The full length of C2 gene from both ToCSV_V22 and ToCSV_V30 were cloned into the MCS of PVX (pGR107, accession number: AY297842) using the restriction enzymes *SalI* and *SmaI*. The constructs were labelled as PVX/C2_V22 and PVX/C2_V30. Primers used for each gene can be found in Table 3.1

Figure 3.1: pGR107, a PVX-based viral vector described by Jones et al. (1999) : Left border (LB), CaMV 35S promoter, RNA dependent RNA polymerase (RdRp), triple gene box block (TGB), multiple clonig site (MCS) with ClaI, SmaI and SalI restriction sites, coat protein (CP), NOS terminator and right border (RB) (adapted from Lacorte et al, (2010))

The constructs were then transformed into *Agrobacterium tumefaciens* strain C58C1 (as described in Chapter 2) and grown on LB plates containing 100 mg/L kanamycin and 50 mg/L rifampicin. The plate was incubated for 48 hours at 28 °C for the selection of transformed cells. Single colonies were isolated from the plates and colony PCR was used to detect the presence of positive colonies. Positive colonies were cultured in liquid LB media containing 100 mg/L kanamycin and 50 mg/L rifampicin. The cultures were used to make glycerol stocks containing 50% glycerol and 500 µl of the culture. The glycerol stocks were stored at -80 °C.

Table 3.3-1: Primers used to amplify V2 and C2 genes from ToCSV_V22 and ToCSV_V30. Amplified fragments are then cloned into PVX (pGR107) using SacI and SmaI.

Primer name	Primer sequence	Fragment size
To12C2F/ SmaI	5'- TA*CCCGGG*ATGCGGTCTTCATCAC -3'	*408 bp*
To12C2R/SalI	5'- CT*GTCGAC*TTAAATACGCTTAAG -3'	

Underlined and italicized sequences represent the restriction sites.

3.3.2. Growing conditions for *N. benthamiana*

N. benthamiana wild type seeds were sown and germinated in jiffies (Jiffy Products S.L (Pvt) Ltd, Sri Lanka) in a tray, sealed with cling wrap, and grown at 25 °C with 16 hours of light and 8 hours of dark photoperiodism. When seedlings were three weeks old, they were transferred into pots with germinating mix made of soil (Culterra) and vermiculite (3:1). The pots were placed into deep trays, covered with cling wrap, and allowed to acclimatize for a week. Plants were agro- inoculated at the four to six leaf stage.

3.3.3. Agro-infiltration and symptom assessing

Acclimatized *N. benthamiana* plants were agro- inoculated at the leaves below the apical meristem. Prior to infiltration, agro stocks of constructs were grown in 5ml liquid LB containing 100 mg/L kanamycin and 50 mg/L rifampicin at 28 °C overnight. The cultures were centrifuged at 4200 xg for 8 min. The pellets were resuspended with infiltration buffer (composed of 10mM $MgCl_2$, 10 mM MES and 200 µM acetosyringone) until they reached an OD_{600} of 1. For two to three hours, the cultures were kept at room temperature. Two leaves below the apical meristem were inoculated with the PVX clones. Plants were agro- inoculated with 100 µl per plant. Four plants were used for each construct (PVX/C2_V22, PVX/C2_V30). Plants inoculated with empty PVX vector were used as negative controls and plants inoculated with ToCSV_V22 and ToCSV_V30 were used as positive controls. Symptom assessment and plant material collection were performed at 7, 14 and 28 dpi. Two leaves below the apical meristem were collected for RNA extraction.

3.3.4. RNA extraction and cDNA synthesis

Liquid nitrogen was used to grind approximately 50 mg of leaf material. The ground sample was resuspended in 1 ml of Tri Reagent (Sigma-Aldrich, St. Louis, USA) and allowed to sit for 10 minutes at room temperature. The sample was centrifuged at 13300 rpm for 15 min at 4°C and the supernatant transferred into a new tube. Chloroform was added to the sample, mixed vigorously, and incubated for 3 min at RT. The sample was centrifuged at 13300 rpm for 15 min at 4°C, the supernatant was removed to a new tube and 500µl of isopropanol added to it. The sample was incubated at RT for 10 min then centrifuged at 13300 rpm for 15 min at 4°C. The supernatant was discarded, and the pellet washed with 75% ethanol, then centrifuged at 7500 g for 5 min at 4°C. The ethanol was removed, and the pellet was allowed to dry for less than 5 min, then resuspended in DEPC water containing RNase inhibitor.

Prior to cDNA synthesis 1µg RNA was treated with RNase-free DNase. To the 1µg RNA, 10X reaction buffer with $MgCl_2$, 1U DNase (RNase Free) and DEPC-treated water were added to a total reaction of 10 µl. The sample was incubated for 30 min at 37 °C. To the sample, EDTA was added and incubated at 65 °C. The treated RNA was used as template for reverse transcriptase (RT). The Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit was used to reverse transcribe the RNA according to the manufacturer's recommendations. To the cDNA sample, 100 pM Oligo $(dT)_{18}$ primer, 10mM dNTP, 5X RT buffer, 20 U/µl RiboLock RNase inhibitor, 200 U/µL RevertAid M-MuLV RT were added to a final reaction of 20 µl. The reaction was incubated at 42 °C for 60 min then terminated by heating at 70°C for 5 min. A RT minus (RT -) reaction, which excluded the RT enzyme, was used as negative control. This first strand cDNA synthesis was used as template directly in qPCR.

3.3.5. Quantification of gene expression using RT-qPCR

To determine the effect of the expression of C2 on the transcription of PVX, reverse transcriptase quantitative PCR (RT-qPCR) was performed. RT-qPCR was used to measure the expression levels of PVX. The reactions were performed in the LightCycler ® 1.5 Realtime system using Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix to a total volume of 20 µl. To the reaction, 10 µl Maxima SYBR green master mix, of 0.3 µM C2 primers (final concentration) (Table 3.2), 2 µl of the synthesized cDNA and nuclease free water were added. RT-qPCR was conducted for 40 cycles. Initial denaturation occurred at 95 °C for 10 min, followed by denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. C2 and PVX CP expression was normalized to the housekeeping elongation factor gene *Ef1α* (Liu et al., 2012).

Table 3.3-2: Primers used to determine C2 and PVX coat protein expression levels by RT-qPCR. Expression levels were normalized to the housekeeping gene *Ef1α*

Primer name	**Sequence**	**Fragment size**
qC2_F	TCCCAATCAAGGTCCAGCAT	124 bp
qC2_R	CGGTGCGTAAATCCATGGTT	
qPVX_CP_F	ATGCTGCCCAAACTGCTG	130 bp
qPVX_CP_R	GTGACAACAGCCTCAGCG	
Elf_F	AGCTTTACCTCCCAAGTCATC	116 bp
Elf_R	AGAACGCCTGTCAATCTTGG	

A standard curve was constructed prior to expression analysis. The standard curve was obtained by serial dilution of PVX/C2_V22, PVX/C2_V30 and PVX cDNA. The cDNA was diluted 10-fold from a starting concentration of 10 ng to 1 pg. The standard curve was constructed using C2 and PVX CP primers and conditions and the primer efficiency was calculated using

the slope. The relative gene expression was determined using the Ct values generated by LightCycler. The Ct values were used in the Pfaffl method to determine the relative gene expression ratio of target gene (PVX CP and C2) in comparison to a reference gene (*Elfα*) (Pfaffl, 2001).

3.3.6. Statistical analysis

A two-way analysis of variance (ANOVA) was performed on the relative expression ratios from qPCR analysis to determine statistical significance between the treatments, and between treatments and controls. Significant differences between infections were calculated with α = 0.05 (*).

3.4. Results

3.4.1. PVX constructs

To determine the role of C2 in pathogenicity in *N. benthamiana*, the wild-type C2 coding sequences from ToCSV_V30 and ToCSV_V22 were amplified by PCR and cloned as *SalI* and *SmaI* fragments into MCS of PVX expression vector (pGR107).

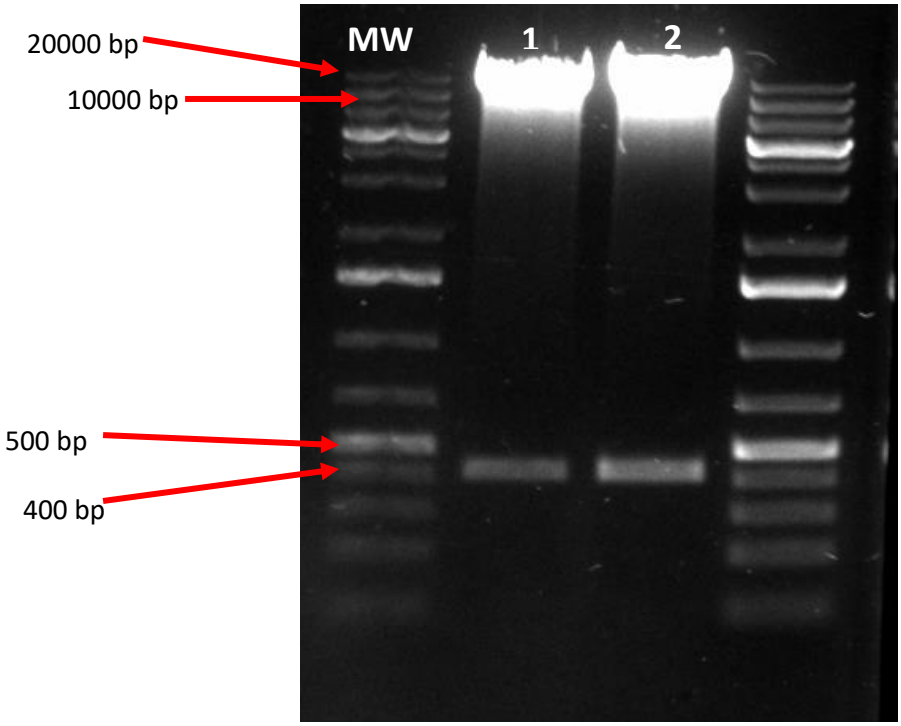

Figure 3.2: PVX expression constructs. Restriction digest confirmation of PVX clones. Fragments were inserted into PVX (10436 bp) with Sal1 and SmaI restriction sites. Restriction digest of PVX clones with C2 fragments (408 bp) amplified from ToCSV_V30 (lane 1), and ToCSV_V22 (lane 2). Molecular weight marker (MW) Thermo Scientific™ O'GeneRuler 1 kb Plus DNA Ladder.

3.4.2. Symptom evaluation

To determine whether C2 of ToCSV is a symptom determinant, Agrobacterium carrying PVX constructs were inoculated into *N. benthamiana* plants at the four to six leaf stage. *N. benthamiana* plants infected with PVX constructs resulted in the induction of symptoms by 7 dpi. Leaves inoculated with PVX/C2_V22 showed severe necrosis (red circle) and leaf curling (yellow circle) in subsequent leaves (Figure 3.3-A). Leaves inoculated with PVX/C2_V30 and PVX (no insert) showed very mild leaf curling, small veinal necrotic lesions and leaf chlorosis (blue arrows) (Figure 3.3- B and C). Plants infected with ToCSV_V30 and ToCSV_V22 showed no symptoms at 7dpi (Figure 3.3-D and E). By 14 dpi all plants infected with PVX, PVX/C2_V22 and PVX/C2_V30 showed symptom recovery in systemic leaves (Figure 3.4), however, leaves inoculated with PVX/C2_V22 showed cell death (yellow circle) and leaf curling and leaves inoculated PVX/C2_V30 continued to show mild necrosis and leaf curling. Plants inoculated with ToCSV_V22 (Figure 3.4-E) showed mild leaf curling and cupping, and vein swelling on systemic leaves (blue circle). Plants inoculated with ToCSV_V30 showed minor leaf curling and vein swelling (blue circle) (Figure 3.4-D). At 28 dpi (, plants inoculated with all the PVX constructs showed no symptom development in newly emerging leaves, showing complete recovery of plants from viral infection (Figure 3.5). Plants inoculated with ToCSV_V22 showed severe symptoms (Figure 3.5-E), including severe leaf curling and severe vein swelling (blue circle), while plants inoculated with ToCSV_V30 milder leaf curling (blue circle) and vein swelling (yellow arrow) (Figure 3.5-D**)**.

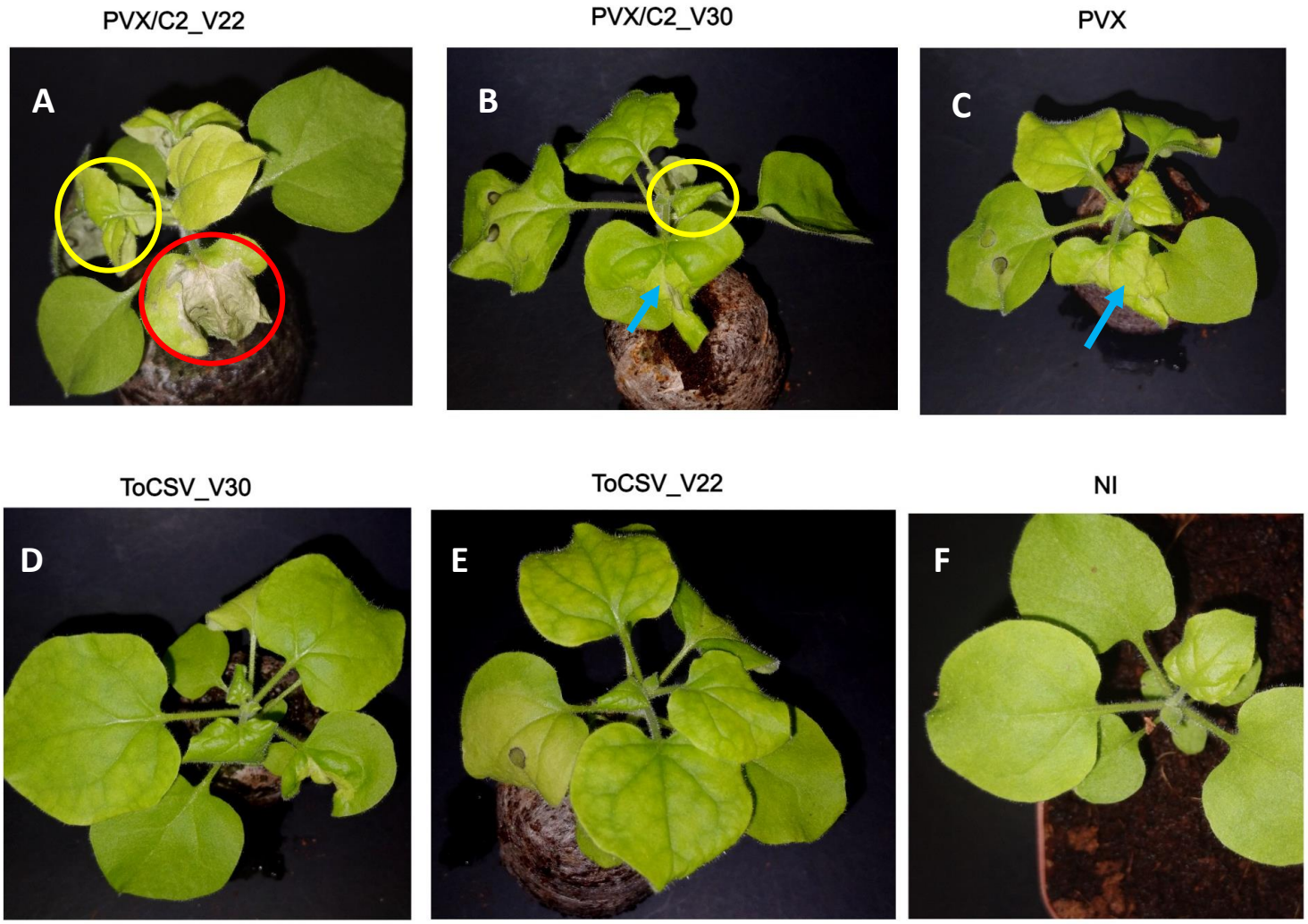
PVX/C2_V22
A
PVX/C2_V30
B
PVX
C
ToCSV_V30
D
ToCSV_V22
E
NI
F

Figure 3.3: Symptoms induced on *N. benthamiana* plants inoculated with PVX expressing C2 ORF at 7 dpi. Plants show symptoms induced by PVX expressing C2 ORF of ToCSV_V30 and ToCSV_V22. Plants inoculated with A) PVX/C2_V22 showed severe necrosis (red circle) on inoculated and leaf curling (yellow circle) on adjacent leaves. Plants inoculated with B) PVX/C2_V30 and C) PVX (no insert) showed very mild leaf curling on inoculated leaves and adjacent leaves (yellow circles, small veinal necrotic lesions and mild leaf chlorosis (blue arrows). Plants infected with D) ToCSV_V30 and E) ToCSV_V22 showed no symptoms. F) Healthy non-inoculated *Nicotiana benthamiana* plants

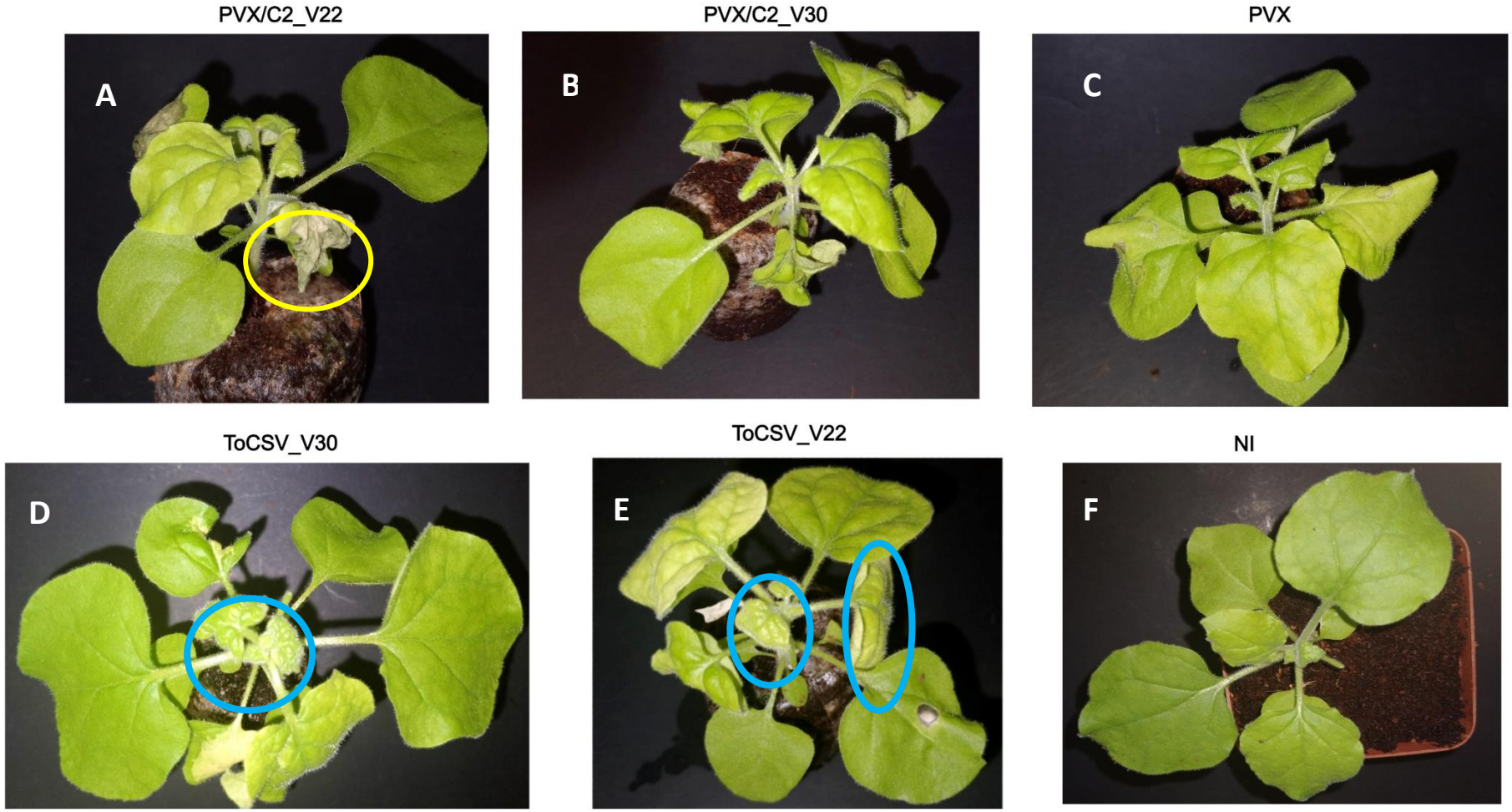

Figure 3.4 : Symptoms induced on *N. benthamiana* plants inoculated with PVX constructs at 14 dpi. At 14 dpi, systemic leaves showed no symptom development indicating recovery, however, the leaves inoculated with **A)** PVX/C2_V22 showed cell death and leaf curling (yellow circle) and leaves inoculated **B)** PVX/C2_V30 continued to show mild necrosis and leaf curling. Plants inoculated with **D)** ToCSV_V30 showed

minor leaf curling and vein swelling (blue circle) on systemic leaves. Plants inoculated with **E)** ToCSV_V22 showed mild leaf curling and cupping, and vein swelling (blue circle). F) Healthy non-inoculated *Nicotiana benthamiana* plants

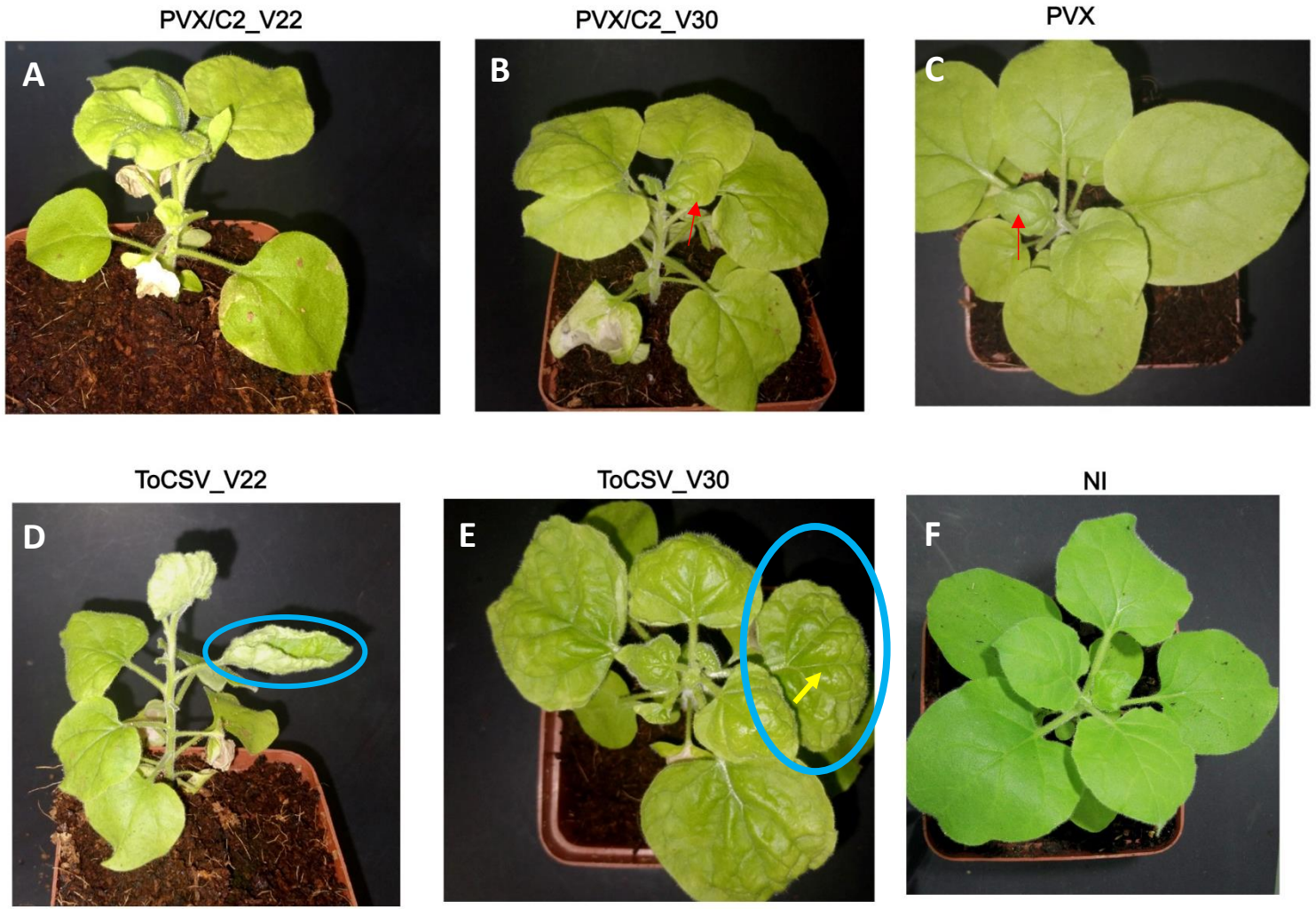

Figure 3.5: Symptom recovery at 28 dpi. Plants inoculated with **A)** PXV/C2_V22, **B)** PVX/C2_V30, **C)** showed no symptom development in newly emerging leaves (red arrows). Plants inoculated with **D)** ToCSV_V22 showed severe symptoms, including severe leaf curling and severe vein swelling (blue circle), while plants inoculated with **E)** ToCSV_V30 had milder leaf curling (blue circle) and vein swelling (yellow arrow). F) Healthy non-inoculated *Nicotiana benthamiana* plants

3.4.3. PVX coat protein and ToCSV C2 expression

To determine the PVX coat protein (CP) and C2 mRNA expression levels in inoculated plants, extracted RNA was used for qPCR analysis using PVX CP and C2 primers and normalized against *elongation factor α* (*Elfα*) gene. The qPCR efficiency was initially determined by constructing standard curves using PVX (Figure 3.6**)** and C2 (Figure 3.7) cDNA at different concentrations. The efficiencies obtained for PVX CP, C2 and Elfα were used in Equation 2.3.7-1**,** Pfaffl method (Pfaffl, 2001) (Chapter 2) to calculate the relative expression of PVX CP (efficiency of 1.972 with a slope of -3.390) and C2 (efficiency of 2.055 with a slope of -3.196) mRNA using non-inoculated wild type *Nicotiana benthamiana* as the calibrator.

To determine whether the presence of C2 has an effect on PVX mRNA accumulation, qPCR analysis was performed to examine the expression levels of PVX CP. The PVX mRNA expression values obtained from plants inoculated with PVX/C2 constructs were compared to expression values of plants inoculated with PVX only (Figure 3.8-A). At 7dpi, plants inoculated with PVX/C2_V22 showed a 109,3% (1,9-fold) increase in PVX CP expression when compared to plants inoculated with PVX only and plants inoculated with PVX/C2_V30 showed a 99% (0,99-fold) increase in PVX CP mRNA expression when compared to plants inoculated with PVX only. By 14dpi, an increase in PVX CP mRNA expression in plants inoculated with PVX/C2_V22 and PVX only could be observed, however, there was no change in PVX CP mRNA expression in plants inoculated with PVX/C2_V30 when compared at 7 dpi. Plants inoculated with PVX/C2_V22 showed a 54% (0,54-fold) increase in PVX CP mRNA expression when compared to plants inoculated with PVX only and plants inoculated with PVX/C2_V30 showed a 14% (0,14X) increase when compared to plants inoculated with PVX only. These results indicate that C2 from both ToCSV_V22 and ToCSV_V30 are virulence factors that increase the replication of PVX at 7 dpi and 14 dpi.

Analysis of C2 mRNA levels in inoculated plants revealed that C2 was expressed by the PVX vector in PVX/C2 constructs. The C2 mRNA expression values from plants inoculated with PVX/C2 constructs were compared to expression values of plants inoculated with PXV only, ToCSV_V22 and ToCSV_V30 (**Figure 3.8-B**). At 7dpi, C2 mRNA was not detected in plants inoculated with PVX only (negative relative expression values), however C2 was detected in plants inoculated with PVX/C2_V22, PVX/C2_V30, ToCSV_V22 and ToCSV_V30. The expression levels showed that plants inoculated with PVX/C2_V22 had a 14,7% (0.,147-fold) decrease in C2 mRNA levels when compared to plants inoculated with ToCSV_V22 and with PVX/C2_V30 had a 18,9% (0,189-fold) decrease in C2 mRNA levels when compared to plants inoculated with ToCSV_V30. By 14 dpi, the C2 expression levels in plants inoculated with PVX/C2_V22 showed a 69,5% (0.695-fold) increase when compared to plants inoculated with ToCSV_V22, however, there was a decrease in expression levels for C2 mRNA in plants inoculated with PVX/C2_V30. This decrease showed a 13,6% reduction in C2 expression levels when compared to plants inoculated with ToCSV_V30. At 28 dpi, there was a decrease in C2 expression levels in plants inoculated with PVX/C2_V22 and an increase in plants inoculated with PVX/C2_V30 when compared to expression levels at 14 dpi. Plants inoculated with PVX/C2_V22 showed a 19,8% (0,198-fold) decrease in C2 expression when compared to plants inoculated with ToCSV_V22 and there was a 28,9% decrease in plants inoculated with

PVX/C2_V30 when compared to plants inoculated with ToCSV_V30. It can also be observed that plants inoculated with PVX/C2_V22 expressed higher levels of C2 mRNA compared to plants inoculated with PXV/C2_V30.

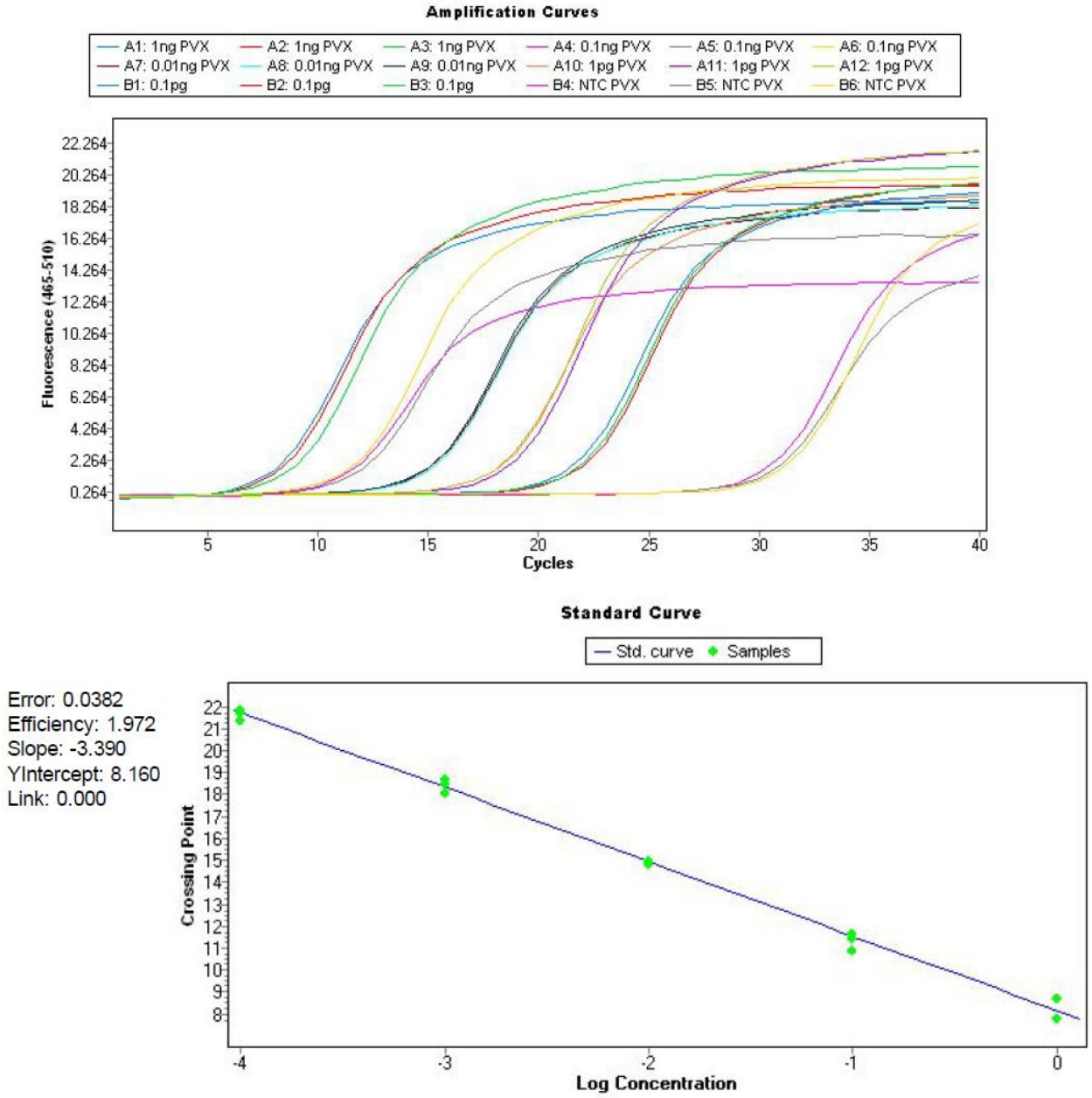

Figure 3.6: Standard curve for PVX coat protein expression. PVX cDNA was serially diluted 10-fold. Diluted cDNA was used to construct a standard curve using the LightCycler II 480 software. Primer efficiency was determined using the slope of the curve. Standard curve obtained a PCR efficiency of 97.2% and a slope -3.390.

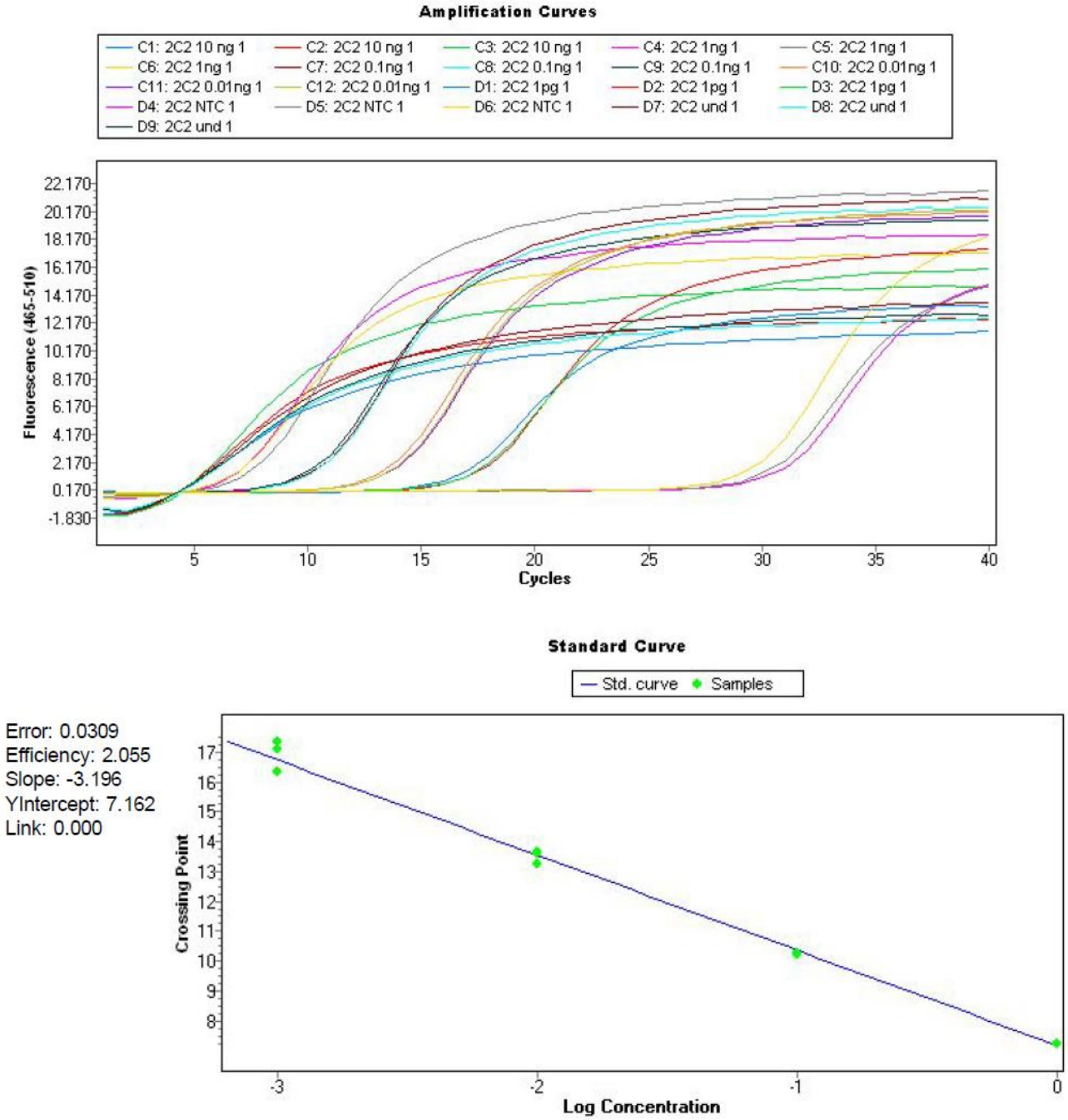

Figure 3.7: Standard curve for C2 expression. C2 cDNA was serially diluted 10-fold. Diluted cDNA was used to construct a standard curve using the LightCycler II 480 software. Primer efficiency was determined using the slope of the curve. Standard curve obtained a PCR efficiency of 105.5% and a slope -3.196.

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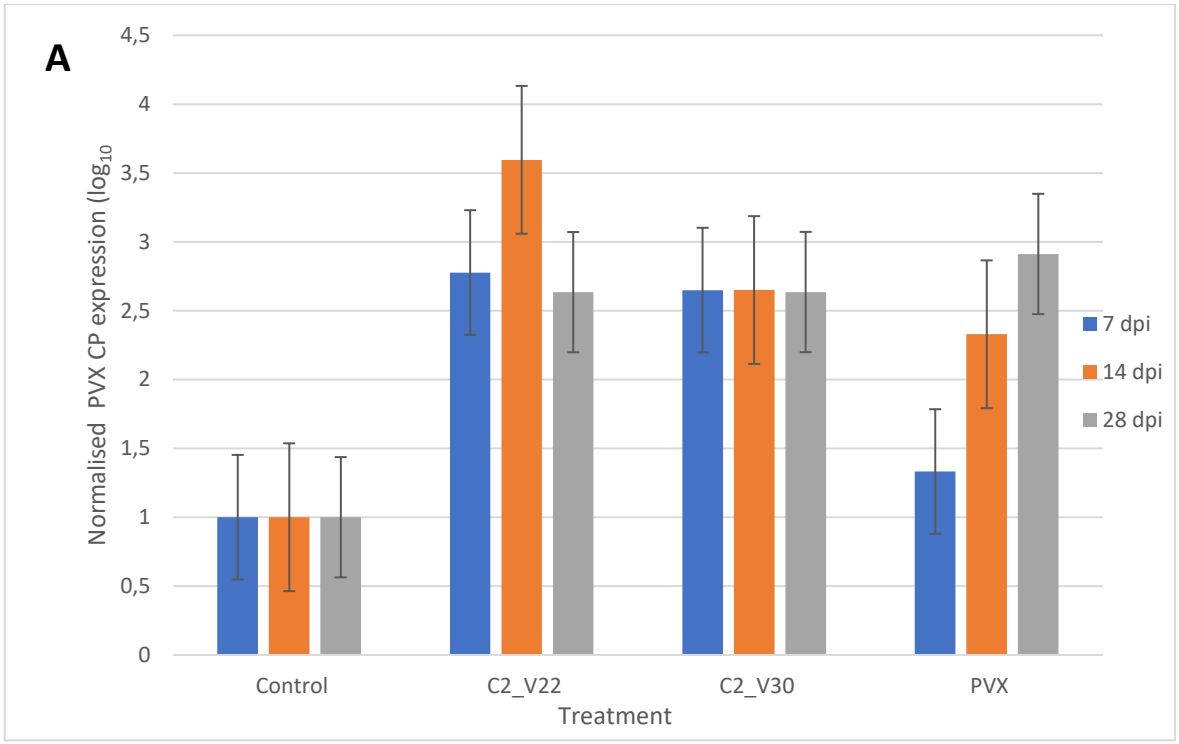

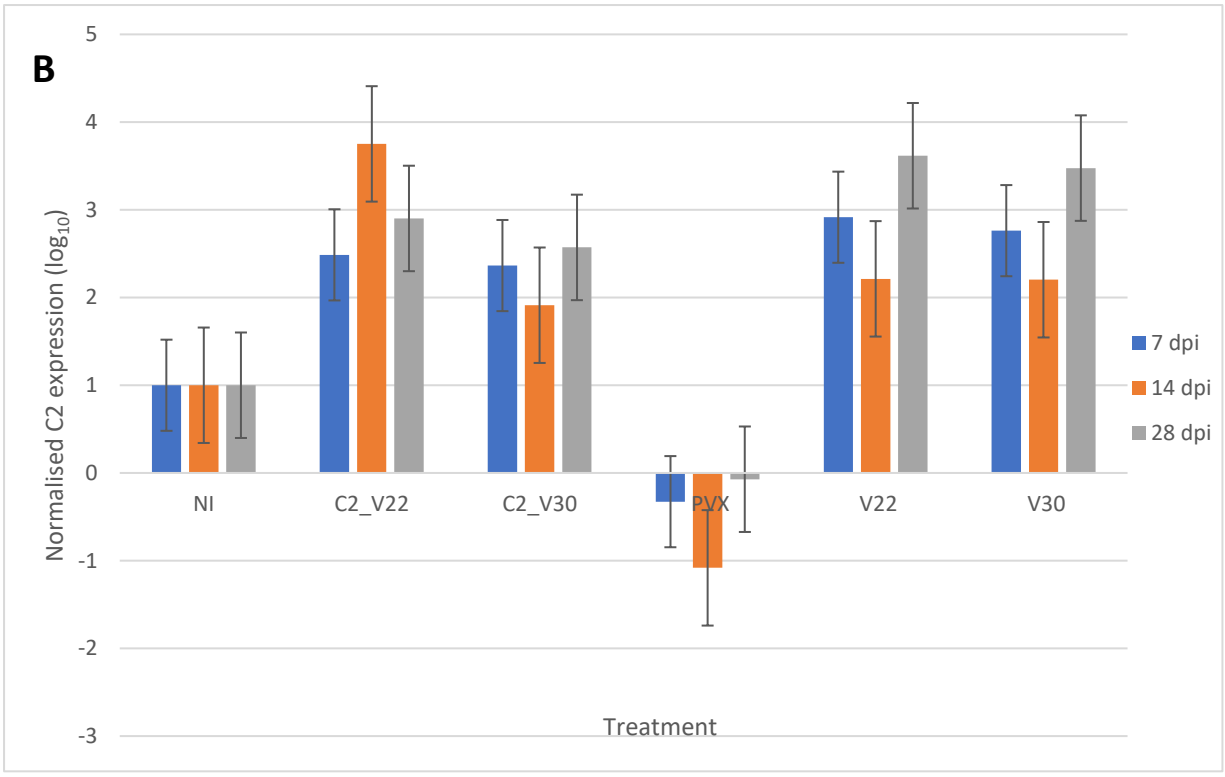

Figure 3.8:**Relative PVX coat protein and C2 expression. A)** Shows the change in relative PVX coat protein expression in plants inoculated with PVX only, PVX/C2_V22 and PVX/C2_V30. **B)** Shows the relative expression of C2 mRNA in plants inoculated with PVX only, PVX/C2_V22, PVX/C2_V30, ToCSV_V22 and ToCSV_V30. PVX CP and C2 mRNA expression values are represented on a log 10 scale. Significant differences between infections were calculated with $\alpha = 0.05$ (*). Error bars show standard error

3.5. Discussion

The expression of viral proteins using viral vectors such as PVX allows for the identification of pathogenicity determinants and to describe additional functions of these viral proteins (Luna et al., 2012). In this study we tested pathogenicity of ToCSV C2 using a PVX expression system. *N. benthamiana* plants inoculated with PVX/C2_V22 showed severe necrosis on inoculated leaves by 7 dpi. However, inoculation of plants with PVX only and PVX/C2_V30 showed only limited veinal necrosis and leaf chlorosis. C2_V22 was able to enhance viral symptoms caused by PVX thus indicating that C2 of ToCSV_V22 is a pathogenicity determinant. Intriguingly, *N. benthamiana* plants inoculated with wild-type ToCSV_V22 showed severe symptoms, while plants inoculated with ToCSV_V30 showed milder symptoms (Figure 3.5). This is not the case in the natural host, tomato, where ToCSV_V30 shows more severe disease symptoms (Esterhuizen, 2012). This suggests that C2 of ToCSV_V22 variant could be responsible for induction of severe symptoms elicited in *N. benthamiana*.

Plants inoculated with the PVX constructs showed symptom recovery in newly emerging leaves by 14 dpi. Plants inoculated with PXV/C2_V22 showed only mild curling in newly merging leaves. Recovery is described as an early symptomatic infection that is followed by a reduction or abolition of symptoms in newly emerging leaves (Wingard ,1928). Both C2_V22 and C2_V30 were unable to cause any systemic symptoms. Although inoculation of plants with PVX constructs did not cause systemic symptoms, analysis of PVX RNA showed that PVX constructs were able to replicate in systemically infected cells Figure 3.8-A) and that PVX was able to express C2 from ToCSV variants (Figure 3.8-B). It has been shown that a reduction of viral RNA levels is not necessary for the induction of symptom recovery (Xin & Ding, 2003). This accumulation of viral RNA and protein could be due to that the sap (phloem cells) from leaves is highly infectious (Wingard, 1928). Increased levels of viral RNA or viral proteins may be required to suppress a symptomatic defense/necrotic response. Another mechanism that may lead to symptom recovery is RNA silencing. There is a strong link between a viral protein's ability to inhibit RNA silencing and its potential to increase the severity of PVX infection (Gong et al., 2021). It has been shown that weak viral suppressors are important in triggering RNA silencing (Martin-Hernandez & Baulcombe, 2008). Our results show that ToCSV C2 weakly suppresses local RNA silencing in transgenic-GFP *Nicotiana benthamiana*, however, C2 was unable to suppress systemic silencing (Chapter 4).

The expression of V2 in PVX from papaya leaf curl virus (PaLCuV) caused cell death in inoculated *N. benthamiana*, however, when co-infiltrated with PVX expressing C2, the C2 protein inhibited the cell death induced by V2 at the site of inoculation (Mubin et al., 2010). Therefore, it is proposed that ToCSV C2 is involved in the initial induction of the host defense response, which is then suppressed by other viral proteins (Li et al., 2015). The C2 protein may require the subsequent expression of other viral proteins to induce a severe systemic phenotypic effect in infected plants. These findings can also be supported by the mRNA levels of PXV CP and C2_V22 (**Figure 3.8.**) which showed an increase of PVX CP and C2 mRNA at 14 dpi, followed by a decrease in expression levels when complete symptom recovery was

observed. A study conducted showed that C2 of the geminivirus TYLCSV can induce severe local necrosis which leads to cell death in *N. benthamiana* (Matić et al., 2016). However, co-infiltration of the C2 gene with V2 and C1 (and a truncated C1), showed that they can prevent local necrosis caused by C2. Our C2 gene inserted into the PVX vector contains overlapping regions from C1 and C3 which could play a role in preventing the systemic spread of necrosis caused by C2. Further analysis of the remaining ToCSV genes will have to be conducted to determine whether they can cause local and systemic necrosis and other symptoms and if they do have an effect on symptoms caused by C2.

Results from previous studies show that different phenotypes may be induced by homologous genes from distinct viruses, implying that these genes may interact with different host factors or comparable host factors to varying degrees (Amin et al., 2011). These interactions may lead to induction of symptoms that vary between viral proteins of different viruses. TrAP of ACMV, CbLCuV and TYLCV induces vein yellowing and leaf curling (Amin et al., 2011). TrAP of MYMIV induces HR at the inoculation site, while showing curling, necrosis and bleaching symptoms on systemic leaves (Ilyas, 2010). However, TrAP of TYLCV did not induce any cell death in infected plants (Matić et al., 2016). Our results only induced severe necrosis (7 dpi) leading to cell death (28 dpi) on *N. benthamiana* leaves inoculated with C2 of ToCSV_V22 variant, whereas plants inoculated with C2 of ToCSV-V30 showed symptoms similar to PXV. Therefore, TrAP of distinct viruses elicits varying symptoms and at varying stages. Similar results can be noticed with other viral proteins. TLCV C1 protein (Rep) has shown to cause local necrotic lesions on inoculated *N. benthamiana* leaves (Selth et al., 2004), however, TYLCV-China and ACMV induce necrotic symptoms on systemic leaves of inoculated *N. benthamiana* plants (Rene Van Wezel et al., 2002). TYLCV V2 expression from a PVX in *N. benthamiana* induced severe leaf curling, leaf yellowing and plant stunting (Amin et al., 2011; Zhao et al., 2018). CLCuKoV and ToLCJV V2 induces systemic cell death, indicating that might target a host defense response (Hussain et al., 2007; Mubin et al., 2010; Sharma & Ikegami, 2010). This indicates that V2 may be a key symptom determinant for TYLCV (Zhao et al., 2021). However, expression of AV2 EACMCV-CM in PVX does not induce necrotic symptoms in *N. benthamiana* (Chowda-Reddy et al., 2009). The expression of V3 protein via PVX, a recently described protein in TYLCV, showed severe symptoms in inoculated *N. benthamiana* plants when compared to PVX alone, with an associated increased accumulation of the PVX CP in systemic leaves(Gong et al., 2021). However, infection with PXV-V3 did not induce any cell death in *N. benthamiana*. Future analysis of other ToCSV ORF will give insight on which genes are involved systemic induction of symptoms in *N. benthamiana*.

The expression of viral proteins using a PVX-based approach allows pathogenicity determinants of begomoviruses to be identified, as shown by the production of new phenotypes that are not commonly attributed with the wild-type viral infection (Sharma et al., 1996). These results could suggest that C2 protein of ToCSV is expressed at an early stage of infection and is required for the initial induction of symptoms which elicits plant host defense responses. The differences in symptom severity induced by C2_V22 (severe necrosis) and C2_V30 (mild veinal necrosis) indicate that C2 protein is important in symptom development induced by both variants in both the natural tomato host and experimental *N.*

benthamiana plants ToCSV_V30 induces severe symptoms in tomato (Figure 2.3, Chapter 2), however, induces mild symptoms in *N. benthamiana* and ToCSV_V22 induces mild symptoms in tomato and severe symptoms in *N. benthamiana*. It is also evident that C2 is important in expression of viral mRNA which was shown in this study to increase PVX CP in local and systemic infection in *N. benthamiana* plants (which showed recovery). Further studies should focus on how C2 of ToCSV interacts host genes to induce symptoms and difference in symptoms between variants. It is also important to note that TrAP is important for the transactivation of late genes such as V2 (Sunter & Bisaro, 1997), which may affect the symptoms induced by C2 during infection with wild-type virus. It would be interesting to determine how C2 interacts with other viral proteins and whether these interactions affect the symptom severity.

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Chapter 4

Investigation of ToCSV C2 as a potential virus suppressor of RNA silencing (VSR) in GFP transgenic *Nicotiana benthamiana*

Abstract

Plants use RNA silencing as a defence mechanism against invading viruses, degrading viral RNAs in a sequence-specific manner. To counter this defense strategy, plant viruses encode genes that suppress RNA silencing. The C2 proteins of begomoviruses have been shown to be a viral suppressor of RNA silencing. In this study we investigated whether C2 genes of ToCSV variants are potential RNA silencing suppressors. In order to achieve this, we used the ability of C2 to prevent silencing of endogenous GFP gene in *N. benthamiana* 16c line. Our results indicated that the C2 gene encoded by both ToCSV_V30 and ToCSV_V22 does not suppress systemic RNA silencing. However, infection with C2 from both variants at 4 dpi did exhibit a weak suppression of local RNA silencing. This weak suppression was supported by a pale yellow fluorescence on the infiltrated leaves and relative GFP mRNA expression that showed a 1.19X increase for C2_V22 and 0.86X increase for C2_V30 when compared to infection with GFP only

4.1. Introduction

Plants have adapted various ways to combat invasion by viruses. One way is using the RNA interference (RNAi) or transcriptional TGS) - /post transcriptional gene silencing (PTGS) pathways. RNA silencing is triggered by double-stranded RNA (dsRNA). This RNA could be endogenous or from invading viral genome (Yadava et al., 2010) . Geminiviruses both induce and are targets of RNA silencing, through overlapping reading frames due to bidirectional transcription of their genes, which leads to dsRNA production (Ramesh et al., 2017). There are three main groups of small RNA, namely small interfering RNA (siRNA), microRNAs (miRNAs) and piwi-interacting RNAs (piRNAs). This grouping is based on the origin of the RNA, their structure, relationship with pathogen effector proteins and their roles (Singh et al., 2015). These dsRNA are cleaved by Dicer enzymes resulting in siRNA, which are amplified by RNA dependent RNA polymerase 2 and/or 6, ensuring the systemic spread of RNA silencing (Ramesh et al., 2017; Yadava et al., 2010). The production of siRNAs has been shown to increase as plants recovered from virus infections. The increase in siRNAs was also shown as temperatures increased and disease symptom severity decreased (Chellappan et al., 2005). Virus induced gene silencing (VIGS) is a form of PTGS used in plants defense mechanism against viruses, mediated by homologous siRNA targeting of viral transcripts (Ramegowda et al., 2014; Senthil-Kumar & Mysore, 2014). The development of VIGS has also been used extensively for studying plant gene functions (Senthil-Kumar & Mysore, 2014) since down regulation of an individual gene is achieved by degrading the transcripts associated with the gene (Baulcombe, 1999).

Various VIGS vectors are used for silencing in different plants. The most common VIGS vectors used in *Nicotiana benthamiana* are derived from viruses such as tobacco rattle virus (TRV) TRV and potato virus X (PVX), which silence endogenous genes expressed in the plant (Senthil-Kumar & Mysore, 2014). Both vectors are derived from the cDNA of the infectious viral components and are placed between a 35S promoter and a NOS terminator (Ahlquist et al., 1984; Cody & Scholthof, 2019; Rahman et al., 2021) . TRV has a bipartite genome namely RNA1 (TRV1) and RNA2 (TRV2). RNA1 encodes genes that are important for viral replication and these include 134 kDa and 194 kDa replicase proteins, a 29-kDa movement protein and a 16 kDa cysteine-rich protein (which has been shown to be important in allowing entry of TRV into meristems and to infect the reproductive organs (Liu, Schiff et al., 2002a; Martin-Hernandez & Baulcombe, 2008). Unlike other VIGS vectors, TRV can infect the meristem which is important for effective PTGS analysis and importantly causes mild symptoms that do not interfere with PTGS phenotype analysis (Burch-Smith et al., 2004). PVX is a monopartite virus encoded with a plus sense-RNA molecule and consist of five ORFs (Hefferon, 2014) . RNA-dependent RNA polymerase (RdRp) is important for viral replication. The triple gene block (TGB, 25kD, 12kD and 8kD) are the central three overlapping ORFs. TGB is important for virus localization surrounding cells. The coat protein (CP) is important for virion assembly, cell to cell infection through the plasmodesmata and systemic important (Larsen & Curtis, 2012). PVX has been used for the expression of full-length proteins, fusion proteins and epitopes of viral particles (Hefferon, 2014; Larsen & Curtis, 2012).

Viruses encode genes that can suppress RNA silencing and are known as viral suppressors of RNA silencing (VSRs). These genes are pathogenicity determinants as they regulate pathogenicity by suppression of host RNA silencing defense mechanisms (Yadava et al., 2010). The AC2 and C2 proteins of bipartite and monopartite geminiviruses, respectively, have been shown to be silencing suppressors of host defense (Trinks et al., 2005; Van Wezel et al., 2002). The silencing of AC2/C2 leads to a large reduction of host siRNA. This reduction could be caused by the interference of AC2 with a component required for the biosynthesis of siRNA, however, AC2 protein does not interact directly with host siRNA or miRNA (Kumar et al., 2015; Kumar & Naqvi, 2016). AC2 protein of tomato leaf curl New Delhi virus (ToLCNDV) and mungbean yellow mosaic India virus (MYMIV) has been shown to affect siRNA synthesis by inhibiting the RNA polymerase activity of RNA dependent RNA polymerase 6 (RDR6), which is involved in the synthesis of single stranded RNA, and it also interacts with AGO1 thus inhibiting AGO-RISC activity, which is involved in the cleaving of RNA transcripts (Kumar et al., 2015; Kumar & Naqvi, 2016). However, the ability of C2 of the monopartite tomato yellow leaf curl virus China(TYLCCNV) to suppress host RNA silencing is inhibited by mutations on cysteine residues in the putative zinc-finger domain (Van Wezel et al., 2002). The zinc-finger domain has been shown to bind ssDNA more strongly than dsDNA (Hartitz et al., 1999).

4.2. Aims

The aim of this study was to determine whether C2 of ToCSV mild (ToCSV_V22) and severe (ToCSV_V30) variants are suppressors of host posttranscriptional gene silencing (PTGS) activity.

4.3. Methodology

4.3.1 Construction of VIGS vectors

The TRV vectors, pTRV1 and pTRV2, were used to design the silencing constructs. The pTRV1 RNA1 has genes that code for RNA-dependent RNA polymerase, movement protein and 16K cysteine rich protein (Y. Liu, Schiff, Marathe, et al., 2002a). The pTRV2 has genes that encode the coat protein and protein required for nematode transmission. These genes on RNA2 were replaced with the multiple cloning sites (MCS) .

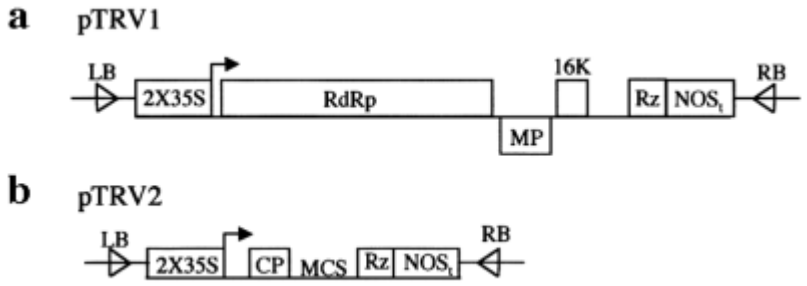

Figure 4.1: TRV-vectors a) pTRV1 and b) pTRV2 with MCS used to insert the full length of C2, mGFP5-ER and p19 using XbaI and SacI restriction enzymes (Liu, Schiff and Dinesh-Kumar, 2002). The TRV based vector as described in (Y. Liu, Schiff, Marathe, et al., 2002b): cDNA clones from TRV were inserted between the duplicated CaMV 35S promoter and nopaline synthase terminator (NOS_t) in a T-DNA vector. RNA dependent RNA polymerase (RdRp) ;16 kDa cysteine rich protein (16K); movement protein (MP); coat protein (CP); left (LB) and right borders (RB) of T-DNA; self-cleaving ribozyme (Rz); multiple cloning sites (MCS).

The full length of C2 ORF from both ToCSV_V22 and ToCSV_V30 were cloned into the MCS of TRV2 using the restriction enzymes *XbaI* and *SacI*. The mGFP5-ER gene (green fluorescent protein-endoplasmic reticulum-mGRF5ER) was cloned into pTRV2. The mGFP5-ER gene was used as it has been shown that *N.benthamiana* 16-C plants contain the sequence of mGFP5-ER with the T-DNA sequence, thus making it at a target of transcriptional and post transcriptional gene silencing by RDR6 (Nuthikattu et al., 2013; Philips et al., 2017) A strong suppressor (p19) encoded by tobamoviruses (from tomato bushy stunt virus, TBSV), cloned into pTRV2 was used as a positive control. Primers used can be found in Table 4.1. The constructs were then transformed into *Agrobacterium tumefaciens* strain C58C1 (as described in Chapter 2) and grown on LB plates containing 100 mg/L kanamycin and 50 mg/L rifampicin. For the purpose of choosing transformed cells, the plate was incubated for 48 hours at 28 °C. From the plates, single colonies were chosen, and colony PCR was used to check for the presence of successful colonies. Positive colonies were cultured in liquid LB media containing 100 mg/L kanamycin and 50 mg/L rifampicin. The cultures were used to make glycerol stocks containing 50% glycerol and 500 µl of the culture. The glycerol stocks were stored at -80 °C.

Table 4-1: Primers used to amplify GFP and p19. Amplified fragments are then cloned into pTRV2 using RE XbaI and SacI

Primer name	Primer sequence	Fragment size
pTRV2/mGFP5ER_F/XbaI	5'-*TCTAGA*CGATGAAGACTAATCTTTTT-3'	792 bp
pTRV2 mGFP5ER_R/ SacI	5'- *GAGCTC*TTAAAGCTCATCATGT-3'	
pTRV2/p19_F/XbaI	5'-GC*TCTAGA*ATGGAACGAGCTATACAAGGAA-3'	519 bp
pTRV2/p19_R/SacI	5'-CG*GAGCTC*TTACTCGCTTTCTTTTTCGAAGG-3'	
pTRV2/C2_F/XbaI	5'-GC*TCTAGA*ATGCGGTCTTCATCAC-3'	408 bp
pTRV2/C2_R/ SacI	5'-CG*GAGCTC*TTAAATACGCTTAAGAAACGA-3'	

Underlined and italicized sequences represent the restriction sites

4.3.2. Growing conditions for *N. benthamiana*

N. benthamiana 16C line (transgenic *N. benthamiana* expressing endogenous GFP) seeds were planted and germinated in Jiffy Products S.L (Pvt) Ltd, Sri Lanka jiffies in a tray, sealed with cling wrap, and allowed to germinate at 25 °C with 16 h of light and 8 h of dark photoperiodism. When seedlings were three-week-old , they were transferred into pots containing germination mix made of Culterra and vermiculite (3:1). The pots were placed into deep trays, covered with cling, and acclimatized for a week. Plants were agro-infiltrated at the six to eight leaf stage.

4.3.3. Agro-infiltration and GFP observation

Acclimatized *N. benthamiana* 16 C line plants were agro-infiltrated at the four weeks when plants had reached the 6 to 8 leaf stage. The leaves were infiltrated below the apical meristem. Prior to infiltration, agro stocks of constructs were grown in 5ml liquid LB containing 100 mg/L kanamycin and 50 mg/L rifampicin at 28 °C overnight. The cultures were centrifuged at 4200 xg for 8 minutes. The pellets were resuspended with infiltration buffer (composed of 10mM $MgCl_2$, 10 mM MES and 200 µM acetosyringone) until they reached an OD_{600} of 0.6. The cultures were incubated at RT for 2-3 hours. Plants were infected with pTRV2 clones (pTRV2_C2_V22 + pTRV1, pTRV2_C2_V30 + pTRV1) and co-inoculated with pTRV1 and pTRV2/mGFP5ER. Equal volumes of pTRV1, pTRV2 clones and GFP were mixed. Four plants were used for each construct. Plants inoculated with only pTRV1 +pTRV2/mGFP5ER were used as a negative control and those inoculated with pTRV1 + pTRV2/p19 were used as positive controls. Three biological replications were performed. Plants expressing GFP were observed under a strong UV lamp,100 Watts (Nightsea Bluestar, Tektite, Trenton, NJ, U.S.A,) in a dark room. Pictures showing local and systemic silencing were taken using a Canon PowerShot camera and UV yellow filters. Plant material was collected at 4, 14 and 28 dpi for analysis. Two leaves below the apical meristem were collected for RNA extraction. Three biological replicates were performed for each construct.

4.3.4. RNA extraction and cDNA

Liquid nitrogen was used to grind about 50 mg of leaf material. The ground sample was resuspended in 1 ml of Tri Reagent (Sigma-Aldrich, St. Louis, USA) and allowed to sit for 10 minutes at room temperature. The sample was centrifuged at 13,300 rpm for 15 min at 4°C and the supernatant transferred into a new tube. Chloroform was added to the sample, mixed vigorously, and incubated for 3 minutes at RT. The sample was centrifuged at 13,300 rpm for 15 min at 4°C, the supernatant was removed to a new tube and 500µl of isopropanol added to it. The sample was incubated at RT for 10 minutes then centrifuged at 13300 rpm for 15 minutes at 4°C. The supernatant was discarded, and the pellet washed with 75% ethanol, then

centrifuged at 7500 g for 5 minutes at 4°C. The ethanol was removed, and the pellet was allowed to dry for less than 10 min, then resuspended in DEPC water containing RNase inhibitor.

Prior to cDNA synthesis 1µg RNA was treated with RNase-free DNase. To the 1µg RNA, 10X reaction buffer with MgCl2, 1U DNase (RNase Free) and DEPC-treated water were added to a total reaction of 10 µl. The sample was incubated for 30 min at 37 °C. To the sample, EDTA was added and incubated at 65 °C. The treated RNA was used as template for reverse transcriptase (RT). The Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit was used to reverse transcribe the RNA according to the manufacturer's recommendations. To the cDNA samples 100 pM Oligo (dT)18 primer, 10mM dNTP, 5X RT buffer, 20 U/µl RiboLock RNase inhibitor, 200 U/µL RevertAid M-MuLV RT were added to a final reaction of 20 µl. The reaction was incubated at 42 °C for 60 minutes then terminated by heating at 70°C for 5 minutes. A -RT reaction was used as negative control. This first strand cDNA synthesis was used as template directly in RT-qPCR.

4.3.5. Quantification of gene expression using RT-qPCR

To determine the effect of the expression of GFP mRNA, reverse transcriptase quantitative PCR (RT-qPCR) was performed using cDNA. RT-qPCR was used to measure the expression of mGFP5ER. The reactions were performed in the LightCycler ® 1.5 Realtime system using Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix to a total volume of 20 µl. To the reaction, 10 µl Maxima SYBR green master mix, 0.3 µM GFP primers (final concentration) (**Table 4-2**), 2 µl of the synthesized cDNA and nuclease free water were added. RT-qPCR was conducted for 40 cycles. Initial denaturation occurred at 95 °C for 10 min, followed by denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. GFP protein expression was normalized to the housekeeping gene *EF1α* (D. Liu et al., 2012).

A standard curve was constructed prior to expression analysis. The standard curve was obtained by serial dilution of pTRV2-mGFP5ER cDNA. The cDNA was diluted using a 10X dilution. The standard curve was constructed and the primer efficiency was calculated using the slope. The relative gene expression was determined using the Ct values generated by LightCycler. The Ct values were used in the Pfaffl method, described in Chapter 2 (Pfaffl, 2001)

Table 4-2: Primers used to determine GFP expression by RT-qPCR

Primer Name	Sequence	Fragment size
qPCR_GFP_F	5'-GGCATCAAAGCCAACTTCAAGACCC-3'	140 bp
qPCR_GFP_R	5'-GCAGATTGTGTGGACAGGTAATGGTTG-3'	

4.3.6. Statistical analysis

A two-way analysis of variance (ANOVA) was performed using the relative expression ratios from qPCR analysis to determine statistical significance between the treatments, and between treatments and controls. Significant differences between infections were calculated with $\alpha = 0.05$ (*).

4.4. Results

4.4.1. TRV silencing constructs

To determine whether C2 is a suppressor of host PTGS activity, the wild-type C2 coding sequencing from ToCSV_V30 and ToCSV_V22 and mGFP5ER were amplified by PCR (**Figure 4.1**) and cloned as *XbaI* and *SacI* fragments into MCS of TRV2 expression vector (**Figure 4.2**). Additionally, *Tomato bushy stunt virus* p19 gene was amplified by PCR and cloned into TRV2. p19 was used as a positive control as it is strong suppressor of PTGS.

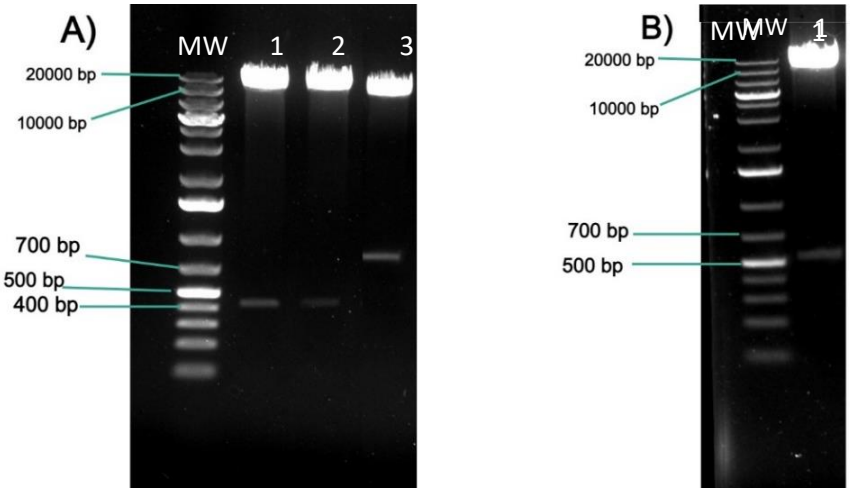

Figure 4.2: TRV constructs. Restriction digest confirmation of TRV clones. Fragments were inserted into TRV (9663 bp) with XbaI and SacI restriction sites. **A)** Restrictions digest of TRV clones with C2 fragments (408 bp) amplified from ToCSV_V30 (lane 1), and ToCSV_V22 (lane 2) and amplified mGFP5ER (lane 3, 792 bp). **B)** Restriction digests of TRV clone with p19 fragment (lane 1). Molecular weight marker (MW) Thermo Scientific™ O'GeneRuler 1 kb Plus DNA Ladder.

4.4.2. Symptom evaluation

To determine whether C2 gene of ToCSV variants is a potential RNA silencing suppressor of PTGS activity of the host plant, GFP-transgenic *N. benthamiana* 16C plants were inoculated with the silencing pTRV2 constructs (pTRV2_C2_V22 + pTRV1, pTRV2_C2_V30 + pTRV1) and

co-inoculated with pTRV1 and pTRV2/GFP5 (single stranded). TBSVp19 gene was inserted into pTRV2 (pTRV2_p19) and used as positive silencing control in the experiment. Results showed that by 4 dpi, silencing could be observed in leaves infiltrated with the vector containing GFP only (pTRV2/GFP). *N. benthamiana* 16-C line plants expressing GFP exhibit strong green fluorescence under UV light, however, GFP silencing is shown by red fluorescence, under UV light, around infiltrated area, and red fluorescence in leaf veins (yellow arrows) which then spreads throughout the whole leaf lamina (**Figure 4.3-C**). When the silencing signal leaves the agro-infiltrated area, a strong decrease in GFP expression is caused by cell-to-cell movement, resulting in a red ring surrounding the infiltrated area (Himber et al., 2003). Red fluorescence could also be seen in leaves co-infiltrated with pTRV2/C2_V22 and pTRV2/GFP, and pTRV2/C2_V30 and pTRV2/GFP with red rings surrounding the infiltrated area (yellow arrow), however, a pale-yellow fluorescence (from a combination of cells expressing green and red fluorescence) (green arrow) can also be seen around these red rings indicating a weak suppression of GFP silencing around the leaves. Plants co-infiltrated with pTRV2_p19 and pTRV2/GFP showed green fluorescence around infiltration patch (**Figure 4.3-E** and **F**). These results indicate that ToCSV C2 gene does not completely suppress local GFP silencing. By 14 dpi, systemic silencing could be observed in plants inoculated with pTRV2/C2 clones (**Figure 4.4-E** and **F**) and GFP (**Figure 4.4**-**C**). These plants showed continuous red fluorescence in systemic leaves. Systemic silencing was evaluated in upper young leaves. However, GFP expression increased in plants co-infiltrated with pTRV2/p19 and pTRV2/GFP, shown by a strong green fluorescence in systemic leaves. Systemic silencing was evaluated up to 28 dpi (**Figure 4.5**). By 28 dpi, plants infiltrated with pTRV2/GFP only (**Figure 4.5**-**C**), pTRV2/C2_V22 and pTRV2/GFP (**Figure 4.5**-**E**) , and pTRV2/C2_V30 and pTRV2/GFP (**Figure 4.5**-**F**) showed complete red fluorescence on systemic leaves. Plants inoculated with pTRV2/p19 and pTRV2/GFP (**Figure 4.5**-**D**) continued to show green fluorescence, however, the intensity decreased when compared to 14 dpi.

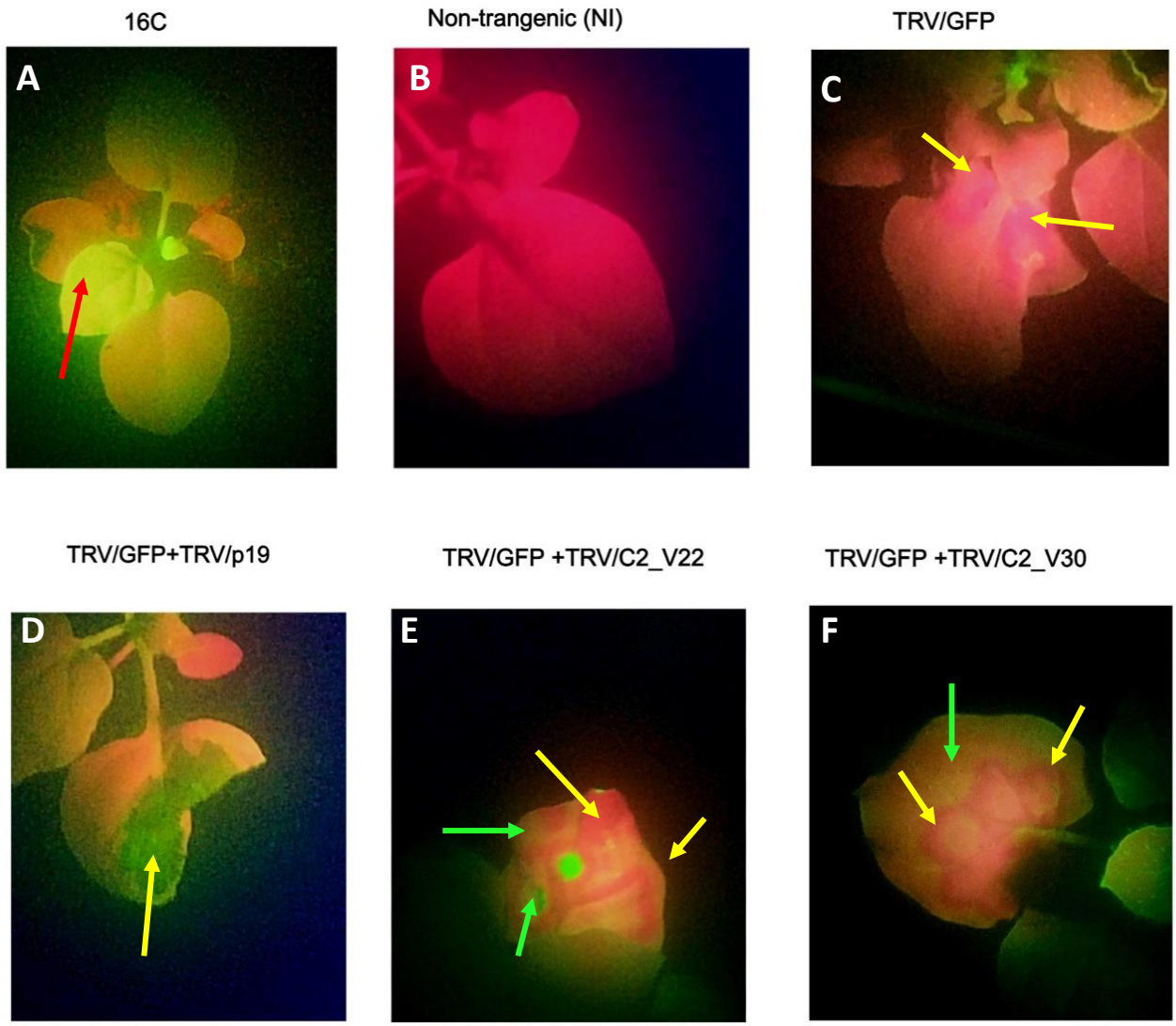

Figure 4.3: Posttransriptional gene silencing (PTGS) of ToCSV C2 gene at 4 dpi. Transgenic Nicotiana benthamiana-GFP (16C) plant leaves subjected to several treatments viewed under UV light. **(A)** Control 16C leaves showing green fluorescence (red arrow) at 4 dpi. **(B)** Wild-type non-transgenic leaves fluorescing red. **(C)** Leaf infiltrated with pTRV2/GFP vector show red fluorescence indicating GFP silencing. (**D**) Leaf infiltrated with pTRV2/p19 and pTRV2/GFP show green fluorescence (yellow arrow) indicating suppression of GFP silencing. (**E**) Leaf infiltrated with pTRV2/C2_V22 and pTRV2/GFP showing red rings around infiltration point, however, pale yellow fluorescence (green arrow) can be seen around these red rings indicating weak expression of GFP fluorescence. (**F**) Leaf infiltrated with pTRV2/C2_V30 and pTRV2/GFP show showing red rings around the infiltrated area and a pale-yellow fluorescence following the red rings.

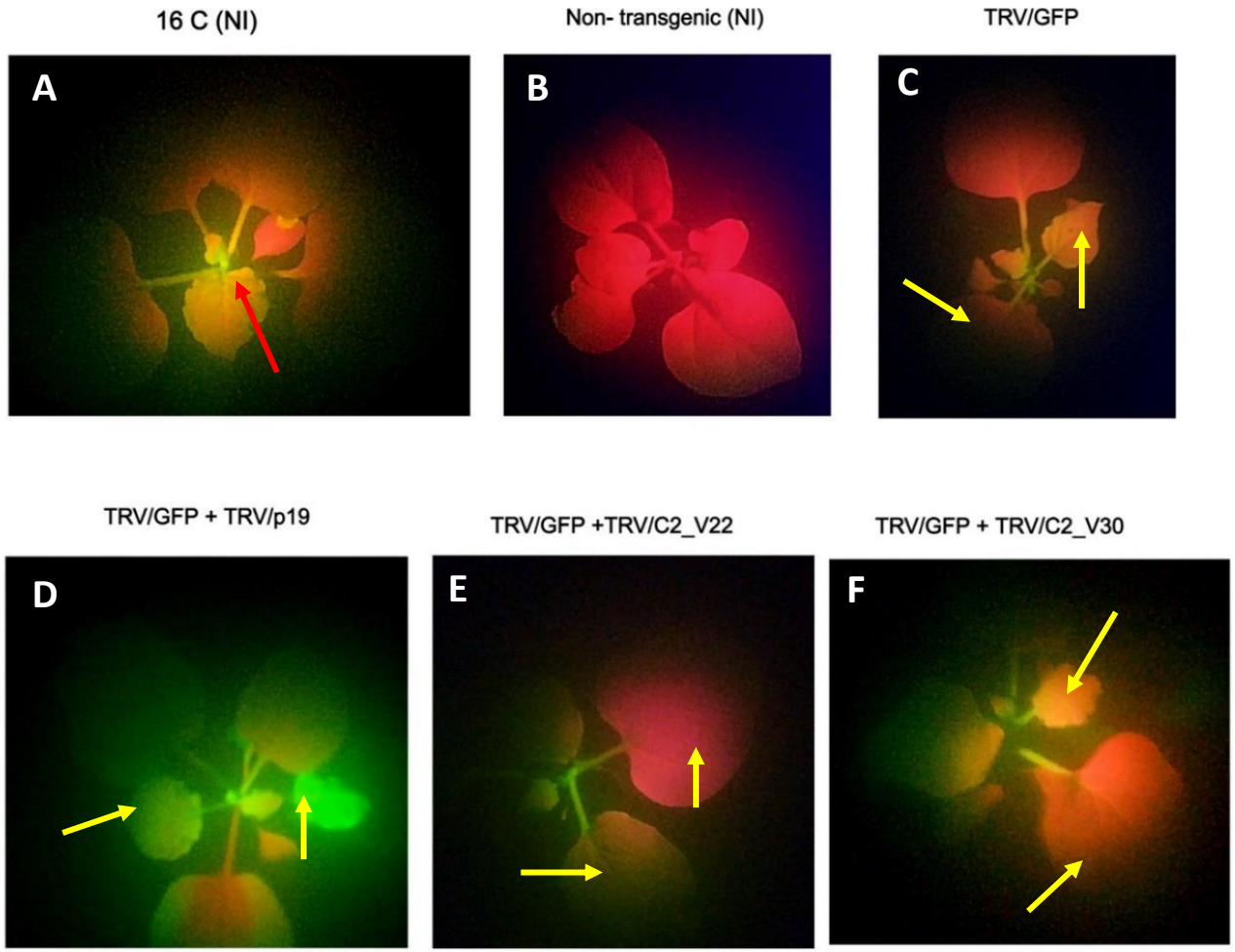

Figure 4.4: Systemic GFP silencing observed at 14 dpi. Transgenic Nicotiana benthamiana-GFP (16C) plant leaves viewed under UV light at 14 dpi. **(A)** Control 16C leaves showing a decrease green fluorescence (red arrow). **(B)** Wild-type non-transgenic leaves fluorescing red. **(C)**Systemic leaves from plants inoculated with pTRV2/GFP vector show red fluorescence (yellow arrow). (**D**) Systemic leaves from plants infiltrated with pTRV2/p19 and pTRV2/GFP show strong green fluorescence (yellow arrow). (**E**)

Systemic leaves of plants inoculated with pTRV2/C2_V22 and pTRV2/GFP showing red fluorescence resulting from the spread of GFP silencing. (**F**) Red fluorescence seen on plants inoculated with pTRV2/C2_V30 and pTRV2/GFP.

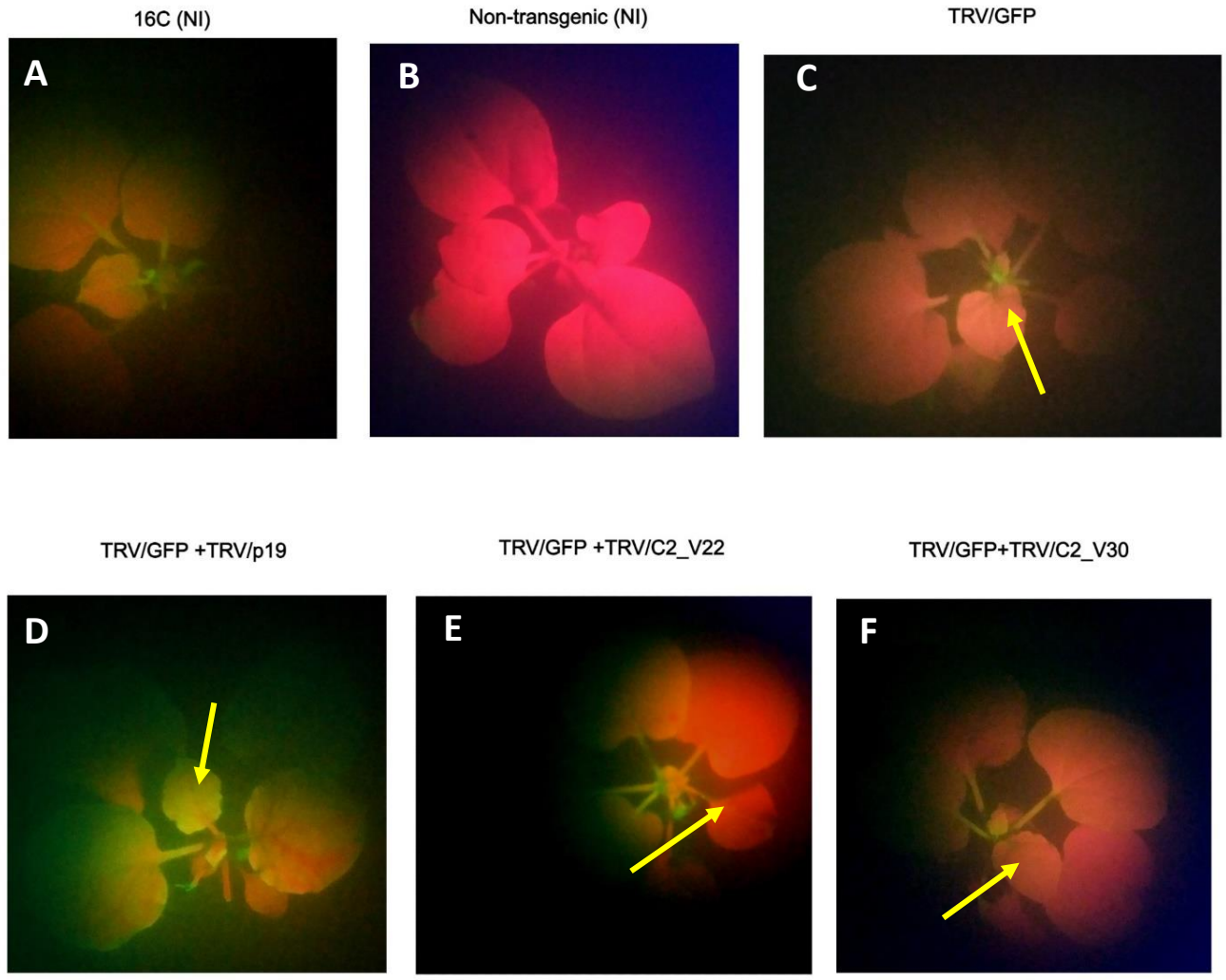

Figure 4.5: Posttranscriptional gene silencing of ToCSV C2 gene at 28 dpi. Transgenic N. benthamiana plants views at 28 dpi under UV light. (A) Control 16C leaves showing a green fluorescence (red arrow). (B) Wild-type non-transgenic leaves fluorescing red. (C)Systemic leaves from plants inoculated with pTRV2/GFP vector show red fluorescence (yellow arrow). (D) Systemic leaves from plants infiltrated with pTRV2/p19 and pTRV2/GFP show reduced green fluorescence (yellow arrow). (E) Systemic leaves of plants inoculated with pTRV2/C2_V22 and pTRV2/GFP showing complete red fluorescence. (F) Red fluorescence seen on plants inoculated with pTRV2/C2_V30 and pTRV2/GFP showing complete GFP silencing

4.4.3. GFP mRNA expression

To determine the GFP mRNA expression levels in inoculated plants, extracted RNA was used for qPCR analysis using GFP primers and normalized against *elongation factor α* (*Elfα*) gene. To determine the qPCR efficiency, a standard curve was constructed using GFP cDNA at different concentrations (Figure 4.6). The efficiencies obtained from GFP and Elfα analysis were used in Equation 2.3.7-1**,** (Pfaffl, 2001) **(**Chapter 2**)** to calculate the relative expression of GFP mRNA using non-inoculated 16c line as the calibrator. The GFP mRNA expression values obtained from plants infected with C2 were compared to expression values of plants infected with GFP only (negative control) and p19 suppressor (positive control) and graphed on a log10 scale (Figure 4.7). At 4 dpi, there was a 118.6% increase in GFP mRNA expression in plants inoculated with pTRV2/C2_V22 when compared to infection with GFP only, however, infection with pTRV2/C2_V22 showed a 55% decrease when compared to infection with p19. Infection with pTRV2/C2_V30 at 4dpi showed an 89% increase in GFP mRNA expression when compared to infection with pTRV2/GFP only and showed a 61% decrease in GFP mRNA expression when compared to infection with p19. At 14 dpi expression of GFP mRNA began to decrease for all inoculations, however GFP expression remained lower for C2 and GFP silencing vectors only when compared to inoculation with p19. GFP expression showed a 67%, 77% and 66% decrease for inoculation with pTRV2/C2_V22, pTRV2/C2_V30 and pTRV2/GFP only, respectively, when compared to inoculation with pTRV2/p19. GFP expression levels were similar for inoculation with pTRV2/C2_V22 and pTRV2/GFP only, showing only a 3.4% difference. By 28 dpi, systemic GFP expression levels decreased greatly in all inoculations, however, silencing suppression was still observed for p19 inoculation. Inoculations with pTRV2/C2_V22, pTRV2/C2_V30 and GFP only showed a 68%, 58% and 51% decrease, respectively, when compared to inoculation with p19. These results indicate that C2 from both variants was not able to suppress systemic RNA silencing, however, weak suppression of local RNA silencing on infiltrated leaves was demonstrated.

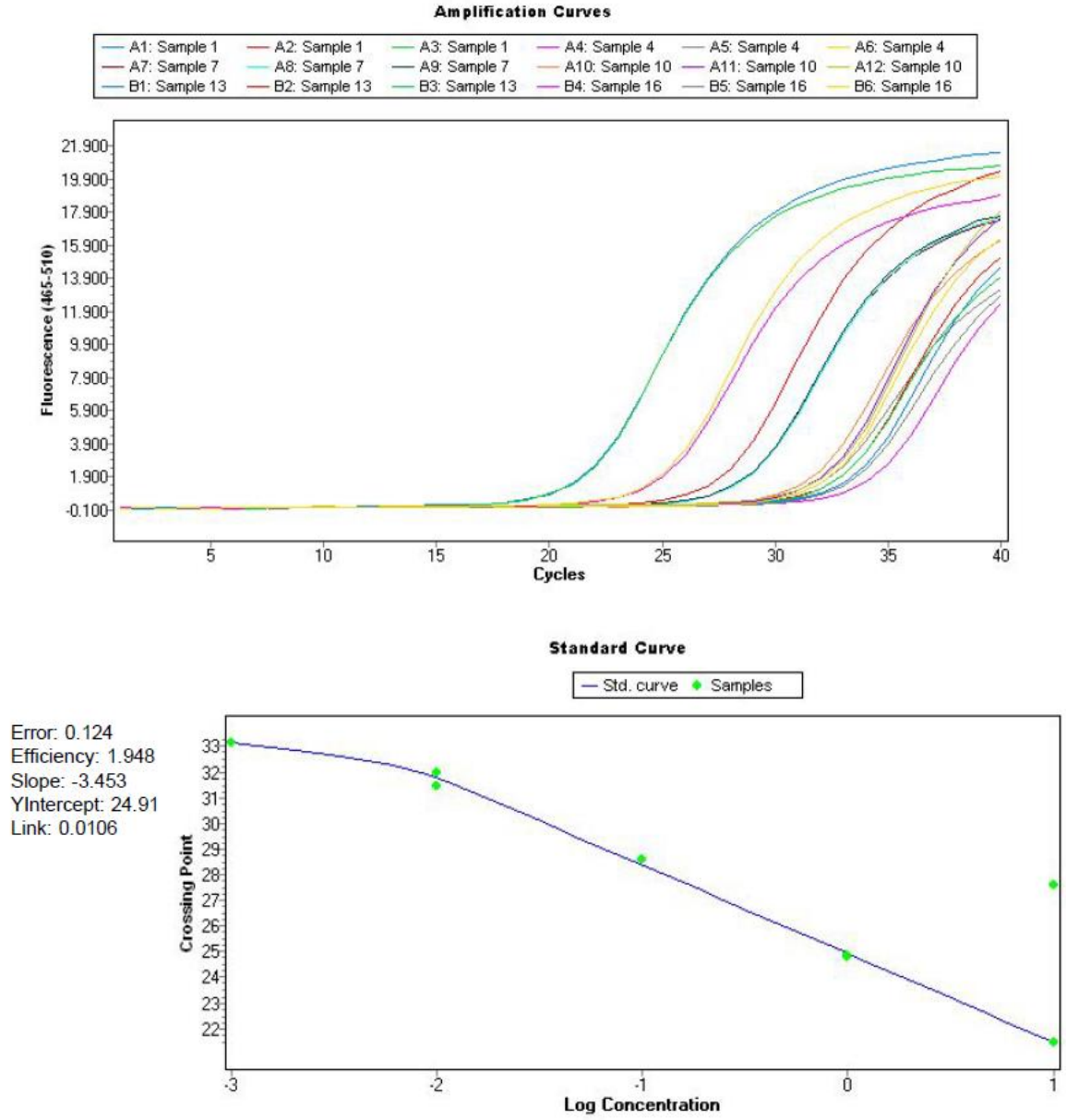

Figure 4.6: Standard curve for GFP expression. GFP cDNA was serially diluted 10-fold. Diluted cDNA was used to construct a standard curve using the LightCycler II 480 software. Primer efficiency was determined using the slope of the curve. Standard curve obtained a PCR efficiency of 94.8% and a slope -3.453.

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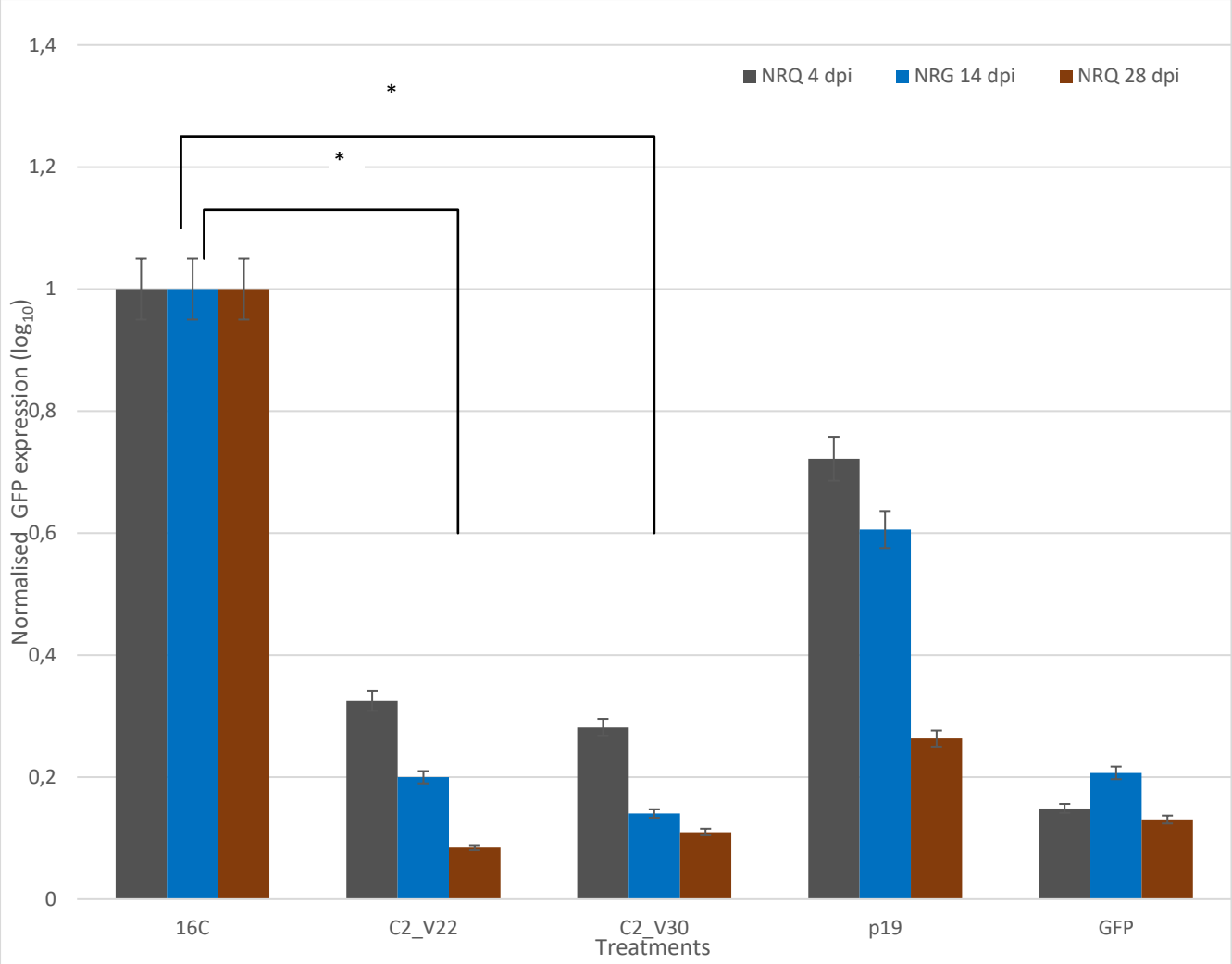

Figure 4.7: Relative GFP expression. The relative GFP mRNA accumulation was measured by RT-qPCR. Silencing of GFP can be observed by lower GFP mRNA expression compared to the positive control (p19) and negative control (non-inoculated 16C line). GFP expression values are represented on a log 10 scale. Significant differences between infections were calculated with $\alpha = 0.05$ (*). Error bars show standard error.

4.5. Discussion

RNA silencing is an antiviral response in plants and is initiated by the production of viral dsRNA. Viruses trigger RNA silencing in various way. Single stranded DNA geminiviruses are able to induce silencing by either producing dsRNA formed from the bidirectional transcription of overlapping sequences (Chellappan et al., 2004), and abundant C1 transcripts may be used as template by host RdRp to produce dsRNA which may trigger silencing. Additionally, the strong fold back structure of geminivirus transcripts due to intramolecular base pairing may be used as templates for DICER to produce siRNA (Dalmay et al., 2000; Vanitharani et al., 2005). Viruses encode RNA silencing suppressors to counter this defense response (Li et al., 2021; Zrachya et al., 2007).

Inoculation of host transgenic plants with virus suppressors can lead to the reversal of silencing that has already developed and/or inhibit the spread of silencing into developing

tissue (Vanitharani et al., 2004). Geminivirus C2 (or TrAP) has been shown to suppress both TGS and PTGS through different systems depending on the related C2 sequence of the viral genome (Teixeira et al., 2021) and therefore was selected in this study to investigate whether C2 genes of ToCSV variants are potential RNA silencing suppressors. In order to achieve this, we used the ability of C2 to prevent silencing of endogenous GFP gene in *N. benthamiana* 16c line. The study was performed in *N. benthamiana* 16c as this is a widely used model plant system to study the activity of PTGS suppressor proteins of plant viruses (Garcia-Ruiz, 2021; Philips et al., 2017; Verchot-Lubicz & Carr, 2008). Our results indicated that the C2 gene encoded by both ToCSV_V30 and ToCSV_V22 does not suppress systemic RNA silencing. However, infection with C2 from both variants at 4 dpi did exhibit a weak suppression of local RNA silencing. This weak suppression was supported by a pale yellow fluorescence on the infiltrated leaves (Figure 4.3) and relative GFP mRNA expression that showed a 1.19X increase for C2_V22 and 0.86X increase for C2_V30 when compared to infection with GFP only (Figure 4.7). ToCSV-V22 exhibits mild symptoms in tomato (Esterhuizen, 2012; Pietersen et al., 2000)and severe symptoms in *N.benthamiana* which would account for the higher GFP expression in this variant. In Chapter 3, C2_V22 also induced more severe symptoms in *N. benthamiana*. The fluorescence began to decrease from 14 dpi, indicating systemic silencing. The weak suppression observed in this study may be an indication that ToCSV C2 is a mild silencing suppressor early in infection or is not effective in the host *N. benthamiana*.A strong link has been shown between a viral protein's ability to inhibit RNA silencing and its potential to increase the severity of viral infection (Gong et al., 2021). Alternatively, silencing could be occurring at different stages in the plant life-cycle (Vanitharani et al., 2004). This was shown by the inoculation of plants with African cassava mosaic virus Cameroon strain (ACMV-[CM]) which was able to strongly suppress GFP silencing in transgenic *N. benthamiana* 16 C line for two weeks but showed a great reduction in suppression thereafter. However, inoculation with individual genes showed that ACMV-[CM] AC4 suppressed silencing for only approximately two weeks, whereas AC2 suppressed silencing at the first two weeks but later showed mild suppression (Vanitharani et al., 2004). The suppression activity of C2 was compared to plants co-infiltrated with pTRV2/p19 and pTRV2/GFP. The tomato bushy stunt virus p19 was used as a positive control as it is a strong suppress of RNA silencing. It has been shown that tobamovirus p19 suppresses RNA silencing by binding and sequestering siRNAs (Lakatos et al., 2004; Roth et al., 2004). In our study, pTRV2/p19 induced strong suppression on inoculated plants at 4 dpi and 14 dpi, however, suppression activity began to decrease at 28 dpi shown by a decrease in GFP fluorescence. This incomplete suppression by p19 is caused by the production of dsRNA of RNA which are involved in activating silencing (Martín-Hernández & Baulcombe, 2008; Qiu et al., 2002). Results in this study C2_V22 and C2_V30 were weak showed to exhibit weak suppression activity compared to p19.

It has been shown that RNA silencing suppressors do not share homology in their sequences or in their viral functions (Vanitharani et al., 2005). C2 of monopartite tomato leaf curl Java virus (ToLCJAV) has been shown not to suppress RNA silencing (Kon et al., 2007). However, in the same study, the upper leaves for GFP transgenic *N. benthamiana* inoculated with PVX-C2/PVX-GFP showed mild suppression of silencing at 21 dpi. This suggested that ToLCJAV-C2 might be a weak suppressor of RNA silencing. Results from this study also appear to suggest

that ToCSV C2 is a very mild VSR and occurs only locally and briefly at around 4 dpi. Expression analysis also showed a slight increase in GFP mRNA with plants inoculated with C2 (Kon et al., 2007). Similar results were observed in GFP transgenic *N. benthamiana* co-infiltrated with bhendi yellow vein mosaic virus (BYVMV) C2. Infiltration with the C2 construct induced mild expression of GFP by 5 dpi, however, by 7 dpi there was loss of GFP expression (Gopal et al., 2007). It is also interesting to note that suppression activity of viral proteins is host dependent (Luna et al., 2012; Shimura et al., 2008), and that ToCSV may not be functional in *N. benthamiana*. Suppression activity of C2 from TYLCV and TYLCSV was analyzed in *N. benthamiana*, tomato and bean (Luna et al., 2012). The results revealed that C2 from both viruses induced a weak suppression in *N. benthamiana*, however, showed strong suppression in tomato and was unable to suppress silencing in bean. Further studies on silencing by ToCSV C2 in tomato are therefore warranted before a conclusion can be made about the putative VSR activity of the C2 gene.

Other ORFs of monopartite geminiviruses have been shown to be silencing suppressors (Fondong, 2013), and could be the case in ToCSV variants. Transgenic *N. benthamiana* 16 line plants inoculated with C2, C4 and V2 of TYLCSV, TYLCV, TYLCV-Mld and TYLCMalV showed that the V2 of TYLCSV, TYLCV and TYLCV-Mld is a strong suppressor of local silencing while V2 of TYLCMalV is a weak suppressor of silencing (Luna et al., 2012). While C2 (expect TYLCSV and TYLCV, showing no suppression) and C4 (except TYLCMalV) of all tested viruses showed weak suppression of local silencing. Intriguingly, it could be observed that these viruses required a strong (V2) and weak (C2 or C4) suppressor. More recently, several other small putative ORFs have been discovered in TYLCV which are transcribed during infection. One of these additional and largest ORF, named V3, showed to be effective in suppressing local and systemic GFP silencing in transgenic *N. benthamiana* 16 C line plants (Gong et al., 2021) However, V3 of mulberry mosaic dwarf-associated virus (MMDaV) cannot suppress silencing in *N. benthamiana* 16 C line plants (Yang et al., 2018) thus indicating that suppression of silencing by VSR is virus-dependent. The C1 (Rep) protein of monopartite mastrevirus wheat dwarf virus (WDV) has been shown to suppress local and systemic silencing in *N. benthamiana* and inhibits the spread of systemic GFP silencing signal (Wang et al., 2014).

Two classes of subviral molecules (betasatellites and the alphasatellites) are frequently associated with begomoviruses, mainly in the Old World (Zhou, 2013). These have been shown to play a role in pathogenicity and are also RNA silencing suppressors (Zhou, 2013). However no satellites to date have been reported to be associated with ToCSV (Esterhuizen, 2012; Pietersen et al., 2008). Betasatellites contain a single ORF, named, βC1, encoding a pathogenicity determinant and like many other viral pathogenicity determinants is able to suppress RNA silencing (Cui et al., 2005; Zhou, 2013). There is evidence that true monopartite begomoviruses can trans-replicate betasatellites, such as TYLCV and Papaya leaf curl China virus (PLCCCV) (Zhou, 2013). There has been no detection of β-satellite associated with TYLCV, however, it has been shown that the Rep protein encoded by TYLCV can trans-replicate the β-satellite component of TYLCCNV and cause more severe symptoms (Zhang et al., 2009). The βC1 of TYLCCNV is able to suppress RNA silencing in *N. benthamiana* 16C line plants and that by suppressing silencing defenses, it critical in symptom induction (Cui et al., 2005). Therefore, ToCSV may be involved in trans-replication of betasatellites associated with other

viruses in mixed infections. Sahu & Sanan-Mishra, (2021) showed that chilli leaf virus (ChiLCV) and its β-satellite, ChiLCB-βC1, suppress RNA silencing. The ChiLCB-βC1 suppresses local silencing in *Nicotiana tabacum* but could not suppress systemic silencing. However, co-infiltration of ChiLCB-βC1 and ChiLCV-CP showed strong suppression of local and systemic RNA silencing. Therefore, ChiLCB-βC1 is important in suppressing systemic silencing in the presence of ChiLCV-CP. According to the findings of this work, future research focusing on all of the other genes in the ToCSV variations should allow for the discovery of which proteins are involved in the significant suppression activity of host RNA silencing and whether they are able to suppress RNA silencing long term.

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Chapter 5

Summary

Globally, tomato is the second most important cultivated crop after potatoes. Through evolution and domestication cultivated tomatoes experienced several genetic bottlenecks due to selection and inbreeding (Dhaliwal et al., 2020). These genetic bottlenecks resulted in limited genetic variation making the crop susceptible to disease and insect epidemics (Miller & Tanksley, 1990; Tanksley & McCouch, 1997). Tomatoes are known to be infected with about 146 viruses belonging to 33 genera (Dhaliwal et al., 2020). Begomovirus infections cause the most damaging effects on tomato production, with tomato yellow leaf curl virus (TYLCV) being the most devastating virus in tropical and subtropical regions (Lapidot & Polston, 2006; Rey et al., 2012). Symptoms induced by TYLCV include severe leaf yellowing, leaf apex cupping, and severe size reduction in normal plant height. These symptoms can have a major impact on the production of the fruits, thus decreasing the quality of commercial tomato produce(Pico' et al., 1996).

The monopartite begomovirus, tomato curly stunt virus (ToCSV), was first discovered in 1997 on infected tomato plants in the Onderberg region and has been identified in seven provinces in South Africa (Esterhuizen, 2012; Pietersen et al., 2000). ToCSV has been shown to be the major tomato infecting begomovirus in South Africa. Symptoms induced by ToCSV were similar to those induced by TYLCV inducing leaf chlorosis, leaf curling, plant stunting and fruit set reduction which lead to severe yield and quality loses (Pietersen et al., 2008). Infection of tomato plants with ToCSV can lead to yield loses of up to 100% (Pietersen et al., 2000) thus making ToCSV a major concern as it affects the South African economy. Two widespread variants of ToCSV were previously described, namely, ToCSV_V30 and ToCSV_V22 (Esterhuizen, 2012). Both variants are made up of 2768 nt of DNA-A and have a genomic structure identical to the Old World monopartite begomovirus with six ORF: V1 (CP), V2 (pre-coat protein), C1 (Rep), C2 (TrAP), C3 (REn) and C4. The genome of ToCSV also consists of a 259 nt IR containing a nonanucleotide motif. It was further shown that ToCSV_V30 causes severe symptoms in tomato and ToCSV_V22 causes mild symptoms in tomato (Esterhuizen, 2012), and therefore it was important to further investigate these two disease phenotypes, which was performed in this study.

The presence (ToCSV V22) or lack (ToCSV V30) of a recombination segment in the V2 region differentiated the two ToCSV variant sequences (Esterhuizen, 2012). A comparison of the remaining ORF showed that both variants had a further 41 single nucleotide difference and 22 amino acid substitutions. These nucleotide modifications were linked to 35 nucleotide mutations in the IR's 3'-end, as well as three, two, seven, and eleven amino acid mutations in the V1, C1, C2, and C3 sequences, respectively (Esterhuizen, 2012). These nucleotide and amino acid difference may contribute to the phenotype differences induced by the two variants in natural host, tomato, and the experimental host, *N. benthamiana.* Therefore, this study aimed to determine what roles pathogenicity determinants V2 (pre-coat protein) and C2 (transcriptional activator protein) encoded by either ToCSV_V30 or ToCSV_V22 variants

contribute to symptom severity in tomato and *N. benthamiana*. This study is significant as it is the first experimental study on ToCSV_V22 and ToCSV_V30 V2 and C2 proteins associated with pathogenicity in the natural host tomato and the experimental host *N. benthamiana*.

In **chapter 2**, mutational studies were carried out to investigate the effect of mutations in the C2 and V2 ORFs of ToCSV variants to determine whether C2 and/or V2 are important for viral replication and viral DNA accumulation in tomato cv. Rooikhaki. It was also of interest to ascertain if V2/C2 were therefore responsible for symptom severity or phenotype differences between the two viral variants. A chimeric infectious clone was produced by swapping the **entire V2 region** of ToCSV_V30 with that of the ToSCV_V22. Inoculation of tomato cv. Rooikhaki with the chimeric infectious clone containing the V2 swap showed no significant difference in symptom severity when compared with symptoms induced by ToCSV_V30, however, the swap induced more severe symptoms in comparison to ToCSV_V22. Interestingly, this demonstrated that V2 plays a role in disease severity in context of the severe ToCSV-V30 variant background. Plants inoculated with the entire V2 swap showed higher levels of viral DNA when compared to inoculation with wild-type ToCSV_V30 and ToCSV_V22, and this result suggests that severity is associated with viral DNA levels in this chimeric swap mutant infected tomato. Further analysis of the V2 region is required in future to determine whether other specific domains in V2 may be responsible for symptom development and severity, and viral DNA accumulation. Previous mutational studies have shown that specific amino acid domains of the V2 region are critical for systemic infection and symptom severity in tomato and tobacco plants (Mubin et al., 2010; Sharma & Ikegami, 2010; Zhao et al., 2020).

Single-site mutations were introduced in regions of the C2 ORF sequences of ToCSV_V30 in order to substitute the amino acids (aa) in the severe ToCSV_V30 with the corresponding ones from the mild V22 variant. Results from agro-inoculation with the single aa mutant clones indicated that the specific amino acids in this region did affect viral DNA replication and were responsible for the differences in symptom severity between the severe ToCSV_V30 and mild ToCSV_V22 variants, since plants inoculated with mutant clones harboring the single aa mutations resembling mild variant V22 showed symptoms similar to those induced by wild type ToCSV_V30. Tomato plants were still able to accumulate viral DNA in infected local and systemic leaves. These results indicate that these single aa variations in the C2 regions are responsible for the symptom severity differences between the two V30 and V22 variants.

Additional analyses were carried out to determine whether C2 of ToCSV_V30 and ToCSV_V22 is a symptom determinant in the experimental host plant, *N. benthamiana*. As a widely used experimental host plant, *N. benthamiana* is susceptible to infection by begomoviruses (Akhtar et al., 2011; Goodin et al., 2008). The use of *N. benthamiana* as an experimental host allows for detection and, identification of plant viruses and their genes (Adkins & Rosskopf, 2002). Infection of *N. benthamiana* with ToCSV variants induced symptoms that differ from those induced in tomato. The severe strain, ToCSV_V30, which induces severe symptoms in tomato, induced mild symptoms in *N. benthamiana*, while the less severe ToCSV_V22 variant induced severe symptoms in *N. benthamiana*. The difference in symptoms between the two hosts

plants induced by the variants could be caused by different interactions of the variants individual proteins with different host-specific components (Osterbaan & Fuchs, 2019).

In **chapter 3,** the possible role of C2-encoded TrAP by both mild and severe variants in pathogenicity was investigated in *Nicotiana benthamiana* by overexpressing *C2* using a PVX expression vector, pGR107. Results showed that C2_V22 was able to enhance viral symptoms in inoculated leaves caused by PVX, however, C2_V30 was unable to alter the symptoms induced by PVX thus indicating that C2 of ToCSV_V22 is a pathogenicity determinant in this host and could be involved in the symptom severity difference between the two variants. This correlates to the more severe symptoms induced by ToCSV_V22 variant in *N. benthamiana*. Expression of C2 protein of ToCSV at an early stage of infection is required for the initial induction of symptoms which likely requires suppression of plant host defense responses. However, this needs to be confirmed in future studies, and in the natural host, tomato where V22 is less severe. This also proposes that pathogenicity of these two geminivirus ToCSV variants is host specific. Host specificity may be due to several factors, including immunity and virus-host protein interactions (Garcia-Ruiz, 2019; Mandadi & Scholthof, 2013) . Intriguingly, plants inoculated with C2 expressed in PVX from both V30 and V22 showed symptom recovery in systemic leaves. C2 has been shown in several other monopartite geminiviruses to be a viral suppressor of RNA silencing (VSR) (Trinks et al., 2005; Van Wezel et al., 2002). Recovery to geminiviral symptoms has been shown to involve RNA silencing and AGO2 in *N. benthamiana* (Silva-Martins et al., 2020), and also in some cases methylation-associated silencing (TGS) of ssDNA geminiviruses (Zhang et al., 2011). The expression of C2 may also be suppressed by other viral genes expressed in late infection, for example the coat protein (Li et al., 2015).

Since it is well documented that C2 in several monopartite begomoviruses is a VSR (Trinks et al., 2005; Van Wezel et al., 2002; Zhang et al., 2011), in **chapter 4** the aim was to investigate if indeed ToCSV C2 was a suppressor of RNA silencing. Results showed that ToCSV C2 is a weak suppressor of local RNA silencing in inoculated transgenic-GFP *N. benthamiana* leaves, however, C2 was unable to suppress systemic silencing. This weak suppression in local leaves might indicate that C2 is a mild VSR required at an early stage of infection or that silencing may occur at different stages of the plant life cycle (Vanitharani et al., 2004). Future research focusing on all of the other genes in the ToCSV variants should identify which proteins are responsible for the significant suppression activity of host RNA silencing.

This study has given insight on the possible roles of two viral genes encoded by ToCSV variants showing different symptom severity and phenotype in different hosts. Further analysis of C2 and V2 may provide knowledge on how these genes interact with various host genes in tomato and *N. benthamiana* to induce different symptoms. Future analysis of nucleotide and amino acid variations of the remaining ToCSV C2 and V2 regions would be valuable. Additionally, the role of other genes/proteins in pathogenicity and symptom severity differences between the two variants need to be explored. The effect of C2 mutants and V2 swap will have to be tested in *N. benthamiana* to determine whether symptom changes affect viral replication in this host. Understanding the biology of ToCSV will give us insight on the

virus-host interactions which may help in establishing strategies to manage virus outbreaks in South African on tomato cultivars

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