Evaluation of resistance to *Tomato curly stunt virus* in tomato

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DECLARATION

I declare that this is my own, unaided work. It is being submitted for the degree of Master of Science in the School of Molecular and Cell Biology, to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Katia Dias

This 19Th day of September 2012.

ABSTRACT

Solanum lycopersicon (the cultivated tomato) is a commodity of great economic importance in South Africa (SA) as well as worldwide. A destructive viral disease known as *Tomato curly stunt virus*, ToCSV-[ZA:Ond:98], belonging to the genus *Begomovirus* has negatively impacted on tomato production in SA. This has brought about the need to develop resistant cultivars to ToCSV. Since all cultivated tomato cultivars are susceptible to ToCSV, resistance genes against the virus found in wild tomato plant species have been introgressed into the cultivated tomato by plant breeding techniques. Wild relatives of tomato were adapted to many pathogens (including viruses) as well as stresses from the surrounding environment. During breeding for improved fruit quality and increased yield, the gene networks giving rise to many biotic and abiotic stress resistances have been lost leaving the domesticated tomato extremely susceptible. Plant breeders have reconstituted some of the gene networks into the cultivated tomato that provide tolerance to stresses including viruses. They have achieved this by the help of marker-assisted selection (MAS), where the associated marker is used as an indirect selection criterion. This is an important process in commercial breeding programs as it allows for a speedy selection of selected traits in the development of tomato hybrids. The defence response to abiotic stresses in plants includes the expression of heat shock proteins (HSPs) that function as stress response proteins, molecular chaperones and proteases which repair or degrade damaged proteins.

The objective of this study was to elucidate the type of resistance mechanism of a tomato inbred line (TAM), to ToCSV. Since TYLCV-IL shows 77% nucleotide identity with ToCSV, molecular markers already established for the detection of resistance genes for TYLCV-IL were used to screen TAM.

The inbred line, TAM, was screened for the absence of any of the known resistant genes to TYLCV-IL using molecular markers already established for the screening of TYCLV-IL resistance genes. TAM was crossed with susceptible cultivar, Rooikhaki, to produce F1 hybrids. These F1 hybrids were selfed to produce an F2 population. Infection trials using ToCSV were conducted using TAM inbred line, F1 hybrids and the F2 population. Since TAM did not have any of the known resistance genes to TYLCV-IL, a possible novel resistance source to ToCSV was speculated. A clue to the resistant mechanism against ToCSV resistance in TAM was indicated by the segregation patterns of the F2 population after inoculation with ToCSV. The results suggest that the resistance is under the control of partially dominant resistant genes.

The level of resistance of commercial South African tomato cultivars (Tyler and Tovi-star) against TYLCV-IL was investigated. The heat shock protein (HSP) profiles of these two SA lines including susceptible cultivar, Rooikhaki, were treated with abiotic stresses (salt and heat) and results were compared with a similar study conducted with TYCLV-IL resistant and susceptible tomato cultivars. Heat shock protein 70 accumulation patterns were similar in that HSP70 was more stable in the resistant cultivars throughout the application when abiotic stresses were applied to the SA resistant and susceptible tomato cultivars as compared to Israel resistant and susceptible breeding lines. A relation between infection severity and the pattern of HSP expression was found. A higher level of HSP 70 in resistant tomato plants could contribute to a lower symptom severity phenotype.

DEDICATION

In memory of a great man, my Dad

João Afonso Dias

1943 – 2006

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RESEARCH OUTPUTS

Conference Presentations

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ABBREVIATIONS

ACMV	*African cassava mosaic virus*
APS-1	Acid phosphatase
BCTV	*Beet curly top virus*
BGYMV	*Bean golden yellow mosaic virus*
BYDV	*Bean yellow dwarf virus*
CAPS	Cleaved amplified polymorphic sequence
CP	Coat protein
CR	Common region
DIG	Digoxigenin
dsDNA	Double stranded DNA
dpi	Days post inoculaion
DSI	Disease severity index
EACMV – [UG]	*East African cassava mosaic virus*
ICTV	International Committee on Taxonomy of Viruses
IR	Intergenic Region
Kb	Kilobases
Kbp	Kilobase pairs
MAS	Marker assisted selection
nm	Nanometers
nt	Nucleotide
NSP	Nuclear shuttle protein
ORF	Open reading frame
ori	Origin of replication
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
QTL	Quantitative trait locus

RCR	Rolling circle replication
RDR	Recombination-dependent-replication
REn	Replication enhancer protein
REP	Replication – associated protein
RFLP	Restriction fragment length polymorphism
SCAR	Sequence – characterised amplified region
SIR	Short intergenic region
ssDNA	Single stranded DNA
TrAP	Transcriptional activator protein
ToCSV	*Tomato curly stunt virus*
TYDV	*Tobacco yellow dwarf virus*
TYLCV	*Tomato yellow leaf curl virus*

BACKGROUND AND MOTIVATION FOR THE STUDY

Tomato (*Solanum lycopersicum*) is an important vegetable in the staple diet of the local population in SA. Additionally, in the neighbouring countries such as Mozambique and Zimbabwe it is also integrated into the maize meal diets as an essential supplement. Frequently, the crop is cultivated by subsistence and resource poor farmers and is consequently one of the key vegetables used for trading locally in the markets. Furthermore, tomato crops are grown commercially, producing employment opportunities for a large number of people. As reported by the Department of Agriculture in 1998, approximately 5465 hectares of tomato were planted, creating direct employment for 16 295 citizens (Department of Agriculture, 2003).

A novel disease affecting tomato plants emerged during 1997 in the tomato producing areas of the Onderberg region of Mpumalanga, South Africa (Pietersen *et al.,* 2000). Since the first report, in 1997, the disease has extended further into tomato growing regions. By March 2000 it was found in the Kwazulu-Natal/Swaziland region (Pietersen *et al.,* 2000). From there, the virus has extended through to Limpopo, Mpumalanga and East London (L. Esterhuizen, personal communication). In addition, Mozambique has also been affected by this newly described geminivirus, *Tomato curly stunt virus* (ToCSV-[ZA:Ond:98]) (Pietersen *et al.,* 2008).

ToCSV is genetically closely related to the *Tomato yellow leaf curl virus* (TYLCV) complex which originated from Israel and the Middle East. However, phylogenetic analysis has revealed that ToCSV is most closely related to *Tobacco leaf curl Zimbabwe virus* (TbLCZV), at 84% nucleotide identity which signifies that ToCSV is a new species in the genus *Begomovirus* (Pietersen *et al.,* 2008). Tomato plants infected with ToCSV show symptoms similar to those induced by TYLCV, including upper leaf yellowing, leaf curling, progressively stunted internodes and reduced fruit set. Up to 100% yield losses have been reported (Pietersen *et al.,* 2000).

ToCSV is transmitted by the whitefly *Bemisia tabaci*, biotype-B; a recent introduction to the tomato producing areas of SA (Brown, 2000). The disease is rapidly spread over great distances by the vector (Cohen et al., 1988); subsequently the control of the spread of ToCSV mainly involves the application of insecticides. However, repeated insecticide use to control *B. tabaci* populations has often resulted in the development of resistance by the insects (Dittrich *et al*., 1990). Genetic resistance in the host plant is a perfect defence against whitefly–transmitted viruses as no chemical contribution is needed and can potentially be long-lasting and stable.

Therefore, the most appealing way to reduce the spread of geminiviruses is by breeding tomatoes that are resistant or tolerant to the viruses. In view of the fact that all tomato (*S. lycopersicum*) cultivars

are particularly vulnerable to TYLCV and other begomoviruses, wild tomato species have been screened for their reaction to the virus. Resistant genes were found and using plant breeding techniques, molecular markers that are linked to the resistant genes have been developed (Ty-1, Ty-2, Ty-3/3a, Ty-4 and Ty-5) (Zamir *et al*., 1994; Hanson *et al*., 2000; Ji and Scott, 2007b; Ji *et al*., 2008; Anbinder *et al*., 2009). These resistant genes have been introgressed into the cultivated tomato, *S. lycopersicum,* using plant breeding and molecular assisted selection (MAS, which includes the use of molecular markers). Following years of breeding efforts elite commercial TYLCV-resistant tomato hybrids are available (Lapidot and Friedmann, 2002).

Little is known about this new ToCSV strain in SA and Mozambique, and resistance or susceptibility screens to this virus are not known. The motivation for this study was to identify the type of resistance of a local inbred line (TAM) that shows a good resistance to TYLCV in Jordan and a high degree of tolerance to ToCSV in this study in SA. The attractive point about TAM is the fact that it has no known Ty-resistance gene markers, and yet shows tolerance/resistance to TYLCV and ToCSV Therefore, in an attempt to reveal the resistance mechanism and the number of genes involved in the resistance, test crosses of TAM with a susceptible line (Rooikhaki) were made and the results investigated.

Specific Aims

-Identify inbred lines from seed company, Sakata Pty (Ltd), breeding program with recessive resistance to ToCSV/TYLCV by inoculation with viruliferous whiteflies.

-Confirm using molecular markers that inbred lines have none of the known *Ty* - genes and evaluate each line for segregation.

-Study the genetics of the resistance to see if it is monogenic or polygenic by making crosses of the inbred lines with each other as well as with a resistant and susceptible line.

-Use the resistant (Tovi-star) and susceptible line (Rooikhaki) to investigate changes in heat shock proteins, which have shown to be involved in induced and basal defence responses in plants when the plants are exposed to ToCSV-[ZA:Ond:98] infection.

-Conduct an infection trial using commercial resistant varieties developed in SA which show a good level of tolerance to ToCSV and infect with TYLCV and ToCSV-[ZA:Ond:98] to make a comparison of the severity of the two viruses.

CHAPTER 1. GENERAL INTRODUCTION

1.1. Tomato, the crop

Worldwide, tomato (*S. esculentum*) is one of the most important vegetables grown. It belongs to the *Solanaceae* family which also contains other familiar crops like potato, peppers and egg-plant (aubergine) (Kalloo, 1991). The wild tomato species has its origins from the South American Andes. It was first brought to Europe by the Spanish during the sixteenth century and soon after introduced to southern and eastern Asia, Africa, the Middle East, South America and Mexico. Thousands of tons of fresh tomatoes are harvested in South Africa on a yearly basis, by subsistence and resource poor farmers, but mainly by commercial farmers (Department of Agriculture, 2003). Tomatoes are extensively used as a fresh vegetable and in the form of an onion-tomato-amaranth stew to supplement the local diet of maize meal. Furthermore tomatoes grown commercially produce employment opportunities to thousands of South Africans. As reported by the Department of Agriculture in 1998, approximately 5465 hectares of tomato was planted, consequently creating direct employment for 16 295 citizens (Department of Agriculture, 2003).

1.1.1. Health benefits of tomatoes

Tomatoes form part of a balanced, cancer-preventing and heart-healthy diet. They are loaded in minerals, vitamins, essential amino acids, sugars and dietary fibres. Most importantly, tomatoes are rich in carotenoids, especially lycopene (Beecher, 1998; Leonardi *et al.,* 2000). Lycopene has been broadly researched for its antioxidant properties, and in humans, it was found to have cancer-preventing properties against a list of cancers. In addition to the antioxidant function of lycopene, which has the capability of protecting the body's cells from oxygen damage and has been correlated to the prevention of heart disease (Agarwal and Rao, 2000).

Tomatoes also contain a great amount of conventional nutrients such as vitamin A and vitamin C, both being antioxidants, subsequently decreasing inflammation and the succession of atherosclerosis (Leonardi *et al.,* 2000). Being a great source of fibre, tomatoes help prevent colon cancer, and by preventing the blood sugar levels from increasing to dangerously high levels, they help with diabetic complications as well and lower high cholesterol levels too (Leonardi *et al.,* 2000). Tomatoes are a very good source of chromium, folate, niacin, potassium and vitamins B6 and K. Vitamin B6 and folate are necessary to convert homocysteine, a possible hazardous chemical, into harmless components. This is imperative since elevated levels of homocysteine are linked to an increased risk of heart attacks and strokes, since the reason that the blood vessel walls are damaged with such high levels. Chromium intake is especially important in diabetic patients to keep their

blood sugar level steady (Rao and Rao, 2003; Leonardi *et al.,* 2000). Niacin lowers high cholesterol levels and has been safely used for this purpose for many years. Potassium lowers blood pressure and reduces the risk of heart disease. Vitamin K is important in sustaining bone health, by anchoring the calcium molecules inside the bone for the correct mineralization to take place (Rao and Rao, 2003).

1.2. Diseases infecting tomato

The global tomato industry is of significant value, with a total annual production of 70 million tons cultivated on 3 million hectares (Rosello *et al.,* 1996). The tomato crop is susceptible to over 200 diseases caused by fungi, bacteria and viruses (Kalloo, 1991). Some important fungal pathogens of tomato include fusarium wilt, caused by *Fusarium oxysporum* f. sp *lycopersici* Sacc. and verticillium wilt caused by *Verticillium albo-atrum* and *Verticillium dahliae* (Kalloo, 1991). In addition, a large number of bacteria are able to infect tomato crops. A few of the diseases caused by bacteria include: Bacterial canker, which is caused by *Clavibacter michiganensis* spp. *michiganensis, Pseudomonas syringae* pv. *tomato* is responsible for bacterial speck of tomato and bacterial spot is caused by *Xanthamonas campestris* pv*. tomato*. These and the many other bacterial diseases are the most economically important in a large number of tomato growing regions of the world (Boudyach *et al*., 2001; Ji *et al*., 2006b). Viral diseases can also cause massive losses to the tomato producing industry (Pico *et al*., 1996; Rosello *et al*., 1996). Many viral diseases of tomato exist, including *Tomato chlorotic mottle virus*, *Tomato golden mosaic virus*, *Tomato rugose mosaic virus* (Abhary *et al.* 2007), *Tomato mottle virus* (Simone *et al*. 1990), *Tomato spotted wilt virus* (German *et al.,* 1992), *Pepino mosaic virus* (van der Vlugt *et al.,* 2000) to name just a few. Several of the most destructive viral diseases of the tomato crop belong to the family *Geminiviridae.* Within this family, *Tomato yellow leaf curl virus* (TYLCV) has been responsible for yield losses of up to 100% in many tropical and subtropical regions (Cohen and Antignus, 1994).

1.3. Geminiviruses

Geminiviruses are plant infecting DNA viruses consisting of a small genome size ~ 2.6 – 3.0 kilobases (kbp) (Gutierrez, 2000). Viruses of the *Geminiviridae* family are insect-transmitted and can infect both monocots and dicots (Moffat, 1999; Gutierrez *et al.,* 2004; Mansoor *et al.,* 2006). Geminiviruses are characterized by one or two circular single-stranded DNA genomes packaged within a geminate virion particle (Gutierrez, 2000; Fauquet *et al.,* 2003). The virion particle is approximately 18-30 nanometers (nm) in size and the single stranded DNA (ssDNA) molecules are 2.5 – 3.0 kbp in length (Gutierrez *et al.,* 2004). Based on genome organisation, host range and insect vectors Geminiviruses

are divided into four different genera (Briddon et al., 1996; Rybicki *et al.,* 2000; Gutierrez et al., 2004). These included *Mastrevirus, Curtovirus, Topocuvirus and Begomovirus* (Fauquet *et al.,* 2008). *Mastrevirus* includes the viruses that have a monopartite genome, consisting of a single DNA component, and are transmitted mainly to monocotyledenous plants by leafhopper vectors, with *Maize streak virus* as the type species of this genera (Fauquet *et al.,* 2003). With the exceptions: *Tobacco yellow dwarf virus* (TYDV) and *Bean yellow dwarf virus* (BYDV), which infect dicotyledenous plants (Gutierrez, 2000). *Mastreviruses* contain two virion-sense and two complementary sense open reading frames (ORFs) with one long intergenic region (IR) and one short intergenic region (SIR) (Gutierrez, 2000). *Curtoviruses* are also transmitted by leafhoppers to dicotyledenous plants. The type species is *Beet curly top virus* (BCTV). Their monopartite genomes consist of three ORFs in the virion-sense with one intergenic region (Gutierrez, 2000). The genus *Topocovirus* has one associated virus namely *Tomato pseudo-curly top virus* (TPCTV), which is transmitted by a treehopper vector to dicotyledenous plants. TPCTV monopartite genome has two ORFs in the virion-sense and two in the complementary-sense. The fourth genus is *Begomovirus,* of which the type species is *Bean golden yellow mosaic virus* (BGYMV) (Varma and Malathi, 2003). Most begomoviruses have bipartite genomes consisting of two DNA components: DNA–A and DNA–B Figure 1.1 and 1.2). However, there are a small number of species like *Tomato yellow leaf curl virus* (TYLCV) that have a single genome corresponding to the DNA–A component of bipartite begomoviruses (Gafni and Epel, 2002). The DNA–A component encodes the coat protein (CP) and four other proteins which are involved in replication and encapsidation on the complementary strand. The DNA–B component encodes proteins involved in viral movement (Figure 1.2) (Gutierrez, 2000).

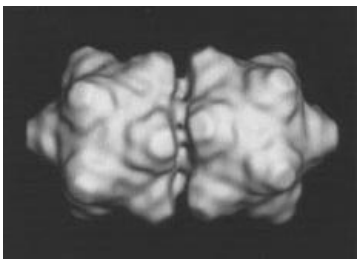

Figure 1.1: Diagram of twinned geminate particles which components vary in size between 2.5 – 3.9 kbp (From Zhang *et al.,* 2001).

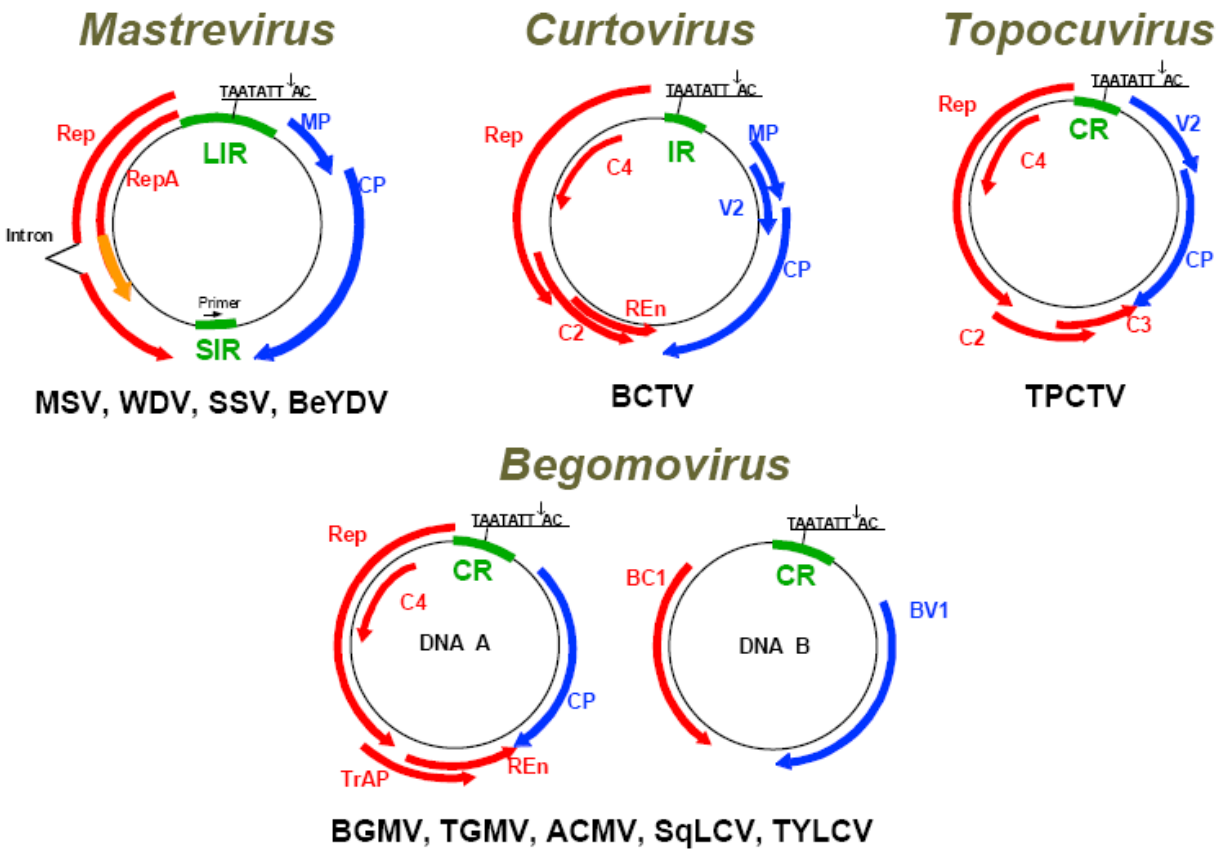

Figure 1.2: The organisations of the four genera that constitute the family *Geminiviridae* (Gutierrez *et al.,* 2004). DNA–A is composed of six ORFs of which CI–C4 ORFs are positioned on the complementary strand whereas the V1 and V2 ORFs are located on the virion-sense strand. C1 ORF encodes for the replication–associated protein (Rep) which is vital in replication of the virus. C2 ORF encodes for a transcriptional activator protein (TrAP) accountable for gene expression. C3 ORF encodes for a replication enhancer protein (REn) which, with Rep, boosts viral replication. V1 ORF encodes for the coat proteins (CP) involved in virus encapsidation and movement. V2 and C4 ORFs encode proteins that induce systemic viral movement as well as symptom initiation. The DNA–B component is composed of two ORFs, the V1 and C1. Open reading frame V1 encodes a nuclear shuttle protein (NSP) which is required for intracellular movement of the viral DNA between the nucleus and cytoplasm. C1 ORF encodes a movement protein essential for the transportation of the viral DNA between cells in the host. The common region (CR) which is approximately 200 bp is a non-coding region. It is made up of a stem-loop structure lined with GC-rich inverted repeats. This loop contains a nanonucleotide sequence (TAATATTAC), which is conserved among all geminiviruses and is the origin of replication (*ori*) (Pico *et al.,* 1996).

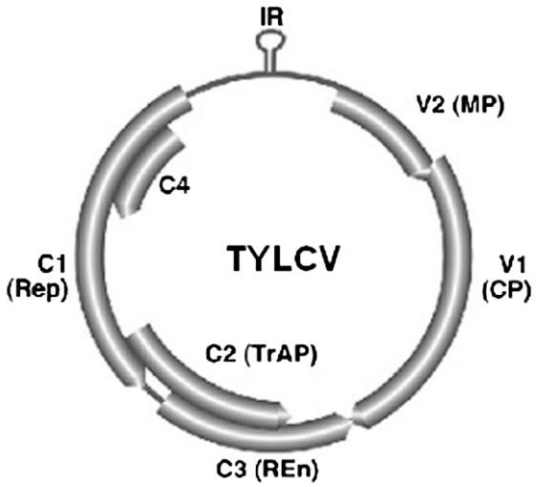

Figure 1.3: The genome of *Tomato yellow leaf curl virus,* a monopartite begomovirus transmitted by the whitefly, *Bemisia tabaci*. Genes of virion sense (V) or complementary sense (C) strand polarity are shown with IR-intergenic region with the conserved inverted repeat sequence TAATATTAC. V1 gene encodes the capsid protein (CP), V2 encodes a movement protein (MP), C1 encodes is the replication initiator protein (Rep), C2 encodes a transcriptional activator protein (TrAP) and C3 is a replication enhancer protein (REn) (Hanley-Bowdoin *et al.,* 1988).

1.3.1. Replication of Geminiviruses

Geminivirus replication is very similar to that of prokaryotic ssDNA phage's and plasmid replication (Baas and Jansz, 1988; Novick, 1998). The geminivirus replication cycle relies exclusively on DNA intermediates and takes place within the nucleus of infected cells. When the geminivirus is transported into host cells by insect vectors, they enter a life cycle of DNA replication, DNA accumulation and virus assembly and spread in the plant (Vanderschuren *et al.,* 2007). Virus movement into the nucleus is completely reliant on the coat protein (Gafni and Epel, 2002). From there, the process can be divided into three stages (Gutierrez, 2000). During the first stage, the ssDNA is converted into double stranded DNA (dsDNA). This product associates with cellular histones to form viral mini chromosomes. The second stage involves rolling circle replication (RCR) of the dsDNA intermediates. The initiator protein Rep, encoded by the C1 is the only virally encoded protein vital for this stage to take place. Rep protein initiates the replication process by introducing a

nick (/) within the 9 bp sequence (TAATATT/AC) which is conserved in all geminiviruses, and is located in the intergenic region (Figure 1.3) (Arguello-Astorga *et al.,* 1994; Stanley, 1995). Following the initiation of replication step, the essential factors needed to complete the rolling circle replication are of cellular origin (Rushing *et al.,* 1987; Horns and Jeske, 1991; Lucy *et al.,* 1996). The third stage in replication is the production and packaging of ssDNA products into viral particles. Once adequate amounts of CP and movement proteins build up, the virion particles are transported to the neighbouring cells, thereby spreading throughout the plant.

1.4. Begomoviruses

The genus *Begomovirus* is the largest genus of the *Geminiviridae* family and encompasses the whitefly-transmitted geminiviruses that infect dicotyledonous plants (Sawangjit *et al.,* 2005). Majority of begomoviruses originating in the New World (mostly from the American continents, including the Caribbean islands), have a bipartite genome made up of two circular ssDNA molecules, DNA–A (2.6kbp) and DNA–B (2.5-2.8kbp). Several others with monopartite genomes have been identified in the Old World (consisting of Asia along with the Indian subcontinent and Africa) (Kheyr-Pour *et al.,* 1991; Navot *et al.,* 1991). These viruses are spread by the whitefly, *B. tabaci* in a circulative and persistent manner. The first begomovirus identified with a single genome equivalent to the DNA –A component of bipartite begomoviruses was TYLCV from Israel (Gafni and Epel, 2002).

Begomoviruses infect many significant agricultural plants globally including bean, cassava, cotton, melon, pepper, potato, squash, tobacco, tomato and watermelon (Czosnek *et al.,* 2002). There are currently 133 officially recognized geminivirus species of which 117 belong to the genus *Begomovirus* (Stanley *et al.,* 2005; Fauquet *et al.,* 2008). Several of the most destructive viral diseases of the tomato crop belong to the family *Geminiviridae*. There are at a minimum, 57 different species of geminiviruses (most of them being begomoviruses) that are able to infect tomato (Table 1.1; Figure 1.5). Amongst the tomato–infecting monopartite geminiviruses, TPCTV is the only affiliate of the genus *Topocuvirus,* while *Tomato leaf roll virus* (ToLRV) is a member of the genus *Curtovirus,* and all remaining other tomato-infecting geminiviuses belong to the *Begomovirus* genus.

The intensification of agriculture and increases in *B. tabaci* populations have been proposed as one of the main causes of the rapid emergences of begomoviruses (Morales and Anderson, 2001; Varma and Malathi, 2003; Xie and Zhou, 2003). With an increase in vector populations worldwide, the associated begomovirus disease epidemics are prominent (Seal *et al.,* 2006). As a result of the

spread of a more prolific, polyphagous biotype of whitefly (B-biotype) serious epidemics have been reported in the western hemisphere and Europe since the 1980s. With this emergence, the spread of begomoviruses has amplified tremendously (Polston and Anderson, 1997; Varma and Malathi, 2003). Other fundamental factors influencing the epidemics include the evolvement of more destructive virus variants, increased movement of infected material and the introduction of more susceptible crop lines (Varma and Malathi, 2003).

Table 1.1: Geminiviruses capable of infecting tomato indicating the Genbank accession number abbreviation and distribution (Abhary *et al.,* 2007).

Virus isolates .	Accession number	Abbreviation	First report on distribution
Chino del tomate virus	DQ347945	CdTV	Mexico
Merremia mosaic leaf curl virus	AF068636	MeMV	Puerto Rico
Potato yellow mosaic Panama virus	Y15034	PYMPV	Panama
Potato yellow mosaic Trinidad virus	AF039031	PYMTV	Trinidad and T Tombago
Potato yellow mosaic virus	D00940	PYMV	Venezuela
Tomato chino La Paz virus	DQ347948	ToChLPV	Mexico
Tomato chlorotic mottle virus	DQ336353	ToCMoV	Brazil
Tomato curly stunt virus	AF261885	ToCSV	South Africa
Tomato golden mosaic virus	K02029	TGMV	Brazil
Tomato golden mottle virus	AF132852	ToGMoV	Guatemala
Tomato leaf curl Arusha virus	DQ519575	ToLCArV	Tanzania
Tomato leaf curl Bangalore virus	AF295401	ToLCBV	India
Tomato leaf curl Bangladesh virus	AF188481	ToLCBDV	Bangladesh
Tomato leaf curl China virus	AJ558119	ToLCCNV	China
Tomato leaf curl Guangdong virus	AY602165	ToLCGuV	China
Tomato leaf curl Guangxi virus	AM236784	ToLCGxV	China
Tomato leaf curl Gujarat virus	AY190290	ToLCGV	India
Tomato leaf curl Hsinchu virus	DQ866131	ToLCHsV	Taiwan
Tomato leaf curl Indonesia virus	AF198018	ToLCIDV	Indonesia
Tomato leaf curl Iran virus	AY297924	ToLCIRV	Iran
Tomato leaf curl Java virus	AB162141	ToLCJV	Indonesia
Tomato leaf curl Joydebpur virus	AJ875159	ToLCJoV	Bangladesh
Tomato leaf curl Karnataka virus	U38239	ToLCKV	India
Tomato leaf curl Laos virus	AF195782	ToLCLV	Laos
Tomato leaf curl Madagascar virus	AJ865338	ToLCMGV	Madagascar
Tomato leaf curl Malaysia virus	AF327436	ToLCMYV	Malaysia
Tomato leaf curl Mali virus	AY502936	ToLCMLV	Mali
Tomato leaf curl Mayotte virus	AJ865341	ToLCYTV	Mayotte
Tomato leaf curl New Delhi virus	DQ169056	ToLCNDV	India
Tomato leaf curl Pakistan virus	AB116884	ToLCPKV	Pakistan
Tomato leaf curl Philippines virus	DQ092867	ToLCPV	Philippines
Tomato leaf curl Sinaloa virus	AJ608286	ToLCSinV	Nicaragua
Tomato leaf curl Sri Lanka virus	AF274349	ToLCSLV	Sri Lanka
Tomato leaf curl Sudan virus	AY044137	ToLCSDV	Sudan
Tomato leaf curl Taiwan virus	U88692	ToLCTWV	Taiwan

Tomato leaf curl Uganda virus	DQ127170	ToLCUV	Uganda
Tomato leaf curl Vietnam virus	AF264063	ToLCVV	Vietnam
Tomato leaf curl virus	S53251	ToLCV	Australia
Tomato mild yellow leaf curl Aragua virus	AY927277	ToMYLCAV	Venezuela
Tomato mosaic Havana virus	Y14874	ToMHV	Cuba
Tomato mottle Taino virus	AF012300	TomoTV	Cuba
Tomato mottle virus	AF291705	ToRMV	Brazil
Tomato rugose mosaic virus	AF291705	ToRMV	Brazil
Tomato severe leaf curl virus	DQ347947	ToSLCV	Mexico
Tomato severe rugose virus	AY029750	ToSRV	Brazil
Tomato yellow leaf curl Axarquia virus	AY227892	TYLCAxV	Spain
Tomato yellow leaf curl China virus	AM050555	TYLCCNV	China
Tomato yellow leaf curl Guangdong virus	AY602166	TYLCGuV	China
Tomato yellow leaf curl Kanchanaburi virus	DQ169054	TYLCKaV	Vietnam
Tomato yellow leaf curl Malaga virus	AF271234	TYLCMalV	Spain
Tomato yellow leaf curl Mali virus	AY502934	TYLCMLV	Mali
Tomato yellow leaf curl Sardinia virus	Z28390	TYLCSV	Italy
Tomato yellow leaf curl Thailand virus	AF206674	TYLCTHV	Myanmar
Tomato yellow leaf curl Vietnam virus	DQ641697	TYLCVNV	Vietnam
Tomato yellow leaf curl virus	X15656	TYLCV	Israel
Tomato yellow margin leaf curl virus	AY508993	TYMLCV	Venezuela
Tomato yellow spot virus	DQ336350	ToYSV	Brazil

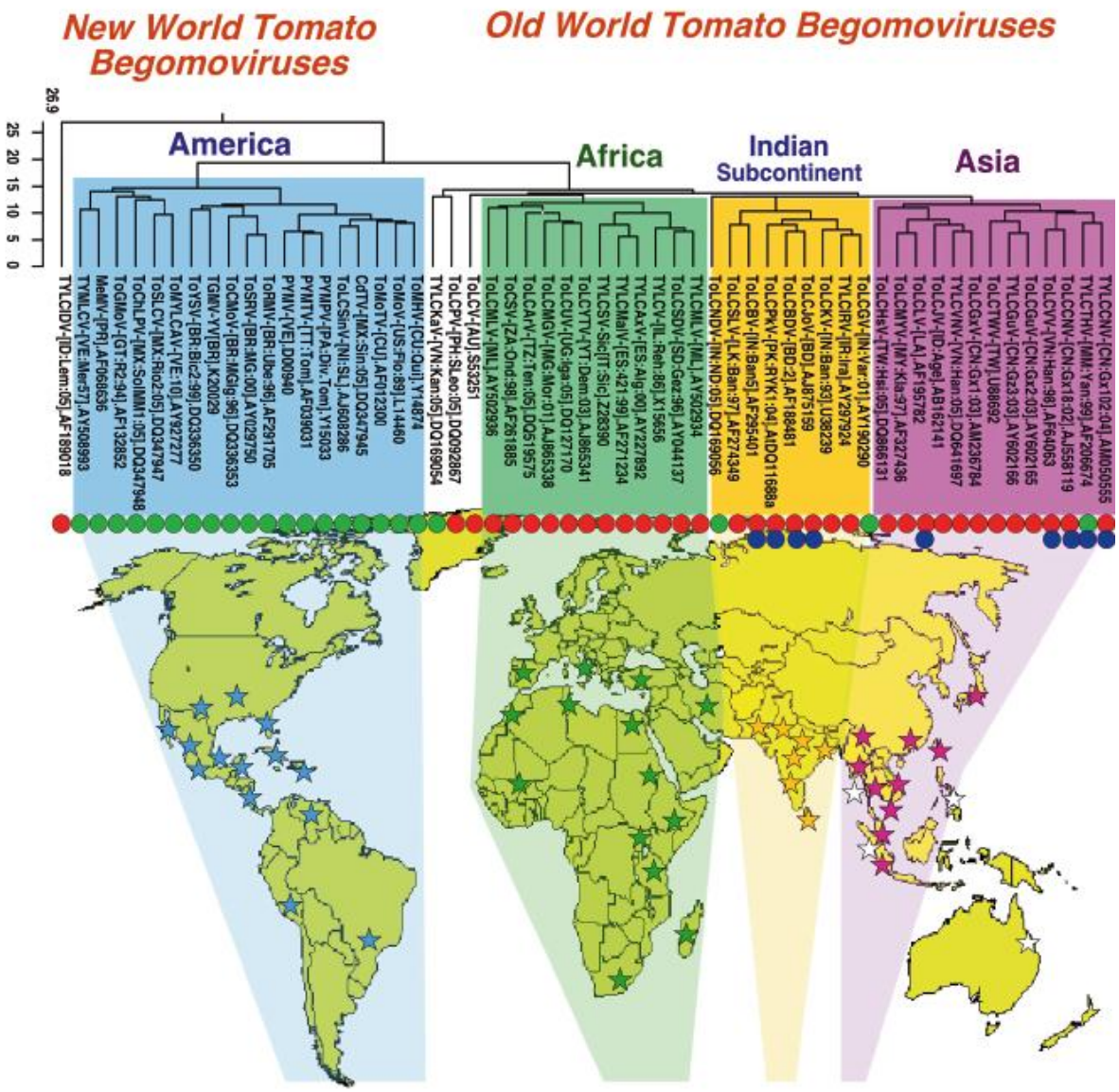

Figure 1.4: The geographical distributions of the 57 begomoviruses (Table 1.1) infecting tomato. They have been divided into two branches, namely: Old World tomato begomoviruses and New World tomato begomoviruses. Within these branches they are further divided into 4 clusters; America, Africa, Indian subcontinent and Asia. The stars on the continents represent the individual viruses and their locations. Monopartite viruses are represented with a red dot, whereas bipartite viruses have a green dot. There are 4 classified viruses (indicated by the white stars) that do not fit in the geographical positions stated namely: *Tomato yellow leaf curl Indonesia virus, Tomato yellow leaf curl Kanchanaburi virus - Vietnam, Tomato leaf curl Phillippines virus, Tomato leaf curl virus – Auatralia* and found in Indonesia, Indochina, Philippines and Australia respectively (Abhary *et al.,* 2007).

1.5. Recombination

Genetic diversity among viruses could occur through mutations or recombination events. Geminiviruses have used recombination as a means of having a selective advantage and perhaps result in the emergence of new geminiviral diseases (Padidam *et al.,* 1999). TYLCV-like viruses are dependent on the replication machinery of the host cell for their replication and transcription in the nucleus of infected cells (Hanley-Bowdoin *et al*., 1999). The production of new virion particles occurs either through RCR or the recombination-dependent-replication (RDR) and consequently promotes new recombinations（Preiss and Jeske, 2003) (Figure 1.4). Begomoviruses are able to bypass the repair mechanism as their replicative forms do not undergo methylation, allowing the inheritance of the mutations (Roossinck, 1997). Generally, the resulting recombinant is naturally selected with its robustness and superior acquisition by the whitefly vector (Monci *et al.,* 2002).

The earliest account of recombination in begomoviruses was when a recombinant virus, *East Africa cassava mosaic virus* – Uganda (EACMV-[UG]) was found to be associated with cassava mosaic pandemic that has been spreading in East Africa since the late 1980s (Zhou *et al.,* 1997; Legg, 1999). The EACMV-[UG] virus originated predominantly from EACMV but a part of its coat protein gene was derived from *African cassava mosaic virus* (ACMV) (Zhou *et al.,* 1997). This is seen as the most devastating genomic recombination event amongst the begomoviruses (between EACMC and ACMC) giving rise to the virulent EACMV-Uganda variant, which has caused a pandemic in East Africa (Harrisson and Robinson, 1999). Mixed infections are a pre-requisite for recombination and are common in geminivirus diseases (Padidam *et al*., 1999). Due to the broad feeding habits of the whiteflies, the geminiviruses they carry are introduced into a large number of plant hosts, many of them already containing other geminiviruses and thus their coexistence in the same plant host gives considerable opportunity for recombination to occur. Other factors that contribute to geminiviruses having a high tendency for recombination include high levels of replication since geminiviruses replicate via a double stranded replicative form and achieve high copy numbers, and an increased host range which has extended immensely due to the biotype B whitefly that feeds on hundreds of host species (Padidam *et al*., 1999).

1.6. Tomato Yellow Leaf Curl Virus Disease

Tomato yellow leaf curl disease (TYLCD) is one of the most devastating plant diseases in the world and is present in 20 countries across the world. Several viral species make up this disease, which is referred to as Tomato yellow leaf curl disease (TYLCD). These belong to six species and fifteen strains of geminiviruses (Fauquet *et al*., 2003).

TYLCD is responsible for enhanced damage to tomato crops and is invading new areas every year. In the 1930s, TYLCD was first identified in Israel and since the 1960s, has become the most infamous disease in the Mediterranean region, sub-Saharan Africa, Caribbean islands, Australia, and in several Asian countries like China, India, and Japan (Czosnek and Laterrot, 1997). Reports of this disease from several US states in the 1990s were recorded (Polston *et al.,* 1999). Numerous geminivirus species belonging to the genus *Begomovirus* have been related to TYLCD, and they are also called TYLCV-like viruses. They are naturally transmitted by the whitefly (*Bemisia tabaci* Gennadius Hemiptera: *Aleyrodidae*) and have a monopartite ssDNA genome (Moriones and Navas-Castillo, 2000). TYLCV was the first identified virus and named after the disease it caused in Israel (Czosnek and Laterrot, 1997). Geminiviruses causing similar symptoms on tomato crops across the globe are referred by the same or similar names. Later, it became apparent that different virus species were causing similar symptoms on tomato crops; therefore, new names were created for these (Mohammad *et al*. 2007). TYLCV and its virus relatives belong to the genus *Begomovirus*, family *Geminiviridae*. Their genomes are strictly monopartite (Kheyr-Pour *et al.,* 1991; Navot *et al*., 1991) and encode six ORFs (Figure 1.3) (Navot *et al*., 1991).

1.7. *Tomato curly stunt virus*: a begomovirus from South Africa

A new destructive TYLCV-like viral disease of tomatoes emerged during 1997 and 1998 in the Onderberg region of South Africa (Pietersen e*t* al., 2000; 2008). The affected tomato plants show symptoms similar to those of TYLCV, such as leaf curling, stunting, foliar chlorosis and reduced fruit set (Pietersen *et al.*, 2000). The ToCSV name was based on observed field symptoms in tomato plants and this novel begomoviral species was called ToCSV-[ZA:Ond:98] (Pietersen *et al.,* 2000) genome sequence analysis of the core coat protein (CP) (GenBank AF261885), which shared less than 86% nucleotide (nt) identity with the most closely related begomovirus *Tobacco leaf curl Zimbabwe virus* (TbLCZV). ToCSV-[ZA:Ond:98] was shown to be transmissible by the biotype B whitefly *B. tabaci*, a recent introduction to the tomato-producing regions of South Africa (Brown, 2000).

The control of ToCSV and TYLCV has been majorly based on insecticide applications against *B. tabaci*. Reducing the use of chemical treatments limits the risk for pesticide contamination for farmers and consumers. The use of resistant varieties is an alternative method to control the effect of the virus on the tomato plants. Classical breeding for disease resistance has been used since the early 20th century as a major method for the control of plant diseases (Scott, 2005). Breeding programs for tomato mainly focused on the transfer of resistance genes from wild *Solanum lycopersicon*, *S. peruvianum* and *S. chilense* (Pilowsky and Cohen, 1974; Michelson *et al.,* 1994) into the

domesticated tomato, with the aim to produce a resistant hybrids with acceptable fruit (Scott *et al.,* 1995; Vidavsky and Czosnek, 1998; Chen *et al.,* 2003; Naraseqowda *et al.,* 2003; Mejia *et al.,* 2005). Selection for TYLCV resistance is based on the absence of symptoms in infested fields (Vidavsky and Czosnek, 1998). These methods involve growing plants to maturity in a field that has been shown to produce 100% infection of susceptible plants (Maxwell *et al.* 2006). Each cycle is time consuming and there can be an incorrect diagnosis of plant resistance due to escapes, therefore in order to facilitate the breeding efforts, molecular markers for resistance genes have been identified. Molecular markers can be used to track the resistance gene through successive generations with polymerase chain reaction (PCR). The available molecular markers for TYLCD are named Ty-1, Ty-2, Ty-3, Ty-3a, Ty-4 and Ty-5 (Zamir *et al*., 1994; Hanson *et al*. 2000; Ji and Scott, 2007a; Ji *et al*., 2008; Anbinder *et al*., 2009).

1.7.1. Epidemiology of *Tomato curly stunt virus*

Tomato curly stunt virus disease occurred mainly around the Strydomblok District close to South African-Mozambique border (Pietersen *et al.,* 2000). It was noted that by March 2000 the disease had spread to the Kwazulu-Natal/Swaziland region (Pietersen and Smith 2002). From there, the virus has extended through to Limpopo in the past few years. It was also identified in Mpumalanga area as of 2006. Finally a recent introduction into the Eastern Cape in 2009 has been reported (Esterhuizen, unpublished). Within the past 10 years, ToCSV-[ZA:Ond:98] has made its way down the east coast of SA (Figure 1.5). The route the virus is taking seems to indicate that it will make its way into the western parts of South Africa soon.

Figure 1.5: The outbreaks of *Tomato curly stunt virus* in South Africa during the period from 1997 to 2009 (Esterhuizen, unpublished).

1.7.2. Disease symptoms and variation

ToCSV causes infected plants to show similar symptoms of TYLCD disease. Infected plants show upper leaf yellowing, reduction in size with curling margins and progressively stunted internodes (Figure 1.6). The flowers are abscised shortly after formation therefore no fruit is borne in the affected tissue, resulting in severe tomato yield losses (up to 100%) (Pietersen *et al.*, 2000).

Variation may occur due to different factors like the host genetic background and the fact that resistant plants have a lower virus titre than susceptible plants (Lapidot *et al*., 2001). Therefore resistant plants will show less severe symptoms than susceptible plants. Environmental conditions affects symptom variation. Infection during cooler conditions (winter) leads to milder symptoms as opposed to infection during the summer time. Normal physiological processes can be repressed while the plants cope with adverse environmental conditions, rendering them more susceptible to viruses. Lapidot and Levy (2008) experimented with different aged tomato seedlings and inoculated them with TYLCV-IL to determine the effects of plant age on the expression of genetic resistance to the virus. They found that plant age at the time of inoculation had no effect on the disease-severity score of susceptible varieties, and a minute effect on the disease-severity score of resistant varieties. In contrast, plant age at the time of inoculation had a significant effect on the total yield of all of the varieties tested, susceptible and resistant plants. Virus-induced yield reduction is the ultimate test for resistance. All tested varieties suffered a significant yield reduction due to inoculation with TYLCV. The lowest yield was produced by plants inoculated at 14 days after sowing (DAS). The susceptible varieties produced essentially no yield following inoculation at 14 DAS, whereas that produced by the resistant varieties varied according to their resistance level.

Figure 1.6: Typical symptoms of a susceptible tomato cultivar,

Rooikhaki, to *Tomato curly stunt virus*.

1.7.3. The whitefly vector – *Bemsia tabaci*

There is approximately 1300 whitefly species, from the *Aleyrodidae* family, in more than 120 genera that have been described; however, only a small number of them spread plant viruses (Byrne and Bellows, 1991). Three species are known as plant virus vectors, namely: *B. tabaci, Trialeurodes vaporariorum* and *T. abutilonia* (Figure 1.7). The most significant of the species, *B. tabaci,* can transmit 100 different viral diseases, belonging to the *Begomovirus* genus, in the tropics and subtropics (Jones, 2003). *Bemisia tabaci* is a pest and virus vector on all continents where agriculture is practiced, where it colonizes agronomically important crops. *Bemisia tabaci* and its different biotypes has become an invasive species, extending their geographic and host range beyond formerly endemic borders. This recent occurrence has been through introductions of *B. tabaci* transported on plants by global trade. The most prevalent and damaging diseases caused by the whitefly is the spread of those from the genus *Begomovirus*, to which all TYLCV strains and species belong (Brown, 2007).

Figure 1.7: A comparison of the two different types of whiteflies; (a) *Trialeurodes vaporariorum* whiteflies are bigger, whiter and their wing positions form a heart shape from above (Bennison, 2001), (b) *Bemisia tabaci* whiteflies are smaller in size, their coloring is more yellow and they hold their wings parallel to their bodies when at rest (Bennison, 2001), (c) both types of whiteflies feed from the underside of the tomato leafs (Esterhuizen, personal communications).

Whiteflies occur in warm climates where they are pests of herbaceous woody plants and are usually pests of protected crops. There are different types of damage caused by whitefly feeding on a plant namely;

- **i) Direct damage**: excessive sap removal from leaves reduces host vitality, growth rate, yield, and causes weakening as well as early wilting of the plant resulting in plant death (Schuster *et al*., 1990; Byrne and Bellows, 1991).

- **ii) Indirect damage**: emission of a sticky secretion (honeydew) which affects leaves functioning and promotes growth of a sooty mould on leaves which then impairs the full functioning of the leaf (Schuster *et al*., 1990; Byrne and Bellows, 1991).

- **iii) Virus transmission** – includes the spread of TYLCV like viruses (Schuster *et al*., 1990; Byrne and Bellows, 1991).

Feeding habits of the whitefly permit transmission of these viruses to other plants (Figure 1.8). The majority of begomoviruses are limited to the phloem of infected plants. Consequently, in order for *B. tabaci* to acquire a begomovirus from an infected plant or to transmit a begomovirus to a host plant, its stylet needs to penetrate between the epidermal and parenchymal cells before piercing the vascular tissues and reaching the phloem that they feed on (Pollard, 1955) (Figure 1.8).

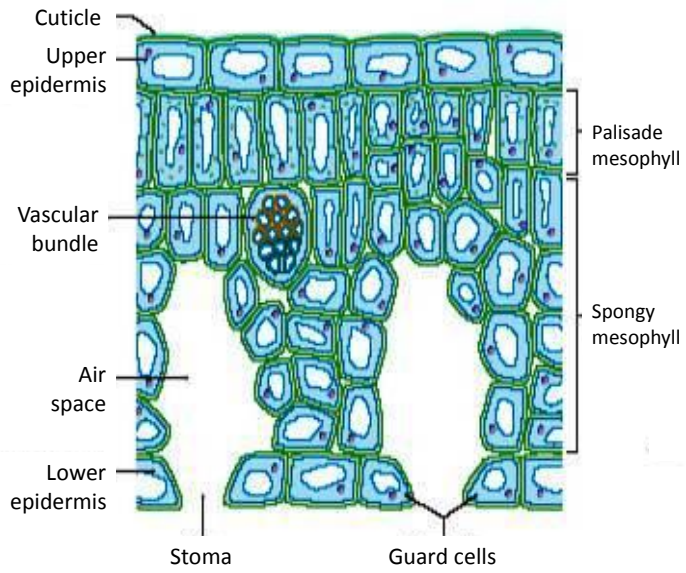

Figure 1.8: A cross section of a plant leaf showing the arrangement of tissues. The vascular bundle is made up of the xylem and phloem. The xylem cells are found in the centre of the bundle and the phloem surrounds these cells. The xylem distributes water to the tissues, the phloem absorbs the sugars produced by photosynthesis and the epidermis retains water in the leaf while permitting the absorption of carbon dioxide (Mauseth, 1988). The whiteflies stylet penetrates

through the cuticle, upper epidermis, palisade mesophyll and into the vascular bundle.
(Image from http://leavingbio.net/TRANSPORT%20OF%20MATERIALS%20IN%20A%20FLOWERING%20PLANT.htm)

An individual whitefly is capable of acquiring TYLCV and transmitting it to host tomato plants. The reported minimum acquisition access period (AAP) (the time that it takes for the insect to acquire the virus into its system) and inoculation access period (IAP) (the time that it takes in order to transmit the virus onto another host while feeding off of it) of TYLCV isolates by *B. tabaci* biotype B was found to be 15 min and 30 min, respectively (Cohen and Harpaz, 1964; Mansour and Al-Musa, 1992; Mehta *et al*., 1994).

1.7.4. Circulative transmission of viruses by *Bemisia tabaci*

Female whiteflies were found to have sixfold higher transmission efficiency than their male counterparts (Cohen and Nitzany, 1966). The virus is circulative and persistent in the whitefly (Cohen and Nitzany, 1966). Once the insect feeds on an infected host plant and obtains the virus, viral spread is rapid, within a few hours, and may continue for the vector's life span. The latent period was found to be from 21 to 24 h. TYLCV transmission efficiency by its vector declines with time (Cohen and Nitzany, 1966). It was found that following TYLCV acquisition, viruliferous whiteflies progressively lose infectivity and about 10 days after completion of the acquisition feeding period, most of the insects are no longer able to transmit the virus. In addition to acquisition by adults, the virus is also acquired by the whitefly larval stages (Cohen and Harpaz, 1964).

TYLCV is transmitted in a circulative manner. *Bemisia tabaci* possesses a stylet that penetrates the plant tissues to reach the phloem from where sugars are transported into the insect body. By their feeding habits of withdrawing plant sap, viruses are easily acquired into the insect's system. Once the virus particles have been consumed from the plant phloem of infected plants, the virions cross the midgut wall barrier, are transported through the haemolymph to the salivary glands and from this point they are once again passed out during feeding, infecting a new host plant (Figure 1.9) (Ghanim *et al.,* 2001).

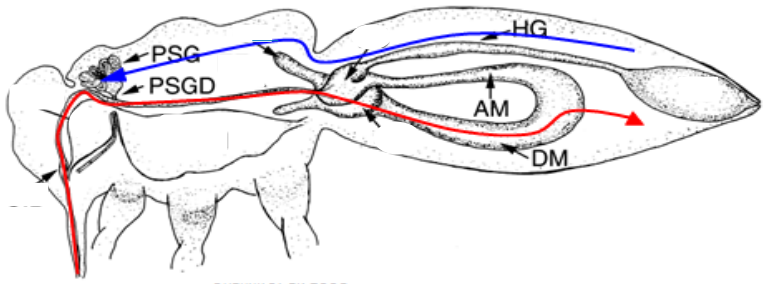

Figure 1.9: The circulative travel route that Begomovirus particles take inside *Bemisia tabaci.* The red arrow shows the uptake of the virus particles, this starts at the stylet, when the insect is feeding on an infected plant. The virions travel into the insect's midgut from which it crosses the midgut wall barrier into the haemolymph. From here, the blue arrow shows the route that the virus particles take through the haemolymph back to the primary salivary gland. When the whitefly feeds on another plant the virions are passed out through the primary salivary gland duct. AM indicates ascending midgut, DM the descending midgut, HG the hindgut; PSG the primary salivary gland and PSGD the primary salivary gland duct Image from Ghanim *et al.* (2001).

1.7.5. Lifecycle of *Bemisia tabaci*

The whitefly lifecycle progression from egg to adult is dependent mainly on temperature. In warm temperatures the whole life cycle may take up to 3 weeks (Figure 1.11), however, in cool weather, it may last up to two months, and if the temperature drops below 17°C, adults will not emerge. The reproduction rate of whiteflies is approximately 160 eggs per female (Byrne and Bellow, 1991).

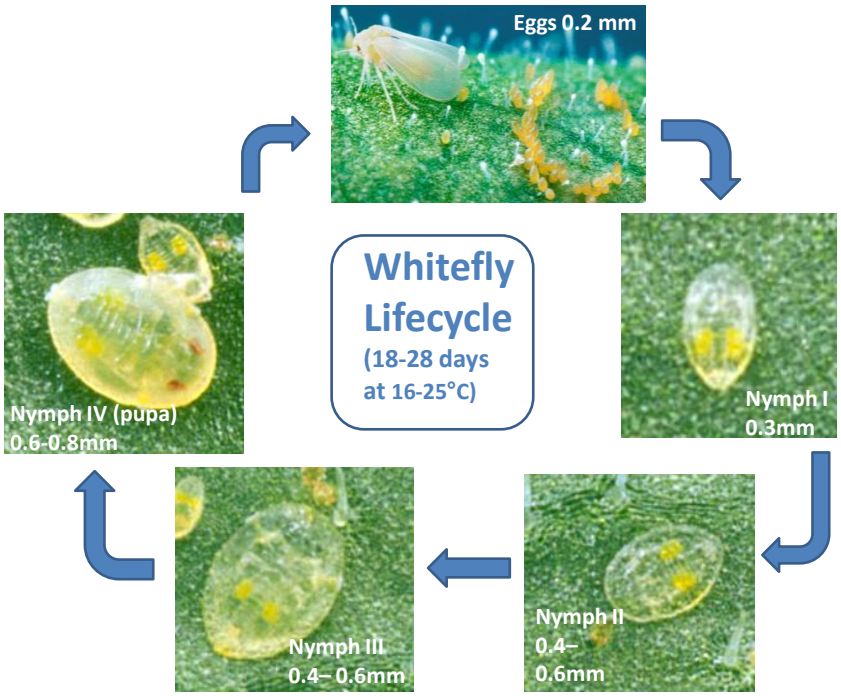

Figure 1.10: The lifecycle of the whitefly (*Bemisia tabaci*). A female may lay roughly 400 eggs during her lifetime. Once the eggs are laid, they go through four different instars before the flying adult insect emerges, ready to spread diseases. Unfertilized eggs give rise to haploid males, while fertilized eggs develop into diploid females. Image from Horowitz and Gerling, (1992).

1.7.6. ToCSV Management

Throughout the last 15 years, TYLCV has been a severe problem for tomato production areas in many parts of the world. In Israel, the virus has been recognized for over 40 years and a large amount of research has been done on the virus (Cohen and Nitzany, 1966). Tomato crops are highly affected in Israel by outbreaks of TYLCV and regardless of daily spraying with insecticides, 100% yield losses have repeatedly been recorded in cases where the whitefly populations were high (Cohen and Antignus, 1994). ToCSV, a relatively new species amongst the many geminiviruses causing leaf curl disease in tomato was identified in SA just over 12 years ago (Pietersen *et al.,* 2000). In SA, yield losses on individual plants ranged from negligible to 100%. (Department of Agriculture Brochure, 2003).

Even though ToCSV is a newly described geminivirus in SA and Mozambique, and minimal information is known about its epidemiology, control strategies applied for TYLCV are likely to be helpful for the control of ToCSV. This suggestion is made based on the fact that: 1.) ToCSV is transmitted by *B. tabaci* biotype B whitefly, as with TYLCV, and has similar transmission properties to TYLCV, 2.) both viruses have similar host ranges, 3.) ToCSV causes a very similar disease to TYLCV on tomato crops, 4.) numerous different tomato cultivars tolerant to TYLCV are also tolerant to ToCSV, 5.) the sequence similarity of the genomes of the two viruses, when compared, is 77% identical.

The control of TYLCV in tomato crops is complicated and costly. Frequently, management techniques are not sufficient and great economic losses are incurred. Numerous approaches have been used in an attempt to decrease losses due to the virus even though only a few are usually effective and some cannot be used in all climates and locations. There is no particular approach to effectively manage TYLCV, therefore combinations of chemical and cultural practices are applied to reduce the number and movement of the whitefly vector and minimize or eliminate inoculum sources (Polston and Lapidot, 2007).

The management approaches used in Florida and Israel for the control of TYLCV are as follows:

- Avoidance in time and space: Planting dates and locations that avoid elevated whitefly populations will have a considerable impact on the prevalence of TYLCV-infected plants. When TYLCV infects plants later in their development, the impact on their yield is significantly reduced. New tomato plantings should not be positioned near any crops that are known or suspected of being hosts of TYLCV nor, should they be located near older fields of tomato, whether they were susceptible or resistant cultivars as the resistant plants act as viral reservoirs for the virus (Lapidot *et al.,* 2001).

- Reflective and yellow plastic mulches: the most successful reflective mulches are partly or totally aluminized and reflect a great deal of daylight, including visible and UV light. This disorients the whiteflies and prevents their landing on the tomato plants. Plastic yellow mulches attract the whiteflies, and subsequently results in their death owing to dehydration induced by the high temperature of the mulch (Cohen and Melamed-Madjar, 1978).

-Physical barriers: Whitefly-proof screens are fine mesh screens over plastic houses that physically inhibit the whiteflies from entering into the greenhouses. UV absorbing plastics and screens when used as greenhouse covers or insect-proof nets, reduce the intensity of UV light and blind the whiteflies which use the UV wavelengths to navigate. These UV absorbing films have been shown to inhibit infiltration of whiteflies into and movement within greenhouses (Antignus *et al.,* 1996).

-Production and use of virus-free transplants: Virus-free transplants should be used in the beginning of the production season. This can be done by acquiring or creating tomato transplants in remote areas away from production fields. The incidence of infected seedlings decreases greatly with the larger the distance between production areas and nurseries (Cohen and Berlinger, 1986).

-Cultural practices: Crop-free periods of 2 months has been applied with success in Israel (Ucko *et al.*, 1998).

Sanitation: Old fields with aged tomato plants can sustain whitefly reproduction and the spread of TYLCV. The elimination of tomato plants immediately after harvest reduces the insect populations and helps diminish the movement of virus into nearby fields (Polston and Lapidot, 2007).

Weed management: Weed species are able to act as reservoirs for TYLCV and they can harbour whitefly populations. For this reason, they should be kept at a bare minimum.

Rouging: By rouging young infected plants in the field early in the season, a vast decrease in the amount of secondary spread within a field can be observed, provided that the infection rate is still relatively low (Polston and Lapidot, 2007).

-Chemical approaches: Viral spread can be in part controlled by the elimination of the vector, however, the control of the whiteflies to a level that will significantly decrease viral infections is difficult. Some of the factors causing the difficulties in managing whiteflies are a wide host range which exceeds 500 species; the presence of immature instar stages and adults on the underside of leaves; the extreme mobility of adults; and the ability of *B. tabaci* to rapidly develop resistance to most classes of existing insecticides (Polston and Anderson, 1997).

-Resistant Cultivars: the use of TYLCV-resistant tomato cultivars, when available, is the best approach to diminish losses due to infection by the virus. It is the most effective and environmentally sound method and therefore breeding TYLCV resistant plants is a very important long-term goal (Polston and Lapidot, 2007).

1.7.7. Tomato Breeding

The quest for tomato improvement began by breeding novel cultivars over 200 years ago in Europe. In the USA, tomato breeding started a century later (Stevens and Rick, 1986; Tigchelaar, 1986). Breeding of tomato included development of multipurpose cultivars suited for fresh market and processing industries. The universal accomplishment of tomato breeding for both markets is to increase fruit yield per unit area. There are other requirements that are important to both industries

namely; disease resistance, broad adaptability, earliness in maturity, ability to set fruit at adverse temperatures, resistance to rain induced cracking, tolerance to major ripe-fruit rots, adequate vine cover, fruit firmness and several other fruit quality characteristics. Important characteristics needed in processing cultivars include compact, determinate plant habit and concentrated flowering and fruit set suitable for once-over machine harvest, ease of fruit separation from the vine and specific fruit quality characteristics such as colour, pH, total acidity, soluble solids, total solids and consistency. Specific traits of interest in fresh market cultivars includes large, round fruit with adequate firmness and shelf-life, uniform fruit size, shape and colour, appearance, no external blemishes or abnormalities, texture, taste and flavour (Stevens and Rick, 1986; Tigchelaar, 1986).

Some major tomato breeding achievements reached are (Foolad, 2007);

- Yield: Higher yield and greater quality are the universal goals of majority of breeding programs. Molecular markers have been created that directly or indirectly are related to yield.
- Fruit quality: Characteristics of interest include flavour, nutritional value, fruit size, shape, colour and firmness.
- Fruit ripening: In industry for fresh consumption, tomatoes are harvested at the 'mature green' stage in order to prevent post harvest damage to fruit which includes physical, biotic or abiotic aspects.
- Insect resistance: The tomato crop has received substantially less attention to insect resistance than disease resistance. However few commercial cultivars have been developed with specific insect resistance.
- Hybrid production: Mainly for the protection of breeder's research investments but also for the combination of important characteristics including disease resistance.
- Disease resistance: Resistance has been identified and characterized for over 30 of the major tomato diseases. Majority of commercial cultivars contain 6 or more disease resistance traits.

1.7.8. Breeding for TYLCV/ToCSV resistance

Different parts of the world have taken numerous management approaches including virus-free tomato seedlings, applying insecticides, using insect-proof netting, and implementing a whitefly-host-free period, to control tomato-infecting begomoviruses. Recently, tomato cultivars possessing different levels of resistance have become available to some regions of the world. Employment of

resistant hybrids presents many advantages and when used with integrated pest-management schemes, these resistant hybrids can guarantee a high rate of success for tomato growers.

1.7.8.1. Main sources of resistance

The tomato crop is susceptible to more than 200 diseases derived from fungi, bacteria, viruses and nematodes (Foolad, 2007). All of the domesticated tomato source germplasm (*S. lycopersicum*), originally tested for resistance to TYLCV, were found to be susceptible (Pilowsky and Cohen, 1974). Therefore, it was essential to screen the majority of the related wild species of tomato for any possible sources of resistance to TYLCV and other begomoviruses.

Solanum pimpinellifolium

Solanum pimpinellifolium is the only red-fruited wild species of tomato. Pilowsky and Cohen (1974) were among the first to study TYLCV-resistance in wild species, finding that resistant plants in several accessions of *S. pimpinellifolium* exist and accounted the resistance to be controlled by a single, incompletely dominant gene in *S. pimpinellifolium* LA121. Similar heritage of resistance was proposed for accession A1921 (Banerjee and Kalloo, 1987). LA121 and LA373 were studied by Hassan *et al.* (1984) who found that resistance was partially recessive. A single dominant gene was coupled with resistance in the accessions hirsute-INRA and LA1478; LA1478 and LA1582 (Geneif, 1984), and PI407543 and PI407544. Partial dominance was proposed for the resistance from PI407555 (Hassan and Abdel-Ati, 1999). The "PIMPERTYLC" population was created by crossing *S. pimpinellifolium* plants from accessions hirsute-INRA and LA1478 (Laterrot, 1992), which had been chosen for resistance in different countries. Although resistance has been identified in various accessions of this wild species, it has not become a major source of resistance in present breeding programs.

Solanum peruvianum

Since the lines derived from *S. pimpinellifolium* LA121 had little vigor and yield, Pilowsky and Cohen (1990) assessed an additional wild species, *S. peruvianum*, and found that a few recessive genes were associated with plants from TYLCV-tolerant accession PI26935. This endeavour brought out the release of TY20, as a moderately resistance cultivar. Consequently, greatly resistant breeding lines such asTY172 and TY197 have been developed in Israel from *S. peruvianum* (PI126926, PI126930, PI390681 and LA441 (Lapidot *et al.,* 1997; Friedmann *et al.,* 1998). At least three genes are involved in the resistance (Lapidot *et al.,* 2000). In Egypt, Hassan *et al.* (1982) found that accessions LA372, LA452, LA462, LA1274, LA1333, LA1373, and CMV sel INRA (PI126926 - PI128648–6), as well as *S.*

peruvianum var. *humifusum* LA385 were very resistant to TYLCV. This group developed a highly resistant line. Genes from *S. peruvianum* are presently deployed in commercially grown hybrids which have provided fine resistance to TYLCV.

Solanum chilense

Resistance genes derived from introgressions with *S. chilense* are important in several breeding programs across the globe (Zakay *et al.,* 1991; Scott *et al.,* 1995; Mejía *et al.,* 2005; Pinón et *al.,* 2005). Zakay *et al.* (1991) reported highly resistant plants from LA1969 to TYLCV, and plants from this accession have also been found to be resistant to TYLCV in Cuba (Pinón *et al.,* 2005) and to *Tomato mottle virus* (ToMoV) and TYLCV in Florida, USA (Scott and Schuster, 1991; Scott *et al.,* 1995). A gene, conferring partially dominant resistance, called *Ty-1,* has been mapped to chromosome 6 and two modifier genes mapped to chromosomes 3 and 7 (Zamir *et al.,* 1994). Resistance from LA1969 has also been introgressed into the cultivated tomato by some private seed companies and the resistance is located in a chromosome 6 region that includes Ty-1 and perhaps an additional linked resistance locus. This introgression appears to offer resistance to a broader collection of begomoviruses than that of Ty-1 alone. *Solanum chilense* accessions LA1932, LA1938, LA1959, LA1960, LA1961, LA1963, LA1968, LA1969, LA2747, LA2774, and LA2779 were found to be resistant to the bipartite begomovirus ToMoV in Florida and used to initiate a program of interspecific crosses (Scott *et al.,* 1995). LA1932, LA2779, and LA1938 have been useful sources of resistance for the tomato breeding program in Florida (Scott et *al.,* 2001). Inheritance studies using LA1932 indicated two loci with primarily additive gene action accounted for the resistance to ToMoV (Griffiths and Scott, 2001). At present, several commercial breeding programs are using resistance genes from the *S. chilense* and horticultural adequate cultivars are being marketed. Among these cultivars are Anastasia, Boludo, and, Llanero in Guatemala.

Solanum habrochaites

Accessions of *S. habrochaites* (*Lycopersicon hirsutum*) LA386, LA1252, LA1295, LA1352, LA1393, LA1624, and LA1691 were found to be greatly resistant to TYLCV (Hassan *et al.,* 1982). Studies on the interaction phenotypes of F1 *S. lycopersicum* cv. VF145-B7879 - LA386 point towards resistance that was dominant (Hassan *et al.,* 1984). A high level of resistance was reported for LA1777. Vidavsky and Czosnek (1998) selected TYLCV-resistant plants from LA386 and LA1777, and crossed them to produce a highly resistant F1 population, which was further used in crosses with *S. lycopersicum.* The resulting tolerant F1 plants were backcrossed to the cultivated tomato. Through a sequence of self-pollinations and selection for resistance to TYLCV, plants with immunity and tolerance were

generated. The study suggested that resistance was controlled by one major dominant gene and several minor ones. A breeding line Ih902 was used to create hybrids, including FAVI9, which has been an key source of resistance for breeding programs in Guatemala (Mejía *et al.,* 2005) and other Middle Eastern countries (Maruthi *et al.,* 2003). Picó *et al*., (2000) found high levels of resistance in *S. habrochaites* too. In India, *S. habrochaites* B6013 was shown to have two epistatic genes controlling resistance to *Tomato leaf curl virus* (Banerjee and Kalloo, 1987). Consequently, these researchers developed line H24 from this accession (Kalloo and Banerjee, 1990) and this line has the Ty-2 resistance, which was originally derived from *S. habrochaites* (Hanson *et al.,* 2000). Line H24 confers specific tolerance to some, but not all strains of TYLCV/ToLCV. Ty-2 resistance was the initial source of resistance used in tomato breeding program at the Asian Vegetable Research and Development Centre (AVRDC) and has been extensively exploited by seed companies across the globe.

Solanum cheesmaniae

A recessive and/or polygenic resistance has been associated with accessions of *S. cheesmaniae* (Hassan *et al.,* 1984). In Egypt, a moderately resistant breeding line (line 44) was derived from an introgression of resistance genes from *S. cheesmaniae* with the commercial cultivar Pakmor (Moustafa and Nakhla, 1990). This species has not been a significant source of resistance in current breeding programs.

Genetic resistance in the host plant is the perfect protection against whitefly-transmitted viruses, given that it needs no chemical involvement and plant isolation and can potentially be stable and longer lasting. Thus, the best way to reduce TYLCV/ToCSV spread is by breeding tomatoes that are resistant or tolerant to the virus. Since all cultivars of tomato (*S. lycopersicum*) are extremely susceptible to TYLCV/ToCSV, wild tomato species have been screened for their response to the virus (Lapidot and Friedmann, 2002). The first attempts at breeding for TYLCV-resistant tomato plants were made in the early 1970s using *S. pimpinellifolium* accession LA121 as the resistant source (Pilowsky and Cohen, 1974). After a few years of repeated efforts to introgress the resistance into the domesticated tomato (*S. lycopersicum*), the resistance level of LA121 was found to be insufficient and efforts were shifted to accessions of *S. peruvianum*, which was found to express a higher level of TYLCV resistance. Indeed, in 1988, the first commercial TYLCV-resistant tomato hybrid TY20 was released and carried resistance derived from L.peruvianum (accession PI126935) that was later determined to be mediated by 5 recessive genes (Pilowsky and Cohen, 1990). The breeding efforts continued, and led to the development of highly TYLCV-resistant lines which do not exhibit symptoms following inoculation with TYLCV (Friedmann *et al.,* 1998; Lapidot *et al.,* 1997). It was

demonstrated that tomato lines expressing a high level of TYLCV resistance serve as a poor inoculum source for the virus (Lapidot *et al.,* 2001). Today, due to the continuous breeding efforts of a number of research groups, elite commercial TYLCV/ToCSV resistant tomato hybrids are available (Lapidot and Friedmann, 2002).

Wild *Lycopersicon* species were screened for their response to the virus, and certain accessions of *L. pimpinellifolium*, *L. cheesmanii, L. hirsurum, L. peruvianum*, and *L. chilense* were found to have naturally occurring resistance, which varied from partial to complete. The disease may be expressed in different accessions with varying degrees of symptom severity. Three categories of response to TYLCV infection were defined by:

Susceptibility where plants contain viral DNA and develop symptoms of the disease,

Tolerant plants contain detectable amounts of viral DNA but are symptomless, and

Resistance cannot be detectable by squash blot hybridization tests and the plants are symptomless.

1.7.8.2. Types of resistance

Resistance can be of either a qualitative or quantitative nature in plants. A qualitative attribute is a monogenic trait which segregates according to Mendelian ratios while a quantitative trait is a polygenic character and has constant segregation. A quantitative trait locus (QTL) is an individual locus that controls the quantitative trait. Resistance to disease can be monogenic or polygenic.

1.7.8.2.1. True resistance

True resistance is disease in the plant that is genetically controlled by the presence of one, a few or many genes. The host and the pathogen are incompatible with each other. This could be as a result of lack of chemical recognition or the host plant can protect itself against the pathogen. There are two kinds of true resistance: partial (or quantitative, polygenic or horizontal resistance) and R gene resistance (also known as race specific, monogenic or vertical resistance).

1.7.8.2.2. Partial, quantitative, polygenic, or horizontal resistance

Partial resistance, also called polygenic resistance is more than likely controlled by several genes. The several genes involved in partial resistance seem to exert their influence by controlling the numerous steps of the physiological processes in the plant that provide the materials and structures that make up the defence mechanisms of the plant. Partial resistance is affected by and may differ significantly more than R gene resistance under different environmental conditions. Overall, partial

resistance does not guard plants from becoming infected but slows the development of individual infection loci on a plant down, thereby slowing down the spread of the disease and the development of epidemics in the field (Agrios, 2005).

1.7.8.2.3. R Gene resistance, race-specific, monogenic, or vertical resistance

Race-specific resistance is always controlled by one or a few genes. These genes (R genes), control a chief step in the recognition of the pathogen by the host plant and consequently play a key role in the expression of resistance. In race-specific resistance, the host and pathogen are mismatched. The host could react with a hypersensitive response, may well show immunity, or may inhibit pathogen reproduction. Frequently, this type of resistance hinders the initial establishment of pathogens that arrive at a field from host plants that lack, or have different, major genes for resistance. Race-specific resistance inhibits the progress of epidemics by restraining the initial inoculums or by limiting reproduction after infection. Varieties with race-specific resistance (monogenic) in general show complete resistance to a specific pathogen under majority of environmental conditions however a few or single point mutations in the pathogen may produce a new race that may infect the previously resistant variety. In contrast, varieties with partial (polygenic) resistance are less stable and may vary in their response to the pathogen under altered environmental conditions, but a pathogen will have to go through many more mutations to fully break down the resistance of the host (Agrios, 2005).

1.7.8.3. Genetic resistance markers

Tomato hybrids are developed using plant genetics and breeding as well as marker-assisted selection (MAS) when it is accessible. Considerable progress has been made in the development of markers for key resistance genes. Currently over 285 morphological, physiological, and disease resistance markers, 36 isozymes and over 1000 restriction fragment length polymorphism (RFLP) markers have been mapped to the 12 individual chromosomes (Sol Genomic Network (SGN), www.sgn.cornell).

MAS in tomato breeding has been used since the early 1980's where selection for nematode resistance was based on the Aps marker which was known for its linkage association between the *Aps-1* locus and nematode resistance. Today MAS is a routine practice in the majority of seed companies with the clear vision of enhancing tomato production (or many simply inherited traits) (Table 1.2) such as joint-less, ripening and carotenoid content (lycopene and beta-carotene). Unfortunately most MAS activities are not reported in public literature. Foolad (2007) conducted a survey of major seed companies in the US, which included Seminis Vegetable Seeds), Syngenta, Sakata and in Europe: De Ruiter, Seminis Vegetable Seeds Holland, and found that MAS was used

routinely in tomato breeding programs for qualitatively inherited disease resistance traits such as fusarium wilt, late blight, root-knot nematode, powdery mildew, TMV, TYLCV etc. (Table 1.2) (Foolad, 2007). MAS was reported to be faster, cheaper and more effective than phenotypic selection (PS).

Table 1.2: Documented traits for which marker- assisted selection and breeding are done in tomato plants (Foolad, 2007).

Traits	Source species	Gene/QTL (Q)	Reference
Bacterial canker	*L. peruvianum*	Q3	Seed companies
	L. hirsutum	*Rcm2.0, Rcm5.1(Q)*	Coaker and Francis (2004)
Bacterial speck	*L. pimpinellifolium*	*Pto*	Yang and Francis (2005)
Bacterial spot	*L. esculentum*	*Rx-3 (Q)*	Yang and Francis (2005)
Bacterial wilt	*L. esculentum*	*2 Q*	Seed companies
Blackmold	*L. cheesmanii*	*Few Q*	Robert *et al.* (2001)
Corky root rot	*L. peruvianum*	*Py-1*	Seed companies
Fusarium wilt	*L. pimpinellifolium*	*I-2C, I-3*	Seed companies, public breeders
Jointless	*L. cheesmanii*		Seed companies
Late blight	*L. pimpinellifolium*	*Ph-3*	Seed companies, public breeders
	L. hirsutum	*4 Q*	Brouwer and Clair (2004)
Lycopene	*L. esculentum*	*Og , cr*	Seed companies
Powdery mildew	*L. chilense, L. hirsutum*	*Lv, Ol-1, Ol-2*	Seed companies
Ripening inhibitor	*L. cheesmanii*	*rin*	Seed companies
Root- knot nematode	*L. peruvianum*	*Mi*	Seed companies, public breeders
Self pruning	*L. esculentum*	*sp*	Seed companies, public breeders
Soluble solids	Not known	Q	Seed companies
Tomato spotted wilt virus	Different species	Few Q	Seed companies
Tobacco mosaic virus	*L. peruvianum*	*Tm-2*	Seed companies
Verticillium wilt	*L. esculentum*	*Ve*	Seed companies, public breeders

MAS can be described as selection for a characteristic not based on the trait, but instead on the genotype of an associated marker. Essentially, the associated marker is used as an indirect selection criterion. The possibility of MAS as an alternative means for crop enhancement has been comprehensively investigated (Ribaut *et al*., 2002; Sevin *et al*., 2004). MAS is currently an important part of many commercial breeding programs as it allows for a speedy development of tomato hybrids. It allows selecting for a trait in seasons or locations where phenotypic selection is not feasible or is costly or ineffective, thus increasing the efficiency of selection and flexibility of a

breeding program. MAS may be less time consuming for traits whose expressions are developmentally regulated and are phenotypically apparent only late in the season. Markers are independent of variation caused by genetic or environmental factors and this offers the advantage of permitting selection for traits such as resistance in the absence of pathogen, which is otherwise required to identify valuable segregants. Trait heritability is the most important factor influencing the efficacy of MAS. The mapping and progress of marker development for resistant genes to begomoviruses would facilitate breeding of hybrids by pyramiding resistance genes from various sources. For example, in the future, resistance genes from the different wild species may be combined to provide higher levels of resistance and to provide resistance to a wider range of begomoviruses.

1.7.8.3.1. Known molecular markers for TYLCV/ToCSV

Ty-1

All tomato cultivars are tremendously susceptible to TYLCV and ToCSV, therefore, wild *Lycopersicon* species have been screened for their reaction to TYLCV (Zakay *et al.,* 1991). Out of four wild *Lycopersicon* species, (*L. pimpinellifolium, L. hirsutum, L. peruvianum* and *L. chilense*) inoculated with TYLCV, the only species that showed no infection symptoms was *L. chilense* after field and controlled greenhouse infections. TYLCV does not spread or replicate in *L. chilense* and therefore the accession LA1969 was used further for breeding tomatoes resistant to TYLCV (Zakay *et al.,* 1991).

Zamir *et al.* (1994) crossed *L. chilense* (LA1969) with *L. esculentum* (cv M82-1-8) in a classical breeding project with the result of mapping and introgressing a TYLCV tolerance gene, from the wild specie *L. chilense* (LA1969). Their results indicated an incompletely- dominant gene for TYLCV tolerance, *TY*-1, which maps to chromosome 6 between TG297 (4.0 cM Tomato-EXPEN 2000 and 6.0 cM Tomato-EXPEN 1992) and TG97 (8.6cM Tomato-EXPEN 1992) (Figure 1.11) (Zamir *et al*., 1994). Many commercial resistant tomato cultivars have the *Ty*-1 gene (De Castro *et al.,* 2007). Minor loci, were mapped to chromosomes 3 and 7. On chromosome 7, a locus which contributed a minor degree to the resistance phenotype, was located near TG61 (9.0 cM). The RFLP markers (TG66 and TG33) on chromosome 3 had only minor association with the resistant interaction phenotype. Michelson *et al*. (1994) found the localization and the inheritance of the *Ty*-1 allele from *L. chilense* LA1969 has led to a tolerant line where the replication and spread is hindered compared to the susceptible near-isogenic line. The *Ty*-1 gene is located in a “hot-spot” for resistance genes as described by Pérez de Castro *et al.* (2007). This “hot-spot” is known to have genes for resistance to *Alfalfa mosaic virus* (*Am* gene, Parrella *et al.,* 2004), powdery mildew (*Ol*-1 gene, Huang *et al.,* 2000),

Cladosporium fulvum (*Cf-4* gene, Thomas *et al.,* 1997), *Ralstonia solanacearum* (Wang *et al.,* 2000), and *Meloidogyne* spp. (*Mi*-1 gene, Sean *et al.,* 2007).

Seeing as *Ty-1* homozygous plants can develop mild disease symptoms, Zamir et *al*. (1994) chose to use the term tolerant instead of resistant. In tolerant lines the viral DNA is restricted to the inoculated leaf and its long distance movement is hindered. However, in susceptible lines, the viral DNA spreads to the nearby leaves and to the roots. This provides some evidence of the resistant mechanism which appears to interfere with the action of the viral movement proteins. In that case, when the amount of inoculum is low, there are sufficient numbers of antiviral factors present to prevent cell-to-cell movement and viral concentration remains low. When the level of inoculum is high, the protection mechanism is not enough, and there is a slow but significant viral accumulation in the plant. This protection mechanism has been found in other related geminiviruses too (Michelson *et al*., 1994; Zamir *et al*., 1994).

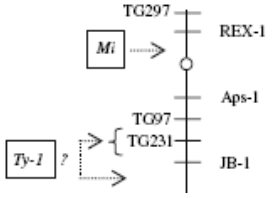

Figure 1.11: The positions of the Ty-1 and Mi markers on chromosome 6 of tomato. The genes Mi, and Ty-1 are shown in frames. The circle represents the centromere of the chromosome. JB-1 is located beyond the TG231 marker (De Castro *et al.,* 2007).

PCR-based markers for the TG97 locus are being used in MAS for research purposes as well as in commercial companies. Another marker that can locate the Ty-1 gene is CAPS (cleaved amplified polymorphic sequences) marker REX-1 (ca. 5.5 cM), which is also related with the *Mi-1* gene for resistance to root-knot nematode (Milo, 2001). The REX-1 fragments for *S. lycopersicum*, *S. peruvianum*, and *S. chilense* have zero, one and two *Taq*I restriction sites, respectively, which can be differentiated by agarose gel electrophoresis. The first isozyme used as a marker for Mi (nematode resistance) was the locus 1 of acid phosphatase (Aps-1). De Castro *et al*. (2007) developed a PCR-based marker for the detection of *Ty*-1 gene using the Aps-1 locus. Plant material tested

corresponded to alleles found in *S. lycopersicum* and *S. peruvianum* showing allele 1 or 2 respectively. However, Aps-1 is not very useful as a marker for *Ty*-1 as it is not tightly linked to this gene, and the presence of other genes, in the same region from different wild species can lead to false positive results. In addition, all species tested have this same allele, except *S. lycopersicum*, so introgressions from other species could lead to false positive results with this marker (De Castro *et al.* 2007).

CT21 is the RFLP (restriction fragment length polymorphism) marker from which JB-1 was designed. All *S. lycopersicum* plant material carrying Ty-1, regardless of being homozygous or heterozygous, showed allele 3 for this marker (which is one allele of JB-1 that is always found associated with *Ty*-1). None of the *S. lycopersicum* plant materials without Ty-1 showed this allele. Plant material which did not carry *Mi* gene, showed allele 1, which is the *S. lycopersicum* allele. In plant material carrying *Mi*, alleles 1 and 2 appeared alternatively; allele 1 was present in lines with the small introgression from *S. peruvianum*, while allele 2 was revealed by lines that retained the larger introgression. Yet, all accessions of *S. peruvianum*, showed allele 3, which is not present in the rest of the wild species analysed like *S. pimpinellifolium* and *S. habrochaites* (De Castro *et al.* 2007).

Ty-2

A co-dominant SCAR (sequence-characterized amplified region) marker for detection of the begomovirus resistance *Ty*-2 locus derived from *S. habrochaites* in tomato germplasm was found by Hanson *et al*. (2000). The introgression was tracked from *S. habrochaites* to the long arm of chromosome 11 between TG36 (84 cM) and TG393 (103 cM), which conferred resistance to TLCV. This resistance gene was identified as *Ty*-2 and was found to be associated with an introgression from TG36 (84 cM) to TG26 (92 cM) (Hanson *et al*., 2000). PCR primers from the SGN website for T0302 (89 cM) marker produce 800 bp band for susceptible plants and a 900 bp band for resistant material (containing the *Ty*-2 introgression). However, it is possible that this marker might not detect all lines that have the *Ty*-2 gene, since it is not known how closely linked this marker is to the *Ty*-2 gene.

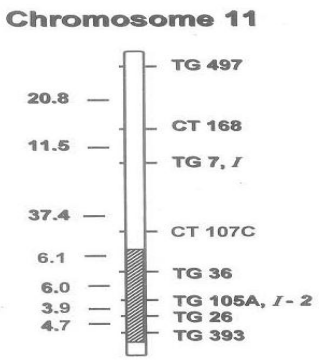

Figure 1.12: Location of *Ty*-2 is shown shaded in between TG36 and TG393 on chromosome 11 of tomato (Hanson *et al.,* 2000).

Ty-3

A partially dominant gene, designated *Ty*-3, was mapped to the marker interval between cLEG-31-P16 (20 cM) and T1079 (27 cM) on the long arm of chromosome 6 (Ji and Scott, 2007a). Scott and his team from the University of Florida have used a number of accessions of *S. chilense* to introgress resistance to TYLCV and *Tomato mottle virus* (ToMoV) into tomato lines (Agrama and Scott, 2006; Scott, 2001). These were assessed in Guatemala (Mejía *et al.,* 2005) and the line Gc9 with the source of resistance from LA2779 and line Gc171 from LA2779/LA1932 were highly resistant to bipartite begomoviruses. The begomovirus resistance *Ty*-1 and *Ty*-3 loci on chromosome 6 for advanced breeding lines derived from LA2779 were mapped between C2_At2g39690 (5.3 cM) to T0834 (32 cM) (*et al.,* 2007b). The partially dominant *Ty*-3 gene was mapped to region between cLEG-31-P16 (20 cM) and T1079 (27 cM) (Ji and Scott, 2006a). The line Gc9 had an introgression from chromosome 6 from the *REX-1* locus (6 cM) to T0834 (32 cM), which would include the loci for *Ty-1* and *Ty-3* (Maxwell, unpublished data). SCAR or CAPS have been developed to detect the *Ty-1* and *Ty-3* loci. Unfortunately, the co-dominant SCAR marker, FLUW25, only detected the Ty-3 introgression from LA2779 and not the introgression from LA1932, line Gc171. The FLUW25 primers amplify DNA in the region of the FER BAC clone (56B23, AY678298) at 25 cM on chromosome 6.

Agrama and Scott (2006) reported three regions that contributed to resistance in breeding lines with introgressions from *S. chilense* LA2779 or LA1932. One region corresponded to the region having the *Ty*-1 locus. Another region was the *Ty*-3 locus, which was mapped to a region between cLEG-31-P16 (20 cM) and T1079 (27 cM) (Ji and Scott, 2006a; Ji *et al.,* 2007b). The third region was near the self-pruning (*sp*) and potato leaf (*c*) loci. Another begomovirus-resistance QTL, derived from an

introgression from *S. pimpinellifolium*, was mapped near the marker TG153 (33 cM; Chagué *et al.,* 1997). Previously Ji *et al.* 2007b reported the development of SCAR and CAPS markers linked to begomovirus resistance genes derived from *S. chilense* on chromosome 6, and they determined that the Ty-3 locus mapped to a region that included the FER locus (25 cM, BAC clone 56B23, AY678298). Maxwell *et al.* (2007) found that the sequences for the G8 gene of the BAC clone 56B23 are different for lines derived from *S. chilense* LA2779 and LA1932. To differentiate the two introgressions, the one from LA2779 is designated Ty-3 and the one from LA1932, Ty-3a. A co-dominant SCAR marker, FLUW25, was reported by Ji *et al.* (2007b), which can detect ty3 (*S. lycopersicum*) and Ty3 loci. The FLUW25 primers amplify DNA in the region of the FER BAC clone (56B23, AY678298) at 25 cM on chromosome 6. The FLUW25 primers detected a fragment of 480 bp from a susceptible line and 640 bp from a begomovirus-resistant line containing an introgression from *S. chilense* LA2779. The heterozygous plant, gave the two sizes, 480 and 640 bp, for the *Ty*3*/ty*3 genotype. However, these primers showed inconsistency by the amplification of an introgression from *S. habrochaites* which also gave a 640 bp band. In another case, they failed to amplify an introgression of a breeding line, Gc171, from San Carlos University, Guatemala, which was known to have an introgression, *Ty*-3a derived from *S. chilense* LA 1932.

An additional primer set, P6-25-F2 and P6-25-R5, that provides co-dominant SCAR markers for detection of the Ty-3 and Ty-3a introgressions and a newly discovered introgression from *S. chilense* LA1969, was designed to give smaller fragments than the FLUW25 primer set (Ji et al., 2007 b). With begomovirus-resistant breeding lines derived from either the *S. chilense* LA2779 source, Gc9, or the Ih902 line (Vidavsky and Czosnek, 1998), the expected 450 bp *Ty*-3 fragment was obtained. A 320 bp *ty*-3 fragment was amplified from breeding lines lacking the introgression from either of these two begomovirus-resistance sources. A 630 bp *Ty*-3a fragment was obtained from lines derived from *S. chilense* LA1932, such as Gc171. Heterozygous hybrids were easily detected with these primers which amplified two fragments corresponding to the *S. lycopersicum ty-3* fragment (320 bp) and either the *Ty*-3 (450 bp) or the *Ty*-3a (630 bp) fragment. The P6-25F2/P6-25R5 primer pair was used to screen several begomovirus-resistant hybrids from different commercial seed companies. Yet, another size PCR fragment of 660 bp was obtained with three commercial hybrids. This fragment was sequenced and had 100% nt identity with the fragment from *S. chilense* LA1969. These two sets of primers detect co-dominant SCAR markers, FLUW25 and P6-25, for the *ty*-3, *Ty*-3, *Ty*-3a and *Ty*-3b loci. It is not known how closely these markers are to the functional *Ty*-3 gene (Ji *et al.,* 2007b), so it is possible that some breeding lines would give false negative or false positive results. It is of interest that the introgressions from three different *S. chilense* accessions have different size introgressions.

Only the *Ty*-3b introgression has 100% nt identity with one of these accessions, LA1969, and this accession has been used as a source of the *Ty*-1 gene in several different laboratories.

The primer pair FER-G8F1/FER-G8R1 gave a 500-bp fragment with M82-1-8 (*S. lycopersicum*), Gc171, and GIh902 (this line has the same introgression as Gc9 for this region (6.5 cM to 32 cM), unpublished data). The sequences were aligned and the sequence for M82-1-8 did not have a *Taq*I site, the one for Gc171 had one site (*Ty*-3a locus) and the for GIh902 two *Taq*I sites (*Ty*-3 locus). These primers matched the sequence of the G8 from nt 171,604 to 172,113 of the FER BAC clone (56B23, AY678298).

Ty-4

Agrama and Scott (2006) described three locations coupled with chromosome 6 in begomovirus resistant lines derived from introgressions form *S. chilense*. A line Gc171 (c = *S. chilense*). The introgression was from *S. chilense* LA1932/LA2779. This line has shown the best resistance in the field in Guatemala. The introgressions associated with Gc171 were of special significance since Gc171 is a resistant phenotype. The only introgression that it had was at FER BAC clone (25 Cm, AY678298) on chromosome 6, and was designated *Ty-3a* locus. Ji and Scott designed a primer pair from a RAPD marker linked to the resistance in lines with introgressions from LA1932 in the lower half of chromosome 3, near 78 Cm (Ji *et al.,* 2008). This new TYLCV resistance locus, designated as *Ty*-4, was mapped to the marker interval between C2_At4g17300 and Ct_At5g60160 on chromosome 3 (Ji *et al.,* 2008).

Ty-5

TY172 has been shown to express a high level of resistance to TYLCV. This line was developed at the Volcani Center in Israel from a cross between *S. lycopersicum* line and a combination of four *S. peruvianum* accessions: PI 126926, PI 126930, PI 390681 and LA0441 (Friedmann *et al.,* 1998). Besides the major QTL discovered on chromosome 4, four minor QTLs were found which influence resistance level. These minor QTLs can be detected by markers C2_at4g34700 on chromosome 1, TG174 on chromosome 7, *SlSUMO* on chromosome 9 and C2_at4g22260 on chromosome 11. Two of these minor QTLs, marked by C2_at4g22260 and *SlSUMO*, displayed a minor additive effect. The QTL marked by *SlSUMO* showed a partial dominance effect, the resistance allele originating from the resistant TY172 line, similar to *SlNAC1*. Interestingly, however, the QTL marked by C2_at4g22260 displayed a dominant effect in which the resistance allele originated from the susceptible line LA1589. Their results suggest that the QTL identified on chromosome 4 can be used in breeding TYLCV resistant cultivars and that the Ty-5 marker is of high utility (Figure 1.14). The major QTL

identified, *Ty-5*, maps to chromosome 4 and not to any of the other chromosomes controlling TYLCV resistance (Anbinder *et al.,* 2009).

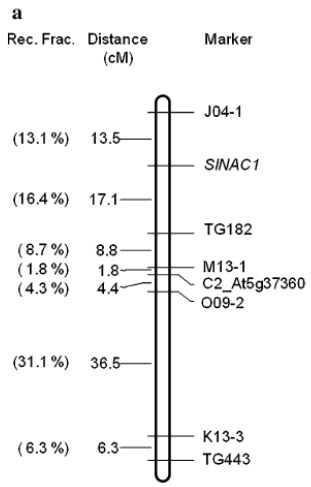

Figure 1.13: Map of chromosome 4, showing the distance in centiMorgan (cM) among the markers analyzed (Anbinder *et al*., 2009).

1.7.9. The plant defence system

Plants offer a nourishing environment and shelter for a wide range of parasites including viruses, bacteria, fungi, nematodes insects and even other plants (McDowell and Dangl, 2000). Pre-existing mechanisms for preventing pathogens invasion exist, such as waxy cuticles and antimicrobial compounds. However, many pathogens can break down these physical barriers. Consequently plants have evolved mechanisms to recognize and counteract these invaders and prevent disease by preventing their replication.

If a plant perceives an attack, by the release of elicitor molecules from the invader that interact with plant receptors, a set of inducible defence responses is deployed which leads to initiation of a signalling pathway (Figure 1.14) (Cohn *et al*., 2001). This includes programmed cell death (known as the hypersensitive response or HR), tissue reinforcement at the infection site, production of anti-microbial metabolites and generation of defence-associated gene expression. An example is the phosphorylation state of the cell which changes; hence, Ca^{+2} ions increase in the cytoplasm and activate an oxidative burst (Ebel and Mithofer, 1998). The oxidative burst is thought to be required for most induced defence responses and is expressed in many plant species (Gorovitz and Czosnek, 2007). As a result, reactive oxygen species (ROS), superoxide radicals (O_2) and peroxides (H_2O_2) are produced. Nitric oxide (NO) collaborates with ROS to trigger transcriptional activation of plant defence genes. These biochemical reactions result in cell wall thickening and cellular damage to both

host and pathogen (Figure 1.14). Additionally, at the site of infection, rapid cell death occurs; this is the HR. The cell death deprives the pathogen of access to nutrients and stops its spread throughout. These defence mechanisms are achieved through the interaction of pathogen avirulence (*avr*) gene products and plant resistance (*R*) gene products; gene-for-gene resistance. In these interactions the outcome of either resistance or susceptibility of the host to a pathogen is determined based on the pathogen genotype and the extent of pathogen virulence observed is conditional on the host genotype (Crute and Pink, 1996). Compatibility, being the widespread pathogen expansion and reproduction in the absence of an efficient host defence response, is the result of a host- pathogen combination unless an allele for resistance at a host locus is distinctively corresponding by an allele for avirulence at a particular pathogen locus. In this situation, the degree of incompatibility (reduced pathogen development and reproduction associated with an effective host defence response) expressed depends on the particular matching gene pair (Crute and Pink, 1996; Dangl and Jones, 2001).

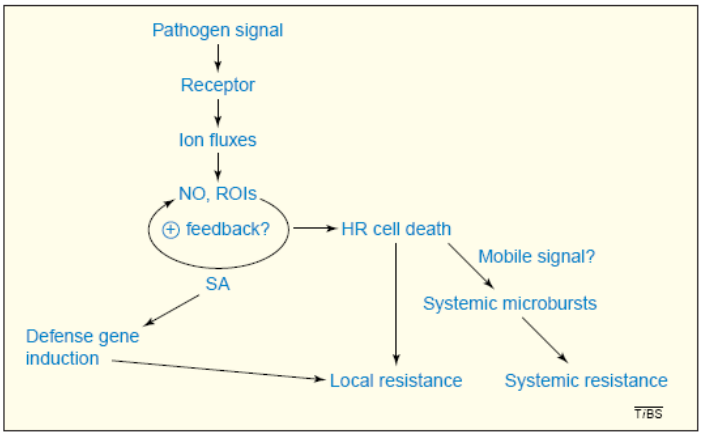

Figure 1.14: Regulation of local and systemic responses by ion fluxes, reactive oxygen intermediates (ROIs), nitric oxide (NO) and salicylic acid (SA). The ion fluxes activate local production of NO and ROIs instantaneously after pathogen detection. These second messengers bring about cell death, defence gene expression, and production of SA and more ROIs, establishing an alleged feedback ring where the response is amplified. In distal parts of the plant, defence responses may be activated by a similar mechanism, involving an unknown mobile signal (McDowell and Dangl, 2000).

In addition to defence mechanisms that are activated upon pathogen attack, plants have two distinct systemic defence mechanisms. The first one is systemic acquired resistance. After pathogen attack salicylic acid (SA) accumulation induces this mechanism and as a result, pathogenesis related (PR) proteins are expressed. SAR is long lasting and is effective against a broad spectrum of pathogens

(Hammond and Jones, 1996). The other mechanism is induced systemic resistance (ISR). It is independent of SA but instead relies on jasmonic acid and ethylene which induce expression of antimicrobial peptides correlated with systemic resistance. The activation of the signalling pathway leads to the induction of many of the pathogenesis-related (PR) proteins. These stress-inducible proteins can directly protect against environmental conditions as well and regulate gene expression and signal transduction (Shinozaki and Shinozaki, 1997). Plant PR proteins are represented by 17 protein families, including ß-1,3-glucanases, chitinases, and peroxidases (Van Loon and Pieterse, 2006). PR proteins have been shown to be directly involved in plant immunity, coupled with protective mechanisms (Pieterse and Van Loon, 1999). Biochemical and physiological studies have produced evidence defining arrays of signalling events in plant basal defence responses, including reversible protein phosphorylation catalyzed by protein kinases and phosphatases (Zhang and Klessig, 2000; DeLong *et al.,* 2002). The mitogen-activated protein kinase (MAPK) cascades are present in higher plants and play an important role in signal transduction in response to biological signals, hormones and to environmental pressures such as wounding, cold, salt, drought, oxidative stress and ozone (Hirt, 1997).

Abiotic and biotic stress of cells in plants brings about the expression of heat shock proteins (HSPs), which function as stress-response proteins and also as molecular chaperones and proteases (Wang *et al*., 2004). HSPs partake in cell revival from stress either by repairing (refolding) or by degrading damaged proteins, in an attempt to restore protein homeostasis. HSPs have been classified into six key families according to their molecular size: HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs (Jolly and Morimoto, 2000). HSPs, chaperones and proteases are known to be expressed in plants when they experience high temperature and a wide range of other environmental insults such as cold, drought, salinity and oxidative stress (Wang *et al*., 2004). Recently, several plant proteases were shown to be induced during drought and salinity, desiccation and high illumination. Many stresses affect chlorophyll levels and degradation of chloroplast proteins, accompanied by the degradation of several photosystem-II (PSII) proteins (which are involved in the oxidizing of H_2O in photosynthesis) (Adam and Clarke, 2002; Voet *et al.,* 2002).

CHAPTER 2. SCREENING FOR RESISTANCE TO *Tomato curly stunt virus* USING MOLECULAR MARKERS DEVELOPED FOR *Tomato yellow leaf curl virus* (TYLCV) isolates

ABSTRACT

The cultivated tomato, *Solanum lycopersicon* is widely grown around the world and constitutes a major agricultural industry. A destructive viral disease of tomatoes emerged during 1998 in South Africa named *Tomato curly stunt virus*. This virus, similar to TYLCV is also a monopartite begomovirus which is spread by the whitefly vector, *Bemisia tabaci* Biotype B. Infected plants show upper leaf yellowing, reduction in size with curling margins, progressively stunted internodes and severe yield losses of up to 100% can incur. The use of resistant varieties is an alternative method to control the detrimental effect of the virus on the tomato plants. Natural genetic resistance in a host plant is the preferred protection mechanism against whitefly-transmitted and other viruses. To date, numerous viral resistance genes have been reported from studies of crops, their wild relatives and the plant model, *Arabidopsis thaliana*. In this study, several inbred tomato lines were screened for markers for TYLCV. Inbred lines were screened for TYLCV-linked resistant gene markers (*Ty*-1*, Ty*-2*, Ty*-3*, Ty*-3a, *Ty*-4), with the aim to identify inbred lines with a high level of disease resistance but with no known Ty marker genes. A wide range of different tomato inbred lines were screened for the presence of all of the known *Ty* marker genes. From our results, an inbred line, named TAM, showing the absence of *Ty*-1*, Ty*-2, *Ty*-3, *Ty*3a and *Ty*-4 genes, was identified. Molecular marker analysis of different generations from TAM, (F5, F6 and F7 hybrids) were infected with ToCSV using viruliferous whiteflies to confirm resistance. TAM showed a high level of resistance to ToCSV. The source of the resistance was unknown, and further studies to elucidate the genetics of the resistance were performed, including test crosses of TAM with a susceptible cultivar, Rooikhaki and the production of F2 population. The F1 hybrids and F2 populations were subsequently infected with ToCSV and the severity of the symptoms of the infection was recorded. The resulting segregation patterns suggested partial dominance in these tomato populations. This resistance is likely to be novel and further studies to elucidate the molecular marker linked to this resistance in TAM would be useful to produce a new marker for use in plant breeding.

2.1. INTRODUCTION

Tomato curly stunt virus is one of the most severe tomato viruses occurring in Southern Africa. This tomato-infecting begomovirus can cause losses of up to 100% in heavily infected tomato fields (Pietersen *et al.,* 2000). Various management approaches to control tomato-infecting begomoviruses include virus-free tomato seedlings, insecticide applications, insect-proof netting, a whitefly-host-free period, and the use of resistant varieties (Ji *et al*., 2007b). Of these, the use of resistant hybrids used in combination with integrated pest-management schemes, is the best approach to minimize the impact of this pathogen (Ji *et al*., 2007b). These resistant tomato varieties are developed by employing intense plant breeding techniques as well as the use of marker-assisted selection (MAS). MAS is a screening process for a characteristic based on the genotype of an associated marker. MAS is an important component of several commercial breeding programs as it allows for a rapid development of tomato hybrids. Due to the constant breeding efforts of a number of research groups, elite commercial TYLCV resistant tomato hybrids are available (Lapidot and Friedmann, 2002). Virus resistant gene-linked markers were developed specifically for tracking the introgression of several resistance genes for TYLCV in plant breeding. The known markers are Ty-1, Ty-2, Ty-3/3a, Ty-4 and Ty-5 (Zamir *et al*., 1994; Hanson *et al*., 2000; Ji *et al*., 2007b; Ji *et al*., 2008; Anbinder *et al*., 2009).

A number of resistant genes have been introgressed into the cultivated tomato for protection against numerous pathogens and viruses. The *Ty* genes which have been shown to confer resistance to the viruses TYLCV and ToCSV have been well discussed in the previous chapter. In addition to conferring resistance to monopartite viruses, the *Ty-1* locus mapped to chromosome 6, has been shown to control tolerance to species belonging to bipartite species too, including: *Tomato mottle virus* (Scott *et al.,* 1996), *Tomato rugose mosaic virus* and *Tomato yellow vein streak virus* (Boiteaux *et al.,* 2007). Apart from these, there are numerous other genes which confer resistance to numerous other tomato plant viruses. The source of resistance to *Alfalfa mosaic virus,* has been identified in three accessions of wild tomato *S. hirsutum* and has been shown to be inherited as a single dominant gene named, *Am* (Parella, 1997) which is located in the cluster of dominant resistant genes on chromosome 6. Resistance to another destructive disease, *Tomato spotted wilt virus* (TSWV), has been derived from *S. chilense* with a single dominant gene, *Sw-7* (Saidi and Warade., 2008). Resistant and tolerant accessions were also introgressed from *S. peruvianum* (*Sw-5* gene) (Saidi and Warade., 2008). Finlay (1953) described five different genes (two dominant and three recessive) for TSWV resistance in tomato (*Swa 1, Sw1, Sw2, Sw3 and Sw4*). A dominant gene for

resistance to TSWV was identified in a SA cultivar, Stevens, denoted *Sw-5*, and mapped to chromosome 9 (Stevens *et al.,* 1992).

Wild tomato accessions have also been screened and used for introgression to confer resistance to fungal pathogens (Stevens and Rick,. 1988; Dickinson *et al.,* 1993). Leaf mold disease on tomato is caused by *Cladosporium fulvum* of which *Cf* genes, conferring resistance, have been introgressed to the cultivated tomato from various wild species such as: *S. pimpinellifolium*, *S. hirsutum* and S*. peruvianum* (Dickinson *et al*., 1993; Hammond-Kosack., and Jones., 1993). Two *Cf* genes, *Cf-2* and *Cf-5* are found on chromosome 6 and *Cf-4* as well as *Cf-9* are located on chromosome 1 (Jones *et al.,* 1993). Furthermore, late blight, caused by infestation with *Phytophthora infestans* in tomato, has led to the transfer of a dominant gene, *Ph-I* from *S. pimpinellifolium* (Richards and Baratt., 1946; Bonde and Murphy., 1952). A single incompletely dominant gene, *Ph-2*, was mapped to chromosome 10 and controls partial resistance to late blight in tomato. Powdery mildew, caused by *Oidium lycopersicum,* is another fungus that affects tomato plants and all cultivated tomatoes are highly susceptible to it. Searching the tomato genus for the occurrence of resistance genes, Lindhout *et al.,* (1994) reported four accessions of *S. hirsutum* and one of *S. peruvianum* to exhibit high levels of resistance to *O. lycopersicum.* An incompletely-dominant gene *Ol-1* found on chromosome 6 of tomato was shown to control resistance to powdery mildew (Van der Beek and Linhout., 1994). In addition, a recessive resistance gene (*Ol-2*) has been reported in *S. lycopersicon*, variety cerasiforme (Ciccarese *et al.,* 1998), located on chromosome 4. Lastly, resistance to root-knot nematodes was incorporated into many commercial tomato varieties, initially introduced from its wild relative *S. peruvianum* and identified as the *Mi-1* gene, located on chromosome 6 (Smith., 1944; Parella *et al.,* 2004).

There has been great progress in the understanding of the molecular mechanisms linked with natural virus resistance genes (Maule *et al*., 2007). The majority of characterized resistance plant genes have provided monogenic dominant resistance (Maule *et al.,* 2007). Most genes characterized at the molecular level present resistance to fungal or bacterial pathogens, however there are fewer such characterized genes conferring resistance to viruses which have been identified from crops such as tomato, potato, tobacco, soybean, bean, and model species *A. thaliana* (Parker and Higgins 1993). The majority fall into the nucleotide binding site-leucine rich repeat (NBS-LRR) class of resistant genes. They operate through a 'gene-for-gene' recognition of pathogen *avr* factors (Maule *et al.,* 2007).

As viruses depend on host factors to complete their infection cycle, the study of these factors has resulted in the identification of mutant alleles that confer recessive resistance to plant viruses in a range of species including tomato, lettuce, pepper, pea, melon, barley and rice (Diaz-Pendon *et al*., 2004; Robaglia and Caranta, 2006). Although recessive resistances can be qualitative, in many cases they are quantitative and/or components of polygenic resistance (Maule *et al*., 2007).

Another source of natural resistance to plant viruses is RNA silencing (innate immunity). It is a process where double stranded RNA (dsRNA) is recognized as a substrate for the targeting and degradation of sequence-homologous RNAs (Brodersen and Voinnet, 2006). Through similar pathways, gene silencing serves a defensive role against pathogen attacks. Plant viruses that have dsRNA as a secondary structural component of their genomic RNA, or as a component of their replication cycle, are therefore susceptible to RNA silencing (Lippman and Martienssen, 2004; Brodersen and Voinnet, 2006).

The first ToCSV infection study was conducted in SA using TYLCV-tolerant tomato accessions and local tomato cultivars (Pietersen and Smith, 2002). The study showed that TYLCV resistant cultivars were good sources of resistance to ToCSV, as they displayed similar resistant levels as local cultivars when infected with ToCSV (Pietersen and Smith, 2002). Such tomato cultivars are valuable for the control of ToCSV in SA.

2.2. GENERAL OBJECTIVES

There is a need to manage the spread and damage caused by ToCSV because of the ever increasing economic significance of tomato as a staple food, and supplement to the local diet of maize meal in South Africa and Mozambique. ToCSV is known to be one of the begomoviruses, which belongs to the TYLCD complex of tomatoes. It severely affects the production of tomatoes in South Africa. The control of ToCSV is generally by using chemicals and cultural practices to control the vector, thereby minimising the spread of the virus. These are difficult and expensive means. The best approach would be to use resistant hybrids. By introgressing resistant (R) genes from wild tomato species into the domesticated tomato through crossings, and screening for virus-responsive genotypes using molecular markers, breeding programs have successfully produced resistant and tolerant lines to TYLCD. Identification of other sources of resistance in tomato would prove useful in the development of additional R markers and widening the genetic potential for plant breeding for R gene traits. The objective of this study was to screen inbred lines from Sakata Vegenetics RSA (Pty) Ltd, using the known molecular markers for resistant genes to TYLCV isolates (*Ty-1, Ty-2, Ty-3, Ty-3a,*

Ty-4), and subsequently, narrow the lines down to one on the basis of having a high resistance level to ToCSV and no known *Ty* marker genes.

The high level of resistance that TAM showed under controlled infection against ToCSV, led us to carry on the study using TAM. Test crosses were made with TAM and a susceptible line, Rooikhaki. Consequently in an attempt to elucidate the type of resistance found in TAM, the F1 hybrids were selfed and an F2 population was generated and when infected with ToCSV, the type of segregation (if any) could be concluded based on the resistance levels of the plants.

Specific Aims

The specific aims in this chapter were to:

- Identify several inbred lines without the known resistant linked genes (*Ty-1, Ty-2, Ty-3/3a, Ty-4*) using molecular marker screening.
- Inoculate these lines with viruliferous whiteflies to confirm their resistance/susceptibility (response) to ToCSV.
- Investigate whether the resistance it is monogenic or polygenic by making test crosses of the inbred line (TAM) with a susceptible line (Rooikhaki).

2.3. MATERIALS AND METHODS

2.3.1. Selection of the inbred lines

Sixteen inbred lines were selected from the breeding program of Sakata Vegenetics RSA (Pty) Ltd (Table 2.1), which had different levels of resistance to TYLCV. These lines were previously exposed to TYLCV-IL in a field trial in Jordan and small ToCSV trials at Sakata Vegenetics (Pty) Ltd in Lanseria with ToCSV, where their response to the virus was recorded using the disease severity index scoring (DSI) system described by Lapidot and Friedmann (2002) (Figure 2.5).

Table 2.1: Sixteen inbred lines, their source, and recorded resistance to *Tomato yellow leaf curl virus* and to *Tomato curly stunt virus*.

INBRED NAME	SOURCE	RESISTANCE TO TYLCV-IL*	RESISTANCE TO ToCSV-[ZA:Ond:98]*
TAA	Tovi Sol	Res	Res
TBL	Tovi Sol	Res	Res
TAM	Elvira	Res	Res
TAN	Elvira	Res	Res
TAP	Elvira	Res	Res
TAF	Anastasia	Res	Res
RQX	Tiway	Mod Res	Res
TCG	Tiway	Res	Res
TCH	Tiway	Res	Res
TBJ	Tovi Green	Res	Res
TCK	Tovi King	Mod Res	Res
RQP	Cornelia	Res	Res
TAQ	Enza914	Mod Res	Res
TAR	Enza914	Mod Res	Res
TCY	XP198-1068	Mod Res	Res
TCM	Tovi Sol	Mod Res	Res

*Res – resistant; Mod Res – moderate resistance

2.3.1.1. Screening for the *Ty* molecular marker-associated genes

In order to confirm some of the results obtained from the Jordan and South African trials, these lines (Table 2.1) were screened using the *Ty-1, Ty-2, Ty-3/3a* TYLCV-resistance linked markers. In addition, a fifth molecular marker *Ty-4* was obtained later in this study, from J. W. Scott, University of Florida, and was used to screen inbred line TAM. Part of the selection process was to confirm the absence of any of these known resistance genes within these sixteen inbred lines.

2.3.1.2. DNA extraction of inbred lines

Two hundred milligrams of fresh leaf tissue was sampled from the newly formed leaves at the apex of the both the infected and control plants 18 days post inoculation. The sampled leaf material was transferred into a 96 well plate (Axygen), where each sample was transferred into its own well. Three to four stainless steel beads (3mm) were added to each well together with 500 µl extraction buffer (100 mM Tris, 50 mM EDTA, 500 mM NaCl and 10 mM ß-mercaptoethanol). The plant tissue was lysed using the tissue lyser (Retsch) for 3 minutes after which the samples were centrifuged for 30 seconds at 4000 rpm. Then 10% SDS (sodium dodecyl sulphate) was added to each well and the samples were incubated at 65°C for 20 minutes and centrifuged for 30 seconds at 4000 rpm followed

by adding 5M potassium acetate to each sample, vortexed and centrifuged for 10 minutes at 4000 rpm at 4°C. Supernatant was removed into a new, clean 96 well plate. Isopropanol (0.5 volume) was added to the supernatant, vortexed and centrifuged for another 10 minutes at 4000 rpm (at 4°C). Without unsettling the pellet, the isopropanol was removed and 500 µl of 70% ETOH (ethanol) was added and the samples were centrifuged for 5 minutes at 4°C at 4000 rpm. Following the spin, the supernatant was removed and the samples were vacuum dried for 45 minutes at 30°C, after which they were re-suspended in 100 µl SABAX water. RNA was digested using RNAse A (10mg/ml) that was incubated for 10 minutes at 37°C.

Ty-1 molecular marker:

The region linked to *Ty-1* resistance gene was amplified using TG97 molecular marker (Zamir *et al*., 1994) for 15 breeding lines (Table 2.1) which excluded TAN. A 25 µl PCR reaction constituted of 1 µl of DNA (522.31 ng/µl) and the following final concentrations of the PCR components: 1x PCR Buffer, 2 mM $MgCl_2$, 0.25 mM of each dNTP, 0.2 µM of each primer and 0.125 units of Taq DNA polymerase (Bioline). The *Ty-1* linked resistant loci was amplified by incubation at 95°C for 2 minutes, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 49°C and 90 seconds at 72°C, and a final extension at 72°C for 10 minutes.

The PCR product (398bp) was digested in a 20 µl reaction with 5 µl of PCR product and the following final concentrations of 0.25U of *Taq*I (Fermentas) and 1x *Taq*I buffer. The mixture was incubated at 65°C for 2 hours. After digestion the samples were subjected to electrophoresis in a 3% agarose gel and stained with ethidium bromide (EtBr) to visualise under ultraviolet light.

Ty-2 molecular marker:

Ty-2 resistant linked locus was amplified using the following TG0302 molecular markers F (forward) 5' – TGG CTC ATC CTG AAG CTG ATA GCG C – 3' and the TG0302 R (reverse) 5' – AGT GTA CAT CCT TGC CAT TGA CT – 3' (Hanson *et al*., 2000) for 15 breeding lines (Table 1) excluding TAN. A 25 µl PCR reaction consisting of 1 µl of DNA, 1x PCR Buffer, 2 mM $MgCl_2$, 0.25 mM of each dNTP, 0,2 µM of each forward and reverse primer and 0.125U of Taq DNA polymerase (Bioline). The *Ty-2* linked resistant gene was amplified by incubation at 95°C for 3 minutes followed by 30 cycles of 1 minute at 94°C, 2 minutes at 55°C and 2 minutes at 72°C, and a final extension at 72°C for 10 minutes.

Ty-3/3a molecular marker:

The region linked to *Ty3* resistance gene was amplified using FER-G8-F molecular marker F (forward) and FER-G8- R molecular marker (reverse) (Ji and Scott., 2007a) for 15 breeding lines excluding TAN (Table 2.1). A 25 µl PCR reaction consisting of 1 µl of DNA and 1x PCR Buffer, 2 mM $MgCl_2$, 0.25 mM of each dNTP, 0.2 µM of each primer and 0.25U of Taq DNA polymerase (Bioline). The PCR conditions included: incubation at 94°C for 3 minutes followed by 35 cycles of 30 seconds at 94°C, 1 minute at 55°C and 1.5 minutes at 72°C, and a final extension at 72°C for 10 minutes.

The PCR product was digested in 20 µl reaction volume with 5 µl of PCR product and 0.25U of *Taq*I (Fermentas) and 1x *Taq*I buffer and incubated at 65°C for 2 hours. After digestion the samples were loaded on a 3% agarose gel and stained with EtBr to visualise the product.

Ty-4 molecular marker:

The region linked to Ty-4 resistance was assayed for using P3-Ty4F1 and P3-Ty4R1 molecular markers (personal communication, J. W. Scott, University of Florida) for breeding line TAM. A PCR reaction volume of 25 µl was made up consisting of 1 µl of DNA and the following final concentrations of the reaction components: 1x PCR Buffer, 2 mM $MgCl_2$, 0.25 mM of each dNTP, 0.2 µM of each reverse and forward and 0.125U Taq DNA polymerase (Bioline). Cycling conditions were an initial denaturation at 94ºC for 3 minutes followed by 35 cycles of 30 seconds at 94ºC, 1 minute at 53ºC and 1.5 minutes at 72ºC, and a final extension at 72ºC for 10 minutes.

2.3.2. Disease resistance screening and controlled greenhouse infection trials

The different generation levels of TAM were screened for resistance to ToCSV as shown in the figure below (figure 2.1). TAM(1) and TAM(2) are from the F6 generation level. TAM(3) is from the F5 generation and TAM(5) is from F7.

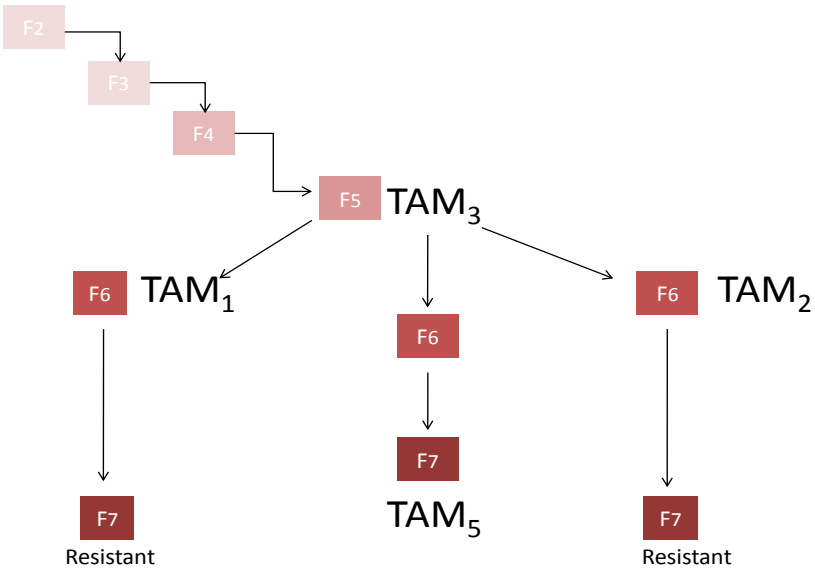

Figure 2.1: The generation map of TAM originating from Elvira. Different generations of TAM are indicated on the map.

2.3.2.1. Sowing

For all the infection trials, seeds were treated before sowing: the appropriate amount of seeds was weighed treated with 0.5% NaOCl (Bleach) solution for 30 minutes followed by a pre-incubation in a 37°C water bath for 10 minutes and then by incubation at 50°C for 25 minutes. After cooling down, the seeds were soaked in a solution of 10% trisodium phosphate ($Na_3\ PO_4 \cdot 12H_2O$) for 20 minutes, where the seeds were periodically agitated. Thereafter the seeds were rinsed under running water and dried at 25°C.

Seedling trays were sterilized and filled with a seedling mix containing a mixture of 8 mm pine bark and peat moss. The seeds of the different varieties were positioned in separate rows, and alternate cavities, where there was one seed per cavity. All the seeds were, subsequently, covered with medium vermiculite. The trays were watered and left inside the seedling tunnel at a temperature between 15°C and 25°C under 80% shade net for 72 hours. After 3 days, the trays were moved onto the seedling beds within the seedling tunnel and left to germinate under the correct irrigation schedules.

2.3.2.2 Infectious clone construction

For the construction of the infectious clone, pCambia2300 (AF234315) binary vector was modified as follows: a 84-bp dsDNA fragment (Figure 2.2a), containing the conserved stem-loop region of ToCSV and a unique SpeI restriction site was synthesized by Geneart (Regensburg, Germany) and

subcloned into pCambia2300 using the EcoRI and BamHI restriction sites indicated in Figure 2.2b. This construct allowed direct cloning of infectious monomeric viral genomes amplified using the SpeI primer set that incorporated a unique SpeI restriction site in the IR region. The full length genome of two ToCSV isolates, ToCSV-[ZA:Mks30:08] as ToCSV variant I and ToCSV-[ZA:Mks22:07] as ToCSV variant II, were amplified by PCR using the SpeI primer set. (Spe-IF: 5'-ATAATAACTAGTCCCCACGCACTATTTTATGTCGAC-3' and Spe-IR: 5'-ATAATAACTAGTTTTTTTTGGGGGCACGGCCATCCG-3'). The viral fragments (~2.7kbp) were cloned into the pGEM-T-Easy vector (Promega) using TA cloning, resulting in a recombinant clone pGEMSpeIV30 and pGEMSpeIV22. Both viral fragments were sequenced in full. Plasmids pGEMSpeIV22 and pGEMSpeIV30 were digested with the SpeI restriction enzyme respectively, to release the full-length genomes. The genomes were ligated to pCAM100 linearized with SpeI and dephosphorylated, resulting in pCam30-VI and pCam22-VII binary plasmids containing the 1.1-mer of the two ToCSV variant genomes. The structure of the two 1.1-mer constructs is shown in Figure 3.1c. After checking for the sense orientation of the inserted genome, recombinant plasmids were selected and purified from E. coli JM109 (Promega) using the alkaline lysis miniprep method in Sambrook *et al.* (1989), introduced into *Agrobacterium tumefaciens* and used for agroinoculation (Esterhuizen, unpublished).

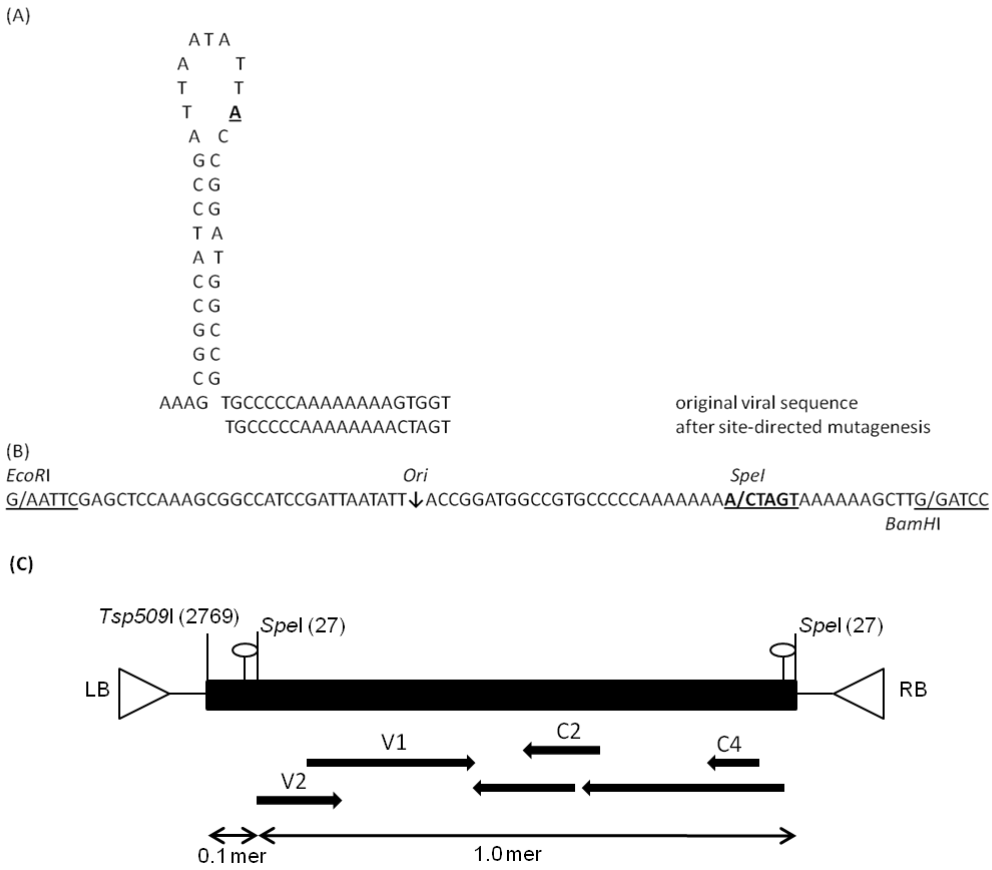

Figure 2.2: Infectious clone construction. (A) Construction of pCambia100. Site-directed mutagenesis at the end of the stem-loop to generate a SpeI site in the ToCSV clones. The underlined bold A nucleotide in the loop indicates the origin of replication. (B) The sequence of the 84-nucleotide insert containing the stem-loop shown in (A) that was placed in the multiple cloning site of the pCambia2300 vector using the EcoRI and BamHi restriction sites indicated. (C) Structure of the 1.1 mer pCam30-VI and pCam22-VII ToCSV infectious clone. LB and RB represent the left and right T-DNA border sequence of pCambia2300 and (ю), the stem-loop forming region. The numbers show nucleotide positions. The first nucleotide at the 5' end of the conserved sequence (TAATATT↓AC) in the stem-loop forming region is designated as nucleotide 1 (Esterhuizen, unpublished).

2.3.2.3 Agroinoculation and analysis of symptoms

Chemically competent cells of *A. tumefaciens* strain C58C1 (Van Larebeke *et al.,* 1984) were prepared and transformed by the freeze-thaw method (Höfgen and Willmitzer *et al.,* 1988), with the agroinfectious clones pCam30-VI and pCam22-VII, respectively. Transformed Agrobacterium cultures were grown in LB agar plates containing 100 µg/ml rifampicin and 100 µg/ml kanamycin at 28°C for 48 h. Ten to fifteen tomato plants (Rooikhaki) per construct were inoculated by using the agro-pick method as described previously (Urbino *et al.,* 2008) Briefly, for each eighteen day old seedling, the

stem was pricked three times at different levels with the tip of a sterile needle previously dipped into the 48 h plated culture. As a negative control, plants were inoculated with *A. tumefaciens* carrying the empty pCambia2300 plasmid. All the agro-inoculated plants were tested for the presence of viral DNA by PCR using the ToCSV specific primer set. Each inoculated plant was inspected for symptoms of virus infection regularly until 21 days post inoculation (DPI) and thereafter on a weekly basis up until 120 DPI.

2.3.2.4 Viruliferous *B. tabaci* whiteflies

Bemisia tabaci biotype B were reared on Rooikhaki plants that were infected with an infectious dimer of ToCSV. The infection of the tomato seedlings was conducted as follows: seedling trays were moved into the presence of the viruliferous whiteflies in the enclosed insectarium. The whiteflies were brushed off once a day, from the older Rooikhaki plants (providing virus source to the vector) and onto the seedlings in the seedling trays. This ensured a more even distribution of *Bemisia tabaci* feeding on the seedlings of approximately five whiteflies per seedling. After the infection period, the whiteflies were brushed off the seedlings and the trays were moved into a whitefly-free enclosed room where the seedlings were drenched with imidacloprid to rid of the vector. The seedlings remained in this enclosed area for 3 days and were drenched daily to ensure there were no live whiteflies.

2.3.2.5 DNA extraction and PCR confirmation of ToCSV infection

DNA was extracted using a modified method of Dellaporta *et al*. 1983. Two hundred milligrams of fresh leaf tissue was sampled from the newly formed leaves at the apex of the both the infected and control plants 18 days post inoculation. The sampled leaf material was transferred into a 96 well plate (Axygen), where each sample was transferred into its own well. Three to four stainless steel beads (3mm) were added to each well together with 500 µl extraction buffer (100 mM Tris, 50 mM EDTA, 500 mM NaCl and 10 mM ß-mercaptoethanol). The plant tissue was lysed using the tissue lyser (Retsch) for 3 minutes after which the samples were centrifuged for 30 seconds at 4000 rpm. Then 10% SDS (sodium dodecyl sulphate) was added to each well and the samples were incubated at 65°C for 20 minutes and centrifuged for 30 seconds at 4000 rpm followed by adding 5 M potassium acetate to each sample, vortexed and centrifuged for 10 minutes at 4000 rpm at 4°C. Supernatant was removed into a new, clean 96 well plate. Isopropanol (0.5 volume) was added to the supernatant, vortexed and centrifuged for another 10 minutes at 4000 rpm (at 4°C). Without unsettling the pellet, the isopropanol was removed and 500 µl of 70% ETOH (ethanol) was added and the samples were centrifuged down for 5 minutes at 4°C @ 4000 rpm. Following the spin, the supernatant was removed and the samples were vacuum dried for 45 minutes at 30°C, after which

they were re-suspended in 100 µl SABAX water. RNA was digested using RNAse A (10 mg/ml) that was incubated for 10 minutes at 37°C.

A set of degenerate primers were used to confirm infection with ToCSV. These primers bind to the coat protein (CP) region (they bind to all begomoviruses) and serve as an identification of positive infection with ToCSV (Accotto *et al*., 2000). The primer pairs were : F (forward) 5'- GCC CAT GTA YCG TAA GCC – 3' and R (reverse) 5' – GGV TTA GAR GCA TGM GTA C – 3' (Accotto *et al*., 2000). A 25 µl PCR reaction consisting of 1 µl of template DNA, 1x PCR Buffer, 2.5 mM $MgCl_2$, 0.1 mM of each dNTP, 0.4 µM of each primer and 0.125U of Taq DNA polymerase (Bioline). The cycling parameters were as follows: initial denaturation at 94°C for 1 minute followed by 35 cycles of 20 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 72°C. After amplification the samples were subjected to electrophoresis in a 1% agarose gel and stained with EtBr to visualise under ultraviolet light (254 nm).

NOTE: SEVERAL INFECTION TRIALS WERE PERFOMED TO SCREEN FOR DISEASE RESISTANCE TO ToCSV.

2.3.2.6. INFECTION TRIAL ONE

This pilot trial was conducted to ascertain the number of days the tomato plants required to be exposed to viruliferous *Bemisia tabaci* biotype B whiteflies in order to produce 100% infection. Ten seed of a known ToCSV susceptible tomato cultivar, Rooikhaki were treated and sown in seedling trays as described above (sections 2.3.2.1). Seventeen and 21 day old seedlings were exposed to viruliferous whiteflies for 8 continuous days.

Eight days post infection the seedlings were transplanted into 9 cm pots and placed inside a greenhouse at 25°C to monitor symptom development daily for a week. The disease severity index was undertaken based on the method developed by Lapidot and Friedman (2002) (figure 2.5) with 0 = no symptoms 1 = slight yellowing and minor curling on margins of the apical leaf 2 = yellowing and minor curling of leaflet ends 3 = wide range of leaf yellowing, curling and cupping, some reduction in size, yet plants continue to develop 4 = very severe plant stunting and yellowing and pronounced cupping and curling plant growth stops. Refer to section 2.3.2.5 for the DNA extraction and PCR confirmation of ToCSV infection.

2.3.2.7. INFECTION TRIAL TWO

TAM inbred line was selected for further resistance screening in greenhouse infection trials with ToCSV infectious clone. Different generations were selected namely, TAM(1) (F6), TAM(2) (F6), TAM(3) (F5) and TAM (5) (F7) (Figure 2.1).

The purpose of this infection trial was to screen various generations of a putative resistant inbred line, TAM (10 seeds of TAM(1), TAM(2), TAM(3) and TAM(5) were planted) (section 2.3.2.1) in order to confirm resistance towards ToCSV.

Fifteen seeds from the susceptible ToCSV cultivar Rooihkaki, ten seeds from the ToCSV resistant control variety RQS, ten seeds of TAM(1), (2), (3) and (5) (Figure 2.1) were treated and sowed in seedling trays (as described previously). Ten seeds of each cultivar were sown as the healthy controls and were not exposed to viruliferous whiteflies. The 18 day old seedlings were inoculated with ToCSV infectious clone for 8 continuous days by caging the seedlings with the viruliferous whiteflies as previously described. The seedlings were transplanted into an insect proof greenhouse at 25°C into 25 L planting bags. DSI ratings were recorded weekly for 7 weeks using DSI index developed by Lapidot and Friedman (2002) (Figure 2.5). DNA was extracted and PCR confirmation of ToCSV infection conducted (Refer to section 2.3.2.5).

2.3.2.8. INFECTION TRIAL THREE

Infection trial three was a repetition of trial two. Ten seeds of each generation of TAM: (TAM(1), TAM(2), TAM(3) and TAM(5)) were treated and sown as described in section 2.3.2.1. Ten seeds of the resistant cultivar RQS and 10 of the susceptible line, Rooikhaki, were included in the trial as resistant and susceptible controls, respectively. At 18 days old, the seedlings were inoculated with ToCSV as described previously for a period of 72 hours. The seedlings were transplanted into 25L planting bags an insect proof greenhouse at 25°C and DSI ratings were recorded weekly for 5 weeks.

The disease severity index was undertaken based on the method developed by Lapidot and Friedman (2002) (Figure 2.5). DNA extraction and PCR confirmation of ToCSV infection was conducted as previously described.

2.3.2.9. INFECTION TRIAL FOUR

A larger infection trial was conducted as an experimental repeat. The seedlings were treated and sown as described in section 2.3.2.1. Eighteen seedlings of TAM(1), 23 seedlings of TAM(2), 26

seedlings of TAM(3), 28 seeds of RQS (resistant control), 28 seeds of Rooikhaki (susceptible control) and 28 seeds of Tyler, a commercial SA-grown resistant variety to ToCSV (used as a resistant control) were used. At 18 days old, the seedlings were inoculated with ToCSV viruliferous whiteflies for a period of 72 hours. DNA extraction and PCR confirmation of ToCSV infection was conducted as previously described (section 2.3.2.5).

Confirmation of ToCSV-[ZA:Ond:98] infection using a Dot Blot

A dot blot was done for all the experimental and control plants in infection trial 4. The full length DNA (2.7kb) of ToCSV was random primed labelled with Digoxigenin-11-dUTP using DIG-High Prime, a 5x concentrated labelling mixture of random hexamers, dNTP mix containing alkali-labile Digoxigenin-11-dUTP, labelling grade Klenow enzyme and an optimized reaction buffer, according to the manufacturer's instructions (Roche). The extracted DNA (8 µl) was blotted onto a positively charged nylon membrane (Amersham, UK). The DNA was cross linked under UV light for 1 minute before the experiment unfolded. The membrane was washed in warm hybridisation buffer (DIG Easy Hyb ready-to-use hybridization solution without formamide, Roche) for 30 minutes with agitation. Fresh hybridisation buffer containing the probe was prepared (according to the manufacturer's instructions) and the membrane was incubated overnight at 60°C with gentle rotation. The membrane was washed with a low stringency buffer (2× SSC, 0.1% SDS) twice for 5 minutes at room temperature. The high stringency buffer (0.5× SSC, 0.1% SDS) was preheated to 60°C and the membrane was washed in it twice for 15 minutes with agitation. The membrane was then washed with washing buffer (0.1 M Maleic acid, 0.15 M NaCl; pH 7.5 (20°C); 0.3% (v/v) Tween 20) after which blocking solution (1× working solution diluted in the 10× blocking solution 1:10 in maleic acid buffer, according to the manufacturer's instructions, Roche) was added and for 30 minutes the membrane was soaked in it. The anti- digoxigenin (antibody) (anti-digoxigenin-AP 1:10 000 (75 mU/ml) diluted in blocking solution) was added to a fresh batch of blocking solution and the membrane was further incubated for 30 minutes. Consequently the membrane was washed twice and equilibrated in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5). The chemiluminescent substrate was added to the blot and it was soaked for 5 minutes at room temperature. The excess liquid was removed and the damp membrane was incubated at 37°C for 10minutes, after which it was exposed to x-ray film for 15 minutes, and the film was consequently developed and the results were observed.

2.3.2.10 INFECTION TRIAL FIVE: THE TEST CROSSES

From results obtained in infection trial 4, we went on to determine the genetics of resistance i.e. dominance/recessive; segregating/stability of the inbred line TAM. This was done by making test crosses.

2.3.2.11 Test crosses of TAM with Rooikhaki to determine the nature of the resistance

Rooikhaki was used as the male plants and TAM was used as the females on which the fruit was grown. At least 6 emasculated flowers of each generation of TAM were pollinated to produce the F1 hybrids of the test crosses, being; TAM(1) x Rooikhaki, TAM(2) x Rooikhaki and TAM(3) x Rooikhaki. The flowers on TAM(1), TAM(2) and TAM(3) plants were emasculated (Figure 2.3) while they were still green and closed. Using a tweezers, the sepals were forced open. The yellow petals were carefully removed exposing the stigma. After two days, the stigma was ready to be pollinated with pollen, from the Rooikhaki plant. Using a self made pollen collector (Figure 2.3e), the pollen was collected from the male flower and the tip of the stigma of the emasculated flower was dipped into the pollen. A flower marker (plastic ring) was used to mark the pollinated flowers. The fruits were left to grow and ripen, after which they were harvested and the seeds were washed, treated and weighed.

2.3.2.12 Production of F1 hybrids

The fruit were left to grow and ripen on the TAM plants before they were harvested and the seeds extracted. The fruit of the test crosses (F1 hybrids) was harvested after which the seeds were extracted as follows: The tomato pulp was collected into a sealable plastic bag and left to ferment for 72 hours in the dark. The pulp was washed, and the viable seeds were separated out and acid treated. The seeds were soaked in a solution of 1.25% hydrochloric acid (HCl) for 30 minutes. Every 10 minutes the seeds were agitated after which they were thoroughly rinsed with water. They were then collected and dried for 30 minutes. After drying, they were weighed, packed and stored in a cold room (10°C).

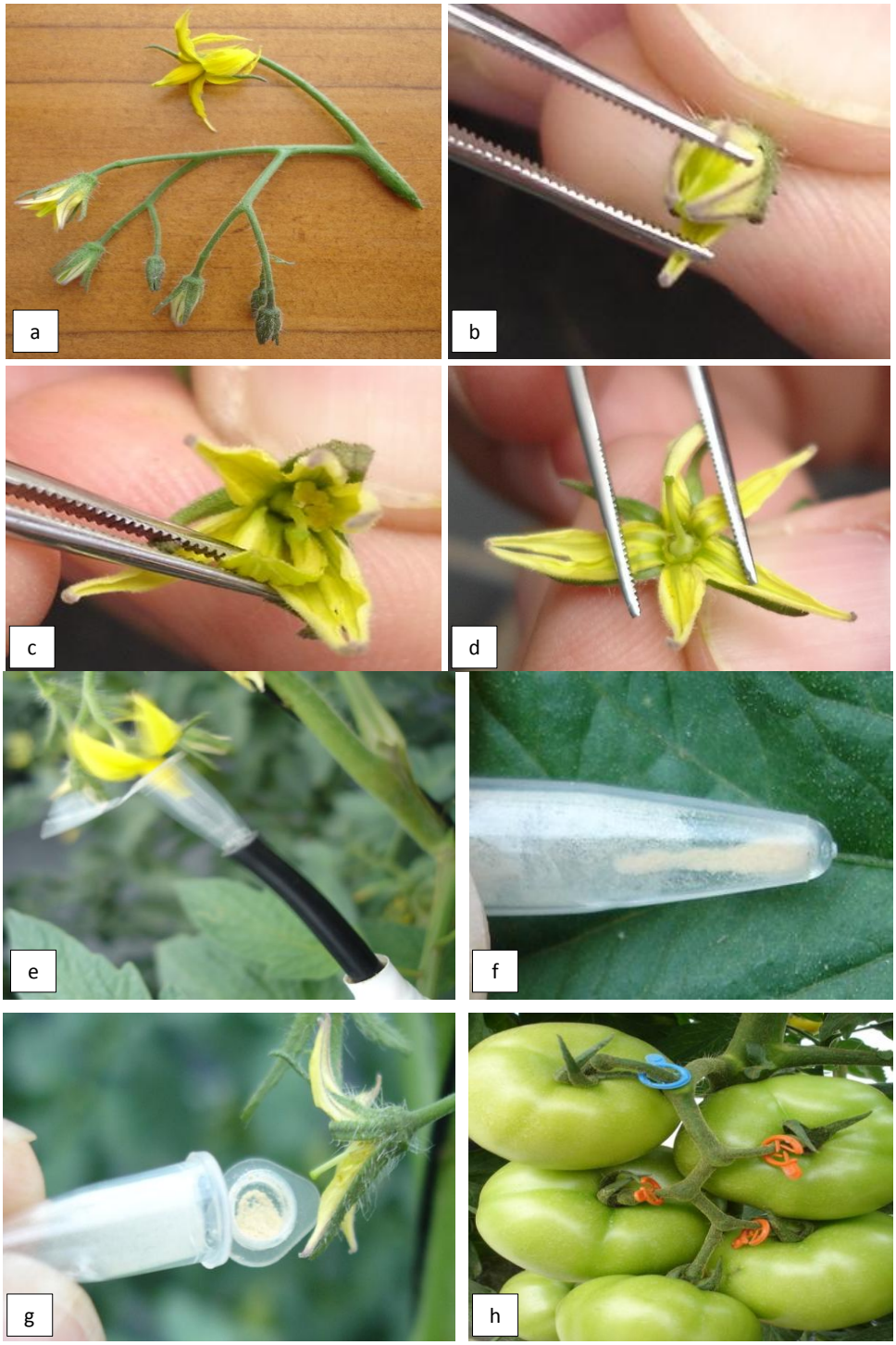

Figure 2.3: Steps to generate a F1 hybrid. a) An unripe tomato flower is chosen b) Opening the flower. c) Emasculation. d) The stigma is exposed. e) Extraction of pollen. f) Collection of pollen. g) Pollination. h) F1 Hybrid.

2.3.2.13 Production of F2 population

Five seeds of each of the F1 test crosses were treated and sown as described previously (section 2.3.2.1.). After 18 days, the seedlings were transplanted into the greenhouse and were left to develop and grow fruit to produce the F2 generations of the test crosses (TAM(1) x Rooikhaki F2, TAM(2) x Rooikhaki F2 and TAM(3) x Rooikhaki F2). Twenty seed of each generation of tomato lines of TAM [TAM(1), TAM(2) and TAM(3)], as well as a commercial cultivar, Tyler, and 30 seeds of the two controls, (RQS, resistant control and Rooikhaki, susceptible control) were treated and sown as previously described (section 2.3.2.1.). Another twenty seeds of each from the test crosses (TAM(1) x Rooikhaki, TAM(2) x Rooikhaki and TAM(3) x Rooikhaki), and a further twenty seed of each from the F2 test crosses were treated and sown as previously described (section 2.3.2.1). The seedlings were infected at 18 days old, for a period of 72 hours using viruliferous whiteflies as previously described. Control plants were grown within the same insect proof greenhouse and were not infected with viruliferous ToCSV whiteflies. The seedlings were transplanted into 25L planting bags an insect proof greenhouse at 25°C. The development of ToCSV symptoms were monitored and recorded weekly using the DSI index (Lapidot and Friedmann., 2002).

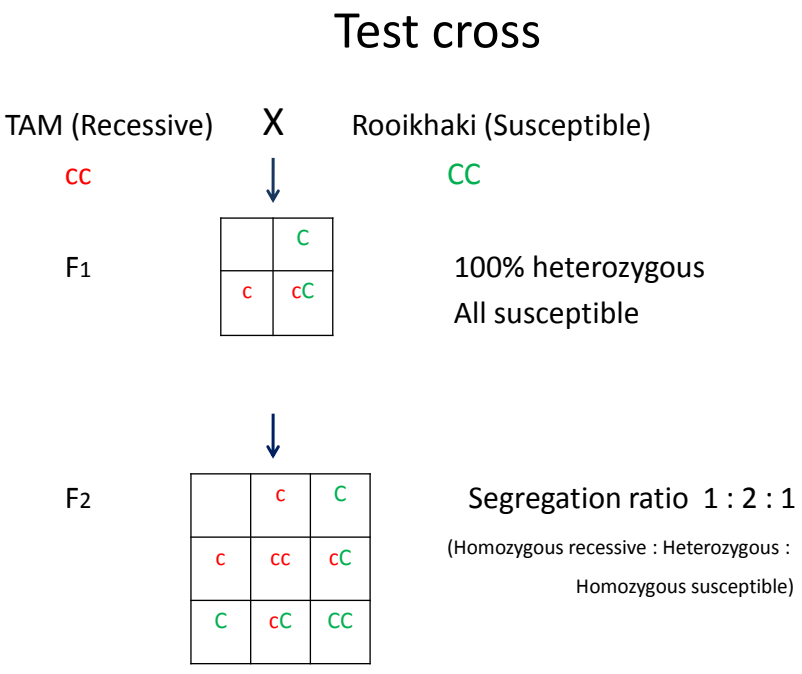

Figure 2.4: The test cross showing the expected ratios of 1:2:1 homozygous: heterozygous: homozygous susceptible, if TAM has recessive resistance.

The expected ratios are shown in Figure 2.4, assuming that the resistance in the inbred line TAM is recessive as suspected. The F1 population, when infected with ToCSV should all be susceptible to

ToCSV as they would have heterozygous alleles. The F2 population, on the other hand should show a ratio of 1:2:1 (Homozygous recessive: Heterozygous: Homozygous recessive). If the segregation patterns match as these, it can be concluded that the resistance is recessive in TAM.

2.3.2.14 PCR confirmation of ToCSV infection

PCR confirmation of ToCSV infection was conducted as described in section 2.3.2.5.

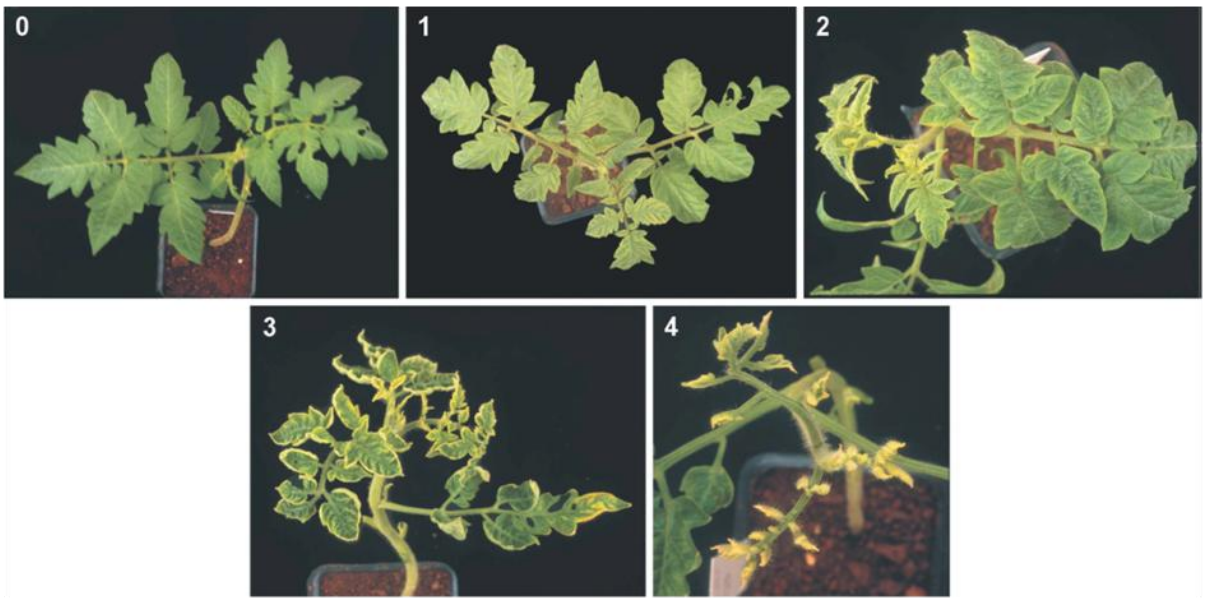

Figure 2.5: Disease severity index according to Lapidot and Friedman (2002).

0 = No visible symptoms

1 = Very slight yellowing and minor curling of leaflet margins on apical leaf

2 = Some yellowing and minor curling of leaflet ends

3 = Wide range of leaf yellowing, curling and cupping, some reduction in size, yet plants continue to develop

4 = Very severe plant stunting and yellowing and pronounced cupping and curling, plant growth stops

2.3.3 Statistical analysis

The differences in the disease severity index among the cultivars were analysed statistically using a one way Analysis of Variance (ANOVA) (SPSS Version 13). Data were tested for normality using Kolmogorov Smirnov Z test and Kruskal wallis (non parametric test) test. The null hypothesis in each infection trial stated that there is a significant difference between the generations of TAM.

2.4. RESULTS

2.4.1. Screening the four tomato inbred lines for reported *Ty* genes associated with resistance to all TYLCV isolates.

Ty-1 molecular marker

Typically, a resistant plant would show two bands at 303 bp and 95 bp, heterozygous plants would show three bands at 398 bp, 303 bp and 95 bp (Figure 2.6b), and susceptible plants would show only one band at 398 bp (Figure 2.6a). None of the plants tested showed Ty-1 resistance (Figure 2.6).

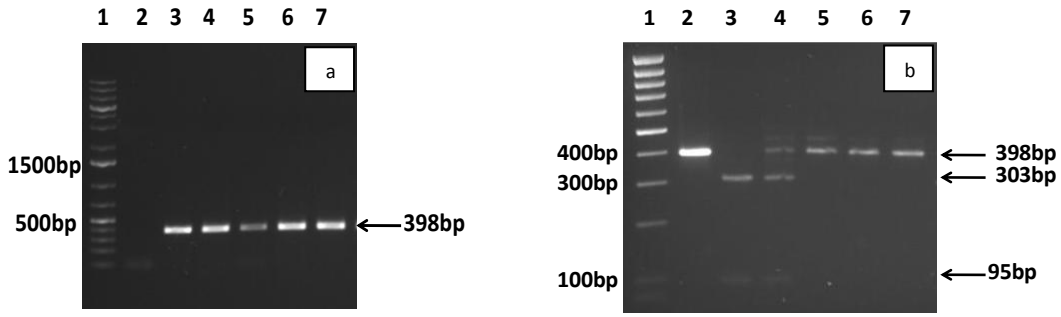

Figure 2.6: *Ty-1* PCR-RFLP with *Taq* I of inbred line of tomato produced (a) 398 bp PCR product that was not cut by *Taq* I by susceptible plants. Lane 1: Fermentas Generuler 1 Kb DNA ladder plus, lane 2: non template control, and with resistant control RQS in lane 3 with PCR-RFLP fragment of 303bp and 95 bp as well as heterozygous plants with PCR-RFLP fragments of 398, 303 and 95 bp in lane 4.
(b) Lane 1: Fermentas ladder; lane 2: undigested PCR product, lane 5-7: with susceptible plants TAA (1), TAM (1) and TAQ (1) in lane 5-7 respectively.

Ty-2 molecular marker

A 850 bp amplicon indicated the absence of the *Ty-2* gene (Figure 2.7). The inbred lines TAA, TAM, TAQ and TAR, and all the generations of each line tested, showed that *Ty-2* linked resistance gene marker was not present in these plants (Figure 2.7).

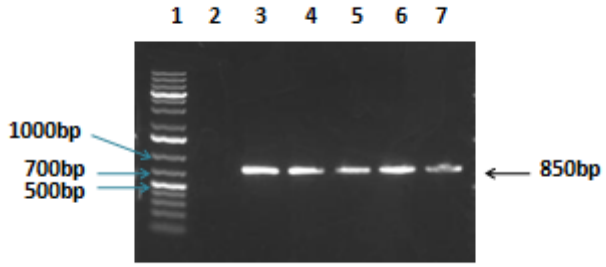

Figure 2.7: Ty-2 PCR product of susceptible plants Rooikhaki, TAA (1), TAM (1), TAQ (1), TAR (1) in lane 3-7 respectively and lane 1 with Fermentas GeneRuler 1kb DNA ladder Plus and non template control in lane 2.

Ty-3/3a molecular marker

After *Taq* I digest, the Ty-3 PCR product produced 250, 200, 50 bp product (Figure 2.8b) whereas Ty-3a PCR product produced 300 and 200bp fragments (Figure 2.8b).

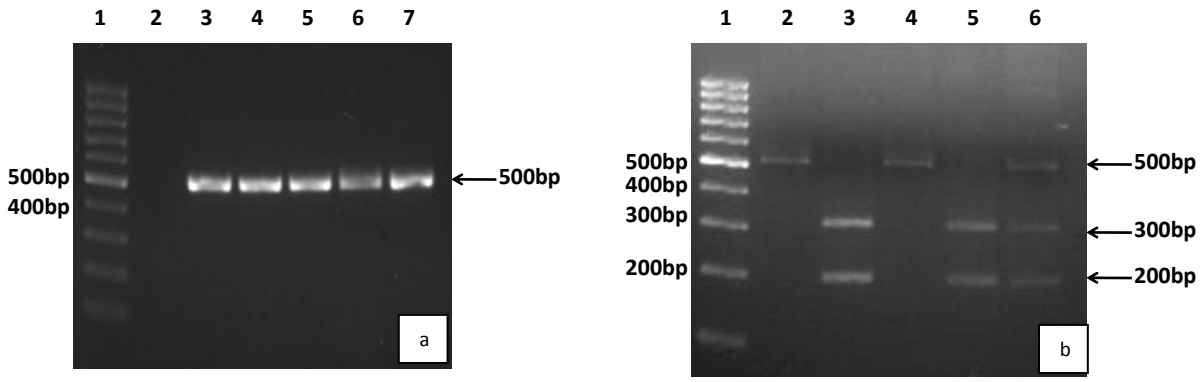

Figure 2.8: PCR-RFLP products using Ty-3/3a molecular markers indicate (a.) susceptible plants in lane 3-7 with RQS, TAA(1), TAM(1), TAQ(1), TAR(1), respectively and Fermentas O' GeneRuler 100bp maker in lane 1 and Ty3a gene control plants in lane 3 and 5, susceptible plant in lane 2 [TAM(1)], 4[TAR(1)] and Ty3 gene control in lane 6.

Ty-4 molecular marker

The amplification of plant DNA would typically show a positive sample with a 500bp amplicon (Figure 2.9). All the tested inbred lines did not have the Ty-4 linked molecular marker resistance gene present (Figure 2.9).

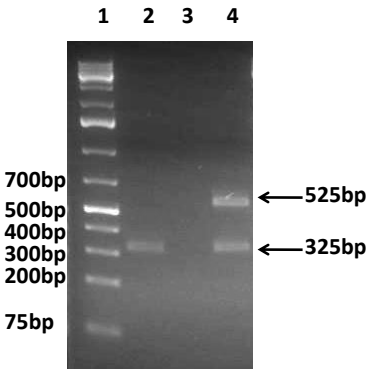

Figure 2.9: Ty-4 PCR with 525 bp product and 325 bp for susceptible plant (lane 2). Lane 4 indicates heterozygous plant and the Fermentas GeneRuler 1kb DNA Ladder Plus in lane 1.

2.4.2. Disease resistance screening and greenhouse infection trials

The agroinoculated Rooikhaki plants started to show symptoms of ToCSV infection such as leaf curling and stunting after 21 days post infection (Figure 2.10). Infection was confirmed by PCR using degenerate primers yielding a positive result showing an amplicon of 500 bp (Figure 2.11).

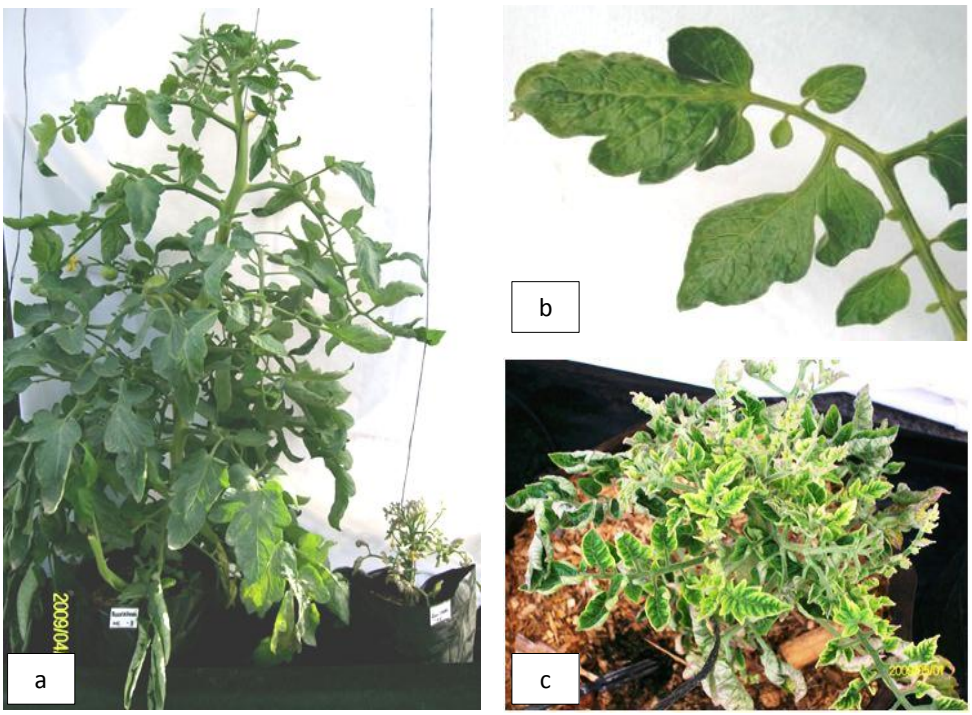

Figure 2.10: Plant symptoms observed in trial one with a.) showing uninfected Rooikhaki plant (left) and infected (right). b.) Healthy Rooikhaki plant. c.) Higher magnification of Rooikhaki infected leaves showing yellowing and upward curling of the leaf margins.

INFECTION TRIAL ONE

This pilot trial was conducted to ascertain the number of days the tomato plants required to be exposed to viruliferous *Bemisia tabaci* biotype B whiteflies in order to produce 100% infection. The uninoculated control Rooikhaki plants were symptomless and healthy. Both 17 and 21 day old Rooikhaki seedlings showed symptoms 21 days post infection. The seedlings scored a maximum DSI of 4 and showed typical signs of infection such as stunting and leaf curling (Figure 2.16). Infection was confirmed by PCR using degenerate primers yielding a positive result showing an amplicon of 500bp (Figure 2.11). No statistical analysis was performed on this trial.

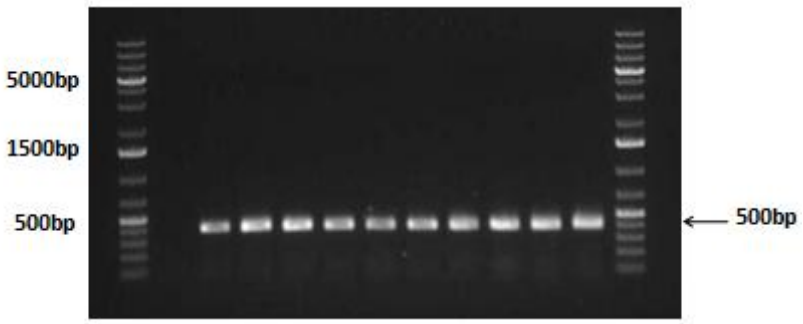

Figure 2.11: Coat protein PCR confirmation of ToCSV using the Ty-primers producing a 500 bp fragment.

INFECTION TRIAL TWO

TAM inbred line was selected for further resistance screening in the greenhouse infection trials. Different generations were selected namely: TAM(1) (F6), TAM(2) (F6), TAM(3) (F5) and TAM(5) (F7). The resistant control, RQS, showed positive results for infection as PCR analysis of the plant's DNA showed a positive 500 bp amplicon (Figure 2.11). RQS displayed the lowest index severity rating (0) with no symptoms on the infected plants (Figure 2.12). Rooikhaki, the susceptible control, scored the highest DSI of 4 (Figure 2.13). TAM (2), an F6 generation level inbred, showed the lowest resistance with an average DSI rating of 1.3. TAM (3), an F5 generation, had the lowest rating in this trial with a score of 0.4. The F6 and F7 generations, TAM (1) and TAM (5) respectively, displayed very similar values of 0.6 and 0.7 (Figure 2.13).

Figure 2.12: ToCSV Infected RQS on the left with a healthy RQS control on the right.

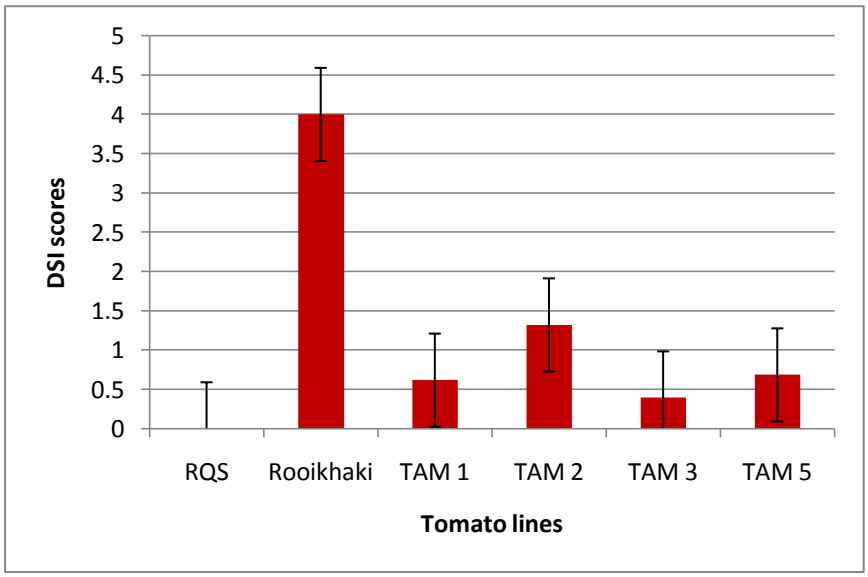

Figure 2.13: The average DSI scores for the pilot infection trial with RQS, Rooikhaki and different generations of TAM.

The DSI ratings from infection trial two are not normally distributed as the null hypothesis is rejected (p-value = 0.000 < 0.05) when the Kolmogorov Smirnov Z test was used. The Kruskal wallis (non

parametric test) shows that there is a significant difference between the DSI scores in the generations of TAM(1), (2) (3) and (5) (Table 2.2). The p-value confirms this, that with a 5% significant level, on average, the DSI ratings of TAM differs across the generations. This is re-iterated by the one way ANOVA (Appendix table A1).

Table 2.2: Descriptive statistics of different TAM plants generations in trial two.

	N	Minimum DSI	Maximum DSI	Median	Mean	Standard Deviation
TAM(1)	28	0	2	0	0.4643	0.6372
TAM(2)	28	0	2	1	1.3214	0.6696
TAM(3)	49	0	1	0	0.2245	0.4216
TAM(5)	49	0	2	0	0.4898	0.5818

P-value = 0.000

N indicates the number of tomato plants used for the statistical analysis from each generation of inbred line TAM.

A pair wise comparison between the generations of TAM, shows that the significant differences lie between TAM(2) and TAM(1), (3), and (5) (p-values = 0.000) (Table 2.2). There is no significant difference between TAM(1) and (5) (p>0.5) (Table 2.2).

Table 2.3: Pair wise comparisons of different generations of TAM in infection trial two.

(I) Generation	(J) Generation	Mean Difference (I-J)	P-value
TAM(1)	TAM(2)	-0.857(*)	0.000
	TAM(3)	0.240	0.451
	TAM(5)	-0.026	1.000
TAM(2)	TAM(1)	0.857(*)	0.000
	TAM(3)	1.097(*)	0.000
	TAM(5)	0.832(*)	0.000
TAM(3)	TAM(1)	-0.240	0.451
	TAM(2)	-1.097	0.000
	TAM(5)	-0.265	0.128
TAM(5)	TAM(1)	0.026	1.000
	TAM(2)	-0.832	0.000
	TAM(3)	0.265	0.128

INFECTION TRIAL THREE

In infection trial three, a repeat of trial two, except DSI was observed for 7 weeks. The resistant control, RQS was positive for the infection as PCR analysis of the plant's DNA showed a positive 500bp amplicon with ToCSV PCR (Figure 2.11). RQS displayed the lowest index severity rating (0) and showed no obvious signs of infection. Rooikhaki, the susceptible control, scored the highest disease severity rating index of 4 (Figure 2.14). TAM (5) showed the most resistance with a low score of 1.1 (Table 2.4) in the first week, and by the 5th week it was the line that displayed the least resistance to ToCSV as compared to the other infected plants [TAM(1), TAM(2) and TAM(3)]. In addition, TAM(2) scored a relatively high DSI rate (1.7) as compared to the other infected plants in the first week of

infection and gradually decreased to 1.3, being the lowest score of the group in week 5 (Table 2.4 and Figure 2.14).

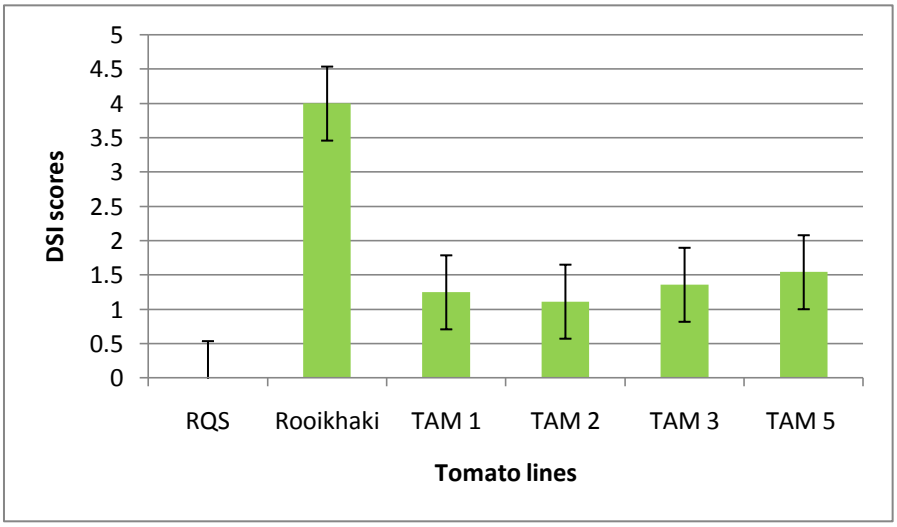

Figure 2.14: The DSI scores from the third trial indicating the values of the DSI scores ranging from 0 to 4 for each of the tomato lines.

Table 2.4: The weekly DSI scores from the third infection trial performed.

		Mean DSI scores				
Tomato lines	**No. of plants**	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 5**
RQS	**10**	0	0	0	0	0
Rooikhaki	**9**	4±1.2	4±1.2	4±1.2	4±1.2	4±1.2
TAM(1)	**8**	1.6±0.5	1±0.8	1.1±0.8	1.3±0.7	1.4±0.7
TAM(2)	**7**	1.7±0.5	1±0.9	0.9±0.8	0.7±0.8	1.3±0.5
TAM(3)	**5**	1.2±0.8	1±0.8	1.4±1	1.6±0.9	1.6±0.7
TAM(5)	**7**	1.1±0.9	1.3±0.8	1.7±1	1.9±0.7	1.7±0.6

± indicates standard deviation

The DSI ratings obtained from the third infection trial show that the values are not normally distributed therefore the null hypothesis is rejected. No significant difference was found between the generations of TAM in this infection trial since the P = 0.078 in the Kruskal wallis test.

Table 2.5: Descriptive statistics of the DSI rating scores for TAM plants in the four generations for infection trial three.

	N	Minimum DSI	Maximum DSI	Median	Mean	Standard Deviation
TAM(1)	50	0	2	1	1.0200	0.8449
TAM(2)	50	0	2	1	0.7800	0.8401
TAM(3)	50	0	3	0	0.6800	0.9781
TAM(5)	50	0	3	1	1.0800	1.0069

P-value = 0.072

N indicates the number of plants of each generation of TAM used for the statistical analysis.

INFECTION TRIAL FOUR

TAM (5) was excluded from infection trial four since it did not display good resistance level to ToCSV in trial three. RQS showed some signs of infection in the first two weeks (DSI rating of 1.4) such as minor leaf curling, thereafter, the scores dropped to 0.2 and the plants continued to develop normally. Rooikhaki scored the highest disease severity rating index of 4 (Figure 2.15). Tyler, a commercial resistant variety, showed resistance to ToCSV, which reflected closely with TAM(1) (Table 2.6). TAM(3) (Figure 2.15) (F5 generation level) displayed the lowest DSI ratings after 5 weeks, demonstrating the highest resistance of the hybrids (Figure 2.15). Infection was confirmed by ToCSV PCR producing a 500 bp (Figure 2.11) for all the infected plants used in this trial. A second confirmation of infection was also obtained by doing a dot-blot assay (Figure 2.16). A positive result (hybridization between ToCSV probe and ToCSV probe in plant material is indicated by a black spot, and all infected plants showed hybridization.

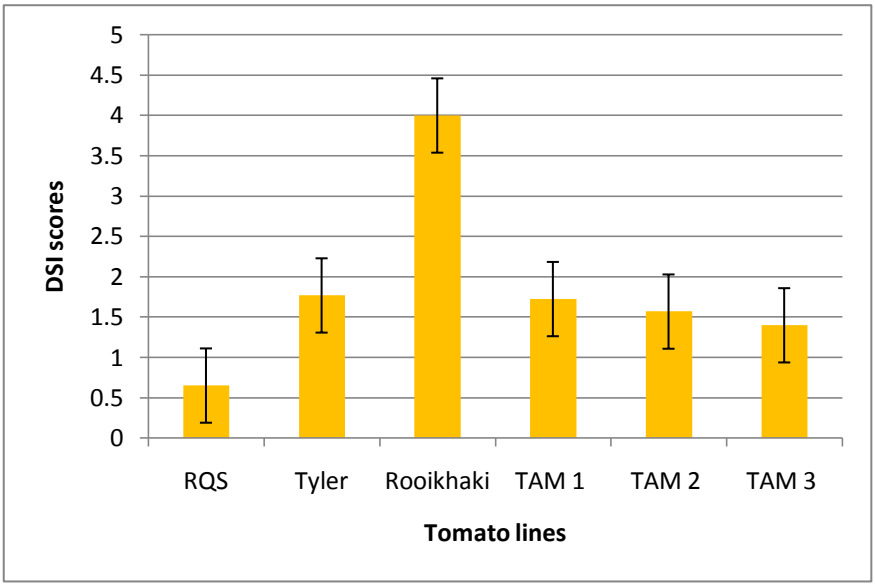

Figure 2.15: ToCSV infectivity results from the fourth trial. The y-axis represents the DSI scores ranging from 0 to 4. Tyler, a commercial resistant line to ToCSV-[ZA:Ond:98] was included in this trial.

Table 2.6: DSI results over 5 weeks of the fourth ToCSV-[ZA:Ond:98] infection trial, which included Tyler, a commercial resistant variety.

		Mean DSI Scores				
Plant code	**No. Of plants**	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 5**
RQS	**30**	1.4±0.7	1.3±0.7	0.2±0.9	0.2±0.5	0.2±0.5
Rooikhaki	**24**	4±0	4±0	4±0	4±0	4±0
Tyler	**28**	1.4±0.9	1.8±1	1.9±0.8	1.9±0.8	1.8±0.8
TAM(1)	**16**	1.1±0.8	1.8±0.5	1.9±0.2	1.8±0.2	1.9±0.2
TAM(2)	**20**	1.1±0.7	1.8±0.5	1.6±0.5	1.6±0.5	1.6±0.5
TAM(3)	**23**	0.9±0.7	1.6±0.6	1.6±0.6	1.4±0.5	1.4±0.5

± indicates the standard deviation

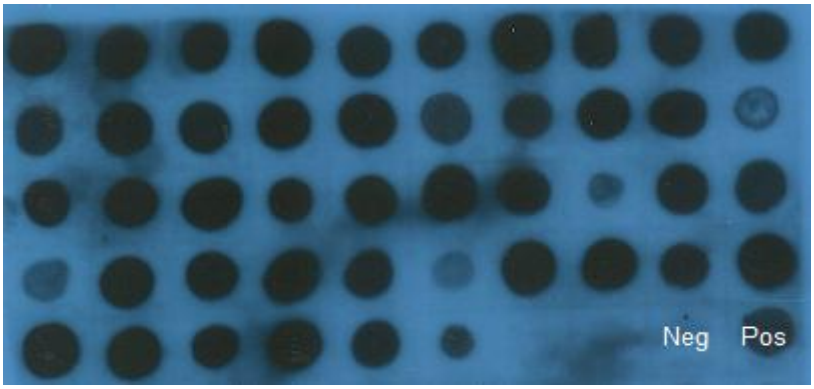

Figure 2.16: Dot blot hybridisation showing the intensity of hybridization between ToCSV probe and ToCSV infected plants.

The DSI ratings obtained from the third infection trial show that the values are not normally distributed. A significant difference was found between the DSI ratings in the generations of TAM(1), (2) and (3), The P-value (P=0.000) confirms that a 5% significant level, on average, the DSI ratings of TAM differ across the generations. This result is also confirmed by the one way ANOVA (Appendix table A3). A pair wise comparison between the generations of TAM, shows that the significant differences lie between TAM(1) and TAM(3) only (P-values = 0.000) (P<0.05) (Table 2.7).

Table 2.7: Descriptive statistics of DSI scoring of the TAM plants in infection trial four.

	N	Minimum	Maximum	Median	Mean	Standard Deviation
TAM(1)	85	0	2	2	1.6353	0.6874
TAM(2)	115	0	2	2	1.3913	0.7689
TAM(3)	130	0	2	1	1.2385	0.7346

P-value = 0.000

N indicates the number of plants of each generation of TAM used for the statistical analysis.

Table 2.8: Pair wise comparisons in infection trial four.

(I) Generation	(J) Generation	Mean Difference (I-J)	P-value
TAM(1)	TAM(2)	0.244	0.063
	TAM(3)	0.397	0.000
TAM(2)	TAM(1)	-0.244	0.063
	TAM(3)	0.153	0.316
TAM(3)	TAM(1)	-0.397	0.000
	TAM(2)	-0.153	0.316

Figure 2.17: Symptom comparison between ToCSV infected TAM(1) (left) with a healthy TAM (1) control.

Figure 2.18: Symptom comparison of TAM(2) infected with ToCSV (left) and healthy TAM(2) control (right).

Figure 2.19: Symptom comparison of ToCSV infected TAM(3) (left) and healthy TAM(3) control.

Figure 2.20: Symptom comparison of Tyler (healthy control is the tall plant in the middle), surrounded by ToCSV infected Tyler plants.

2.4.3. Infection trials of the test crosses of TAM with Rooikhaki

The uninoculated Tovi-star and Rooikhaki plants (resistant and susceptible control plants respectively) were healthy and tested negative for the presence of ToCSV. Tovi-star positive control plants continued to grow with slight symptoms and tested positive for the presence of the virus (DSI=1). Rooikhaki positive controls showed intense ToCSV infection symptoms such as stunting and widespread leaf curling. Clear differences in symptom phenotype among individual tomato plants were observed in the F1 hybrids (Rooikhaki crosses with TAM(1), (2) and (3) respectively (Figure 2.21 -2.23).

TAM(1) x RK F1 had the highest average DSI score of 2.6±1, followed by TAM(3) x RK F1 with a score of 1.1±1 and TAM(2) x RK F1 with a score of 1.0±1 (Table 2.9). Certain plants showed pronounced stunting in their growth, while others continued to develop. The F1's, did not show 100% susceptibility as was expected, if TAM had recessive resistance to ToCSV (Figure 2.4).

Figure 2.21: ToCSV infection of F1 hybrid cross between TAM(1) and Rooikhaki. The Tomato plant on the far right is the uninfected control (indicated with the arrow).

Figure 2.22: ToCSV infected plants of F1 hybrids of cross between TAM(2) x Rooikhaki. The uninfected control is shown on the far right.

Figure 2.23: ToCSV infected F1 hybrid plants of the cross TAM(3) x Rooikhaki. The control is indicated on the far right.

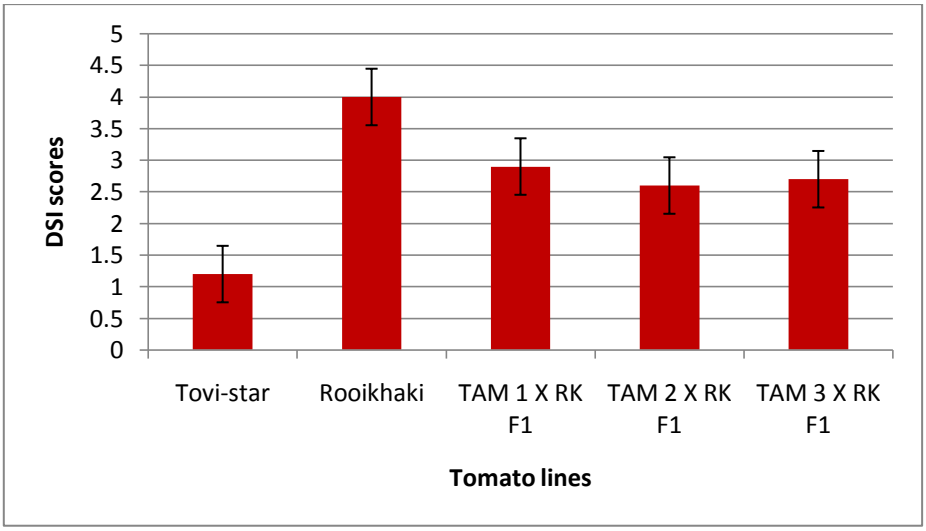

Figure 2.24: The mean DSI scores for the infection of the test crosses (F1's). Tovi-star is a commercial resistant variety that was included in the trial as a resistant control and Rooikhaki as susceptible control. The Y-axis indicates the DSI scores from 0-4.

The DSI ratings from this infection trial are not normally distributed as the null hypothesis is rejected (p-value = 0.000, P < 0.05) when the Kolmogorov Smirnov Z test was used. The Kruskal wallis (non parametric test) shows that there is no significant difference between the DSI ratings in this trial.

Table 2.9: Descriptive statistics of interactions of ToCSV infected F1 hybrids of the test crosses.

	N*	Minimum	Maximum	Median	Mean	Standard Deviation
TAM(1) x RK F1	20	0	4	3	2,7500	0.7864
TAM(2) x RK F1	20	1	3	2	2,1500	0.6708
TAM(3) x RK F1	20	1	3	2	2,3000	0.6569

P-value = 0.006

*N indicates the number of plants used from each test cross.

Table 2.10: Pair wise comparisons in interactions of the ToCSV infected F1 hybrid test crosses.

(I) Type	(J) Type	Mean Difference (I-J)	P-value
TAM(1) x RK F1	TAM(2) x RK F1	0.600	0.029
	TAM(3) x RK F1	0.450	0.147
TAM(2) x RK F1	TAM(1) x RK F1	-0.600	0.029
	TAM(3) x RK F1	-0.150	1.000
TAM(3) x RK F1	TAM(1) x RK F1	-0.450	0.147
	TAM(2) x RK F1	0.150	1.000

2.4.3.1. Infection trials of the F2 population of the test crosses of TAM with Rooikhaki

In this infection trial the F1 hybrids between TAM and Rooikhaki was selfed to produce an F2 population. The results varied between the three F2 populations. Although the F2 population of TAM 2 x Rooikhaki showed a stable resistance to ToCSV relative to the other crosses they had the highest infection DSI scores (DSI= 2.6±0.5). There was no correlation with the results obtained and the expected ratio of 1:2:1 (homozygous recessive: heterozygous: homozygous susceptible) (Figure 2.4).

Figure 2.25: ToCSV Infection of TAM(1) X Rooikhaki F2 with uninfected control plant on the far right (indicated with arrow).

Figure 2.26: TAM(2) x Rooikhaki F2 infected with ToCSV. An uninfected control plant is shown on the far right (indicated with arrow).

Figure 2.27: ToCSV infected TAM(3) x Rooikhaki F2 with uninfected control plant on the far right (indicated with arrow).

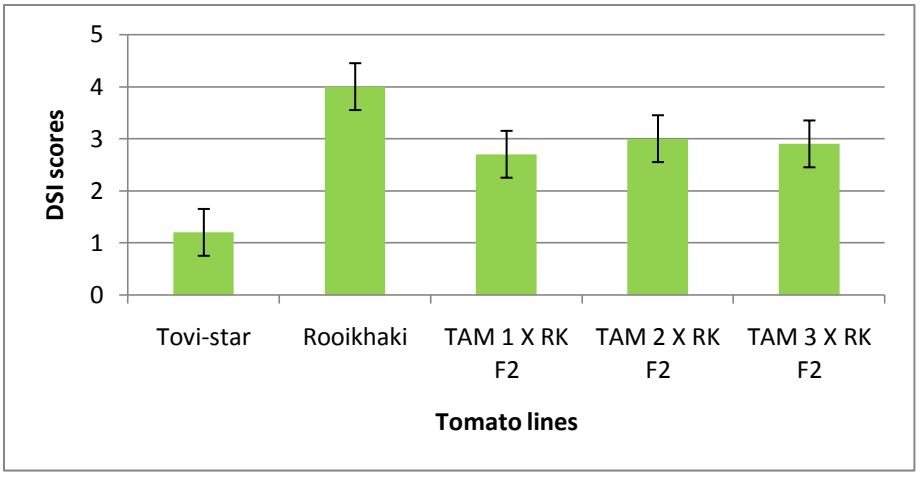

Figure 2.28: The mean DSI scores for the infection of the F2 population from F1 hybrid crosses with Rooikhaki. DSI scoring from 0-4 is shown on the Y-axis.

The DSI ratings from this infection trial are not normally distributed as we reject the null hypothesis (P-value = 0.000, $P < 0.05$) in the Kolmogorov Smirnov Z test. The Kruskal wallis (non parametric test) shows that there is no significant difference between the DSI ratings in this infection trial, since $P > 0.05$ (Table2.11).

Table 2.11: Descriptive statistics of interactions of the F2's of the test crosses.

	N	Minimum	Maximum	Median	Mean	Standard Deviation
TAM(1) x RK F2	20	0	3	2	2.2500	0.9105
TAM(2) x RK F2	20	2	3	3	2.7500	0.4443
TAM(3) x RK F2	20	2	3	3	2.6500	0.4894

P-value = 0.098

N indicates the number of plants used from each test cross.

Table 2.12: Pairwise comparisons in interactions of the F2 population.

(I) Type	(J) Type	Mean Difference (I-J)	P-value
TAM(1) x RK F2	TAM(2) x RK F2	-0.500	0.054
	TAM(3) x RK F2	-0.400	0.169
TAM(2) x RK F2	TAM(1) x RK F2	0.500	0.054
	TAM(3) x RK F2	0.100	1.000
TAM(3) x RK F2	TAM(1) x RK F2	0.400	0.169

The overview of the results obtained in the different generations of TAM as well as the test crosses is reported in Table 2.13. TAM(1), (2) and (3) show that the majority of individual plants scored DSI ratings from 0- 2. The F1 population of the test crosses showed varied responses of resistance as a number of plants in each generation were symptomless (and scored DSI ratings of 0), but there were also a numer of plants that scored ratings of 2 and 3. The F2 population of the test crosses also showed varied DSI ratings from 2-3. The most stable generation was the TAM(2) X Rooikhaki F2 where all infected plants were at the same height (Figure 2.26) and scored similar DSI scores (Table 2.13).

Table 2.13: Response to *Tomato curly stunt virus* infection in the different generation levels of TAM including the test crosses, F1 and F2 population. The type of resistance is suggested based on the DSI scoring and the height difference of the plants in the same group.

Generations and crosses of TAM		Number of plants per DSI score					Avg DSI scores	Resistance
		0	**1**	**2**	**3**	**4**		
TAM (1)	**F6**	8	6	29	2	0	1±0.8	Stable
TAM (2)	**F6**	15	11	19	0	0	1.1±0.8	Stable
TAM (3)	**F5**	19	16	3	1	0	0.6±0.7	Stable
TAM (1) X RK	**F1**	1	0	2	6	1	2.6±1.1	Partial Dominance
TAM (2) X RK	**F1**	5	0	5	0	0	1±1	Partial Dominance
TAM (3) X RK	**F1**	5	0	4	1	0	1.1±1.2	Partial Dominance
TAM (1) X RK	**F2**	2	0	6	2	0	1.8±1	Segregating
TAM (2) X RK	**F2**	0	0	5	5	0	2.6±0.5	Stable
TAM (3) X RK	**F2**	0	0	6	4	0	2.4±0.5	Segregating

± indicates the standard deviation

2.5. DISCUSSION

In our study, we demonstrated that the inbred line TAM does not have any of the known Ty-resistant linked genes (Figures 2.6- 2.9). From previous trials conducted by Sakata Vegenetics RSA (Pty) Ltd. in Jordan, TAM showed good resistance to field challenge to TYLCV-IL. Pietersen and Smith (2002) found that TYLCV-IL tolerant tomato accessions also have tolerance to ToCSV. Our results for TAM concur with their findings as ToCSV infection greenhouse trials showed that TAM has a certain level of resistance to this virus (with mean DSI scores ranging from 0.9 -1.9 as opposed to Rooikhaki, the susceptible control, which scored a mean DSI of 4). The level of resistance of each generation of TAM differed between each infection trial, however, what can be concluded is that inbred line TAM has a good level of resistance to ToCSV infection as the average mean DSI scores did not exceed DSI=2.

The absence of the *Ty*-linked resistance gene markers in the inbred line TAM suggests that the resistance source could be novel and perhaps recessive. Garcia-Cano and his colleagues (2008) at the CSIC (Consejo Superior de Investigaciones Cientificos), in Spain, have shown that three tomato inbred lines, not carrying any of the known resistant gene-linked molecular markers, are resistant to monopartite begomoviruses associated with the Tomato yellow leaf curl disease (TYLCD) namely; TYLCV-IL (*Tomato yellow leaf curl virus* Israel), TYLCSV (*Tomato yellow leaf curl Sardinia virus,* Italy), TYLCMalV (*Tomato yellow leaf curl Malaga virus*, Spain) and TYLCAxV (*Tomato yellow leaf curl Axarquia virus*, Spain). The resistance, they found was linked to a major recessive locus with epistatic interactions controlling the resistance to TYLCD. Interestingly, these inbred lines are also resistant to bipartite begomoviruses in Brazil and have now been shown to be resistant to TYLCD associated diseases. Another discovery of a recessive allele (*tgr*-1) conditioning tomato resistance to geminivirus infection, specifically TLCV (*Tomato leaf curl virus*), a monopartite virus, was made by Bian and colleagues (2007). They found the resistance is controlled by a single recessive allele which they called *tgr-1*. In fact, recessive genes have been linked to resistance to many plant viruses (Diaz-Pendon *et al*., 2004) and have been attributed to the plants lacking some essential factor(s) required for virus replication or movement. Therefore it is possible that resistance to tomato begomovirsues may be polygenic and include both recessive and dominant mechanisms.

In all the infection trials conducted in this study, susceptible and resistant control plants were used. The susceptible, Rooikhaki showed full symptoms after inoculation (Figure 2.10) in all the infection trials conducted. In accord with Pietersen and Smith (2002), Rooikhaki seedlings were used as the susceptible controls as they do not have any resistance-linked genes present and are extremely susceptible to ToCSV (Figure 2.10). The whiteflies were reared on Rooikhaki, as it is known that more

virus accumulates in a susceptible host plant than a resistant one (Lapidot and Friedmann, 2002). Therefore, rearing the whiteflies on Rooikhaki plants ensured they had a maximum amount of transmissible virus at all times.

The advantage of infecting Rooikhaki with an infectious clone of ToCSV as opposed to natural infection by viruliferous whiteflies in the field is that the emergence of resistant breaking viral strains is halted. The frequency of such occurrences depends on the error rate of the polymerase with which the viral genome is replicated. The frequency of mutations therefore is high (Urbino *et al*., 2008). In this way the infectious whiteflies were reared on Rooikhaki plants inoculated with the infectious clone (Figure 2.2) which ensured that the strain used in all the infection trial was consistent and there were no introduced mutations that we were not aware of.

The resistant control, RQS (which has *Ty-1* homozygous genes), showed no symptom development in infection trial three. In contrast, it displayed mild symptoms in the first two weeks after infection with ToCSV in infection trial four (Table 2.6). However in the third week of the fourth infection trial, the plants recovered and they continued to develop normally (Figure 2.12). Plants which have *Ty-1* homozygous genes have been shown to develop mild disease symptoms to TYLCV-IL (Zamir *et al*. 1994). We have shown that the same is true for ToCSV and propose that since the infections were not controlled (with consistent numbers of whiteflies feeding off each seedling), there is a possibility that in the fourth infection trial the plants experienced a very high inoculum pressure leading to a display of symptoms on RQS in the first few weeks post infection.

In the fourth infection trial, a commercially available tolerant line (Tyler) was included (Figure 2.20). Results from the trial showed similar results, with respect to symptom severity (DSI of 1.8), compared with TAM (1) (Table 2.6). The final DSI of 1.8 indicates that Tyler is tolerant to ToCSV, but not fully resistant. Gilberston *et al*. (2007) found that high whitefly pressure and TYLCV-IL virus pressure may be able to overcome levels of resistance in a commercial cultivar, Gempride, a cultivar known to be highly resistant to TYLCV-IL, has been reported in southern Europe. Lapidot and Friedmann (2002) and Lapidot et al. (1997), found that some resistances subside under early or severe infection pressure. In the TAM infectivity trials from this research, the number of whiteflies feeding per seedling was not controlled and the source plants (Rooikhaki) containing ToCSV were present with the whiteflies continuously throughout the infection period. Consequently, the whiteflies were constantly infected with ToCSV. The virus inoculum pressure therefore is believed to have been very high. In essence, Lapidot *et al*. (1997) described a good controlled inoculation being one that consists of allowing whiteflies to feed for 48 h on infected source plants (acquisition access period), followed by exposing young tomato seedlings to large numbers of viruliferous whiteflies

(approximately 30–50 whiteflies per plant) which, upon feeding on the plants, transfer the virus with close to 100% efficiency (all susceptible controls become infected with TYLCV-IL) (Lapidot *et al.,* 1997). Therefore the infection rate and virus pressure were not equal throughout all the seedlings and some may have been more infected than others, skewing the results somewhat. This could have been the case with the resistant control used in infection trial four with Tyler.

There are several examples of the incomplete or partial dominance of genes for resistance to various viral, bacterial and fungal pathogens. These include for example, the L^{l} and L^{3} genes of *Capsicum chinese* which are effective against the virus *Tomato mottle virus* (Boukema, 1980), the *Dm6* gene of lettuce effective against *Bremia lactuca* (Crute and Norwood, 1986), the *Pto* gene of tomato effective against *Pseudomonas syringae* (Carland and Staskawicz, 1993), several alleles of the *Mla* locus of barley effective against *Erisyphe graminis* (Jahoor *et al.* 1993); and the *Arabidopsis thaliana* gene *RPP5* effective against *Peronospora parasitica* (Parker and Higgins, 1993). The expression of partial resistance is dependent, to some degree, on the environmental conditions, the physiological stage of the plants and the aggressiveness of the pathogen (Laterrot, 1975). An effective approach for the study of complex disease resistances is by the use of molecular markers (Young, 1996). All regions of the genome can be analysed, by the development of saturated linkage maps (Tanksley, 1993). In tomato, the construction of a saturated RFLP linkage map (Tanksley *et al.,* 1989) has allowed numerous disease resistance genes to be mapped. Such approaches have been productively used in tomato for the isolation of *Pto* and *Prf*, two genes that confer resistance in tomato to *P. syringae.*

This study demonstrated that the inbred line TAM, known to be resistant to TYLCV-IL, was also resistant to ToCSV. The source of the resistance is unknown, and further studies to elucidate the genetics of the resistance included test crosses of TAM with a susceptible cultivar, Rooikhaki, and the production of F2's to conclude whether the resistance is stable or segregating.

A test cross with Rooikhaki was performed and F1's produced. The inoculated F1's showed a level of resistance intermediate between the resistant and the susceptible parents and had mean DSI scores ranging from 2.7 to 2.9 (Table 2.13), indicating that resistance was inherited as an incompletely dominant trait and is not a recessive resistance trait, as expected (Figure 2.4). Table 2.9-2.10 shows the statistical results where no significant difference between the DSI scores of the F1's hybrids was shown. Interestingly, the F2 population scored high DSI values (DSI= 2.7–3), however, their segregation patterns varied. TAM (2) X Rooikhaki F2 showed a stable pattern of resistance as all the infected plants were of the same height and all individual plants scored a DSI of 3 (Table 2.13). It was

found that, although TAM (2) X Rooikhaki F2 plants showed no segregation for resistance, they were all stable for resistance. There was no significant difference in their DSI scores between the F2 populations. In all the F2 population generations, the expected ratio of 1:2:1 (homozygous resistant: heterozygous: homozygous susceptible) was not seen. This supports the finding that the resistance of TAM is not recessive, as it was hypothesised, but rather partial dominant resistance is suggested.

The genes conferring the resistance to ToCSV have not been identified yet. Future work should include the full characterization of the genes involved in the resistance and subsequently, the creation of a tightly linked molecular marker to the gene/s involved in the resistance of tomato line TAM. The future identification of additional novel resistance loci for begomovirus resistance will prove potentially invaluable for the tomato breeding industry, not only in South Africa, but worldwide, as tomato-infecting begomoviruses are a serious threat to production in many countries.

CHAPTER 3. INVESTIGATION OF THE STRESS RESPONSE OF RESISTANT SOUTH AFRICAN TOMATO LINES TO *Tomato curly stunt virus*

ABSTRACT

Tomato curly stunt virus, a whitefly transmitted virus of tomato, was discovered in South Africa during 1998. Investigations showed that ToCSV is related to TYLCV-IL (originating from Israel) with a 77% homology in their phylogenetic sequences. These viruses share similarities such as their genome organisation (circular, single stranded DNA with a monopartite genome), infectivity symptoms (leaf curling, stunting etc.) and the mode of transmission (vector-*Bemisia tabaci*). Both ToCSV and TYLCV are viruses that induce a number of stresses that limit plant growth and crop production and which elicit biochemical and physiological responses that may induce tolerance. One of the defence response to abiotic stresses in plants includes the expression of heat shock proteins (HSPs). These function as stress response proteins, molecular chaperones or proteases. They repair or degrade damaged proteins in order to restore protein homeostasis. The re-establishment of stress protein stability increases tolerance to abiotic and biotic stresses in plants. The aim of this study was to compare the severity of infection of the two closely related viruses using commercial resistant varieties (Tyler and Tovi-Star) and a susceptible cultivar Rooikhaki, grown in South Africa, by conducting an infection trial with TYLCV-IL using viruliferous whiteflies. Viral accumulation was monitored using semi-quantitative PCR. Another aim of this study was to compare the difference in heat shock protein levels of South African tomato cultivars, resistant to ToCSV, with those of TYLCV-IL resistant breeding lines, when exposed to abiotic stresses of salt and heat shock treatment. Our study demonstrated that Tyler and Tovi-Star infected with TYLCV-IL showed disease severity index (DSI) scores below 1.5, whereas Rooikhaki had a mean DSI score of 3.5. In contrast to ToCSV infection, (previous infection trial; chapter 2), Rooikhaki infected with TYLCV-IL continued to grow and the infection symptoms were significantly milder. Semi-quantitative PCR results of the overall pattern of TYLCV-IL DNA accumulation demonstrated that viral DNA levels in the 3 South African (SA) cultivars peaked at 14 to 21 days post inoculation (dpi) and decreased thereafter. In contrast, the TYLCV-IL accumulated steadily in the Israeli resistant and susceptible lines (R-IL and S-IL respectively). Heat shock protein 70 accumulation patterns were similar when abiotic stresses were applied to the South African resistant and susceptible tomato cultivars, as compared to Israeli resistant and susceptible breeding lines. The levels of HSP70 have been shown, in previous studies, to accumulate in a more stable manner in TYLCV resistant plants than susceptible (R-IL and S-IL) throughout the treatment of the stresses. TYLCV symptoms are milder than those of ToCSV Rooikhaki which is a susceptible cultivar in SA. It could be speculated from this study that there may

be a correlation between the severity of infection (disease response) and pattern of HSP induction. A more enduring HSP70 level in resistant tomatoes could contribute to a lower severity symptom phenotype. This study offers potential opportunities to develop broad-based genetic markers for biotic-abiotic resistance, and warrants further investigation in other geminivirus-tomato pathosystems.

3.1. INTRODUCTION

Plants are hosts to a variety of pathogens. Since they lack an active immune system, plants have evolved different strategies to combat both individual pathogen strains and changing pathogen populations (Afzal *et al.,* 2008). The wild relatives of tomato were well adapted to many pathogens and environmental stresses. However during breeding for increased yield and fruit quality, the majority of the gene networks conferring resistance to stress have been lost. Consequently, domesticated agricultural crops are susceptible to many biotic and abiotic stresses. Introgressions of chromosomal fragments from wild species into the domesticated tomato have been successful to restore the stress-resistant genes into superior hybrids. Breeders have accomplished the reconstitution of some of the gene networks that provide tolerance to stresses such as viruses, drought and salinity (Tal and Shannon, 1983). In a study by Gorovits and Czosnek, (2008) an attempt to link abiotic stress responses in tomato with broad or innate immunity was undertaken. Results demonstrated a clear correlation with accumulation of HSPs and resistance to TYLCV-IL. A collection of highly conserved proteins, Heat Shock Proteins (HSPs) (chaperones and proteases) has been developed to cope with environmental stresses. Chaperones control the folding of proteins and with ATP-dependant proteases, and form the cellular protein quality control system. Many work together to correct protein folding and repair, while others associate with proteolytic components to degrade damaged proteins that could accumulate to dangerously high levels (Parsell and Lindquist, 1993). HSPs also participate in cell recovery or by degrading them in order to restore protein homeostasis and promoting cell survival (Pareek *et al.* 1995; Katiyar-Afarwal *et al.* 2003). HSP's are expressed in plants experiencing a variety of stresses including high and cold temperatures, drought, salinity osmotic sock, oxidative stress and pathogen attacks (Wang *et al.* 2004).

Usually, the quantity of virus detectable in resistant and breeding lines is much less than that in the susceptible plants (Gorovitz and Czosnek, 2008). Genetic studies have shown that a number of genes provide the resistance phenotype. These genes are expressed as quantitative trait loci (QTL) and using DNA markers, have been mapped to tomato chromosomes (Griffiths and Scott, 2001). The

molecular source of resistance to TYLCV-IL including the physiological state of susceptible vs. resistant plants, before and after inoculation is not entirely known. To gain some insight into resistance mechanisms, molecular reactions of tomato plants to biotic and abiotic stresses have been studied (Gorovitz and Czosnek, 2008). Pathogens, including viruses are considered as a specific type of stress as they can elicit similar recognition and signalling responses as abiotic stress. A stress response begins when a plant recognizes stress at the cellular level. By activating signal transduction pathways that send out information within the individual cell and throughout the plant, changes in the expression level of numerous gene networks takes place. Consequently plants respond to biotic and abiotic stresses by activation of R-gene mediated and signal transduction pathways (Bolwell, 1999).

3.2. GENERAL OBJECTIVES

In our previous study (chapter 2) it was demonstrated that line TAM does not have any of the known Ty- resistant-linked genes, yet demonstrated a high level of resistance under controlled infection against ToCSV. This line was also shown to be resistant to TYLCV-IL in a field study conducted in Jordan. Since TYLCV shares many properties, including a similar disease-response phenotype and a nucleotide sequence identity of 77% with ToCSV, we thought it useful to investigate the level of resistance of commercial South African (SA) tomato cultivars (Tyler and Tovi-Star) against TYLCV-IL, and compare this to ToCSV. Furthermore, these two selected SA commercial resistant cultivars and one susceptible variety (Rooikhaki) were treated with abiotic stresses (salt and heat) and their heat shock protein 70 levels (HSP70) were studied and compared with a similar study conducted with a TYLCV-IL-resistant and susceptible Israeli line.

3.3. MATERIALS AND METHODS

3.3.1. Infectivity trial

3.3.1.1. Sowing of the seeds

Seedling trays were filled with potting soil so that each cavity was three quarters full. Ten seeds of the resistant SA varieties, Tovi-star and Tyler, and Rooikhaki (susceptible), were positioned in separate rows where one seed was sown per cavity. All the seeds were subsequently covered with more potting soil. The trays were watered and left in the greenhouse for seed germination. When the seedlings reached their 3-4 leaf stage (21 days after sowing), they were transferred into pots

with potting soil for further development. At their 6 leaf stage (5 weeks after sowing), the seedlings were moved, in their pots, into the presence of the TYLCV-IL infected whiteflies. The number of infected whiteflies feeding off each seedling was not controlled and therefore the amount of inoculum in each seedling would have varied.

3.3.1.2. Infection trial

The source of TYLCV virus was acquired in the field where infected tomato plants are readily available. The plants were tested by PCR for the presence of TYLCV and if positive, were brought into the greenhouse where the whiteflies were housed. By feeding on the infected plants, all the whiteflies became infectious with TYLCV. The seedlings were introduced into the greenhouse and remained in the presence of the infectious whiteflies throughout the duration of the experiment (five weeks). The development of TYLCV-IL symptoms were monitored and recorded at 11dpi using the severity (DSI) scores developed by Lapidot and Friedmann (2002).

3.3.1.3. DNA extraction

Leaf tissues (100 mg) was ground twice with a drill homogenizer in 500 µl extraction buffer (100mM Tris HCl, pH 8.0, 5OmM EDTA, 500Mm NaCl (sodium chloride), 10Mm DTT (Dithiothreitol), 2% polyvinyl-pyrrolidone). Samples were incubated with 10% SDS (Sodium dodecyl sulphate) for 15 mins at 65°C. Potassium acetate (160µl) was added and the samples were centrifuged for 10 min at 12 000 rpm. The supernatant was recovered and treated twice with an equal volume of phenol/chloroform (1:1), followed by vortex and centrifugation at 12 000 rpm at 4°C for 15 min, and once by chloroform. DNA was recovered from the supernatant by ethanol precipitation. The samples were treated with RNase (25 µg/ml) for 20 min at 37°C.

3.3.1.4. Confirmation of infection using PCR

TYLCV-IL coat protein (CP) DNA was detected using PCR. Each reaction contained 0.02µg DNA template, and 1X PCR Buffer, 2 mM $MgCl_2$, 0.1 mM of each dNTP, 0.2 µM of each primer and 5 units of Taq DNA polymerase (Bioline). The cycling parameters were as follows: initial incubation at 94°C for 30 seconds followed by 35 cycles of 30 seconds at 59°C, 30 seconds at 55°C and 30 seconds at 72°C and a final extension of 5 min at 72°C. The primers used were: F (forward) 5'-ATA CTT GGA CAC CTA ATG GC– 3' (nt 61-80) and R (reverse) 5' –TTG TAA GGG CCC GTG ACT– 3' (nt 473-456) which produces a 412 bp amplicon.

3.3.1.5. Semi-quantitative PCR

Using the TYLCV-IL CP primers and a conventional PCR machine, semi-quantitative PCR was undertaken to estimate the relative amount of virus present in the different tomato lines (Tovi-star, Tyler and Rooikhaki) at 14, 21, 28 and 35 days after inoculation (dpi). The concentration of DNA of each cultivar was measured using a nanodrop and diluted accordingly with ddH_2O in order to have all the samples as close to each other's concntrations (ng/µl) as possible. The primer pairs and the conditions used are the same as described above (section 3.3.1.4.). At 18, 20, 23, 26, 28, 30 and 35 cycles for each line and each dpi. One PCR tube for each of the reactions for each line was removed from the PCR machine and stored at 4°C until the full cycle of the PCR was completed. All the samples were run together on a 1% agarose gel and the bands were visualised under UV light using EtBr (ethidium bromide).

3.3.2. Determination of heat shock protein induction in a resistant and susceptible South African tomato cultivar in response to abiotic stress (salt and heat)

3.3.2.1. Abiotic stress treatments

Leaf material (100mg) was sampled from a resistant (Tovi-star) and susceptible (Rooikhaki) cultivar, weighed, cut up into small pieces and exposed to an abiotic stress treatment. The leaves were exposed to salt treatment (0.5M NaCl) as well as heat shock (45°C). In both treatments, the samples were continuously agitated. At different time points; 10, 30, 60 and 120 min for the salt treatment and 10, 30, 60, 120 and 360 min for the heat shock treatment, samples were removed from the stress source and stored in the fridge until the protein was extracted. As a control non-treated sample, leaves were collected directly off the tomato plant, but not exposed to any stresses and protein was extracted.

3.3.2.2. Total protein extraction from tomato leaves

Leaf tissue (75 mg) was collected and stored in the freezer for an hour prior to beginning the extractions. The frozen tissue was ground using a drill homogenizer in 700 µl of sample buffer (0.01 M Tris, 87% glycerol, 20% SDS, 4 M urea and bromophenol blue). Subsequently the samples were boiled for 10 min in a waterbath and centrifuged at 12 000 rpm for 20 min. The upper phase was removed and stored at -20°C until run on a polyacrylamide (PAGE) gel.

3.3.2.3. Polyacrylamide gel electrophoresis of extracted proteins

A 12% separating (pH 8.8) and stacking PAGE gel (Ph 6.8) were prepared (Tris-buffer, 30% acrylamide, 20% ammonium persulfate, TEMED) and poured into the BIORAD wet tank blotting system. A glycine running buffer (50 mM Tris-HCl, 50 mM Tris, 384 mM Glycine, 0.2% SDS) of pH 8.0 was used to run the samples. The samples were run at 5 mA for 30 min, after which the voltage was adjusted to 10 mA for the remainder of the run.

3.3.2.4. Trans-blotting of the proteins

Nitrocellulose membrane (Amersham) and the Whatman paper were soaked in the transfer buffer (48 mM Tris, 39 mM Glycine, 10% SDS and 20% Methanol). The proteins were transferred from the gel onto the nitrocellulose membrane using the Trans-blot semi-dry electrophoretic transfer cell (BIORAD). The transfer was run for an hour at 140 volts and 100 mA. Once the transfer was complete, the membrane was dried and ready for immunodetection.

3.3.2.5. Immunodetection of tomato HSP70

The membrane was washed in full cream milk for 15 min after which every 10 min it was rinsed with clean water (dh_2O). Subsequently, the membrane was washed in washing buffer (I M Tris-HCl, pH 7.5, 5 M NaCl, Tween). The antibody, anti-Hsp/Hsc70 (Stressgen Biotechnologies), was diluted in washing buffer (1: 5000), and the membrane submerged in this solution while shaking for 10 min on a rotational shaker after which it was stored in the fridge overnight in the antibody solution. The following day the membrane was washed three times using clean washing buffer (Stressgen Biotechnologies) each time, with agitation, for 10 min. The membrane was transferred into the secondary antibody solution (goat peroxidase-coupled secondary antibody from Stressgen Biotechnologies) diluted in washing buffer (1: 80000) for an hour with shaking. Subsequently, the membrane was washed with washing buffer three times, each time, with agitation, for 10 min. Goat peroxidase-coupled secondary antibody (Stressgen Biotechnologies) incubation was followed by the enhanced chemiluminescence detection (Amersham) according to the manufacturer's instructions.

Results from this study were compared to a similar study undertaken at the University of Jerusalem, Israel with a susceptible (S-IL) and resistant (R-IL) Israeli breeding lines subjected to identical heat and salt abiotic stress regimes (Gorovits and Czosnek, 2008).The S-IL and R-IL breeding lines originated from crosses between a F1 hybrid between *S. habrochaites* accessions LA386 x LA1777 and a commercial variety. Backcross followed by repeated selfings allowed selection for a resistant and a susceptible line (R-IL and S-IL) (Vidavsky and Czosnek, 1998).

3.4. RESULTS

3.4.1. Infectivity of TYLCV-IL in South African tomato breeding cultivars compared with infection results with ToCSV-[ZA:Ond:98] (from Chapter 2).

3.4.1.1. Infectivity trial: Challenge of selected South African breeding lines with TYLCV-IL

The susceptible cultivar, Rooikhaki, showed symptoms of TYLCV-IL infection such as leaf yellowing, and curling (Figure 3.1). Despite being infected with the virus, the plants continued to develop further and there was not a great difference in plant height when compared to the uninoculated control plant (Figure 3.1).

Figure 3.1: Infected TYLCV-IL Rooikhaki (left) and healthy Rooikhaki control (right) thirty five days after infection.

Tyler, a commercial resistant variety from South Africa, infected with TYLCV-IL showed distinct yellowing in the leaves with limited leaf curling in the newer leaves after thirty five days (Figure 3.2). The infected plant continued to grow, and no significant difference in height was observed.

Figure 3.2: Cultivar Tyler infected with TYLCV-IL (left) and the healthy uninoculated control (right). The newer leaves of TYLCV-IL plant (left) show limited leaf curling (indicated by red arrow) thirty five days post infection.

Tovi-star, another commercial resistant variety available in South Africa (Figure 3.3), showed minimal symptoms of TYLCV-IL infection. The infected plant continued to develop normally and only a few leaves showed yellowing. The infected plants were scored using a DSI score system developed by Lapidot and Friedman (2002).

Figure 3.3: Tovi-star cultivar infected with TYLCV-IL (left) and the uninfected Tovi-star control (right) thirty five days post infection.

Table 3.1: The mean DSI scores of the South African cultivars that were challenged with TYLCV-IL.

Number of plants per DSI score						
Cultivar	**0**	**1**	**2**	**3**	**4**	**Mean DSI scores***
Rooikhaki				5	5	3.5±0.5
Tyler		5	4			1.4±0.5
Tovi- Star		9				1±0

*Mean DSI scores showing standard deviation

The commercial resistant varieties, Tyler and Tovi-Star infected with TYLCV-IL showed DSI scores below 1.5 (Table 3.1). The susceptible cultivar Rooikhaki had a mean score of 3.5. In comparison with ToCSV from South Africa, (previous infection trial; chapter 2), Rooikhaki infected with TYLCV-IL continued to develop and the infection symptoms were milder (Figure 3.4). Rooikhaki infected with TYLCV-IL showed slight yellowing and curling of the leaves (Figure 3.4a), while Rooikhaki infected with ToCSV shows severe stunting where no more plant development is seen (Figure 3.4b).

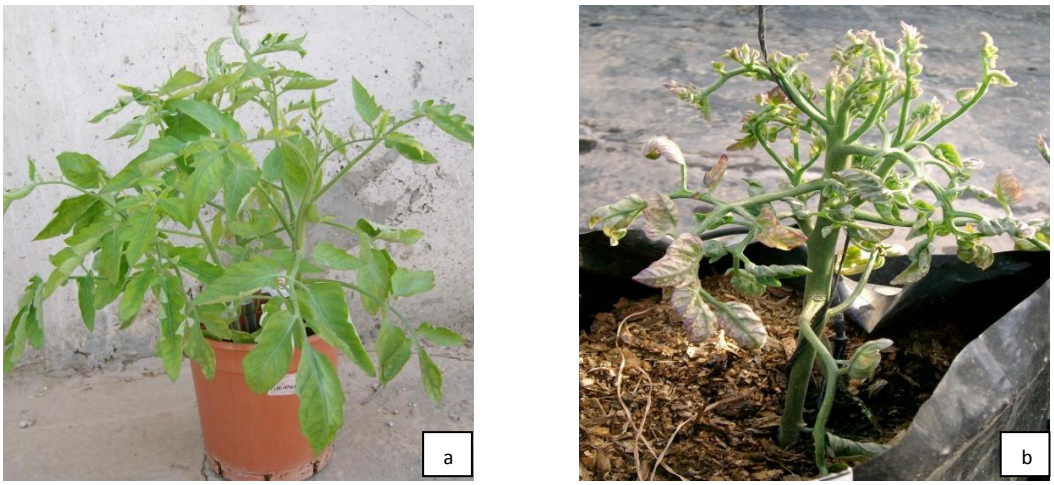

Figure 3.4: Disease symptoms of (a) Rooikhaki cultivar infected with TYLCV-IL, and (b) Rooikhaki infected plant infected with ToCSV.

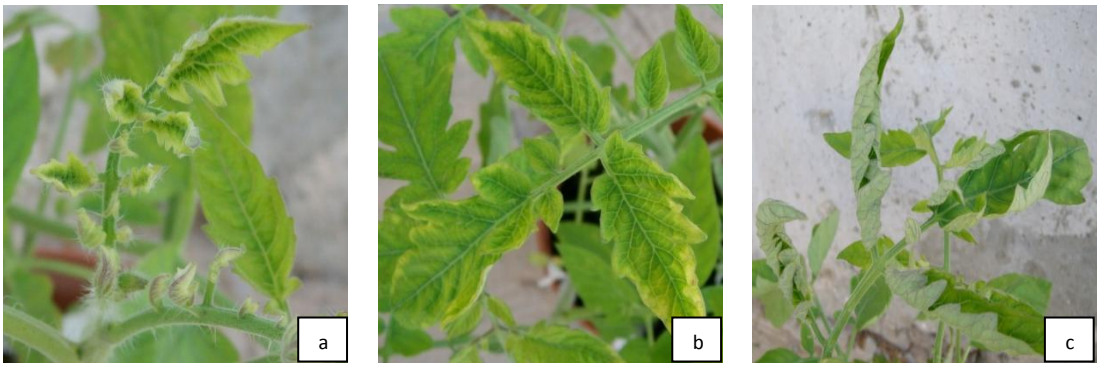

Figure 3.5: Symptoms on Rooikhaki infected with TYLCV-IL showing (a) newly formed leaves that do not develop to full size, (b) pronounced yellowing of the outer edges of the leaves and (c) older leaves show severe curling.

3.4.1.2. Confirmation of TYLCV-IL infection using PCR

PCR was performed using the coat protein primers for TYLCV-IL (Figure 3.6). A positive PCR result using these primers resulted in an amplicon of 412bp that was obtained for all infected plant in this study.

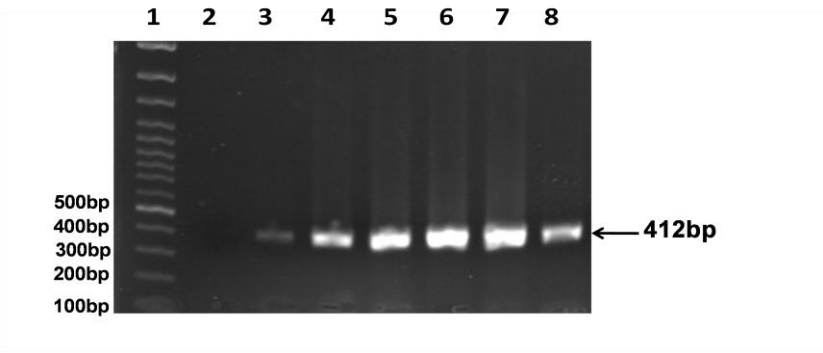

Figure 3.6: PCR confirmation of infection using the coat protein primers for TYLCV-IL on a 1.5% agarose gel. Lane 1: Fermentas O' GeneRuler 100 bp marker. Lane 2: Non template control. Lane 3-8: TYLCV-IL positive samples showing the 412 bp fragment.

3.4.1.3. Semi-quantitative PCR to measure TYLCV-IL viral accumulation

(a)Virus accumulation in susceptible and resistant SA cultivars

The progression of viral DNA accumulation over 35 PCR cycles was measured in two resistant and one susceptible SA tomato variety at 4 time points post infection. Semi-quantitative PCR showed that TYLCV-IL was detected after 23 cycles in the South African susceptible line, Rooikhaki at 14 and 21 days after infection, but DNA accumulation was only detected after 26 cycles at 28 and 35 dpi (Figure 3.7). The SA commercial resistant varieties, Tyler and Tovi-Star, showed similar results compared with Rooikhaki at 14 and 21 days after infection, but DNA levels were much lower at the 23 cycle in the resistant varieties. At 28 and 35 dpi viral DNA was detected at 26 cycles for Tyler and at 28 cycles for Tovi-Star (Figure 3.7). Viral DNA levels of Rooikhaki were lower at 26dpi compared to the resistant cultivar Tyler.

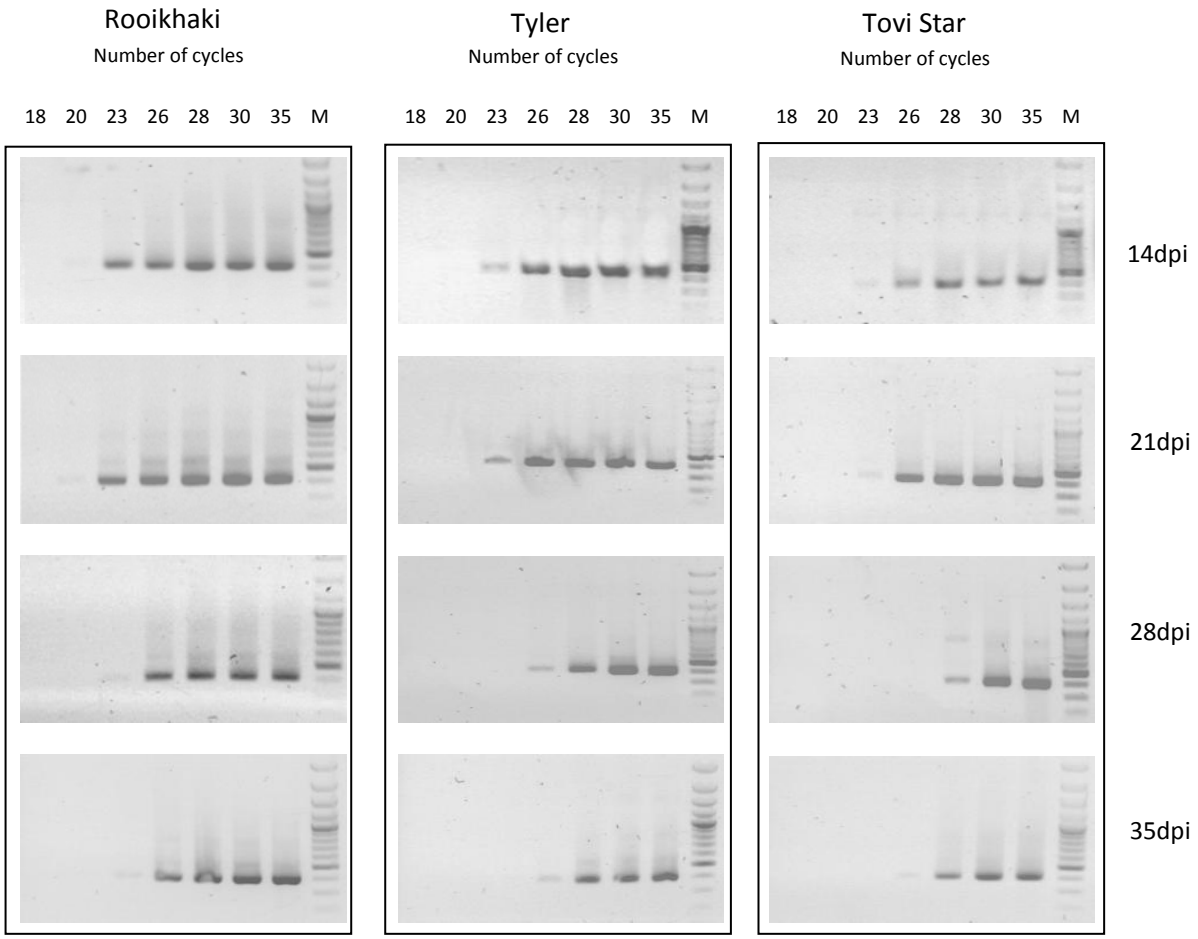

Figure 3.7: Semi-quantitative PCR (observed from cycle 18-35) of TYLCV-IL in South African tomato lines, Rooikhaki, Tyler and Tovi-Star detected at different times after infection (dpi). M indicates the Fermentas 1 kb molecular marker.

(b) Comparison between the SA and Israeli susceptible cultivars (Rooikhaki and S-IL respectively)

The Israeli susceptible breeding line showed virus DNA amplification at 26 cycles (Figure 3.8) 14 dpi, but the amount of virus present was lower than that of Rooikhaki (Figure 3.7). However, at 28 and 35 dpi, Rooikhaki viral DNA accumulation was only seen from cycle 26 (Figure 3.7), whereas the Israeli susceptible line showed amplification of TYLCV-IL is visible from cycle 18 (Figure 3.8). Viral replication was high at 49 dpi, where PCR amplification was detected as early as 15 cycles in this Israeli susceptible variety.

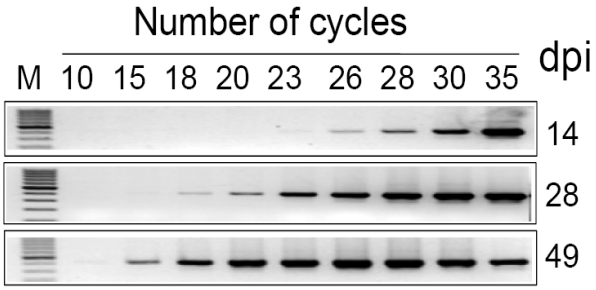

Figure 3.8: Semi-quantitative PCR (observed in cycles 10 -35) of S-IL, a susceptible Israeli breeding line observed at different days post infection (dpi).

3.4.2. Comparison of heat shock protein (HSP70) induction in resistant and susceptible SA tomato lines and Israeli breeding lines in response to abiotic stress (salt and heat)

Applied abiotic stresses (heat and salt treatment) to the South African resistant and susceptible tomato lines, (Tovi-star and Rooikhaki respectively), showed that HSP70 was more stable in the resistant cultivars throughout the application of the stresses since similar amount of HSP 70 was detected for 2 hours (salt) and 6 hours (heat) (Figure 3.9 and Figure 3.11). Similarly, results of the previous studies conducted by Gorovits and Czosnek, (2008), show that Hsp70 is more stable in TYLCV resistant than susceptible plants (Figure 3.10 and Figure 3.12).

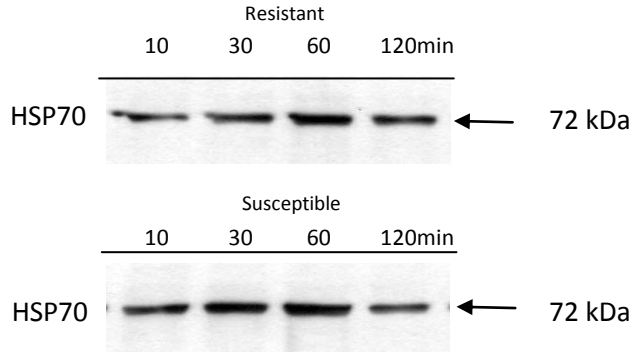

Figure 3.9: Cellular HSP70 patterns in susceptible (Rooikhaki) and resistant (Tovi-star) South African tomato lines in response to salt treatment.

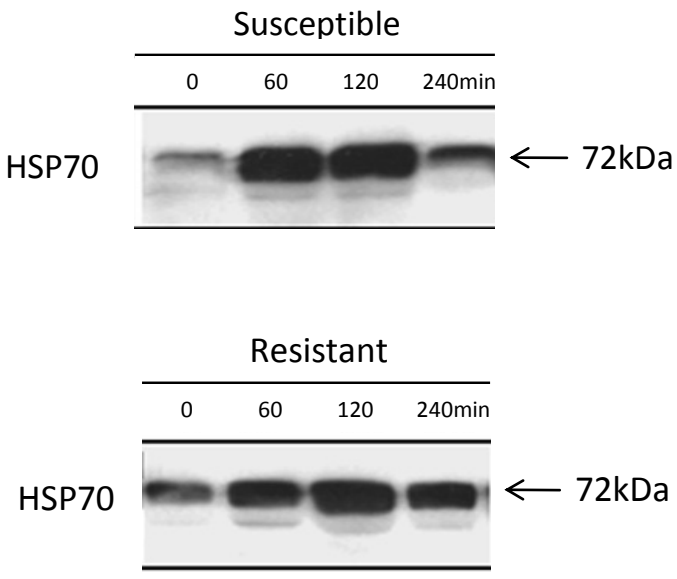

Figure 3.10: Changes in cellular HSP70 patterns in susceptible (S-IL) and resistant (R-IL) tomato lines in response to salt treatment.

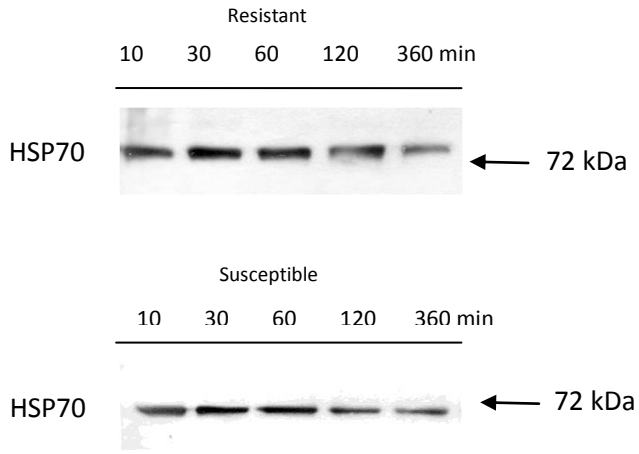

Figure 3.11: HSP70 cellular concentration in resistant (Tovi- star) and susceptible (Rooikhaki) plants in response to heat treatment (45°C).

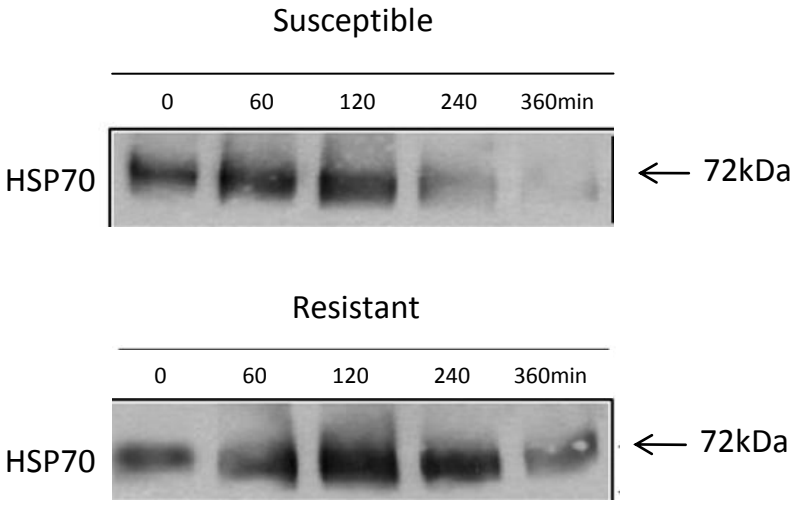

Figure 3.12: Cellular HSP70 concentration in S-IL (susceptible) and R-IL (resistant) plants following heat shock treatment at 45°C.

3.5. DISCUSSION

Plants have developed tactics to recognize and to manage harmful conditions, whether biotic (attacks by pathogens, including fungi insects and viruses) or abiotic (including temperature, water, and salt) stresses. In the majority of cases the defence responses brought about are regulated by a complex of signal transduction pathways. A rapid response to stresses determines the probability of survival of the threatened plants (Gorovits and Czosnek, 2008). In this study, we have subjected two SA tomato lines (resistant commercial Tovi-Star and the susceptible cultivar Rooikhaki) to abiotic stresses: heat and salt treatments. It has been shown previously that Hsp 70 is more stable in TYLCV-IL resistant than susceptible plants (Gorovits and Czosnek, 2008). Figures 3.9 and 3.11 in this study showed that a similar response was obtained when Tovi-star and Rooikhaki plants were subjected to salt stress (0.5M NaCl) and to heat shock (45^{o}C). Results indicated that HSP 70 was more stable in the resistant cultivar throughout the application of the stresses. Therefore, the response to these abiotic stresses in the South African lines could also correlate with their level of tolerance to TYLCV-IL. These results are similar to those observed with resistant and susceptible Israeli tomato lines, where there was a correlation between resistance to abiotic stresses and TYLCV-IL infection (Gorovits and Czosnek, 2008). Evidence that more potent stress response genes in resistant plants may originate from the wild *S. habrochaites* LA1777 was presented by Gorovits and Czosnek, (2008). After exposure to stresses, the members of the HSP family play a key role in re-establishing cellular homeostasis. The higher abundance of HSP 70 in resistant tomato lines as opposed to susceptible lines could be one of the reasons of higher tolerance to stresses. It appears that virus-resistant plants have a superior buffer-capacity to cope with stress than susceptible plants.

Since a comparison of TYLCV and ToCSV has never been previously reported, an infectivity trial with TYLCV and SA resistant and susceptible cultivars was conducted. A direct comparison of the results could not be undertaken as both experiments were not conducted in parallel under identical virus delivery regimes. The ToCSV infection trials were carried out such that the tomato seedlings were inoculated under high inoculum pressure for only three days after which they were removed from the presence of the whiteflies and were allowed to develop further in a greenhouse, compared with TYLCV infected seedlings that were caged with the infected whiteflies for the duration of the experiment (five weeks). Nonetheless, despite the fact that there were differences in the delivery of virus inoculums between the ToCSV and the TYLCV trials, certain observations could be made. The most evident observation was that Rooikhaki, a very susceptible cultivar to ToCSV, continued to grow under high inoculum pressure with TYLCV, and showed a milder phenotype compared to ToCSV. ToCSV appears to be more virulent (showing a higher DSI score) than TYLCV. Figure 3.4

showed the extreme stunting of Rooikhaki after ToCSV infection compared to the continued growth development with a TYLCV infected plant. The commercial cultivar Tovi-star, showed the highest resistance to TYLCV (Figure 3.3). Tyler, another commercial cultivar, showed intense yellowing (Figure 3.2), however the development of the plants did not seem to be greatly hindered. Pietersen and Smith (2002) reported that TYLCV tolerant tomato accessions also have tolerance to ToCSV as indicated by this study.

There was a clear difference in TYLCV accumulation in the susceptible cultivar from Israel and Rooikhaki from SA. Semi-quantitative PCR indicated that the virus level peaked early in infection in Rooikhaki (14 to 21 days) and decreased thereafter. In contrast, virus accumulation steadily increased in the Israeli cultivar S-IL (Figure 3.8). Therefore, comparative semi-quantitative PCR data of viral DNA replication in the SA susceptible cultivar, Rooikhaki, and the Israeli breeding line suggests that the Israeli cultivar is more susceptible. Tyler and Tovi star (resistant) showed similar patterns as Rooikhaki where there was a decrease in viral accumulation as time progressed. Correlating virus levels in this study with symptom severity scores from the previous infection trials, Tovi-Star showed the most resistance (DSI score of 1) when infected with TYLCV (Table 3.1). Tyler and Rooikhaki also showed some degree of resistance (1.4 and 3.5 DSI scores respectively). This indicates that susceptible plants accumulate virus sooner than resistant plants, with higher virus titres. This is in agreement with Lapidot *et al*. (2001), where he found that the host genetic background influences the viral accumulation where resistant plants have a lower virus titre than susceptible plants.

In conclusion, the response of tomato lines to TYLCV-IL infection of South African lines differs from Israeli lines, with regard to symptom severity (DSI) and virus accumulation patterns. The difference could be due to introgressions from wild species that provided resistance, even through Tovi-star and the Israeli resistant line are both tolerant to TYLCV-IL, suggesting that the mechanism of resistance seems to involve other factors bedsides HSPs. Heat shock proteins are only one response to biotic virus stress. Resistance to geminiviruses will likely be multigenic. Several loci on the chromosome 6 of tomato have been identified with resistance to TYLCV using *Solanum chilense* (Zamir *et al.*, 1997; Agrama and Scott 2006) and *S. pimpinellifolium* (Chague *et al.,* 1997). Another locus associated with resistance to TLCV originating from *S. habrochaites* has been mapped to the long arm of chromosome 11 (Hanson *et al.*, 2000). Further research on the genetic mechanisms involved in tomato geminiviruses is warranted for the development of DNA markers for resistance to stresses in tomato lines and cultivars.

CHAPTER 4: GENERAL CONCLUSIONS

The losses to the susceptibility of tomato to ToCSV are high therefore control measures are very important. Conventional methods include cultural practices, pesticides and physical barriers to eliminate infection of crops. However the use of resistant varieties brought about by classical breeding is an alternative method to disease control. The genetic difference between ToCSV-[ZA:0nd:98] and TYLCV-IL suggests that they may share a number of biological properties and therefore control strategies.

In this study it was shown that inbred line TAM has a source of resistance that is possibly novel. TAM showed a high level of resistance to ToCSV and TYLCV but contains none of the known Ty-resistant linked genes. Test crosses of TAM with Rooikhaki indicated in the segregation pattern that the F1 hybrids genetic control is partially dominant and the characteristics in the F2 population indicated that the test cross of TAM (2) and Rooikhaki show stable resistance.

Future studies should include a test cross of TAM with Rooikhaki, thereafter, a single F1 plant should be self pollinated o create F2 progeny and crossed with both parents to create reciprocal backcross (BC) progenies. The parents, F1, F2 and BC progenies should be evaluated for their response to ToCSV infection. Random amplified polymorphic DNA (RAPD) primers would then be used to screen the parents. Those generating repeatable polymorphisms between the parental DNA's must be used to genotype segregating F2 progeny to identify putative markers associated with ToCSV resistance. The markers associated with ToCSV resistance should then be tested using BC1 progeny as well. Consequently, restriction fragment length polymorphism maps are able to be constructed. Qualitative analysis of resistance test data can be done by grouping phenotypic classes and testing segregation ratios by Chi-square analyses.

We have also shown that TYLCV-IL is less severe in symptom phenotype in commercial tomato varieties from South Africa than from Israel. This suggests that TYLCV-IL tolerant cultivars may be useful for the control of ToCSV but require evaluation prior to release in breeding programs in South Africa.

In future, larger field trials are needed to confirm the resistance of inbred line TAM to ToCSV and TYLCV-IL. The genes conferring resistance have not yet been identified therefore it is important that future studies include the full characterization of the genes in the resistance and creation of a tightly linked molecular marker to the gene/s involved in the tomato line TAM.

REFERENCES

Abhary, M., Patil, B., Fauquet, C., (2007). Molecular biodiversity, taxonomy and nomenclature of Tomato yellow leaf curl-like viruses. In Czosnek, Henryk (Ed). Tomato Yellow Leaf Curl Virus Disease, Management, Molecular Biology, Breeding for Resistance (p85-118). Dordrecht, The Netherlands: Springer.

Accotto, G., Navas- Castillo, J., Noris, E., Moriones, E., Louro, D., (2000). Typing of *Tomato yellow leaf curl virus* in Europe. European Journal of Plant Pathology. 106: 179-186.

Adam, Z., and Clarke, A., (2002). Cutting edge of chloroplast proteolysis. Trends in Plant Science 7: 451-456.

Afzal, A., Wood, A., and Lightfoot, D., (2008). Plant receptor-like serine threonine kinases: roles in signalling and plant defence. Molecular Plant-Microbe Interactions 21: 507- 517.

Agarwal, S., and Rao, A., (2000). Tomato lycopene and its role in human health and chronic diseases. Canadian Medical Association Journal 19: 739-744.

Agrama, H. A., and Scott, J. W., (2006). Quantitative trait loci for tomato yellow leaf curl virus and tomato mottle virus resistance in tomato. Journal of the American Society for Horticultural Science 131: 267-272.

Agrios, G., (2005). Plant Pathology. 5th edition. Academic Press p136-160.

Ali, N., Mulwa, R., Norton, M., Skirvin, R., (2007). Radical disinfestations protocol eliminates in vitro contamination in Guava (*Psidium guajava* L.) seeds. Plant cell tissue organ culture. 91: 295-298.

Anbinder,I., Reuveni, M., Azari, R., Paran, I., Nahon, S., Shlomo, H., Chen, L., Lapidot, M., Levin, I., (2009). Molecular dissection of *Tomato leaf curl virus* resistance in tomato line TY172 derived from *Solanum peruvianum*. Theory of Applied Genetics 119: 519-530.

Antignus, Y., Mor, N., Joseph, R. B., Lapidot, M., and Cohen, S., (1996). Ultraviolet-absorbing plastic sheets protect crops from insect pests and from virus diseases vectored by insects. Environmental Entomology 25: 919– 924.

Arguello-Astorga, G. R., Guevara-Gonzalez, R. G., Herrera-Estrella, L. R., Rivera-Bustamante, R. F. (1994). Geminivirus replication origins have a group-specific organization of iterative elements: a model for replication. Virology 203: 90-100.

Baas, P. D., and Jansz, H. S., (1988). Single-strand DNA phage origins. Current Topics in Microbiology and Immunology. 136: 31– 69.

Banerjee, M. K., and Kallo, G., (1987). Inheritance of tomato leaf curl virus resistance in *Lycopersicon* hirsutum. Euphytica 36: 581–584.

Beecher, G., (1998). Nutrient content of tomatoes and tomato products, nutrient content of tomatoes. Proceedings of the Society of Experimental Biology and Medicine 218: 98-100.

Bennison, J., (2001). Protected herbs: control of glasshouse whitefly and leafhoppers within IPM programmes. Final report for Hoticulture Development Company project PC 178. http://www.hdc.org.uk/herbs/page.asp?id=6#.

Benor, S., and Zhang, M., (2008). Assessment of genetic variation in tomato (*Solanum lycopersicum* L.) inbred lines using SSR molecular markers. Journal of Genetics and Genomics 35: 373-379.

Bian, X., Thomas, M., Rasheed, S., Saeed, M., Hanson, P., De Barro, P., Rezaian, M., (2007). A Recessive Allele (tgr-1) Conditioning tomato resistance to geminivirus infection is associated with impaired viral movement. Phytopathology 97: 930-937.

Boiteaux, L., Oliveira, V., Silva, C., Makishima, N., Nagata, A., Fonseca, M., Giordano, L., (2007). Reaction of tomato hybrids carrying the *Ty-1* locus to Brazilian bipartite *Begomovirus* species. Horticultura Brasileira. Brasília 25: 020- 023.

Bolwell, G. P., (1999). Role of active oxygen species and NO in plant defence responses. Current Opinion in Plant Biology 2: 287– 294.

Bonde, R., and Murphy, E., (1952). Resistance of certain tomato varieties and crosses to late blight. Maine Agricultural Experiment Station Research Bulletin No. 497.

Bosco, D., and Caciagli, P., (1998). Bionomics and ecology of *Bemisia tabaci* (Sternorrhyncha: Aleyrodidae) in Italy. European Journal of Entomology 95: 519– 527.

Boudyach, E. H., Fatmi, M., Akhayat, O., Benizri, E., Aoumar, A. B., (2001). Selection of antagonistic bacteria of *Clavibacter michiganensis* spp. *michiganensis* and evaluation of their efficiency against bacterial canker of tomato. Biocontrol Science and Technology 11: 141- 149.

Boukema, I., (1980). Allelism of genes controlling resistance to TMV in *Capsicum* L. Euphytica 29: 433- 439.

Briddon, R. W., Bedford, I. D., Tsai, J. H., Markham, P. G., (1996). Analysis of the nucleotide sequence of the treehopper-transmitted geminivirus, *Tomato pseudo-curly top virus*, suggests a recombinant origin. Virology 219: 387- 394.

Brodersen, P., and Voinnet, O., (2006). The diversity of RNA silencing pathways in plants. Trends in Genetics 22: 268- 280.

Brouwer, D. J., and Clair, D. A., (2004). Fine mapping of three quantitative trait loci for late blight resistance in tomato using near isogenic lines (NILs) and sub-NILs. Theoretical and Applied Genetics 108: 4, 628- 638.

Brown, J. K., (2000). Molecular markers for the identification and global tracking of whitefly vector-begomovirus complexes. Virus Research 71: 233- 260.

Brown, J., K., (2007). The Bemisia tabaci complex: genetic and phenotypic variation and relevance to begomovirus-vector interactions. In Czosnek, H (Ed). Tomato Yellow Leaf Curl Virus Disease, Management, Molecular Biology, Breeding for Resistance. (p25- 56). Dordrecht, The Netherlands: Springer.

Byrne, D., and Bellows, T., (1991). Whitefly biology. Annual Review of Entomology 36: 431– 457.

Caciagli, P., and Bosco, D., (1996). Quantitative determination of *Tomato yellow leaf curl* geminivirus DNA by chemiluminescent assay using digoxigenin-labelled probes. Journal of Virological Methods 57: 19– 29.

Caciagli, P., and Bosco, D. (1997). Quantitation over time of tomato yellow leaf curl geminivirus DNA in its whitefly vector. Phytopathology 87: 610– 613.

Carland, F., and Staskawicz, B., (1993). Genetic characterization of the *Pto* locus of tomato: Semi-dominance and segregation of resistance to *Pseudomonas syringae* pv. *tomato* and sensitivity to the insecticide fenthion. Molecular and General Genetics 239: 17- 27.

Chagué, V., Mercier, J. C., Guenard, M., de Courcel, A., and Vedel, F., (1997). Identification of RAPD markers linked to a locus involved in quantitative resistance to TYLCV in tomato by bulked segregant analysis. Theoretical and Applied Genetics. 95: 671- 677.

Chen, T., Hanson, M., Kuo, G., Opena, T., (2003). Genetic improvement of summer fresh market tomatoes. Journal of the Agricultural Association of China 4: 83- 102.

Chiang, B., T., Maxwell, D., Green, S., (1994). Leaf curl virus in Taiwan. Tomato Leaf Curl News. No. 5: 3.

Ciccarese, F., Amendauni, M., Shiavone, D., and Cirulli, M., (1998(. Occurence and inheritance of resistance to powdery mildew (*Oidium lycopersicum*) in *Lycopersicon* species. Plant Pathology 47: 417- 419.

Coaker, G., L., and Francis, D., M., (2004). Mapping, genetic effects, and epistatic interaction of two bacterial canker resistance QTLs from *Lycopersicon hirsutum.* Theoretical and Applied Genetics 108: 1047- 1055.

Cohen, S., and Harpaz, I., (1964). Periodic, rather than continual acquisition of a new tomato virus by its vector, the tobacco whitefly (*Bemisia tabaci* Gennadius). Entomologia Experimentalis et Applicata 7: 155– 166.

Cohen, S., and Nitzany, F., E., (1966). Transmission and host range of *Tomato yellow leaf curl virus*. Phytopathology 56: 1127– 1131.

Cohen, S., and Melamed-Madjar, V., (1978). Prevention by soil mulching of the spread of *Tomato yellow leaf curl virus* transmitted by *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) in Israel. Bulletin of Entomological Research 68: 465– 470.

Cohen, S., and Berlinger, M. J., (1986). Transmission and cultural control of whitefly-borne viruses. Agriculture, Ecosystems and Environment 17: 89– 97.

Cohen, S., Kern, J., Harpaz, I., Ben Joseph, R., (1988). Epidemiological studies of the *Tomato yellow leaf curl virus* (TYLCV) in the Jordan Valley, Israel. Phytoparasitica 16: 259– 270.

Cohen, S., and Antignus, Y., (1994). *Tomato yellow leaf curl virus*, a whitefly-borne geminivirus of tomatoes. Advances in Disease and Vector Resistance 10: 259- 288.

Cohn, J., Sessa, G., Martin, G., (2001). Innate immunity in plants. Current Opinion in Immunology 13: 55- 62.

Crute, I., and Norwood, J., (1986). Gene-dosage effects on the relationship between *Bremia lactucae* (downy mildew) and *Lactuca sativa* (lettuce): The relevance to a mechanistic understanding of host parasite specificity. Physiological and Molecular Plant Pathology 29: 133– 122.

Crute, I., and Pink, A., (1996). Genetics and utilization of pathogen resistance in plants. The Plant Cell 8: 1747- 1755.

Czosnek, H., Navot, N., Laterrot, H. (1990). Geographical distribution of tomato yellow leaf curl virus. A first survey using a specific DNA probe. Phytopathologia Mediterranea 29: 1– 6.

Czosnek, H. and Laterrot, H., (1997). A worldwide survey of tomato yellow leaf curl viruses. Archives of Virology 142: 1391– 1406.

Czosnek, H., Ghanim, M., Ghanim, M. (2002). The circulative pathway of begomoviruses in the whitefly vector *Bemisia tabaci*– insights from studies with *Tomato yellow leaf curl virus*. Annals of Applied Biology 140: 215- 231.

Dalmon, A., Cailly, M., David, C., (2000). Comparison of serological and molecular techniques for detection of tomato yellow leaf curl begomovirus. Bulletin of European and Mediterranean Plant Protection Organisation 30: 457– 462.

Dangl, J., and Jones, J., (2001). Plant pathogens and integrated defence response to infection. Nature 411: 826-833.

De Castro, A., Blanca, J., Diez, M., Vinals, F., (2007). Identification of a CAPS marker tightly linked to the Tomato yellow leaf curl disease resistance gene *Ty-1* in tomato. European Journal of Plant Pathology 117: 347- 356.

Dellaporta, S. L., Wood, J., Hicks, J. B., (1983). A plant DNA minipreperation: Version II. Plant molecular biology reporter 1: 19- 21.

DeLong, A., Mockaitis, K., Christensen, S., (2002). Proteing phosphorylation the delivery of and response to auxin signals. Plant Molecular Biology 49: 285- 303.

Department of Agriculture (2003), Directorate Agricultural Information Services. Tomato curly stunt begomovirus, a new pathogen of tomatoes in South Africa. Technical manual no. 1 (p1- 6). Published by Department of agriculture. Retrieved from: http://www.nda.agric.za/docs/GenPub/tomatovirus.pdf .

Diaz-Pendon, J., Truniger, V., Nieto, C., Garcia-Mas, J., Bendahmane, A., Aranda, M., (2004). Advances in understanding recessive resistance to plant viruses. Molecular Plant Pathology 5**:** 223- 233.

Dickinson, M., Jones, D., and Jones, J., (1993). Molecular evidence for close linkage between the *Cf-2/ Cf-5* and *Mi* loci in tomato. Molecular Plant- Microbe Interactions 6: 341- 347.

Dittrich, V., Ernst, G. H., Ruesh, O., Uk, S., (1990). Resistance mechanisms in sweetpotato whitefly

(Homoptera: *Aleyrodidae*) populations from Sudan, Turkey, Guatemala, and Nicaragua. Journal of Economic Entomology 83: 1665– 1670.

Dry, I. B., Krake, L. R., Ridgen, J. E., Ali Rezaian, A., (1997). A novel subviral agent associated with a geminivirus: The first report of a DNA satellite. Proceedings of the National Academy of Sciences U.S.A 94: 7088- 7093.

Ebel, J., and Mithofer, A., (1998). Early events in the elicitation of plant defence. Planta 206: 335-348.

Fauquet, C. M., Bisaro, D. M., Briddon, R. W., Brown, J. K., Harrison, B. D., Rybicki, E. P., Stenger, D. C., Stanley, J., (2003). Revision of taxonomic criteria for species demarcation in the family *Geminiviridae,* and an updated list of begomovirus species. Archives of Virology 148: 405- 421.

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

Finlay, K,. (1953). Inheritance of *Spotted wilt virus* resistance in tomato. Australian Journal of Biological Sciences 6: 153- 163.

Foolad, M. R., (2007). Genome mapping and molecular breeding of tomato, review article. International Journal of Plant Genomics. doi: 10.1155/2007/64358.

Friedmann, M., Lapidot, M., Cohen, S., Pilowsky, M., (1998). A novel source of resistance to *Tomato yellow leaf curl virus* exhibiting a symptomless reaction to viral infection. Journal of the American Society for Horticultural Science 123: 1004– 1006.

Gafni, Y., and Epel, B. L., (2002). The role of host and viral proteins in intra- and inter- cellular trafficking of Geminiviruses. Physiological and Molecular Plant Pathology 60: 231- 241.

Garcia-Cano, E., Resende, R., Boiteux, L., Giordano, L., Fernandez-Munoz, R., Moriones, E., (2008). Phenotypic expression, stability, and inheritance of a recessive resistance to monopartite begomoviruses associated with tomato yellow yeaf curl disease in tomato. Phytopathology 98: 618- 627.

Garcia, B., Graham, E., Jensen, K., Hanson, P., Mejia, L., Maxwell, D., (2007). Co-dominant SCAR marker for detection of the begomovirus-resistance *Ty2* locus derived from *Solanum habrochaites* in tomato germplasm. Tomato Genetics Cooperative Report 57: 2007. Retrieved from http://tgc.ifas.ufl.edu/vol57/vol57.pdf .

Garcia-Ruiz, H., and Murphy, J., (2001). Age-related resistance in bell pepper to *Cucumber mosaic virus*. Annals of Applied Biology 139: 307– 317.

Geneif, A. A., (1984). Breeding for resistance to tomato leaf curl virus in tomatoes in the Sudan. Acta Horticulturae 143: 469- 484.

Ghanim, M., Rosell, R. C., Campbell, L. R., Czosnek, H., Brown, J. K., Ullman, D. E., (2001). Digestive, salivary and reproductive organs of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) type B. Journal of Morphology 248: 22– 40.

Gilbertson, R., Rojas, M., Kon, T., and Jaquez, J., (2007). Introduction of Tomato yellow leaf curl virus

into the Dominican Republic: the development of a successful integrated pest management strategy. In Czosnek, H (Ed). Tomato Yellow Leaf Curl Virus Disease, Management, Molecular Biology, Breeding for Resistance (p279– 303). Dordrecht, The Netherlands: Springer.

Gill, R. J., (1990). The morphology of whiteflies. In D. Gerling (Ed.), Whiteflies: Their Bionomics, Pest Status, and Management. Andover, Hants, England: Intercept p13– 45.

Griffiths, P. D., and Scott, J. W., (2001). Inheritance and linkage of *Tomato mottle virus* resistance genes derived from *Lycopersicon chilense* accession LA1932. Journal of the American Society for Horticultural Science 126: 462– 467.

Gorovitz, R., and Czosnek, H., (2008). Expression of stress gene networks of tomato lines susceptible and resistant to *Tomato yellow leaf curl virus* in response to abiotic stresses. Plant Physiology and Biochemistry 46: 482- 492.

Gutierrez, C., (2000). Geminiviruses and the plant cell cycle. Plant Molecular Biology 43: 763- 772.

Gutierrez , C., Ramirez-Parra, E., Castellano, M. M., Sanz-Burgos, A. P., Luque, A., Missich, R., (2004). Geminivirus DNA replication and cell cycle interactions. Veterinary Microbiology 98: 111- 119.

Hammond-Kosack, K., and Jones, J., (1996a). Inducible plant defence mechanisms and resistance gene function. Plant Cell 8: 1773- 1791.

Hammond-Kosack, K., and Jones, J., (1996b). Incomplete dominance of tomato *Cf* genes for resistance to *Cladosporium fulvum*. Molecular Plant-Microbe Interactions 7: 58- 70.

Hanley-Bowdoin, L., Elmer, J. S., Rogers, S. G., (1988). Transient expression of heterologous RNAs using *Tomato golden mosaic virus*. Nucleic Acids Research 16: 10511– 10528.

Hanley-Bowdoin, L., Settlage, S. B., Orozoo, B. M., Nagar, S., Robertson, D., (1999). Geminiviruses: Models for plant DNA replication, transcription, and cell cycle regulation. Critical Reviews in Plant Sciences 18: 71– 106.

Hanson, P. M., Bernacchi, D., Green, S., Tanksley, S. D., Muniyappa, V., Padmaja, A. S., Chen, H. M., Kuo, G., Fang, D., Chen, J. T. (2000). Mapping a wild tomato introgression associated with *Tomato yellow leaf curl virus* resistance in a cultivated tomato line. Journal of the American Society for Horticultural Science 125: 15– 20.

Harrisson, B., D., and Robinson, D. J., (1999). Natural genomic and antigenic variation in whitefly-transmitted Geminiviruses using sequence comparisons. Journal of General Virology 76: 246-263.

Hartl, F., U., and Hayer-Hartl, M., (2002). Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 295: 1852– 8 (Review).

Hassan, A. A., Mazyad, H. M., Moustafa, S. E., Nakhla, M. K., (1982). Assessment of tomato yellow leaf curl virus resistance in the genus *Lycopersicon.* Horticultural Research in Egypt 9: 13– 116.

Hassan, A. A., Mazyad, H. M., Moustafa, S. E., Nassar, S. H., Nakhla, M. K., Sims,W. L., (1984). Inheritance of resistance to *Tomato yellow leaf curl virus* derived from *Lycopersicon cheesmanii* and *Lycopersicon hirsutum.* HortScience 19: 574– 575.

Hassan, A., A., and Abdel-Ati, K. E. A., (1999). Genetics of tomato yellow leaf curl virus tolerance derived from *Lycopersicon pimpinellifolium* and *Lycopersicon pennellii.* Horticulural Research in Egypt 26: 323- 338.

Hirt, H., (1997). Multiple roles of MAP kinases in plant signal transduction. Trends in Plant Science 2: 11-15.

Hofgen, R., and Willmitzer, L., (1988). Storage of competent cells for Agrobacterium transformation. Nucleic Acids Research 16: 169- 170.

Horns, T., and Jeske, H., (1991). Localization of *Abutilon mosaic virus* (AbMV) DNA within the leaf tissue by *in situ* hybridization. Virology 181: 580– 588.

Horowitz, A. R., and Gerling, D., (1992). Seasonal variations of sex ratio in *Bemisia tabaci* on cotton in Israel. Environmental Entomology 21: 556– 559.

Huang, C. C., Cui, Y. Y., Weng, C. R., Zabel, P., Lindhout, P., (2000). Development of diagnostic PCR markers closely linked to the tomato powdery mildew resistance gene *Ol-1* on chromosome 6 of tomato. Theoretical and applied Genetics 101: 918- 924.

Jahoor, A., Jacobi, A., Schuller, C., Fishbeck, G., (1993). Genetical and RFLP studies at the *Mla* locus conferring powdery mildew resistance in barley. Theoretical and Applied Genetics : 713- 718.

Ji, Y., and Scott, J. W., (2006a). Development of breeder friendly markers for begomovirus resistance genes derived from *Solanum chilense*. Proceedings of the tomato breeders table, Tampa, Florida, USA. roundtable06.ifas.ufl.edu/Schedule.htm

Ji, Y., Campbell, H. L., Kloepper, J. W., Jones, J., B., Suslow, T. V., Wilson, M., (2006b). Integrated biological control of bacterial speck and spot of tomato under field conditions using foliar biological control agents and plant growth-promoting rhizobacteria. Biological Control 36: 358- 367.

Ji, Y., and Scott, J., (2006). *Ty-3*, a begomovirus resistance locus linked to *Ty-1* on chromosome 6 of tomato. Tomato Genetics Cooperative Report 56: 22- 25.

Ji,Y., Schuster, J., Scott, J., (2007a). *Ty-3,* a begomovirus resistance locus near the *Tomato yellow leaf curl virus* resistance locus *Ty-1* on chromosome 6 of tomato. Molecular Breeding 20: 271- 284.

Ji, Y., Scott, J., Hanson, P., Graham, E., Maxwell, D., (2007b). Sources of resistance, inheritance, and location of genetic loci conferring resistance to members of the tomato-infecting begomoviruses. In Czosnek, H (Ed). Tomato Yellow Leaf Curl Virus Disease, Management, Molecular Biology, Breeding for Resistance (p347-361). Dordrecht, The Netherlands: Springer.

Ji, Y., Scott, J., Maxwell, D., Schuster, D. (2008). *Ty-4*, a tomato *yellow leaf curl virus* resistance gene on chromosome 3 of tomato. Tomato Genetics Cooperative Report 58*:* 29– 31.

Johnson, J., (1946). Soil steaming for disease control. Soil Science 61: 83- 92.

Jolly, C., and Morimoto, R., (2000). Role of the heat shock response and molecular chaperones in oncogenesis and cell death. Journal of the National Cancer Institute 92: 1564- 1572.

Jones, D., Dickinson, M., Balinut-Kurti P., Dixon, M., and Jones, J., (1993). Two complex loci revealed by classical and RFLP mapping of the *Cf-2, Cf-4, Cf-5* and *Cf-9* leaf mold resistance genes in tomato. Molecular Plant-Microbe Interactions. 6: 348- 357.

Jones, D. R., (2003). Plant viruses transmitted by whiteflies. European Journal of Plant Pathology 109: 195– 219.

Kalloo, G., (1991). Disease resistance in tomatoes. Genetic improvement of tomato (p99- 108). New York: Springer.

Kalloo, G., and Banerjee, M. K., (1990). Transfer of tomato leaf curl virus resistance from L*ycopersicon hirsutum* to *L. esculentum.* Plant Breeding 105: 156– 159.

Katiyar-Agarwal, S., Agarwal, M., and Grover, A., (2003). Heat-tolerant basmati rice engineered by over-expression of hsp101. Plant Molecular Biology. 51: 677– 686.

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

Lapidot, M., Friedmann, M., Lachman, O., Yehezkel, A., Nahon, S., Cohen, S., Pilowsky, M., (1997). Comparison of resistance level to tomato yellow leaf curl virus among commercial cultivars and breeding lines. Plant Disease 81: 1425– 1428.

Lapidot, M., Goldray, O., Ben-Joseph, R., Cohen, S., Friedmann, M., Shlomo, A., Nahon, S., Chen, L., Pilowsky, M., (2000). Breeding tomatoes for resistance to tomato yellow leaf curl begomovirus. Bulletin of European and Mediterranean Plant Protection Organisation 30: 317– 321.

Lapidot, M., Friedmann, M., Pilowsky, M., Ben-Joseph, R., Cohen, S., (2001). Effect of host plant resistance to *Tomato yellow leaf curl virus* (TYLCV) on virus acquisition and transmission by its whitefly vector. Phytopathology 91: 1209- 1213.

Lapidot, M., and Friedmann, M., (2002). Breeding for resistance to whitefly-transmitted geminiviruses. Annals of Applied Biology 140: 109– 127.

Lapidot, M., and Levy, D., (2008). Effect of plant age at inoculation on expression of genetic resistance to tomato yellow leaf curl virus. Archives of Virology 153: 171- 179.

Laterrot, H., (1992). Resistance genitors to *Tomato yellow leaf curl virus* (TYLCV). Tomato Leaf Curl Newsletter 1: 2– 4.

Laterrot, H., (1975). Selection pur la resistance au mildiou, *Phytophthora infestans* Mont., de Bary chez la tomate. Ann. Amelior. Plant 25**:** 129 – 149.

Laterrot, H., and Moretti, A., (1994). The chiltylic populations of the EEC-DGX programme. Tomato Leaf Curl Newsletter 5: 2.

Legg, J. P., (1999). Emergence, spread and strategies for controlling the pandemic of *Cassava mosaic virus* disease in east and central Africa. Crop Protection 18: 627– 637.

Leonardi, C., Ambrosino, P., Esposito, F., Fogliano, V., (2000). Antioxidant activity and carotenoid and tomatine content in different typologies of fresh consumption tomatoes. Journal of Agriculture and Food Chemistry 48: 4723- 4727.

Levy, D., and Lapidot, M., (2007). Effect of plant age at inoculation on expression of genetic resistance to *Tomato yellow leaf curl virus*. Archives of Virology 153: 171- 179.

Lindhout, P., Pet, G., Van der Beek, J., (1994). Screening wild *Lycopersicon* species for resistance to powdery mildew (*Oidium lycopersicum*). Euphytica 72: 43- 49.

Lippman, Z., and Martienssen, R., (2004). The role of RNA interference in heterochromatic silencing. Nature 431: 364- 370.

Loebenstein, G., (1972). Localization and induced resistance in virus-infected plants. Annual Review of Phytopathology 10: 177– 206.

Lucy, A. P., Boulton, M. I., Davies, J. W., Maule, A. J., (1996). Tissue specificity of *Zea mays* infection by *Maize streak virus*. Molecular Plant-Microbe Interactions 9: 22– 31.

Mansour, A., and Al-Musa, A., (1992). *Tomato yellow leaf curl virus*: host range and vector-virus relationships. Plant Pathology 41: 122– 125.

Maruthi, M. N., Czosnek, H., Vidavski, F., Tarba, S. Y., Milo, J., Leviatov, S., Venkatesh, H. M., Padmaja, A. S., Kukarni, R. S., Muniyappa, V., (2003). Comparison of resistance to *Tomato leaf curl virus* (India) and *Tomato yellow leaf curl virus* (Israel) among wild species, breeding lines and hybrids. European Journal of Plant Pathology 109: 1– 11.

Maule, A., Caranta, C., Boulton, M., (2007). Sources of natural resistance to plant viruses: status and prospects. Molecular Plant Pathology 8: 223- 231.

Mauseth, J., (1988). Plant anatomy. The Benjamin/Cummings Publishing Company, Inc. Canada. P6-10.

Maxwell, D., Garcia, B., Martin, C., Salus, M., Jensen, K., Mejia, L., (2006). PCR-based methods for tagging begomovirus-resistance genes in tomatoes. Retrieved from www.plantpath.wisc.edu/Geminivirus ResistantTomatoes/Markers.

Maxwell, D. P., Martin, C. T., Garcia, B. E., Salus, M., S. Jensen, K. S., Havey, M. J., Mejia, L., (2007). Markers for tomato chromosomes. Retrieved from www.plantpath.wisc.edu/GeminivirusResistantTomatoes.

McDowell, J., and Dangl, J., (2000). Signal transduction in the plant immune response. Trends in Biochemical Sciences 25: 79- 82.

Mehta, P., Wyman, J. A., Nakhla, M. K., Maxwell, D. P., (1994). Transmission of tomato yellow leaf curl geminivirus by *Bemisia tabaci* (Homoptera: Aleyrodidae). Journal of Economic Entomology 87: 1291– 1297.

Mejia, L., Teni, R., Vidavsky, F., Czosnek, H., Lapidot, M., Nakhla, K., Maxwell, P., (2005). Evaluation of tomato germplasm and selection of breeding lines for resistance to begomoviruses in Guatemala Acta Horticulturae 659: 251- 255.

Michelson, I., Zamir, D., Czosnek, H., (1994). Accumulation and translocation of *Tomato yellow leaf curl virus* in a *Lycopersicon esculentum* breeding line containing the *L. chilense* TYLCV tolerance gene *Ty-1*. Phytopathology 84: 928- 933.

Milligan, S. B., Bodeau, J., Yaghoobi, J., Kaloshian, I., Zabel, P., (1998). The root knot nematode-resistance gene *Mi* from tomato is a member of the leucine zipper, nucleotide binding, leucine rich repeat family of plant genes. Plant Cell 10: 1307– 1319.

Milo, J., (2001). The PCR-based marker REX-1, linked to the gene Mi, can be used as a marker to TYLCV tolerance. Tomato Breeders Roundtable. Retrieved from www.oardc.ohiostate.du/tomato/TBRT%202001%20Abstracts.pdf.

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

Mohammad, A., Basavarprabhu, L., Fauquet, C., (2007). Tomato yellow leaf curl virus disease, management, molecular biology, breeding for resistance. (Henryk Czosnek) p85- 113

Monci, F., Sanchez-Campos, S., Navas-Castillo, J., Moriones, E., (2002). A natural recombinant between the geminiviruses *Tomato yellow leaf curl Sardinia virus* and *Tomato yellow leaf curl virus* exhibits a novel pathogenic phenotype and is becoming prevalent in Spanish populations. Virology 303: 317– 326.

Monsoor, S., Zafar Y., Briddon W. R., (2006). Geminivirus disease complexes: the threat is spreading. Trends in Plant Science 11: 209- 212.

Morales, F. J., and Anderson, P. K. (2001). The emergence and dissemination of whitefly-transmitted geminiviruses in Latin America. Archives of Virology 146: 415– 441.

Moreau, P., Philippe, T., Jocelyne, O., Laterrot, H., Grimesley, N., (1998). Genetic mapping of *Ph-2*, a single locus controlling partial resistance to *Phytophtora infestans* in tomato. Molecular Plant-Microbe Interactions 11: 259- 269.

Moriones, E., Aramburu, J., Riudavets, J., Arno, J., Lavina, A., (1998). Effect of plant age at time of infection by *Tomato spotted wilt topovirus* on the yield of field-grown tomato. European Journal of Plant Pathology 104: 295– 300.

Moriones, E. and Navas-Castillo, J., (2000). *Tomato yellow leaf curl virus*, an emerging virus complex causing epidemics worldwide. Virus Research 71: 123– 134.

Moustafa, S. E., and Nakhla, M. K., (1990). An attempt to develop a new tomato variety resistant to *Tomato yellow leaf curl virus* (TYLCV). The Journal of Agricultural Science 21: 167– 184.

Mueller, L. A., Solow, T. H., Taylor, N., Skwarecki, B., Buels, R., Binns, J., Lin, C. W., Wright, M. H., Ahrens, R., Wang, Y., Herbst, E. V., Keyder, V. R., Menda, N., Zamir, D., Tanksley, S. D., (2005). The SOL genomics network. A comparative resource for *Solanaceae* biology and beyond. Plant Physiology 138: 1310– 1317.

Pareek, A., Singla, S., L., and Grover, A., (1995). Immunological evidence for accumulation of two high-molecular-weight (104 and 90 kDa) HSPs in response to different stresses in rice and in response to high temperature stress in diverse plant genera. Plant Molecular Biology 29: 293– 301.

Parella. G., Laterrot, H., Marchoux, G., and Gebre-Selassie, K., (1997). Screening *Lycopersicon* accessions for resistance to *Alfalfa mosaic virus*. Journal of Genetics and Breeding 51: 75- 78.

Parker, J., and Higgins, V., (1993). Phenotypic characterisation and molecular mapping of the *Arabidopsis thaliana* locus, *RPP5*, determining disease resistance to *Peronospora parasitica*. The Plant Journal 4: 821– 831.

Parsell, D. A., and Lindquist, S., (1993). The role of heat-shock proteins in thermotolerance. Philosophical Transactions of the Royal Society B: Biological Sciences 339: 279– 285.

Nakhla, M. K., and Maxwell, D. P. (1998). Epidemiology and management of tomato yellow leaf curl disease. In Hadid, A., Khetarpal, R., and Koganezawa, H (Eds). Plant virus disease control (p565– 583). Maine, USA: APS Press.

Nakhla, M., Sorenson, A., Mejia, L., Ramirez, P., Karkashian, J., Maxwell, D., (2005). Molecular characterization of tomato-infecting Begomoviruses in central America and development of DNA-based detection methods. Acta Horticulturae 69: 277- 288.

Narasegowda, M., Czosnek, H., Vidavsky, F., Tarba, S., Milo, J., Leviatov, S., Mallithimmaiah, V., Seetharam, P., Subbappa, R., Muniyappa, V., (2003). Comparison of resistance to *Tomato leaf curl virus* (India) and *Tomato yellow leaf curl virus* among *Lycopersicon* wild species, breeding lines and hybrids. European Journal of Plant Pathology 109: 1- 11.

Navot, N., Pichersky, E., Zeidan, M., Zamir, D., Czosnek, H., (1991). *Tomato yellow leaf curl virus*: a whitefly-transmitted geminivirus with a single genomic component. Virology 185: 151– 161.

Nombela, G., Williamson, V. M., Muniz, M., (2003). The root-knot nematode-resistance gene *Mi-1.2* of tomato is responsible for resistance against the whitefly *Bemisia tabaci*. Molecular and Plant- Microbe Interactions 16**:** 645– 649.

Novick, R. P., (1998). Contrasting lifestyles of rolling-circle phages and plasmids. Trends in Biochemical Sciences 23: 434– 438.

Padidam, M., Sawyer, S., Fauquet, C. M., (1999). Possible emergence of new geminiviruses by frequent recombination. Virology 256: 218- 225.

Parrella, G., Moretti, A., Gognalons, P., Lesage, M.-L., Marchoux, G., Gebre-Selassie, K., Caranta, C. (2004). The *Am* gene controlling resistance to *Alfalfa mosaic virus* in tomato is located in the cluster of dominant resistance genes on chromosome 6. Phytopathology 94: 345- 350.

Picó, B., Sifres, A., Elía, M., Díez, M. J., Nuez, F., (2000). Searching for new resistance sources to *Tomato yellow leaf curl virus* within a highly variable wild *Lycopersicon* genetic pool. Acta Physiologiae Plantarum 22: 344– 350.

Pico, B., Diez, M. J., Nuez, F., (1996). Viral Diseases causing the greatest economic losses of tomato crops. II. The *Tomato leaf curl virus* – a review. Scientia Horticulturae 67: 151- 196.

Pieterse, C., and Van Loon, L., (1999). Salicylic acid-independent plant defence pathways. Trends in Plant Science 4: 52- 58.

Pietersen, G., Idris, A., M., Kruger, K., Brown, J. K., (2000). *Tomato curly stunt virus*, a new begomovirus of tomato within the *Tomato yellow leaf curl virus-IS* cluster in South Africa. Plant Disease 84: 810.

Pietersen, G., and Smith, M. F., (2002). *Tomato yellow leaf virus* resistant tomatoes show resistance to *Tomato curly stunt virus*. Plant Disease 86: 528– 534.

Pietersen, G., Idris, A., M., Kruger, K., Brown, J. K., (2008). Characterization of *Tomato curly stunt virus*: a new tomato-infecting begomovirus from South Africa. Plant Pathology 10: 1365- 3059.

Pilowsky, M., and Cohen, S., (1974). Inheritance of resistance to *Tomato yellow leaf curl virus* in tomatoes. Phytopathology 64: 632- 635.

Pilowsky, M., and Cohen, S., (1990). Tolerance to *Tomato yellow leaf curl virus* derived from *Lycopersicon peruvianum*. Plant Disease 74: 248– 250.

Pinon, M., Gómez, O., Cornide, M. T., (2005). RFLP analysis of Cuban tomato breeding lines with resistance to *Tomato yellow leaf curl virus*. Acta Horticulturae 695: 273– 276.

Pringle, C. R. (1999). Virus taxonomy – 1999 – The universal system of virus taxonomy, updated to include the new proposals ratified by the International Committee on Taxonomy of Viruses during 1998. Archives of Virology 139: 49- 77.

Pollard, D. G., (1955). Feeding habits of the cotton whitefly. Annals of Applied Biology 43: 664– 671.

Polston, J. E., McGovern, R. J., Brown, L. G., (1999). Introduction of *Tomato yellow leaf curl virus* in Florida and implications for the spread of this and other geminiviruses of tomato. Plant Disease 83: 984– 988.

Polston, J. E., Anderson, P. L., (1997). The emergence of whitefly transmitted geminiviruses in tomato in the Western Hemisphere. Plant Disease 81: 1358– 1369.

Polston, J. E., and Lapidot, M., (2007). Management of Tomato yellow leaf curl virus - US and Israel perspectives. In Czosnek, H., (Ed). Tomato Yellow Leaf Curl Virus Disease, Management, Molecular Biology, Breeding for resistance (p251- 262). Dordrecht, The Netherlands: Springer.

Preiss, W., and Jeske, H., (2003). Multitasking in replication is common among geminiviruses. Journal of Virology 77: 2972– 2980.

Rao, A., and Rao, L., (2003). Lycopene and human health. National genomics and functional foods 1: 35- 44.

Robaglia, C., and Caranta, C., (2006). Translation initiation factors: a weak link in plant RNA virus infection. Trends in Plant Science 11: 40- 45.

Ribaut, J. M., Jiang, C., Hoisington, D., (2002). Simulation experiments on efficiencies of gene introgression by backcrossing. Crop Science 42: 557– 565.

Richards, M., and Barratt, R., (1946). A partial survey of the genus *Lycopersicon* for resistance to *Phytopthora infestans*. Plant Disease 30: 16- 20.

Robert, V. J. M., West, M. A. L., Inai, S., (2001). Marker-assisted introgression of blackmold resistance QTL alleles from wild *Lycopersicon cheesmanii* to cultivated tomato (*L. esculentum)* and evaluation of QTL phenotypic effects. Molecular Breeding 8: 217- 233

Roossinck, M. J., (1997). Mechanisms of plant virus evolution. Annual Review of Phytopathology 35: 191– 209.

Rosello, S., Diez, M. J., Nuez, F., (1996). Viral diseases causing the greatest economic losses to the tomato crop. I. The *Tomato spotted wilt virus* – a review. Scientia Horticulturae 67: 117- 150.

Rossi, M., Goggin, F. L., Milligan, S. .B, Kaloshian, I., Ullman, D. E., (1998). The nematode resistance gene Mi of tomato confers resistance against the potato aphid. Proceedings of the National Academy of Sciences USA 95**:** 9750– 9754.

Rushing, A. E., Sunter, G. Gardiner, W. E., Dute, R. R., Bisaro, D. M., (1987). Ultra structural aspects of *Tomato golden mosaic virus* infection in tobacco. Phytopathology 77: 1231– 1236.

Rybicki, E. P., Briddon, R. W., Fauquet, C. M., Maxwell, D. P., Harrison, B. D., Markham, P. G., Stanley, J., (2000). *Geminiviridae.* In van Regenmortel, M., H., V., Fauquet, C., M., Bishop, D., H., L., Carstens, E., Estes, M., K., Lemon, S., Maniloff, J., Mayo, M., A., McGeoch, D., Pringle, C., R., Wickner, R., B., (Eds). Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses (P285- 297). New York: Academic Press.

Rushing, A. E., Sunter, G., Gardiner, W. E., Dute, R. R., Bisaro, D. M., (1987). Ultra structural aspects of *Tomato golden mosaic virus* infection in tobacco. Phytopathology 77: 1231– 1236.

Saidi, M., and Warade, S., (2008). Tomato breeding for resistance to *Tomato spotted wilt virus* (TSWV): An overview of conventional and molecular approaches. Journal of Genetics and Plant Breeding 44: 83- 92.

Sambrook, J., Fritsch, E. F., Maniatis, T., (1989). Molecular cloning: a laboratory manual, 2nd edition. Cold Spring Harbor Press, New York.

Sanchez, M., Valencia, C., Ciruelos, A., Latorre, A., Gallegos, C., (2003). Rheological properties of tomato paste: Influence of the addition of tomato slurry. Journal of Food Science 68: 551- 554.

Sawangjit, S., Chatchawankanphanich, O., Pissawan, C., Attathom, T., Dale, J., Attathom, S., (2005). Molecular characterization of tomato-infecting begomoviruses in Thailand. Virus Research 109: 1- 8.

Schuster, D. J., Muller, T. F., Kring, J. B., Price, J. F., (1990). Relationship of the sweetpotato whitefly to a new tomato fruit disorder in Florida. Horticultural Science 25: 1618– 1620.

Scott, J. W., and Schuster, D. J., (1991). Screening of accessions for resistance to Florida tomato geminivirus. Tomato Genetics Cooperative Report 41: 48– 50.

Scott, J., Stevens, M., Barten, M., Thorne, R., Polston, J., Schuster D., Serra, C., (1995). Introgression of resistance to whitefly-transmitted geminiviruses from *Lycopersicon chilense* to tomato. In Gerling, D., and Mayer, T., (Eds). Taxonomy, Biology, Damage control and management (p357-367). Andover, United Kingdom: Intercept Ltd.

Scott, J., W. (2001). Geminivirus resistance derived from *Lycopersicum chilense* accessions LA1932,

LA1938, and LA2779. Tomato Breeders Round Table. Retrieved from www.oardc.ohio-state.edu/tomato/TBRT%202001%20Abstracts.pdf.

Scott, J, (2005) Perspectives on tomato disease resistance breeding: past, present, and future. Acta Horticulturae (ISHS) 695: 217- 224.

Seal, S. E., van den Bosch, F., Jeger, M. J., (2006) Factors influencing begomovirus evolution and their increasing global significance: implications for sustainable control Critical Reviews in Plant Sciences 25: 23- 46.

Sean, S., Telleen, A. C., Williamson, V. M., (2007). Introgressed and endogenous Mi-1 gene clusters in tomato differ by complex rearrangements in flanking sequences and show sequence exchange and diversifying selection among homologues. Theory of Applied Genetics 114: 1289- 1302.

Servin, B., Martin, O. C., M´ezard, M., Hospital, F., (2004). Towards a theory of marker-assisted gene pyramiding. Genetics 168: 513– 523.

Simone, G. W., Brown, J. K., Hiebert, E., Cullen, R. E., (1990). New geminivirus epidemic in Florida tomatoes and peppers. Phytopathology 80: 1063.

Sinozaki, K., Shinozaki, Y. (1997). Gene expression and signal transduction in water-stress response. Plant Physiology 115: 327- 334.

Smit, G., and Parlevliet, J., (1990). Mature plant resistance of barley to barely leaf rust, another type of resistance. Euphytica 50: 159– 162.

Smith, P., (1994). Embryo culture of a tomato species hybrid. American Society for Horticultural Science 44: 414- 416.

Soler, S., Jose Diez, M., Nuez, F., (1998). Effect of temperature regime and growth stage interaction on pattern of virus presence in TSWV-resistance accessions of *Capsicum chinense*. Plant Disease 82: 1199– 1204.

Spassova, M., Prins, T., Folkertsma, R., Klein-Lankhorst, R., Hille, J., Goldbach, R., Prins, M., (2001). Tomato gene *Sw-5* is a member of the coiled coil, nucleotide binding, leucine rich repeat class of plant resistance genes and confers resistance to TSWV in tobacco. Molecular Breeding 7: 151- 161.

Stanley, J., (1995). Analysis of *African cassava mosaic virus* recombinants suggests strand nicking occurs within the conserved nanonucleotide motif during the initiation of rolling circle DNA replication. Virology 190: 506- 509.

Stanley, J., Bisaro, D. M., Briddon, R. W Brown, J., K., Fauquet, C. M., Harrison, B. D., Rybicki, E. P., Stenger, D. C., (2005). In Fauquet, C., M., Mayo, M., A., Maniloff, J., Desselberg, U., Ball, L., A., (Eds). VIIIth Report of the International Committee on Taxonomy of Viruses. *Virus Taxonomy* (p301- 326). London, United Kingdom: Elsevier/Academic Press.

Stevens, M. A., and Rick, C. M., (1986). Genetics and breeding in the tomato crop: A Scientific basis for Improvement. In Rudich, J., and Atherton, J., G. (Ed). The tomato crop: a scientific basis for improvement (p35- 109). The Netherlands: Springer.

Stevens, M., and Rick, C., (1988). Genetics and breeding. In Atherton, J., and Rudich, J., (Eds). The tomato crop (p35- 109). London, United Kingdom: Chapmean and Hall.

Stevens, M., Scott, S., Gergerich, R., (1992). Inheritance of a gene for resistance to *Tomato spotted wilt virus* (TSWV) from L*ycopersicon peruvianum*. Euphytica 59: 9- 17.

Stevens, M., Scott, S., Gergerich, R., (1994). Evaluation of seven *Lycopersicon* species for resistance to *Tomato spotted wilt virus* (TSWV). Euphytica 80: 79- 84.

Tal, M., and Shannon, M. C., (1983). Salt tolerance in two wild relatives of the cultivated tomato: responses of *Lycopersicon esculentum*, *L. cheesmanii*, *L. peruvianum*, *Solanum pennelli*, and F1 hybrids of high salinity. Australian Journal of Plant Physiology 10: 109– 117.

Tanksley, S., Young, N., Paterson, A., Bonierbale, M., (1989). RFLP mapping in plant breeding: new tools for an old science. Biotechnology 7: 257- 264.

Tanksley, S., (1993). Mapping polygenes. Annual Reviews of Genetics 27**:** 205– 233.

Thomas, C. M., Jones, D. A., Parniske, M., Harrison, K., Balint-Kurti, P. J., Hatzixanthis, K., Jones, J. D. G., (1997). Characterization of the tomato *Cf-4* gene for resistance to *Cladosporium fulvum* identifies sequences that determine recognitional specificity in *Cf-4* and *Cf-9*. Plant Cell 9: 2209- 2224.

Tigchelaar, E. C., (1986). Tomato breeding. In Bassett, M., (Ed). Breeding for Vegetable Crops (p135- 171). Connecticut, USA: AVI Westport.

Ucko, O., Cohen, S., Ben-Joseph, R., (1998). Prevention of virus epidemics by a crop-free period in the Arava region of Israel. Phytoparasitica 26: 313– 321.

Urbino, C., Thebaud, G., Granier, M., Blanc, S., Peterschmitt, M., (2008). A novel cloning strategy for isolating, genotyping and phenotyping genetic variants of geminiviruses. Virology 5: 135.

Van der Beek, G., Pet, G., and Lindhout, P., (1994). Resistance to powdery mildew (*Oidium lycopersicum*) in *Lycopersicon hirsutum* is controlled by an incompletely-dominant gene *Ol-1* on chromosome 6. Theory of Applied Genetics 89: 467- 473.

Van der Vlugt, R., Stijger, C., Verhoeven, J., Verhoeven, J., Lesemann, D., (2000). First report of *Pepino mosaic virus* on tomato. Plant Disease 84: 103.

Van Larebeke, N., Engler, G., Holsters, M., van den Elsacker, S., Zaenen, I., Schilperoort, R., Schell, J., (1974). Large plasmid in *Agrobacterium tumefaciencs* essential for crown gall inducing ability. Nature 252: 169- 170.

Van Loon, and L., Pieterse, C., (2006). Significance of inducible defence related proteins in infected plants. Annual Reviews of Phytopathology 44: 135- 162.

Vanderschuren, H., Stupak, M., Futterer, J., Gruissem, W., Zhang, P., (2007). Engineering resistance to geminiviruses – review and perspectives. Plant Biotechnology Journal 5: 207- 220.

Varma, A., and Malathi, V., (2003). Emerging geminivirus problems: a serious threat to crop production. Annals of Applied Biology 142: 145- 164.

Vidavsky, F., and Czosnek, H., (1998). Tomato breeding lines immune and tolerant to *Tomato yellow leaf curl virus* (TYLCV) issued from *Lycopersicon hirsutum*. Phytopathology 88: 910- 914.

Voet, D., Voet, J., Pratt, C., (2002). Fundamentals of Biochemistry, Upgrade Edition (p540- 548). USA: John Wiley and Sons, Inc.

Wang, J. F., Olivier, J., Thoquet, P., Mangin, B., Sauviac, L., Grimsley, N. H., (2000). Resistance of tomato line Hawaii7996 to *Ralstonia solanacearum* Pss4 in Taiwan is controlled mainly by a major strain-specific locus. Molecular and Plant-Microbe Interactions 13: 6- 13.

Wang, W., Vinocur, B., Shoseyov, O., Altman, A., (2004). Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends in Plant Science 9: 244- 252.

Xie, Y., and Zhou, X.-P., (2003). Molecular characterization of *Squash leaf curl Yunnan virus*, a new begomovirus and evidence for recombination. Archives of Virology 148: 2047– 2054.

Yang, W., and Francis, D. M., (2005). Marker-assisted selection for combining resistance to bacterial spot and bacterial speck in tomato. Journal of the American Society for Horticultural Science 130: 716- 721.

Young, N., (1996). QTL mapping and quantitative disease resistance in plants. Annual Review of Phytopathology 34: 479– 501.

Zakay, Y., Navot, N., Zeidan, M., Kedar, N., Rabinowitch, H. D., Czosnek, H., Zamir D., (1991) Screening *Lycopersicon* accessions for resistance to the *Tomato yellow leaf curl virus*: presence of viral DNA and symptom development. Plant Disease 75: 279- 281.

Zamir, D., Michelson, I. E., Zakay, Y., Navot, N., Zeidan, M., Sarfatti, M., Eshed, Y., Harel, E., Pleban, T., van Oss, H., Kedar, N., Rabinowitz, H. D., Czosnek, H., (1994). Mapping and introgression of a *Tomato yellow leaf curl virus* tolerance gene, 5-1. Theory and Applied Genetics 88: 141- 146.

Zhang, W., Olson, N., Baker, T., Faulkner, L., McKenna, M., Boulton, M., Davies, J., McKenna, R., (2001). Structure of the *Maize streak virus* geminate particle. Virology 279: 471- 477.

Zhang, S., and Klessig, D., (2000). Pathogen-induced MAPkinases in tobacco. Results and Problems in Cell Differentiation 27: 65- 84.

Zhou, X., Liu, Y., Calvert, L., Munoz, D., Otim-Nape, G. W., Robinson, D. J., Harrison, B. D., (1997). Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. Journal of General Virology 78: 2101– 2111.

APPENDIX

One way ANOVA for infection trials two to five

A one way ANOVA (Analysis of Variance) was carried out four each trial. All the tests were performed with 5% significant level in SPSS (Statistical Package for Social Sciences) version 13.

Table A1: A one way ANOVA of infection trial two.

ANOVA Trial two					
	Sum of Squares	df	Mean Square	F	P-value
Between Groups	5.460	3	1.820	2.147	0.096
Within Groups	166.120	196	0.848		
Total	171.580	199			

Table A2: A one way ANOVA of infection trial three.

ANOVA Trial three					
	Sum of Squares	df	Mean Square	F	P-value
Between Groups	8.095	2	4.047	7.490	0.001
Within Groups	176.693	327	0.540		
Total	184.788	329			

Table A3: A one way ANOVA of infection trial four.

ANOVA Trial four					
	Sum of Squares	df	Mean Square	F	P-value
Between Groups	1.217	2	0.608	1.720	0.184
Within Groups	41.375	117	0.354		
Total	42.592	119			

Table A4: A one way ANOVA of infection trial five (TAM x Rooikhaki F1 hybrid)

ANOVA					
	Sum of Squares	df	Mean Square	F	P-value
Between Groups	3.900	2	1.950	3.900	0.026
Within Groups	28.500	57	0.500		
Total	32.400	59			

Table A5: A one way ANOVA of infection trial five (TAM x Rooikhaki F2 hybrid).

ANOVA					
	Sum of Squares	df	Mean Square	F	P-value
Between Groups	2.800	2	1.400	3.318	0.053
Within Groups	24.050	57	0.422		
Total	26.850	59			