CHARACTERISATION OF CITRUS TRISTEZA VIRUS VARIANTS AND THEIR INFLUENCE ON SYMPTOM EXPRESSION IN GRAPEFRUIT

by

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Declaration

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This dissertation includes 2 original papers published in peer-reviewed journals and 1 unpublished publication. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

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ABSTRACT

Citrus tristeza virus (CTV), a member of the family *Closteroviridae*, was responsible for large scale destruction of citrus, especially in the Americas, due to tristeza disease and necessitated a production switch to less sensitive rootstocks. CTV however continues to affect citrus through the stem-pitting disease phenotype which is especially problematic in sweet orange, grapefruit and lime cultivars. In South Africa, the productive lifespan of grapefruit trees was severely affected by stem-pitting, requiring early tree replacement with an associated lag in production. This affect was later mitigated by applying cross-protection, a management strategy using non-stem-pitting sources of CTV, but without prior knowledge of which CTV strains were responsible for stem-pitting or which strains were present in the cross-protection sources. To understand the disease and unravel mechanisms underlying cross-protection, it is necessary to characterise CTV strains to investigate both virus-host- and strain-interactions. The aim of this study was firstly to identify single-strain isolates belonging to different strains, to characterise them biologically and to determine full-genome sequences. These characterised CTV isolates were further used in a complementation study to investigate possible synergistic interactions affecting stem-pitting. Complete viral genomes of eight single-strain isolates were determined during the study. Two commercial grapefruit cultivars, 'Star Ruby' and 'Marsh', were used in a glasshouse trial to evaluate the ability of specific strains to induce stem-pitting in single or mixed infections. Evaluation over four years showed that symptom expression of mild strains did not result in altered symptom expression when in combination with each other. Importantly demonstrating that there was no additive effect on stem-pitting expression with multiple isolates. Relative quantitation of the strains in 'Marsh' and 'Star Ruby' plants indicated that the individual strain concentrations were not significantly altered when in combination with the other strains. A valuable discovery made within this project was the characterisation of two variants of the T68 strain, derived from the same GFMS12 source, but displaying differences in stem-pitting severity in grapefruit. This finding demonstrates the co-existence of severe and mild variants of the same strain in one source and provides an explanation for the presumed strain segregation event observed for the GFMS12 cross-protection source that resulted in the discontinuation of the source for use in cross-protection of grapefruit. The characterisation of these variants will further assist in the identification of the sequence determinants for stem-pitting in grapefruit.

OPSOMMING

Sitrus tristeza virus (CTV), 'n lid van die familie *Closteroviridae*, was verantwoordelik vir grootskaalse vernietiging van sitrus, weens tristeza siekte en het 'n produksie veradering na minder sensitiewe onderstamme genoodsaak. CTV het egter steeds 'n nadelige invloed op sitrus weens stamgleuf wat veral problematies is in soetlemoen, pomelos en lemmetjie kultivars. In Suid-Afrika is die produktiewe lewensduur van pomelo-bome verlaag weens stamgleuf, wat vroeë boomvervanging met 'n gepaardgaande produksieverlaging vereis het. Hierdie invloed is later verminder deur die toepassing van kruisbeskerming, 'n bestuurstrategie waar ligte bronne van CTV toegepas word, maar sonder voorafgaande kennis van watter CTV-rasse verantwoordelik is vir stamgleuf of watter rasse teenwoordig is in die bronne. Om die siekte te verstaan en meganismes onderliggend aan kruisbeskerming te ontleed, is dit nodig om CTV-rasse te karakteriseer om beide virus-gasheer- en rasinteraksies te ondersoek. Die doel van hierdie studie was om eerstens enkel-ras isolate wat aan verskillende rasse behoort, te identifiseer, om hulle biologies te karakteriseer en om volledige-genoom-nukleotiedvolgordes te bepaal. Hierdie gekarakteriseerde CTV-isolate is verder gebruik in 'n komplementeringsstudie om moontlike sinergistiese interaksies te ondersoek wat stamgleuf beïnvloed. Vollengte virale genome van agt enkelras-isolate is tydens die studie bepaal. Twee kommersiële pomelo kultivars, 'Star Ruby' en 'Marsh', is in 'n glashuisproef gebruik om die vermoë van spesifieke rasse te evalueer om stamgleuf in enkel- of gemengde infeksies te veroorsaak. Evaluering oor vier jaar het getoon dat simptoom uitdrukking van ligte rasse nie gelei het tot 'n veranderde simptoom uitdrukking wanneer hul in kombinasie met mekaar voorgekom het nie. Dit is belangrik om aan te toon dat daar geen toevoegende effek op stamgleuf uitdrukking met veelvoudige isolate was nie. Relatiewe hoeveelheids bepaling van die rasse in 'Marsh' en 'Star Ruby' plante het aangedui dat die individuele raskonsentrasies nie beduidend verander in kombinasie met die ander rasse nie. 'n Waardevolle ontdekking wat in hierdie projek gemaak is, was die karakterisering van twee variante van die T68-ras, afkomstig van dieselfde GFMS12-bron, maar met verskille in stamgleuf uitdrukking in pomelo's. Hierdie bevinding demonstreer die gelyktydige bestaan van strawwe en ligte variante van dieselfde ras in een bron en verskaf 'n verduideliking vir die vermoedelike ras segregasie gebeurtenis waargeneem in die GFMS12 kruisbeskerming bron, wat gelei het tot die staking van die bron vir gebruik as kruisbeskerming van pomelo's. Die karakterisering van hierdie variante sal verder help met die identifisering van die volgorde-bepaling van stamgleuf in pomelo's.

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The Creator God

John 1:3
"All things were made through Him, and without Him was not anything made that was made."

Jerimiah 32:17
"For of Him, and through Him, and to Him, are all things: to whom be glory for ever. Amen."

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1 INTRODUCTION

1.1 GENERAL INTRODUCTION

Citrus is grown either for the fresh fruit market or for juice production and South Africa is the 13th largest citrus producer worldwide. Local production is geared largely for the fresh fruit export market, making South Africa the third largest fresh citrus exporter. In 2017 South Africa produced 2.3 million tons of citrus with 76% of fruit produced, being exported, 18% processed and 6% consumed by the local market. Citrus production occurs in all provinces apart from the Free State and Gauteng provinces, with the largest production areas located in the Limpopo and the Eastern Cape provinces [1].

Grapefruit represents 7% of total citrus production worldwide and globally, 8.3 million tons of grapefruit were produced in 2016 [2]. China is the top producer of grapefruit in the world and South Africa the sixth largest, but South Africa is the largest fresh grapefruit exporter. The grapefruit sector represents 13% of the total South African citrus exports [1]. The major grapefruit production areas are found in the Limpopo and Mpumalanga provinces and in 2017 a total of 7886 ha was under grapefruit production, 56% in Limpopo and 20% in Mpumalanga. 'Star Ruby' was the most popular cultivar with 83% of total grapefruit hectares planted, followed by Marsh at 13% [1].

The early beginnings of the citrus industry in South Africa was severely hampered due to quick decline [5], a disease caused by citrus tristeza virus (CTV) when sour orange, a CTV sensitive rootstock, is used. A change to CTV tolerant rootstocks remedied the quick decline problem, but the production of grapefruit was still severely impacted by CTV. Severe stem-pitting, a different disease associated with CTV, significantly reduced the productive lifespan of grapefruit trees with the most popular cultivar, 'Star Ruby', affected the most [6]. A biological intervention, referred to as mild-strain cross-protection was successfully implemented for grapefruit [4]. This management practice entails the application of non-pathogenic CTV sources to propagation material, which mitigates the effects of severe field strains. However, this intervention was not always successful and changes to the CTV sources used, were required [7]. This application was, and still is, informed

solely on empirical field trial testing of various CTV sources, without an understanding of the strains or mechanisms involved.

The genetic and biological complexity of CTV has only recently been realised [3] and the capability to develop strain diagnostics follows the characterisation of strains. The understanding of the diversity of strains now enables further research to determine the strain components involved in disease expression and also those that play a role in cross-protection. In this study, single strains were identified and investigated for pathogenicity and possible synergism in commercial grapefruit cultivars. The most widely planted cultivars in South Africa, 'Star Ruby' and 'Marsh' were used for this investigation. 'Star Ruby' is a red-fleshed cultivar and 'Marsh' is a white cultivar. 'Star Ruby' is also known to be more sensitive to stem-pitting of CTV. This study purposed to improve the understanding of strains impacting stem-pitting disease in commercial grapefruit.

1.2 PROJECT AIMS AND OBJECTIVES

The aim of the study was to identify and characterise single-strain CTV isolates and variants of a single-strain to investigate their effect, singly or in combination on stem-pitting expression in commercial grapefruit cultivars.

The following objectives were set out to achieve this aim:

- Develop diagnostic assays to detect all known CTV strains.
- Identify and/or isolate CTV single-strain isolates.
- Characterise single-strain isolates by full-genome sequence determination and comprehensive biological characterisation on a standardised host range.
- Develop strain-specific RT-qPCR assays for relative quantification of five CTV isolates.
- Evaluate symptom expression of strains, inoculated singly and in various combinations, in two commercial grapefruit cultivars over time.
- Investigate possible strain interactions by determining individual strain concentrations in the constructed populations by using RT-qPCR.
- Interrogate biological and genetic differences two variants of one strain, derived from the same source plant.
- Evaluate the effect of variants of a strain on stem-pitting in grapefruit, in a glasshouse trial and an existing field trial.

1.3 CHAPTER LAYOUT

The dissertation contains six chapters that are introduced, concluded and referenced individually. A general introduction is followed by a literature overview, three research chapters and a general conclusion.

Chapter 1: Introduction

General introduction, aims and objectives of the study and the chapter layout of the thesis are provided. The scientific outputs generated during the study are stated and the contributions by the author are stated.

Chapter 2: Literature Review

A literature overview is presented pertaining to citrus, with a focus on grapefruit and the impact of CTV on this sector. The biology, genetic diversity of the virus and detection methodologies are reviewed.

Chapter 3: Molecular and biological characterisation of single-strain CTV isolates

In this chapter, improvements to CTV strain-specific diagnostics are reported which enable detection of all known CTV strains. Additionally, seven single-strain isolates were identified, biologically characterised and complete genomes determined. This study was foundational to identify isolates that could be used in a complementation study in Chapter 4.

Chapter 4: CTV single-strain isolates in single and mixed infections: symptom expression and virus concentration

This chapter reports a glasshouse trial of two commercial grapefruit cultivars, performed to investigate whether strains, in various combinations, alter stem-pitting expression. A four year biological evaluation indicated that complex populations of mild and moderate stem-pitting strains did not influence stem-pitting expression. Also reported is the development of strain-specific real-time assays and their use to investigate whether individual strain concentrations were changed in various strain populations. Strains were found to propagate within certain concentration ranges, independent of population structure, but concentrations did differ between the two grapefruit hosts.

Chapter 5: T68 variants of GFMS12 differ in stem-pitting severity in grapefruit

This chapter reports, the isolation, biological characterisation and full-genome determination of a severe stem-pitting variant and is compared to a mild stem-pitting variant of the T68 strain, derived from the same source plant. Variants of a single-strain were diagnostically differentiated and their effects on stem-pitting were investigated in grapefruit, in a glasshouse trial and in an existing field trial. This is the first report of the characterisation CTV variants of a strain, originating from the same parental population and which display altered stem-pitting severity.

Chapter 6: Conclusion

This chapter provides general concluding remarks and future research prospects.

1.4 RESEARCH OUTPUTS

The following publications and conference proceedings were generated during the study. Research contributions are listed.

1.4.1 Publications

- **Cook G**, van Vuuren SP, Breytenbach JHJ, Burger JT, Maree HJ (2016) Expanded Strain-Specific RT-PCR Assay for Differential Detection of Currently Known *Citrus Tristeza Virus* Strains: a Useful Screening Tool. Journal of Phytopathology 164:847-851
  This paper describes the strain-specific assays developed to improve existing diagnostics which is an outcome of Chapter 3 and is in its entirety the work of GC.

- **Cook G**, van Vuuren SP, Breytenbach JHJ, Steyn C, Burger JT, Maree HJ (2016) Characterization of Citrus tristeza virus Single-Variant Sources in Grapefruit in Greenhouse and Field Trials. Plant Disease 100:2251-2256
  This paper presents full-genome sequence and biological characterisation of single-strain CTV isolates. The work presented was part of the PhD study and an outcome of Chapter 3. It includes the work of GC apart from the field trial that was initiated by SPvV. Field data was generated by JHJB and GC performed the analysis on the field data.

1.4.2 Manuscripts in preparation

Cook G, Breytenbach JHJ, van Vuuren, SP, Coetzee, B, Steyn C, Clase, R, Burger JT and Maree HJ

Variants of the T68 strain of citrus tristeza virus from the GFMS12 source differ in stem-pitting expression in grapefruit. In preparation.

This paper is part of the PhD study and reports the results of Chapter 5. GC did all the work apart from the NGS sequence verification done by BC and the generation of the field trial data. The field trial was initiated by SPvV and field data was generated by JHJB. GC performed the analysis on the field data.

1.4.3 Conference presentations (presenter underlined)

19th Conference of the International Organization *of* Citrus Virologists, Kruger National Park, South Africa. 28 July - 2 August 2013

- G. Cook, V.Z. Maqutu, J.H.J. Breytenbach and S.P. van Vuuren. Profiling of the South African citrus tristeza virus pre-immunisation and trial sources used for cross-protection by means of an RT-PCR genotype system.
  This presentation included the development and use of CTV-strain diagnostics which is an outcome of Chapter 3 and is in its entirety the work of GC.

20th Conference of the International Organization *of* Citrus Virologists, Chongqing, China, 10-15 April 2016.

- J.H.J. Breytenbach, G. Cook and S.P. van Vuuren. Field performance of various CTV cross-protection sources in grapefruit in different climatic conditions.
  Interpretation of the field trial results was reliant on CTV strain determination. Strain diagnostics, developed in Chapter 3, was an integral part of the analysis for the presentation. Strain variants, identified in Chapter 5, are included in these trials. GC did the strain determination and analysis of field trial data.

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2 LITERATURE REVIEW

2.1 CITRUS ORIGINS AND THE STORY OF GRAPEFRUIT

Citrus has been cultivated for centuries in South East Asia with its origin probably in a region that is currently north eastern India, northern Burma (Myanmar) and north western Yunnan province in China [122]. Although the genus *Citrus* is diverse, fossils discovered in Yunnan indicates that the genus has a single progenitor in the Miocene epoch [123], followed by further speciation events [122]. Whole genome phylogeny of existing citrus types shows introgression of three major ancestral species; pure mandarins (*Citrus reticulata* Blanco), citron (*C. medica* L.) and pummelo (*C. maxima* (Burm.)), but also a wild citrus species, *C. micrantha* and Ngami kumquat (*Fortunella margarita)*. These gave rise to commercial citrus including oranges, limes, lemons, grapefruit and other citrus types which are all hybrids of these ancestral species [122].

Citrus belongs to the family *Rutaceae* and is placed in the subfamily *Aurantioideae* together with the genera; *Fortunella*, *Poncirus*, *Eremocitrus* and *Microcitrus*, however Wu et al. [122] show that *Fortunella*, *Eremocitrus* and *Microcitrus* should be considered species of *Citrus* with *Poncirus* remaining a separate genus. There is little consensus in citrus taxonomy and a number of nomenclature systems exist, but the system proposed by Mabberley [63] best accommodates the insights gained through genomic studies, which show that admixtures of ancestral species have led to the various citrus types by indicating hybridization of genomes as per example; sweet orange (*Citrus* × *sinensis* (L.) Osbeck) with the "×" denoting that sweet orange is a hybrid. The nomenclature system proposed by Mabberley is used further in this dissertation.

Grapefruit (*C.* × *paradisi* (Macfad.)) arose through a hybridisation event between sweet orange and pumelo in Barbados in the 17th or 18th century [108]. The prefix "grape" possibly refers to the fruit that hang in small clusters, suggestive of grapes, but others think this is improbable and therefore uncertainty exists regarding the origin of the name. The fruit's unique bitter flavour is due to a flavour compound, naringin, which is more concentrated in grapefruit compared to other citrus types.

Grapefruit first gained popularity in the United States. It was commercial planted in Florida in the late 19th century and subsequently successfully cultivated in the Rio Grande Valley of Texas, in

Arizona and also in California. A range of cultivars were developed over time with different internal flesh colours ranging from white, pink to red. Marsh is the only white fleshed cultivar of worldwide commercial significance, but numerous pigmented cultivars are traded. Pink grapefruit cultivars were selected from bud mutations on white grapefruit trees and cultivars were additionally developed by irradiating seeds of existing varieties which gave rise to Star Ruby, the most deeply pigmented grapefruit cultivar to date [128]. Star Ruby demonstrated inferior production characteristics in the USA and was therefore not widely planted, but the history of Star Ruby in South Africa is different. The cultivar was introduced as seed in the mid 1970's and various selections were made from the original seed source. Trees of the South African Star Ruby selection grow precociously and have a different growth habit to the original Star Ruby [129]. It is the most popular grapefruit cultivar grown in South Africa with the only detrimental attribute being its' sensitivity to citrus tristeza virus [108].

2.2 CITRUS TRISTEZA VIRUS

2.2.1 A destructive pathogen

Citrus tristeza virus (CTV) belongs to the family *Closteroviridae* and genus *Closterovirus* [66]. CTV virions are long, flexuous particles, approximately 2000 × 11 nm in size, phloem restricted [57, 104] and comprise two coat proteins, CP and CPm, covering 95 and 5% of the particle length, respectively, with CPm covering only one end of the particle known either as the 'head' or 'tail' [29]. Initially this structure was referred to as the 'tail' but later studies showed that this structure is at the 5' end of the genome suggesting that it is rather the 'head' of the particle. In this dissertation this structure is referred to as the 'head' (Figure 1).

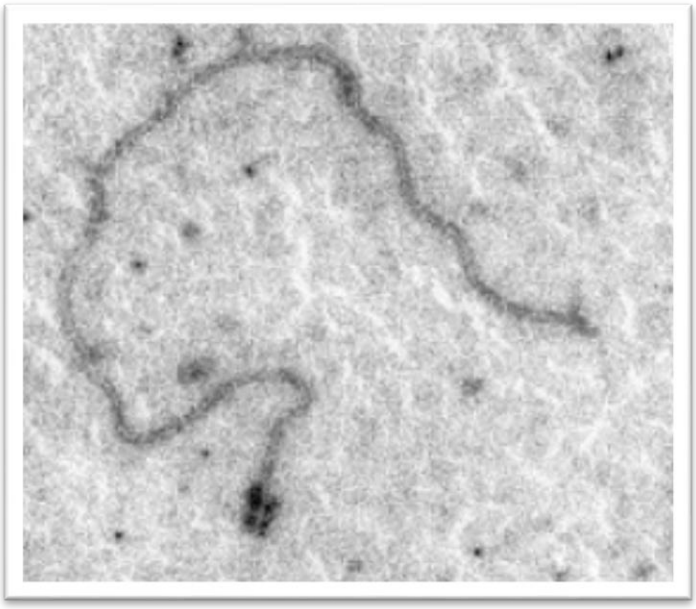

Figure 1. Electron micrograph of a CTV virion. (Reproduced from Niblett et al. [82]). The head structure is enhanced by immunogold labelling of CPm.

CTV was responsible for great losses in many major citrus producing countries [76, 94]. In the order of 100 million trees were lost, mostly in the America's, but also in a number of European and Mediterranean countries as a result of tristeza disease, alternately known as quick decline [76]. Tristeza was considered the most destructive disease of citrus prior to the spread of Huánglóngbìng to the Americas. The tristeza epidemic was inadvertently caused by interventions to control phytophtora root rot. The phytophtora resistant rootstock, sour orange (*Citrus* × *aurantium* L.), became widely used and was the favoured rootstock for citrus production in the Mediterranean region and the Americas due to its adaptability to a wide range of soil types, as well as the superior fruit quality imparted by the rootstock [6, 76]. Unfortunately, sour orange is the only rootstock susceptible to tristeza disease which occurs when either sweet orange, grapefruit or mandarin scions are propagated on sour orange rootstocks in the presence of certain CTV strains. Anatomical studies showed phloem sieve element necrosis below the bud union, girdling the tree and disrupting sap flow to the roots and ultimately causing rapid decline and death of the tree [109]. The tristeza epidemic necessitated drastic intervention and a production switch to less sensitive rootstocks.

CTV however continues to affect citrus through the stem-pitting disease phenotype. Stem-pitting ascribed to CTV was reported on grapefruit [70, 84] and sweet orange [14, 79, 92], but also on other citrus types, notably acid limes [51]. Removal of the bark shows distinctive pits and grooves in the underlying wood with the inner surface of the bark displaying corresponding projections [10, 109] (Figure 2). Many stem-pitting phenotypes are found, ranging from grooves to fine porous wood pitting [37]. These symptoms can be observed externally as deep grooves on the tree trunk and limbs, or the trunk may display a knotted appearance when severe porous wood pitting is present. Tree decline associated with stem-pitting resembles that of tristeza, but the progression is gradual and seldom results in the death of the tree. The affected trees display poor condition, often yielding small, unmarketable fruit. Wood depressions or pits form at sites of disrupted vascular growth, whereas surrounding tissue develops normally. Aberrant differentiation of the cambium layer was observed with a lack of normal xylem production and an over production of phloem and phloem parenchyma tissue growing into the wood depressions [10, 109]. Very little is understood regarding the induction of stem-pitting, but more CTV inclusion bodies were observed in the phloem tissue in the wood depressions than in phloem tissue with normal appearance [10].

2.2.2 The impact of CTV on South African citrus

Sour orange was used as the principal rootstock in the initial phases of the establishment of the South African citrus industry, but major constraints were experienced with its use [65]. In 1947 a virus was suggested as the cause of tree decline [83] and these symptoms were indicative that CTV was present in South Africa early in the industry's existence. The quick decline problem was managed by a change to CTV tolerant rootstocks such as rough lemon and later trifoliate hybrid types. CTV however remained a limiting factor in the production of grapefruit due to severe stem-pitting (Figure 3) [69, 71, 84]. Decline in production and fruit size resulted in early tree replacement and associated financial losses. Prior to any intervention strategies, the average lifespan of white grapefruit cultivars was reduced to 15 years and that of red cultivars to 10 years due to severe stem-pitting [64].

Figure 2. Stem-pitting grooves in wood with corresponding projections in the bark.

Figure 3. Severe stem-pitting field symptoms on grapefruit. Severe stem-pitting on branch (A) and tree trunk (B), which causes tree decline with resulting twig dieback and small fruit (C).

2.2.3 Genome organisation and protein functions

CTV has a single-stranded, positive-sense RNA genome and has the largest known plant viral RNA genome of approximately 19300 nucleotides (nt) [7, 55]. The genome comprises 12 open reading frames (ORFs) and two untranslated regions (UTRs) at the 5' and 3' termini [55]. The *replication gene block* and *quintuple gene block* gene clusters, common to members of the family *Closteroviridae*, are evident in the CTV genome (Figure 4). Proteins of the replication gene block are required for RNA replication and those of the quintuple gene block for cell-to cell movement [27].

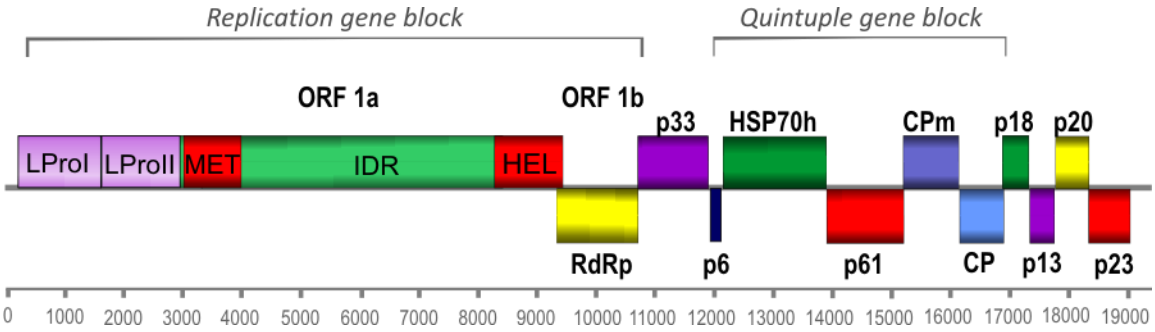

Figure 4. Schematic diagram of the genome organisation of CTV. Adapted from Dawson et al. [23] and Dolja et al. [27]. The boxes show the positioning of each of 12 ORFs. L-Pro, papain leader proteases; MET, methyl transferase-like domain; IDR, large inter domain region; HEL, helicase-like domain; RdRp, RNA-dependent RNA polymerase domain; HSP70h, heat shock protein homologue; CPm and CP, minor and major coat proteins.

The *replication gene block* comprises the 5'-half of the genome and consists of ORF1a and 1b that are expressed as two polyproteins from the genomic RNA (gRNA). ORF1a includes domains for two papain leader proteases (L-Pro), a type I methyltransferase (MT) and an RNA helicase (HEL). These gene products are all required for RNA replication [105]. ORF1b is expressed by means of a +1 ribosomal frame shift and encodes the RNA-dependent RNA polymerase (RdRp) [55].

The *quintuple gene block* is a set of five signature ORFs that express major (CP~ p25) and minor (CPm ~ p27) coat proteins, a heat shock protein 70-homologue (HSP70h ~ p65), an HSP90h (p61) and a ≈6-kDa protein (p6) [27]. Encapsidation of RNA initiates at the 5'-end with CPm. Both HSP70h (p65) and HSP90h (p61) are required for virion assembly, and together with the CPm encapsidation, are limited to the 5'-RNA region and the first 5 % of the virion or head. The

remaining genomic RNA is encapsidated by the CP. It is possible that p65 and p61 are additionally incorporated in the virion assembly at the transition zone between CPm and CP, but this has not been definitively demonstrated for CTV [107]. The HSP70h of *Beet yellows virus* was associated with intercellular plasmodesmata and is considered one of the movement proteins [27]. The primary function of a virion is to protect the virus genome, but it is thought that the head structure found with closteroviruses, uniquely facilitate cell-to-cell movement. Virion assembly and virus cell-to-cell transport are therefore closely correlated in closteroviruses [27]. The full function of the other signature protein (p6) in CTV is unclear but it is required for systemic infection [111].

Five additional ORFs are present in the 3'-half of the genome of CTV including p20, a homologue to p21 of BYV, and four unique ORFs, including p33, p18, p13 and p23, with no homologues found in other closteroviruses [27]. The p23 protein is an RNA binding protein [61] which controls the synthesis of positive sense RNAs [106] and also acts as a silencing suppressor, as does p20 and CP [62]. Three proteins; p33, p18 and p13, are required for systemic infection in some, but not all citrus hosts [112] and are additionally associated with stem-pitting expression [113].

The ten genes in the 3' genome region are expressed by 3' co-terminal sub-genomic messenger RNAs (sg mRNAs) [47].

2.2.4 CTV diversity and strains

Terminology describing plant viruses has not been used consistently in CTV related literature and a few terms are defined here for use in this dissertation. A field isolate is referred to as a source and an isolate is a derivation of a source, obtained by a single isolation event such as a single aphid transmission. Strains are not uniform genetic entities, but are groups of isolates that share close nucleotide sequence homology and are clearly differentiated from other groups based on sequence identity. The demarcations for strains of CTV are discussed later. CTV isolates within strains, although showing close sequence identity, may display phenotypic diversity. Variants are derived from the same parental isolate and display small differences in nucleotide sequences between each other.

Diversity is driven by mutations introduced during replication and recombination events [34, 50]. RdRp-replicating RNA viruses are prone to mutation which can be introduced during genome

replication [50]. This inherency to diversify ensures survival of a virus as changes allow the virus to adapt to changing environments such as new hosts [112], evade plant resistance mechanisms [119] and alter pathogenicity [58].

A distinctive sequence divergence is observed with CTV, where genomes sort into two clearly separated lineages with high sequence divergence in the 5'-half of the genome and a gradual increase in sequence identity toward the 3'-region [48]. This sequence divergence was observed with the first two full-length CTV genomes sequenced; strains T36 [55] and VT [68]. The first 1-9100 nucleotides showed a 71% sequence identity and the following 9101-19226 nucleotides shared 87% identity. The gradual change over the genome suggests that the diversification is not due to a recent recombination event between two divergent isolates, but is a result of a longer evolution period [68]. Within the two distinct lineages, further divergence is found with at least seven phylogenetic clades defined, which differ by 10-20% at the nucleotide level and are referred to as strains [40]. The currently recognised strains are T36, VT, T30, T3, HA16-5, T68 and RB. Recently two isolates were described that potentially justify the recognition of an eighth strain, S1 [124].

CTV is most often found naturally as a complex of strains [11, 18, 97, 121], a result of the longevity of citrus trees. New infections are continuously introduced by aphid transmissions and the diversity accumulates over time. Vertical virus transmission by clonal propagation allows the introduction of the virus to new environments, thus further expanding the diversity. Complex CTV populations, in long-term persistent infections, allow for the generation of abundant genetic diversity in a single host plant [100, 118, 120]. Co-replicating genomes undergo numerous recombination events, a phenomenon commonly observed in positive-sense RNA plant viruses [15]. In the process gene domains are exchanged and proteins are reconstituted, altering phenotypic expression.

2.2.5 Transmission

CTV is not seed transmitted [72], but is graft transmissible and infections are perpetuated by graft propagation. The early movement of citrus from its origin to other world regions would have been by fruit and seed and CTV would not have been dispersed by that means, but in the late 19th century, with advances in shipping, entire plants could be transported to new destinations. This period is likely the initial dissemination of both CTV and aphid vectors globally [76].

CTV is transmitted naturally by a number of aphid species in a stylet-borne or semi-persistent manner [8]. The brown citrus aphid, *Toxoptera citricida* (Kirk.) is the most efficient CTV vector [125], but CTV is also transmitted by *Aphis gossypii* (Glov.), *A. spiraecola* (Patch), (formerly *A. citricola* (v. d. Goot)), and *T. aurantii* (B.de.F.) [90]. *A. gossypii* was shown to be 6-25% less efficient in transmitting CTV compared to *T. citricida* [125] and *A. spiraecola* and *T. aurantii*.transmit CTV at low rates [90]. Both *T. citricida* and *A. gossypii* are endemic in South Africa [75, 110], necessitating aphid control during citrus production.

Aphid transmission of CTV affects both the establishment and change in CTV strain populations in orchards. The vector-virus interaction is not a coincidental association and transmission efficiency is influenced by specific interactions between the aphid species and CTV. The minor coat protein of CTV binds to the lining of the aphid cibarium (space anterior to the mouth cavity) by means of a protein-carbohydrate interaction. The presence of both p61 and p65 reduces the binding of virions and virion retention is therefore determined by an interplay of the three virus-encoded proteins [56].

Differences in transmission of CTV isolates from the same acquisition host were reported [4, 39] and transmission efficiencies ranged from 7-100% [89]. Nucleotide sequence variation in the p61 and p65 genes influence the efficacy of aphid transmission, independent of virus accumulation [44]. Polymorphisms in these genes provide a possible explanation for variable transmission of strains, a phenomenon otherwise referred to as genetic bottlenecking. However, two studies also suggest possible complementation of strains in aphid transmission in that some strains, which are poorly transmissible, are assisted by the presence of other, more transmissible strains, to be efficiently transmitted [12, 46].

2.2.6 Host pathogen interactions

The natural host range of CTV is primarily the genera *Citrus* and *Fortunella*, but also the closely related *Poncirus trifoliata* (L.) Raf., initially considered resistant to CTV infection due to a *Ctv* resistance gene [25], but infection by the RB strain was shown to overcome the resistance [21, 38]. *P. trifoliata* resistance to certain strains is not uniform in the plant as strains excluded from above ground plants parts, were detected in roots, demonstrating tissue tropism beyond the phloem limitation observed with CTV [41]. Strain-specific limitation of CTV to roots was demonstrated not only in trifoliate orange, but also 'Sun Chu Sha' mandarin and 'Swingle' citrumelo [41].

Systemic virus infection requires initial cell infection, virus replication following infection, cell-to-cell and long-distance movement of virions. These requirements are dependent on numerous interactions of the virus with cellular host components [22, 23]. CTV infections are restricted to phloem-associated cells, either companion or phloem parenchyma cells, where the virus transverses cell walls to adjacent cells, creating clusters of infected cells. Long distance movement occurs when the virus enters the phloem sieve elements from these nucleated cells, travels through the sieve channels and exits at a further point into phloem associated cells again [23]. The more susceptible a host is, the more cells form part of an infection focus, whereas in less susceptible hosts infection foci consist of fewer, often single cells [30].

Three non-conserved CTV genes, p33, p18 and p13, are not required for systemic infection of some citrus hosts, but are essential for infection of other citrus hosts [112]. It is assumed that acquisition of these genes extended the host range of CTV and the influence of these genes indicate complex virus-plant interactions.

Sustained infection of a virus is dependent on the virus' ability to evade or circumvent plant defence responses. The mechanism of RNA silencing or RNA interference (RNAi) is a well-recognised plant defence system [28] and viruses encode silencing suppressors that counteract the RNAi plant defence mechanisms [88]. Three silencing suppressors were identified for CTV and include the proteins expressed by CP, p23 and p20. Suppression of intercellular silencing is effected by both CP and p20 expression, while intracellular silencing suppression is effected by p23 and p20 expression [62].

As early as 1955, variation in symptom expression in different citrus hosts was ascribed to the existence of different components of the virus [70, 74]. The identification of CTV strains gave insight to the complex phenotypic expression, but symptom expression was clearly also reliant on the citrus host [37]. Three disease syndromes are recognised for CTV, including quick decline, stem-pitting and seedling yellows [74]. The symptoms of quick decline and stem-pitting were discussed earlier in this chapter. Seedling yellows (SY) however, is not regarded detrimental to citrus production, but is a useful phenotype for biological characterization. SY expression is often indicative of decline inducing isolates and symptoms are observed only at the seedling stage in sour orange, grapefruit and lemons. New growth of infected seedlings exhibit distinctive leaf chlorosis symptoms and mild stunting, but can also result in severe stunting depending on the isolate [8, 73]. The expression of the three symptoms is dependent on both citrus host and CTV isolate.

The 3'-terminal genome region, including the p23 gene and the 3'UTR, of the T36 strain was associated with both seedling yellows and decline, but specific sequence determinants were not identified [1, 24]. It is not known whether the same 3' region of other decline-inducing strains, apart from T36, are similarly associated with both seedling yellows and decline [24]. The genetics determining stem-pitting may be intricate and likely involves the interrelated expression of three genes, p33, p18 and p13 which are unique to CTV, but the mechanism is not yet understood [113].

2.2.7 Disease management

It was through the international movement of citrus that CTV disease outbreaks had occurred. Therefore, quarantine and budwood certification programmes, which are operational in most major citrus producing countries, are crucial to prevent introduction of CTV to virus-free areas or to avoid new strain introductions where CTV is endemic [117]. Attempts to control severe CTV outbreaks by eradication programmes, conducted in California (USA) and Israel, were not able to eradicate CTV in the long term, in the presence of efficient aphid vectors, but did manage to suppress disease incidence in some areas [6, 8, 87].

The tristeza outbreaks in the 1930s through to the 1980s lead to a requisite change of rootstock use, from sour orange to other more CTV tolerant rootstocks [76]. This was the only intervention

possible to enable continued citrus production in CTV prevalent regions and meant the discontinuation of a horticulturally superior rootstock.

In the presence of widespread, severe stem-pitting in sweet orange [14, 53, 92, 102], grapefruit [19, 33] and lime [51], a disease management strategy called mild-strain cross-protection was investigated and implemented in a number of countries including Australia, Argentina, Brazil, Japan, Peru and South Africa [20]. Cross-protection is an approach whereby 'mild' CTV sources are intentionally introduced in propagation material to mitigate the effects of severe, field-challenge strains of the same virus. Although cross-protection was developed first for other crops and commercially tested for tomato, cacao, passionfruit and Zucchini, the practice was discontinued due to various factors, but it is in citrus that cross-protection is still successfully applied [20]. The mild sources currently used for cross-protection were empirically selected and tested. Isolates were obtained from symptomless trees, found in severely affected orchards or by passaging isolates through various hosts, followed by glasshouse and field testing [78, 91, 93, 114]. The selection process is lengthy and only a few candidate isolates were found [77, 95]. Lee et al. [59] suggested four criteria that define a good cross-protection source; (1) the isolate should express mild or no symptoms on the intended citrus host, but also on other citrus species, (2) the isolate should be applied in similar environmental conditions from which it was isolated, (3) the isolate should translocate efficiently and at high titre in the plant and (4) be aphid transmissible. Despite the arduous selection process for a suitable source, the success of the Brazilian cross-protection programme is well documented for the protection of millions of 'Pera' sweet orange trees with the IAC isolate [77, 96] in addition to another source, 'Citrovita', found optimal for cooler production areas [103]. In Peru, sweet orange and 'Key' lime production was saved from the brink of collapse by its cross-protection programme which is still operational today [9]. The Australian and South African grapefruit cross-protection programmes are also two enduring success stories [13, 64] , but not without challenges and the original South African source used, GFMS12, was replaced due to suspected strain segregation that lead to severe stem-pitting [115].

Cross-protection field trials in Florida to control decline on sour orange showed promise, but the trials were lost in a severe freeze event and the work was discontinued [60]. Other attempts to cross protect against decline isolates were unsuccessful [96].

The mechanics of cross-protection is a challenging research topic. Superinfection exclusion (SIE) has been proposed as the possible mechanism behind CTV cross-protection. Using an infectious T36 clone labelled with a green fluorescence protein (GFP) it was demonstrated that infection with the clone was not possible if the plant contained a primary infection of a natural T36 isolate, but this exclusion did not occur with heterologous strains [31]. Achieving SIE requires the expression of a functional p33 protein [32], suggesting that RNA silencing probably does not explain SIE. Further research indicated that it is not only p33 that determines exclusion, but homologous sequence determinants in the 5'region of the CTV genome are also required to effect SIE [3]. The SIE model system was tested under field conditions and was not as successful as the glasshouse trials. The fitness of primary and challenge isolates determined which isolate persisted. It was further demonstrated that superinfection was sometimes evident in roots but not in shoots which meant that SIE was not always functional in the whole plant [45]. To definitively confirm that cross-protection is achieved by this mechanism, both mild and severe isolates of a strain need to be identified to demonstrate a natural state scenario.

2.2.8 Detection methodologies and applications

The development of detection methods for CTV followed essentially the same progression as for most plant viruses. Electron microscopy and biological indexing were the first means of detection. The diversity observed in the symptom expression of CTV between isolates and different citrus types is a unique complication in CTV pathology which led to the use of the standardized 'Garnsey' host range to characterise isolates and determine pathogenicity [36]. This host range is still regarded as the standard for biological characterisation of an isolate. Serological tests using polyclonal antibodies in SDS-immunodiffusion [35] and enzyme linked immuno-sorbent assay (ELISA) [5] were developed, followed later by the production of monoclonal antibodies (MCAs) [86, 116]. One particular antibody, MCA13, was useful as it reacted with most decline and severe stem-pitting isolates, but not with non-decline and mild stem-pitting isolates [86] and was used extensively in the Californian CTV eradication programme [87]. However, determination of whether a reactive sample is a decline or a stem-pitting isolate was not possible and non-reactive samples were either infected with mild strains or un-infected, requiring additional tests for clarification [82]. The epitope, detected by MCA13, is located in the major coat protein (CP) and

a single, non-synonymous nucleotide change was found to be responsible for the differentiation. A bi-directional RT-PCR was later developed, targeting the single nucleotide polymorphism in the CP. Severe and mild isolates could then be identified simultaneously. Additionally, the method also confirmed the presence of CTV in the presence of only mild strains and thus bridged the limitations of the MCA13 ELISA [85]. RT-PCR became the routine diagnostic methodology thereafter [67].

The first RNA based technique used was the analysis of double stranded RNA patterns and qualitative difference could be seen between infected plants, which pointed to the diversity known to exist for CTV [26]. The challenge for CTV diagnostics, beyond mere detection, was to distinguish strains. Initial efforts used methods including restriction fragment length polymorphism (RFLP), hybridization with group-specific probes, single-strand conformation Polymorphism (SSCP) and cloning and sequencing of amplification products of various genome regions [82]. The laborious nature of these techniques meant that is was not possible to analyse large sample numbers. An RT-PCR method, using genetic markers targeting four genome regions, generated specific marker patterns for known strains, but failed to identify isolates which generated atypical marker patterns. The atypical patterns indicated the existence of even greater diversity than was known at the time [49]. Further attempts were made to differentiate severe and mild isolates using RT-PCR, but the tests were also not sufficiently inclusive to accommodate the full range of CTV diversity [52, 101, 126]. A multiplex RT-PCR was developed to simultaneously detect five strains [98], but the discovery of additional strains required further assays [16] and as more strains are identified [124], continual additions to strain detection assays are required.

The next frontier was to quantify strains for population studies to investigate population dynamics and strain interactions. Real-time quantitation opened the door for this research and population studies were able to show differences in relative titres of strains within a source [2, 42], the influence of temperature on virus population structure [17], demonstrate complementation between strains [43] and yet, many interactions remain to be explored.

The advancements in next generation sequencing (NGS) technology made it a valuable tool for plant virus discovery [80, 81, 99]. It is now commonly used for accurate, full-genome sequence

determination of isolates and new strains [124, 127] and with the significant advances in this technology, its application for routine diagnostics is eminent [54].

2.3 CONCLUSION

CTV has the largest known plant viral RNA genome. Given centuries of citrus cultivation, this virus has acquired extensive genetic diversity that enabled it to infect all known citrus and express the diversity of symptoms observed. CTV has caused devastating disease outbreaks when strains, occurring as latent entities in some citrus species, were introduced to different citrus genetic backgrounds. Control interventions to address these events were often complicated by the existence of effective aphid vectors. However, mild-strain cross-protection has been successful in a number of instances to mitigate stem-pitting disease. To understand the mechanism(s) operational in cross-protection, characterisation of the strains involved in stem-pitting and further insight into strain interactions are required. The other challenge is to elucidate virus and host factors involved in pathogenesis. These insights will assist in pre-empting disease outbreaks and to develop and understand control interventions.

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3 MOLECULAR AND BIOLOGICAL CHARACTERISATION OF SINGLE-STRAIN CTV ISOLATES

3.1 INTRODUCTION

Seven strains of *Citrus tristeza virus* (CTV) are currently recognized [19] and another tentative strain proposed [46] based only on nucleotide sequence divergence. Phenotypic expression for most published CTV genomes is poorly defined, partly due to CTV naturally occurring as mixed strain populations, but also due to the time and resources required for isolation and biological assessment on citrus hosts. CTV symptom expression is as varied and complex as the genetic diversity observed for the virus itself [16, 19] and contributing further to this complexity is the diversity of the citrus host [45].

Three primary disease phenotypes are induced by CTV and are referred to as syndromes. The first to be described was decline, occurring when sweet orange (*Citrus* × *sinensis* (L.) Osbeck), grapefruit (*C.* × *paradisi* (Macfad.)) or mandarin (*C. reticulata*) scions are grafted to the sour orange (*C.* × *aurantium* L.) rootstock in the presence of certain CTV strains. Rapid tree decline and death occur when phloem necrosis at the bud-union restricts normal sap flow between the scion and rootstock [34]. Stem-pitting (SP) syndrome, primarily found on acid limes (*C.* × *aurantifolia* (Christm.) Swingle), grapefruit and sweet orange is caused by the disruption of normal vascular tissue development and aberrant growth occurs with pits forming in the wood at the sites of disrupted growth [7, 34]. Trees displaying SP do not normally die, but tree growth and vigour are affected, consequently impacting production. ‘Seedling yellows’ (SY), the third syndrome, is observed only at the seedling stage of sour orange, grapefruit and lemons [26]. New growth of infected seedlings exhibits a distinctive foliar chlorosis with either mild or severe stunting. The SY syndrome is not regarded detrimental to citrus production [6], but is a useful phenotype for biological characterisation and is frequently indicative of decline inducing isolates [16].

All isolates of the T36 strain induce decline, indisputably associating this strain with the decline syndrome [13]. Conversely, isolates of the T30 strain are not associated with decline. Despite association of some strains with specific phenotypes, strains are not definitive demarcations of all phenotypes. Substantial variability in pathogenicity within strains was reported in addition to

different strains inducing similar symptoms [21]. It is therefore not possible to describe the phenotype or severity of an isolate based on strain determination.

The 'Garnsey' host range was developed as a standardized biological evaluation system to determine relative severity of CTV isolates and enables the evaluation of the three disease syndromes of CTV; decline, stem-pitting and seedling yellows. The economic impact of each reaction is reflected by using a weighted scoring of symptoms in five defined citrus hosts. The 'Garnsey' host range remains the accepted standard for biological characterisation of CTV isolates [15].

Apart from the 'Garnsey' host range to define isolate severity, a monoclonal antibody, MCA13, reacting to decline-, seedling yellows- and stem pitting-inducing isolates was used to differentiate severe and mild strains [28]. The phenotypes of MCA13-reactive isolates can however not be specified and nonreactive samples are unresolved as either mild or uninfected, limiting its usefulness [27]. This antibody was later shown to react to isolates of the 'Resistance Breaking' (RB) strain and also to isolates of the tentative S1 strain, which were all classified as mild according to 'Garnsey' host reactions [46, 48]. Definitive differentiation between mild and severe stains using MCA13 is therefore not possible.

Specific sequence determinants for the three disease syndromes are not resolved, although the 3'-terminal region, including the p23 gene and the 3'-untranslated region (UTR), was associated with both seedling yellows and decline [1, 14]. Determinants for stem-pitting are also unknown with three non-conserved genes, p33, p18 and p13, implicated [37]. The inability to identify strain severity, either serologically or by molecular markers, means that biological characterization remains the only conclusive method to determine pathogenicity.

Isolation of single-strain sources are required to link genomes to specific phenotypes. Various methods have been used to separate CTV components from field sources. Aphids have been widely used for the purpose of strain separation and isolation [2, 8, 29, 38, 39, 41] and this is possible due to the uneven distribution of CTV strains observed within a single plant [12, 49] and to differences in specific virus proteins that influence the efficacy of transmission [20]. Graft transmission was also shown to effect separation of component strains [44] and specifically by passaging through different hosts [35].

Identification of single-strain isolates is reliant on comprehensive strain-specific detection assays to screen isolates. No assay targeting a single genome region is optimal for strain diagnostics as potential recombinant genomes cannot be identified, but until technologies such as High Throughput Sequencing (HTS) are more widely accessible and pipelines for strain differentiation are in place, simpler screening tools are still useful.

CTV genomes are most divergent within the 5' region [19, 25, 33] and is therefore the preferred target for strain differentiation [32]. Various 3' genomic regions were used for strain detection, but due to lower sequence divergence, differentiation of all known strains is not always possible [47] and sequencing is required to discriminate strains in less divergent regions [48]. Strain-specific RT-PCR assays, targeting 5' genomic regions were developed to detect five CTV strains [32], but the assays did not allow for detection of the RB and HA16-5 strains subsequently identified. The T36 assay also non-specifically amplified isolates of the RB strain and was therefore not reliable [9].

Augmentations to the strain-specific assays of Roy et al. [32] were made in the current study, which allowed for the differential detection of additional strains. The assays were expanded to facilitate differentiation of two variant groups of the RB strain as well as detection of the HA16-5 strain. A specific T36 assay was also developed (Appendix A1)[i] [9].

Moreover, seven CTV isolates, identified as single-strain sources, were biologically characterised using the 'Garnsey' host range and the complete genomes of these strains were determined by Sanger sequencing. A glasshouse trial was performed using four of these isolates in four grapefruit cultivars to evaluate the symptom expression and translocation in the hosts. This was done to determine whether these strains are pathogenic in grapefruit and to identify non-pathogenic single-strain sources that can be trialled further as potential cross protection sources.

[i] Appendix A1. Cook G, van Vuuren SP, Breytenbach JHJ, Burger JT, Maree HJ (2016) Expanded Strain-Specific RT-PCR Assay for Differential Detection of Currently Known *Citrus Tristeza Virus* Strains: a Useful Screening Tool. Journal of Phytopathology 164:847-851.

The development of CTV strain-specific RT-PCR assays are detailed.

3.2 MATERIALS AND METHODS

3.2.1 Determination of single-strain status of CTV isolates

Seven sub-isolated CTV sources were characterised in this study as listed in Table 1. The T3-KB isolate was isolated in the course of the study by means of single-aphid transmission from a source that additionally contained *Citrus dwarfing viroid* (CDVd) [43]. A non-viruliferous colony of *Toxoptera citricida* was established by placing apterous, field-collected aphids on actively growing virus-free Mexican lime plants in an insect cage in a laboratory maintained at 20- 25°C. Aphids were transferred three times within 24 h intervals to new plants after initial colony establishment. For transmission, 20-30 aphids were transferred to the source plant and allowed an acquisition feeding period of 24 h. Thereafter, aphids were placed individually on virus-free 'Mexican' lime plants for an inoculation access period of 24 h. Aphids were killed with an appropriate insecticide and plants maintained further in a glasshouse. CTV transmission was confirmed one month later. Single aphid transmissions were previously used to obtain sub-isolates GFMS12-8 and LMS6-6 [39] as well as sub-isolates B390-5, B389-1 and B389-4. The Maxi isolate was obtained as an escape in a shoot-tip grafting process to render plants virus-free.

Table 1. CTV sub-isolates used in the study, their origin and available literature references.

Isolate	Origin	Reference
B389-1	Sub-isolate obtained by single aphid transmission from a CTV source, GFMS14, obtained from 'Nartia' white grapefruit. Sub-isolation done in Beltsville, USA.	
B389-4	Sub-isolate obtained by single aphid transmission from a CTV source, GFMS14, obtained from 'Nartia' white grapefruit. Sub-isolation done in Beltsville, USA.	
B390-5	Sub-isolate obtained by single aphid transmission from a 'Mouton' Valencia source. Sub-isolation done in Beltsville, USA.	[10]
LMS6-6	Sub-isolate obtained by single aphid transmission from the LMS6 CTV source applied as a cross-protecting source for sweet orange in South Africa.	[10, 39]
GFMS12-8	Sub-isolate obtained by single aphid transmission from a CTV source, GFMS12, previously used as a cross-protection source for grapefruit.	[39]
Maxi	Sub-isolated in a shoot-tip grafting process from a Valencia source.	[10]
T3-KB	Single aphid transmission from a Valencia source that additionally contained CDVd.	[43]

The sources were bark-inoculated to a citrus host range including 'Madam Vinous' sweet orange, sour orange, 'Mexican' lime and 'Duncan' grapefruit for CTV strain determination. Four citrus hosts were used to allow detection of strains that might be suppressed in one or more of the hosts and potentially go undetected.

Each inoculated host plant was tested with eight strain-specific RT-PCRs, four of which were developed in this study and include those targeting strains HA16-5, RB (two variant groups) and T36 [9], while assays for strains T68, VT, T3 and T30 were those of Roy et al. [32]. Primer details are provided in Table 2. The PCR amplicons were Sanger sequenced as confirmation. RNA was extracted as previously described using an acid phenol extraction method from either bark scrapings and/or leaf midribs [9]. For more details regarding these protocols see Appendix A1.

Single-strain status was further interrogated using degenerate primers to amplify a mid-genome region to allow detection of possible mixtures. RT-PCR amplification and direct sequencing of a 1 535 bp product was performed for each isolate using primers;

CTVmid-F, 5'GAACCGGCTCGYGTTCGGCGT3'

CTVmid-R, 5'GCAAACATCYYGACTCAACTACC3'

The CTV sources were indexed for the presence of other locally found pathogens and diseases by both biological indexing and RT-PCR / PCR for detection of citrus exocortis viroid (CEVd), hop stunt viroid (HSVd), citrus dwarfing viroid (CDVd), citrus bent leaf viroid (CBLVd), citrus bark cracking viroid CBCVd), citrus viroid V (CVd V), citrus psorosis virus (CPsV), Impietratura, citrus tatter leaf virus (CTLV) and '*Candidatus* Liberibacter africanus'. Details of these routine indexing methodologies including the citrus hosts used, the duration of indexing and PCR primers are recorded in Appendix B1[ii].

[ii] Appendix B1. Details of indexing methods used to confirm absence of other pathogens in the CTV sources.

Table 2. Species and strain-specific primer sequences used in a two-step PCR

CTV strain	Polarity	Primer sequences from 5' to 3'	Annealing temp. (°C)	Product size (bp)
Primers of this study:				
CTV generic	Sense	TCT GAT TGA AGT GGA CGG AAT AAG	62	157
	Antisense	GCT TAG ACC AAC GAG AGG ATA		
RB: group1 [a]	Sense	AGT GGT GGA GAT TAC GTT G	60	628
	Antisense	TAC ACG CGA CAA ATC GAG		
RB: group 2 [b]	Sense	CGG AAG GGA CTA CGT GGT	60	658
	Antisense	CGT TTG CAC GGG TTC AAT G		
T36	Sense	GGT GTA AGG AAG CGT GTG TCG CAT TTA	66	537
	Antisense	ACC TGC ACC GTC TAA CAA CAT CAT CG		
HA16-5	Sense 1	TAG GAA GGG TCA CTG CCC TGA CA	56	610
	Antisense	GTA AGT ATC TAA AAC CAG GAG		
	Sense 2	CGA CAA GTG CAT TAC GTC TCA G		176
Primers of Roy et al. 2010:				
B165 (T68)	Sense	GTT AAG AAG GAT CAC CAT CTT GAC GTT GA	59	510
	Antisense	AAA ATG CAC TGT AAC AAG ACC CGA CTC		
T3	Sense	GTT ATC ACG CCT AAA GTT TGG TAC CAC T	60	409
	Antisense	CAT GAC ATC GAA GAT AGC CGA AGC		
VT	Sense	TTT GAA AAT GGT GAT GAT TTC GCC GTC A	60	302
	Antisense	GAC ACC GGA ACT GCY TGA ACA GAA T		
T30	Sense	TGT TGC GAA ACT AGT TGA CCC TAC TG	60	206
	Antisense	TAG TGG GCA GAG TGC CAA AAG AGA T		

[a] Based on exact sequence matches RB group 1 primers will detect NZRB-TH28 [FJ525433], NZRB-M12 [FJ525431], NZRB-G90 [FJ525432], B389-4 [MH051718], CA-RB-115 [KU361340] and HA18-9 [GQ454869].
[b] Based on exact sequence matches RB group 2 primers will detect NZRB-TH30 [FJ525434], NZRB-M17 [FJ525435], Taiwan- Pum/SP/T1[JX266712], DSST-17 [MH186146], B390-5 [KU883265], B389-1 [MH051717], Crete 1825 [KF90813] and CA-RB-AT35 [KU358530].

3.2.2 Full-genome sequence determination

Full-genome sequences of each CTV isolate were obtained by amplifying overlapping genome regions of approximately 1500 bp and direct Sanger sequencing. Overlapping primer sets were designed for VT, T68, HA16-5, RB and T3 strains from existing sequences available on GenBank (Appendix B2)[iii].

RNA was extracted from CTV-inoculated 'Mexican' lime by an acid phenol extraction [9]. Total RNA (0.5-1μg) was reverse-transcribed to cDNA. RNA was denatured at 65°C for 3 min with 0.2µg Random Hexamer Primer (Thermo Scientific, MA, USA) and placed on ice for 1min. Further reaction components were added to the denatured RNA, including reaction buffer, dNTPs (1mM final conc.), 100U RevertAid H Minus, Reverse Transcriptase (Thermo Scientific) and 10U RiboLock RNase Inhibitor (Thermo Scientific) in a 20µl total reaction volume. Reverse transcription was done at 25°C for 5 min followed by 42°C for 60 min and inactivation 70°C for 10 min.

PCR amplification was done in 25µl reaction volumes using KAPA HiFi HotStart ReadyMix (KAPA Biosystems, MA, USA) according to manufacturer's instructions and 0.3µM of each primer. PCR products were gel-purified using the Zymoclean™ Gel DNA recovery kit (Zymo Research Corporation, CA, USA) and bi-directionally sequenced. To generate the complete genomes, low-quality bases were removed and the overlapping sequences were aligned using BioEdit [18]. The most distal 5' and 3' primer sequences were included in the sequences, although these regions could not be verified by overlapping sequences. Closest sequence identity of genomes were determined using BLAST [3]. A Neighbor Network of 71 complete CTV genomes available in GenBank, including eight genomes determined in this study, was constructed. Genome sequences were aligned using the FFT-NS-2 strategy in MAFFT (v7.408) [23]. The FASTA alignment was converted to Nexus format using ALTER [17] and the Neighbor Network was constructed with SplitsTree v4.16.6 using default parameters [22].

[iii] Appendix B2. Overlapping primers used for full-genome amplification of strains VT, T68, HA16-5, RB and T3.

3.2.3 Characterisation of single-strain CTV isolates on the 'Garnsey' host range

Seven CTV isolates were inoculated to a full 'Garnsey' biological indicator host range with four to six replicates of each host for each isolate assessed. Inoculation was done by grafting a bark piece from the source plant to each scion. Side branches were removed and one shoot was allowed to grow from the top bud. Plants were maintained in an aphid-free polycarbonate tunnel equipped with wet wall cooling. Temperatures were recorded in the tunnel over the duration of the trial using a Tinytag data logger (Gemini Data Loggers (UK) Ltd). Each inoculated plant was tested for successful transmission by RT-PCR as described above and symptom expression recorded over a period of seven months. Foliar symptoms such as vein clearing, leaf curl and seedling yellows were observed during and after growth flushes. Stunting was recorded in relation to un-inoculated control plants. Stem-pitting was observed by stripping the bark, eased by steaming the cut stems in an autoclave for 10 min prior to bark removal. The symptom rating scale was 0 (no symptoms), 1 (mild), 2 (moderate) and 3 (severe) for all symptoms as described for 'Garnsey' virulence indexing [15]. Symptoms scored per host were as follows: 'Mexican' lime; vein clearing and stem-pitting (SP), sweet orange on sour orange; stunting/decline, sour orange; seedling yellows (SY) and stunting; 'Duncan' grapefruit; SY, SP and stunting; and 'Madam Vinous' sweet orange; SP and stunting. The individual component scores for symptoms on each host were averaged and the composite score multiplied by the weight factor for each host [15] as indicated in Table 3. The final disease index was obtained by adding the scores for each citrus host.

3.2.4 Evaluation of four CTV single-strain isolates in four grapefruit cultivars

3.2.4.1 Symptom assessment

Four single-strain CTV isolates, Maxi, GFMS12-8, LMS6-6 and B390-5 were evaluated in two pigmented; 'Star Ruby' and 'Nel Ruby', and two white; 'Marsh' and 'Duncan', grapefruit cultivars for their ability to induce stem-pitting. Virus-free rough lemon (*C.* × *jambhiri* Lush.) seedlings were planted singly in 3 L planting bags and maintained in an aphid-free polycarbonate tunnel equipped with wet wall cooling. Virus free scions of each grapefruit were bud-grafted to rootstocks according to normal nursery practice. CTV isolates were inoculated separately to five plants of

each cultivar and a minimum of four plants were left as uninoculated controls. Inoculation was done by grafting two bark chips of the source plant to the scion. All plants were inoculated at the same height and the scions were cut back approximately 10 cm above each inoculation point. One shoot was then allowed to grow from the top bud. Temperatures were recorded in the tunnel over the duration of the trial using a Tinytag data logger (Gemini Data Loggers (UK) Ltd). Plants were cut-back at various intervals to evaluate stem-pitting and one shoot of new growth was allowed to grow out each time. The first cut back was seven months after inoculation, followed by 10-, 12-, and 9-month intervals. An alternate stem-pitting evaluation scale, depicted in Figure 1, was used to rate stem-pitting severity in grapefruit to enable better differentiation of symptom variation.

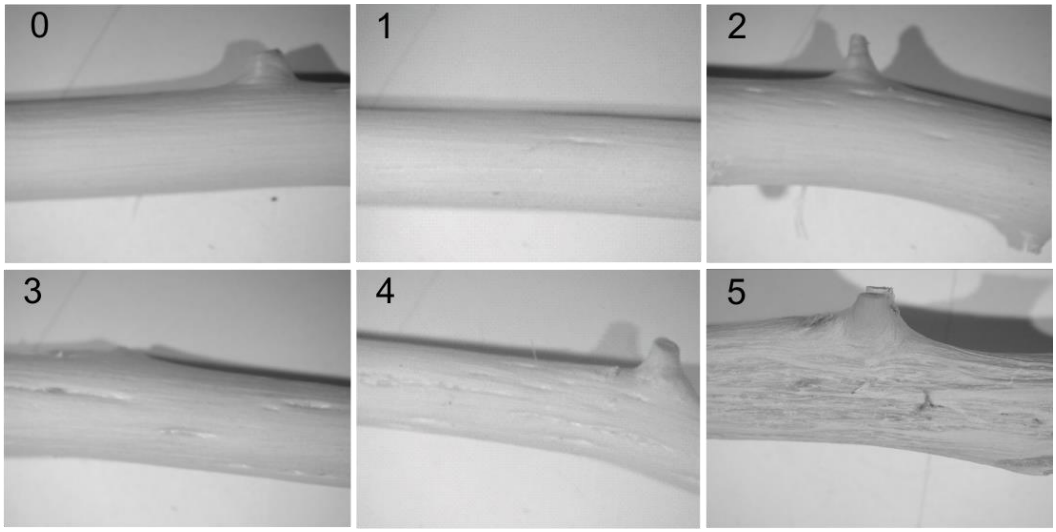


Figure 1. Rating scale for stem-pitting severity in grapefruit; 0 = no stem-pitting, 1 = few (less than 10) shallow pits over the length of the cut stem, 2 = numerous (more than 10) shallow pits, 3 = few deep pits, 4 = frequent deep pits in close proximity to each other and covering a whole section of the stem 5 = honeycomb-like pitting or porous wood pitting.

3.2.4.2 Strain translocation and titre determination by ELISA

Translocation and titre of the four CTV isolates were monitored in the four grapefruit cultivars by semi-quantitative ELISAs in the first growth period of the trial. Leaves of each plant were sampled at three different sites and intervals post-inoculation: (i) seven weeks post-inoculation (wpi), 15 cm above the inoculation point; (ii) 13 wpi, 30 cm above inoculation point; and (iii) 24 wpi, at the

top of the plant. The SRA78900 CTV ELISA reagent set (Agdia, Inc., Elkhart, IN, USA) was used as per supplier's protocol. A total of 400 mg of leaf material including midribs were used per sample and were macerated in 4 ml of general extraction buffer in maceration bags, using a tissue homogenizer (Agdia) attached to a bench drill press. Absorbance values were recorded at 405 nm after 30 min incubation at room temperature using an ELX800 automated microplate reader (BIO-TEK®, Vermont, USA).

3.3 RESULTS AND DISCUSSION

3.3.1 Single-strain confirmation

CTV isolates; Maxi, GFMS12-8, LMS6-6, B389-1, B389-4, B390-5 and T3-KB were shown to be single-strain sources determined by testing four citrus hosts inoculated with each isolate using strain-specific RT-PCRs as well as direct sequencing of PCR amplicons using degenerate primers which yielded single variant sequences for each amplification. No other pathogens were detected in these sources using biological indexing and molecular diagnostic assays.

Sub-isolates B389-1, B389-4 and B390-5 were identified as RB strains. B389-4 amplified with the group 1 differential RB primers, whereas B389-1 and B390-5 amplified with the group 2 differential RB primers [9]. Sub-isolates GFMS12-8, LMS6-6 and Maxi and T3-KB were identified as T68, HA16-5, VT and T3 strains respectively.

3.3.2 Full-genome sequences

Complete genome nucleotide sequences of single-strain isolates, B389-1, B389-4, B390-5, Maxi, LMS6-6, T3-KB, GFMS12-8 and GFMS12-1.3 were compiled and deposited in GenBank under the accession numbers (MH051717, MH051718, KU883265, KU883266, KU883267, MH051719, MK033511 and MK033510), respectively (Appendix A2)[iv]. A Neighbor Network construction of these full-length genomes and those available on GenBank, is presented in Figure 2 which displays

[iv] Appendix A2. Cook G, van Vuuren SP, Breytenbach JHJ, Steyn C, Burger JT, Maree HJ (2016) Characterization of *Citrus tristeza virus* Single-Variant Sources in Grapefruit in Greenhouse and Field Trials. Plant Disease 100:2251-2256

Genomes of isolates B390-5, Maxi and LMS6-6 are reported together with the biological characterisation of these isolates and GFMS12-8.

the positioning of each genome in relation to other available CTV genomes, based on sequence interrelatedness.

Isolates B389-1 and B390-5 of the RB strain, share a 99% sequence identity to each other and RB isolates; CSL01 (KY110737), CA-RB-AT35 (KU358530), Taiwan-Pum/SP/T1 (JX266712), Crete 1825 (KF908013) and DSST-17 (MH186146). The neighbor network construction illustrates that these seven genomes form a monophyletic group detached from other genomes in the RB clade. Isolate B389-4 shared closest sequence identity (99%) to NZRB-TH28 (FJ25433) and NZRB-M12 (FJ525431) which clustered in a separate group to that of B389-1 and B390-5. These two RB clusters can be differentiated in RT-PCR using the RB2 and RB1 primer sets of Cook et al. [9].

Isolate Maxi is a VT variant with closest sequence identity (96%) to Kpg3 (HM573451), an isolate associated with mandarin decline from India. The genome of T3-KB has closest sequence identity (97%) to isolate T3 (KC525952). The VT and T3 clades show close association. Maxi and Kpg3 genomes show specific interrelatedness with the genomes of T3 isolates.

The genome of isolate LMS6-6 was most similar to HA16-5 (GQ454870) (96%). LMS6-6 clusters in a group with HA16-5 and TaiwanPum/M/T5 (JX266713) in the neighbor network construction and validates the status of the HA16-5 clade as a separate CTV strain with three full-length genomes clustering apart from the other strains.

The full-genomes characterised for T68 sub-isolates derived from the CTV source, GFMS12, include GFMS12-8, GFMS12-1.3 and CTZA3 (KC333868). These genomes display close sequence identity with each other and are most similar to the type member of the strain, T68-1 (JQ965169). Nucleotide and amino acid differences between sub-isolates GFMS12-8 and GFMS12-1.3 are discussed with in chapter 5 of this study.

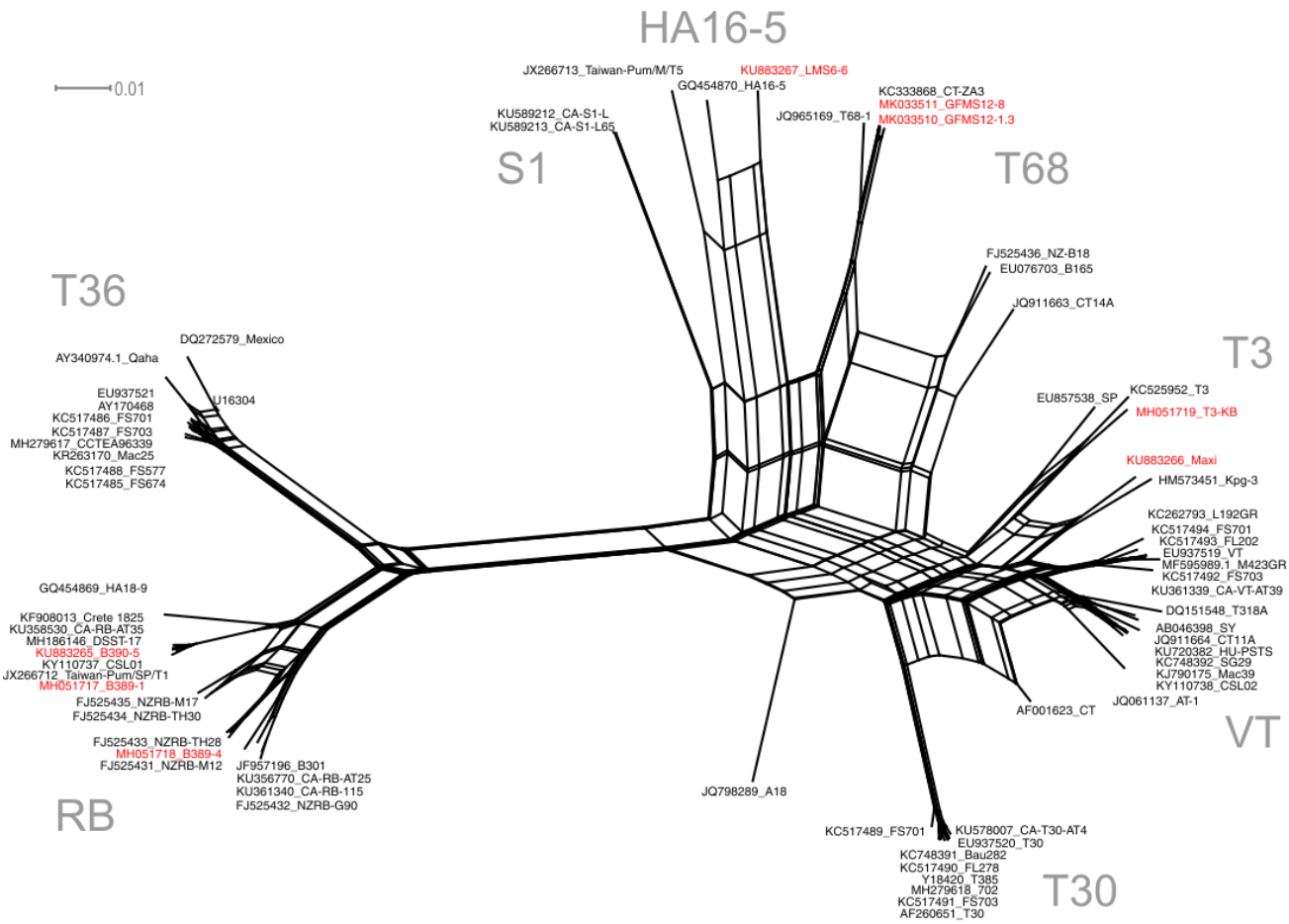


Figure 2. A Neighbor Network construction of complete genomes of citrus tristeza virus including genomes of this study indicated in red text; B389-1, B389-4, B390-5, GFMS12-8, Maxi, LMS6-6, T3-KB and GFMS12-1.3. Strain clusters are indicated in grey text.

3.3.3 Disease ratings on the 'Garnsey' host range

Single-strain isolates inoculated on the 'Garnsey' hosts were evaluated for their ability to induce symptoms over a period of seven months. The average temperature within the growth tunnel over the assessment period was 21°C, with average day and night temperatures of 26°C and 17°C, respectively. Midday temperatures exceeded 35°C at times. Table 3 summarizes the reactions of each isolate on the various citrus hosts and Figure 3 (A-H) shows the symptoms observed on 'Mexican' lime (ML), the sweet on sour host (SW/SO), sour orange (SO) and 'Duncan' grapefruit (DGF). No stem-pitting was observed on 'Madam Vinous' sweet orange with any of the isolates used in the study.

Table 3. Virulence indexing of single-strain CTV isolates based the 'Garnsey' host range disease index per host and cumulative score (ΣDI).

CTV Isolate	Strain	Average DI per citrus host[z]					
		ML (×1)	SW/SO (×2)	SO (×3)	DGF (×4)	MV (×5)	ΣDI
Un-inoculated control	….	0	0	0	0	0	0
B389-1	RB	1.5	0	0.5	1.0	0	3.0
B389-4	RB	1	0	0.6	0	0	1.6
B390-5	RB	1.5	0	0	1.3	0	2.8
GFMS12-8	T68	2.2	0	0	2.9	0	5.1
Maxi	VT	3	0	0	1.9	0	4.9
LMS6-6	HA16-5	1.4	0	2	3.4	0	6.9
T3-KB	T3	2.9	5	4.1	8.3	0	20.4

[z] Individual symptom were rated as 0 = no symptoms, 1 = mild, 2 = moderate and 3 = severe. The component scores for individual symptoms for each host were averaged and the composite score multiplied by the weight factor for each host as indicated. Symptoms scored per host were as follows: ML = 'Mexican' lime, vein clearing and stem-pitting (SP); SW/SO = sweet orange/sour orange, stunting/decline; SO = sour orange, seedling yellows (SY) and stunting; DGF = 'Duncan' grapefruit, SY, SP stunting; and MV = 'Madam Vinous', SP and stunting.

T3-KB obtained an index score of 20.4 due to severe and moderate seedling yellows in DGF and SO respectively, as well as its ability to induce decline in the SW/SO host. This sub-isolate was obtained from a source containing a mixture of a T3 strain and CDVd, which induced severe stem-pitting (SP) typical of porous wood pitting in grapefruit, however mild to no SP was observed in DGF in this trial using the sub-isolate that excluded CDVd. Severe stunting observed with DGF was associated with severe seedling yellows (SY) and not stem-pitting. It is unclear whether the

difference in stem-pitting observed between the original source and the sub-isolate is due to the presence/absence of CDVd or that a non-SP variant was sub-isolated that does not induce SP on DGF.

RB sub-isolates B389-1 and B390-5 obtained index scores of 3 and 2.8 respectively, similar scores to the 3.3 cumulative score reported for RB isolate CA-RB-AT35 [48], with close sequence identity to these isolates. The two sub-isolates generated similar host scores in ML and DGF to that reported for CA-RB-AT35, however, SP was observed on DGF for B389-1 and B390-5 and not observed with CA-RB-AT35 [48], indicating that either minor sequence differences between these genomes, or differences in trial conditions affected symptom expression.

The virulence index obtained for isolate B389-4, an RB isolate that grouped apart from B389-1 and B390-5 in the neighbor network analysis was also low at 1.6, but no SP was observed on DGF.

RB sub-isolates B389-1 and B389-4 showed yellowing of flush, atypical for SY on some, but not all SO plants as shown in Figure 3E. These symptoms were recorded as mild SY. Mild forms of SY are seemingly difficult to identify on SO [16].

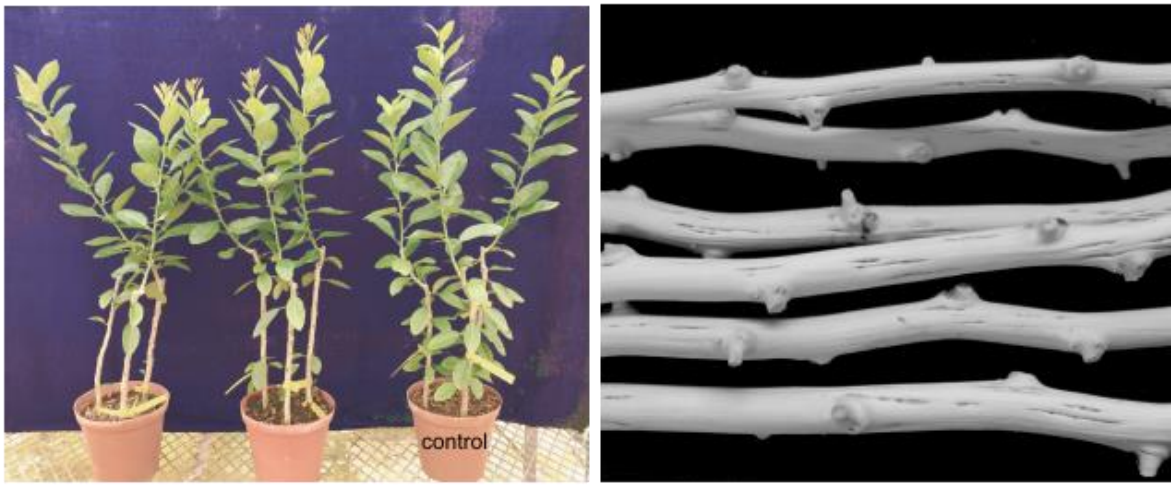
LMS6-6 showed no SP on either grapefruit or sweet orange, but did develop SY on both DGF and SO. No decline symptoms were observed on the SW/SO host. Combined, these reactions resulted in a total virulence index of 6.9.

Isolate Maxi induced severe SP on ML, mild SP on DGF and none on sweet orange (SW). The relatively low index score of 4.9 is attributed to the lack of SY and decline and no SP on SW. Similarly, GFMS12-8 showed moderate and mild SP in ML and DGF respectively, none on SW and also no SY or decline. The resulting virulence index obtained for this isolate was 5.1.

The original description of the ‘Garnsey’ range did not delineate the cumulative disease index and therefore it remains a relative score with a possible maximum score of 45 [15]. Severe field strains such as the Capão Bonita strain from Brazil, associated with severe SP on sweet orange, was awarded a score of 45 and an isolate from Reunion, associated with severe field symptoms, obtained a disease index of 26 [15]. Intuitively therefore, scores below 10 could be considered mild. With this assumption, isolates tested in this study, apart from T3-KB are characterised as mild isolates and can be further evaluated for their potential as cross-protection sources.

A. Mexican lime

B389-1

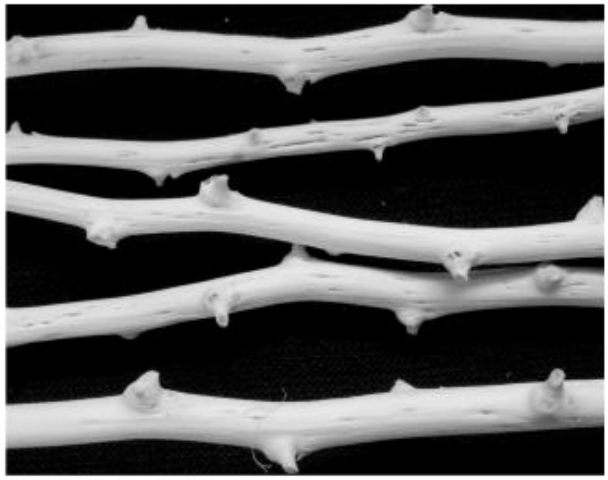


B389-4

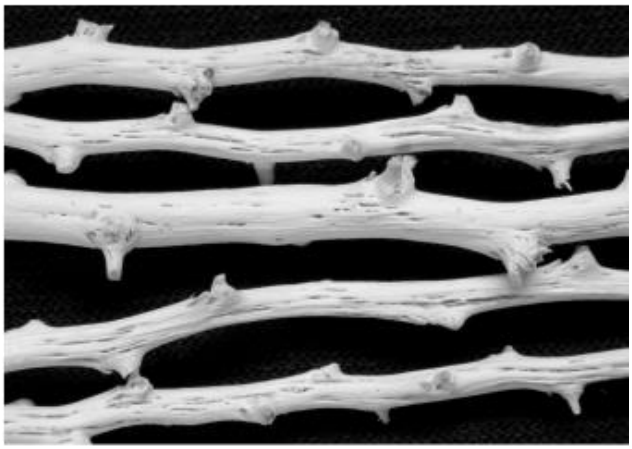
B390-5

B. Mexican lime

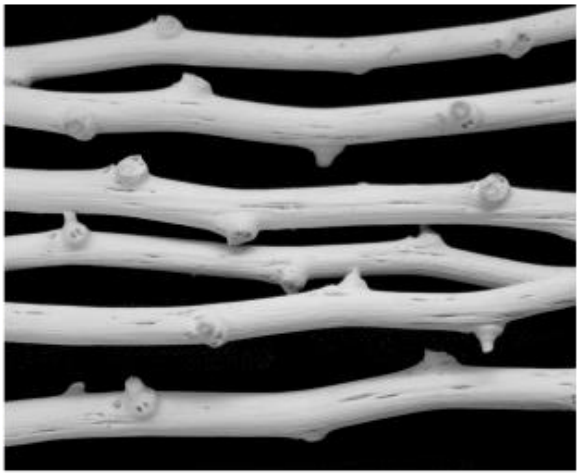
GFMS12-8



'Maxi'



LMS6-6



C. Mexican lime

T3-KB

D. Sweet on Sour orange

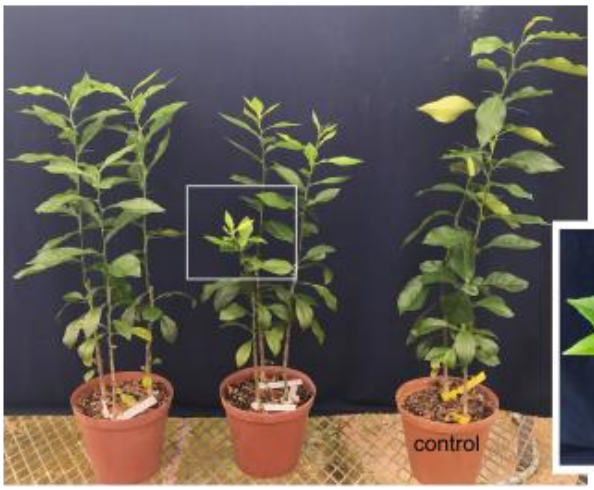
T3-KB

E. Sour orange

B389-1

B389-4

LMS6-6



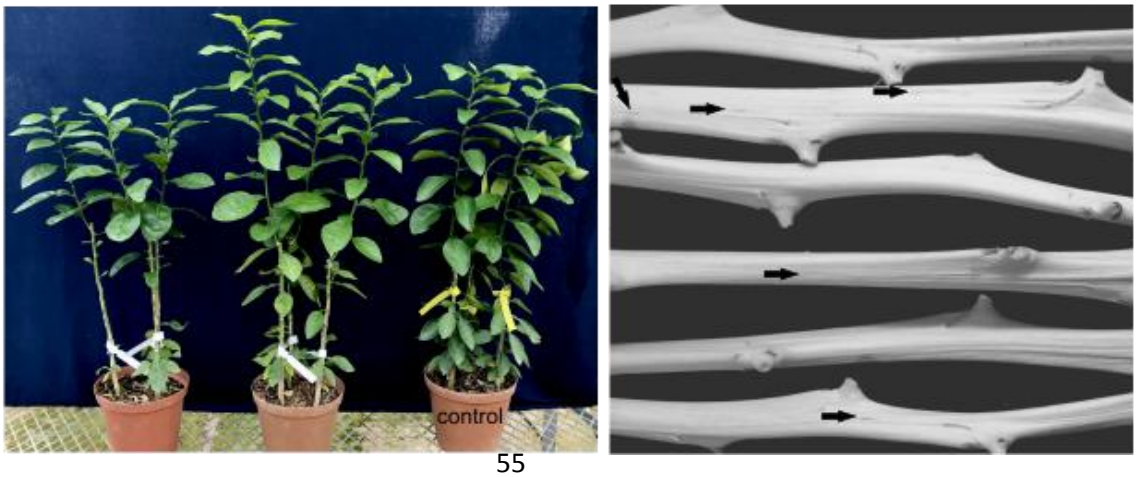
T3-KB

F. Duncan grapefruit

B389-1

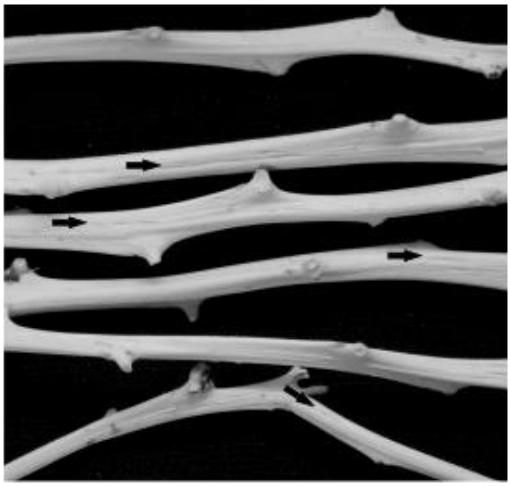
B389-4

B390-5

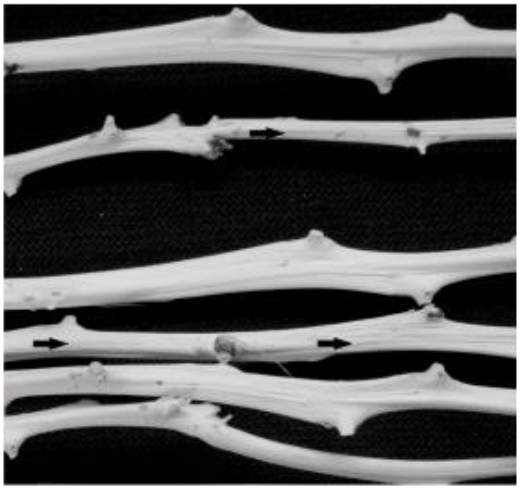


G. Duncan grapefruit

GFMS12-8



'Maxi'



LMS6-6

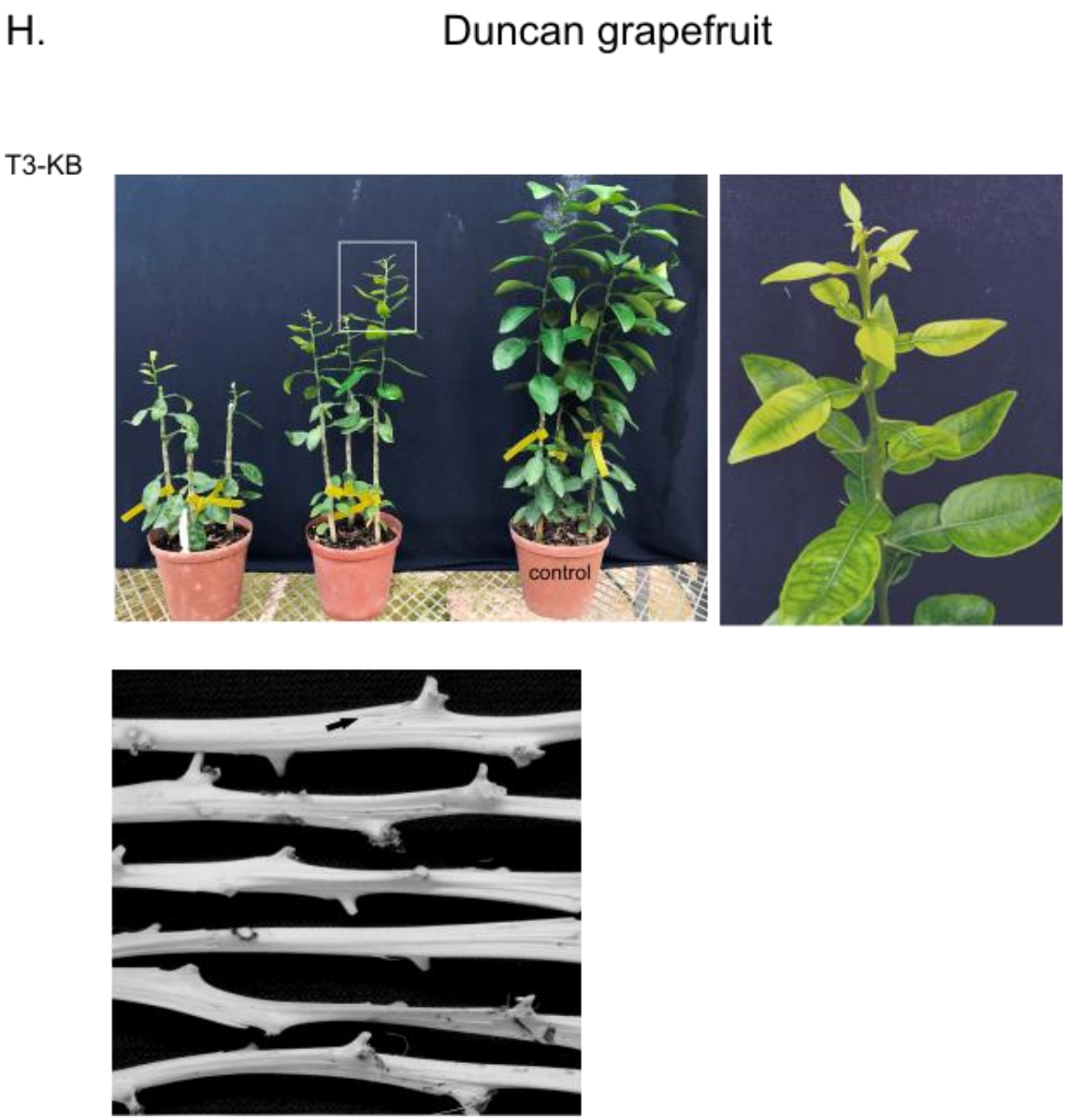


Figure 3. Symptoms induced by single-strain isolates on 'Garnsey' hosts; 'Mexican' lime (**A- C**), sweet on sour orange (**D**), sour orange (**E**) and 'Duncan' grapefruit (**F-H**). Stem-pitting (SP) is indicated by arrows where mild SP was observed.

A. RB isolates, B389-1, B389-4 and B390-5 on 'Mexican' lime. **B**. GFMS12-8, Maxi and LMS6-6 on 'Mexican' lime. **C.** Isolate T3-KB showing irregular stunting and associated stem-pitting on 'Mexican' lime. **D**. Isolate T3-KB showing decline symptoms on the sweet on sour orange host. **E**. Isolates B389-1 and B389-4 showing yellowing of flush on isolated sour orange plants, atypical of seedling yellows. LMS6-6 and T3-KB showing mild seedling yellows. **F**. RB isolates, B389-1, B389-4 and B390-5 on 'Duncan' grapefruit **G**. Isolates, GFMS12-8, Maxi, and LMS6-6 on 'Duncan' grapefruit with stem-pitting and seedling yellows of LMS6-6 emphasized. **H**. Isolate T3-KB on 'Duncan' grapefruit with mild SP and seedling yellows emphasized.

3.3.4 Single-strain isolates in four grapefruit cultivars

3.3.4.1 Symptom assessment

Stem-pitting induced by four single-strain CTV isolates, B390-5 (RB), GFMS12-8 (T68), LMS6-6 (HA16-5) and Maxi (VT) in four grapefruit cultivars, 'Duncan', 'Marsh', 'Nel Ruby' and 'Star Ruby', was recorded over three evaluation periods and results are graphically presented in Figure 4. Stem-pitting over the first growth period was minimal to none and significantly less compared to the subsequent evaluation periods, therefore data for this period was excluded from the analyses. Temperatures recorded over the duration of the trial are summarized in Table 4 according to the various growth periods.

Only isolate LMS6-6 did not induce stem-pitting in any of the grapefruit hosts at any stage of the evaluation. Isolates, B390-5, Maxi and GFMS12-8, caused stem-pitting at various assessment periods, but stem-pitting severity was not consistent over the various evaluation periods. Additionally, stem-pitting was not distributed evenly over the length of each stem, but was observed only in some growth flushes. Noteworthy was that stem-pitting induced by isolate B390-5 was most severe in the third growth period in all four grapefruit hosts. The effect of the Maxi isolate on stem-pitting was also varied over the different evaluation periods, but differed to that of B390-5. The same trends were observed across the various grapefruit hosts with this isolate as well, where severity was higher with each subsequent evaluation period.

No severe porous wood pitting was obtained with any of the isolates in any of the grapefruit hosts. Combined stem-pitting data over the duration of the trial showed no statistical differences in average stem-pitting values between isolates B390-5, Maxi and GFMS12, presented in Table 5. These results indicate that these three isolates are similar in virulence in grapefruit and cause mild to moderate stem-pitting in these hosts, but the timing of symptom expression severity varied. The isolates therefore differ in their effect on the citrus hosts at different stages of the plant's lifecycle or under different environmental conditions. Seasonal factors such as daylight and temperature influence plant growth [30], as well as a plant's response to virus infection. Temperature specifically, was shown to influence stem-pitting expression of CTV where elevated temperatures attenuated CTV symptoms [31] and more CTV particles were observed in plants held at 22°C

compared to plants maintained at 30-36°C [4, 5]. The influence of temperature on virus expression can in part be explained by the effect of temperature on the RNA silencing-mediated defence of plants. Lower temperatures are linked to lower levels of small interfering (si) RNA and related increases in virus accumulation and symptom expression [36, 42].

Average temperatures recorded for the first growth period were higher than those for subsequent periods and no winter months were included in this assessment period. The higher temperatures are a likely explanation for the poor stem-pitting expression observed in this evaluation period.

It is unclear which other factors may have influenced the variant symptom expression of the different strains over the duration of the trial, but autumn, winter and spring temperatures of the third assessment period were lower on average compared to those of the second and fourth assessment period. The lower temperatures in these periods might therefore be associated with the increased stem-pitting observed with isolate B390-5 in the third evaluation period. This trend was not observed with GFMS12-8 or Maxi where stem-pitting rates were higher with each subsequent evaluation period. This anomaly was previously observed where temperature changes did not have the same effect on all CTV strains [11].

The GFMS12 CTV source was formerly used as a cross-protection source for grapefruit in South Africa until probable segregation of strains gave rise to severe stem-pitting [40]. It was not determined which strains or variants were originally responsible for the severe stem-pitting or how the original CTV population structure of GFMS12 changed over time. It was therefore relevant to characterise sub-isolate GFMS12-8 as a component of the GFMS12 source. No severe wood porous pitting was observed at any stage of the evaluation that would associate this isolate with the severe symptoms later found with GFMS12.

The evaluation of the effect of different single-strain isolates in various grapefruit hosts over time, underscores the complexity of CTV as a pathogen in the citrus host. Results show that factors apart from the virus isolate influence stem-pitting severity, including host cultivar and temperature, but these alone do not sufficiently explain the variations observed, indicating additional unaccounted factors.

Table 4. Temperature data (°C) recorded during the grapefruit trial. The growth periods relate to growth of the plant prior to cut-back. Average seasonal temperatures and the presence of stem-pitting for each growth period is indicated.

				Duration of each growth period			Autumn (March-May)	Winter (June-Aug)	Spring (Sept-Nov)	Summer (Dec-Feb)
Growth period		Duration (months)	Stem-pitting presence	Average temperature (°C)						
				Total	Day	Night	Total	Total	Total	Total
1	Oct 2012 - Apr 2013	7	minimal	24	28	20	22	–	24	26
2	May 2013 - Feb 2014	10	yes	21	25	17	20	17	22	26
3	Mar 2014 - Feb 2015	12	yes	21	24	17	19	16	22	26
4	Mar 2015 - Nov 2015	9	yes	21	25	18	22	17	24	–

Table 5. Average stem-pitting values over three growth periods obtained with four CTV strains in 'Duncan', 'Marsh', 'Nel Ruby' and 'Star Ruby' grapefruit.

	Stem-pitting [x]			
CTV Isolate (strain)	Duncan	Marsh	Nel Ruby	Star Ruby
B390-5 (RB)	1.9	2.2	1.5	1.3
Maxi (VT)	1.5	1.2	1.6	1.4
GFMS12-8 (T68)	1.0	1.3	0.9	1.3
LMS 6-6 (HA16-5)	0.0	0.0	0.0	0.0
Control	0.0	0.0	0.0	0.0

[x] Data presented are the means of five trees per treatment recorded over three evaluation periods. The rating scale ranged from 0 (no stem-pitting) to 5 (severe).

Figure 4. Means (n = 5) stem-pitting severity in four grapefruit cultivars inoculated with four CTV single-strain isolates, B390-5, GFMS12-8, LMS6-6 and Maxi over three evaluation periods. A = Duncan, B = Marsh, C = Nel Ruby and D = Star Ruby. Growth period 2 = (May 2013 - Feb 2014), 3 = (Mar 2014 - Feb 2015) and 4 = (Mar 2015 - Nov 2015). Standard error bars are shown.

3.3.4.2 Strain translocation and titre

Average absorbance values for three semi-quantitative CTV ELISA tests performed at specific intervals PI on four grapefruit cultivars infected with the four single-strain sources are presented in Figure 5. Results show that isolate B390-5 reached high titres in the four grapefruit cultivars within the first two testing periods at seven and 13 weeks PI, as well as at 24 weeks PI, demonstrating efficient translocation and virus multiplication in these hosts. Sub-isolate GFMS12-8 was detected at each testing period, however titres were lower than B390-5 in all cases. Virus titre of the Maxi isolate, obtained at both seven and 13 weeks, were comparatively low in all grapefruit cultivars, but were higher at 24 weeks in both 'Duncan' and 'Star Ruby', but not in 'Marsh' and 'Nel Ruby'. Isolate LMS6-6 reached high titres at the 24-week sampling point, but titres varied at seven and 13 weeks and were higher in the two white grapefruit varieties, 'Duncan' and 'Marsh', compared to the red varieties, 'Nel Ruby 'and 'Star Ruby'.

Parameters that define a good cross-protecting source, apart from disease mitigation, include the ability of the virus to rapidly translocate to all parts of the plant and induce no or mild symptoms in the target cultivar, but also in other citrus types [24]. The ability of the virus to rapidly translocate in the host is important to ensure that the virus is proliferated in the bud-wood material supplied to industry.

Isolate B390-5 was the only single-strain isolate tested that translocated efficiently and that was consistently detected at high titre in the four grapefruit hosts. Stem-pitting over the duration of the trial ranged from mild to moderate but no severe porous wood pitting was observed at any stage, which makes this isolate a good candidate to evaluate as a cross-protection source for grapefruit. Low titre and variable translocation of isolates GFMS12-8, LMS6-6 and Maxi in the grapefruit hosts diminish their potential value as cross-protection sources for grapefruit.

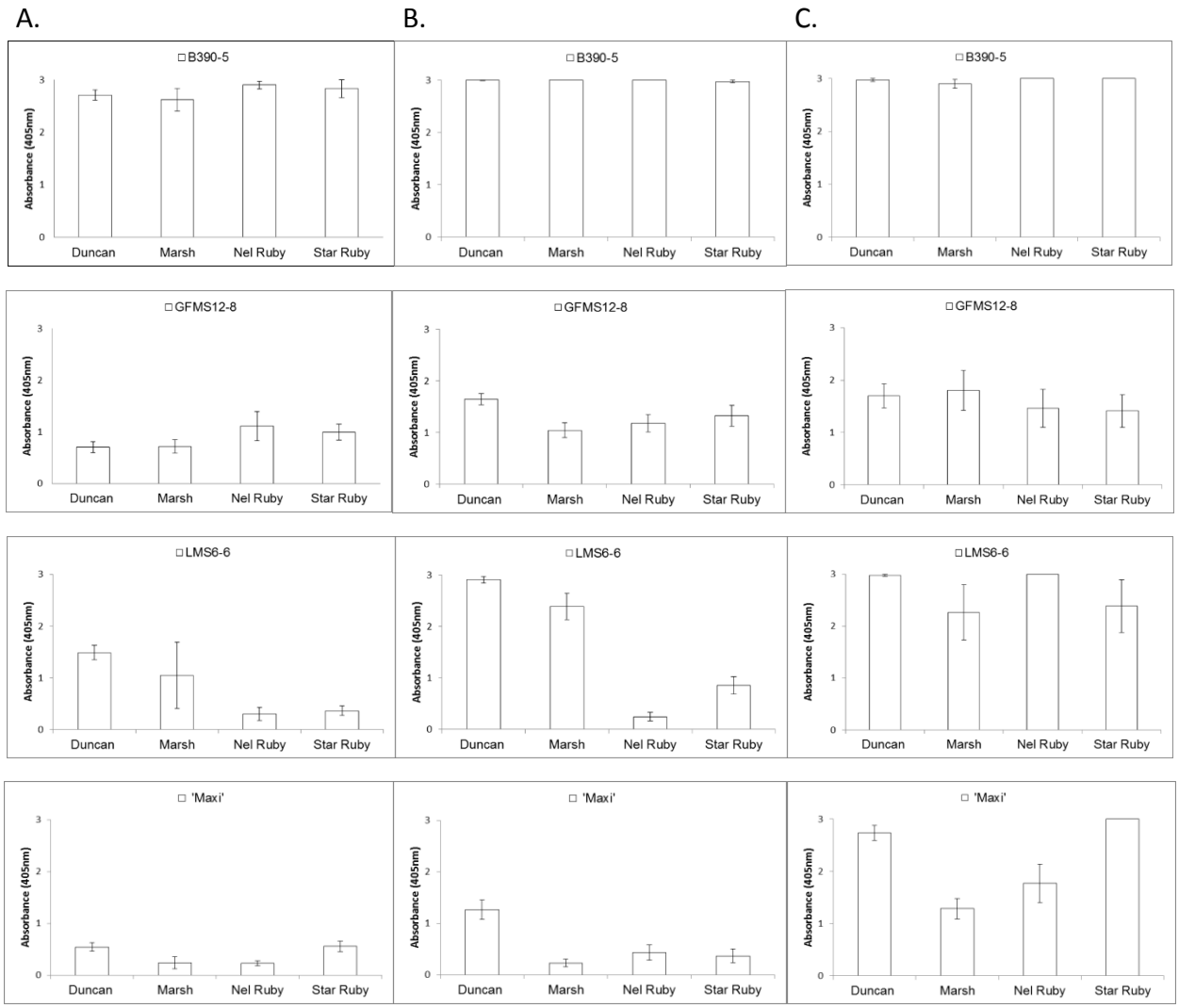


Figure 5. Average (n = 5) absorbance values at 405nm obtained after 30 min for CTV ELISA tests of grapefruit varieties Duncan, Marsh, Nel Ruby and Star Ruby inoculated with CTV isolates B390-5, GFMS12-8, LMS6-6 and Maxi. Leaves were sampled (A) seven weeks post inoculation (PI), 15cm above inoculation point, (B) 13 weeks PI, 30cm above inoculation point and (C) 24 weeks PI at the top of the plant. Standard error bars of means are shown.

3.4 CONCLUSION

CTV strain-specific detection assays were improved to facilitate detection of known strains and were used to identify single-strain isolates. The single-strain status of seven isolates were confirmed by amplification of a mid-genome region from four citrus hosts using degenerate primers. These isolates were also biologically characterised on the full complement of the 'Garnsey' citrus host range and full-genome sequences were determined. Four of these isolates were characterised on a grapefruit host range and their virulence assessed in a glasshouse trial.

These comprehensively characterised CTV isolates are valuable tools as reference isolates of defined genomes and known phenotypes. They can be used in comparative analyses with other single-strain isolates, to determine relative severity. Since pathogenicity determinants of CTV are not defined, identification of isolates of equivalent strains, which might differ in virulence to those of this study, can assist in the identification of genetic determinants of pathogenicity. These isolates are also useful for complementation studies investigating population dynamics.

Ultimately, the isolates can be used to identify components useful for cross-protection. Field studies using the single-strain isolates will demonstrate whether the isolates have value as cross-protection sources. If single-strain sources are shown to be effective in cross-protection by mitigating the effect of severe strains, their use would simplify monitoring transmission of cross-protection isolates compared to mixed strain populations.

3.5 REFERENCES

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4 CTV SINGLE-STRAIN ISOLATES IN SINGLE AND MIXED INFECTIONS: SYMPTOM EXPRESSION AND VIRUS CONCENTRATION

4.1 INTRODUCTION

Perennial crops are continually exposed to infections introduced by insect vectors from surrounding field sources. CTV is therefore mostly found as mixed strain populations and seldom as single-strain infections. The effects of complex populations on a host are difficult to predict and empirical studies are needed to determine the effect of population structure on disease expression.

Quantitative PCR (qPCR) is widely used in plant virus population studies [1, 4, 7, 10, 15, 16]. Absolute real-time quantitation for CTV was described [6, 22, 23], but the ability to determine relative differences in strain concentrations enables insight into strain dynamics and interactions [4, 15-17]. The presence of numerous CTV strains and a diverse natural host suggests that many interactions are possible.

The reliability of qPCR is dependent on careful consideration of a number of factors that influence reaction efficiencies and correct interpretation of results [9]. Accurate template preparation and standardization is foundational to precise qPCR. To adjust for template discrepancies, stably expressed genes, responsible for normal cellular functions, are used to normalize quantitative data. These reference genes should be unaffected by any other factors and be constitutively expressed. In reality however, gene expression is regulated and may be impacted by linkage to expression of other genes and may additionally be influenced by other factors such as tissue type, developmental stage or environmental conditions. Therefore, care should be given to the selection of reference genes within a specific experimental design [9]. The use of more than one reference gene for normalization is advocated to adjust for possible expression differences and ideally a minimum of three reference genes should be used [24]. Statistical methods have been developed to assess gene expression stability and to assist in the selection of appropriate reference genes [2, 20, 24].

qPCR was used to demonstrate that CTV strains accumulate to specific equilibria in some hosts, irrespective of the population complexity [4, 16], but this was not true for all citrus hosts and strain concentrations were also altered by co-infections of certain strains [4]. Strain accumulation was

additionally shown to differ between isolates with the same strain composition and is suggestive of differences in isolate fitness [16]. Differences in tissue tropism of strains was also demonstrated [17, 25]. The understanding of principles that dictate CTV strain dynamics is limited and although some trends have been established, they are not consistent in all scenarios and cannot be assumed for every citrus host and CTV population.

This study was done to investigate the effects that strain populations have on stem-pitting expression in two commercial grapefruit cultivars by using well characterised strains to construct the populations. Additionally, relative quantitation of the strains was done to interrogate inter- and intra-strain dynamics.

4.2 MATERIALS AND METHODS

4.2.1 Pathogenicity assessment of strains and strain combinations

Two commercial grapefruit cultivars (*C.* × *paradisi* (Macfad.)), 'Star Ruby' and 'Marsh' were used in the trial. Virus-free rough lemon (*Citrus* × *jambhiri* Lush.) seedlings were planted singly in 3-liter planting bags and were maintained in an aphid-free polycarbonate tunnel with wet-wall cooling. Virus-free scions were bud-grafted to rootstocks according to normal nursery practices.

Five single-strain isolates were used for a population study including 'Maxi' (VT strain), GFMS12-8 (T68 strain), LMS6-6 (HA16-5 strain), B389-1 (RB strain, group 1) and B389-4 (RB strain, group 2). The isolates were inoculated by patch-grafting bark of the source plants to the grapefruit scions. Thirty-one different combinations were inoculated with four replicates. Trees left un-inoculated served as controls. The scions were cut back four internodes above the last inoculation point at three weeks post-inoculation and one shoot of new growth was allowed to grow from the top bud.

Transmission success was determined by testing each plant using the strain-specific RT-PCRs described in Chapter 3 [12]. Tests were repeated for confirmation.

Plants were cut back at yearly intervals for stem-pitting evaluation after which one shoot of new growth was again allowed to grow. Bark was removed from the cut stems and evaluated for stem-pitting severity using the rating scale from 0 to 5 as outlined in Figure 1 of Chapter 3. The scale

was as follows; 0 = no stem-pitting, 1 = few (less than 10) shallow pits over the length of the cut-stem, 2 = numerous (more than 10) shallow pits, 3 = few deep pits, 4 = frequent deep pits in close proximity to each other and covering a whole section of the stem 5 = honeycomb-like pitting or porous wood pitting. At the final cut-back in 2018, all samples from each season since 2015 were simultaneously evaluated to ensure uniformity of the evaluation. Statistical analysis using the Friedman repeated measures was done to compare combined stem-pitting over all treatments across years for both 'Star Ruby' and 'Marsh'. Average stem-pitting between treatments were compared for each yearly assessment period using the non-parametric Kruskal-Wallis ANOVA and pairwise comparisons of means were carried our using the Games-Howell nonparametric test (Statistica)[v].

4.2.2 Total RNA extraction

Total RNA was isolated using an acid phenol extraction buffer comprising 38% sodium acetate-saturated phenol (pH 5.0), 0.8M guanidine isothiocyanate, 0.4M ammonium thiocyanate, 0.1M sodium acetate (pH 5.0) and 5% (v/v) glycerol. The single stem of each tree was cut back at the same height and bark was removed from the first 5cm of the bottom end of each stem. Each bark sample (800mg) was macerated in 5ml extraction buffer in a maceration bag (Agdia Inc., Elkhart, IN, USA) using a tissue homogenizer (Agdia) attached to a bench drill press. Homogenates were transferred to microcentrifuge tubes and incubated for 5 min on ice and centrifuged at 12 000g for 5 min at 4°C. The aqueous phase was transferred to a new tube and extracted twice with chloroform. From the final aqueous phase, 800µl was precipitated at room temperature by the addition of 200µl isopropanol and 200µl 4M LiCl for 10 min and centrifuged at 12 000g for 15 min. The pellet was rinsed in 75% ethanol and resuspended in 50µl nuclease-free water.

Total RNA (40µl) was treated with 1U DNase I, RNase-free (Thermo Scientific, Massachusetts, USA) using buffer with $MgCl_2$ according to manufacturer's instructions in 50µl reaction volumes. 450µl of nuclease-free H_2O was added to the DNAse treatment mixture and a phenol:chloroform (5:1) extraction was performed followed by an ethanol and sodium acetate precipitation (2.5 volumes of 100% ethanol and 0.1 volumes of 3M Sodium acetate (pH5.2)). Pellets were washed

[v] Statistical analyses were performed by the Centre for Statistical Consultation, Stellenbosch University.

with 75% ethanol, dried and resuspended in 40 µl nuclease-free H_2O. Nucleic acid concentrations were measured with the Nanophotometer Pearl (IMPLEN, Munich, Germany) at 260nm. Purity of the total RNA was assessed by the 260/280nm and 260/230nm ratios and RNA integrity verified by agarose gel electrophoresis.

4.2.3 Development of CTV strain-specific RT-qPCR assays

Strain-specific primers (IDT, Coralville, USA) presented in Table 1 were designed and optimized for real-time PCR using SYBR Green detection and validated for the isolates used in the study. Primers were designed within open reading frame 1a (ORF1a) in a region spanning the leader protease II and methyltransferase domains. Primers for universal CTV detection targeting the 3' UTR were previously reported [12]. Specificity of the primers were confirmed by testing cross-reactivity in end-point PCR reactions using GoTaq®Hot Start Green Master Mix (Promega Corp., Madison, WI, USA) and real-time PCR using SensiFAST SYBR No-ROX Kit (Bioline, Taunton, USA). Five-fold dilution series of cDNA were prepared separately for each strain. The Rotor-gene Q software version 2.2.3 (Qiagen) was used to calculate the reaction efficiency for each assay.

Relative quantitation was performed using three citrus genes as described by Mafra et al. [19] for normalization and include ubiquitin protein ligase 7 (UPL7), glyceraldehyde-3-phosphate dehydrogenase C2 (GAPC2) and ubiquitin conjugating enzyme 9 (UBC9). Primer details are provided in Table 2.

Table 1. Primers used for CTV generic and isolate specific qPCR assays and qPCR parameters.

Isolate (strain)	Primer sequence	Product size (bp)	Annealing temperature (°C)	Primer concentration (nM)
B389-4 (RB group 1)[a]	F: GAGAGTGGTGGAGATTACGTTG	150	62	300
	R:AACATCCGTCATAGTCGCGGCGTAGC			
B389-1 (RB group 2)[a]	F:CGAGAGCGGAAGGGACTACGTG	197	65	350
	R:CAATCTCAACATCGGGAACGCAGT			
GFMS12-8 (T68)	F:AGGTAAACTCCCCAAATCGGTGTG	212	64	350
	R:CGACAGACGAGCCAAAACTATGCG			
LMS6-6 (HA16-5)	F:AAGGTAAACTCCCAGGATCGGTGC	282	65	350
	R:TACACGCCACGAACTGAGACGTAATG			
Maxi (VT)	F:CGCAGGTAGCGATTATTCGGACGTA	142	60	375
	R:CTTTACTAAGTTGATGATGACGAACG			
Universal CTV	F:TCTGATTGAAGTGGACGGAATAAG	157	62	250
	R:GCTTAGACCAACGAGAGGATA			

[a] differential amplification of variants of the RB strain.

Table 2. Citrus reference gene primers and qPCR parameters.

	Primer sequence	Annealing temperature (°C)	Primer concentration (nM)
GAPC2	F:TCTTGCCTGCTTTGAATGGA	62	375
	R:GTGAGGTCAACCACTGCGACAT		
UBC9	F:GCCTCCCAAGGTAGCATTCAG	62	375
	R:GTAAGAGCAGGACTCCATTGTTC		
UPL7	F:CAAAGAAGTGCAGCGAGAGA	62	375
	R:TCAGGAACAGCAAAAGCAAG		

Acronyms: GAPC2 = Glyceraldehyde-3-phosphate dehydrogenase C2, UBC9 = Ubiquitin conjugating enzyme 9, UPL7 = Ubiquitin-protein ligase 7

4.2.4 Relative quantitation of CTV components

RNA was extracted from the same sampling point on each tree. The single stem of each tree was cut back at the same height and bark was removed from the first 5 cm of the bottom end of each stem for RNA extraction.

One microgram total RNA of each sample was reverse-transcribed to cDNA. The RNA was denatured at 65°C for 3 min with 0.2µg Random Hexamer Primer (Thermo Scientific, MA, USA) and placed on ice for 1 min. Further reaction components were added to the denatured RNA including reaction buffer, dNTPs (1 mM final conc.), 100U RevertAid H Minus, Reverse Transcriptase (Thermo Scientific) and 10U RiboLock RNase Inhibitor (Thermo Scientific). The final reaction volume was 20µl. Reverse transcription was done at 25°C for 5 min followed by 42°C for 60 min in a Multigene Optimax thermal cycler (Labnet International Inc., Edison NJ, USA). Samples were heated for 10 min at 70°C to terminate the RT reaction.

Two microlitres of each cDNA sample were pooled to make a representative mixture of all the samples and either a five or six point 5-fold dilution series was prepared to construct standard curves for the generic CTV and the citrus reference gene assays. Five µl cDNA prepared from samples containing the same single-strain (single infection samples) were pooled and either a five or six point 5-fold dilution series prepared to construct a standard curve for each strain. Reactions were run in the Rotor-Gene Q thermal cycler (Qiagen, Venlo, Netherlands) and efficiencies for each assay determined. Arbitrary values were assigned to the universal CTV and reference gene standard curves. The second dilution point of each strain-specific dilution series was run in the universal CTV PCR and the values read off from the standard curve were used to calibrate each strain-specific standard curve. This allowed for the comparison of concentrations between strains.

Complementary DNA of each sample was diluted 1:24 and treated as the unknown samples for quantitation. All cDNA dilutions were stored at -20°C.

Each sample was tested using the universal CTV and the relevant strain-specific assays. Quantitative PCRs were performed in the Rotor-Gene Q thermal cycler using the SensiFAST SYBR No-ROX Kit. Reactions contained 2× SensiFAST SYBR mix, nuclease-free H_2O and forward and reverse primers at concentrations provided in Tables 1 and 2. Each reaction was performed with lµl of diluted cDNA in a final reaction volume of 12.5µl. CTV negative plant

controls and no-template controls and were included. All reactions were performed in triplicate in Qiagen Rotor-Gene Q 0.1 ml tube-and-cap strips. Cycling parameters included an initial activation of 95°C for 3 min and 35 cycles of 95°C for 15 s, primer-specific annealing temperature for 15 s (Table 1 and 2) and 72°C for 15 s. Acquisition on the green channel was recorded at the end of the extension step. Melting curve analysis of PCR amplicons was performed with temperatures ranging from 65°C to 95°C with a 1°C increase in temperature every 5 s to identify primer-dimers and non-specific amplification. The second dilution point of the relevant standard curve was included in all runs to normalize inter-assay variation and the adjustment were done after importing the primer-specific standard curve in each run. Rotor Gene run files were exported as .csv files for further analysis.

Virus concentration ratios (VCR) for each sample were calculated using the geometric mean of the triplicate reactions and normalization using a reference gene index (RGI) which is the geometric mean of the three reference genes [7].(*The term VCR is used to refer to the CTV universal assay and strain concentration ratio (SCR) when referring to the strain-specific assays*). To simplify the analyses, data was uploaded to Harbin [8], a web-based software application, to perform the calculations.

The expression stability of the reference genes was validated using the (Cq) data from 96 Star Ruby and 91 Marsh samples in BestKeeper [20].

Point plots of sample VCR values for the universal CTV quantitative analysis for ‘Star Ruby’ and ‘Marsh’ were drawn in R [21]. Statistical analyses for each strain-specific assay was done to test for differences between treatments using the Games-Howell nonparametric test with a 95% confidence interval (XLSTAT 2018: Addinsoft, Paris, France)[vi].

[vi] Statistical analysis was performed by Mr S. van der Westhuizen, Department of Genetics, Stellenbosch University.

4.3 RESULTS AND DISCUSSION

4.3.1 *Pathogenicity assessment of single and mixed infections*

'Star Ruby' and 'Marsh' plants were inoculated with five single-strain isolates in various combinations and evaluated for stem-pitting (SP) severity yearly, for four years. Not all inoculations were successful and treatments where sufficient replicates were available were used for statistical analyses. Transmission success to 'Marsh' grapefruit was poorer than to 'Star Ruby'. Despite successful graft take, transmission of the RB1 isolate was unsuccessful in ten plants and of RB2 in ten plants of 'Star Ruby'. In 'Marsh', 19 transmissions of RB2 failed and were co-inoculations with RB1. In 80% of the failed transmissions the two RB isolates were co-inoculated. Additionally, VT transmission to 15 'Marsh' plants was unsuccessful.

Symptom expression of single-strains, GFMS12-8 (T68), Maxi (VT) and LMS6-6 (HA16-5) was similar to findings in the grapefruit trial reported in Chapter 3. Mild to moderate SP was observed with GFMS12-8 and Maxi, whereas LMS6-6 did not induce SP. However, the two RB variants, B389-1 (RB2) and B389-4 (RB1) differed in expression from isolate B390-5 (RB2), used in the previous trial, and displayed mild to no SP whereas isolate B390-5 was previously associated with mild to moderate SP.

The average SP differences between treatments for each of four years are graphically presented in Figure 1 for 'Star Ruby' and 'Marsh'. (*The average stem-pitting residuals from ANOVA were not normally distributed and therefore not used as the statistical test, but the least square means plots in Figure 1 illustrates the differences between the inoculations better than the corresponding Box-plots of the Kruskal-Wallis ANOVA test.*)

Single infections of HA16-5, RB1 and RB2, or any of these strains in combination with each other, consistently yielded little or no SP over the four years in both grapefruit cultivars and confirms the mild status of these isolates in grapefruit. Variation in symptom expression over the different evaluation periods for strains, T68 and VT as well as treatment combinations are seen in Figure 1. This fluctuation was similarly observed for these isolates of T68 and VT in the grapefruit trial reported in Chapter 3. SP severity fluctuated between mild to moderate over the observation period, but severe porous wood pitting did not occur.

Yearly SP averages over all treatments differed between 'Star Ruby' and 'Marsh' and were consistently higher in 'Star Ruby', demonstrating a greater sensitivity of the cultivar to CTV (Table 3). Average SP severity was highest in 2018 in 'Star Ruby', but in 'Marsh' higher average SP values were recorded in 2017, indicating different responses of the cultivars to CTV under the same environmental conditions.

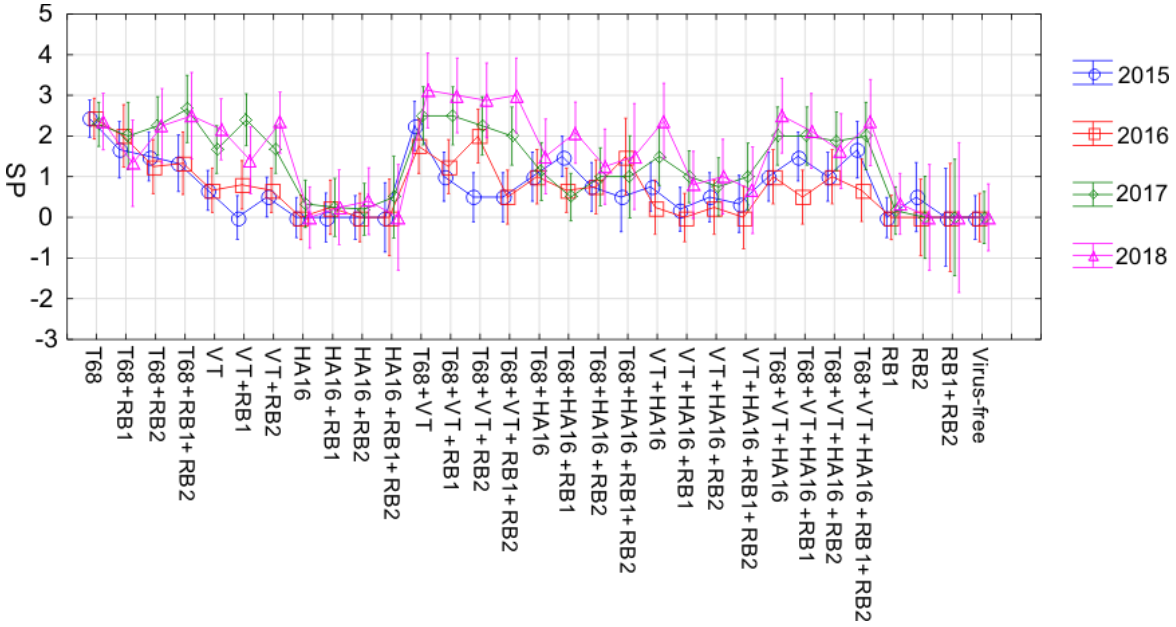
Table 3. Average stem-pitting of all treatments for 'Star Ruby' and 'Marsh' from 2015 to 2018. Chi square and p values of the Friedman ANOVA are presented.

		Stem-pitting Mean	***Std Dev***
			$\chi^2 = 96.08, p = 0.000$
Star Ruby	2015	0.78	0.9
	2016	0.74	0.9
	2017	1.35	1.1
	2018	1.58	1.3
			$\chi^2 = 84.06, p = 0.000$
Marsh	2015	0.47	0.7
	2016	0.19	0.4
	2017	1.01	0.9
	2018	0.65	0.7

The purpose of this trial was to investigate possible strain interactions impacting SP expression. Some treatment combinations with strain T68 showed lower SP in 'Star Ruby' compared to the single infection treatment of T68 in the first two evaluation periods of 2015 and 2016. These observations were not statistically supported and the trend was not observed further in subsequent evaluations. No treatment combinations that included strain T68 displayed statistically different SP in either 'Star Ruby' or 'Marsh' compared to the single infection treatment of T68. Similarly, no differences in SP were observed with the VT strain in any combination treatments compared to the single infection treatment. It is notable that SP was not amplified in treatment combinations containing both T68 and VT, the two isolates associated with SP. The individual effects of each strain were therefore not additive in combination. The statistical summaries are presented in Appendix B3[vii].

[vii] Appendix B3. Statistical summaries of stem-pitting results.
Statistical analyses were performed by the Centre for Statistical Consultation, Stellenbosch University.

A.



B.

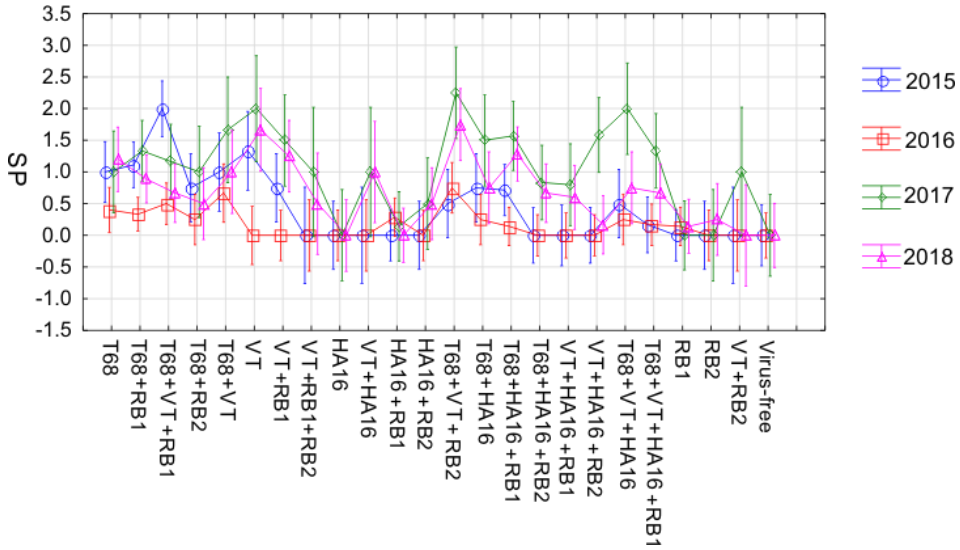


Figure 1. Average stem-pitting rates for 'Star Ruby' (A) and 'Marsh' (B) grapefruit inoculated with various combinations of four strains (two variants of the RB strain) in four consecutive yearly evaluations. Vertical bars denote 95% least squares confidence intervals for treatment means.
Rating scale: 0 = no stem-pitting, 1 = few (less than 10) shallow pits over the length of the cut stem, 2 = numerous (more than 10) shallow pits, 3 = few deep pits, 4 = frequent deep pits in close proximity to each other and covering a whole section of the stem, 5 = honeycomb-like pitting or porous wood pitting.

4.3.2 CTV and strain-specific RT-qPCR assays

The primers developed for the strain-specific assays did not detect the other strains in either end point or real-time specificity tests. Each standard curve consisted of six, five-fold dilution points apart from the VT standard curve that consisted of five dilution points due to low concentration of the strain in the pooled cDNA mixture. The PCR efficiencies for the optimized assays were high, as shown in Table 4. PCR cycles were limited to 35 as primer-dimers developed occasionally in later cycles and the limitation of the cycle numbers avoided primer-dimer detection even though they were easily distinguishable from the amplicons on melt profiles.

Table 4. PCR assay efficiencies, coefficients of determination (r^2) and slopes of standard curves as calculated by Rotor-Gene Q software.

PCR	*Efficiency*	*R^2 value*	*Slope*
HA16-5	1.03	0.998	-3.24
RB1	1.04	0.997	-3.22
RB2	1.06	0.997	-3.19
T68	0.96	0.998	-3.42
VT	1.07	0.998	-3.16
CTV	0.99	0.996	-3.36
GAPC2	0.98	0.992	-3.36
UBC9	0.99	0.988	-3.35
UPL7	1.02	0.981	-3.28

4.3.3 Reference gene stability

Citrus references genes, GAPC2, UPL7 and UBC9, used in the study were previously assessed and their expression stability determined under various conditions [19]. Reference gene stability was validated in this study using BestKeeper and the descriptive statistics for the individual reference genes, as well as the BestKeeper indices, are presented in Table 5. Data for ‘Star Ruby’ and ‘Marsh’ samples are presented separately to indicate variations in stability between the cultivars. Expression stability is observed in Cq variations described by standard deviations (SD) and coefficient of variances (CV). Genes with SD values higher than 1 are considered inconsistent [20]. The three reference genes used were shown to be sufficiently stable in this study, with

GAPC2 being the most stable and UBC9 being the least stable in both cultivars. Similar expression stability were previously found for these three genes using geNorm, however NormFinder ranked UPL7 more stable than GAPC2 [19]. The BestKeeper index for three genes showed lower variation than for the individual reference genes in Star Ruby samples. For Marsh samples, the BestKeeper index for the three genes showed lower variation compared to individual stability of UPL7 and UBC9, but was not better than GAPC2. Overall, the results validate the use of the three reference genes for normalization.

Table 5. Descriptive statistics for the Cq values obtained for the three reference genes used as well as the BestKeeper indices computed with the same descriptive parameters. Data for Star Ruby and Marsh samples are presented separately.

	Star Ruby				***Marsh***			
	GAPC2	**UPL7**	**UBC9**	*BestKeeper (n = 3)*	**GAPC2**	**UPL7**	**UBC9**	*BestKeeper (n = 3)*
n	96	96	96	96	91	91	91	91
Geometric mean [Cq]	19.20	25.22	19.79	21.24	19.55	25.63	20.52	21.74
Arithmetic mean [Cq]	19.21	25.23	19.81	21.25	19.56	25.65	20.56	21.76
Minimum [Cq]	17.70	23.58	17.70	19.72	17.58	22.84	17.45	19.48
Maximum [Cq]	20.73	27.27	21.85	22.56	21.14	27.99	23.00	23.57
Std Dev [± Cq]	**0.48**	**0.66**	**0.70**	**0.42**	**0.46**	**0.84**	**0.99**	**0.63**
CV [% Cq]	**2.51**	**2.60**	**3.54**	**1.96**	**2.36**	**3.26**	**4.79**	**2.91**
Minimum [x-fold]	-2.78	-3.17	-4.20	2.87	-3.84	-7.11	-8.26	4.78
Maximum [x-fold]	2.85	4.23	4.14	2.50	2.97	5.25	5.51	3.55
Std Dev [± x-fold]	**1.39**	**1.57**	**1.61**	**1.33**	**1.37**	**1.77**	**1.96**	**1.55**

Abbreviations: *n* = number of samples, *Std Dev*. = standard deviation, *CV* = coefficient of variance and *x-fold* = expression regulation coefficient.

4.3.4 Relative quantitation of CTV components

Relative quantitation was performed for each sample using a universal CTV assay to gauge the combined CTV concentrations of the various treatments. The results are graphically presented in Figure 2 and the individual virus concentration ratios (VCR) are represented as points in the plot. Overall higher average VCRs were obtained in 'Marsh' compared to 'Star Ruby' with total averages of 1.9 and 1.1 respectively. This is an inverse correlation with the average stem-pitting severity for these grapefruit cultivars.

One ‘Star Ruby’ (SR75) and one ‘Marsh’ (M108) sample obtained VCRs outside the range of the universal CTV standard curve. These data points are included although the values are unreliable, but they do indicate elevated VCRs beyond those of other samples. These samples are discussed further with the strain-specific quantitation. ‘Star Ruby’ treatment combinations T68+HA16-5, T68+HA16-5+RB1 and T68+HA16-5+RB2 showed greater variability in VCRs within these treatments than observed for other treatments.

The strain concentration ratio (SCR) was determined for each strain present in a sample as confirmed by repeated strain-specific RT-PCRs. Table 6 shows the average SCRs over all the treatments for each strain. The average SCRs for all strains were higher in ‘Marsh’ compared to ‘Star Ruby’, although the treatments were not exactly the same for the two grapefruit cultivars. The T68 isolate, GFMS12-8, and the RB isolates, B389-1 and B389-4, were originally isolated from white grapefruit and the higher SCRs, together with lower SP expression, could indicate that these isolates are more adapted to white grapefruit.

Table 6. Average strain concentration ratios over all treatments in ‘Star Ruby’ and ‘Marsh’ grapefruit.

Strain	***Marsh***	***Star Ruby***
RB1	1.23 a[x]	0.73 a
HA16-5	1.04 ab	0.93 a
T68	0.96 a	0.57 a
RB2	0.45 bc	0.06 b
VT	0.31 c	0.13 b

[x] The same letters indicate the means do not differ significantly.

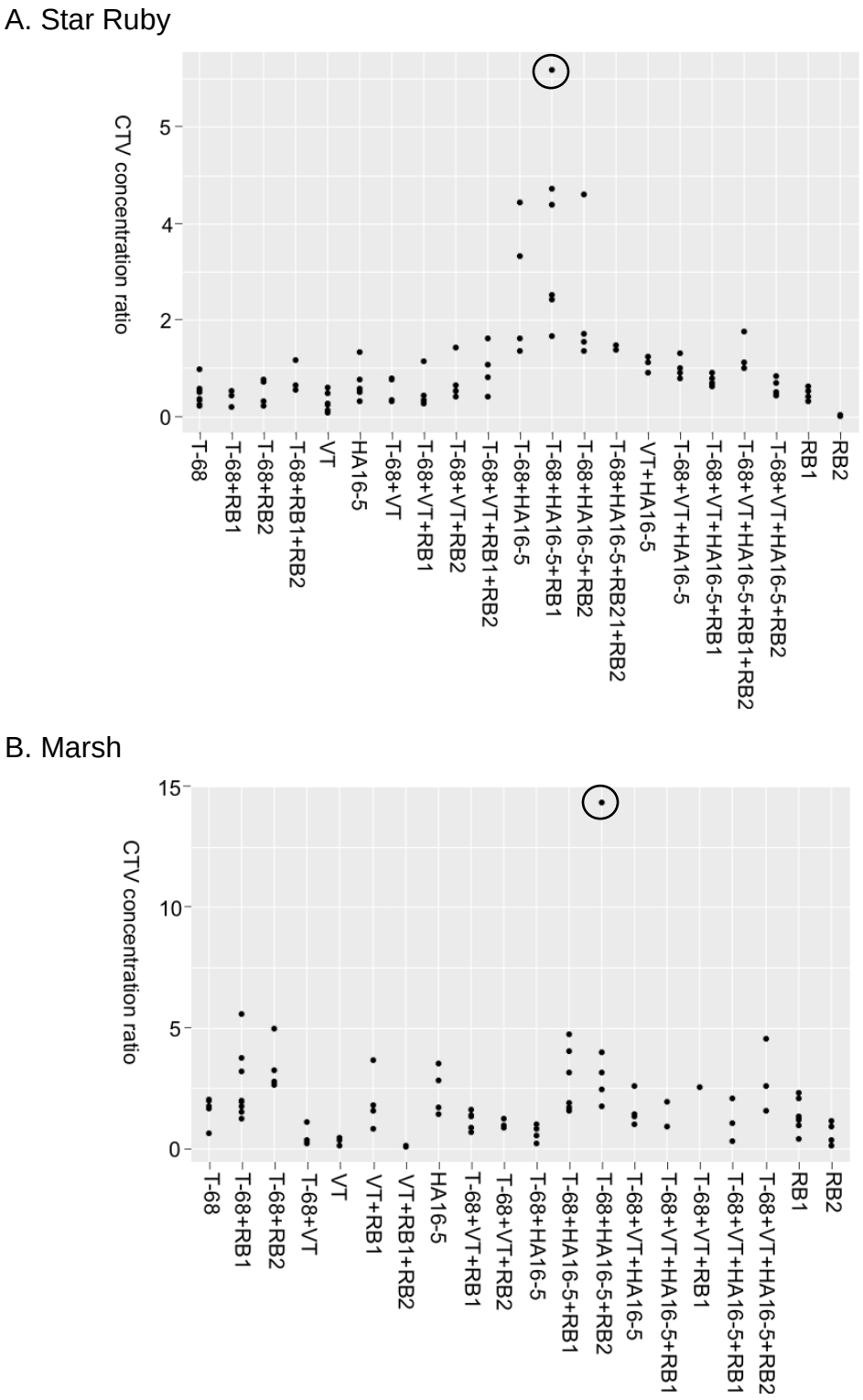


Figure 2. Point plot of CTV virus concentration ratios (VCR) of Star Ruby (A) and Marsh (B) samples. *Encircled VCR values are unreliable as they were not inside the range of the universal CTV standard curve.*

To investigate possible strain interactions, discernible by altered strain concentrations, quantitative analyses of single infection treatments were compared to treatments containing the same strains in combination with the other strains. The statistical summaries of these comparisons are presented in Appendix B4[viii] . Variances within treatments were high and results were not suggestive of specific interactions. Variances over all treatments for each strain are depicted in the graphs of Figure 3 for "Star Ruby' and "Marsh'. Each of the five strains were found within certain concentration ranges depending on the strain and the host, but isolated samples had concentrations beyond these ranges. In both 'Star Ruby' and 'Marsh', strains VT and RB2 were found at lower concentrations than strains T68, HA16-5 and RB1(Table 6, Figure 3). Samples indicated in the universal CTV assay with elevated VCRs are also indicated in the strain-specific assays in Figure 3. 'Star Ruby' sample 75 is the encircled sample in Figure 2A with strain components (T68+HA16-5+RB1) and each component strain shows an elevated SCR compared to the other samples. Similarly, two of three components of 'Marsh' sample M108 (T68+HA16-5+RB2), the encircled sample in Figure 2B, show elevated SCRs, as do the two strain components of sample M24 (VT+RB1), also indicated. Sample M35 is a HA16-5 single infection sample. These observations indicate that strains are not in homeostasis and concentration fluctuations can occur concurrently with other strains in a population.

Previous CTV population studies demonstrated that strain concentrations stabilized over time and reached equilibrium within a host species [4, 16] and that strain ratios were maintained when fluctuations in concentrations were observed [4]. In this study, strains were not found in equilibrium and ratios between strains were not maintained. The different findings can be due to the different strains and host species used in the various studies, but more evident is the fact that concentration differences between strains of this study were substantially less pronounced compared to logarithmic differences observed between strains used in previous studies [4, 16]. Since the concentration differences between strains of this study were not logarithmic and concentrations within strains were found within comparatively narrow fluctuation ranges, a certain equilibrium can be assumed. A further factor that differed between the studies was the sampling strategy. In Harper et al. [16] sampling was not done at specific or equivalent points on the plant

[viii] Appendix B4. Statistical summaries of strain quantitation results.
Statistical analysis was performed by Mr S. van der Westhuizen, Department of Genetics, Stellenbosch University.

as was done for this study, but samples were taken from various plant sites and pooled. The sampling strategy would probably average out variances similar to those observed in this study.

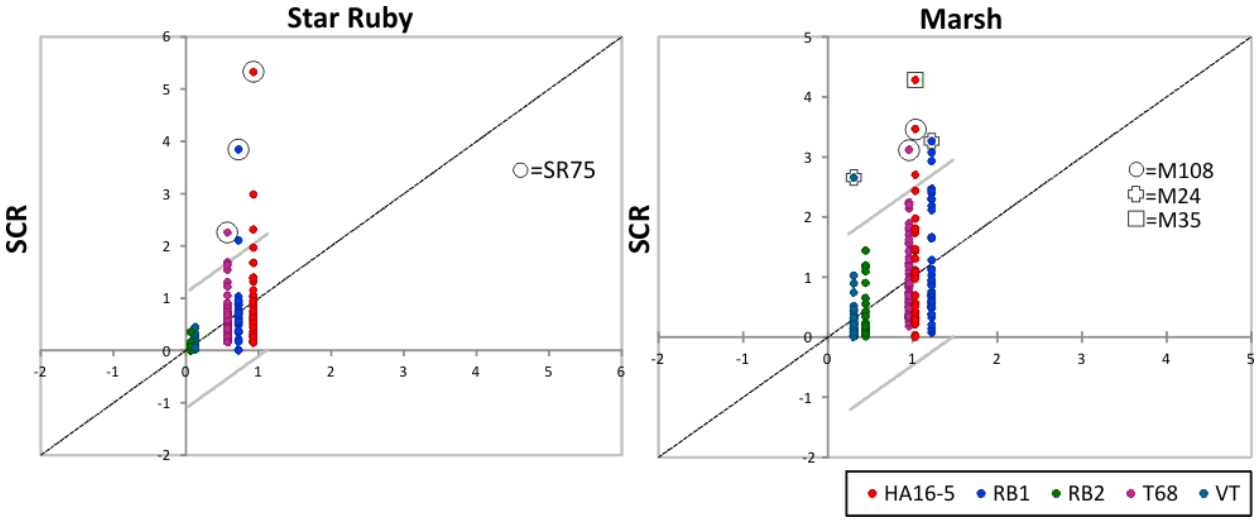


Figure 3. **Graphs depicting the variability in strain concentration ratios (SCR) of all treatments.** Graphs are basic linear models with the SCR plotted on the vertical axis and the treatment means on the horizontal axis. The parallel grey lines demarcate 95% confidence intervals. Specific samples are indicated.

The plant hosts' siRNA defence response to viral infection [3] and the silencing suppression capability of the virus to counteract the host's response [11], is doubtless a dynamic system with the two mechanisms in tension. This counteracting interplay between the two systems can likely account for the variances observed in this study as the silencing suppression of the virus overcomes the plants' defence at times. One study reported that two different CTV strains were found in close proximity of each other within the plant and were even found to occupy the same cells on occasion [5]. This leads to the assumption that the strains would be subject to the same mediated response of the host at the same time and could explain the simultaneous elevation of component strains within some samples, as shown in Figure 5.

All plants of this study were tested twice for their component stains to verify the population structure after inoculation. The two RB variants of this study, B389-1 and B389-4, were simultaneously detected in some, but not all plants that were dually inoculated. Co-infection with both variants was only detected in 40% of plants where they were co-inoculated. In fact, 90% of the failed transmissions of these two isolates were where they were co-inoculated to the same

plants. Samples that were included in the quantitative analyses and that were confirmed to contain both RB variants are listed in Table 7. Nevertheless, in the quantitative analyses either a single variant was detected or the other variant was poorly detected in these plants. These results suggest that the variants did not co-exist at the points of sampling. These RB isolates were originally obtained by single aphid transmissions from the same source plant, GFMS14 (Chapter 3), and were therefore previously also components of a population within a single plant.

Table 7. Strain concentration ratios (SCR) of the two RB variants in grapefruit samples containing both variants.

		SCR	
Sample	**Treatment**	**RB1 (B389-4)**	**RB2 (B389-1)**
SR14	T68-1 + RB1 + RB2	0	0.35
SR16	T68-1 + RB1 + RB2	0.6	0.06
SR17	T68-1 + RB1 + RB2	0.96	0
SR64	T68-1 + VT + RB1 + RB2	0.88	0
SR65	T68-1 + VT + RB1 + RB2	0.35	0
SR66	T68-1 + VT + RB1 + RB2	0.51	0
SR67	T68-1 + VT + RB1 + RB2	0.67	0
SR81	T68-1 + HA16-5 + RB1 +RB2	0.57	0
SR82	T68-1 + HA16-5 + RB1 +RB2	0.64	0
SR112	T68-1 + VT + HA16-5 + RB1 +RB2	0.61	0
SR113	T68-1 + VT + HA16-5 + RB1 +RB2	1.03	0
SR114	T68-1 + VT + HA16-5 + RB1 +RB2	0	0.06
M31	VT + RB1 + RB2	0	0.07
M32	VT + RB1 + RB2	0	0.13

The occurrence of variants of a strain in a single plant might challenge the principle of superinfection exclusion (SIE) whereby a primary infection by a strain excludes a secondary infection by challenge isolates of the same strain [13]. However, if the infections occur simultaneously, the homologous viruses can either co-exist in the plant, or the one component will displace the other. Superinfection by a homologous stain was in fact demonstrated under field conditions with both primary infection and the secondary challenge of the same strain simultaneously detected. It was shown that a primary isolate with lower fitness does not necessarily exclude infection by another, well-adapted isolate of the same strain [17]. Therefore, although

variants of RB were previously found as co-existing components within sources [12, 14], results of this study may further suggest that this coexistence in the host occurs by spatial segregation, similar to that of plum pox virus (PPV) where variants of PPV in a peach tree were shown to exist as spatially separated populations, able to expand and colonize other plant regions, but still as confined populations within the host plant [18].

4.4 CONCLUSION

Considerable genetic diversity has been demonstrated for CTV and the effects of different CTV populations on commercial cultivars are unknown until empirically tested.

This study aimed to investigate the influence of CTV populations, constructed using various combinations of well characterised single-strain isolates, on symptom expression in commercial grapefruit cultivars. Potential inter-strain and intra-strain interactions were investigated by quantitative determination of strain concentrations. Five isolates belonging to four CTV strains; T68, VT, HA16-5 and RB, including two isolates of the RB strain, were used. These isolates were previously characterised as mild isolates, although the T68 and VT isolates induced mild to moderate stem-pitting on grapefruit. Yearly evaluation of these constructed populations, over four years, showed that symptom expression of these mild strains did not result in altered symptom expression when in combination with each other. Stem-pitting was not more severe with coinfections of the T68 and VT isolates. Their individual stem-pitting abilities were not additive in combination, neither were their effects reduced in combination with any of the other mild strains that were not associated with stem-pitting in grapefruit.

Real-time, quantitative, strain-specific assays were developed and calibrated using a universal CTV assay in order to determine the concentrations of the strains relative to each other and enabled the quantitative determination of strain components of the constructed populations.

Overall, the strains were found within specific concentration ranges that differed between the two hosts. Since the order of magnitude of these ranges were similar for the strains of this study, concentration fluctuations were more visible and showed that these fluctuations can occur concurrently with other strains in the population.

Individual strain concentrations were not affected by the presence of heterologous strains in any of the strain combinations, but variants of the RB strain appeared to be in tension and were not detected simultaneously, suggesting spatial separation in the plant.

This study was not able to demonstrate strain interactions that impacted symptom expression in any additive manner, as either a reduction or an increase in stem-pitting. Therefore, complex mixtures of mild strains did not adversely affect the cultivars tested. Neither were inter-strain interactions, resulting in altered strain concentrations observed, but probable intra-strain segregation was noted. Information gained from this study further informs factors that impact both disease expression and CTV population dynamics, specifically in commercial grapefruit cultivars.

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5 T68 VARIANTS OF GFMS12 DIFFER IN STEM-PITTING SEVERITY IN GRAPEFRUIT

5.1 INTRODUCTION

Mild strain cross-protection research in South Africa was initiated prior to 1970 as a means to retain the commercial viability of grapefruit that was severely affected at the time by citrus tristeza virus (CTV) stem-pitting [6, 20]. A CTV isolate was obtained from a white grapefruit tree on the farm 'Nartia' in the Western Cape Province of South Africa in 1972. The tree was productive and displayed no stem-pitting [26]. The exact origin of the tree is unclear and the cultivar unknown, but it is believed that it originated from bud-wood that was imported to South Africa from California in the 1930s.

The 'Nartia' isolate was evaluated in various grapefruit field trials as a cross-protection source and was effective against natural challenges, but succumbed to stem-pitting when artificially challenged with a severe source [17]. At the inception of the South African Citrus Improvement Scheme in 1980, all citrus budwood supplied through the CIS was pre-immunised with the 'Nartia' mild isolate, later referred to as GFMS12 [18]. The effectiveness of this source in its cross-protection ability varied, dependent on the climatic region. Grapefruit planted in humid, hot areas succumbed to severe stem-pitting, suggesting environmental influences on symptom expression [17].

Severe stem-pitting was later also observed in some 'Star Ruby' grapefruit bud-wood source trees, which contained GFMS12. This occurrence was suggestive of a segregation event within the CTV population [23].

Single aphid transmissions (SATs) performed from the GFMS12 source to 'Mexican' lime seedlings generated nine sub-isolates that were biologically characterised by van Vuuren et al (2000). These sub-isolates were found to differ in stem-pitting severity in both 'Mexican' lime and 'Marsh' grapefruit. No differences were observed between these isolates in either restriction fragment length polymorphism (RFLP) or single-strand conformation polymorphism (SSCP) patterns of the coat protein gene [22].

The original GFMS12 source was later propagated on ‘Duncan’ grapefruit and maintained at the Nelspruit facility of Citrus Research International (CRI), but developed severe stem-pitting over time.

The nine sub-isolates showing varied symptom expression in ‘Marsh’ grapefruit and the severe stem-pitting observed after a host transfer from the original source, indicated that a component(s) of this source induced severe stem-pitting in grapefruit. This study aimed to biologically characterise components of GFMS12 and to compare the full-genomes of mild and severe stem-pitting variants.

5.2 MATERIALS AND METHODS

5.2.1 GFMS12 propagation sources and sub-isolates

The original ‘Nartia’ or GFMS12 source collected in 1972 [26] was established on a Volkameriana lemon rootstock (C. × *volkameriana*) and is still maintained at the Agricultural Research Council Institute of Tropical and Sub-tropical Crops (ARC-ITSC) in Nelspruit, Mpumalanga in an insect-proof tunnel. The source was further propagated in ‘Duncan’ grapefruit and maintained in a glasshouse at the CRI facility in Nelspruit. However, this propagation plant displayed severe stem-pitting (SP) after a number of years (Figure 1).

Single aphid transmissions from the original GFMS12 source plant were performed. The source was first inoculated to ‘Mexican’ lime, which was used as the acquisition host and from there, single aphids were transferred to ‘Mexican’ lime receptor plants. Nine sub-isolates of GFMS12 were obtained and biologically characterised on ‘Mexican’ lime and ‘Marsh’ grapefruit [22]. Three of these sub-isolates; GFMS12-7, GFMS12-8 and GFMS12-9 are maintained in ‘Mexican’ lime at CRI, Nelspruit.

Figure 1. GFMS12 propagation sources showing absence and presence of stem-pitting. (A) Original GFMS12 in 'Nartia' grapefruit at ARC-ITSC, Nelspruit and (B) GFMS12 in 'Duncan' grapefruit at CRI, Nelspruit.

5.2.2 Sub-isolation of GFMS12-1.3

A bark patch of the GFMS12 source plant showing severe SP was grafted to an insecticide- and virus-free 'Mexican' lime seedling for aphid transmissions. Approximately 20-30 non-viruliferous aphids, *Toxoptera citricida* (Kirk.), were transferred to this acquisition host and allowed an acquisition feeding period of 24 h. Thereafter 4-5 aphids were placed on a single 'Mexican' lime seedling for an inoculation access period of 24 h and aphids were physically removed thereafter. Receptor plants were tested for CTV transmission after one month with the generic CTV RT-PCR assay [5]. A second, single aphid transmission was done from one of these positive plants and a positive plant from the second aphid transmission was maintained as isolate GFMS12-1.3 in an insect proof glasshouse.

5.2.3 Strain determination of propagation sources and sub-isolates of GFMS12

The original GFMS12 source plant was tested, using CTV strain-specific RT-PCRs, to determine the strain components. Six samples were taken at different positions of the tree and bark of green twigs was used to extract RNA. The CRI GFMS12 propagation plant and sub-isolates GFMS12-

1.3, GFMS12-7 and GFMS12-9 were also tested using the same strain-specific RT-PCR assays. Extractions and RT-PCR were done as previously reported [5]. The strain determination of GFMS12-8 was documented in Chapter 3.

5.2.4 Biological Evaluation of GFMS12-1.3 and GFMS12-8

Isolate GFMS12-8 was previously tested on the full range of 'Garnsey' citrus hosts [9] as reported in Chapter 3. Nonetheless, two T68 sub-isolates derived from different propagation sources of GFMS12, including GFMS12-8 and GFMS12-1.3, were comparatively tested on the 'Garnsey' host range. The isolates were inoculated to the citrus hosts with four to six replicates for each host, dependent on availability. Inoculations were done by grafting bark pieces from the respective 'Mexican' lime source plants to each scion. Side branches were removed and a single shoot was allowed to grow from the top bud of each plant. Plants were maintained in an aphid-free glasshouse with average day temperatures ranging between 24-27°C and average night temperatures between 12-20°C. Midday temperatures exceeded 35°C at times. Each inoculated plant was tested for successful transmission by RT-PCR using the generic CTV assay [5]. Symptom expression was recorded over six months. Another set of Duncan seedlings were inoculated as a validation test and evaluated for SP after four months. The individual component scores for symptoms on each 'Garnsey' host were averaged and the composite score multiplied by the weight factor for each host. The final disease index was obtained by adding the scores for each citrus host [9].

Isolate GFMS12-1.3 was additionally inoculated to 'Star Ruby' grapefruit on Rough lemon rootstocks. Limited plants were available and GFMS12-8 was not tested in parallel, but was tested extensively on this host previously as reported in Chapter 3.

5.2.5 Full-genome sequence determination of GFMS12-1.3 and GFMS12-8

Full-genome sequences for isolates GFMS12-8 and GFMS12-1.3 were obtained by amplifying overlapping genome segments of approximately 1500bp and direct Sanger sequencing as described in Chapter 3. The primers used for strain T68 amplifications are provided in Appendix B2[ix].

[ix]Appendix B2. Overlapping primers used for full-genome amplification of various CTV strains, T68 included.

Both sequences were verified by Next Generation Sequencing (NGS) at the Genetics Department of Stellenbosch University. Independent RNA extractions were performed for this purpose. Total RNA was extracted from the phloem material using a CTAB method [25] and shipped on dry ice to an NGS sevice provider (Macrogen Inc., South Korea). The RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). A ribosome-depleted RNA library was prepared using the TruSeq Stranded Total RNA LT Plant Sample Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions and sequenced on the Illumina NovaSeq, generating 100bp paired-end reads. NGS reads were mapped to the Sanger generated genomes for GMFS12-8 and GMFS12-1.3 using default parameters in CLC genomics Workbench 10.1.1 (CLCbio, Qiagen). A consensus sequence was extracted from the alignment and then aligned with the Sanger-generated sequence. At the positions where the consensus differed from the Sanger sequence, an estimate of the NGS nucleotide ratios at those positions, were made.

NGS reads for GMFS12-8 and GMFS12-1.3 were also *de novo* assembled using default parameters in CLC genomics Workbench 10.1.1. Assembled contigs were identified using command line BLAST and contigs corresponding to CTV were aligned to the respective Sanger-generated sequences of GMFS12-8 and GMFS12-1.3 [x].

Further sequence alignments and translations were performed in CLC Sequence Viewer 7.6 and closest sequence identity to other CTV genomes was determined using BLAST [1].

5.2.6 Diagnostic differentiation of T68 variants

Primers were designed to amplify a region encompassing five single nucleotide polymorphisms (SNPs) found within a short section of ORF p33 of the GFMS12 T68 variants. Amplicons of this region were Sanger sequenced to identify the dominant variants found in the GFMS12 source plants and sub-isolates.

Reverse transcription reactions were random primed as detailed in Chapter 4. PCR was done using primers T68_J+(5'-GATGTTGGTTATAATGCTGCCGA-3') and T68_p33R1(5'-ATTTTCGGATATCGTTTGTGTGC-3') at 0.5µM each and Q5 Hot Start High Fidelity Master

[x] NGS verification of sequences was done by Dr. B. Coetzee at the Genetics Department, Stellenbosch University.

Mix (New England Biolabs Inc, MA, USA) in 20 µl reaction volumes as per supplier's protocol. Cycling conditions included an initial denaturation step at 98°C for 30 sec followed by 35 cycles of 98°C for 5 sec, 60°C for 30 sec 72°C for 30 sec. A final extension of 72°C for 5 min was done. Amplicons of 377 bp were generated and gel extracted using the Zymoclean™ Gel DNA recovery kit (Zymo Research Corporation, CA, USA). Direct Sanger sequencing was done using a forward reaction only.

5.2.7 Field evaluation of GFMS12 and sub-isolates in 'Star Ruby' grapefruit

Virus-free 'Star Ruby' trees were prepared on 'Troyer' citrange rootstocks according to normal nursery practices. Scions were inoculated when they were approximately pencil thickness by patch-grafting the CTV sources. Only information relevant to the CTV sources discussed in this study are presented and include GFMS12 and the sub-isolates, GFMS12-7 and GFMS12-9. The GFMS12 propagation source maintained at CRI was used to inoculate this trial. Enzyme linked immunosorbent assay (ELISA) was used to confirm CTV transmission, three months post-inoculation and was done as detailed in Chapter 3. The trees were planted in 2007 in the Letsitele area in the Limpopo Province according to a randomised block design and included five replicates for each treatment and five un-inoculated control trees[xi].

5.2.8 Stem-pitting evaluation and production performance

The trees were evaluated annually for the development of SP as observed externally on the trunk using a severity scale from 0 to 3, where (0) represents a smooth trunk with no visible pitting, (1) represents one to three grooves on the stem, (2) indicates multiple grooves and (3) is severe SP resulting in the tree trunk having a knotted appearance (Appendix B5)[xii]. As a final assessment after 10 years in the field, bark flaps of approximately 70cm^2 were removed from the trunk of each tree to assess SP in the wood. These bark windows were photographed.

Tree canopy volumes were determined yearly using the formula V = S2(πh -1.046S), where S is canopy radius and h is the height of the fruit bearing canopy [3].

[xi]The Star Ruby field trial was initiated by Dr. S.P. van Vuuren (CRI) and work pertaining to the management of the field trial, external stem-pitting evaluation, canopy volume determination and harvesting was conducted by Mr. J.H.J. Breytenbach (CRI).

[xii] Appendix B5. External stem-pitting severity scale for field trial evaluations.

Fruit was harvested annually and yield (kg) per tree determined. The fruit size distribution was determined at harvest according to export size categories, and fruit of 86 mm in diameter and smaller was regarded as small fruit. A four-year cumulative yield per tree for the sixth to the ninth year was calculated.

Statistical analyses of tree canopy volume differences between treatments were done using ANOVA and Fisher's least significant difference test with 95% confidence interval. Differences in the percentage of small fruit between isolates were analysed using the non-parametric Kruskal-Wallis ANOVA and pairwise comparisons of means were done using Dunn's test. Statistical analyses were done in XLSTAT 2015 (Addinsoft, Paris, France).

5.2.9 CTV strain analysis of field trial trees

RNA was extracted from cambium scrapings of each bark flap, removed at the final evaluation, and samples were tested to determine the CTV strain components present after ten years in the field, using strain-specific RT-PCR assays previously described [5]. The samples were also tested for the dominant T68 variant present in each tree by amplification and Sanger sequencing of the p33 region as described above.

5.3 RESULTS AND DISCUSSION

5.3.1 Strain determination of GFMS12 propagation sources and sub-isolates

Only strain T68 was detected in the original source plant of GFMS12 by sampling at six different positions of the tree and strain-specific RT-PCR analyses. Similarly, only strain T68 was detected in the CRI propagation source of GFMS12.

Sub-isolate GFMS12-1.3 was confirmed as a T68 single-strain isolate by means of RT-PCR strain-specific assays. Additional sequencing of an amplification product using degenerate, mid-genome primers substantiated the single-strain status of the sub-isolate.

Single infections of the T68 strain were also shown for sub-isolates GFMS12-7 and GFMS12-9.

5.3.2 Biological Evaluation of GFMS12-1.3 and GFMS12-8

Both T68 sub-isolates GFMS12-1.3 and GFMS12-8 were rated as mild strains according to the 'Garnsey' disease index as they are both non-decline isolates that do not induce seedling yellows on either sour orange or 'Duncan' grapefruit, nor do they induce SP on sweet orange. The sweet orange SP phenotype is regarded as more severe than the grapefruit SP phenotype and is weighted accordingly in the disease index. However, GFMS12-1.3 induced moderate to severe SP on 'Duncan' grapefruit as shown in Figure 2A and the disease index therefore under- represents the significance of this phenotype for the purpose of this study. Differences in scores in 'Duncan' grapefruit were obtained for the sub-isolates and the disease index for sub-isolate GFMS12-1.3 twice that of GFMS12-8 (Table 1).

The 'Garnsey' disease index score for GFMS12-8 was 3.2 in this trial, compared to 5.1 obtained in the previous trial (Chapter 3). Symptom expression can vary slightly, depending on environmental conditions, the age of the seedlings inoculated and other seasonal influences as reported in Chapter 3 and 4 of this study. However, this isolate consistently expressed as a mild isolate in this study, and in a previous study [22].

Two of the three inoculations of GFMS12-1.3 to 'Star Ruby' plants were successful and severe SP was observed as is shown in Figure 2C. Although GFMS12-1.3 was not tested in parallel with GFMS12-8 in 'Star Ruby' due to the limited availability of plants, the same SP severity was not observed for GFMS12-8 in numerous evaluations in 'Star Ruby' grapefruit as reported in both Chapter 3 and Chapter 4 of this study. A direct comparison of sub-isolates GFMS12-1.3 and GFMS12-8 was therefore only done in 'Duncan' grapefruit and they are therefore differentiated by their relative SP severity in this host, but the severity of "GFMS12-1.3 on 'Star Ruby' is also indicative of differences in expression in 'Star Ruby.

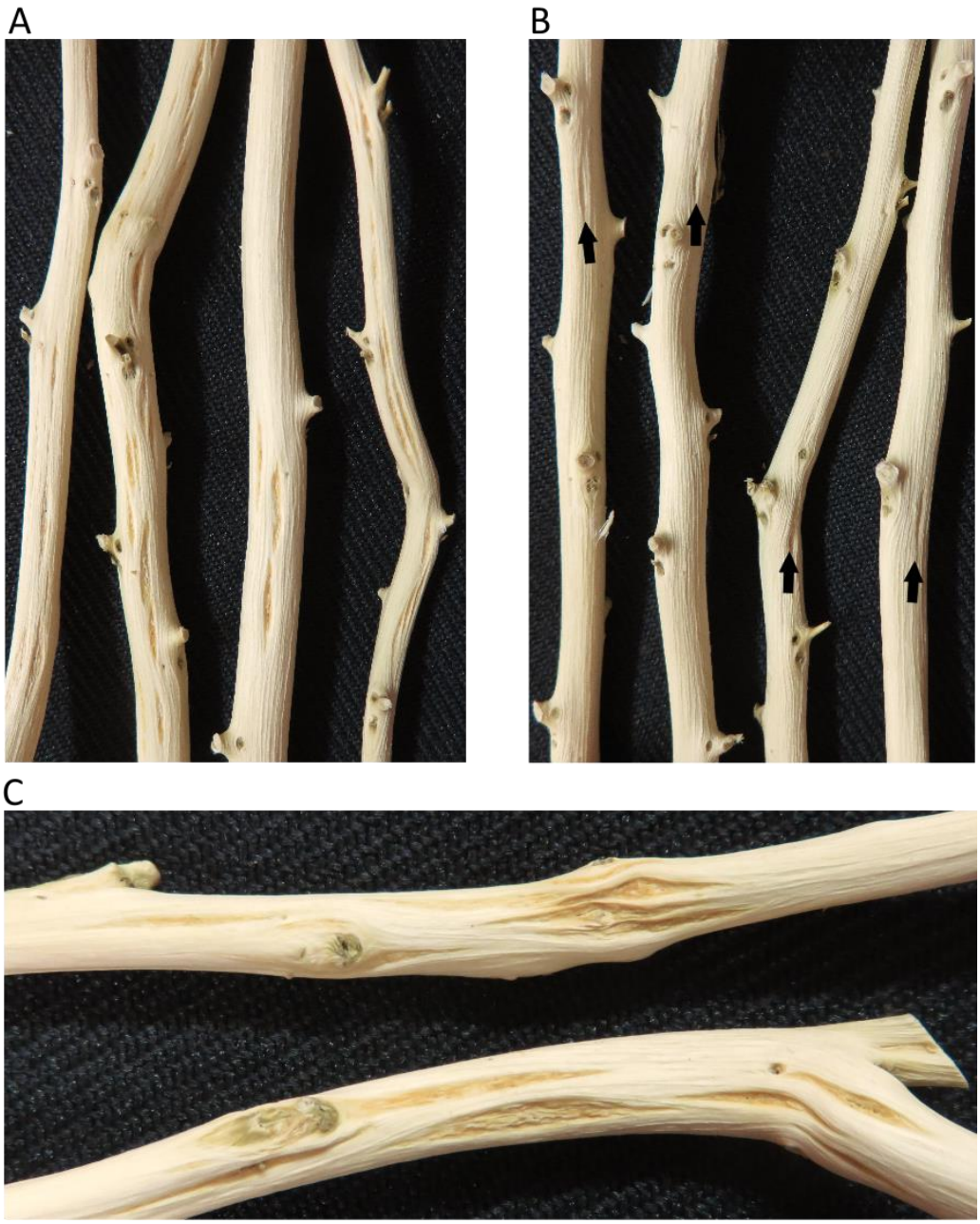

Figure 2. Stem-pitting of sub-isolates GFMS12-1.3 and GFMS12-8. (A) GFMS12-1.3 and (B) GFMS12-8 in 'Duncan' grapefruit, four months after inoculation (C) GFMS12-1.3 in 'Star Ruby' grapefruit. *Arrows indicate less prominent SP*.

Table 1. Virulence indexing of single-strain CTV isolates based the 'Garnsey' host range disease index per host and cumulative score (ΣDI).

CTV Isolate	Strain	Average DI per citrus host[z]					
		ML (×1)	SW/SO (×2)	SO (×3)	DGF (×4)	MV (×5)	ΣDI
Un-inoculated control	….	0	0	0	0	0	0
GFMS12-8	T68	1.9	0	0	1.3	0	3.2
GFMS12-1.3	T68	2.3	0	0	4.6	0	6.9

[z] Individual symptom were rated as 0 = no symptoms, 1 = mild, 2 = moderate and 3 = severe. The component scores for individual symptoms for each host were averaged and the composite score multiplied by the weight factor for each host as indicated. Symptoms scored per host were as follows: ML = 'Mexican' lime, vein clearing and stem-pitting (SP); SW/SO = sweet orange/sour orange, stunting/decline; SO = sour orange, seedling yellows (SY) and stunting; DGF = 'Duncan' grapefruit, SY, SP stunting; and MV = 'Madam Vinous', SP and stunting.

5.3.3 Sequence analysis of T68 sub-isolates GFMS12-8 and GFMS12 1.3

Complete genome nucleotide sequences for sub-isolates GFMS12-1.3 and GFMS12-8 were compiled and validated independently by NGS[xiii]. These sequences were deposited in GenBank under the accession numbers MK033510 and MK033511, respectively. The sequence of GFMS12-8 (MK033511) shares 99.9% identity with CT-ZA3 (KC333868) and refers to the same isolate and they are therefore confirmation sequences. The GFMS12-8 (MK033511) sequence has 11 nucleotide degeneracies not reported in CTZA3 (KC333868). Both these sequences share 99.7% sequence identity with GFMS12-1.3 (MK033510). The type member of the strain, T68-1 (JQ965169), is also closely related to the three above sequences and shares 97.3% nucleotide identity with them. These close similarities are depicted in Figure 2 of Chapter 3 in a Neighbor Network construction of complete CTV genomes.

Nucleotide degeneracies were found in both sequences at a few positions, either in both NGS data and Sanger sequences, or only in one of the data sets. Where Sanger sequencing indicated a single nucleotide, the most prevalent nucleotide found with NGS at the position, agreed with the Sanger call. At positions where Sanger sequencing indicated a degeneracy and NGS yielded a single nucleotide, it was consistent with the Sanger degeneracy. The genome positions of nucleotide

[xiii] NGS verification of sequences was done by Dr. B. Coetzee at the Genetics Department, Stellenbosch University.

degeneracies of isolates GFMS12-8 and GFMS12-1.3 are shown in Figure 3. More ambiguities were found in the GFMS12-8 genome compared to the genome of GFMS12-1.3.

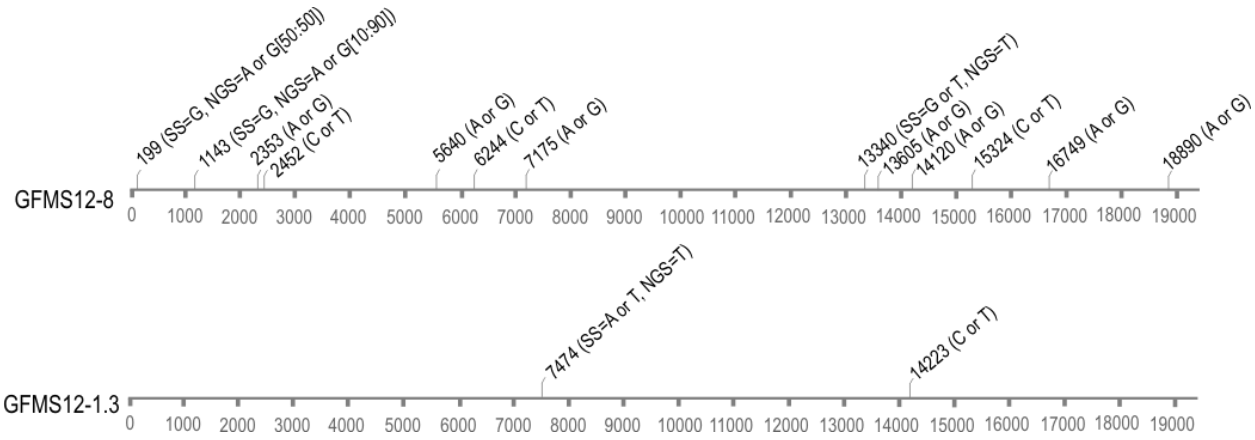

Figure 3. Nucleotide ambiguities on the GFMS12-8 and GFMS12-1.3 genomes. The genome position and ambiguities found are indicated. SS = nucleotide(s) found with Sanger sequencing and NGS = nucleotide(s) found with next generation sequencing.

The differences between the genomes of GFMS12-8 and GFMS12-1.3 were interrogated and 39 SNPs were found over the length of the genome as indicated in Figure 4. Eighteen SNPs are present in open reading frame 1a (ORF1a) of which 10 were non-synonymous. Two synonymous SNPs were present in the RNA-dependent RNA polymerase (RdRp), five SNPs in ORF p65 of which four were non-synonymous, three non-synonymous SNPs in ORF p61, three synonymous SNPs in ORF p27, one synonymous and one non-synonymous SNP was found in the p20 and p23 ORF, respectively. A further six SNPs were observed in ORF p33 of which five were non-synonymous and these were in relatively close proximity to each other. This section was targeted as a diagnostic region to discriminate the T68 variants.

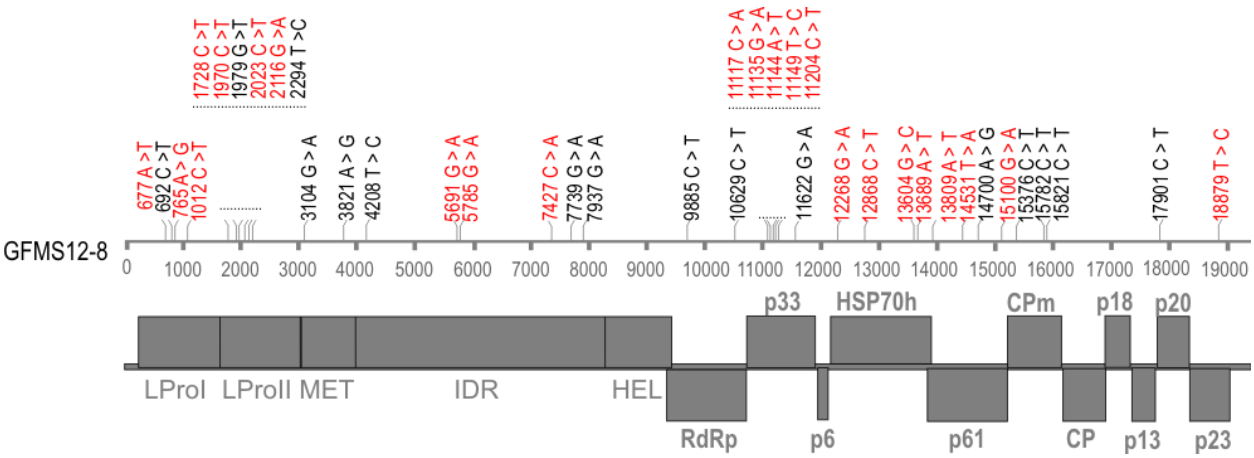

Figure 4. Single nucleotide polymorphisms (SNPs) between genomes of GFMS12-8 and GFMS12-1.3. Nucleotide differences and positions are indicated on the GFMS12-8 genome. *Non-synonymous nucleotides are indicated in red*. Boxes show the relative positioning of the ORFs.

Pathogenicity determinants for SP have not been defined, but ORFs p33, p18 and p13 were implicated and seemingly influence SP by the combined expression of these genes [21]. It is noteworthy therefore, that five non-synonymous SNPs were found in ORF p33. It will be of value to further investigate this region as a SP determinant. Apart from p33, SNPs were found in clusters in ORF1a in both the LProI and LProII domains. Leader proteases of closteroviruses were shown to also function in virus infection and cell-to-cell movement and can therefore, possibly be implicated in pathogenicity [16, 19].

5.3.4 Diagnostic differentiation of T68 variants

The transmission of T68 variants, GFMS12-1.3 and GFMS12-8, inoculated to 'Mexican' lime and 'Duncan' grapefruit was confirmed by amplification and sequencing of the targeted p33 region. Figure 5 shows a sequence alignment of a section of the amplified region for each of the plants and shows the SNPs that differentiate GFMS12-1.3 and GFMS12-8. The detection of the five SNPs was consistently found in the respective inoculated plants of two citrus hosts. This indicates that these nucleotide differences were stably maintained over the six-month evaluation period. The two successfully inoculated Star Ruby plants (Figure 2) also contained the five SNPs that is consistent with GFMS12-1.3.

Figure 5. Nucleotide sequence alignment showing the p33 diagnostic region for T68 variant discrimination in samples of 'Duncan' grapefruit and 'Mexican' lime (MxL) inoculated with either GFMS12-1.3 or GFMS12-8. The equivalent regions of the respective GenBank sequences for the two variants are included. Dots indicate equivalent nucleotides.

Figure 6. Nucleotide sequence alignment showing the p33 diagnostic region for T68 variant discrimination in samples of two GFMS12 propagation plants and the GFMS12 sub-isolates. The equivalent regions of the GenBank sequences for GFMS12-1.3 and GFMS12-8 are included. Dots indicate equivalent nucleotides.

The p33 differentiating fragment was amplified and sequenced for the two GFMS12 sources (the original source at ARC-ITSC and the propagation source at CRI), as well as the sub-isolates GFMS12-7 and GFMS12-9. The CRI GFMS12 propagated source, displaying severe stem-pitting (Figure 1B), yielded exactly the same nucleotide sequence as sub-isolate GFMS12-1.3, which was derived from the source (Figure 6), indicating that GFMS12-1.3 is the dominant T68 variant in the CRI GFMS12 source plant.

The original GFMS12 source plant, at ARC-ITSC, is maintained in a drum and is larger than the other source plants. For that reason, six separate branches were sampled in order to detect possible sectorial differences in the plant. The six samples yielded the same sequence. This consensus sequence is shown in the alignment of Figure 6 and differs from both that of GFMS12-1.3 and GFMS12-8. Three of the five SNP positions are the same as GFMS12-1.3 and the other two are

the same as GFMS12-8 and indicates the presence of a dominant sequence variant that differs from the GFMS12 source plant maintained at CRI.

The equivalent sequence fragments for sub-isolates GFMS12-7 and GFMS12-9 also differ from the above sources, with an additional SNP observed for GFMS12-7 (Figure 6).

Five variants of the T68 strain were identified in various sub-isolates and source plants of GFMS12. This demonstrates the existence of an array of variants present in the parental source, some of which were separated by host transfers and single aphid transmissions and identified in this study. This is suggestive of the quasispecies concept where numerous sequence variants are found with viruses that replicate with high error rates. A virus therefore develops heterogeneity over time and variants are found that oscillate around a master sequence [8]. The formation of quasispecies have biological significance as a mechanism enabling adaptability and modulation of the expression of phenotypes [7]. As environments change, these mutations become targets for selection and can become dominant. In the case of plant viruses, these genetic variations may enable a virus to infect new hosts, improve the potential to be transmitted by arthropod vectors and may also result in altered pathogenesis.

5.3.5 Field evaluation of GFMS12 sub-isolates in 'Star Ruby' grapefruit

The progression of stem-pitting in 'Star Ruby' field trees, inoculated with GFMS12 and sub-isolates, GFMS12-7 and GFMS12-9 is presented in Figure 7. Trees inoculated with GFMS12 and sub-isolate GFMS12-7 displayed severe SP, significantly more than control trees and trees inoculated with GFMS12-9.

SP was visible externally on tree trunks, shortly after planting, and increased in severity over the years. The severe SP observed in GFMS12 inoculated trees correlated to diminished tree growth (Table 2) and was associated with a high percentage of small fruit (Table 3). One tree, inoculated with GFMS12, died after 5 years in the field due to the severe SP. GMFS12-7 showed moderate to severe SP, but this was not correlated to a reduction in tree growth.

Removal of bark flaps to observe SP in the wood showed differences in SP severity depending on the treatment as shown in Figure 8. All trees containing GFMS12 displayed severe porous wood pitting, while trees containing sub-isolate GFMS12-7 showed comparable, but less severe SP in

most replicates. GFMS12-9 was associated with less SP compared to either GFMS12 or GFMS12-7 and mild SP was observed in some control trees. These observations correlated with the external stem-pitting evaluations.

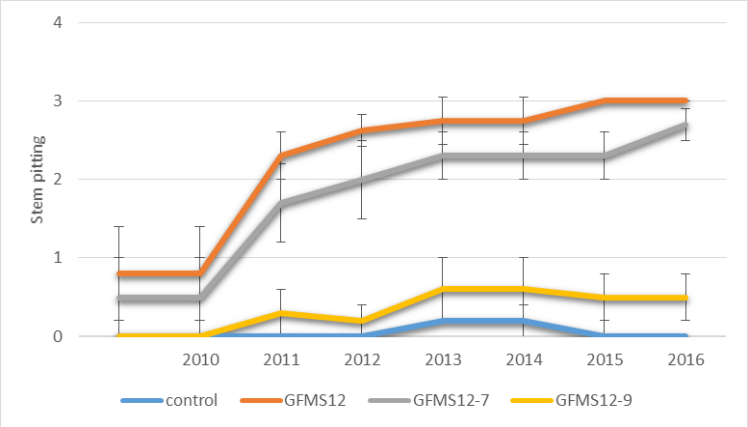

Figure 7. Average stem-pitting progression in 'Star' Ruby grapefruit field trees inoculated with GFMS12 and sub-isolates GFMS12-7 and GFMS12-9. Standard error bars are shown. Rating scale: 0 = Smooth trunk; 1 = one to three grooves on stem (mild); 2 = multiple grooves (moderate); 3 = severe SP resulting in the tree trunk having a knotted appearance

Table 2. Average tree canopy volumes for 'Star Ruby' grapefruit field trees inoculated with GFMS12 and sub-isolates GFMS12-7 and GFMS12-9, eight years after planting.

Treatment	*n*	Canopy volume (m^3)	
Control	5	24	A
GFMS12-7	5	20	A
GFMS12-9	5	18	A
GFMS12	4	11	B
Prob F treat [z]		*0.01*	

Treatments with the same letters do not differ statistically.

[z] *Probability value from analysis of variance for differences between treatments.*

Table 3. Average percentage of small fruit obtained during the 2014 to 2016 harvests of 'Star Ruby' grapefruit trees inoculated with GFMS12 and sub-isolates GFMS12-7 and GFMS12-9.

	Percentage (%) small fruit		
Treatment	**2014**	**2015**	**2016**
GFMS12	69 A	92 A	83 A
GFMS12-7	40 A B	59 A B	39 A B
GFMS12-9	36 A B	38 A B	37 A B
Control	21 B	19 B	19 B
p-value:	*0.048*	*0.003*	*0.040*

Treatments with the same letters do not differ statistically.

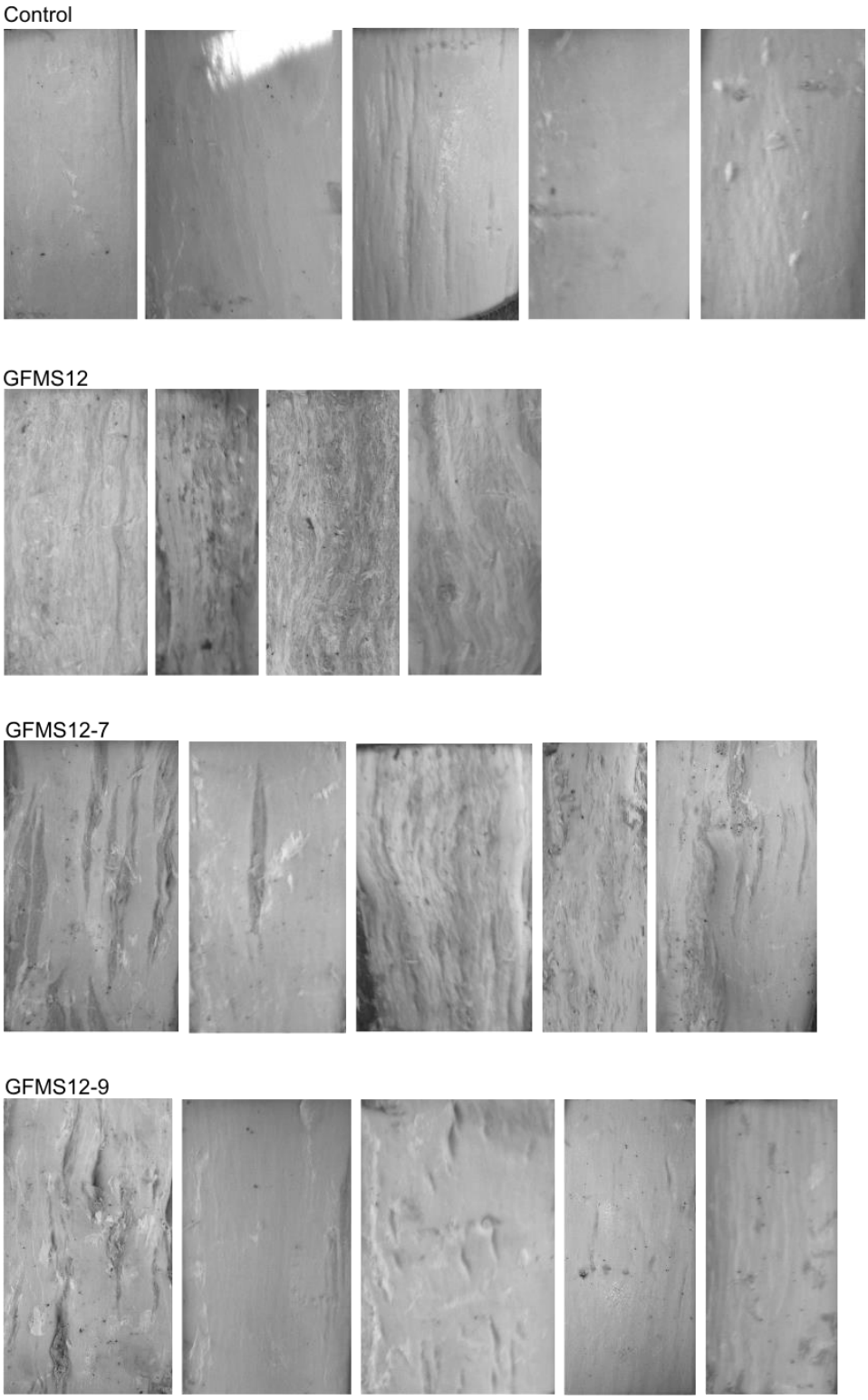

Figure 8. Stem-pitting visible in the wood of 'Star Ruby' grapefruit trees inoculated with GFMS12 and sub-isolates GFMS12-7 and GFMS12-9, ten years after planting. Bark windows on un-inoculated control trees are also shown.

5.3.6 CTV analysis of 'Star Ruby' field trees

Strain-specific RT-PCR results, presented in Table 4, show that aphid transmission of CTV had occurred. The RB, VT and HA16-5 strains were not detected as components of the inoculation sources, but were detected in the trees ten years after planting. Field transmission of strain T68 to control trees had also occurred. Nonetheless, the SP observed within replicate trees of each treatment was similar and field transmission to the control trees did not result in severe SP in any of the replicates. This indicates that the SP observed was mostly due to the primary infections. This was also substantiated by the disease progression of GFMS12 and GFMS12-7 as shown in Figure 7, where early SP onset and progression was consistent between replicates of these treatments.

Table 4. CTV strains detected in 'Star Ruby" field trees, ten years after planting.

	CTV strains							
Tree no.- inoculated source	**RB1**	**RB2**	**VT**	**T68**	**HA16-5**	**T3**	**T30**	**T36**
5 - control	+	+	+	+	+	-	-	-
8 - control	+	+	(+)	-	(+)	-	-	-
9 - GFMS12-9	+	+	(+)	+	(+)	-	-	-
13 - control	+	+	(+)	(+)	-	-	-	-
15 - GFMS12-9	+	+	(+)	(+)	-	-	-	-
22 - GFMS12-9	+	+	(+)	+	(+)	-	-	-
28 - GFMS12-9	+	+	(+)	+	(+)	-	-	-
30 - control	+	+	-	(+)	(+)	-	-	-
35 - control	+	+	+	+	(+)	-	-	-
38- GFMS12-9	+	+	(+)	+	-	-	-	-
41 - GFMS12	+	+	(+)	+	(+)	-	-	-
42 - GFMS12-7	+	(+)	-	+	(+)	-	-	-
45 - GFMS12	+	+	-	+	-	-	-	-
46 - GFMS12-7	+	+	(+)	+	(+)	-	-	-
50 - GFMS12-7	+	+	(+)	+	-	-	-	-
56 - GFMS12	-	+	(+)	+	-	-	-	-
57 - GFMS12-7	+	+	-	+	-	-	-	-
63 - GFMS12	+	+	(+)	+	+	-	-	-
64 - GFMS12-7	+	+	(+)	+	-	-	-	-

[+ = positive amplification, (+) = weak amplification, – = no amplification]

The p33 region of the T68 strain was amplified and sequenced for each trial tree and an alignment of these sequences is presented in Figure 9. The consensus sequences associated with the isolates used for inoculation were consistently detected in the respective plants. Variant GFMS12-1.3 was the dominant variant detected in the GFMS12-inoculated plants and is therefore associated with the severe SP observed, although the presence of other CTV components cannot be discounted.

It is unfortunate that only isolates GFMS12-7 and GFMS12-9 were included in this trial and not GFMS12-8. However, sub-isolates of GFMS12, including GFMS12-1 to -9 were previously tested in 'Marsh' grapefruit in a glasshouse trial where GFMS12-8 was associated with minimal SP, GFMS12-9 induced slightly more SP, but GFMS12-7 was associated with significantly more SP than either GFMS12-8 or GFMS12-9 [22]. The results of the field trial of this study, correlate with the finding that isolate GFMS12-7 is associated with more severe SP than GFMS12-9. The sequencing results further demonstrate that these sub-isolates are distinct variants of the T68 strain and are consistently found as the dominant sequences in the field trees as inoculated. A nucleotide degeneracy is observed for GFMS12-9 at the one SNP in the field trees (Figure 9).

Transmission of strain T68 to four control trees was detected (Table 4). Sequences of the p33 diagnostic region indicate that the GFMS12-9 variant was transmitted in each case. The trees were planted randomly within the orchard rows and aphid transmission should therefore not have been influenced by tree proximity. It is possible that GFMS12-9 is more aphid transmissible that the other variants. Control trees showed mild SP at the final evaluation (Figure 8), although symptom expression would depend on the time the infection occurred.

Figure 10 shows the translation alignment of the p33 diagnostic region for the field samples and reference sequences, as in Figure 9. Five amino acid changes are seen in this region for the various T68 variants. Although this region could be used to differentiate the variants, it has not been demonstrated that the changes in ORF p33 impact SP expression. Other SNPs were also detected in the full-genome analysis of the GFMS12-1.3 and GFMS12-8 variants. Full-genome determinations of GFMS12-7 and GFMS12-9 were not done in this study and were therefore not available for comparisons.

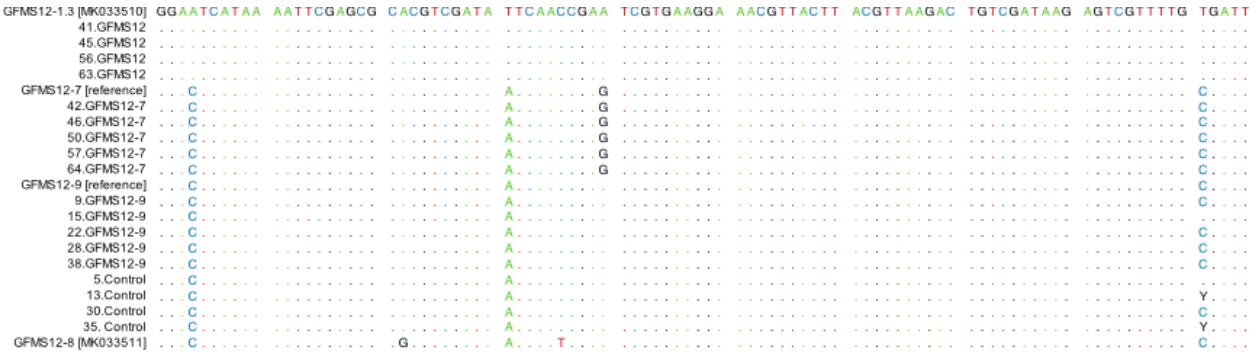

Figure 9. Nucleotide sequence alignment showing the p33 diagnostic region for T68 variant discrimination in 'Star Ruby' field samples inoculated with the GFMS12 and sub-isolates GFMS12-7 and GFMS12-9. T68 field transmissions to control trees are included. The equivalent regions of the GenBank sequences for GFMS12-1.3 & GFMS12-8 and reference sequences for GFMS12-7 & GFMS12-9 are shown. Dots indicate equivalent nucleotides.

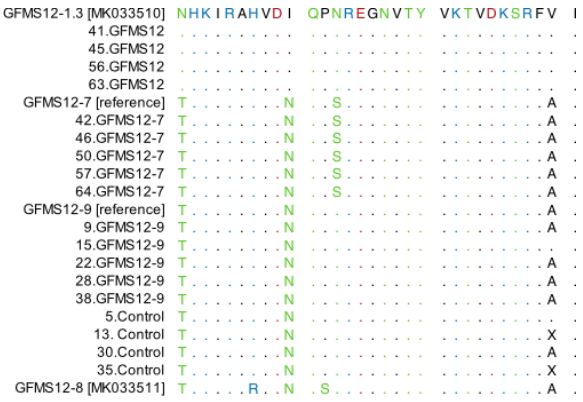

Figure 10. Amino acid sequence alignment of the p33 diagnostic region for T68 variant discrimination in 'Star Ruby' field samples inoculated with the GFMS12 and sub-isolates GFMS12-7 and GFMS12-9. T68 field transmissions to control trees are included. The equivalent regions of the GenBank sequences for GFMS12-1.3 & GFMS12-8 and reference sequences for GFMS12-7 & GFMS12-9 are shown. Dots indicate equivalent nucleotides.

5.4 CONCLUSION

Sequence determination of CTV genomes and diagnostic capabilities to distinguish strains, facilitate the linkage of biological expression to specific genetic components. Characterisation of CTV sources, used for cross-protection, is required to develop an understanding of components and mechanisms underlying cross-protection to be able to apply effective cross-protection agents and to monitor their transmission. An improved understanding of these aspects is of unique importance when problems occur with the implementation of this management intervention, as was encountered with the use of GFMS12 for cross-protection in grapefruit.

Characterisation of CTV sources, used in cross-protection, was previously reliant on the sub-isolation and biological characterisation of components of these populations, but lacked the current strain-identification diagnostic capabilities [22]. The maintenance of isolates and sub-isolates of GFMS12 provided the opportunity to understand the possible reasons for the failure of GFMS12 as a cross-protection source in grapefruit. The separation of strain variants of the original GFMS12 source was facilitated by probable changes in environmental conditions during plant maintenance, host changes and aphid transmission.

Two processes are understood to determine the genetic structure of a virus population consisting of quasispecies and are referred to as selection and genetic drift. The process of selection results in an altered frequency of variants in a changed environment. An event such as a virus infecting a new host can result in genetic drift called the ‘founder effect’. This happens when a different population emerges when a small number of variants are randomly sampled from the original population. These founder populations generally demonstrate lower population diversity [8]. It is relevant to note that sub-isolate GFMS12-8, which was obtained in the late 1990s displayed more nucleotide ambiguities than GFMS12-1.3, sub-isolated in 2017. The older sub-isolate shows greater diversity, likely due to quasispecies formation over a longer period, whereas GFMS12-1.3, still in a founder population phase, has greater homogeneity.

This study demonstrated that the original GFMS12 contains the T68 strain, but that an assortment of variants was derived from this source, displaying different stem-pitting phenotypes. Two of these sub-isolates were characterised by full-genome sequence determination and biological evaluation on a citrus host range. Although sub-isolates GFMS12-1.3 and GFMS12-8 differed in stem-pitting severity in grapefruit, only 39 SNPs were found between the genomes. This would suggest that only minor sequence differences were responsible for the significant difference in symptom expression.

Symptom evaluation of GFMS12-1.3 in ‘Star Ruby’ grapefruit could not be done in parallel with GFMS12-8 and is a limitation of this study. Nonetheless, the two isolates were compared in ‘Duncan’ grapefruit, a

standard indicator host. Isolate GFMS12-8 was extensively tested in two previous trials in 'Star Ruby' and no severe SP was observed. The full-genome sequencing of isolates GFMS12-7 and GFMS12-9 will follow in future investigations.

The intra-strain heterogeneity of GFMS12, as demonstrated in this study, is known to be driven by the error prone replication of the RNA-dependent RNA polymerase (RdRp) [12]. It was shown that different T68 variants dominated in different populations and that the original GFMS12 population structure was most likely altered by host changes, allowing different variants to emerge and dominate. This was especially evident with the transfer of the GFMS12 source from the original 'Nartia' to 'Duncan' grapefruit, which resulted in a phenotypic change from mild to severe SP. A host transfer to 'Mexican' lime and subsequent aphid transmissions, also effected the separation of various T68 variants from the original population.

The phenomenon that different hosts alter strain population structures has previously been demonstrated for CTV [2, 4, 10]. However, a diverse strain population was not detected in the original GFMS12 source plant, but rather a single-strain, T68. The existence of quasispecies, better explains the sub-isolation of genetic variants of the same strain from the GFMS12. This is the first report of the characterisation CTV variants of one strain, originating from the same parental population and which display altered SP severity.

Of significance is that minor nucleotide changes resulted in significant phenotypic changes. This is widely documented for viruses of annual crops [13, 15, 24], but such evidence is limited for CTV [11]. Clusters of SNPs observed in certain domains are important leads to investigate as possible SP determinants.

Glasshouse trials are inadequate to properly evaluate the effects of CTV on commercial cultivars as symptom expression is not as rapid as in more sensitive indicator hosts. The impact of infections is often only fully realised after a number of years in the field, as environmental conditions, influencing disease expression, cannot be replicated in glasshouse trials. Analysis of field trial trees, inoculated with various isolates of the T68 strain, confirmed that distinct T68 variants differ in SP severity in grapefruit. It is unfortunate that GFMS12-8 was not included in the field trial.

Results from this study support the notion that there was a segregation event in GFMS12 in 'Star Ruby' bud-wood source trees [23], which was probably effected by the host change from the 'Nartia' white grapefruit to the red cultivar, 'Star Ruby'. Over time, a severe variant, likely a minor component in the original source, became the dominant variant in the new host.

This study also underscores some principles important to consider when implementing cross-protection as a management strategy and include the requirement that isolates should be shown to be stable and should not differ in pathogenicity in other citrus types or cultivars [14].

Apart from clarifying the probable cause of the 'breakdown' of a cross-protection source, insight is gained into the diversity that can develop within a single strain in a population and that minor sequence changes can result in altered phenotypic expression.

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6 CONCLUSION

Citrus tristeza virus (CTV) is a unique plant virus that has the potential to induce three main phenotypes in citrus, referred to as syndromes, two of these are the disease phenotypes, quick decline and stem-pitting. Seedling yellows, the third phenotype, is useful as a diagnostic phenotype with little commercial relevance. The diverse symptom expression of CTV can be attributed to the diversity in both the virus and the citrus host. Three major ancestral species of citrus gave rise to the common citrus types known today [21]. In part, combinations of these varied genetic backgrounds determine the CTV induced phenotypes displayed. Additionally, CTV exhibits its own unique genome diversity that contributes to the diverse symptom expression of this virus. The most significant contribution to diversity in CTV is the divergence in the 5'-half of CTV genomes, which separates isolates into two distinct linages [12]. Further differentiation into seven [7], possibly eight [22] phylogenetic clades or strains, is evident. Within the demarcations of strains, additional genetic and phenotypic variation exist.

Stem-pitting in grapefruit was a production limiting factor in South Africa that lead to the application of mild-strain cross-protection to mitigate the impact of the disease [15], but this application was not always successful. Pre-immunised grapefruit trees in humid, hot regions succumbed to severe stem-pitting, suggesting environmental influences on symptom expression [14]. A further complication arose when severe stem-pitting was observed in some of the bud-wood source trees, containing the CTV GFMS12 cross-protection source. These incidences necessitated a change in the CTV source applied for cross-protection of grapefruit [20]. Improved understanding of the components involved in disease expression is required to apply effective cross-protection and also to address failures encountered with this management strategy. Three CTV genes were associated with stem-pitting [19], however the specific interactions, mechanisms or sequence determinants that trigger stem-pitting are still unknown. Due to the lack of defined pathogenicity determinants, and the need to understand which CTV components are involved in disease expression, this study aimed to investigate the impact of different strains on stem-pitting in grapefruit, both as single infections and in combination. Additionally, sequence variants of the T68 strain, showing different stem-pitting phenotypes, were identified and the impact on stem-pitting in both glasshouse and field trials studied.

Advancements in CTV research led to the characterisation of numerous CTV genomes, establishing the need for comprehensive diagnostic capabilities to distinguish strains. For this purpose, strain-specific RT-PCR detection assays were improved to facilitate detection of known strains. An assay to detect a novel isolate at the time, HA16-5 [16], was developed and assisted in the identification of similar genomes locally. This strain was then also identified as a component of the cross-protection source, LMS6, applied to sweet orange cultivars in South Africa [4]. Further, the available diagnostic assays are not able to differentiate strains of one major CTV linage, which includes the T36 and RB strains [18]. An additional assay was developed to specifically detect the T36 strain. Isolates of the RB strain diverge into two separate clusters, designated RB1 and 2, in addition to two recombinant genomes which also cluster in this group. In this study, two assays were developed which could differentiate variants from the two RB clusters [4]. Using these assays, variants from both RB1 and 2 were detected in current cross-protection sources used for grapefruit (GFMS35) and sweet orange cultivars (LMS6) [4]. The development of strain-specific diagnostic assays was a significant advancement that enabled the identification of strain components of the cross-protection sources and which provided a means to monitor transmission of the individual components.

These expanded set of assays were used in this study to identify single-strain isolates as well as confirm the strain status of inoculated trial plants. In total, eight single-strain isolates were identified and used in this study. These isolates were biologically characterised and full-genome sequences determined which make them useful research tools and reference isolates. One isolate of this study was the third reported genome that clustered separately as a clade and was therefore validation for the seventh strain, designated as the HA16-5 strain. These eight genomes contribute to the greater understanding of the diversity of CTV, phylogenetically and biologically.

Characterisation of CTV diversity adds to the broader understanding of the virus, but ultimately, such information should have relevance to the commercial impact on the crop. The influence of the host is a significant factor in disease expression and reliance on symptom expression in indicator hosts may not reflect expression in commercial cultivars. Different strains were evaluated singly and in various combinations in commercial grapefruit cultivars to evaluate the expression of deleterious symptoms of CTV populations over time. Evaluation over a four year period confirmed that the isolates used, induced mild-to moderate stem-pitting in the two grapefruit

cultivars and that symptom expression did fluctuate depending on seasonal influences which could in part be related to temperature, but was likely not the only influence. Nonetheless, stem-pitting expression did not significantly change over the period of the trial evaluation. Symptom expression of mild strains did not result in altered symptom expression when in combination with each other. Neither did the severity of stem-pitting increase with coinfections of moderate stem-pitting isolates. Importantly demonstrating that there was no additive effect on stem-pitting expression with multiple isolates. Synergism between these isolates was not detected since stem-pitting was not reduced for mild to moderate stem-pitting isolates, when in combination with any of the other mild strains. This does however not exclude the possibility of complementation between heterologous CTV strains, but interactions will be determined by the isolates, the operational interaction and the host [8, 11]. Synergistic interactions between heterologous strains was an avenue of investigation to detect possible mechanisms that might explain effective cross-protection, but was not demonstrated in this study. The 'Star Ruby' cultivar developed more stem-pitting than the less sensitive 'Marsh' cultivar, confirming the relative cultivar sensitivities.

The strain-specific RT-qPCR assays were developed to quantify strain components of constructed populations to investigate inter- and intra-strain dynamics. This quantitative analysis was done with the purpose to investigate possible strain interactions. Strains were however found to propagate within certain concentration ranges, independent of the population structure. Individual strain concentrations were not significantly affected by the presence of heterologous strains in any of the combinations. However, two variants of the RB strain appeared to be in tension and were not detected simultaneously, which suggested possible spatial separation in the plant. These two isolates were also poorly transmitted during the inoculation process when co-inoculated. This is suggestive of the super-infection exclusion principle [5], but if this was the reason for the poor co-transmission and lack of simultaneous detection of the two variants, it is not a mechanism that was able to achieve total exclusion of these homologous strains, a finding previously demonstrated [10]. Strain concentrations did differ between the two grapefruit hosts and strains were generally detected at higher average concentrations in 'Marsh' compared to 'Star Ruby'. This was an inverse correlation with the average stem-pitting severity found for these cultivars and suggests that elevated strain concentrations are not necessarily coupled to increased symptom severity. The findings of this study are limited to the CTV isolates evaluated in two grapefruit cultivars, but are

informative and add to an increasing understanding of CTV population structures and strain dynamics [2, 3, 9].

The sub-isolation and diagnostic differentiation of variants displaying both genetic and phenotypic differences led to the discovery of intra-strain heterogeneity in a single population and is indicative of the quasispecies concept, where viruses develop genetic heterogeneity over time, but still share significant genetic commonality [6]. This study uniquely demonstrates these principles and allowed for clarification and understanding of historical biological results found for the GFMS12 source. Results of this study suggest that certain variants most likely became dominant after the source was transferred to the sensitive 'Star Ruby' host and that different environmental factors also impacted this population 'shift' to a severe stem-pitting variant. This is the first report characterising CTV variants of a strain, naturally derived from the same parental population and showing altered pathogenicity. Importantly, these results will enable further investigation of specific genome regions as pathogenicity determinants of CTV stem-pitting in grapefruit.

Further credence of the functioning of the super-infection exclusion principle [5] is found in the field results obtained with the T68 variants of GFMS12. The dominant variants, as they were introduced, remained stable. Field cross-infection between variants that might have resulted in population shifts, were not detected, despite evidence of field transmission.

In general, the results have practical implications and underscore a number of prescribed principles for cross-protection application [1, 13]. The cross-protection source used, should be derived from the same citrus type, even cultivar, to which it will be applied and should preferentially be used in the same climatic region from which it was sourced. The source should show stability and preferably be an isolated variant [17].

This study contributed to improved diagnostic capabilities to detect the genetic diversity found in CTV. Further, a broader understanding of the biological significance of the genetic variation found in CTV, specifically in the grapefruit host, was developed, which will inform the application of biological interventions of disease control, such as cross-protection. Avenues for investigation are also opened to study possible pathogenicity determinants of CTV stem-pitting in grapefruit.

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APPENDIX A1

Publication

Cook G, van Vuuren SP, Breytenbach JHJ, Burger JT, Maree HJ (2016) Expanded Strain-Specific RT-PCR Assay for Differential Detection of Currently Known *Citrus Tristeza Virus* Strains: a Useful Screening Tool. Journal of Phytopathology 164:847-851

Journal of Phytopathology

SHORT COMMUNICATION

Expanded Strain-Specific RT-PCR Assay for Differential Detection of Currently Known *Citrus Tristeza Virus* Strains: a Useful Screening Tool

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Abstract

Genotypic characterization of *Citrus tristeza virus* (CTV) strains has progressed significantly, but their phenotypic expression is poorly established as CTV naturally occurs as mixed-strain populations. A screening system for the analysis of mixed-strain populations is required for population studies and the correlation with symptom expression. In this study, a published CTV strain-specific detection assay was expanded and improved to facilitate detection of currently known CTV strains. Supplementary RT-PCR assays were developed for two variant groups of the RB strain and the HA16-5 strain, and assays for the T36 strain and generic CTV detection were improved. The value of the strain-specific assays was shown by the ability to identify the strain components of two CTV cross-protecting sources, GFMS35 and LMS6, used in the South African budwood certification scheme and to demonstrate the segregation of strains in budwood source trees.

Introduction

Citrus tristeza virus (CTV), a member of the family Closteroviridae, has been responsible for significant losses in citrus production worldwide (Moreno et al. 2008). At least seven phylogenetic clades or strains of CTV have been identified (Harper 2013); however, the interactions of strains and their effect on host symptom expression are poorly understood as they mostly occur as mixed populations in addition to citrus being a genetically diverse crop. The term 'genotype' has been used as a phylogenetic concept describing genetically similar genomes that cluster together. Due to the very complex diversification of the CTV genomes, the genotypic and phenotypic associations are poorly understood. Harper (2013) proposed that within CTV classification, members within distinct phylogenetic lineages, which share common ancestries, be classified as strains. The term 'strains' therefore, used throughout this manuscript, refers to these distinct lineages.

Some citrus-producing countries have circumvented the negative effects of CTV by applying cross-protection, a management strategy using mild-strain sources of the virus to reduce the deleterious effects of secondary infections, introduced by aphid vectors. This approach has significantly extended the productive life of grapefruit (*C. paradisi* Macfad) varieties in South Africa from about 10 and 15 years for pigmented and white varieties, respectively (Marais 1994), to approximately 25 years (CGA 2014). Brazil (Salibe et al. 2002), Peru (Bederski et al. 2005) and Australia (Broadbent et al. 1991) also apply cross-protection for CTV and report diminished expression of disease symptoms and improved production. It is unknown which strain(s) are important for CTV cross-protection or whether mechanisms other than superinfection exclusion (Folimonova et al. 2010) contribute to cross-protection. CTV sources used for cross-protection are mostly uncharacterized with regard to their strain composition due to the complexity of mixed populations and genome diversity found

within CTV. To facilitate population studies, comprehensive strain-specific assays are required for screening. No assay will be optimal for characterization unless full-genome determination of mixed populations is possible and technologies such as next-generation sequencing (NGS) are more widely accessible and pipelines for strain differentiation are in place. Simpler tools are still currently required for screening populations. A number of approaches have been followed to characterize CTV populations, each with its own set of limitations. The approach of Hilf et al. (2005) uses multiple molecular markers (MMMs) at specific genome regions to accommodate diversity that may occur across the genome, but the MMMs are unable to identify specific strains solely on the amplification profiles and sequencing is required for strain allocation (Wu et al. 2013). CTV genomes are most divergent within the 5′ halves and allow for strain differentiation (Roy et al. 2010). No specific sector on the CTV genome is informative enough to differentiate all strains. For that reason, a strain-specific RT-PCR assay (Roy et al. 2010) was able to differentiate the currently known CTV strains by targeting various 5′ genome positions. With the subsequent discovery of additional CTV strains such as HA16-5 and the inability of this RT-PCR assay to differentiate between T36 and RB strains of CTV, updates to the assay were required. In this study, the strain-specific RT-PCR assay was improved to enable the differential detection of all currently known CTV strains.

This expanded assay was used to identify the CTV strains in the South African cross-protecting sources, GFMS35, used for cross-protection of all grapefruit varieties, and LMS6, used for cross-protection of limes (*C. aurantifolia* (Cristm.) Swingle) and sweet orange (*C. sinensis* (L.) Osbeck). Strain segregation in grapefruit budwood source trees was also detected. The expanded system is regarded as a useful screening tool for population studies and for diagnostics required in certification programmes as presented here. Limitations, such as the inability to detect recombinant genomes, are acknowledged. If additional genotypes are discovered, which are not detected by the current assay, the system will require further development.

Materials and Methods

The South African CTV cross-protection sources are maintained at two facilities in secure glasshouses, and the primary grapefruit budwood source trees are maintained in insect-proof tunnels. The budwood source trees were all pre-inoculated with the GFMS35 CTV source and comprise four trees of 'Star Ruby' (red variety), five of 'Marsh' (white variety) and one of 'Flame' (red variety). Individual trees were sampled at four cardinal points to avoid detection errors due to possible sectorial distribution within the plant.

RNA was isolated using an acid–phenol extraction buffer comprising 38% sodium acetate-saturated phenol (pH 5.0), 0.8 M guanidine isothiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate (pH 5.0) and 5% (v/v) glycerol. Bark shavings and/or leaf midribs (500 mg) were placed in maceration bags (Agdia Inc., Elkhart, IN, USA) and macerated in 5 ml of the extraction buffer using a power homogenizer. Samples were incubated for 5 min on ice, and 2 ml of each homogenate was transferred to a microcentrifuge tube and centrifuged at 12 000 ***g*** for 5 min at 4°C. The aqueous phase was transferred to a new tube and extracted twice with chloroform. From the final aqueous phase, 800 μl was precipitated at room temperature by the addition of 200 μl isopropanol and 200 μl 4 M LiCl for 10 min and centrifuged at 12 000 ***g*** for 15 min. The pellet was rinsed in 75% ethanol and resuspended in 100 μl nuclease-free water.

Strain-specific primers for the detection of strains T68, T3, VT and T30 (Roy et al. 2010) were used in this study and detailed in Table 1. Alternative generic CTV primers were developed that do not contain degenerate bases and target conserved regions in the 3′ non-coding region. The T36 primers were replaced with primers that do not cross-amplify the closely related RB variants and bind within the interdomain region of open reading frame (ORF)1a. Two other primer sets were added that differentially amplify variants within the RB clade. The RB group 1 primer sequences match genotypes NZRB-TH28 [FJ525433], NZRB-M12 [FJ525431], NZRB-G90 [FJ525432] and HA18-9 [GQ454869], whereas the RB group 2 primer sequences match genotypes NZRB-TH30 [FJ525434], NZRB-M17 [FJ525435] and Taiwan-Pum/SP/T1 [JX2 66712]. Primers to detect strain HA16-5 [GQ454870] and an additional sense primer that will detect both HA16-5 and Taiwan-Pum/M/T5 [JX266713] were developed. Primers for RB group 1, RB group 2 and HA16-5 all amplify portions of the LProII domain of ORF1a. Details of the replacement and additional primers used are provided in Table 1.

Two-step RT-PCRs were performed. Synthesis of cDNA was performed using RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific Inc., Waltham, MA, USA) with modifications to the manufacturer's instructions. RNA template (0.5–1 μg total RNA) and antisense primer were incubated together at 65°C for 3 min and chilled on ice prior to

the addition of the other reaction components. Forty units of RT enzyme and 10 units of RiboLock RNase Inhibitor (Thermo Fisher Scientific) were used per reaction. Reverse transcription was performed at 50°C for 60 min followed by inactivation at 85°C for 5 min. PCRs were performed in a total reaction volume of 20 μl using the GoTaq® Hot Start Green Master Mix (Promega Corp., Madison, WI, USA) and 2 μl cDNA. Cycling parameters were 95°C for 3 min followed by 35 cycles of 95°C for 20 s, 30 s at specific annealing temperature (indicated in Table 1), 72°C for 20 s and a final extension of 72°C for 5 min. Positive controls for each genotype, apart from T36, were from various plant sources which tested positive and for which the amplifications were confirmed by sequencing. The T36 clone, SP6-CTV 947-2 (Tatineni et al. 2003), was used as a positive control in the T36 assay. PCR products were gel purified using the Zymoclean™ Gel DNA recovery kit (Zymo Research, Irvine, CA, USA). Direct sequencing was performed with each strain-specific primer pair in both orientations. Overlapping sequences were aligned and low-quality bases and primer sequences removed using BioEdit (Hall 1999). Closest sequence identity was determined using blast (Altschul et al. 1990).

Results and Discussion

An improved CTV strain-specific RT-PCR assay is described that was used to determine the strain

Table 1 Species and strain-specific primer sequences used in a two-step RT-PCR to amplify *Citrus tristeza virus* (CTV) RNA

CTV strain	Polarity	Primer sequences from 5′ to 3′	Annealing temp. (°C)	Accession no. for nucleotide position	Nucleotide positions of primer	Product size (bp)
Primers of this study						
CTV generic	Sense	TCT GAT TGA AGT GGA CGG AAT AAG	62	NC_001661	19 019	157
	Antisense	GCT TAG ACC AAC GAG AGG ATA			19 155	
RB: group1[a]	Sense	AGT GGT GGA GAT TAC GTT G	60	FJ525433	1974	628
	Antisense	TAC ACG CGA CAA ATC GAG			2584	
RB: group 2[b]	Sense	CGG AAG GGA CTA CGT GGT	60	FJ525434	1976	658
	Antisense	CGT TTG CAC GGG TTC AAT G			2615	
T36	Sense	GGT GTA AGG AAG CGT GTG TCG CAT TTA	66	NC_001661	5641	537
	Antisense	ACC TGC ACC GTC TAA CAA CAT CAT CG			6152	
HA16-5	Sense 1	TAG GAA GGG TCA CTG CCC TGA CA		GQ454870	2128	610
	Antisense	GTA AGT ATC TAA AAC CAG GAG	56		2717	
	Sense 2	CGA CAA GTG CAT TAC GTC TCA G			2563	176
Primers as per (Roy et al. 2010)						
B165 (T68)	Sense	GTT AAG AAG GAT CAC CAT CTT GAC GTT GA	59			510
	Antisense	AAA ATG CAC TGT AAC AAG ACC CGA CTC				
T3	Sense	GTT ATC ACG CCT AAA GTT TGG TAC CAC T	60			409
	Antisense	CAT GAC ATC GAA GAT AGC CGA AGC				
VT	Sense	TTT GAA AAT GGT GAT GAT TTC GCC GTC A	60			302
	Antisense	GAC ACC GGA ACT GCY TGA ACA GAA T				
T30	Sense	TGT TGC GAA ACT AGT TGA CCC TAC TG	60			206
	Antisense	TAG TGG GCA GAG TGC CAA AAG AGA T				

[a]RB group 1 includes genotypes NZRB-TH28, NZRB-M12, NZRB-G90 and HA18-9.
[b]RB group 2 includes genotypes NZRB-TH30, NZRB-M17 and Taiwan-Pum/SP/T1.

Table 2 The *Citrus tristeza virus* (CTV) strains detected in two South African cross-protecting sources using various strain-specific primers and the NCBI accession numbers for the sequences of the positive amplification products

	CTV strain							
CTV source	T68	RB1	RB2	HA16-5	VT	T30	T3	T36
GFMS35	[KP721477][a]	[KP721478]	[KP721479]	–[b]	–	–	–	–
LMS6	[KP721480]	[KP721481]	[KP721482]	[KP721483]	–	–	–	–

[a]NCBI accession number of the nucleotide sequence of positive amplifications.
[b]–, indicates no amplification.

Table 3 The *Citrus tristeza virus* (CTV) strains detected in grapefruit budwood source trees pre-inoculated with GFMS35

	CTV strain							
Grapefruit variety	T68	RB1	RB2	HA16-5	VT	T30	T3	T36
Star Ruby	–	4/4[a]	4/4	–[b]	–	–	–	–
Marsh	–	5/5	5/5	–	–	–	–	–
Flame	–	1/1	1/1	–	–	–	–	–

[a]Number of trees positive/number of trees tested.
[b]–, indicates no amplification.

composition profiles of two cross-protection sources and the grapefruit budwood source trees. The oldest maintenance plants of the GFMS35 and LMS6 CTV cross-protection sources, kept at two different facilities, tested positive for the same CTV strains. Both contained strain T68 and two variants of the RB strain group, but in addition, LMS6 also contained strain HA16-5. Strain identifications were validated by sequencing the amplicons. Sequences were deposited in GenBank under the accession numbers KP721477–KP721483. Table 2 indicates the strains detected in the two sources and their respective accession numbers. The additional RB strain-specific tests were able to differentially amplify two RB strain variants from the mixed-CTV populations of GFMS35 and LMS6. The RB group 1 amplicon sequences of both the CTV sources showed closest identity to the RB isolate NZRB-TH28 [FJ525433] in a BLAST search. The RB group 1 amplicon sequence obtained from the GFMS35 source was 99% homologous to NZRB-TH28, while LMS6 had 98% homology. The RB group 2 amplicon sequence obtained from the LMS6 CTV source was 100% homologous to RB isolates Crete 1825 [KF908013] and Taiwan-Pum/SP/T1 [JX266712], while GFMS35 was 99% homologous to the same isolates. The sequences for the T68 amplicons from both GFMS35 and LMS6 were 99% homologous to CT-ZA3 [KC333868] and CT-ZA2 [KC3 33869]. Sequences of the HA16-5 amplification from LMS6 showed closest homology (99%) to HA16-5 [GQ454870].

The T36 primer set described in this report did not cross-amplify any of the RB strains in the sources tested, indicating greater specificity than those previously published (Roy et al. 2010).

The primary grapefruit budwood source trees, pre-inoculated with GFMS35, all tested positive for the two RB strain variants and negative for strain T68 which is a component of the original source. These findings are summarized in Table 3. The strains detected in these budwood source trees differed from the original source plants and show segregation of a strain in the three grapefruit varieties tested. These results demonstrate that the maintenance of mixed-strain populations is challenging as various host transfers, host selection of strains and varied strain distribution in the host can all facilitate strain segregation. This is further complicated in the propagation of budwood for industry supply where the continued effectiveness of the cross-protection programme is required. It has not been determined which strain(s) are required for CTV cross-protection or whether mechanisms other than superinfection exclusion (Folimonova et al. 2010) contribute to cross-protection. The impact that the differential segregation of the T68 strain in the budwood source trees may have on the cross-protection effectiveness requires further investigation. No severe stem pitting has been reported from orchards planted with trees derived from the tested budwood source trees without the T68 strain component, present in the original source plants.

The ability to differentiate between the subclades in the RB strain (Harper et al. 2010) is useful to further investigate the biological significance of these variants, especially in cross-protection. The demonstration that variants of a strain are able to co-infect a single host challenges the superinfection exclusion principle (Folimonova et al. 2010). Full-genome studies are required to validate this observation.

The enhanced CTV strain-specific assay presented in this study was shown to be useful for screening purposes to investigate strain profiles of mixed infections and will also enable the monitoring of CTV strain transmission within the budwood multiplication scheme.

Acknowledgements

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APPENDIX A2

Publication

Cook G, van Vuuren SP, Breytenbach JHJ, Steyn C, Burger JT, Maree HJ (2016) Characterization of Citrus tristeza virus Single-Variant Sources in Grapefruit in Greenhouse and Field Trials. Plant Disease 100:2251-2256

e-Xtra*

Characterization of *Citrus tristeza virus* Single-Variant Sources in Grapefruit in Greenhouse and Field Trials

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Abstract

Cook, G., van Vuuren, S. P., Breytenbach, J. H. J., Steyn, C., Burger, J. T., and Maree, H. J. 2016. Characterization of *Citrus tristeza virus* single-variant sources in grapefruit in greenhouse and field trials. Plant Dis. 100:2251-2256.

Citrus tristeza virus (CTV) is endemic to southern Africa and the stem pitting syndrome that it causes was a limiting factor in grapefruit production prior to the introduction of cross-protection in the Citrus Improvement Scheme. This disease mitigation strategy, using various field-derived CTV sources, has significantly extended the productive lifespan of grapefruit orchards in South Africa. CTV commonly occurs as a population of various strains, masking the phenotypic effect of individual strains. Likewise, current South African CTV cross-protection sources are strain mixtures, obscuring an understanding of which strains are influencing cross-protection. The severity of various CTV strains has mostly been assessed on sensitive indicator hosts, but their effect on commercial varieties has seldom been investigated. Single-variant CTV isolates were used to investigate the phenotypic expression of CTV strains in commercial grapefruit varieties as well as CTV indicator hosts. They were biologically characterized for their ability to cause stem pitting and their rate of translocation and titer in the different hosts, monitored by enzyme-linked immunosorbent assay. Complete genome sequences for three CTV strain variants were generated. Isolates of CTV strains VT, T68, RB, and HA16-5 did not induce severe stem pitting in four grapefruit hosts in a glasshouse trial. Viral titers of the strains differed in the grapefruit hosts, but the RB isolate reached a higher titer in the grapefruit hosts compared with the VT, T68, and HA16-5 isolates. Additionally, horticultural assessment of two grapefruit varieties inoculated with the RB isolate in two field trials demonstrated that mild stem pitting did not negatively influence the horticultural performance of the grapefruit trees over an eight-year assessment period. 'Star Ruby' trees containing the CTV source GFMS35 showed less stem pitting than trees inoculated with the RB isolate, but had smaller canopy volumes and lower yields than trees containing the RB isolate. This suggests that the influence of CTV sources on tree performance is not limited to the effect of stem pitting.

Citrus tristeza virus (CTV) is a member of the family *Closteroviridae*, with an approximately 19.3-Kb genome (Karasev et al. 1995) that displays significant sequence variation among currently known strains (Harper 2013). The genotypic characterization of CTV variants has progressed significantly, but their phenotypic expression in various citrus varieties is less defined. Fundamental work in CTV is mainly done on sensitive biological indicator hosts in which symptom expression is faster and more definitive. However, these results cannot be extrapolated to commercial citrus varieties with varying CTV susceptibility, as symptom expression can be host-dependent.

CTV can cause a stem pitting syndrome in the trunks and limbs of grapefruit (*Citrus paradisi*) trees that are established on rootstocks other than sour orange (*C. aurantium*). The resulting disruption of vascular flow leads to a gradual tree decline and is often associated with lower yield and a decrease in fruit size, impacting exportability of the fruit. This is contrasted to the quick decline syndrome or 'Tristeza' associated with trees grafted on sour orange rootstocks. This stem pitting syndrome necessitates earlier tree rotation resulting in financial losses associated with tree replacement and production lag. Different phenotypes of stem pitting are found in citrus, including large grooves visible on tree trunks and limbs, 'honey-comb' stem pitting, referring to a high density of small pits, which is more damaging to the tree, and a spectrum between these two phenotypes (Moreno and Garnsey 2010). The requirements for stem pitting development are not fully understood, but expression of the CTV p33, p13, and possibly the p18 genes appear to be involved (Tatineni and Dawson 2012). Expression of the same genes by CTV strains in varying combinations is required for infection of certain citrus species (Tatineni et al. 2011). These findings imply very specific interactions of strains with various citrus hosts that influence host susceptibility.

Apart from South Africa, 'mild-strain' cross-protection is successfully applied to reduce the effect of CTV stem pitting on commercial citrus by a number of citrus-producing countries, including Australia (Broadbent et al. 1991), Brazil (Salibe et al. 2002), and Peru (Bederski et al. 2005). CTV control by eradication of infected trees is not feasible in southern Africa due to the endemic presence of the brown citrus aphid *Toxoptera citricida*, the most efficient aphid vector of CTV. The South African Citrus Improvement Scheme implemented cross-protection to minimize losses incurred due to stem pitting, primarily in grapefruit. This management strategy has significantly extended the productive lifespan of grapefruit orchards in South Africa by minimizing losses incurred due to stem pitting (Marais 1994).

A strain-specific exclusion mechanism, superinfection exclusion (SIE), has been demonstrated and proposed as a possible mechanism for cross-protection (Folimonova et al. 2010). It was established that, once a plant is infected with a certain strain, a challenge virus of the same strain is not able to infect the plant. This exclusion is not applicable to dissimilar strains and relies on a homologous p33 viral protein of the same strain (Folimonova 2012). Additionally, it was demonstrated that two leader proteases, L1 and L2, are also involved in SIE (Atallah et al. 2016). This mechanism has not been completely resolved on a biological level, as the existence of a 'mild' variant of a pathogenic strain is required to prevent infection of 'severe' variants. If this is the mechanism at work in cross-protection, ultimate

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Nucleotide sequence data is available in the GenBank database under accession numbers KU883265, KU883266 and KU883267.

*The *e*-Xtra logo stands for "electronic extra" and indicates that a supplementary figure and a supplementary table are published online.

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cross-protection would require a 'mild' variant of each pathogenic strain for effective cross-protection. The requirement for the existence of 'severe' and 'mild' variants of strains has not been demonstrated in CTV. It is, therefore, important to resolve which strains and strain variants are pathogenic in a specific citrus host and which mitigate disease expression in cross-protection. Current cross-protecting sources used in the South African industry were empirically selected and comprise strain mixtures (Cook et al. 2016), which makes it difficult to ascribe cross-protection to specific strains.

Given the above, empirical studies are required to investigate the influence of CTV variants in specific citrus varieties. CTV components of various sources were previously subisolated by single aphid transmission (SAT), and one single-strain isolate was identified after conclusion of a shoot-tip grafting process. These CTV isolates were characterized as single-variant sources in this study. Complete genome sequencing was complemented by biological assessment on a grapefruit host range to evaluate the pathogenicity, translocation, and titer of each variant in four grapefruit varieties. One of these subisolates was included in grapefruit cross-protection field trials and horticultural data regarding canopy volume, yield, and fruit size of trees inoculated with this source were compared with other CTV sources used in these trials. The CTV sources used could, unfortunately, not be assessed for their effectiveness to cross-protect against pathogenic strains in these trials due to a lack of natural challenge at these sites.

The characterization of single-variant CTV sources is foundational to further complementation and cross-protection studies, and the genome characterizations also add to the known diversity of CTV variants. This work provides biological data of characterized strain-variants on commercial grapefruit varieties and indicator hosts.

Materials and Methods

Biological and molecular strain characterization. Four CTV sources were characterized: (i) 'Maxi', derived from Valencia orange (*Citrus sinensis)*, subisolated in a shoot-tip grafting process, (ii) GFMS12-8, a subisolate obtained by SAT from the cross-protection source GFMS12 (originally derived from 'Nartia' grapefruit) (van Vuuren et al. 2000), (iii) LMS6-6, subisolated by SAT from the cross-protection source LMS6 (originally obtained from 'Mexican' lime [*C. aurantifolia*]) (van Vuuren et al. 2000), and (iv) B390-5, a subisolate obtained by SAT from a 'Mouton' Valencia orange source (L. J. Marais, Citrus Research International research records). The presence of other viruses, citrus viroids, and '*Candidatus* Liberibacter africanus' were excluded by biological indexing, reverse transcription-polymerase chain reaction (RT-PCR), and PCR (Supplementary Table S1).

Each source was bark-inoculated onto a citrus host range including 'Madam Vinous' sweet orange, sour orange, 'Mexican' lime and 'Duncan' grapefruit. The host range was used to determine single variant status by eliminating the possible suppression of strains in certain hosts. Each host was tested with eight strain-specific RT-PCRs and PCR amplicons sequenced as previously described (Cook et al. 2016). Single-variant status was further verified by using degenerate primers to amplify a midgenome region containing sufficient sequence variability to allow for differentiation of strains (Roy and Brlansky 2010). Amplification and direct Sanger sequencing of a 1,535-bp product was done for each isolate using primers CTVmid-F, 5′GAACCGGCTCGYGTTCGGCGT3′, and CTVmid-R, 5′GCAAACATCYYGACTCAACTACC3′, to allow detection of possible mixtures. Reactions were performed as above and primer annealing was at 60°C.

Direct Sanger sequencing of overlapping amplicons of approximately 1,500 bp was used to generate complete genome sequences of the single-strain variants, except for GFMS12-8, which was previously determined (KC333868) (Zablocki and Pietersen 2014). Overlapping primer sets were designed for the VT, HA16-5, and RB strains based on the strains identified with the strain-specific RT-PCRs for 'Maxi', LMS6-6, and B390-5, respectively. RNA was extracted from the 'Mexican' lime hosts for sequencing. RT was done as above and PCR amplification was done using KAPA HiFi HotStart ReadyMix (KAPA Biosystems), as per supplier's protocol. PCR products were gel-purified using the ZYMOCLEAN Gel DNA recovery kit (Zymo Research Corporation) and were sequenced in both directions. To generate the complete genomes, low-quality bases were removed and the overlapping sequences were aligned using BioEdit (Hall 1999). The most distal 5′ and 3′ primer sequences were included in the sequences, as these regions could not be verified by overlapping sequences. Closest sequence identity of genomes were determined using BLAST (Altschul et al. 1990).

A neighbor network of the complete genomes of the four CTV isolates and 47 CTV complete genome sequences available on GenBank was constructed. Alignments were done using CLC Main workbench 7 (Qiagen). The network was constructed with SplitsTree v4 (Huson and Bryant 2006), which uses the "Unrooted Equal angle" algorithm (Dress and Huson 2004) with equal-daylight and box-opening optimization (Gambette and Huson 2008).

Host-range symptom assessment. The presence of stem pitting was assessed in two red, 'Star Ruby' and 'Nel Ruby', and two white, 'Marsh' and 'Duncan', grapefruit varieties as well as 'Mexican' lime, the biological indicator host. Virus-free rough lemon (*C. jambhiri*) seedlings were planted singly in 3-liter planting bags and were maintained in an aphid-free polycarbonate tunnel equipped with wet-wall cooling. Virus-free scions of the grapefruit varieties were bud-grafted to rootstocks according to normal nursery practices and 'Mexican' lime plants were grown from seed. The four single variants were inoculated separately to five plants of each variety and a minimum of four plants were left as uninoculated controls. Inoculation was done by patch-grafting two bark chips of the source plant to the scion. All plants were inoculated at the same height and, after inoculation, the scions were cut back approximately 10 cm above the inoculation points and one shoot of the new growth was allowed to grow from the top bud. Plants were cut back at four intervals and were evaluated for stem pitting and one shoot of new growth was allowed to grow out each time. The first cut back was after 6 months followed by 10-, 11-, and 8-month intervals.

Enzyme-linked immunosorbent assay (ELISA). Translocation and titer differences of the CTV single variants were monitored in the grapefruit and 'Mexican' lime hosts by semiquantitative ELISA tests. Leaves of each plant were sampled at three different postinoculation intervals: (i) 7 weeks postinoculation (wpi), 15 cm above the inoculation point; (ii) 13 wpi, 30 cm above inoculation point; and (iii) 24 wpi, at the top of the plant. The SRA 78900 CTV ELISA reagent set (Agdia, Inc.) was used as per supplier's protocol. A total of 400 mg of leaf material including midribs were used per sample and were macerated in 4-ml of general extraction buffer in maceration bags, using a power homogenizer. Results were recorded by measuring absorbance values at 405 nm after 30 min incubation at room temperature.

Field trial assessment of B390-5 on grapefruit. Isolate B390-5 was assessed for field performance in two grapefruit field trials, evaluating potential CTV cross-protection sources. Trial trees were prepared by bud-grafting virus-free 'Star Ruby' and 'Marsh' to 'Troyer' citrange rootstocks. Scions were inoculated by patch-grafting with the various CTV sources including B390-5, GFMS12, and GFMS35, the standard cross-protection source currently used for grapefruit in South Africa. GFMS12 was previously used as the grapefruit cross-protection source (van Vuuren and Manicom 2005). Control trees were not inoculated. Inoculations were confirmed by ELISA 3 months post-inoculation. The trees were planted in 2007 in two different sites, according to a randomized block design with five replicates for each treatment. The 'Star Ruby' trial was planted in the Letsitele area in Limpopo province (South Africa) and the 'Marsh' trial in the Malelane area in Mpumalanga province (South Africa). These regions differ in climate. Letsitele is a hot and dry region, whereas the Malelane region is hot and humid. The trees were evaluated annually for the development of stem pitting, and tree canopy volumes were determined using the formula $V = S^2(\pi h - 1.046S)$, where S is canopy radius and h is the height of the fruit-bearing canopy (Burger et al. 1970). Fruit yield (kilograms per tree) was measured annually and a 3-year

cumulative yield per tree for the sixth to eighth year obtained. Fruit size distribution was determined at harvest according to export size categories, and fruit of 83 mm in diameter and smaller was regarded as small fruit. Stem pitting was evaluated externally, using a severity scale of 0 to 3, where 0 represents a smooth trunk with no visible pits, 1 represents one to three grooves on the stem, 2 indicates multiple grooves, and 3 is severe stem pitting in which the tree trunk has a knotted appearance (Supplementary Fig. S1). This knotted trunk is associated with honey-comb stem pitting that can be seen by the removal of the bark, but bark removal was not done at each evaluation point. Stem pitting ratings and tree canopy volume measurements for the eighth year in the field are presented. Calculations for analysis of variance and least significant difference (Fisher's least square difference) were performed using XLSTAT 2015.5.01.23039 (Addinsoft).

Table 1. Stem pitting presence on four grapefruit varieties and the 'Mexican' lime indicator host inoculated with the *Citrus tristeza virus* (CTV) single-variant sources[z]

CTV single-variant	'Duncan'	'Marsh'	'Nel Ruby'	'Star Ruby'	'Mexican' lime
B390-5	+	+	+	+	+
'Maxi'	+	+	+	+	+
LMS6-6	–	–	–	–	+
GFMS12-8	+	+	+	+	+
Control	–	–	–	–	–

[z] + means stem pitting observed and – means no STEM pitting observed.

Results

Biological and molecular strain characterization. The four sources, B390-5, GFMS12-8, LMS6-6, and 'Maxi', were shown to be CTV single variants by using strain-specific RT-PCRs. Each source tested positive for one strain only in 'Duncan' grapefruit, sour orange, 'Mexican' lime, and 'Madam Vinous' sweet orange. Isolate B390-5 tested positive for the RB strain and was amplified with the group 2 differential RB primers (Cook et al. 2016). Isolate GFMS12-8 tested positive for strain T68, LMS6-6 for strain HA16-5, and 'Maxi' for strain VT. No other pathogens were detected in these sources, and direct sequencing of amplicons obtained using degenerate primers yielded single-variant sequences only. No stem pitting or stunting was induced by any of these isolates on either 'Madam vinous', the sweet orange host, or on sour orange. No 'seedling yellows' symptom was observed on the indicators, 'Duncan' grapefruit or sour orange, with these sources. All the isolates induced stem pitting on 'Mexican' lime and only LMS6-6 did not induce stem pitting on 'Duncan grapefruit'. These stem pitting results were similar to those found in the host range assessment (Table 1).

Complete genome sequences of three single-variant isolates, B390-5, 'Maxi', and LMS6-6 were generated and deposited in GenBank under the accession numbers KU883265, KU883266, and KU883267, respectively. A neighbor network reconstruction of the full-length genomes and other fully sequenced CTV strains (Fig. 1) displays the positioning of each single-variant in a different strain. The network shows clear separation of strains T36 and RB from the rest of the network, with the other groups being more loosely associated and interrelated to each other.

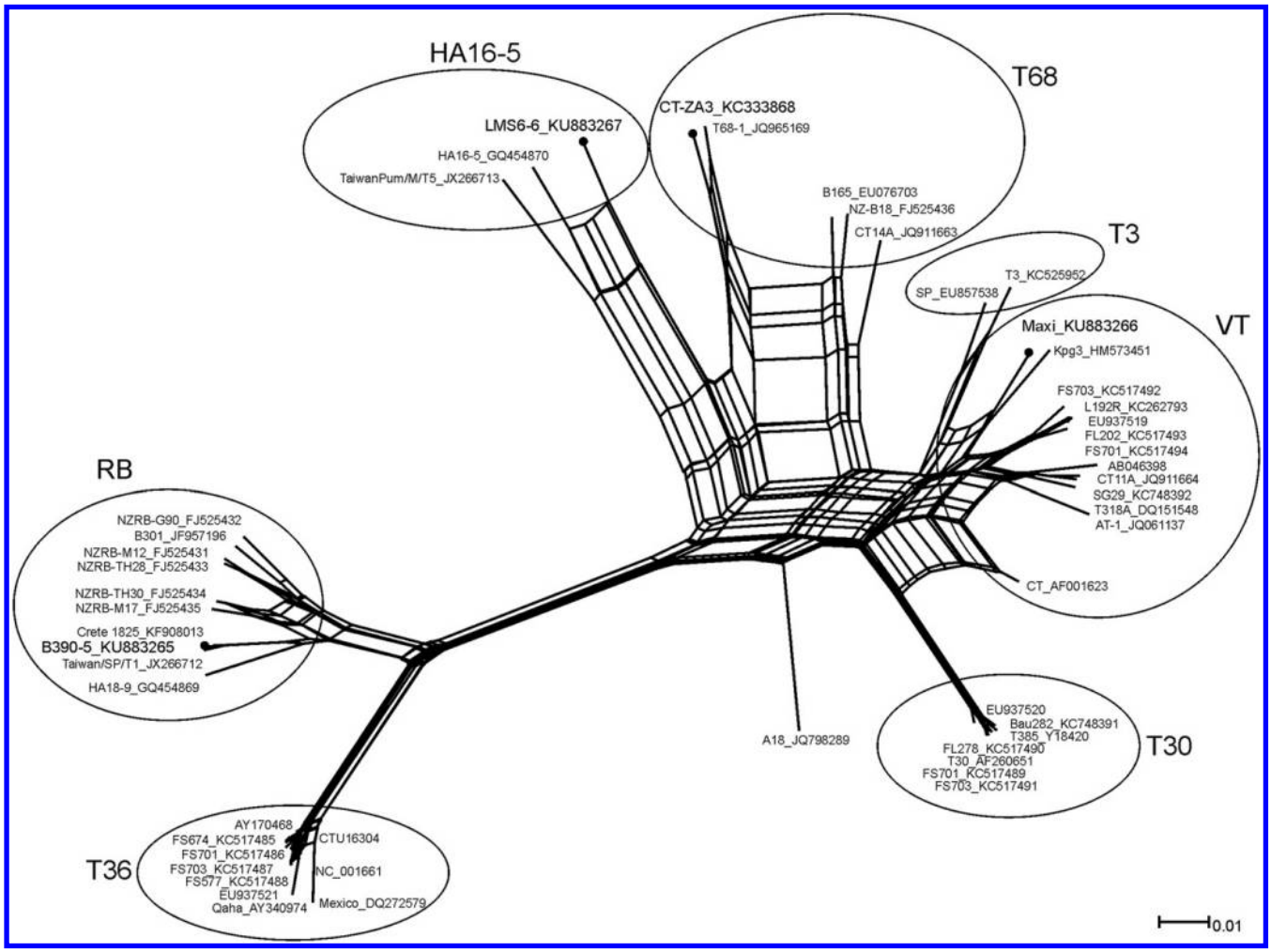

Fig. 1. Neighbor network reconstruction of the complete genomes of *Citrus tristeza virus*, including B390-5, GFMS12-8 (CT-ZA3), 'Maxi', and LMS6-6.

Isolate B390-5 was shown to be a variant in the RB strain and shares a 99% homology with Taiwan-Pum/SP/T1 (JX266712.1) and Crete 1825 (KF908013.1). The neighbor network construction of the full-length genomes illustrates these three isolates as a close grouping apart from the NZRB isolates and the Hawaiian isolate HA18-9 (GQ454869), which has a 3′-terminal half that has closer homology to HA16-5 than to the NZRB isolates (Melzer et al. 2010). Isolate 'Maxi' is a VT variant with closest homology (96%) to Kpg3 (HM573451.1) and LMS6-6 was most similar to strain HA16-5 (GQ454870.1) (96%). The genome of LMS6-6 clusters in a group with HA16-5 and TaiwanPum/M/T5 (JX266713) in the neighbor network construction, apart from the other strains. The GFMS12-8 genome was previously determined to be a T68 variant (Zablocki and Pietersen 2014).

Host-range symptom assessment. Stem pitting was observed in the four grapefruit varieties as well as in 'Mexican' lime inoculated with the single-variant CTV isolates as presented in Table 1. Only isolate LMS6-6 did not induce stem pitting in the grapefruit hosts. Stem pitting expression in grapefruit varieties was less pronounced than in 'Mexican' lime, the biological indicator host, and was unevenly distributed over the length of the shoots. The three isolates, B390-5, 'Maxi', and GFMS12-8, caused frequent stem pits at various assessment periods, but the frequency was not consistent and a rating scale could not produce a reliable indication of stem pitting severity due to this variation. No severe honey-comb stem pitting was obtained with any of these isolates in any of the host plants.

ELISA. Mean absorbance values of three semiquantitative CTV ELISA tests of four grapefruit varieties with the single-variant sources done at specific postinoculation intervals are presented in Figure 2. Results obtained for the four grapefruit varieties inoculated with isolate B390-5 showed that this CTV variant reached high titers in all four cultivars within the first two testing periods at 7 and 13 wpi as well as at 24 wpi, demonstrating efficient translocation and propagation within these grapefruit varieties. Subisolate GFMS12-8 was detected in all grapefruit varieties at each testing period, but titers were lower than B390-5 in all cases. Viral titer of the 'Maxi' isolate, obtained at both 7 and 13 weeks, were comparatively low in all grapefruit varieties, but titers were higher at 24 weeks, when the isolate was detected at high titers in both 'Duncan' and 'Star Ruby' but not in 'Marsh' and 'Nel Ruby'. Isolate LMS6-6 reached high titers at the 24-week sampling point, but detection varied at 7 and 13 weeks when detection levels were higher in the two white grapefruit varieties 'Duncan' and 'Marsh', compared with the red varieties 'Nel Ruby 'and 'Star Ruby'. Viral detection levels in 'Mexican' lime did not follow the same patterns as in the grapefruit varieties (Fig. 2), with noticeable differences obtained with isolates LMS6-6 and 'Maxi'.

Field trial assessment of B390-5 on grapefruit. As presented in Tables 2 and 3, 'Star Ruby' and 'Marsh' trees infected with isolate B390-5 performed well compared with the other treatments. Stem pitting observed with isolate B390-5 on 'Star Ruby' was within an acceptable range in which tree performance was not impeded. The 'Star Ruby' trees were significantly larger than those containing

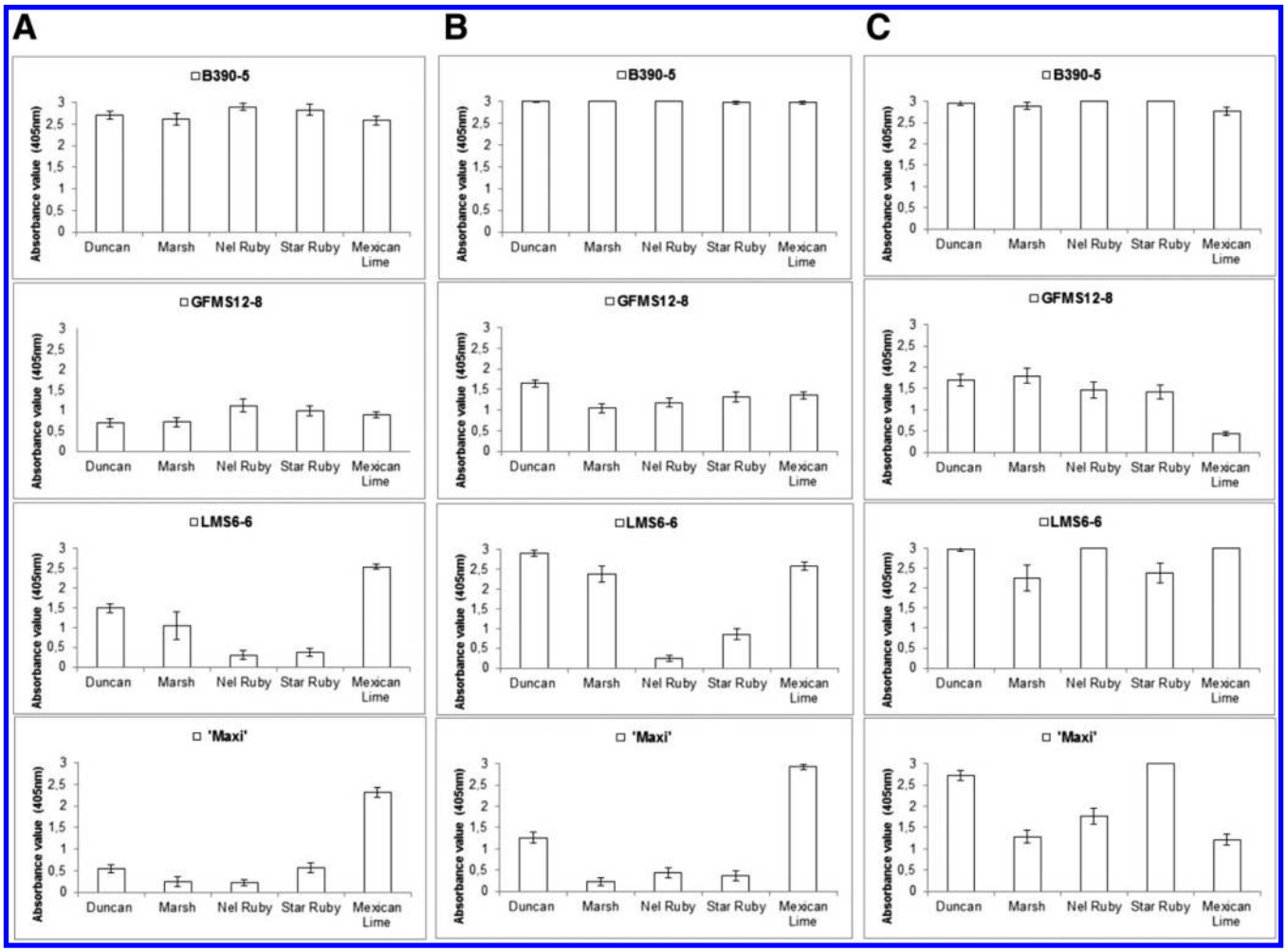

Fig. 2. Mean ($n = 5$) absorbance values at 405 nm obtained after 30 min for *Citrus tristeza virus* (CTV) enzyme-linked immunosorbent assay tests of grapefruit varieties 'Duncan', 'Marsh', 'Nel Ruby', and 'Star Ruby' inoculated with single-variant isolates B390-5, GFMS12-8, LMS6-6, and 'Maxi', compared with the 'Mexican' lime (CTV indicator host). Leaves were sampled **A,** 7 weeks postinoculation (wpi), 15 cm above inoculation point, **B,** 13 wpi, 30 cm above inoculation point, and **C,** 24 wpi at the top of the plant. Standard error bars are shown.

GFMS35 or GFMS12 and the 3-year average cumulative yield was significantly higher than the other treatments. Similarly, mild stem pitting was observed on the 'Marsh' trees, which did not impede tree growth or influence yield. In contrast, however, 'Star Ruby' and 'Marsh' trees inoculated with the GFMS12 source developed severe stem pitting. The stem pitting in 'Star Ruby' inhibited tree growth and decreased fruit size significantly. GFMS12 also induced severe stem pitting on 'Marsh', and this did correlate to a decrease in canopy volume but not as significantly as seen on the more sensitive 'Star Ruby'.

Discussion

The ability of CTV strains or variants to induce severe stem pitting that can diminish yield and fruit size, and conversely, the ability to reduce disease expression with cross-protection strategies is important for the grapefruit industry. Parameters that define a good cross-protecting source, apart from disease mitigation, include the ability of the virus to rapidly translocate to all parts of the plant and induce no or mild symptoms in the target cultivar but, also, in other citrus types (Lee et al. 1987). The ability of the virus to rapidly translocate in the host is important to ensure that the virus is proliferated in the bud-wood material supplied to industry. Isolate B390-5 was the only single-strain variant of the four tested that translocated rapidly and that was consistently detected at high titers in four grapefruit varieties, which makes this isolate a good candidate to evaluate further as a cross-protection source for grapefruit. Low titer and variable translocation of isolates GFMS12-8, LMS6-6, and 'Maxi' in the grapefruit hosts diminish their potential value as candidates for cross-protection sources in grapefruit.

Single-variant isolate GFMS12-8, sequenced as CT-ZA3, a T68 variant (Zablocki and Pietersen 2014), induced stem pitting in both grapefruit and 'Mexican' lime. This isolate was subisolated from the GFMS12 source, which was previously used as a cross-protection source for grapefruit in South Africa until probable segregation of strains gave rise to severe stem pitting (van Vuuren and Manicom 2005). It is still unclear which strains or variants were responsible for the severe stem pitting and how the original GFMS12 virus population structure changed over time. It was, therefore, relevant to determine the pathogenicity of components of this source, which includes GFMS12-8. Although frequent stem pitting was obtained with this isolate in the glasshouse, the frequency was not consistent in the various evaluations. No severe honey-comb stem pitting was observed that would associate this variant with the severe symptoms that were later obtained with GFMS12.

The complete genome of the 'Maxi' isolate confirmed it to be a variant of the VT strain, with closest homology (96%) to the Indian isolate Kpg3, a mandarin (*C. reticulata*) decline isolate, which was characterized on a host range excluding grapefruit. Stunting and chlorosis was observed in sweet orange, but no stem pitting was seen in any of the hosts, including two lime varieties (Biswas 2010). The 'Maxi' isolate did, however, cause stem pitting in 'Mexican' lime, indicating probable phenotypic differences between these isolates. As with GFMS12-8, no severe honey-comb stem pitting was obtained with the 'Maxi' isolate, which would have classed this variant as a severe pathogen of grapefruit. LMS6-6 induced no stem pitting in any of the grapefruit varieties and does not appear to be detrimental to grapefruit, based on the glasshouse trial. The genome sequence of LMS6-6 also validates the status of the HA16-5 clade as a separate CTV strain, as three full-length genomes are now characterized and cluster apart from the other strains.

Isolate B390-5 showed closest sequence homology (99%) to two isolates, Taiwan-Pum/SP/T1 and Crete 1825. Taiwan-Pum/SP was subisolated from a field isolate, CTV-D, by aphid transmission (Tsai et al. 1993). The stem pitting induced by CTV-D was described as numerous deep linear pits showing gumming on grapefruit and pummelo, which was associated with shortened internodes and stunting. Coalescing pits formed grooves in the stem surface that displayed a fluted appearance (Su 1981), but it is uncertain whether this original field isolate was a single-strain source. The subisolate, Taiwan-Pum/SP, caused stem pitting on pummelo but no stem pitting on 'Mexican' lime after two-month intervals in glasshouse trials; however, assessments that this variant was solely responsible for severe grapefruit dwarfing and stem pitting in the field is unconfirmed (Tsai et al. 1993). No associated symptoms were reported or biological data presented with the report of the Crete 1825 genome sequencing (Owen et al. 2014), although severity was assumed based on the first report of the CTV-D field isolate (Su 1981). Contrary to findings obtained with Taiwan-Pum/SP, isolate B390-5 induced stem pitting on 'Mexican' lime in the current study. Assessment periods in this study were longer than those reported for the Taiwan-Pum/SP isolate and evaluations were done at intervals of six months to a year, which could account for the difference in reaction in 'Mexican' lime. Stem pitting was obtained in all grapefruit cultivars and, similar to isolates 'Maxi' and GFMS12-8, no severe honey-comb stem pitting was observed.

Evaluation of field trees inoculated with isolate B390-5 in two grapefruit trials, showed that, although the isolate was associated with stem pitting, it did not impede tree growth, yield, or fruit size during the 8-year evaluation period. Horticulturally, both 'Star Ruby' and 'Marsh' trees containing this isolate performed better than those with GFMS35, the current cross-protection source, when comparing canopy volume, production, and fruit size. 'Star Ruby' trees containing GFMS35 showed less stem pitting than trees inoculated with isolate B390-5, but canopy volumes and yields were smaller than trees containing the RB isolate. This suggests that the influence of CTV on tree performance might not be limited to the effect of stem pitting only. The cross-protecting ability of isolate B390-5 could, however, not be assessed in these trials, as no severe challenge was observed in either trial, based on the absence of any externally visible stem

Table 2. Stem pitting severity, tree size, 3-year cumulative yield, and % small fruit of 'Star Ruby' grapefruit trial trees inoculated with various *Citrus tristeza virus* (CTV) sources, 8 years after planting

	Stem pitting rating and production measurements[w]			
Treatment[x]	Stem pitting[y]	Canopy volume (m^3)	Cumulative yield (kg/tree)	% small fruit (8th year)
B390-5	0.8 B	22 A	524 A	7 C
Control	0.0 B	21 AB	445 B	3 C
GFMS35	0.5 B	15 BC	343 C	28 B
GFMS12	3.0 A	11 C	347 C	64 A
Prob F treat[z]	0.0001	0.012	0.0003	0.0001

[w] Data presented are the means of five trees per treatment. Treatments with the same letters for Fisher least square difference do not statistically differ.
[x] CTV sources applied and uninoculated control.
[y] rating scale: 0 = smooth trunk with no visible pits, 1 = one to three grooves on the stem, 2 = multiple grooves that do not coalesce, and 3 = severe stem pitting in which numerous grooves coalesce to form a knotted appearance.
[z] Probability value from analysis of variance for differences between treatments.

Table 3. Stem pitting severity, tree size, 3-year cumulative yield, and % small fruit of 'Marsh' grapefruit trial trees inoculated with various *Citrus tristeza virus* (CTV) sources, 8 years after planting

	Stem pitting rating and production measurements[w]			
Treatment[x]	Stem pitting[y]	Canopy volume (m^3)	Cumulative yield (kg/tree)	% small fruit (8th year)
B390-5	0.6 B	31 A	347 A	5 A
Control	0.0 C	30 A	290 A	5 A
GFMS35	0.0 C	23 AB	267 A	8 A
GFMS12	2.8 A	21 B	256 A	14 A
Prob F treat[z]	0.0001	0.042	–	–

[w] Data presented are the means of five trees per treatment. Treatments with the same letters for Fisher least square difference do not statistically differ.
[x] CTV sources applied and uninoculated control.
[y] rating scale: 0 = smooth trunk with no visible pits, 1 = one to three grooves on the stem, 2 = multiple grooves that do not coalesce and 3 = severe stem pitting in which numerous grooves coalesce to form a knotted appearance.
[z] Probability value from analysis of variance for differences between treatments.

pitting on the control trees. The ability to moderate the effects of severe strains in cross-protection can only be confirmed by challenge experiments and in field trials in which the challenge pressure of severe strains is high.

Severe stem pitting and stunting was noticeable on field trees inoculated with GFMS12, a severe source, 3 years after planting (data not presented). Given that stem pitting assessment periods in the glasshouse trial were just less than a year, the stem pitting results can only be used as indicative of possible detrimental influence, unless very severe stem pitting was consistently found that induced stunting or other observable negative effects, which was not the case in this study. Conversely, though, and as demonstrated with isolate B390-5, stem pitting observed in the glasshouse and field trials did not always result in a negative influence on the overall performance of the trees.

The effects that CTV strains and variants may have on tree health and production of commercial varieties cannot be extrapolated from results obtained from short-term glasshouse trials. Although these may be indicative, the impact of CTV strains and variants can ultimately only be determined in long-term field trials under various climatic conditions.

Single-variant CTV sources of VT, RB, T68, and HA16-5 strains were identified and characterized in grapefruit hosts. These isolates did not induce severe stem pitting in commercial grapefruit varieties in a glasshouse trial. Of further significance is that results obtained with the RB isolate B390-5 did not support the detrimental findings in grapefruit, previously reported for the homologous isolate Taiwan-Pum/SP/T1 (Tsai et al. 1993). Two field trials testing two grapefruit varieties demonstrated no deleterious horticultural effects associated with this isolate in either 'Star Ruby' or 'Marsh' grapefruit. The single-variant sources characterized are valuable for further complementation studies to identify components required for cross-protection in grapefruit.

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APPENDIX B1

Routine diagnostic methods for detection of citrus viruses, viroids and systemic bacteria (Chapter 3 page 40)

Diagnostic techniques used for detection of citrus viruses, viroids and systemic bacteria:

Table 1. Biological indexing for citrus pathogens, the relevant citrus host and the duration of the evaluation.

Pathogen / Disease	Biological indexing host / confirmation test	Duration of biological indexing
Citrus viroids: citrus exocortis viroid (CEVd); hop stunt viroid (HSVd), citrus dwarfing viroid (CDVd), citrus bent leaf viroid (CBLVd), citrus bark cracking viroid CBCVd), citrus viroid V (CVd V)	'Etrog' citron Arizona 861-S-1 RT-PCR	3-6 months
citrus psorosis virus (CPsV)	'Madam Vinous' sweet orange RT-PCR	12 months
Impietratura	'Duncan' grapefruit / 'Madam Vinous' sweet orange	12 months
citrus tatter leaf virus (CTLV)	'Troyer' citrange RT-PCR	6 months
'*Candidatus* Liberibacter africanus'	'Madam Vinous' sweet orange PCR	6 months

Table 2. PCR primer details, annealing temeratures and expected amplicon size

Pathogen	Primers	Primer Sequence	Annealing temp. (ºC)	Product size (bp)
CBLVd [5]	CBLVd R2	TTCGTCGACGACGACCAGTC	57	234
	CBLVd F2	CCCTTCACCCGAGCGCTGCTT		
HSVd	Mike2 R	GACGAACCGAGAGGTGATG	57	185
	Mike2 F	GACTTACCTGAGAAAGGAGCC		
CDVd	CM3 R	TCGACGACGACAGGTAAGTT	56	295
	CP3 F	CGAAGGCAGCTAAGTTGGTGA		
CVd IV [1]	SL3	GGGTAGTTTCTATCTCAG	52	264
	F3	GGTGGATACAACTCTTGGG		
CEVd	CEV R2	TCTCCGCTGGACGCCAGTGA	60	160
	CEV F	CTTGAGGTTCCTGTGGTGCT		
CVd V	CVd V R	CAACGTCCGCTCGACTAGC	62	130
	CVd V F	GTCGACGAAGGCCGGTGAGC		
CPsV	CPsV-FP R	ATTCTGCCATCTGGAGTGAGG	64	200
	CPsV-FP F	GARTCCCTGATGCCATTGCTGGA		
CTLV [4]	CTLV-AM R	TAGAAAAACCACACTAACCCGGAAATGC	60	456
	CTLV-AP F	CCTGAATTGAAAACCTTTGCTGCCACTT		
Laf /Las [3]	J5	ACAAAAGCAGAAATAGCACGAACAA	57	669/703
	A2	TATAAAGGTTGACCTTTCGAGTTT		

Reverse Transcription and RT-PCR were done using reagents and protocols as previously described [2].

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APPENDIX B2

Overlapping sequencing primer sets for full-genome determination of CTV strains

(Chapter 3 page 42)
(Chapter 5 page 95)

Overlapping primer sets for full genome amplification of CTV strains:

RB Strain	....|....| ....|....| ....| 5 15 25	Approx. amplicon size
RB A+	AATTTCHMAA ATTCAACCTG TTC..	
CTV942R	GAAACCGTAC GRGGACGGT.	**940**
RB A+	AATTTCHMAA ATTCAACCTG TTC..	
RB A-	GCCCAGTRCT CTACGCAC..	**1400**
RB B+	CACTTCCGTC GTCAGCGGAC	
RB B-	GACAAACGAG CYAGCAGTAT G....	**1470**
RB C+	TCGGAGAAGT CGTARTCGTC GT...	
RB C-	TTCGTGTTTA CSGCACCAAC T....	**1400**
RB D+	GGAGAATCAT CTCGTTCGTA AAC..	
RB D-	GCTTGAGTAA CAGTTCCAAC AC...	**1500**
RB E+	AGAGAGGTAA CACTTCCGAA ATC..	
RB E-	TCCCATTTCC ACGAAGACCA GG...	**1370**
RB F+	CTCGATACTT TCCACCTCTA G....	
RB F-	GCCTCGCCGA ACACGCAAAC C....	**1390**
RB G+	CTTGGTGGAA ACGGCAACG.	
RB G-	CTGAGATATT TCAGTTTGAG AATT.	**1380**
RB H+	GCGAACTCGT TCTTACGTGC T....	
RB H-	CGACAGAAGG TTTTCTTGTA GC...	**1300**
RB I+	TCGAAGAATC ATATTGTGGT TGC..	
RB I-	CAAAGTCCTG TGTTATGTCC TTA..	**1500**
RB J+	TGCTTAGAAA CAGGCTTTGA GAC..	
RB J-	CTTCTTCGAT TGTACGACTT TGA..	**1300**
RB K+	TTGCGACTGA TGATGTAGAA GAC..	
RB K-	GATACAGTTT RCTAATCCCA TAA..	**1430**
RB L+	CGCTGTTAGA AGTTCAGGTG GTG..	
RB L-	TCATCAAGGA CTTCAACGGC ATAC.	**1300**
RB M+	CGTTGGACCA CACTCTGAGT TC...	
RB M-	GGTGGAGGTG TGATTGACAC	**1350**
RB N+	CGATCTCTTC AGTTAGGTTA C....	
RB N-	GTCCTTATCT TTGTCAGGTA AGT..	**1320**
RB O+	CGTTAATYGA CGACTCTTGA TAT..	
RB O-	CGAAATTAGC CAAATCGTAA CAT..	**1380**
RB P+	TACTTGTGTG CGGATTTCTT GAC..	
RB P-	GACTTGTGTT CACCCATCTC	**1280**
CTVall Q+	GCGAGCTTAC TTTAGTGTTA	
CTVall Q-	TGGACCTATG TTGGC.....	**1350**

HA16-5-strain	....|....| ....|....| ....| 5 15 25	Approx. amplicon size
HA16-5 A+	AATTTCGATT CAAATTCACC CGT..	
CTV942R	GAAACCGTAC GRGGACGGT.	**940**
HA16-5 A+	AATTTCGATT CAAATTCACC CGT..	
HA16-5 A-	ACGCATCGGA ACCTTCAAAG AAT..	**1460**
HA16-5 B+	GGTCAATGTT ACGTCCGTCA C....	
HA16-5 B-	CAAATGTACC CCAAATGGAA ATG..	**1420**
HA16-5 C+	AAATTGCGGG ACACTTCGGC GT...	
HA16-5 C-	CTTAGCGAAA CCATCTCTAC AGT..	**1400**
HA16-5 D+	TGCTACATGA CCGTGGTTAC	
HA16-5 D-	CACGTTACGA AGACACTCCA GC...	**1360**
HA16-5 E+	GCTAAAGTAT AGTACCGTTC AC...	
HA16-5 E-	CACCTCCCAT TCCCACGAAG AC...	**1110**
HA16-5 F+	CACGGGTCTC TTCACAACTC G....	
HA16-5 F-	CGCAACGCCA AGGTCTTCG.	**1480**
HA16-5 G+	CTTCAGAGCA TTTTTAACGG TC...	
HA16-5 G-	CCGCTTTACA AGAGGTGAAT T....	**1480**
HA16-5 H+	AGGAAAATGG TTGCGAAACA C....	
HA16-5 9H-	AAGCACGGGG AATACATAGC	**1430**
CTVmidF	GAACCGGCTC GYGTTCGGCG T....	
HA16-5 I-	AATACGTCTT GGTTTTCGCT CGC..	**1540**
HA16-5 J+	GAAGTTTGCT AATTTGATCT GC...	
HA16-5 J-	GCGTGAGTGC CGTAAGGAG.	**1340**
RB K+	TTGCGACTGA TGATGTAGAA GAC..	
HA16-5 K	GTCTCTACCG CCTAAGTTCA T....	**1300**
HA16-5 L+	TCCACTTTAC CTAAGTTGAG TTC..	
HA16-5 L-	TACATCCAAC TTCATGCGGG TC...	**1460**
HA16-5 M+	AACGTGTCGT TGGACCACAC T....	
HA16-5 M-	TCATCAGTCT TAGGAAGTAT TGT..	**1490**
HA16-5 N+	TCAATCGACT TAGAAGAGGT TC...	
HA16-5 N-	GCACATTCTA AATCAGTCAA GC...	**1490**
HA16-5 O+	GGTATTGGTA ACCGTACTAA C....	
HA16-5 O-	GACTTGTGTT CACCCATCTC	**1420**
CTVall Q+	GCGAGCTTAC TTTAGTGTTA	
CTVall Q-	TGGACCTATG TTGGC.....	**1350**

VT strain	....|....| ....|....| ....| 5 15 25	Approx. amplicon size
CTV5endFVT	AATTTCTCAA ATTCACCCGT A...	
VT140R	TGCGTACAGC GAAAGTCGAG GA..	**140**
CTV942R	GAAACCGTAC GRGGACGGT.	**940**
VT A-	ACWGAAAGAT CGACGCGCCT	**1420**
VT B+	CTGYTTGTGG GAGTCGTC..	
VT B-	AAGTGTACYG TAACGAGACC	**1440**
VT C+	TTGCGGGACA CGTCGGCGTC	
VT C-	TCACACCGAC GATAGCGTAA CC…	**1480**
VT D+	GTTGGACGCC CGTGAGTC..	
CTV5427R	AGTTYGATCC YACTTCCATA G....	**1650**
VT E+	TTTTTRCYGC TGTTTGGGAC	
VT E-	CACTCTATGA AAGTGTATAT CG...	**1500**
VT F+	TTCTCTTTCC AGGTTCTTGG	
VT F-	CTCCAACCTG CTTAGTGC..	**1380**
VT G+	CGAAGTGTTG AACTCTTTGG AC...	
VT G-	CTTACCAAAC GAACCTTCTT ATA..	**1490**
VT H+	ACTGCTAACA CTGGTAGTAT CG...	
VT H-	GTAACCAAAG AGTTCCCAAT CCA..	**1440**
VT I+	TGACGAATGC TGGGTTGGC.	
VT I-	GTTGATTATC RAYGTGCGCT C....	**1290**
VT J+	GGTAGTTGAG TCGAGATGTT TGC..	
VT J-	CAACCRACCC ACCGTTTCAA	**1380**
VT K+	TTCGACGGTG GCTATGGCTA C....	
VT K-	GTCAAAGCTA AAACTAAAGC GGC..	**1540**
VT L+	ATGACTCTCG TGACCAAAGT	
VT L-	ACAAACATCC CTGCCCAACG C....	**1370**
VT M+	AGTGTTGCCT GTGGTGTTAA	
VT M-	ATCGCGTAAG TTAAGAAGCT C....	**1500**
VT N+	CCAAAGCTGG GACTCCGCAT	
VT N-	TAGTAGTACC AAAAAGAACC TTA..	**1240**
VT O+	AATGTCAGGC AGCTTGGGAA AT...	
VT O-	GATGTCGAGA AGTATTCGCA G....	**1200**
CTVall Q+	GCGAGCTTAC TTTAGTGTTA	
CTVall Q-	TGGACCTATG TTGGC.....	**1350**
CTVF	TCTGATTGAA GTGGACGGAA TAAG.	
CTVall Q-	TGGACCTATG TTGGC.....	**290**

T3 strain	....|....| ....|....| ....| 5 15 25	Approx. amplicon size
CTV5endFVT	AATTTCTCAA ATTCACCCGT A....	
CTV942R	GAAACCGTAC GRGGACGGT.	**940**
T3 A+	GCAGAGACTT CCTTTCGTGA T....	
T3 A-	GCAGAGACTT CCTTTCGTGA T....	**860**
VT B+	CTGYTTGTGG GAGTCGTC..	
VT B-	AAGTGTACYG TAACGAGACC	**1440**
VT C+	TTGCGGGACA CGTCGGCGTC	
VT C-	TCACACCGAC GATAGCGTAA CC...	**1480**
VT D+	GTTGGACGCC CGTGAGTC..	
CTV5427R	AGTTYGATCC YACTTCCATA G....	**1650**
T3F+	ATTTCATCTG CGTGCAGGTC AC...	
VT E-	CACTCTATGA AAGTGTATAT CG...	**3100**
T3 F+	GCGTATTCGA CGACTCCAG.	
T3 F-	CGATCCAAAC GAAATGGAAC	**1340**
VT G+	CGAAGTGTTG AACTCTTTGG AC...	
VT G-	CTTACCAAAC GAACCTTCTT ATA..	**1500**
VT H+	ACTGCTAACA CTGGTAGTAT CG...	
VT H-	GTAACCAAAG AGTTCCCAAT CCA..	**1460**
CTVmidF	GAACCGGCTC GYGTTCGGCG T....	
CTVmidR	GCAAACATCY YGACTCAACT ACC..	**1550**
T3 I+	GACAAGTCTC AAGATTTGTT C....	
T3 I-	CCATTCACGG GTGGGCTTAC	**1180**
VT J+	GGTAGTTGAG TCGAGATGTT TGC..	
VT J-	CAACCRACCC ACCGTTTCAA	**1380**
VT K+	TTCGACGGTG GCTATGGCTA C....	
VT K-	GTCAAAGCTA AAACTAAAGC GGC..	**1540**
VT L+	ATGACTCTCG TGACCAAAGT	
VT L-	ACAAACATCC CTGCCCAACG C....	**1370**
VT M+	AGTGTTGCCT GTGGTGTTAA	
VT M-	ATCGCGTAAG TTAAGAAGCT C....	**1500**
VT N+	CCAAAGCTGG GACTCCGCAT	
VT N-	TAGTAGTACC AAAAAGAACC TTA..	**1240**
VT O+	AATGTCAGGC AGCTTGGGAA AT...	
VT O-	GATGTCGAGA AGTATTCGCA G....	**1200**
CTVall Q+	GCGAGCTTAC TTTAGTGTTA	
CTVall Q-	TGGACCTATG TTGGC.....	**1350**
CTVF	TCTGATTGAA GTGGACGGAA TAAG.	
CTVall Q-	TGGACCTATG TTGGC.....	**290**

T68 strain	....|....| ....|....| ....| 5 15 25	Approx. amplicon size
CTV5endFVT	AATTTCTCAA ATTCACCCGT A...	
CTV942R	GAAACCGTAC GRGGACGGT.	**940**
T68 A+	CTGCTTCGAT GGTCGCCGTC	
T68 A-	CAAACCGAGT GGCACAAATC ATC..	**1200**
T68 B+	TTGCTTGTGG GAGTCGTC..	
T68 B-	AAATGCACTG TAACAAGACC	**1440**
T68 C+	CGGGTGTTGAA ATTGCGGGAT AC...	
T68 C-	GAGTAGGTGTG TGTATACGCT G....	**1340**
T68 D+	GTTACATGACC GTGGTTACG.	
T68 D-	GACAGTGACGA AAATTGAATC G....	**1280**
T68 E+	CTGGACTTGTT GTGCGAGAAG G....	
T68 E-	CAACGCTGTCG GCGGTAAGTC	**1350**
T68 F+	GGCGAGTTGTC ATGTACCGCA G....	
T68 F-	GAATCGTCACT CTCAACGACT G....	**1510**
T68 G+	GTTGCGAGTTC GTAACGCTAA CG...	
T68 G-	GTAAATTCTAC AATGGCTCCG ATC..	**1340**
T68 H+	CACAAGCGGAG ATTTCTCAAC G....	
T68 H-	ACCTTAGCATC ACGTTTCAC.	**1400**
CTVmidF	GAACCGGCTC GYGTTCGGCG T....	
CTVmidR	GCAAACATCY YGACTCAACT ACC..	**1550**
T68 I+	TGACGAACGC TGGGTTGGC	
T68 I-	CCGCTTACTG GTGGGCTTAC	**1270**
T68 J+	GATGTTGGTT ATAATGCTGC CGA..	
T68 J-	CAAATCTTTG TAAAACGAGC CAG..	**1230**
T68 K+	CAAAGTCGCA CGATTGAAGA GGA..	
T68 K-	CAGACACAAA AGGTACAGTT TCC..	**1510**
VT L+	ATGACTCTCG TGACCAAAGT	
VT L-	ACAAACATCC CTGCCCAACG C....	**1370**
VT M+	AGTGTTGCCT GTGGTGTTAA	
VT M-	ATCGCGTAAG TTAAGAAGCT C....	**1500**
VT N+	CCAAAGCTGG GACTCCGCAT	
VT N-	TAGTAGTACC AAAAAGAACC TTA..	**1240**
VT O+	AATGTCAGGC AGCTTGGGAA AT...	
VT O-	GATGTCGAGA AGTATTCGCA G....	**1200**
CTVall Q+	GCGAGCTTAC TTTAGTGTTA	
CTVall Q-	TGGACCTATG TTGGC.....	**1350**
CTVF	TCTGATTGAA GTGGACGGAA TAAG.	
CTVall Q-	TGGACCTATG TTGGC.....	**290**

APPENDIX B3

Statistical summaries of stem pitting results in ‘Star Ruby’ and ‘Marsh’ grapefruit: 2015-2018

(Chapter 4 page 79)

Stem pitting on 'Star Ruby'

Statistical summary and pairwise comparisons of treatments using the Games-Howell nonparametric test

2015

Treatment	N	Mean	Std. dev	Groups	
HA16-5	6	0.00	0.00	A	
HA16-5 + RB1	4	0.00	0.00	A	
HA16-5 + RB2	5	0.00	0.00	A	
HA16-5 + RB1 + RB2	2	0.00	0.00	A	
RB1	6	0.00	0.00	A	
Virus-free	5	0.00	0.00	A	
VT + RB1	6	0.17	0.41	A	
VT + HA16-5 + RB1	5	0.20	0.45	A	B
VT + HA16-5 + RB1 +RB2	3	0.33	0.58	A	B
VT + RB2	6	0.50	0.55	A	B
T68 + VT + RB2	4	0.50	0.58	A	B
T68 + VT + RB1 + RB2	4	0.50	0.58	A	B
T68 + HA16-5 + RB1 +RB2	2	0.50	0.71	A	B
VT + HA16-5 + RB2	4	0.50	0.58	A	B
RB2	2	0.50	0.71	A	B
VT	5	0.60	0.55	A	B
T68 + HA16-5 + RB2	4	0.75	0.50	A	B
VT + HA16-5	4	0.75	0.50	A	B
T68 + VT + RB1	4	1.00	0.00	A	B
T68 + HA16-5	4	1.00	0.00	A	B
T68 + VT + HA16-5	4	1.00	0.00	A	B
T68 + VT + HA16-5 + RB2	4	1.00	0.00	A	B
T68 + HA16-5 + RB1	6	1.50	1.38	A	B
T68 + VT + HA16-5 + RB1	4	1.50	1.00	A	B
T68 + RB1 + RB2	3	1.33	0.58	A	B
T68 + RB2	4	1.50	0.58	A	B
T68 + RB1	3	1.67	0.58	A	B
T68 + VT + HA16-5 + RB1 +RB2	3	1.67	0.58	A	B
T68	7	2.43	1.13		B
T68 + VT	4	2.25	0.96		B

2016

Treatment	N	Mean	Std. dev	Groups	
HA16-5	6	0.00	0.00	A	
HA16-5 + RB2	5	0.00	0.00	A	
HA16-5 + RB1 + RB2	2	0.00	0.00	A	
VT + HA16-5 + RB1	5	0.00	0.00	A	
VT + HA16-5 + RB1 +RB2	3	0.00	0.00	A	
RB1	6	0.00	0.00	A	
RB2	2	0.00	0.00	A	
Virus-free	5	0.00	0.00	A	
HA16-5 + RB1	4	0.25	0.50	A	B
VT + HA16-5	4	0.25	0.50	A	B
VT + HA16-5 + RB2	4	0.25	0.50	A	B
VT	5	0.60	1.34	A	B
T68 + VT + RB1 + RB2	4	0.50	0.58	A	B
T68 + VT + HA16-5 + RB1	4	0.50	0.58	A	B
VT + RB2	6	0.67	0.52	A	B
T68 + HA16-5 + RB1	6	0.67	0.52	A	B
T68 + VT + HA16-5 + RB1 +RB2	3	0.67	0.58	A	B
T68 + HA16-5 + RB2	4	0.75	0.50	A	B
VT + RB1	6	0.83	0.75	A	B
T68 + HA16-5	4	1.00	0.82	A	B
T68 + VT + HA16-5	4	1.00	0.82	A	B
T68 + VT + HA16-5 + RB2	4	1.00	0.00	A	B
T68 + RB2	4	1.25	0.50	A	B
T68 + VT + RB1	4	1.25	0.50	A	B
T68 + RB1 + RB2	3	1.33	0.58	A	B
T68 + HA16-5 + RB1 +RB2	2	1.50	0.71	A	B
T68 + VT	4	1.75	0.96	A	B
T68 + VT + RB2	4	2.00	1.15	A	B
T68 + RB1	3	2.00	1.00	A	B
T68	7	2.43	1.27		B

2017

Treatment	N	Mean	Std. dev	Groups	
RB2	2	0.00	0.00	A	
Virus-free	5	0.00	0.00	A	
RB1	6	0.17	0.41	A	
HA16-5 + RB2	5	0.20	0.45	A	
HA16-5 + RB1	4	0.25	0.50	A	
HA16-5	6	0.33	0.52	A	
HA16-5 + RB1 + RB2	2	0.50	0.71	A	
T68 + HA16-5 + RB1	6	0.50	0.55	A	
VT + HA16-5 + RB2	4	1.00	0.00	A	
VT + HA16-5 + RB1 +RB2	3	1.00	0.00	A	
T68 + HA16-5 + RB2	4	0.75	0.50	A	
T68 + HA16-5 + RB1 +RB2	2	1.00	1.00	A	
VT + HA16-5 + RB1	5	1.00	0.00	A	
T68 + HA16-5	4	1.13	1.03	A	
VT + HA16-5	4	1.50	0.58	A	
VT	5	1.60	1.14	A	
VT + RB2	6	1.67	0.82	A	
T68 + VT + HA16-5 + RB2	4	1.88	1.18	A	
T68 + RB1	3	2.00	1.00	A	
T68 + VT + HA16-5 + RB1 +RB2	3	2.00	1.00	A	
T68 + VT + RB1 + RB2	4	2.00	0.82	A	
T68 + VT + HA16-5 + RB1	4	2.00	0.82	A	
T68 + VT + HA16-5	4	2.00	0.00	A	
T68 + RB2	4	2.25	0.96	A	
T68 + VT + RB2	4	2.25	0.96	A	
T68	7	2.29	0.76	A	
T68 + VT	4	2.50	1.29	A	
VT + RB1	6	2.33	0.52	A	
T68 + VT + RB1	4	2.50	0.58	A	
T68 + RB1 + RB2	3	2.67	0.58	A	

2018

Treatment	N	Mean	Std. dev	Groups	
HA16-5	6	0.00	0.00	A	
HA16-5 + RB1 + RB2	2	0.00	0.00	A	B
RB2	2	0.00	0.00	A	B
Virus-free	5	0.00	0.00	A	B
HA16-5 + RB1	4	0.25	0.50	A	B
RB1	6	0.33	0.52	A	B
HA16-5 + RB2	5	0.40	0.89	A	B
VT + HA16-5 + RB1 +RB2	3	0.67	0.58	A	B
VT + HA16-5 + RB1	5	0.80	0.84	A	B
VT + HA16-5 + RB2	4	1.00	0.00	A	B
T68 + HA16-5 + RB2	4	0.67	0.58	A	B
T68 + RB1	3	1.33	0.58	A	B
T68 + HA16-5 + RB1 +RB2	2	1.50	2.12	A	B
VT + RB1	6	1.50	1.05	A	B
T68 + HA16-5	4	1.50	0.58	A	B
T68 + VT + HA16-5 + RB2	4	1.63	1.25	A	B
T68 + HA16-5 + RB1	6	2.08	1.36	A	B
VT + RB2	6	2.33	1.63	A	B
T68 + VT + HA16-5 + RB1	4	2.13	0.85	A	B
VT	5	2.20	0.76	A	B
T68 + RB2	4	2.25	0.96	A	B
T68 + VT + HA16-5 + RB1 +RB2	3	2.33	1.15	A	B
T68	7	2.36	1.03	A	B
VT + HA16-5	4	2.38	0.95	A	B
T68 + RB1 + RB2	3	2.50	0.50	A	B
T68 + VT + HA16-5	4	2.50	0.58	A	B
T68 + VT + RB1	4	3.00	1.58	A	B
T68 + VT + RB2	4	2.88	1.31	A	B
T68 + VT + RB1 + RB2	4	3.00	0.82	A	B
T68 + VT	4	3.13	0.25		B

Stem pitting on 'Marsh'

Statistical summary and pairwise comparisons of treatments using the Games-Howell nonparametric test

2015

Treatment	N	Mean	Std. dev	Groups
VT + RB2	2	0.00	0.00	A
VT + RB1 + RB2	2	0.00	0.00	A
HA16-5	4	0.00	0.00	A
HA16-5 + RB1	7	0.00	0.00	A
HA16-5 + RB2	4	0.00	0.00	A
T68 + HA16-5 + RB2	6	0.00	0.00	A
VT + HA16-5	2	0.00	0.00	A
VT + HA16-5 + RB1	5	0.00	0.00	A
VT + HA16-5 + RB2	6	0.00	0.00	A
RB1	7	0.00	0.00	A
RB2	4	0.00	0.00	A
Virus-free	5	0.00	0.00	A
T68 + VT + HA16-5 + RB1	6	0.17	0.41	A
T68 + VT + RB2	4	0.50	0.58	A
T68 + VT + HA16-5	4	0.50	0.58	A
T68 + HA16-5	4	0.75	0.96	A
T68 + HA16-5 + RB1	7	0.71	0.76	A
T68 + RB2	4	0.75	0.50	A
VT + RB1	4	0.75	0.50	A
T68 + VT	3	1.00	1.00	A
T68 + RB1	9	1.11	0.93	A
T68	5	1.00	0.71	A
VT	3	1.33	0.58	A
T68 + VT + RB1	6	2.00	0.89	A

2016

Treatment	N	Mean	Std. dev	Groups
VT	3	0.00	0.00	A
VT + RB1	4	0.00	0.00	A
VT + RB2	2	0.00	0.00	A
VT + RB1 + RB2	2	0.00	0.00	A
HA16-5	4	0.00	0.00	A
HA16-5 + RB2	4	0.00	0.00	A
T68 + HA16-5 + RB2	6	0.00	0.00	A
VT + HA16-5	2	0.00	0.00	A
VT + HA16-5 + RB1	5	0.00	0.00	A
VT + HA16-5 + RB2	6	0.00	0.00	A
RB2	4	0.00	0.00	A
Virus-free	5	0.00	0.00	A
T68 + HA16-5 + RB1	7	0.14	0.38	A
RB1	7	0.14	0.38	A
T68 + VT + HA16-5 + RB1	6	0.17	0.41	A
T68	5	0.40	0.89	A
T68 + RB2	4	0.25	0.50	A
T68 + HA16-5	4	0.25	0.50	A
T68 + VT + HA16-5	4	0.25	0.50	A
HA16-5 + RB1	7	0.29	0.49	A
T68 + RB1	9	0.33	0.50	A
T68 + VT + RB1	6	0.50	0.55	A
T68 + VT	3	0.67	0.58	A
T68 + VT + RB2	4	0.75	0.50	A

2017

Treatment	N	Mean	Std. dev	Groups
HA16-5	4	0.00	0.00	A
RB1	7	0.00	0.00	A
RB2	4	0.00	0.00	A
Virus-free	5	0.00	0.00	A
HA16-5 + RB1	7	0.14	0.38	A
HA16-5 + RB2	4	0.50	0.58	A
VT + HA16-5 + RB1	5	0.80	0.84	A
T68 + HA16-5 + RB2	6	0.83	0.75	A
VT + HA16-5	2	1.00	1.41	A
T68 + RB2	4	1.00	0.82	A
T68	5	1.00	0.00	A
VT + RB2	2	1.00	0.00	A
VT + RB1 + RB2	2	1.00	0.00	A
T68 + VT + HA16-5 + RB1	6	1.33	1.51	A
T68 + VT + RB1	6	1.17	0.75	A
T68 + RB1	9	1.33	0.71	A
VT + HA16-5 + RB2	6	1.58	1.11	A
VT + RB1	4	1.50	0.58	A
T68 + HA16-5	4	1.50	0.58	A
T68 + HA16-5 + RB1	7	1.57	0.79	A
T68 + VT	3	1.67	0.58	A
VT	3	2.00	1.00	A
T68 + VT + HA16-5	4	2.00	0.82	A
T68 + VT + RB2	4	2.25	0.50	A

2018

Treatment	N	Mean	Std. dev	Groups
VT + RB2	2	0.00	0.00	A
HA16-5	4	0.00	0.00	A
HA16-5 + RB1	7	0.00	0.00	A
Virus-free	5	0.00	0.00	A
RB1	7	0.14	0.38	A
VT + HA16-5 + RB2	6	0.17	0.41	A
RB2	4	0.25	0.50	A
T68 + RB2	4	0.50	0.58	A
VT + RB1 + RB2	2	0.50	0.71	A
HA16-5 + RB2	4	0.50	0.58	A
VT + HA16-5 + RB1	5	0.60	0.55	A
T68 + HA16-5	4	0.75	0.96	A
T68 + VT + RB1	6	0.67	0.52	A
T68 + HA16-5 + RB2	6	0.67	0.52	A
T68 + VT + HA16-5 + RB1	6	0.67	0.52	A
T68 + VT + HA16-5	4	0.75	0.50	A
T68 + RB1	9	0.89	0.78	A
VT + HA16-5	2	1.00	1.41	A
T68 + VT	3	1.00	1.00	A
T68 + HA16-5 + RB1	7	1.29	0.76	A
T68	5	1.20	0.45	A
VT + RB1	4	1.25	0.50	A
VT	3	1.67	0.58	A
T68 + VT + RB2	4	1.75	0.50	A

APPENDIX B4

Statistical summaries of strain quantitation results

(Chapter 4 page 85)

Quantitative strain specific analyses

Statistical summary and pairwise comparisons of treatments using the Games-Howell nonparametric test

Star Ruby

'Star Ruby' treatments	Concentration ratio (LS means)	Standard deviation	Groups	
HA16-5				
T68 + HA16-5 + RB1	2.00	1.68	A	
T68 + HA16-5	1.72	1.12	A	
T68 + HA16-5 + RB2	1.27	0.33	A	
VT + HA16-5	0.89	0.36	A	
T68 + VT + HA16-5	0.66	0.34	A	
HA16-5	0.65	0.35	A	
T68 + HA16-5 + RB1 +RB2	0.61	0.18	A	
T68 + VT + HA16-5 + RB1 +RB2	0.46	0.26	A	
T68 + VT + HA16-5 + RB2	0.20	0.06	A	
T68 + VT + HA16-5 + RB1	0.19	0.03	A	
RB1				
T68 + HA16-5 + RB1	1.51	1.26	A	
RB1	0.73	0.23	A	
T68 + HA16-5 + RB1 +RB2	0.61	0.05	A	
T68 + VT + RB1 + RB2	0.60	0.23	A	
T68 + VT + HA16-5 + RB1 +RB2	0.55	0.52	A	
T68 + VT + HA16-5 + RB1	0.52	0.13	A	
T68 + RB1 + RB2	0.52	0.49	A	
T68 + RB1	0.51	0.31	A	
T68 + VT + RB1	0.40	0.33	A	
T68				
T68 + HA16-5	1.07	0.67	A	
T68 + HA16-5 + RB1	1.02	0.67	A	
T68 + VT + RB2	0.71	0.57	A	
T68 + RB1 + RB2	0.67	0.33	A	
T68 + VT	0.67	0.26	A	
T68 + HA16-5 + RB2	0.63	0.39	A	
T68 + RB2	0.61	0.23	A	
T68 + VT + RB1 + RB2	0.55	0.18	A	
T68	0.52	0.24	A	
T68 + VT + HA16-5	0.46	0.13	A	
T68 + HA16-5 + RB1 +RB2	0.40	0.04	A	
T68 + VT + RB1	0.39	0.22	A	
T68 + VT + HA16-5 + RB1 +RB2	0.36	0.12	A	
T68 + RB1	0.35	0.18	A	
T68 + VT + HA16-5 + RB2	0.21	0.04	A	
T68 + VT + HA16-5 + RB1	0.21	0.03	A	
VT				
VT	0.23	0.09	A	
T68 + VT + HA16-5	0.21	0.17	A	B
T68 + VT	0.20	0.17	A	B
T68 + VT + RB2	0.15	0.08	A	B
T68 + VT + RB1	0.12	0.13	A	B
VT + HA16-5	0.10	0.02	A	B
T68 + VT + RB1 + RB2	0.09	0.07	A	B
T68 + VT + HA16-5 + RB1 +RB2	0.09	0.07	A	B
T68 + VT + HA16-5 + RB1	0.04	0.03		B
T68 + VT + HA16-5 + RB2	0.04	0.01		B
RB2				
T68 + HA16-5 + RB2	0.15	0.14	A	
T68 + RB1 + RB2	0.14	0.19	A	
T68 + VT + RB2	0.08	0.04	A	
T68 + RB2	0.04	0.02	A	
T68 + VT + HA16-5 + RB1 +RB2	0.02	0.04	A	
T68 + VT + HA16-5 + RB2	0.02	0.00	A	
RB2	0.02	0.01	A	
T68 + VT + RB1 + RB2	0.00	0.00	A	
T68 + HA16-5 + RB1 +RB2	0.00	0.00	A	

Marsh

'Marsh' treatments	Concentration ratio (LS means)	Standard deviation	Groups	
HA16-5				
HA16-5	2.56	1.19	A	
T68 + HA16-5 + RB2	1.78	1.26	A	B
T68 + VT + HA16-5 + RB2	1.12	0.79	A	B
T68 + HA16-5 + RB1	1.00	0.42	A	B
T68 + VT + HA16-5	0.48	0.41	A	B
T68 + HA16-5	0.33	0.25		B
T68 + VT + HA16-5 + RB1	0.29	0.06		B
RB1				
RB1	2.09	0.98	A	
VT + RB1	1.92	1.06	A	B
T68 + HA16-5 + RB1	1.60	0.74	A	B
T68 + RB1	1.10	0.55	A	B
T68 + VT + RB1	0.54	0.21		B
T68 + VT + HA16-5 + RB1	0.45	0.16		B
VT + RB1 + RB2	0.10	0.04		B
T68				
T68 + HA16-5 + RB2	1.53	0.92	A	
T68 + RB2	1.50	0.60	A	
T68 + HA16-5 + RB1	1.39	0.70	A	
T68	1.36	0.52	A	
T68 + RB1	1.16	0.51	A	
T68 + VT + HA16-5 + RB2	1.02	0.64	A	
T68 + VT + RB2	0.67	0.20	A	
T68 + VT + RB1	0.59	0.23	A	
T68 + VT + HA16-5	0.47	0.28	A	
T68 + VT	0.46	0.17	A	
T68 + VT + HA16-5 + RB1	0.42	0.14	A	
T68 + HA16-5	0.39	0.21	A	
VT				
VT + RB1	1.20	1.00	A	
VT	0.50	0.36	A	
T68 + VT + RB1	0.28	0.18	A	
T68 + VT + RB2	0.20	0.08	A	
T68 + VT + HA16-5	0.18	0.11	A	
T68 + VT	0.11	0.18	A	
T68 + VT + HA16-5 + RB1	0.11	0.09	A	
T68 + VT + HA16-5 + RB2	0.03	0.04	A	
VT + RB1 + RB2	0.02	0.01	A	
RB2				
RB2	0.77	0.45	A	
T68 + RB2	0.71	0.40	A	
T68 + VT + HA16-5 + RB2	0.52	0.79	A	
T68 + HA16-5 + RB2	0.25	0.18	A	
T68 + VT + RB2	0.06	0.04	A	

APPENDIX B5

External stem-pitting severity scale for field trial evaluations

(Chapter 5 page 97)

External stem pitting severity scale for field trial evaluations

0 = a smooth trunk with no visible grooves

1 = Mild stem pitting; one to three grooves on the stem and scaffold branches

2 = Moderate stem pitting; multiple grooves on the stem and scaffold branches

3 = Severe stem pitting; numerous grooves merge, the trunk displays a knotted appearance and is often accompanied by twig die-back

1. Mild

2. Moderate

3. Severe