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Bethlehem University Faculty of Science

# **Biotechnology Master Program**

# Collapse of Resistance to Tomato Yellow Leaf Curl Virus in Tomato

# upon Silencing the *Elongation factor 1-alpha* Gene

By

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In Partial Fulfillment of the Requirements for the Degree

Master of Science

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The undersigned hereby certify that they have read and recommend to the Faculty of Science at Bethlehem University and the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University for acceptance a thesis entitled:

# Collapse of Resistance to *Tomato Yellow Leaf Curl Virus* in Tomato upon Silencing the *Elongation factor 1-alpha* Gene

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology

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# Collapse of Resistance to *Tomato Yellow Leaf Curl Virus* in Tomato upon Silencing the *Elongation factor 1-alpha* Gene

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#### ABSTRACT

*Tomato yellow leaf curl virus* (TYLCV) is a geminivirus belongs to the genus *Begomovirus* from *Geminiviridae* family, which is the causal agent of tomato yellow leaf curl disease (TYLCD); the most devastating viral disease attacking tomato worldwide. Whitefly *Bemisia tabaci* is the vector of TYLCV, transmitting the virus in a circulative persistent manner. However TYLCD is a major threat and an international problem for tomato production. Its management still problematically expensive and with limited options.

In order to determine genes involved in tomato resistance to *Tomato yellow leaf curl virus* (TYLCV), previous scientific studies compared cDNA libraries from susceptible (S) and resistant (R) tomato lines from the same breeding program. Hypothesizing that the genes preferentially expressed in R line are possibly part of the network(s) sustaining resistance to TYLCV. Among the genes preferentially expressed in R plants and over expressed following TYLCV infection was the *Elongation factor 1-alpha* gene which encodes the translational Elongation factor 1-alpha protein. The encoded protein responsible for the GTP-dependent binding of aminoacyl-tRNAs to ribosomes during translation.

In this study, we followed a reverse-genetics approach in our attempt to identify genes involved in TYLCV resistance. We applied the most flexible system in this area of gene functionality research, which is the virus-induced gene silencing (VIGS). Silencing the *Elongation factor 1-alpha* gene in R plants using the *Tobacco Rattle Virus* (TRV) vector, then inoculating these R plants with TYLCV outcomes in resistance collapse. The collapse of resistance was evaluated through

monitoring the development of the typical disease symptoms as in the case of infected susceptible plants. As well as the accumulation and spread of the virus which measured quantitatively by PCR. Therefore, this study demonstrated the key role of the *Elongation factor 1-alpha* gene in sustaining the resistance against TYLCV, most probably by inhibiting virus replication and/or movement.

# انحدار المقاومة لمرض اصفرار و تجعد القمم النامية في الطماطم بعد إسكات ألجين Elongation factor 1-alpha

### ملخص

فيروس مرض اصفرار و تجعد القمم النامية في الطماطم من عائلة الفيروسات التوأمية، هو المسبب لمرض اصفرار و تجعد القمم النامية في الطماطم و الذي ينجم عنه خسائر فادحة و يعتبر اشد أمراض الطماطم الفيروسية فتكا و شراسة. الذبابة البيضاء هي الناقل لهذا الفيروس، حيث تقوم بنقله من نبات لآخر بأسلوب الدوري المستمر. على الرغم من أهمية هذا المرض كمصدر تهديد لمحصول الطماطم واعتباره مشكلة اقتصادية على المستوى العالمي، فإن إدارة المرض ما تزال غير مجدية عمليا من حيث التكلفة أو الخيارات المتاحة و التي تنحصر بالمكافحة الكيماوية للناقل و عزل المحصول عن الناقل بوسائل ميكانيكية.

تعتبر تربية سلاسل النسب المقاومة و المتحملة لهذا المرض من أنجع سبل المكافحة المتوفرة. البنية الجينية لهذه السلاسل تحوي الجينات المانحة لصفة المقاومة و التي لم تحدد من قبل. فهذا البحث يشكل جزء من مجهود علمي كبير يرنو لتحقيق هدف معرفة الجينات المكونة لصفة المقاومة لهذا المرض في سلاسل النسب الحاملة لهذه الصفة. من خلال مقارنة التعبيرات الجينية المختلفة و التي تتغير أي يحدث لها زيادة في التعبير الجيني- بعد الإصابة بالمرض و ذلك ضمن مجهود علمي سل سل نتيجة لتلك الدر اسات السابقة فقد تم تحديد الجينات التي زاد تعبير ها الجيني بعد الإصابة بالفيروس. و من ضمن تلك الجينات كان ألجين المبحوث في هذه الدر اسة و المسمى Elongation factor1-alpha.

هذا ألجين يعطي بروتين يحمل نفس الاسم و هو بروتين هام و حيوي في عملية الترجمة من جين إلى بروتين. خلال هذه الدراسة تم إتباع أسلوب الوراثة الرجعية في معرفة وظيفة و أهمية ألجين، بحيث يتم إسكات ألجين و من ثم النظر في ما ينتج عن ذلك الإسكات في ما يخص الوظيفة المفترضة لذلك ألجين على مستوى الخلية و الكائن الحي و هو النبات في هذه الدراسة. من بين الأنظمة المتبعة في أسلوب الوراثة الرجعية، تم اختيار نظام الإسكات المُستحث بواسطة الفيروس و الذي يوظف فيروس التبغ المُجلجل للقيام بالمهمة. بعد القيام بعملية الإسكات وفق النظام المذكور و من ثم تلقيح النبات بالفيروس نتج عن ذلك انحدار لصفة المقاومة في تلك النباتات و الذي تمثل في ظهور واضح للأعراض المعيارية للمرض كالتي تظهر في حالة سلاسل نسب الطماطم الحساسة للمرض و تعاظم التراكم و الانتشار للفيروس في النباتات المصابة. بناءا على ذلك، يتضح جليا الدور المحوري لهذا ألجين في إحداث و استدامة صفة المقاومة لما المربح و من تم تلقيح النباتات بالفيروس أن هذا الدور المحوري لهذا ألجين في إحداث و الذي تمثل في ظهور واضح للأعراض المعيارية المرض كالتي تظهر أن هذا الدور المحوري لهذا ألجين في إحداث و استدامة صفة المقاومة لمالاسل النسب الحاملة لهذه الصفة، و من المرجح

# DECLARATION

I declare that the Master Thesis entitled:

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is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

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# DEDICATION

То

my lovely daughter

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# LIST OF ABBREVIATIONS

| ABBREVIATION | DESCRIPTION                                   |  |
|--------------|---|--|
| %            | Percentage                                    |  |
| aa-tRNA      | aminoacyl-tRNAs                               |  |
| ALSV         | Apple latent spherical virus                  |  |
| BLAST        | Basic Local Alignment Search Tool             |  |
| bp           | base pair                                     |  |
| C            | Complementary sense orientation               |  |
| cDNA         | complementary Deoxyribonucleic Acid           |  |
| СР           | Capsid Protein                                |  |
| Ct           | threshold cycle                               |  |
| °C           | Celsius degree centigrade                     |  |
| DCL          | Dicer-like enzyme                             |  |
| DNA          | Deoxyribonucleic Acid                         |  |
| dpi          | days post inoculation                         |  |
| dsRNA        | double-stranded RNA                           |  |
| EDTA         | Ethzlene diamine tetra-acetic acid            |  |
| EF1a         | Elongation Factor 1-alpha                     |  |
| EMSA         | Electrophoretic Mobility Shift Assay          |  |
| ER           | Endoplasmic Reticulum                         |  |
| et al.       | et alii (and others)                          |  |
| Fig          | Figure  |  |
| GO           | Gene Ontology                                 |  |
| Hsp          | Heat shock protein                            |  |
| IR           | Intergenic Region                             |  |
| kDa          | kilo Dalton                                   |  |
| Mb           | Mega base pair                                |  |
| MCS          | Multiple Cloning Site                         |  |
| mg           | milligram                                     |  |
| miRNA        | microRNA                                      |  |
| ml           | milliliter                                    |  |
| μl           | microliter                                    |  |
| mRNA         | messenger RNA                                 |  |
| NCBI         | National Center for Biotechnology Information |  |
| ng           | nanogram                                      |  |
| NLS          | Nuclear Localization Sequence                 |  |
| nm           | nanometer                                     |  |
| nt           | nucleotide                                    |  |
| OD           | Optical Density                               |  |

| ORF        | Open Reading Frame                         |  |
|------------|--|--|
| PCR        | Polymerase Chain Reaction                  |  |
| PTGS       | Post Transcriptional Gene Silencing        |  |
| PTM        | Post-Translational Modification            |  |
| PVX        | Potato virus X                             |  |
| qRT-PCR    | quantitative Real Time-PCR                 |  |
| R          | Resistant                                  |  |
| RdRP       | RNA dependent RNA Polymerase               |  |
| REn        | Replication Enhancer protein               |  |
| Rep        | Replication associated protein             |  |
| RISC       | RNA-Induced Silencing Complex              |  |
| RNA        | Ribonucleic acid                           |  |
| rpm        | Round per minute                           |  |
| RT-PCR     | Real Time-PCR                              |  |
| S          | Susceptible                                |  |
| siRNA      | short interfering RNA                      |  |
| SNP        | Single Nucleotide Polymorphism             |  |
| SOL        | International Solanaceae Genome Project    |  |
| ssDNA      | single-stranded DNA                        |  |
| ssRNA      | single-stranded RNA                        |  |
| TBE buffer | Tris/Borate/EDTA buffer                    |  |
| ТЕМР       | Temperature                                |  |
| TGMS       | Tomato golden mosaic virus                 |  |
| TGS        | Transcriptional Gene Silencing             |  |
| TMV        | Tobacco mosaic virus                       |  |
| TrAP       | Transcription Activator Protein            |  |
| tRNA       | transfer RNA                               |  |
| TRV        | Tobacco rattle virus                       |  |
| TYLCCNV    | Tomato yellow leaf curl China virus        |  |
| TYLCD      | Tomato yellow leaf curl disease            |  |
| TYLCSV     | Tomato yellow leaf curl Sardinia virus     |  |
| TYLCV      | Tomato yellow leaf curl virus              |  |
| UBA1       | Ubiquitin-activating gene                  |  |
| V          | Virion sense orientation                   |  |
| VIGS       | Virus-Induced Gene Silencing               |  |
| X-gal      | 5-Bromo-4-chloro-3-indolyl-ß-D-galactoside |  |

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#### **CHAPTER ONE**

# **INTRODUCTION**

#### 1.1 Tomato Yellow Leaf Curl Virus (TYLCV)

*Tomato yellow leaf curl virus* (TYLCV) is a geminivirus belongs to the genus *Begomovirus* from *Geminiviridae* family. *Geminiviridae* consists the largest family of plant DNA viruses which is characterized by the circular single stranded DNA genomes that encapsidated in twinned quasi isometric particles. *Begomovirus* is the largest genus among the four genera of *Geminiviridae*, whereas these genera are classified according to their genomes organization and hosts range (Hanssen et al., 2010). TYLCV is the causal agent of Tomato Yellow Leaf Curl Disease (TYLCD), which is the most devastating viral disease afflicting tomato worldwide including Palestine and most countries of the Middle East (Hanssen et al., 2010). Whenever TYLCD affects young plants, they might produce few -if any marketable- fruits while losses can reach up to 100% of the potential production. Although TYLCV was first identified in 1961, historical records reveal that a disease with symptoms similar to TYLCD in the tomato fields was already reported in mandatory Palestine since 1930s. Indeed, tomato production in the Middle East has been harshly affected from the 1970s to date (Ghanim & Czosnek, 2000; Hanssen et al., 2010).

Although TYLCV can exhibit distinct symptoms in tomato, it can also establish symptomless infections in both wild and cultivated species, however the host in both cases can serve as a virus reservoir. Therefore, whiteflies still able to acquire and transmit the virus from the infected nonsymptomatic plants despite lacking TYLCV-induced disease symptoms (Czosnek & Ghanim, 2012). TYLCD management rely mainly on controlling whitefly -which is the insect vector of TYLCV- either chemically by using pesticides

and/or physically by isolating the production atmosphere with mesh fences (Akad et al., 2007). Additionally, TYLCV resistant tomato lines have been developed in several countries over the last two decades, usually through introgression breeding programs from wild species of the *Solanum* Genus (Castro et al., 2012). However, resistant lines are often insufficient commercially due to a linkage with poor fruits quality. Likewise, the tolerant commercial cultivars often collapse under severe infection pressure and require protection during early growth stages. Moreover, even if these cultivars tolerated the infection and produced yield along with the TYLCV presence, they still support the replication of TYLCV and can act as a reservoir of TYLCV against other susceptible crops (Shepherd et al., 2009; Castro et al., 2012).

#### 1.1.1Genome Organization of TYLCV

TYLCV possess a ssDNA genome of about 2.7 kb in size, which encodes six partially overlapping open reading frames (ORFs) with bidirectional organization as in figure1.1. Two of these ORFs (V1 and V2) are in the virion sense orientation. V1 encodes the Capsid Protein (CP), which is involved, as in other geminiviruses, in a number of processes during the life cycle of the virus (Harrison et al., 2002; Glick et al., 2009). While its primary function is the encapsidation of ssDNA and forming virus particles to protect the viral DNA during transmission by the insect vector (Morilla et al., 2006). Interestingly, point mutations in TYLCV CP cause loss of infectivity or loss of whitefly transmissibility (Luna et al., 2011). On the same strand, V2 encodes a distinct motion-related protein, which is involved in cell-to-cell viral movement and spread, for that V2 is considered as a pathogenicity gene (Harrison et al., 2002; Morilla et al., 2006; Glick et al., 2009).

**Fig 1.1: TYLCV genome organization**. The open reading frames are designated V (virion sense orientation) or C (complementary sense orientation) in addition to the intergenic region.

Source: (Glick et al., 2009).



The other four ORFs (C1-C4) are in the complementary sense orientation; C1 encodes a replication associated protein (Rep) which is the essential protein required for viral DNA replication, as it initiate and terminate plus-strand replication through specific binding to a DNA sequence motive located in the intergenic region (IR) during origin-of-replication recognition (Glick et al., 2009). C2 encodes a transcription activator protein (TrAP) which functions as a suppressor of post transcriptional gene silencing (PTGS) in the host plant cells, hence it is considered as a pathogenicity gene (Morilla et al., 2006). C3 encodes a replication enhancer protein (REn) which is required for the efficient viral DNA replication and it also enhances viral DNA accumulation for approximately 50 folds (Morilla et al., 2006; Glick et al., 2009). The fourth gene C4 encodes C4 protein (also known as AC4) which consist of a small ORF located entirely within the Rep ORF but in a different reading frame. C4 protein is considered as a pathogenicity gene, as it together with C3 (TrAP) protein play an important role in the infection process through post transcriptional gene silencing (PTGS) suppression and the induction of necrosis in the host plant cells (Morilla et al., 2006; Luna et al., 2011). Between the two transcriptional units, there is an intergenic region (IR) of approximately 300-nt length, which contains key elements for replication and transcription of the viral genome. These key elements comprise the origin of replication and viral promoters (Morilla et al., 2006; Briddon et al., 2010).

# **1.1.2 Virion Structure**

TYLCV is characterized by its unique capsid morphology, which consists of two geminate (twinned) incomplete icosahedral particles as shown in figure 1.2. The particles are approximately 38 nm in length and 22 nm in diameter. The capsid contains 22 pentameric capsomers made of 110 capsid proteins. Each capsid protein is made of 260 amino acids (Harrison et al., 2002).



Bar = 100 nm

# 1.1.3 TYLCV Replication

Replication of TYLCV, like all geminiviruses, occurs in the nuclei of infected cells through using a combination of a rolling circle mechanism and recombination mediated replication (Ivanov & Ma, 2012). Since geminiviruses do not encode their own DNA polymerases, they rely on the nuclear DNA replication machinery in a similar approach to some mammalian DNA tumor viruses (Morilla et al., 2006). Geminivirus infection starts by inducing the host DNA replication machinery by activating host genes that are required for DNA replication (Glick et al., 2009). Numerous interactions between geminiviruses Rep protein and plant cellular proteins have been identified by biochemical (affinity chromatography) or genetic (yeast two-hybrid system) assays. These interactions involve

mainly with phosphorylating the host DNA replication machinery proteins in order to accomplish the transition of the cell to the synthesis phase during geminiviruses infection development (Boulton, 2002; Ivanov & Ma, 2012). As both replication and transcription occur in the nucleus, importation of the viral DNA or virions into and out the nucleus of the host cell is essential for successful completion of the virus life cycle (Harrison et al., 2002; Morilla et al., 2006).

#### 1.1.4 TYLCV Nuclear, Cellular and Systemic Movement

Since TYLCV enters the host cell without encoded viral proteins, just with its genome and the CP. Thus, movement to the nucleus -where TYLCVs, like all other geminiviruses, transcribe and replicate their genome- must for that reason be entirely dependent on the CP and the host transport mechanisms (Harrison et al., 2002). Several substantial experiments have provided insight into the mechanism by which the CP may function in the intracellular movement of the TYLCV genome (Boulton et al., 2002; Morilla et al., 2006; García-Andrés et al., 2009). These experiments localized the TYLCV CP into the nuclei of host cells and shown that the transport of the TYLCV CP into the nuclei was an active, energy-dependent process. Other experiments showed that a Nuclear Localization Sequence (NLS) resides in the TYLCV CP can facilitate nuclear import (Lapidot et al., 2007). Moreover, recent studies confirmed the karyophilic nature of the TYLCV CP, which suggest that TYLCV CP would interact with karyopherin  $\alpha$  (a protein that serves as a nuclear shuttle for NLS-bearing proteins) through its NLS (Yaakov et al., 2011). Furthermore, this interaction of a tomato karyopherin  $\alpha$  with TYLCV CP was verified in a yeast two-hybrid system study. The results indicated that a tomato karyopherin  $\alpha$  which specifically interacts with CP, most likely mediating its nuclear import by a karyopherin αdependent mechanism (Yaakov et al., 2011; Ivanov & Ma, 2012). Another requirement for

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the TYLCV CP to function as a nuclear shuttle protein for the viral genome is the ssDNAbinding ability, which was certainly demonstrated by the Electrophoretic Mobility Shift Assays (EMSA). The EMSA assay can be used to detect protein interactions with DNA and can be qualitatively used to identify sequence-specific DNA-binding proteins (García-Andrés et al., 2009; Yaakov et al., 2011).

Upon entry into the nucleus, TYLCV replicates, producing both single-stranded and double-stranded forms of the viral genome. Once viral DNA has begun to replicate in the nucleus, the newly synthesized CP will carry out two distinct functions: the encapsidation of ssDNA into virions, and the nuclear export of the infectious form of the virus (Ivanov & Ma, 2012). However, to move from cell to cell, the virus must be able to leave the host cell nucleus and be transported to the plasmodesmata, and through them to adjacent cells to repeat the infection cycle (Harrison et al., 2002).

A functional analysis to characterize the proteins involved in the intracellular movement of TYLCV to the cell periphery in order to be transported to adjacent cells, demonstrated that the CP together with two adjuvant proteins are involved in that process of viral DNA delivery either as virions or as nucleoprotein complexes (Gafni & Epel, 2002; Yaakov et al., 2011). Then, the virus must overcome the cell wall to reach the stage of cell-to-cell movement. Accordingly, the virus encodes movement protein (MP) which biochemically mediate with plasmodesmata to facilitate the transport process (Ivanov & Ma, 2012). Building on these concrete and recently accumulated data, Gafni and Epel (2002) suggested a model for geminiviruses (including TYLCV) intra- and intercellular movement, in which the movement of geminiviruses is strictly and inevitably dependent on the CP (Gafni & Epel, 2002).

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#### 1.1.5 Vector of TYLCV

Whitefly *Bemisia tabaci* is the vector of TYLCV, which has a wide host range and feeds by sucking plant sap from leaves undersurface. Whitefly transmits TYLCV to tomato in a circulative persistent manner; routing to the salivary gland from the digestive track through hemolymph (Ghanim & Czosnek, 2000). Usually, whitefly can acquire the virus after feeding on infected plants for 15 to 30 minutes, and can transmit the virus after 24 hours of incubation. Viruliferous whiteflies retain the virus till adults life end and can transmit the virus to its progeny for two generations (Czosnek & Ghanim, 2012). More interesting, TYLCV can be transmitted from one whitefly to another in a sex-dependent manner, which leads to an increase in the virus inoculation-potential of a certain whitefly population (Ghanim & Czosnek, 2000; Hanssen et al., 2010).

#### **1.2 Breeding Efforts to Generate TYLCV Resistant Tomato Lines**

Planting TYLCV resistant or tolerant tomato lines is an achievable and environmentally reasonable strategy when combined with chemical and physical control methods in an integrative management of the disease. Hence, breeding for TYLCV resistance probably remained the most important long-lasting TYLCV remedy. A controversial issue deserves mentioning here, is the consensus definition of a TYLCV resistant line, since most commercial lines conceived as resistant are tolerant lines indeed (Glick et al., 2009). In essence, a certain line can be named resistant to TYLCV if it can suppress its multiplication and accumulation as well as the development of disease symptoms with infinitesimal effect of the infection on the total yield quantitatively and qualitatively. While tolerant lines can exhibit nonsymptomatic appearance with negligible productivity reduction, but with similar levels of TYLCV multiplication to the susceptible lines (Castro et al., 2012).

On the practical side, screening procedures for TYLCV resistance is necessary for all breeding programs aimed at developing tomato lines resistant to TYLCV. On the same side, selecting plants solely on the basis of the presence or absence of symptoms in infected fields, without taking into account the time of inoculation and the levels of viral inoculums would lead to substantially biased and non-standardized lines (Castro et al., 2012). Finally, it is not a mysterious revelation that the resistance genes will consist a prospective powerhouse in controlling TYLCV, essentially when those genes are identified (Anand et al., 2012).

# **1.2.1 Classical Breeding for Resistance**

Since early 1970s, classical breeding programs have attempted to introduce TYLCV genetic resistance or tolerance traits against TYLCV into the cultivars of domesticated tomato *Solanum lycopersicum* from wild *Solanum* species, (Glick et al., 2009). Through introgression of those traits, chromosomal fragments from the wild species have been loaded onto the commercial lines. Consequently, these chromosomal fragments can be identified with certain polymorphic DNA markers (Vidavski et al., 2008; Castro et al., 2012). As a result, five major loci (Ty1-Ty5) from wild tomato species, which associated with resistance or tolerance to TYLCV have been identified (Vidavski et al., 2008). Regardless of the hard efforts of different research groups to develop TYLCV resistant lines, the available lines still develop symptoms and yield losses under the conditions of high inoculum pressure and early infections (Castro et al., 2012). Therefore, research groups should incorporate more of the different available genetic sources into their TYLCV resistance breeding programs.

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#### **1.2.2 Genetic Engineering Resistance**

Recently, several strategies and techniques have been implemented to engineer TYLCV resistant lines. Most of these strategies and techniques based on the concept of introducing and expressing viral sequences in the host plants in order to interfere with the virus life cycle, which is also called the concept of pathogen-derived resistance (Yang et al., 2004). For instance, the post-transcriptional gene silencing (PTGS) against TYLCV was induced as a result of many attempts for transforming tomato plants with constructs that express either sense or antisense RNA from the TYLCV replication-associated (Rep) gene sequences (Russo & Slack, 1998; Yang et al., 2004). On the other hand, the vector-virus interrelations have been exploited to generate TYLCV resistant lines. For instance, transforming tomato plants to express the whitefly protein GroEL in their phloem would trap the virus since TYLCV avoids destruction in the hemolymph of whitefly by interacting with GroEL. Upon that, TYLCV particles will be trapped in the plants phloem by GroEL i.e. inhibiting virus replication and movement, thus rendering the plants resistant (Akad et al., 2007). Therefore, generating transgenic tomato plants appears to be a more promising way of obtaining resistance to TYLCV, though still confronting regulationrestrictions in numerous countries (Mehrotra & Goyal, 2013).

#### **1.3 Gene Silencing in Plants**

In general, gene silencing in plants can occur at both transcriptional and posttranscriptional levels (Vanitharani et al., 2005). Silencing through transcription suppression is called transcriptional gene silencing (TGS), while silencing through mRNA degradation is called post-transcriptional gene silencing (PTGS) (Vaucheret & Fagard, 2001). Initially, TGS is related to the regulation of transposons through DNA methylation in the nucleus, whereas PTGS was shown to encounter virus infections through double-stranded RNA in the cytoplasm (Vaucheret & Fagard, 2001; Alvarado & Scholthof, 2009).

At the beginning, the mechanism of TGS was originally thought to be the DNA methylation of promoter sequences which either suppresses the promoter through blocking its fundamental interactions with the transcription factors, or through attracting the chromatin-remodeling-proteins; which in turn could lead to the heterochromatinization of promoter sequences (Vaucheret & Fagard, 2001; Chellappan et al., 2004; Vanitharani et al., 2005). Recently, numerous studies reported that TGS like PTGS, can be triggered by either dsRNA or viruses and then finish without changing the methylation pattern of the silenced gene (Raja et al., 2010; Garcia-ruiz et al., 2010; Bai et al., 2012). Lately, upon piles of evidence, TGS and PTGS were not considered as entirely separate pathways. Since major breakthroughs in the attempt to discriminate between TGS and PTGS established the finding that viruses and transgenes encoding certain dsRNAs which can induce either TGS or PTGS of a homologous transgene (Dalmay et al., 2000; Vaucheret & Fagard, 2001; Raja et al., 2012).

#### **1.3.1 Post Transcriptional Gene Silencing (PTGS)**

RNA silencing phenomenon was first discovered in plants and termed post-transcriptional gene silencing (PTGS), while designated different terms in other eukaryotic kingdoms (Wu, 2013). Generally, RNA silencing involves suppression of the gene expression by sequence-specific interactions with RNA at the post-transcriptional level in diverse eukaryotes (Vanitharani et al., 2005). In plants, post-transcriptional gene silencing (PTGS) acts as a natural antiviral defense system and plays several roles in either genome

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preservation or development (Alvarado & Scholthof, 2009). During the past decade, there was considerable evidence of PTGS suppression by various viruses as a prerequisite for establishing a viral infection in plants (Hohn & Vazquez, 2011). However, viruses which have no double-stranded RNA phase in their replication cycle (in particular, the nuclear-replicating geminiviruses including TYLCV) are able to induce and suppress the PTGS and become targets for PTGS (Alvarado & Scholthof, 2009).

There are at least two different pathways of the RNA gene silencing in plants: cytoplasmic short interfering (siRNA) silencing and the silencing of endogenous mRNA by microRNAs (miRNA) (Brodersen & Voinnet, 2006). Though both siRNA and miRNA are processed inside the cell through the cleavage of long double-stranded RNAs into small RNAs by a ribonuclease-III enzyme called Dicer, then these small RNAs are incorporated into a complex called RNA-induced silencing complex (RISC) (Jiang et al., 2011; Bratkovič et al., 2012). miRNAs are endogenous, non-coding RNAs with a 18-25 nucleotide length, exist both in plants and in animals (Voinnet, 2009). These small RNA fragments work as the specificity determinant by being incorporated into the RISC endonuclease, which degrades mRNAs in a sequence-specific manner or inhibits protein translation (Voinnet, 2009). On the other side, siRNAs are exogenous double-stranded RNA that is taken up by cells or entering by means of vectors like viruses, with a 21–26 nucleotide length (Brodersen & Voinnet, 2006). Four Dicer-like (DCL) enzymes have been identified in the model plants with distinct functions. DCL1 is involved in miRNA biogenesis, DCL2 has been connected with viral siRNA production and DCL3 is required for retroelement and transposon siRNA production as well as chromatin silencing (Shimura & Pantaleo, 2011; Mccue & Slotkin, 2012). The function of DCL4 is apparently related to siRNA production and some other processes, but not yet verified (Vanitharani et al., 2005; Brodersen & Voinnet, 2006).

Within the context of this thesis, I will focus on the ability of geminiviruses to trigger and suppress the induced PTGS in order to replicate and induce disease symptoms in the infected plants (Raja et al., 2010). Paradoxically, geminiviruses can equally stimulate and turn into targets of gene silencing. More-detailed analysis regarding siRNA is initiated by dsRNAs which could be viral replication intermediates or ssRNAs that became dsRNA by host-encoded RNA-dependent RNA polymerase (RdRP) (Preuss et al., 2008; Mccue & Slotkin, 2012).

### 1.3.1.1 Virus-derived siRNA as an Antivirus Defense Mechanism in Plants

In plants, some virus-host interactions naturally lead to host recovery. This natural recovery phenomenon is similar to RNA-mediated virus resistance, which is unusual for geminiviruses (Wang et al., 2012). Upon inoculation experiments, such recovery of symptoms is associated with the production of virus-derived siRNAs and accompanied by a reduction in the levels of viral DNA (Raja et al., 2010). Those consequences started one week post-inoculation and became abundant in the newly grown symptomless recovered leaves. An investigation of the composition of siRNAs has revealed that the majority of these siRNAs were derived from a region that corresponds to the 3'end of C1 gene which overlaps with 5' of the C2 gene (Wang et al., 2012). Therefore, it is probable that in infected plants, the presence of virus-specific siRNAs may have silenced the corresponding mRNAs, which in turn delayed viral replication and movement. Accordingly, a reduction in both virus titer and symptoms in the newly developed leaves occurred (Garcia-ruiz et al., 2010).

Since symptoms recovery is not the common plant response against virus infection; virusderived siRNAs implicating virus-induced PTGS have been shown for geminiviruses and for RNA viruses, while the infected plants do not recover (Wang et al., 2012). Though the non-recovery type phenomenon initiated through the geminivirus-host interactions are able to trigger the host PTGS, however, the produced amount of siRNAs was less than 10% when compared with the amount that accumulated with the recovery type virus (Chellappan et al., 2004; Alvarado & Scholthof, 2009). These findings indicate that the ongoing silencing of viral RNAs can occur in successful virus infections and may not be sufficient to lead to a recovery or may that these viruses encode strong silencing suppressors.

#### 1.3.1.2 Suppressing the Induced RNA Silencing

Currently, more than thirty RNA silencing-suppressor proteins which counteract the antiviral RNA silencing have been identified in several plant and animal viruses (Alvarado & Scholthof, 2009). The identified suppressor proteins do not share sequence homology. However, these suppressor proteins may target similar or different steps of the RNA silencing pathway (Wu et al., 2010). Recent comprehensive analyses of key representatives from geminiviruses including *Tomato yellow leaf curl China virus* (TYLCCNV) showed that C4 and C2 genes have the capacity to suppress the induced-PTGS with varied suppression activity (Hohn & Vazquez, 2011; Zhang et al., 2012). Moreover, the suppression activity for C4 was obviously stronger when compared with C2 gene. This indicates that geminiviruses have evolved variable methods to interact with their hosts (Vanitharani et al., 2005; Shimura & Pantaleo, 2011). The above mentioned studies along with previous ones concluded that these proteins play different roles and can target different steps in the silencing pathway(s), or could interact with different host proteins

which collectively contribute to the suppression of RNA silencing (Alvarado & Scholthof, 2009; Wu et al., 2010).

# 1.3.2 Virus-induced Gene Silencing

The sweeping efforts of genomic sequencing provide fundamental raw data for studying genomes (Kushalappa & Gunnaiah, 2013). These new data require nonconventional tools to specify genes functionality, while conventional genetic tools are mostly restricted to model plants (Aoki et al., 2013). In particular, reproducible protocols for stable genetic transformation are extremely challenging to be established for the majority of non-model plant species (Tzfira & Citovsky, 2006). Accordingly, this thesis is part of a research project to identify TYLCV resistance genes and to study their counter-pathogenicity roles by reverse-genetics, including virus-induced gene silencing (VIGS) technique.

VIGS is a type of RNA silencing that is initiated by virus-vectors carrying the host-derived sequence inserts -from the host genes-, later those inserts will initiate targeting host genes corresponding to the inserts and leading to the degradation of homologous mRNAs, then all that interpreted into suppressing a gene expression. In details, TRV- treatment by itself did not has effect on the subsequent TYLCV infection as it neither enhance nor depress the virus spread or any other typical aspect of TYLCD; which demonstrating that TRV did not initiate cross-protection against TYLCV (Czosnek et al., 2013). Therefore, with the potential to silence certain specific genes and to study the rapid loss-of-function VIGS is a powerful technique and it has already been employed successfully in a wide range of plant species including both monocots and dicots (Purkayastha & Dasgupta, 2009). Nowadays, many recent improvements have enabled the use of VIGS as the tool of choice for functional genomics for an increasing number of plant species, while gene functions of

these plant species are extremely laborious to analyze by conventional methods (Senthilkumar & Mysore, 2011).

# 1.3.2.1 Implementation of the Virus-induced Gene Silencing

VIGS, in general, is implemented by cloning a 150-350 bp exon DNA insert of the target gene into a virus vector. Insert length is a critical factor in the silencing efficiency of VIGS. The transgenic viral vector is introduced into *Agrobacterium* and then to the host plants by means of agroinfiltration (Purkayastha & Dasgupta, 2009; Senthil-kumar & Mysore, 2011). It worth mentioning that the presence of a virus vector may interfere with the metabolism of the host plant. Subsequently this may affect the results of studies aiming at investigating plant-microbe interaction. In addition to the finding that the genotype of some plant species can affect the performance of VIGS insert. For this reason, appropriate standardization and optimization of the VIGS protocol is required for each plant species (Martínez-priego et al., 2008; Romero et al., 2011).

Later, during viral replication, double-stranded RNAs from the viral genome are formed, including the gene of interest. These double-stranded RNAs are then going to be chopped into siRNAs by the plant Dicer-like enzymes, thus activating the post-transcriptional gene silencing (PTGS). As a result, mRNAs of the targeted gene are degraded leading to silencing the gene of interest (Purkayastha & Dasgupta, 2009).

#### **1.3.2.2** *Tobacco Rattle Virus* (TRV)

*Tobacco rattle virus* (TRV) is a bipartite single-stranded RNA plant virus with a positive sense strand. It is the type-member of the genus *Tobravirus*. The genomic RNA1 (TRV I) encodes RNA-dependent RNA polymerase (RdRp), a cell-to-cell movement protein and

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the third called 16 kDa protein. Whereas the genomic RNA2 (TRV II) encodes the coat protein and containing the Multiple Cloning Site (MCS); at the MCS as elaborated in figure1.3, an insert of the gene of interest is supposed to be ligated. Another characteristic of TRV is that the ability of RNA1 to replicate and spread within the host plant in the absence of RNA2 (Martínez-priego et al., 2008). Currently, *Tobacco rattle virus* (TRV) is the most common VIGS vector, and therefore is the vector of choice in this research project to identify TYLCV resistance genes. Because VIGS technique is dependent on the host range of the used virus-vectors which are usually limited, the wide host range of TRV complete the potency of VIGS through erasing that limitation. Together, TRV-induced gene silencing is considered as a powerful tool in the field of functional genomics with high silencing efficiency (Ratcliff et al., 2001; Kumar et al., 2012).

Several comparative feasibility studies of other VIGS vectors, mainly *Tobacco mosaic virus* (TMV), *Potato virus X* (PVX) and *Tomato golden mosaic virus* (TGMS) in comparison with TRV in silencing either a certain key gene among different host plants, or silencing different genes within a certain species host plant, concluded the TRV superiority whenever the host specificity is not a barrier (Ratcliff et al., 2001; Jing-di et al., 2012). However, the wide host range of TRV does not mean that it can be used for all plant species thus appropriate VIGS vectors must be developed to fill the gap. In order to develop new VIGS vectors, researchers may chose to develop a specific vector for the desired plant species from a suitable virus that infects the same species. For example, *Apple latent spherical virus* (ALSV) is being developed as a VIGS vector that cover a broad host range (Gilchrist & Haughn, 2010; Senthil-kumar & Mysore, 2011).



**Fig 1.3: Maps of TRVI and TRVII. (A)** TRVII vector with the silencing insert of the gene of interest. **(B)** Map of the TRV binary vectors; TRV cDNA clones of RNA1 (TRVI) and RNA2 (TRVII) were cloned in between two CaMV 35S promoters (2X35S) and NOS terminator (NOSt) T-DNA vector. TRVI contains RNA-dependent RNA polymerase (RdRp), movement protein (MP), and a 16 kDa cysteine-rich protein (16K). TRVII contains the coat protein (CP) and multiple cloning site (MCS). Rz refers to a self-cleaving ribozyme and LB and RB are left and right borders of T-DNA which are shared by both vectors.

Source: (Eybishtz, Assaf. Protocol for Gene Silencing, 2009 [Unpublished Document])

### 1.4 Overview of the Targeted Gene for Silencing

Preceding studies have identified preferential expression of genes in the resistant tomato line (R) in relation to the susceptible line (S), whereas both resistant and susceptible tomato lines were generated from the same breeding program (Eybishtz et al., 2009). One of these genes, namely the *Elongation factor1-alpha* gene which encodes the translational Elongation factor1-alpha (EF1 $\alpha$ ) protein will be the focus of this study. This gene is preferentially expressed in R plants and up-regulated after TYLCV infection.

In general, eukaryotic protein synthesis is commonly occurring in three stages: initiation, elongation and termination. Each stage requires the action of not only the ribosome, mRNA and aminoacyl-tRNAs (aa-tRNA), but also a series of proteins that facilitate each step. During protein biosynthesis, the step of aa-tRNA delivery is catalyzed by the eukaryotic EF1 $\alpha$  protein, as it promotes the GTP-dependent binding of aa-tRNA to the A-site of the ribosome (Ursin et al., 1991).

# 1.4.1 Secondary Roles of the *Elongation Factor 1-alpha*

Away from its function in translation elongation,  $EF1\alpha$  protein have various and significant secondary functions which include but not restricted to quality surveillance of the newly synthesized proteins, protein degradation through ubiquitin-dependent pathways and actincytoskeleton organization (Li et al., 2009; Sasikumar et al., 2012; Hwang et al., 2013).

Since EF1 $\alpha$  is one of the most abundant cellular proteins, and because viruses exploit host proteins as well as their own proteins in order to replicate, exploiting EF1 $\alpha$  by many positive-strand RNA viruses in their replication is a highly probable fate (Ursin et al., 1991). Generally, these positive-strand RNA viruses utilize the EF1 $\alpha$  in their replication via two ordinary routes. In some cases EF1 $\alpha$  interacts directly with the 3'-terminal end of the viral RNA; most likely due to the fact that the projected secondary structure of the 3'ends of the viral RNA is similar to the tRNA structure, whereas tRNA is the ordinary binding-partner of EF1 $\alpha$  (Thivierge et al., 2008). In other cases, the EF1 $\alpha$  interacts with the viral RNA-dependent RNA polymerase (RdRp), which is the essential polypeptide for synthesizing RNA from single-stranded templates of the viral RNA. Trying to speculate the importance of the EF1 $\alpha$  for replication of RNA viruses, and after considering the fact that the viral replication cycle is a chronicle series of events, the EF1 $\alpha$  might be involved in keeping that order (Sasikumar et al., 2012). The third route for positive-strand RNA viruses to utilize the EF1 $\alpha$  protein in their replication is a recently studied route comprises membranous induced-vesicles. Positive-strand RNA viruses generally assemble their RNA replication complexes on the intracellular membranes in association with membranevesicles formation (Thivierge et al., 2008). In accordance with that, several recent studies have ascertained that viral polyproteins from the positive-strand RNA viruses induce the formation of cytoplasmic vesicles, some of these vesicles are derived from the Endoplasmic Reticulum (ER) (Beauchemin et al., 2007; Li et al., 2009; Hwang et al., 2013). In addition, these studies revealed the presence of the EF1 $\alpha$  protein in the inducedvesicles during the infection cycle of the investigated viruses. Upon that, these induced vesicles are supposed to shelter the virus replication complex and the EF1 $\alpha$  protein along with at least other two translation factors (Li et al., 2009; Hwang et al., 2013).

For those viruses where EF1 $\alpha$  protein interacts with both the viral RdRp and the vesicleinducing viral polyproteins, the EF1 $\alpha$  is speculated to help sequestering the virus replication complex into vesicles. In other words, the EF1 $\alpha$  can help in fastening the components of the virus replication complex onto the induced-vesicles membrane through modification of phospholipids and eventually by mediating the vesicle formation (Li et al., 2009).

# **PROBLEM STATEMENT AND OBJECTIVES**

Naturally, there are wild tomato species having resistance against TYLCV, and those species have been recruited in the breeding programs to produce TYLCV resistant lines. The genes of these TYLCV resistant lines which conferring them the trait of resistance against TYLCV are not yet identified. While identifying the resistance genes is considered an imperative priority for the interested researchers in order to unfold the molecular basis of the resistance against TYLCV. Subsequent to identifying the genes, researchers can understand TYLCV-host interactions and ultimately can confront the disease.

The specific objectives of this research are:

• To silence the *Elongation factor1-alpha* gene as a putative TYLCV resistance gene in TYLCV-resistant tomato plants.

• To determine if silencing that gene will collapse the resistance of these plants.

# **MATERIALS AND METHODS**

# **3.1 Materials**

### **3.1.1 Source of Tomato Plants**

Two inbred tomato lines were used, line 902 is resistant (R) to TYLCV, while line 906-4 is susceptible (S). These two lines were kindly supplied from Prof. Henryk Czosnek, The Otto Warburg Minerva Center for Agricultural Biotechnology and the Institute of Plant Sciences and Genetics in Agriculture, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot 76100, Israel. Both lines were produced from a breeding program aimed to introgress resistance to TYLCV from *Solanum habrochaites* -as a source for wild tomato resistance- into the susceptible domesticated tomato *Solanum lycopersicum* (Vidavski et al., 2008). Upon whitefly-mediated inoculation either in the lab or in the field, plants of R line remain symptomless while S line plants present leaf curling, yellowing and stunting.



**Fig 3.1:** TYLCV infected susceptible (S) and resistant (R) tomato plants in the field; typical symptoms in S plants and symptomless appearance of R plants.

Source: (Eybishtz et al., 2009).

#### 3.2 Methods

#### **3.2.1 Differentiation of R from S Plants by SNP-PCR**

To avoid possible mixing of the seeds, previous work of Gorovits & Czosnek (2007) located a single nucleotide polymorphism (SNP) which found in a *Mun*I restriction site within an intron of the *Heat shock protein 70 (hsp70)* gene; the SNP can be used to distinguish between R and S plants (Gorovits & Czosnek, 2007). That SNP is not linked to resistance, and can be PCR-amplified using the primers HSP17F (5'-GTCGCCATG-AATCCTATTAACACCG-3') and HSP678R (5'-CCCAGTTTGATGTCACTCTGTAC-3') (Eybishtz, et al., 2010).

Ten seeds from each line (R and S) were germinated in trays in a controlled growth room for 25 days then transferred into disposable plastic cups and were still growing in the same controlled growth room. One week after transfer, DNA was extracted from the five most vigorous and healthy plants of R and S using QIAGEN plant DNA extraction kit (QIAGEN, Germany). Later, these PCR parameters were followed: one cycle at 95°C for 2 minutes; 30 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, followed by one cycle at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 5 minutes. Then, the PCR products were incubated with the restriction enzyme *Mun*I according to manufacturer instructions (New England BioLabs®, USA). Afterward, 10  $\mu$ I of the incubated PCR-products were subjected to electrophoresis in 1.5% agarose gel in Tris-Borate-EDTA (TBE) buffer and stained with ethidium bromide.

### **3.2.2 Primers Design**

The sequence of *Elongation factor1-alpha* gene was retrieved from Sol Genomics Network (<u>http://solgenomics.net</u>), under Unigene-ID number SGN-U232161. Then, AmplifX 1.5.4

software was used to design the primers within one of the exons of the gene, while ApE-A plasmid editor software was used to identify the open reading frames (ORFs), and to assure the absence of restriction sites for *Xba*I and *Kpn*I. Since both of these restriction enzymes will be used for cloning the silencing insert into the silencing vector (TRV II) later.

# 3.2.3 PCR Amplification of the Silencing Insert

The designed primer pair 4F3-F (5'-CTTTGGCCCTACTGGTTTGACA-3') and 4F3-R (5'-GAGGCAACATAACCACGCTT-3') were used to amplify the genomic DNA template of the silencing insert. Then, the PCR reaction performed according to these parameters: one cycle at 95°C for 3 minutes, 30 cycles at 94°C for 35 seconds, 62°C for 30 seconds, and 72°C for 40 seconds, followed by one cycle at 72°C for 5 minutes. The PCR mix was in a total volume of 20  $\mu$ l, containing 0.4  $\mu$ M of each primer, about 100 ng of the genomic DNA and using AccuPower® PCR PreMix (Bioneer, South Korea). Afterward, 5  $\mu$ l of the PCR products were subjected to electrophoresis in 2% agarose gel in TBE buffer and stained with ethidium bromide.

### 3.2.4 Cloning the Silencing Insert into TOPOII Vector

The PCR product was directly cloned into TOPOII vector (TA cloning) according to the manufacturer instructions (TOPO® TA Cloning® Dual Promoter Kit, Invitrogen<sup>™</sup>, USA). Briefly, the insert and the vector at 3:1 ration were allowed to ligate by incubating the reaction mixture for 1 hour at 25°C, followed by 5 hours at 15°C.

#### 3.2.5 Transforming Competent Cells with the Recombinant TOPOII Vector

*E.coli*  $DH5\alpha$  competent cells (BioSuper Competent Cells, Bio-Lab Ltd., Israel) were transformed by the recombinant TOPOII vectors via heat shock transformation. That

transformation accomplished by adding 6  $\mu$ l of the recombinant TOPOII vector mixture to 40  $\mu$ l of the competent cells then mixing gently while keeping the tubes on ice. Next, tubes kept on ice for 30 minutes before a heat shock for 60 seconds at 42°C in a hot water bath. After that, tubes returned immediately on ice for 5 minutes, and then under the hood, 1 ml of LB liquid media -without antibiotics- was added on each tube. Afterward, tubes incubated for 1 hour at 37°C with shacking. Finally, transformants were smeared on four LB media plates containing 100  $\mu$ g/ml ampicillin, and 40  $\mu$ l/ml of X-gal. Afterward, plates incubated at 37°C for 24 hours.

#### **3.2.6 Preparation of the Recombinant TRVII Silencing Vector**

Following subsections describe cloning the silencing insert of EF1a into TRVII vector.

### 3.2.6.1 Retrieving the Silencing Insert from TOPOII Vector and Sequencing it

White colonies were picked by pipette tips and transferred to Eppendorf tubes with 100  $\mu$ l ultra pure water, in order to be used for a screening PCR to confirm the presence of the silencing insert in the transformed colonies. At the same time, plates must be labeled at the locations where the selected colonies were taken for screening. The screening PCR was performed exactly the same as in the section 3.2.3 concerning PCR amplification of the silencing insert. Upon the screening PCR results, the transformed *E.coli DH5a* cells were grown in liquid LB with 100  $\mu$ g/ml ampicillin at 37°C under shacking. Then plasmid miniprep has been made according to manufacturer instructions (Wizard® Plus SV Minipreps DNA Purification System, Promega, USA). Then, the purified plasmid was sequenced using the insert amplification primers at Hereditary Research Laboratory in Bethlehem University by Sanger sequence to assure the identity of the insert. Afterward, the plasmid was digested with *Xba*I and *Kpn*I according to the manufacturer instructions (New England BioLabs®, USA). Then, the digested products were subjected to electrophoresis in 1.5%

agarose gel for 1 hour and the insert was purified from the gel by QIAquick® Gel Extraction Kit and according to manufacturer instructions (QIAGEN, Germany).

# 3.2.6.2 Opening the Silencing Vector

TRVII vectors were isolated from a 24 hour-old culture of *E. coli* according to manufacturer instructions (Wizard® Plus SV Minipreps DNA Purification System, Promega, USA). Next, the plasmid was incubated with the same restriction enzymes *Xba*I and *Kpn*I according to the manufacturer instructions (New England BioLabs®, USA) to open it. Then, the opened plasmid was subjected to electrophoresis in 1.5% agarose gel for 1 hour and the band was cut and purified using QIAquick® Gel Extraction Kit and according to manufacturer instructions (QIAGEN, Germany).

#### 3.2.6.3 Ligation of the Silencing Insert into the Open TRVII Vector

The silencing insert of the targeted gene was ligated into the *XbaI / Kpn*I cut open TRVII vector using T4 DNA Ligase Kit (New England BioLabs®, USA) and according to the manufacturer instructions. Then, the reaction mixture incubated at the thermal cycler for 2 hours at 20°C, followed by 14 hours at 16°C.

#### 3.2.7 Introducing the Recombinant TRVII into Agrobacterium

The recombinant TRVII vector was introduced into *Agrobacterium* LBA4404 competent cells as follows. First, *Agrobacterium* cells were grown in 5 ml YEB media containing 100  $\mu$ g/ml rifampicin for 48 hours at 28°C with shaking. Then, 2 ml from the grown cells solution were centrifuged for 10 minutes at 4500 rpm. The pelleted cells were resuspended into 0.5 ml YEB media (without antibiotics) and kept on ice. Next, 20  $\mu$ l of the prepared TRVII vector was added to the *Agrobacterium* cells-solution tube, then mixed and kept on

ice for 5 minutes. Afterward, the tubes were transferred to liquid nitrogen and kept for 5 minutes, then the tubes re-transferred to the incubator for 5 minutes at 37°C. After that, 1 ml of YEB media without antibiotics was added, and the *Agrobacterium* cells were grown for 4 hours at 28°C with shaking. The grown cells were pelleted by centrifugation for 5 minutes at 4500 rpm. After that, 80  $\mu$ l of YEB media without antibiotics was added, then mixed and plated on YEB agar plates which supplemented with 100  $\mu$ g/ml ampicillin and 100  $\mu$ g/ml rifampicin. Finally, the plates were incubated for 48 hours at 28°C. Colonies were checked for positive transformation by means of PCR. The transformed colonies were transferred into YEB liquid media and incubated at 28°C for 48 hours under shaking.

# 3.2.8 Agroinfection of Tomato Plants with Agrobacterium

Two stocks of *Agrobacterium* cells (the first containing TRVI and the second containing the recombinant TRVII with the silencing insert) were grown for 48 hours at 28°C under shacking in 20 ml YEB liquid media containing 50  $\mu$ g/ml rifampicin and either 12  $\mu$ g/ml kanamycin or ampicillin for TRVI and TRVII, respectively. After that, the optical density (OD<sub>600</sub>) of the bacterial growth cultures of both TRVI and TRVII were measured. Proper adjustment of the OD by dilution with YEB medium was made when needed in order to obtain an OD between 1.2 and 1.5 for both cultures. Then, the bacterial cultures were centrifuged at 2,800 rpm for 20 minutes and the pelleted cells were resuspended in 10ml of YEB medium using a pipette. Afterward, the two cultures were mixed at a ratio of 1:1 and were kept on ice till use. Consequently, small cuts with a sterile scalpel were made on all the leaves undersurface of the 28 days old tomato plants, and then the *Agrobacterium* mixture was deposited onto the cut area by using blunted-tip syringe, accompanied with gentle finger pressing on the drop. About 2 ml of the mixture was applied for each plant: 1

ml on the leaves and 1 ml on the soil. Finally, the plants were grown in a controlled growth room with a regime of 16 hours light, for 7 days before inoculating them with TYLCV.

### 3.2.9 Inoculating the Tomato Plants with Viruliferous Whiteflies

One week after agroinfection, the plants were inoculated with TYLCV by caging them with viruliferous whiteflies for 3 days in specially-designed cages (about 50 insects per plant). After the 3 days period, insects were exterminated with imidacloprid 0.05% (Confidor®, Bayer Crop Science, Germany). After confirming that the plants are free from whitefly, they moved back into the controlled growth room for continuing growth.

### 3.2.10 Sample Collection for DNA and RNA Extraction

In order to quantify TYLCV replication and the expression of target host gene in the agroinfected R plants, two young leaves were collected weekly from each plant including controls. As in the timeline schedule below, samples were collected weekly starting directly before the agroinfection. Symptoms monitoring was started from the third week after TYLCV inoculation. Also, collected leaves were labeled and stored at -80°C till use.





Source: (Eybishtz, Assaf. Protocol for Gene Silencing, 2009 [Unpublished Document])

#### **3.2.11 Estimation of TYLCV Quantities**

A general screening by PCR on DNA extracted from leaves collected 5 weeks after TYLCV inoculation was carried out to select plants with high virus amounts for further investigations. A pair of primers for the virus coat protein and another pair targeting the housekeeping gene  $\beta$ -actin were used. The primers were designed using the AmplifX 1.5.4 software to amplify a 67-bp fragment of the TYLCV coat protein gene. The designed primers were: TY2480-F (5'-TCCACGTAGGTCTTGACATCTG-3') and TY2547-R (5'-AGGTCAGCACATTTCCATCC-3'). While, a 180-bp fragment of the tomato β-actin gene was amplified using the primer pair ACT771-F (5'-GGAAAAGCTTGCCTATGTGG-3') and ACT951-R (5'-CCTGCAGCTTCCATACCAAT-3') (Evbishtz et al., 2009). The PCR parameters were: one cycle at 94°C for 3 minutes, then 31 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, followed by one cycle at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 3 minutes. The PCR mix was in a total volume of 20 µl, containing 0.4 µM of each primer, 100 ng of the tomato genomic DNA and using AccuPower® PCR PreMix (Bioneer, South Korea). Then, 15 µl from each PCR product was subjected to electrophoresis in 2% agarose gel in TBE buffer and stained with ethidium bromide.

#### 3.2.12 Quantitative Measurement of the Targeted Gene Transcripts

#### **3.2.12.1 RNA Extraction**

To measure the amount of transcripts of the targeted gene by quantitative Real Time-PCR (qRT-PCR), RNA was extracted from the leaves which were collected 1, 2 and 3 weeks after TYLCV inoculation by using QIAGEN® RNeasy Plant Mini Kit (QIAGEN, Germany) and following the manufacturer instructions. In order to eliminate genomic DNA carry over, on-column DNase digestion was performed using QIAGEN® RNase-Free DNase Set (Catalog no.79254, QIAGEN, Germany).

#### **3.2.12.2 cDNA Synthesis**

The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was used according to the manufacturer instructions for the synthesis of cDNA using hexamer primers. As equal volumes of the same concentration from the RNA samples must be used to eliminate concentration differences in the templates, RNA concentrations in the products of RNA extraction were measured, then proper dilutions with ultra pure water were made to get 1000 ng of RNA in the 10  $\mu$ l cDNA reaction solution. Upon manufacturer instructions, the thermal cycler program comprised: first step at 25°C for 10 minutes, second step at 37°C for 2 hours, and the third step at 85°C for 5 minutes.

# **3.2.12.3** Quantification of the Targeted Gene by Real Time-PCR

The qRT-PCR was performed in a AB7300 Real-Time PCR System® (Applied Biosystems, USA) using SYBR® *Premix ExTaqII*<sup>TM</sup> from TAKARA (Catalog no. RR82SW, TAKARA, Japan). Specific primers to amplify the targeted gene were designed using AmplifX 1.5.4 software and the NCBI Primer-BLAST utility was used for checking the primers specificity versus database of consensus sequences concerning *Solanum lycopersicum* (taxid:4081). A 61-bp fragment was amplified by using the primer pair Rt4F3-F (5'-AGTCTGTAGAGATGCACCACGA-3') and Rt4F3-R (5'-AACCCAACATT-GTCACCAGGGA-3') in both silenced and non-silenced plants in order to compare the expression of the silenced EF1 $\alpha$  gene. The thermal cycler program was: first step (as a hot start step) at 95°C for 30 seconds, followed by second step which consisting from 40 cycles of 95°C for 5 seconds, 60°C for 31 seconds, then the melting curve step of 95°C for 15 seconds.

The qRT-PCR mix was in a total volume of 20  $\mu$ l, containing 0.8  $\mu$ M of each primer, 2  $\mu$ l of the synthesized cDNA solution that was diluted 1:1 with ultra pure water to simplify pipetting and 0.4  $\mu$ l of ROX dye 50X as a reference dye. MicroAmp® Optical 96-Well Reaction Plate with Barcode® (Applied Biosystems, USA) were used and sealed with MicroAmp® Caps (Applied Biosystems, USA) according to the manufacturer instructions. Moreover, the reactions were conducted in duplication (two technical replicates for each experimented plant of the five R biological replicates).

#### 3.2.13 Semi-quantitative PCR of TYLCV

Semi-quantitative PCR of DNA extracted from leaves collected 5 weeks after TYLCV inoculation was carried out to compare TYLCV quantities between the silenced and non-silenced plants in reference to the housekeeping gene  $\beta$ -actin. cDNA of the reference housekeeping gene were synthesized from RNA extracted from leaves which collected also at the fifth week after TYLCV inoculation. The same procedural steps of RNA extraction and cDNA synthesis for the targeted-gene transcripts in sections 3.2.12.1 and 3.2.12.2 were followed with the  $\beta$ -actin gene transcripts. A pair of primers for TYLCV coat protein and another pair targeting the housekeeping gene  $\beta$ -actin were used. The primers were the same pairs that used to estimate TYLCV quantities in section 3.2.11. The semi-quantitative PCR reactions were grouped within two batches with different number of cycles.

The parameters for the first batch of reactions were: one cycle at 94°C for 3 minutes, then 25 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. While the parameters for the second batch of reactions were: one cycle at 94°C for 3 minutes, then 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. The PCR mix was in a total volume of 20  $\mu$ l, containing 0.4  $\mu$ M of each primer, 100 ng of tomato

genomic DNA for TYLCV reactions (equal volumes of the same DNA concentration from the samples were used) and corresponding concentration of cDNA for  $\beta$ -actin reactions. Furthermore, the AccuPower® PCR PreMix (Bioneer, South Korea) was used. Then, 15 µl from each semi-quantitative PCR product was subjected to electrophoresis in 2% agarose gel in TBE buffer and stained with ethidium bromide.

| PRIMER<br>NAME     | PRIMER SEQUENCE           | MELTING<br>TEMP.<br>°C | PRODUCT<br>SIZE<br>(BP) |
|--------------------|---------------------------|------------------------|-------------------------|
| HSP17(F)           | GTCGCCATGAATCCTATTAACACCG | 65.2                   | 780                     |
| HSP678( <b>R</b> ) | CCCAGTTTGATGTCACTCTGTAC   | 57.2                   |                         |
| 4F3( <b>F</b> )    | CTTTGGCCCTACTGGTTTGACA    | 62.1                   | 144                     |
| 4F3( <b>R</b> )    | GAGGCAACATAACCACGCTT      | 58.6                   |                         |
| TY2480(F)          | TCCACGTAGGTCTTGACATCTG    | 58.7                   | 67                      |
| TY2547( <b>R</b> ) | AGGTCAGCACATTTCCATCC      | 58.4                   |                         |
| ACT771( <b>F</b> ) | GGAAAAGCTTGCCTATGTGG      | 58.2                   | 180                     |
| ACT951( <b>R</b> ) | CCTGCAGCTTCCATACCAAT      | 58.6                   |                         |
| Rt4F3(F)           | AGTCTGTAGAGATGCACCACGA    | 58.4                   | 61                      |
| Rt4F3( <b>R</b> )  | AACCCAACATTGTCACCAGGGA    | 64.3                   |                         |

**Table 3.1**: The PCR primers used in this study.

# **CHAPTER FOUR**

# RESULTS

# 4.1 Sequence Identification of EF1a

The BLAST results of the sequence of the cloned PCR fragment of the *Elongation factor1alpha* (EF1 $\alpha$ ) gene from the resistant (R) plants against the NCBI database (figure 4.1) showed high identity (99%) with the corresponding exon of the same gene of *L*. *esculentum* (accession number X53043.1).

```
GTGACCTTTGGCCCTACTGGTTTGACAACTGAAGTCAAGTCTGTAGAGATGCACCACGAA
Query 2
                                                     61
         Sbjct 3329
         GTGACCTTTGGCCCTACTGGTTTGACAACTGAAGTCAAGTCTGTTGAGATGCACCACGAA
                                                     3388
Query 62
         GCTCTCCAGGAGGCACTCCCTGGTGACAATGTTGGGTTCAATGTTAAGAATGTTGCTGTT
                                                     121
         sbjct 3389 GCTCTCCAGGAGGCACTCCCTGGTGACAATGTTGGGTTCAATGTTAAGAATGTTGCTGTT 3448
Query 122
         AAGGATCTTAAGCGTGGTTATGTTGCCTCAAACTCCAAGG-TGACC
                                           166
         sbjct 3449 AAGGATCTTAAGCGTGGTTATGTTGCCTCAAACTCCAAGGATGACC
                                           3494
```

**Fig 4.1:** Sequence identification of the cloned fragment of *Elongation factor1-alpha* (EF1 $\alpha$ ) gene from R plants. Upper sequence is the silencing insert of EF1 $\alpha$  gene, and lower sequence is the corresponding exon of the same gene of *L. esculentum* (accession number X53043.1).

### 4.2 PCR Results

### 4.2.1 Differentiation of R from S by SNP-PCR

To avoid mixing of the R and S seeds and to make sure that the correct tomato lines are employed in the subsequent experiments, they were distinguished by a single nucleotide polymorphism (SNP) found in a *Mun*I restriction site within an intron of the *hsp70* gene. The DNA fragment containing that SNP was PCR-amplified, and the PCR products were incubated with the restriction enzyme *Mun*I according to manufacturer instructions (New England BioLabs®, USA). As in figure 4.2, PCR products of R plants were digested as they contain the *Mun*I site while PCR products of S plants are not.



Fig 4.2: SNP-PCR products of five R and five S individual plants, M: 100bp ladder.

# 4.2.2 PCR Amplification of the Silencing Insert

The designed silencing insert (144 bp of one of the exons of EF1 $\alpha$  gene) was amplified from the genomic DNA of R plants. PCR products were subjected to electrophoresis. Specific and intense band in each lane was observed, which is as an indicator of a good nucleic acids quality for the subsequent cloning steps (figure 4.3).



Fig 4.3: PCR products of the designed silencing insert from  $EF1\alpha$  gene, amplified from five different R plants, M: 100bp ladder.

#### 4.2.3 Quantification of EF1α Gene by Real Time-PCR

To measure the amount of transcripts of EF1a gene, RNA was extracted from leaves which were collected at 1, 2 and 3 weeks after TYLCV inoculation and cDNAs were synthesized. The quantitative Real Time-PCR (qRT-PCR) of 5 silenced R plants (5 biological replicates), each tested twice (2 technical replicates) showed variations in the silencing efficiency of  $EF1\alpha$  gene. In RT-PCR the reaction is detected by accumulating a fluorescent signal and that detection is measured by the threshold cycle (Ct) which is the number of required cycles for the fluorescent signal to cross the threshold of the background level. Ct values are inversely proportional to the amount of template in the sample. Thus, for the 5 silenced R plants each Ct value of a certain plant with one cycle lower than a different plant means the amount of EF1a transcript templates in the first plant is double the amount in the second plant which has one cycle higher of Ct value. Upon that, silencing of  $EF1\alpha$ gene reached around 90% in plant number 5 from the second week after TYLCV inoculation as in figure 4.6, that in comparison with the non-silenced control plant. The silencing efficiency was more pronounced when the EF1 $\alpha$  transcripts quantified at one and two weeks after TYLCV inoculation than when quantified at the third week (figures 4.5 and 4.6). In the latter case, there was no significant difference in the amount of  $EF1\alpha$ transcripts between the silenced plants and the non-silenced control plant (figure 4.7).



Fig 4.4: Approximate histogram of silencing the  $EF1\alpha$  gene among R plants from the first three weeks after TYLCV inoculation in comparison with a non-silenced control plant.

Results



Fig 4.5: qRT-PCR curves of representative sample replicates from EF1 $\alpha$ -silenced R plants and non-silenced control plant replicates at the first week after TYLCV inoculation.



**Fig 4.6:** qRT-PCR curves of representative sample replicates from EF1α-silenced R plants and non-silenced control plant replicates at the second week after TYLCV inoculation.



Fig 4.7: qRT-PCR curves of representative sample replicates from EF1 $\alpha$ -silenced R plants and non-silenced control plant replicates at the third week after TYLCV inoculation.



**Fig 4.8:** The dissociation curve plot of our results which is properly typical. Since dissociation curves can detect the nonspecific amplification products.

#### 4.2.4 Semi-quantitative PCR of TYLCV

Increased TYLCV replication in the cells of EF1 $\alpha$ -silenced plants is an indicator of the collapse of resistance in these plants. Semi-quantitative PCR of total DNA as well as synthesized cDNA that were extracted from leaves collected at 5 weeks after TYLCV inoculation showed differences in bands intensity of the PCR products among the silenced plants. These differences were appeared after 25 cycles, then solidified after 30 cycles of amplification. Compared to the non-silenced control plant (WF), the band intensity of the PCR products from plants number 3, 4, 5 and 6 was higher (figure 4.9). However, plants number 1 and 2 showed band intensities similar to that of the non-silenced control plant (WF). Whereas the band intensity of the PCR products from transcripts of the housekeeping gene  $\beta$ -actin were similar among all the plants.



**Fig 4.9:** Semi-quantitative PCR to compare TYLCV quantities within 6 EF1 $\alpha$ -silenced plants and a WF control (non-silenced control plant with TYLCV inoculation) in reference to the  $\beta$ -actin as a house keeping gene, NTC: non-template control. Since the band intensity indicates the amount of the template, it is an indicator of resistance collapse upon EF1 $\alpha$  gene silencing in the plants number 3, 4, 5 and 6.

# **4.3 Symptomatic Results**

# 4.3.1 Typical TYLCD Symptoms Development

Typical TYLCD symptoms on EF1 $\alpha$ -silenced R plants appeared four weeks after TYLCV inoculation and become more sever one week later (figure 4.10). These symptoms include yellowing and curling of the leaves.



Fig 4.10: A comparative picture of both silenced and non-silenced tomato R plants at the fifth week after TYLCV-inoculation. Since typical symptoms appeared obviously on TYLCV-resistant tomato plant, it is an indicator of resistance collapse upon EF1 $\alpha$  gene silencing.

#### **CHAPTER FIVE**

# **DISCUSSION**

#### 5.1 The Resistant and Susceptible Lines

In this study, line 902 which is TYLCV resistant (R) was used to study the molecular basis of its resistance to TYLCV. Line 902 and line 906-4 which is TYLCV susceptible (S) were produced from the same breeding program aiming at the introgression of TYLCV resistance into a susceptible domesticated cultivar of tomato (Vidavski & Czosnek 1998). Two resistant accessions of S. habrochaites which are LA1777 and LA386 which were derived from the parent TYLCV-resistant line H902 through a series of self-pollinations and selections (Vidavski et al., 2008). The two accessions were crossed and F1 population with high TYLCV resistance was produced. Upon that, the F1 plants were further crossed with the domesticated tomato S. lycopersicum. Then, part of the produced F1 plants, which demonstrated resistance to TYLCV, was backcrossed with commercial lines (Vidavski & Czosnek 1998). After backcrossing, resistant plants were selected upon field and laboratory tests for TYLCV resistance. The field test results were measured by virus quantification and symptoms evaluation -during the first month after inoculation. Similar tests were conducted on the other part of F1 plants, which proved to be susceptible to the virus (Vidavski & Czosnek 1998). Upon TYLCV infection, the susceptible-line plants presented typical disease symptoms, while resistant-line plants remained symptomless with significantly lower virus quantities (Czosnek et al., 2013). Therefore, we have chosen these two lines for our study because they have similar genes pool (line 902 and line 906-4) and minimum genetic variations. Since differentiating R and S plants by phenotypic traits is laborious and time-consuming, a single nucleotide polymorphism (SNP) which found in the MunI restriction site within an intron of hsp70 gene was developed to distinguish between the R and S plants that used in this study (Gorovits & Czosnek, 2007). The DNA fragment which contained that SNP was PCR-amplified, and digested with MunI (figure 4.2). This SNP is not linked to resistance and was not identified in other TYLCV-resistant tomato lines (Evbishtz et al., 2010). Also, this R-specific SNP was traced back to accession LA1777 from S. habrochaites by examining a range of plants which were selected at different stages of that breeding program (Vidavski and Czosnek, 1998). In a previous work by Eybishtz et al. (2009), cDNA libraries from R and S plants were compared before and after TYLCV infection. Upon that comparison, 69 genes were found to be preferentially overexpressed in R plants than in S plants. Among them, 19 genes were found to be more preferentially-expressed in TYLCV-infected R plants than non-infected R plants. Moreover, 18 genes were found to be preferentially-expressed in S plants than R plants (Eybishtz et al., 2009). Among the 69 identified genes, 25 genes have been silenced and only 5 genes out of them led to the collapse of resistance upon silencing (Czosnek et al., 2013). *Elongation factor 1-alpha* (EF1a) gene which is the subject of this study was one of the 19 genes that are more preferentially-expressed in TYLCV infected R plants than non-infected R plants.

#### **5.2** Silencing the EF1α Gene

In this study, TRV-VIGS presented the proper silencing system. Congruent with this study results, VIGS may give variable silencing levels of the targeted gene depending on the insert and the plant growth conditions. Also, VIGS may result in uneven or localized silencing or lack of silencing in certain tissues which is mainly due to inefficient virus movement. These problems can be alleviated by maintaining environmental conditions favoring systemic virus movement, and more importantly by choosing appropriate viral vectors that do not suppress or weaken the virus ability of multiplication and spread

(Senthil-kumar & Mysore, 2011). In this study, the most common VIGS vector which is Tobacco rattle virus (TRV) was chosen (Gilchrist & Haughn, 2010). Since VIGS depends on the host range of the used vector which is usually limited, the wide host-range of TRV completed the potency of VIGS approach during our study. Although permanent silencing of genes by VIGS could be achieved by Agrobacterium-mediated transformation of plant cells, this approach is laborious, expensive and time-consuming. Therefore, we followed the transient silencing approach in out attempt to silence  $EF1\alpha$  gene, since several weeks of silencing the EF1 $\alpha$  gene is enough to study its effect on TYLCV resistance. Furthermore, the limitation of uneven or localized silencing was solved by sufficiently-representative sampling throughout the period of five weeks after TYLCV inoculation. TYLCV was introduced into the silenced plants by caging them with viruliferous whiteflies for 3 days. Although a shorter period is enough to inoculate the plants with TYLCV, the plants were caged for 3 days in order to insure that the plants acquired sufficient inoculums of TYLCV. Since this method of inoculation mimics the natural process of TYLCV infection in the field it was chosen to be followed in this study. Furthermore, safety regulations were implemented when dealing with the whiteflies throughout the experiments including whiteflies extermination.

The amounts of EF1 $\alpha$  transcripts were measured weekly during the first 3 weeks after TYLCV-inoculation. According to several previous studies, measuring at the second week after TYLCV-inoculation represent the best time to measure the silenced gene transcripts (Eybishtz et al., 2010; Sade et al., 2012; Czosnek et al., 2013). As already mentioned in the results chapter, the systemic spread of the silencing-signal was peaked after 20 days of silencing the gene. Since silencing the EF1 $\alpha$  gene associated with TYLCV resistance-collapse, this study estimations to quantify viral accumulation shown that TYLCV was

detectable at the first week after TYLCV inoculation. Later, viral quantities exponentially and steadily increased up to the fifth week after TYLCV inoculation.

Real Time-PCR based on SYBR Green II chemistry has been successfully applied to detect EF1a gene transcripts. SYBR II offered exceedingly reproducible with high-sensitivity detection RT-PCR analyses by inhibiting nonspecific amplifications through a hot-start PCR enzyme that uses an anti-Taq antibody. Also, SYBR II reduced the PCR-inhibition (which results from residual mRNAs when using cDNA as a template) by a heat resistant RNase H. Generally, in RT-PCR the reaction is detected by accumulating a fluorescent signal. And the threshold cycle (Ct) is the number of the required cycles for the fluorescent signal to cross the threshold of the background level. Ct values are inversely proportional to the amount of template in the sample. Thus, lower Ct value means greater amount of template in the sample. In that relation, a sample with twice starting amount of templates will get Ct value with one cycle earlier. Accordingly, Ct value of this study results shown that the expression of EF1 $\alpha$  gene was knocked down to 90% in some samples (figure 4.4). While silencing is uneven within the samples, RT-PCR results revealed that samples with strong  $EF1\alpha$  gene-silencing correlated with significant increase in TYLCV quantities. Thus, inverse proportionality was clear between  $EF1\alpha$  gene-transcripts and TYLCV accumulation. Regarding symptoms development on the silenced and TYLCV infected plants, the symptoms were TYLCD-typical and dramatically exposed at the fourth week and exacerbated at the fifth week after TYLCV inoculation. As in the figure 4.10, symptoms on the silenced plants were as severe as the susceptible plants symptoms. Later, after the fifth week, plants growth was ceased and in some plants the early-senescence appearance was noticed.

Discussion

#### 5.3 *Elongation factor1-alpha* Gene

In this study, sequence of the silenced gene was retrieved from the International Solanaceae Genome Project (SOL) data. Gene ontology (GO) analysis of the 69 candidate resistance genes (with differential expression) revealed that many of them are associated with cellular membranes (Czosnek et al., 2013). This fact indicates the membranes participation in sustaining resistance via transportation and/or signaling-transduction activities. Therefore, many researchers were interested in studying membrane-associated resistance genes. However, subsequent research findings for *Pectin methylesterase* as a membrane-associated gene but not a resistant gene refuted the notion of considering each membrane-associated gene from the candidate genes as a potential resistant gene (Eybishtz et al., 2009). On the other hand, non-membranous genes with housekeeping functions could break the resistance as already confirmed in the case of *Chlorophyll a-b binding* protein 7 and Thioredoxin peroxidase findings (Czosnek et al., 2013). Several secondary functions have been reported for EF1a, which in general related to protein posttranslational modifications (PTM). A secondary function of  $EF1\alpha$  was recently reported which is related to virus-host interactions. In fact,  $EF1\alpha$  reported to interact exclusively with the positive-strand RNA viruses. In this relation, many positive-strand RNA viruses utilize EF1 $\alpha$  in their replication; whereas EF1 $\alpha$  can interact directly with the 3' terminal end of the viral RNA or otherwise EF1α could interacts with the viral RNA-dependent RNA polymerase (RdRp) (Yamaji et al., 2006; Thivierge et al., 2008; Sasikumar et al., 2012). Furthermore, positive-strand RNA viruses might utilize  $EF1\alpha$  in their replication through mediating the generation of membrane induced-vesicles.

Alternatively, functions of the main resistance genes against a close relative to TYLCV - which is *Tomato yellow leaf curl Sardinia virus* (TYLCSV) are related to post-translation

#### Discussion

modification (PTM) processes. In a similar work for specifying the genes related to resistance against TYLCSV upon infecting a transgenic line of the model plant Nicotiana benthamiana (Luna et al., 2011), 44% of the genes which implicated with TYLCSV infection were actually PTM genes and essentially performing ubiquitination-related functions (Czosnek et al., 2013). Those PTM genes are postulated to provide a mechanism of quick response to various stimuli in an efficient way when compared with the transcriptional activation mechanisms. Thus, it is not unexpected for the PTM genes to interfere with the TYLCV infection. Ubiquitination has a well-established participation in the PTMs throughout diverse plant as well as animal viral infections (Chuang et al., 2005; Tan et al., 2007). Also, evidence from TYLCSV studies confirmed the essential roles of ubiquitination within the host to respond against assailant viruses. Specifically, silencing the *Ubiquiting-activating* gene (UBA1) in tomato (which encodes an enzyme that catalyzes the first step in ubiquitin conjugation) promoted the TYLCSV infection (Lozano-Durán et al., 2011). Therefore, and since a literature-search did not reveal a possible role of EF1 $\alpha$ with such merely studied single-stranded DNA viruses (Bennett et al., 2008). This study results could collectively suggest that EF1a gene-product might be involved in directing the viral transcribed proteins from the ribosome to the proteasome for degradation.

### **5.4 TYLCV-Host Interactions**

Principally, the limited understanding of the interactions between TYLCV and tomato require referring to the biological models of the virus-host interactions in order to speculate the effects of this study results on understanding the molecular basis of TYLCV resistance. The identified TYLCV-host interactions are restricted to virus entry into the nucleus of the host cell and the interactions between the virus Rep protein and the plant retinoblastoma-related protein to induce host cell division (Eybishtz et al., 2010). Contextually, TYLCV

resistance was hypothesized to be conferred by several genes, and these genes were supposed to be organized in a hierarchically-linked network of defense (Czosnek et al., 2013). Consequently, silencing EF1 $\alpha$  gene would probably down-regulate other genes which are located downstream within that network. To verify the hierarchy of TYLCV resistance genes in a network and whether they are positioned at critical junctions or nods; the transcriptomic profiles for three resistance genes were analyzed after co-silencing (Czosnek et al., 2013). Accordingly, Lipocalin-like gene expression was evaluated in R plants with silenced Hexose transporter LeHT1 gene; whereas the Lipocalin-like gene expression was completely repressed. However, silencing Lipocalin-like gene did not repress *Hexose transporter LeHT1* gene expression. Therefore, the *Lipocalin-like* gene assumed to be located downstream of Hexose transporter LeHT1 gene in the network. Likewise, silencing the Permease I-like gene did not repress the expression of either Lipocalin-like gene or Hexose transporter LeHT1 gene; while silencing Lipocalin-like gene or Hexose transporter LeHT1 gene did not affect the Permease I-like gene expression. Therefore, the *Permease I-like* gene was proposed to be located on a different branch of the network (Adi et al., 2012; Sade et al., 2012; Czosnek et al., 2013). Since a chromosomal location of a certain R gene may provide a clue for the hierarchical organization of the network. EF1 $\alpha$  gene locates on chromosome number six, thus it would be possible that the EF1α gene was introgressed on a separate chromosomal fragment during breeding and selection for resistance. Eventually, as future antiviral therapies will probably be developed to block the interaction of a viral protein with an essential cellular target (Kuiken et al., 2006). Or might be developed to possibly interfere at a given point of the host cell network in order to counteract the virus, identifying the commonalities and/or specificities of the TYLCV-host interactions is fundamentally essential to pave the way ahead of those future antiviral therapies.

# **5.5 Prospective Work**

Since TYLCV resistance conferred by a number of genes, and these genes were hypothesized to be hierarchically organized in networks. Prospective efforts might be aimed at the positional localization of EF1 $\alpha$  gene within the resistance network through combinations of co-silencing between EF1 $\alpha$  gene and the other confirmed resistance genes in order to definitely specify which genes are located down- or upstream of EF1 $\alpha$  within that network.

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