Characterization of major resistance genes to Tomato Yellow Leaf Curl Virus

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This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences

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Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 19th of June, 2013
at 11 a.m. in the Aula.

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Characterization of major resistance genes to Tomato Yellow Leaf Curl Virus
161 pages.

PhD Thesis Wageningen University, Wageningen, NL (2013)
With references, with summaries in Dutch and English
ISBN: 978-94-6173-561-4

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Chapter 1

General introduction

Plants are crucial for life on earth, via photosynthesis they produce oxygen and organic compounds. The organic compounds are the basis for almost all ecosystems and oxygen is essential for all respiring organisms. To survive plants must cope with a variety of abiotic stresses such as extremes in temperature, humidity, pH, salinity and competition with neighboring plants for light and nutrients. On top of these abiotic stresses there are biotic stresses to deal with, which vary from grazing vertebrates to small phloem feeding insects, and on a smaller scale oomycetes, bacteria, nematodes, fungi and viruses (Agrios, 2005). Mankind has learned to efficiently make use of plants via agriculture, but a mix of the described abiotic and biotic stresses are a constant threat to crop production worldwide, especially so to field grown crops. In tomato production Tomato Yellow Leaf Curl Disease (TYLCD), caused by *Tomato yellow leaf curl virus* (TYLCV), has been one of the most devastating viral diseases in the last two decades both in open field and greenhouse production and is nowadays still a major threat (Hanssen et al., 2010).

The genome organization and life cycle of TYLCV

Most plant infecting viruses contain a (+) single-stranded RNA genome, while a much smaller group contains a DNA genome. The latter are classified into three families: *Nanoviridae*, *Caulimoviridae* and *Geminiviridae*. The *Geminiviridae* is the largest family and their genomes comprise one or two circular, single-stranded (ss)DNA components that, hence their naming, are encapsidated into particles of geminate morphology. Geminiviruses can infect dicots and monocots and are transmitted by various species of leafhoppers, treehoppers or the single whitefly species Bemisia tabaci. Based on their genome organization, insect vector and host range members are classified into four genera, i.e. *Curtovirus*, *Topocuvirus*, *Mastrevirus* and *Begomovirus* (Fauquet et al., 2008). The genus *Begomovirus* contains more than 200 species, only infect dicotyledonous plants and are transmitted by the phloem feeding whitefly *Bemisia tabaci*. Most viruses belonging to the *Begomovirus* genus possess a bipartite genome, with two circular ssDNA molecules, DNA-A (2.6-2.8 kb) and DNA-B (2.5-2.8 kb). The TYLCV begomovirus, however, is a true monopartite virus, with all genes coded from one ssDNA molecule of 2.7-2.8 kb (Figure 1). Like most viruses, TYLCV relies on host cell components for completion of its infection cycle (Hanley-Bowdoin et al., 2004). The replication relies solely on DNA intermediates and is proposed to involve complementary strand replication (CSR), rolling circle replication (RCR) and/or recombination-dependent replication (RDR) (Gutierrez, 1999; Hanley-Bowdoin et al., 1999). It starts with the conversion of the ssDNA into double stranded (ds)DNA by cellular factors. These dsDNA intermediates associate with histone proteins to form mini chromosomes, which mediate viral

replication and transcription (Pilartz and Jeske, 1992, 2003). In the second stage the viral replication initatior protein, Rep, is essential. It binds to a specific sequence, the iteron, in the Intergenic Region (IR) and thereby introduces a nick (Laufs et al., 1995). By this it initiates the RCR by the host cell DNA polymerases and produces new ssDNA products that can enter the replication pool or can be encapsidated to produce new virions. An excellent description of this process is presented by Gutierrez, 1999.

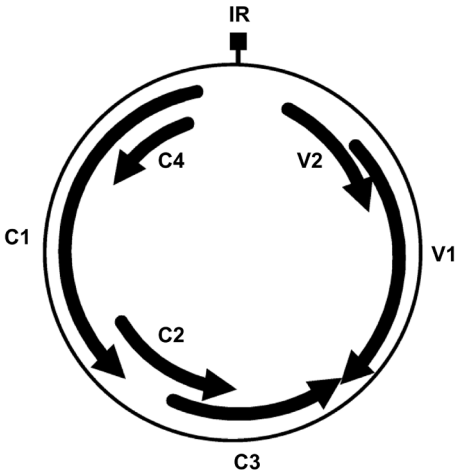

Figure 1. Schematic genome organization of a typical TYLCV.

A monopartite single stranded TYLCV is usually 2.7 to 2.8 kb. Genes code in two directions, virion sense (V) or complementary sense (C). The intergenic region is indicated (IR). V1 encodes the capsid protein (CP), V2 the movement protein (MP), C1 the replication initiator protein (Rep), C2

Recombination plays a key role in begomovirus genome evolution and recombinants arise in high frequency during mixed infections of two related strains (García-Andrés et al., 2007; Lefeuvre et al., 2007; van der Walt et al., 2009). The TYLCV genome contains six open reading frames (ORFs) that are partially overlapping and organized in two transcriptional units, separated by an approximately 200 nucleotides non-coding intergenic region (IR). The IR contains the promoters for the virion-sense (V) genes V1 and V2 and for the complementary-sense (C) genes C1 and C4. The other two complementary genes (C2 and C3) have their promoter within the C1 gene. For all six proteins at least one function has been described, however viral proteins often have multiple roles in the infectious cycle and novel functions are still being discovered. An overview of all six proteins and their function(s) is given in the next paragraph, followed by a more detailed description on the silencing suppressor activity of C2, C4 and V2.

V1 encodes the capsid protein (CP) and is the only known structural protein of the virion particle. CP is indispensable for systemic infection, particle formation, and insect transmission (Noris et al., 1998). Besides being required for transmission, it also

determines insect vector specificity (Briddon et al., 1990) and is shown to interact with the GroEL homolog of whitefly endosymbiotic bacteria. This interaction is essential for TYLCV circulative transmission (Morin et al., 1999; Morin et al., 2000). TYLCV movement (cell-to-cell and long-distance) is assured by the genes encoded by V1 (CP), C4 and the other v-sense gene V2. V2 codes for the pre-coat protein which is also called movement protein (PCP or MP), and when disrupted does not provide systemic infection of tomato (Wartig et al., 1997). The protein also exhibits RNA-silencing suppressor activity and is able to interact with the host papain-like cysteine protease CYP1, a protein involved in defence against multiple diverse plant pathogens. The exact function of this interaction is not known yet but it is speculated to facilitate viral invasion and/or spread (Bar-Ziv et al., 2012).

C1 encodes for the replication initiator protein (Rep), the only viral protein known to be essential for viral DNA replication (Desbiez et al., 1995). It recognizes a sequence in the IR followed by sequence specific DNA cleavage. The protein additionally recruits host cell proteins and triggers initiation of viral DNA-strand synthesis. C2 encodes the transcriptional activator protein (TrAP) and, hence its name, activates transcription. TrAP is essential for infectivity in tomato (Wartig et al., 1997) and has also been described as silencing suppressor (Voinnet et al., 1999). The third complementary sense gene C3 codes for the Replication enhancer protein (REn). REn is not necessary for replication but it greatly enhances symptoms and viral accumulation (Settlage et al., 2005). It interacts with Rep and with tomato proliferating cell nuclear antigen (PCNA). The latter is proposed to induce the assembly of the plant replisome close to the virus origin of replication (Castillo et al., 2003). The last complementary sense gene C4 is entirely embedded within the C1 ORF and upon mutation hampers systemical movement of TYLCV (Jupin et al., 1994). More recently C4 has been shown to additionally exhibit RNA-silencing suppression activity (Vanitharani et al., 2004).

Transmission of TYLCV

TYLCV and most geminiviruses are phloem limited. In nature TYLCV is exclusively transmitted by the whitefly *Bemisia tabaci*, its only known vector. Whiteflies feed on the phloem sap, by penetrating the vascular tissue with an organ named stylet, and from there they pick up TYLCV. Already thirty minutes after this 'acquisition' feeding, TYLCV genomic DNA can be detected in 15% of the whitefly individuals and after 8 hours 100% of the whiteflies test positive for viral content. For whiteflies that feed on leafs containing high viral amounts even within two hours 100% can be reached (Zeidan and Czosnek, 1991; Kashina et al., 2007). TYLCV is transmitted by whiteflies in a persistent

and circulative manner, the virus thus does not replicate within its vector. Whiteflies cannot transmit the virus immediately after uptake because the virus first needs to pass several barriers, i.e. translocate from the digestive tract system to the salivery glands from where it is transmitted during feeding. This so called latency period approximately takes 8 hours for TYLCV but large variations have been reported (Czosnek, 2007).

In tomato plants TYLCV symptoms begin to appear 2-3 weeks after exposure to a viruliferous whitefly. Symptoms start with slight yellowing of leaflet margins in apical leaves and during a later stage followed with upward curling and cupping of leaflets (Figure 2). After a month severe curling, cupping and yellowing of top leaves are visible, while plants stop growing completely and flowers and fruits are abscised (Lapidot, 2007).TYLCV infections may lead to yield losses ranging up to 100%, ad has been reported in many countries (Polston and Anderson, 1997; Varma and Malathi, 2003).

Figure 2. TYLCV symptoms on susceptible tomato cultivar Moneymaker

Typical symptoms like leaf yellowing and curling are visible. Picture is taken four weeks after TYLCV inoculation.

TYLCV has first been observed in the Jordan Valley in 1929, but later described and published by (Cohen and Harpaz, 1964). The rapid evolution of virus variants, the massive increase of vector populations (with the appearance of whitefly 'B' biotype) and the introduction of modern human agricultural practices have contributed to a rapid and wordwide spread of the virus (Varma and Malathi, 2003). Control measurements for TYLCV are usually focused on reducing or avoiding whitefly vector populations; heavy spray of insecticides and/or the use of nets in isolated greenhouses are common practices (Polston and Lapidot, 2007). Breeding TYLCV-resistant tomato cultivars, as part of an integrated management approach, is an economically and environmentally sustainable alternative that reduces pesticide usage.

Sources for TYLCV resistance breeding

Resistance against TYLCV exists in nature. However, all cultivated tomato germplasm has been reported as susceptible to TYLCV infection; no resistance or tolerance genes from *S. lycopersicum* have been found. Hence, breeding for resistance to TYLCV has mainly been focused on the introgression of resistance/tolerance genes from wild tomato relatives. Since the rapid and wordwide spread of TYLCV, many screenings for resistance have been performed by researchers worldwide, using different inoculation and symptom scoring methods. Several sources of TYLCV resistance have been reported including accessions of *S. pimpinellifolium*, *S. chmielewski*, *S. glandulosum*, *S. lycopersicoides*, *S. habrochaites*, *S. chilense* and *S. peruvianum*. Some of these resistant accessions have been used for introgression breeding and until now five major resistance/tolerance genes have been identified and mapped. In most studies the term resistance gene is used for these five loci. This can be confusing because one would easily conclude there is no viral replication in plants having these genes. However, none of the identified genes so far has been associated with an HR and in almost all resistant/tolerant tomato lines tested viral titers can be detected, usually at lower levels if compared with fully susceptible lines (Pico et al., 1999; Pico et al., 2000; Narasegowda Maruthi et al., 2003; Perez de Castro et al., 2005). Recently, mapping of these loci (*Ty-1* to *Ty-5*) conferring resistance to TYLCV has been facilitated with the release of the tomato genome sequence (Consortium, 2012).

Ty-1: *S. chilense* accession LA1969 is the donor of *Ty-1*, the most widely used TYLCV resistance gene in breeding programs worldwide. It has been first mapped by (Zamir et al., 1994), and later on further mapped by (Pérez de Castro et al., 2007).

Ty-2: (Hanson et al., 2006) reported the presence of an introgression derived from *S. habrochaites* accession B6013 (Hanson et al., 2000) in the tomato line H24 with TYLCV resistance. Within this introgression, the *Ty-2* gene is located and later on further fine mapped to the long arm of chromosome 11 (Ji et al., 2009b).

Ty-3: A resistant locus has been identified in *S. chilense* LA2779 and LA1932 through screening of TYLCV-resistant breeding lines. *Ty-3* has been mapped in the long arm of tomato chromosome 6, and has been described to also confer partial resistance to the bipartite begomovirus Tomato mottle virus (ToMoV). (Ji et al., 2007).

Ty-4: This TYLCV resistance locus has been mapped to the long arm of chromosome 3 and is originating from *S. chilense* LA1932. The locus is considered to be a minor one as it only accounted for 16% of the resistance when combined with *Ty-3* (Ji et al., 2009a).

Ty-5: Breeding line TY172, derived from crosses between four different accessions of *S. peruvianum* (PI 126926, PI 126930, PI 390681, LA0441) and the cultivated tomato, has

been reported as highly resistant to TYLCV. Association analysis showed that resistance is controlled by a major QTL located in chromosome 4 which accounts for 39.7 to 46.6% of the variation of the resistance (Anbinder et al., 2009). Genetic control of this major QTL has been suggested as partially dominant. Interestingly, a marker strongly associated with this major QTL is based on the sequence of a gene that is a member of the NAC-domain protein family. This gene has been shown to interact with the viral replication enhancer protein of another tomato-infecting begomovirus, *Tomato leaf curl virus* (Selth et al., 2005).

Recently the recessive resistance in the cultivar Tyking has been shown to co-localize with the resistance in TY172 (Hutton et al., 2012). The authors suggest that one of the populations used by Anbinder (2009) also showed recessive gene action and that the locus in TY172 should therefore be renamed to *ty-5*. They also speculate that *tcm-1* and *tgr-1*, which are also recessive resistance genes, describe *ty-5* as well (Giordano et al., 2005; Bian et al., 2007). Further research with all these lines has to be done to prove if these genes are all allelic.

Genes involved in TYLCV resistance

TYLCV resistance is a complex trait and only few genes are known to be involved. A recent successful approach has compared cDNA libraries of a susceptible and a resistant tomato line carrying a *S. habrochaites* introgression before and after TYLCV inoculation. Approximately 70 genes have been found to differentially express in the two youngest true leaves in a cDNA pool of plants 1, 3, 5 and 7 days after inoculation. Of these genes, three have been confirmed via a virus-induced gene silencing (VIGS) approach to be required for TYLCV resistance, i.e. a lipocalin-like protein (*SlVRSLip*), a Permease I-like protein and a hexose transporter *LeHT1*. *SlVRSLip* functions downstream in the same network as *LeHT1*, while Permease I-like functions in a different gene network (Eybishtz et al., 2009b; Eybishtz et al., 2009a; Sade et al., 2012).

A reverse genetics approach has also been undertaken to identify host genes involved in geminivirus resistance. By combining TYLCV infection and VIGS in transgenic 2IRGFP *N. benthamiana* plants 15 new host genes potentially involved in Tomato Yellow Leaf Curl Sardinia Virus (TYLCSV) infection have been found. Interestingly, almost half of the genes described to affect TYLSCV infection play a role in posttranslational modifications like rubylation, ubiquitination, phosphorylation, acetylation or protein folding (Lozano-Durán et al., 2011).

RNA silencing

Whether a virus can successfully infect a host depends on many factors. Plants have multiple layers of defense, for instance trichomes can act as a first layer of defense and prevent viruliferous insects to feed on a leaf and thus avoid viral transmission (Simmons and Gurr, 2005). Another more specific layer of defense are the dominantly inherited Resistance (R) genes, plant genes that specifically recognize a (component of a) virus and that are often involved in a hypersensitive response (HR). This HR leads to localized cell death which prevents virus spread throughout the whole plant (extensively reviewed in Kang et al., 2005). Examples of cloned R-genes directed against viruses in crop plants are *Rx2* for *Potato virus X* (Bendahmane et al., 2000), *Sw-5* for tospoviruses in tomato (Brommonschenkel et al., 2000) and the *I* locus for *Bean common mosaic virus* (Vallejos et al., 2006). In total approximately 200 virus resistance genes have been described in plants and about half of them is recessively inherited. This recessive nature is more common for viruses than for other plant pathogens like fungi or oomycetes, for which true R-genes are the major part of resistance genes known. Most of these recessive genes are linked to the translation initiation complex and negatively affect the viral RNA replication cycle (Truniger et al., 2009).

A more general antiviral mechanism present in plants is RNA silencing (also called RNA interference, RNAi). RNA silencing is a homology based mechanism that is conserved in eukaryotes and involved in regulation of gene expression and in defense against alien nucleic acids such as transposons, transgenes and also viruses. The RNA silencing machinery (also referred to as RNA interference or RNAi) has multiple essential players including RNA dependent RNA polymerases (RDR), Argonaute (AGO) proteins, and ribonuclease Dicer-like (DCL) proteins. The machinery is triggered by dsRNA molecules from an exogenous or endogenous source. These molecules may harbor a perfect or imperfect double-stranded nature and are processed by DCL into small interfering (si)RNA molecules of ~21-26 nucleotides (Bernstein et al., 2001). After unwinding one strand of the small RNA duplex molecules, the so-called guide strand, is uploaded into an RNA-induced silencing complex (RISC). This enables the RISC to sense and target RNA molecules with sequence complementarity to the guide strand for degradation or translational arrest by means of the core Argonaute (AGO) protein. The primary siRNAs may also act as primers for host RDRs for the conversion of RNA target sequences into new long dsRNAs from which new, secondary siRNAs will be produced. This not only leads to an amplification of the siRNA signal but also results in so-called transitive silencing, a spread of siRNA molecules to other (flanking) parts of the RNA target sequence (Sijen et al., 2001). The amplification of siRNAs is necessary for a strong

RNAi response and Arabidopsis RDR1, 2 and 6, and orthologs of these genes, have been demonstrated to be involved in this amplification. Plants in which these genes have been knocked-out exhibit higher susceptibility to various plant RNA viruses (Schwach et al., 2005; Diaz-Pendon et al., 2007; Pandey et al., 2008; Garcia-Ruiz et al., 2010; Wang et al., 2010).

RNA silencing and Geminiviruses

Viral transcripts of geminiviruses are targeted by Post Transcriptional Gene Silencing (PTGS) as described above, but to defend against DNA viruses plants also employs transcriptional gene silencing (TGS). TGS is based on siRNA directed methylation of viral DNA and results in inhibition of viral transcription and replication. For geminiviruses first proof for this came from experiments using *Nicotiana tabacum* protoplasts transfected with methylated *Tomato golden mosaic virus* and in which only low levels of viral transcription and replication were observed (Brough et al., 1992). More recently methylation deficient *Arabidopsis thaliana* mutants showed hyper susceptibility upon challenging with the begomovirus *Cabbage leaf curl virus* (CaLCuV) and the curtovirus *Beet curly top virus* (BCTV) (Raja et al., 2008). Vice versa, recovery of *Capsicum annuum* plants from infections with *Pepper golden mosaic virus* and of watermelon from *Cucurbit leaf crumple virus* in both cases associated with enhanced viral methylation (Hagen et al., 2008; Rodriguez-Negrete et al., 2009). Altogether, these studies indicated the importance of DNA methylation, and so of TGS, as a defense strategy against geminiviral invasions.

Silencing suppression by geminiviruses

As a counter defense plant viruses have evolved RNA silencing suppressor proteins (RSS) and because DNA viruses are targeted by PTGS and TGS they are postulated to suppress both mechanisms (Hohn and Vazquez, 2011). Meanwhile, for many members of the *geminiviridae*, *caulimoviridae* and *nanoviridae*, RSS have been described, (Hohn and Vazquez, 2011). For geminiviruses the multifunctional C2 protein of African Cassava Mosaic Virus (ACMV) has been the first RSS identified (Voinnet et al., 1999). This protein exerts RSS activity by stimulating transcription of host genes that negatively regulate silencing pathways (van Wezel et al., 2002; Dong et al., 2003; Trinks et al., 2005), and by the inhibition of adenosine kinase, which suppresses local silencing (Wang et al., 2003; Wang et al., 2005; Yang et al., 2007).

The next protein that has been identified to contain RSS activity is C4 from cassava geminiviruses. (Vanitharani et al., 2004). The protein is able to suppress PTGS by sequestering single stranded siRNAs and thereby prevents RISC activation and/or

enhances siRNA degradation (Chellappan et al., 2005).

In plants, SGS3 is known to be required for RDR6 dependent conversion of single stranded RNA into double stranded RNA (Mourrain et al., 2000; Vaucheret, 2006). SGS3 has been described as the target of the third identified begomovirus RSS, namely the V2 protein. The latter protein directly interacts with tomato SGS3 in planta and this interaction is proposed to be required for suppression of gene silencing (Glick et al., 2008). In another study V2 is shown to bind dsRNA and able to outcompete SGS3 for this, suggesting that V2 functions as RSS by preventing SGS3 from accessing substrate RNAs (Fukunaga and Doudna, 2009).

Most of the studies describing RSS activity use transient assays in *N. benthamiana*. However, a recent study tested all three RSS proteins from different strains, namely three TYLCV strains and one *Tomato yellow leaf curl sardinia* (TYLCSV), in bean as well as in tomato. While tomato is a host for all viruses, bean is only a host for TYLCV. The results showed that suppressors from TYLCV are functional in both hosts but the RSS proteins from TYLCSV are only functional in tomato. Based on this, the authors hypothesized that a difference in host range between these viruses is (partly) due to the ability of their respective RSS to suppress silencing in those hosts (Luna et al., 2012).

Outline of this thesis

The aim of this thesis was to fine map, clone and characterize the TYLCV resistance genes *Ty-1* and *Ty-3* which are both located on chromosome 6. At the start of this research, these genes were roughly mapped to a region where multiple other resistance genes are located (e.g. *Mi* for root knot nematodes, *Cf* for *Cladosporum fulvum* and the *Ol*-genes for *Oidum lycopersicum*). However, the absence of a clear consensus on their exact locations on chromosome 6 gave breeders a lot of headaches because it made introgression breeding for this trait very difficult. While breeders are challenged to pyramid all these clustered resistance genes into elite tomato cultivars this region was also known to be prone to recombination suppression which hampered introgression. For this reason we hypothesized that chromosomal rearrangements and/or the location of the *Ty-1* and *Ty-3* genes in the pericentromeric region caused the recombination suppression, similarly as was described earlier for *Mi-1* (Kaloshian et al., 1998). Fine mapping *Ty-1* and *Ty-3* would not only enable breeders to more efficiently employ the genes in breeding programs, but cloning of these genes would also elucidate the first geminivirus resistance genes and possibly provide a glimpse at the underlying resistance mechanism. Considering that *Ty-1*/*Ty-3* are not associated with an HR, the genes likely do not code for the 'classical' NBS-LRR R gene but instead affect replication and/or

movement of the virus. Prior to the fine mapping of *Ty-1* and *Ty-3*, the recombination behavior of the chromosomal region where *Ty-1* is introgressed, was analyzed (Chapter 2). Using Fluorescence in situ Hybridization (FISH) analysis we show chromosomal rearrangements between *S. lycopersicum* and *S. chilense* LA1969 in the *Ty-1* introgression are the cause for recombination suppression. The *Ty-1* gene is shown to be located outside of the rearrangements and is mapped to an interval overlapping with the reported *Ty-3* region. The use of FISH as diagnostic tool in introgression breeding is discussed as well. In Chapter 3 we further fine map *Ty-1* and *Ty-3* to a similar small region with only few candidate genes. Using Virus Induced Gene Silencing (VIGS) the candidate genes are tested and it is shown that *Ty-1* and *Ty-3* are allelic and code for a DFDGD-class RNA-Dependent RNA Polymerases, a class of genes for which as yet no function is described before.

Chapter 4 describes further exploration of the *Ty-1* and *Ty-3* alleles. Using VIGS it is shown that the *Ty-1*/*Ty-3* allele is responsible for TYLCV resistance in multiple tomato lines harboring *S. chilense* introgressions from multiple different accessions. The allelic variation of the gene is also explored using genomic sequences from almost 100 different tomato lines.

The last experimental chapter (Chapter 5) gives a first insight into the mechanistic action of *Ty-1*/*Ty-3*. It is tested whether *Ty-1*/*Ty-3* acts against TYLCV only or to a broader range of (gemini)viruses. Because of its gene function it is plausible that *Ty-1*/*Ty-3* is involved in the siRNA pathway and using northern blot analysis the small RNA fraction from resistant and susceptible TYLCV challenged plants are analyzed. In light of transcriptional gene silencing the methylation status of TYLCV is also investigated using the same plant material.

Finally in Chapter 6 all the results obtained are summarized and discussed in the context of current knowledge. Also some future perspectives on TYLCV resistance research are presented.

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Chapter 2

Chromosomal rearrangements between tomato and *Solanum chilense* hamper mapping and breeding of the TYLCV resistance gene *Ty-1*

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Published as
Maarten G. Verlaan, Dóra Szinay, Samuel F. Hutton, Hans de Jong, Richard Kormelink, Richard G.F. Visser, John W. Scott, Yuling Bai (2011) Chromosomal rearrangements between tomato and *Solanum chilense* hamper mapping and breeding of the TYLCV resistance gene *Ty-1*, The Plant Journal 68: 1093-1103

Summary

Tomato yellow leaf curl disease, a devastating disease of tomato, is caused by a complex of begomoviruses generally referred to as Tomato yellow leaf curl virus (TYLCV). Almost all breeding for TYLCV resistance has been based on the introgression of the *Ty-1* resistance locus derived from *Solanum chilense* LA1969. Knowledge about the exact location of *Ty-1* on tomato chromosome 6 will help in understanding the genomic organization of the *Ty-1* locus. In this study, we analyze the chromosomal rearrangement and recombination behavior of the chromosomal region where *Ty-1* is introgressed. Nineteen markers on tomato chromosome 6 were used in F2 populations obtained from two commercial hybrids and showed the presence of a large introgression in both. Fluorescence in situ Hybridization (FISH) analysis revealed two chromosomal rearrangements between *S. lycopersicum* and *S. chilense* LA1969 in the *Ty-1* introgression. Further, a large-scale recombinant screening in the two F2 populations was done, and 30 recombinants in the *Ty-1* introgression were identified. All recombination events were located on the long arm beyond the inversions, showing that recombination in the inverted region was absent. Disease tests on progenies of informative recombinants with TYLCV mapped *Ty-1* to the long arm between markers MSc05732-4 and MSc05732-14, an interval overlapping with the reported *Ty-3* region, which led to the indication that *Ty-1* and *Ty-3* may be allelic. With this study we prove that FISH can be used as a diagnostic tool to aid in the accurate mapping of genes that were introgressed from wild species into cultivated tomato.

Introduction

Tomato yellow leaf curl disease (TYLCD) is one of the most devastating diseases in tomato in the last decades (Moriones and Navas-Castillo, 2000). TYLCD is a viral disease caused by tomato yellow leaf curl viruses (TYLCV) which all belong to the *Begomovirus* genus within the *Geminiviridae* (Fauquet, 2005). Whereas most begomoviruses (geminiviruses) contain a bi-partite circular, single-stranded (ss) DNA genome, TYLCV only contains one circular ssDNA of about 2.7 kb. Its genome has six partially overlapping open reading frames (ORFs) that are bi-directionally organized and separated by an Intergenic Region (IR) of approximately 200 nucleotides (Gronenborn, 2007). Geminiviruses easily recombine during mixed infections, which not only leads to new variants and diversification within the TYLCV cluster, but also makes its taxonomic classification more and more complex (Monci et al., 2002; García-Andrés et al., 2007; García-Andrés et al., 2009). Meanwhile, at least eleven TYLCV species are reported and a standardized system has been set up to assist in a transparent and useful nomenclature for *Geminiviridae* including newly identified species and strains (Fauquet et al., 2008).

TYLCV is widespread in warm and (sub)tropical regions worldwide and is a limiting factor for tomato production (Cohen and Lapidot, 2007). The disease is still spreading with recent outbreaks reported in California and Hawaii, USA, and in China (Rojas et al., 2007; Zhang et al., 2009; Melzer et al.). TYLCV infections lead to stunting, yellowing, leaf curling and flower abortion. When plants are infected at a young stage crop losses up to 100% may occur (Varma and Malathi, 2003). The virus has a large host range, including many economically important crops like tomato, *Nicotiana tabacum* (tobacco), *Capsicum annuum* (pepper) and *Solanum tuberosum* (potato) (Polston and Anderson, 1997) and is transmitted by the sweetpotato whitefly *Bemisia tabaci.*

Disease management of TYLCV, aimed at controlling the whitefly insect vector, is expensive and labor intensive, and management includes insecticide applications sometimes combined with physical barriers like polyethylene sheets or large plants like sorghum (*Sorghum bicolor*) (Hilje et al., 2001; Palumbo et al., 2001). More recently, whitefly insecticide resistance has been reported (Horowitz et al., 2007), stressing the need for alternative management strategies, such as to breed TYLCV resistant tomato cultivars. Whereas domesticated tomatoes (*Solanum lycopersicum*) are susceptible to TYLCV, high levels of resistance were found in several wild tomato species including *S. pimpinellifolium*, *S. peruvianum*, *S. chilense*, *S. habrochaites* and *S. cheesmaniae* (Ji et al., 2007b). Some of these have been used for intensive genetic studies which have so far lead to the mapping of five TYLCV resistance genes (Table 1).

Table 1. Mapped TYLCV resistance loci that are identified from wild Solanum species.

Gene	Genetic source		Chromosome	Reference
	Accession/ Line[a]	Species		
Ty-1	LA1969	*S. chilense*	6 (pericentromere region)	(Zamir et al., 1994)
Ty-2	B6013	*S. habrochaites*	11	(Hanson et al., 2006)
Ty-3	LA1932, LA2779	*S. chilense*	6 (long arm)	(Ji et al., 2007a)
Ty-4	LA1932	*S. chilense*	3	(Ji et al., 2009)
Ty-5	TY172	*S. peruvianum*	4	(Anbinder et al., 2009)

Currently, five loci (*Ty-1* to *Ty-5*) for TYLCV resistance are available for commercial breeding (Ji et al., 2007b; Anbinder et al., 2009). The *Ty-1* locus from *S. chilense* LA1969 was the first mapped TYLCV resistance locus (Zamir et al., 1994) and was shown to be linked with the *Ty-3* locus on chromosome 6 (Zamir et al., 1994; Ji et al., 2007a). In many (commercial) breeding programs worldwide, *Ty-1* has been introgressed into cultivated tomatoes and these cultivars are for sale on the market (Ji et al., 2007b). However, the *Ty-1* introgression in these cultivars is generally accompanied by undesired horticultural traits (such as autonecrosis, http://www.faqs.org/patents/app/20100212048), a phenomenon that is known as linkage drag. *Ty-1* was first mapped to the pericentromere of tomato chromosome 6 (Zamir et al., 1994), but follow up studies with newly developed molecular markers tightly linked to *Ty-1* presented contradictory results on the genetic position of the *Ty-1* locus. In one study *Ty-1* was linked to the REX-1 locus in the Mi-1 gene cluster, suggesting that *Ty-1* is located on the short arm of chromosome 6 (Milo, 2001). In another study, Pérez de Castro et al. (2007) reported linkage of *Ty-1* to marker CT21 which is located below the centromere on the long arm. So far, the exact position of *Ty-1* has not been determined nor the underlying gene elucidated (Pérez de Castro et al., 2007).

Mapping of genes in the pericentromere is very inaccurate due to suppression of recombination. One example is the mapping of *Mi-1*, a tomato gene conferring resistance to three different pathogens, which is located in the pericentromere in the short arm of tomato chromosome 6. Suppression of recombination was reported in the F2 populations derived from interspecific crosses between *S. lycopersicum* and *S. peruvianum*, which were used for cloning the *Mi-1* gene (Kaloshian et al., 1998). Considering the report of linkage between *Ty-1* and the *Mi-1* gene (Milo, 2001), the failure of efforts to fine map *Ty-1* and

to reduce the introgression size is likely due to the suppression of recombination in this region. Although causes for the recombination suppression are not known, the location of a target-gene in pericentromere heterochromatic regions and/or chromosomal rearrangement(s) between cultivated and wild tomatoes may play a role in this (Tang et al., 2008; Szinay et al., 2010).

Recently, FISH has been shown to facilitate genetic mapping by visualization of physical locations of bacterial artificial chromosomes (BACs) on pachytene chromosomes (Szinay, 2010). Moreover, cross-species FISH has been successfully applied to detect chromosomal rearrangements between *Solanum* species (Iovene et al., 2008; Tang et al., 2008; Lou et al.; Szinay et al., 2010). In this study, we applied BAC-FISH and large-scale recombinant screening to analyze the chromosomal structure and recombination behavior in the chromosomal region where *Ty-1* is introgressed. Markers that cover the large *S. chilense Ty-1* introgressions in tomato hybrids have been developed and used for large-scale recombinant screenings on F2 populations derived from two hybrids. Our results demonstrate that *Ty-1* is located on the long arm of tomato chromosome 6 near the *Ty-3* locus. The failure of previous efforts to precisely locate *Ty-1* was caused by low marker coverage in combination with severe recombination suppression in the previously reported *Ty-1* region, which is the result of chromosomal inversions between *S. lycopersicum* and *S. chilense* LA1969.

Figure 1.

MM (A), a susceptible F2 plant (B) and a resistant F2 plant (C). Photos were taken two weeks after TYLCV whitefly infection. Clear TYLCV symptoms (yellow and curly leaves) are visible in MM (A) and the susceptible F2 plant (B).

Results

Large introgression fragments are present in cultivars carrying *Ty-1*

Two F2 populations (P-1 and P-2), each having an introgression from *S. chilense* LA1969 and segregating for TYLCV resistance, were used to more precisely map the Ty-1 locus. The presence of the *Ty-1* introgression was verified in P-1 by challenging a small set of F2 plants (n=45), along with the susceptible control Moneymaker (MM), with viruliferous whiteflies. Three weeks after infestation, TYLCV symptoms, i.e. yellowing and curling of the leaves, were clearly visible on MM plants (Figure 1). From population 1, 15 F2 plants showed symptoms and were scored as susceptible (S) while 30 plants remained symptomless and were scored as resistant (R) (Figure 1). Five markers linked to *Ty-1*, i.e. REX-1, Aps-1, TG97, TG231 and JB-1 (Table S1), ((Pérez de Castro et al., 2007)), were applied to this set of plants. All markers showed a homozygous or heterozygous *S. chilense* genotype for the resistant plants and a *S. lycopersicum* genotype for the susceptible plants. The same markers were also applied in P-2 and all showed similar polymorphisms among F2 plants, indicating the presence of a *Ty-1* carrying *S. chilense* introgression in each population.

In order to determine the size of the introgression in this material, molecular markers were designed from the tomato genome sequence information. Eighteen BACs physically mapped to the *Ty-1* region on chromosome 6 (Figure 2) (Peters et al., 2009) were targeted for marker development, and Cleaved Amplified Polymorphic Sequences

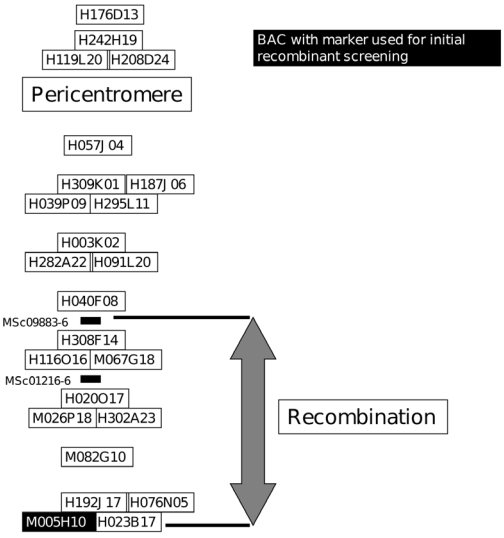

Figure 2. Physical map of BACs in the Ty-1 region on chromosome 6 based on FISH experiments (Tang et al., 2008; Peters et al., 2009).

The following BACs were used for marker development: H304P16*, H242H19, H119L20, H208D24, H057J04, H039P09, H309K01*, H295L11, H187J06, H091L20, H040F08*, H116O16, H308F14, M067G18*, M026P18*, H302A23*, M082G10*, and M005H10*. The BACs marked with a star were successfully converted into a CAPS marker (see Table S1 for details). The grey arrow indicates the region with recombination events in population 1 (Table 2).

(CAPS) markers were successfully developed from 9 of these (Table S1). Tomato genome scaffold sequences were used to design additional markers corresponding to gaps between the BAC-contigs (Figure 2 and Table S1).

Based on polymorphisms between two DNA-pools that were made containing DNA of either 10 R or 10 S F2 plants, the introgression in population 1 spans the region between BAC H304P16 and BAC M005H10. The introgression thus covers a part of the short arm, the centromere and a part of the long arm of chromosome 6 (Figure 2). Markers derived from these two BACs were applied in population 2, and the same marker polymorphisms were observed, suggesting a similar-sized introgression in both populations. Since both F2 populations are derived from commercial F1 hybrids, our results clearly demonstrate that a large chromosomal fragment from *S. chilense* carrying *Ty-1* was introgressed into cultivated tomatoes. This region spans, according to the latest tomato sequence release (WGS2.31), approximately 30 million base pairs.

Suppression of recombination in the *S. chilense* introgression region

To more precisely map *Ty-1*, an initial screening was performed by genotyping approximately 3000 plants from population 1 with markers M-H304P16 and M-M005H10, which flank the chromosomal region where *Ty-1* and *Ty-3* are located according to previous studies (Zamir et al., 1994; Ji et al., 2007a; Pérez de Castro et al., 2007). A total of 26 recombinants between these two markers were identified and further genotyped with additional markers in the region (Table 2). Results revealed that all recombination events occurred downstream of marker MSc09883-6 (Figure 2). Surprisingly, no recombinants were found between markers M-H304P16 and MSc09883-6, an interval corresponding to more than 60% of the physical distance between markers M-H304P16 and M-M005H10, indicating a severe suppression of recombination in this region.

To test whether this suppression was population specific, two other populations (P-2 and P-3) were used for recombinant screening. In P-2, where *Ty-1* is present, only four recombination events between M-H304P16 and M-M005H10 were identified by screening 1600 F2 plants. Again, no recombination events occurred between markers M-H304P16 and MSc09883-6, confirming the suppression of recombination within this region in populations derived from S. chilense LA1969.

In the *Ty-3* population (P-3), approximately 6000 plants were screened with markers Mi23 and M-M005H10, which yielded 150 recombinants. Fifty-four of these recombination events were identified between markers Mi23 and TG97, but only six occurred within the nearly 17 Mb interval between M-H304P16 and C2_At5g61510. No recombination events were observed within the nearly 6 Mb region between markers

Table 2. Genotypes of CAPS markers from recombinants between M304P16-2 and M005H10 identified in F2 population-1 and used in disease tests.

Recombinant	M-H304P16	M-H309K01	M-H271L05	M-H040F08	MSc 09883-6	M-M067G18	T1563	MSc 01216-6	M-M026P18	M-H302A23	M-M082G10	MSc 05732-3	MSc05732-4	MSc05732-14	MSc05732-18	PG9	C2At3g11210	M-M005H10	Result of disease test on F3 progenies
O-C11	h	h	h	h	h	h	h	h	h	h	h	h	h	d	h	c	b	b	Segregating
K-A5	h	h	h	h	h	h	h	h	h	h	h	h	h	d	a	a	a	a	All susceptible
U-A2	h	h	h	h	h	h	h	h	h	h	h	h	h	d	a	a	a	a	Segregating
Z-G9	b	b	b	b	b	b	b	b	b	b	b	b	b	b	h	c	h	h	All resistant
M-G6	h	h	h	h	h	h	a	h	h	h	h	h	b	b	b	c	b	b	Segregating
W-G5	a	a	a	a	a	a	a	a	a	a	a	a	a	d	h	c	h	h	Segregating
M-A7	b	b	b	b	b	b	b	b	b	b	b	b	b	d	h	c	h	h	Segregating
U-F6	b	b	b	b	b	b	b	b	b	b	b	h	h	d	h	c	h	h	Segregating
J-D10	a	a	a	a	a	a	a	a	a	a	h	h	h	d	h	c	h	h	Segregating
S-F7	b	b	b	b	b	b	b	b	b	b	h	h	h	d	h	c	h	h	Segregating
V-A9	h	h	h	h	h	h	h	h	h	a	a	a	a	d	a	a	a	a	All susceptible
K-D1	b	b	b	b	b	b	b	b	h	h	h	h	h	d	h	c	h	h	Segregating
AC-E7	h	h	h	h	h	h	h	h	b	b	b	b	b	b	b	c	b	b	All resistant
L-D5	a	a	a	a	a	a	a	h	h	h	h	h	h	d	h	c	h	h	Segregating
R-G10	a	a	a	a	a	h	h	h	h	h	h	h	h	d	h	c	h	h	Segregating
Z-D8	a	a	a	a	a	h	h	h	h	h	h	h	h	d	h	c	h	h	Segregating
R-C2	b	b	b	b	b	h	h	h	h	h	h	h	h	d	h	c	h	h	Segregating
T-E11	h	h	h	h	h	b	b	b	b	b	b	b	b	b	b	c	b	b	All resistant

a, Homozygous for *Solanum lycopersicum* allele.
b, Homozygous for *Solanum chilense* allele.
h, Heterozygous.
d, Either a or h.
c, Either b or h.

C2_At5g61510 and TG97. Clearly, recombination is also severely suppressed in this region in this *Ty-3* carrying population.

Chromosomal rearrangements in the *S. chilense* introgression

In order to check whether the suppression of recombination is caused by chromosomal rearrangements between homologous chromosomes in the *Ty-1* introgression, we performed FISH experiments using five selected BACs (Figure 3) that had been used in previous BAC-FISH experiments (Tang et al., 2008). Cot100 repeat blocking was applied in order to guarantee locus specific signals. Nine F2 plants of population 1 were used, of which three were homozygous for *S. chilense* alleles in the *Ty-1* introgression ('b' plants), three homozygous for *S. lycopersicum* alleles ('a' plants) and three heterozygous ('ht' plants). On the pachytene chromosome of the 'a' plants (Figure 3a and 3d), the BACs hybridized to the expected locations as in cv. MM and cv. Heinz 1706; two on the short

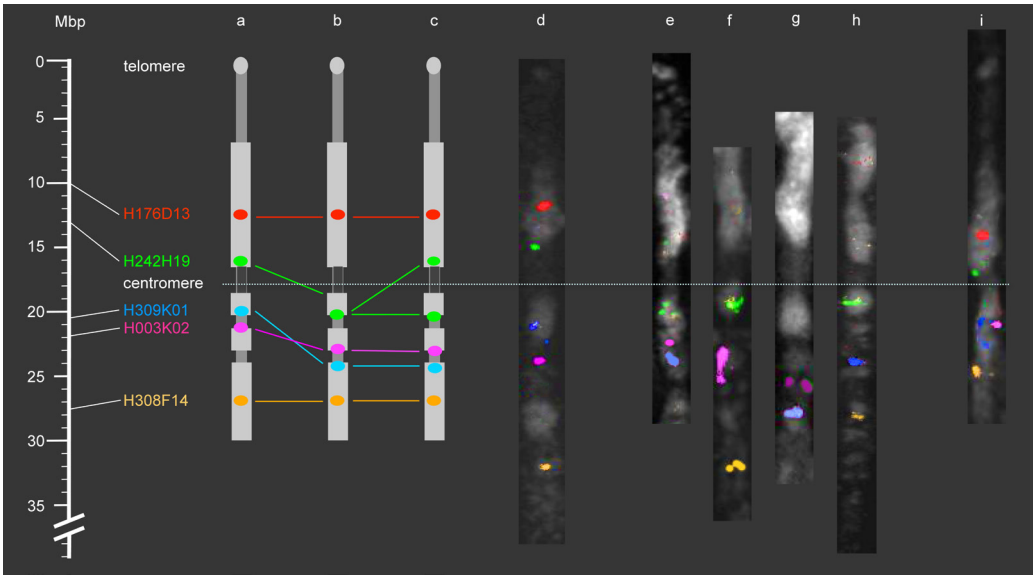

Figure 3. Schematic drawing (a to c) and BAC-FISH images (d to i) of five BACs on pachytene chromosomes of F2 plants selected from population 1. a, d) Representative F2 plant homozygous for *S. lycopersicum* alleles in the *S. chilense* introgression; b, e, f, g, h) Multiple images from one representative F2 plant homozygous for *S. chilense* alleles in the *S. chilense* introgression; c, i) Representative F2 plant heterozygous in the *S. chilense* introgression.

Physical positions (in bp) of these five BACs are shown on the left column and BACs were highlighted with different colors. BAC H242H19 is located above the centromere in 'a' plants (a) and below the centromere in 'b' plants (b); inverted order of BAC H309K01 and 003K02 between 'a' and 'b' plants (a and b); and multiple signals of BAC H242H19 and H309K01 in 'ht' plants (c and i).

arm and three on the long arm (Figure 3). On the 'b' plants (Figure 3b and 3e/f/g/h), we could not obtain an image where all 5 BACs gave signals on the same chromosome. We, therefore, compared BAC locations by combining different representative images obtained from the same plant (Figure 3e/f/g/h). Since each BAC is present on at least two images, relative positions of all the five BACs are determined. For BAC H176D13, it was difficult to obtain a clear signal in 'b' plants, which may be due to the highly condensed heterochromatin of "b" plants. BAC H242H19, which localized on the short arm above the centromere in 'a' plants, showed a signal on the long arm below the centromere in 'b' plants. BAC H309K01 and H003K02 were located on the long arm with an inverted order between 'a' and 'b' plants. BAC H308F14, most distal in the long arm pericentromere heterochromatin, was syntenic between 'a' and 'b' plants. On the 'ht' plants (Figure 3c and 3i), BAC H176D13 and H308F14 each gave a locus specific signal. BAC H242H19 gave two signals: one on the short arm and one on the long arm. For the other two BACs, whose positions were inverted between 'a' and 'b' plants, multiple signals appeared on the 'ht' plants indicating that paring between homologous chromosomes was interrupted. The results altogether suggested the occurrence of two chromosomal inversions between *S. chilense* LA1969 (the donor species of the *Ty-1* locus) and *S. lycopersicum*, i.e. one involving the centromere, as shown by BAC H242H19, and the other one on the long arm pericentromere heterochromatin, as shown by BACs H309K01 and H003K02 (Figure 3). Both inversions localized to the chromosomal region where suppression of recombination was observed (Table 2, Figure 2). The failure in hybridizing BAC H176D13 on "b" plants is likely the result of inefficient Cot100 blocking due to the highly degree of (mostly gypsy type) retrotransposable elements in the heterochromatin of the short arm (Figure 3e/f/g/h) or by undiscovered complex chromosome rearrangements in this region.

To verify the FISH results obtained in the F2 plants, it was also tried to paint the BACs on *S. chilense* LA1969 (donor of *Ty-1*) and on *S. chilense* LA2779 (donor of *Ty-3*). These experiments failed because in almost two years we could not obtain young flower buds in the right stage for FISH. Therefore we painted the same BACs onto TY52 and Su09E941-164-1, which carry *Ty-1* and *Ty-3* introgressions respectively. Based on our marker data (Table 3), TY52 harbors a large introgression that spans the inverted regions, and FISH data confirmed in this line that BAC H242H19 was on the long arm and that BACs H309K01 and H003K02 were inverted compared to "a" plants. Su09E941-164-1 has a small introgression likely below the inversions (Table 3), and the BAC order was collinear between MM and Su09E941-164-1 in the FISH experiments.

Table 3. Marker genotypes of a fixed *Ty-1* and a fixed *Ty-3* line

Genotypes of CAPS markers in TY52 and Su09E941-164-1 in which *Ty-1* or *Ty-3* is introgressed, respectively. The *S. chilense* LA1969 introgression in TY52 ends between markers MSc05732-4 and MSc05732-14, where we have mapped *Ty-1*. The *Ty-3* introgression in Su09E941-164-1 begins between these two markers.

	C2_At4g01900	C2_At5g61510	M-H309K01	M-H040F08	M-H302A23	MSc05732-4	MSc05732-14	PG3	MSc05732-18	PG9	C2_At3g11210	M-M005H10
TY52 (*Ty-1*)	b	b	nd	b+	b	b	a	a	a	a	a+	a
LA1969	b+	b	b	b+	nd	b	b+	b+	b	b+	b	b+
Su09E941-164-1 (*Ty-3*)	a	a	a	a+	a	a	b	b+	b	b+	b	b+
LA2779	b+	b	b	b+	b	nd	b+	b+	b+	b	b	b+

a	**Homozygous for** *Solanum lycopersicum* **allele**
b	**Homozygous for** *Solanum chilense* **allele**
nd	not determined

+ indicates this allele is specific for this line and different from the alleles in F2-population 1

Ty-1 maps near the *Ty-3* locus

To further localize the *Ty-1* locus, disease assays were performed on selfed progenies of the recombinants of P-1. In total, 18 informative F3 families were challenged by agroinoculation with an infectious TYLCV clone. Approximately 4 weeks after agroinoculation, MM plants showed clear TYLCV symptoms. One F3 family homozygous for the *S. chilense Ty-1* introgression was used as a resistant control. All plants of the resistant control were symptomless, and plants of recombinant F3 families were unambiguously scored as either resistant or susceptible. Although autonecrosis is known for some *Ty-1* cultivars, the resistant plants in our test showed a MM-like morphological phenotype. Previous reports locate *Ty-1* to the pericentromere, above the *Ty-3* locus towards the centromere. According to this position, the markers M-H304P16 and MSc09983-6 (Table 2, Figure 4) should flank the resistance gene, and F3 families of the recombinants Z-G9, M-A7, U-F6, S-F7, K-D1 and R-C2 should breed true for resistance, while families of W-G5, J-D10, L-D5, R-G10 and Z-D8 should all be susceptible. However, only Z-G9 bred true for resistance, while all other families segregated for

resistance, showing that Ty-1 is actually distal of marker MSc05732-4 and located in the region where Ty-3 is mapped (Figure 4). This result was supported by the analysis of the V-A9 and K-A5 families that, according to the reported *Ty-1* position, should have segregated for resistance, but in fact bred true for susceptibility. Moreover, segregation in the U-A2 and M-G6 families places *Ty-1* above marker MSc05732-14 (Table 2). Results from all other tested recombinant families support the location of *Ty-1* between marker MSc05732-4 and MSc05732-14 (Table 2), a marker interval that partly overlaps with the mapped marker interval of *Ty-3*.

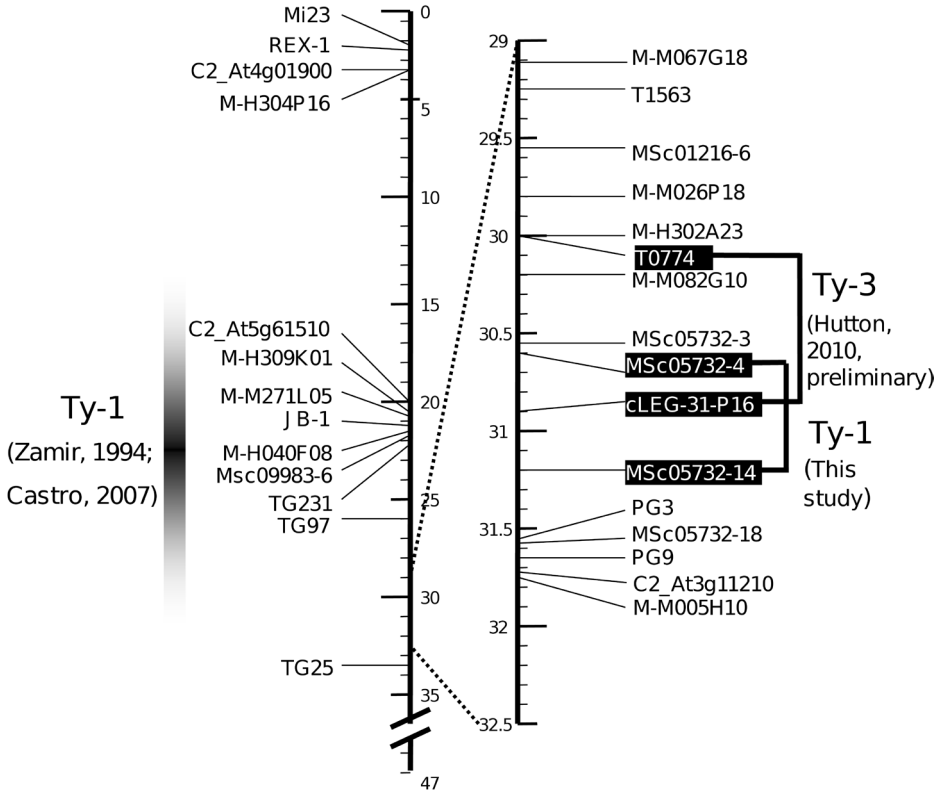

Figure 4. Schematic physical maps of the short arm, the centromere and a part of the long arm of chromosome 6. Numbers given represent millions of basepairs. The position of the markers was based on BLAST results on the Tomato WGS 2.31 Chromosomes database.

Discussion

Although five TYLCV resistance loci are available, introgressions of *Ty-1* from *S. chilense* LA1969 have been so far a major focus in breeding programs (Zamir et al., 1994; Ji et al., 2007a; Pérez de Castro et al., 2007; Vidavski, 2007). A lack of knowledge about the exact location of *Ty-1* on tomato chromosome 6 has hindered efforts to reduce the size of the *Ty-1* introgression. Consequently, *Ty-1* introgressions are often accompanied by linked detrimental traits. In this study, FISH and large-scale recombinant screening was applied to analyze the chromosomal structure and recombination behavior in the chromosomal region where *Ty-1* is introgressed. Our results show that precise mapping of *Ty-1* has been hampered by chromosomal inversions between *S. lycopersicum* and *S. chilense* LA1969 and that *Ty-1* is actually located on the long arm of tomato chromosome 6, near the chromosomal region where *Ty-3* is mapped. Moreover, our study demonstrates that FISH is not only a very helpful tool to reveal the occurrence of chromosomal rearrangements but also a diagnostic technique if one wants to correctly map introgressed genes from wild species.

***Ty-1* and *Ty-3* are likely located in an overlapping chromosomal region**

Results presented here map *Ty-1* between markers MSc05732-4 and MSc05732-14, a region of approximately 600 Kb. This interval was surprising, as it is nearly 5 Mb below the reported map position. Zamir, et al. (1994) originally mapped *Ty-1* to an approximately 40 cM introgression (spanning the restriction fragment length polymorphism (RFLP) markers TG297, TG97 and TG119, as well as the *Mi* locus for *S. peruvianum*-derived nematode resistance). These authors also identified a resistant RIL with an LA1969 introgression spanning the upper portion of this introgression to TG97 but lacking the lower region represented by TG119. They assumed that the tolerance of some *S. peruvianum* accessions to TYLCV might be based on *Ty-1*, so different nematode-resistant lines containing *Mi* introgressions from TG297 to TG97 were examined for their response to the virus, but all were susceptible. Based on these findings, it was concluded that *Ty-1* was linked to TG97 and located below the marker. This conclusion, however, did not consider that TYLCV resistance in *S. peruvianum* accessions might be conferred by a locus other than *Ty-1*; this is confirmed by the recent mapping of *Ty-5* from *S. peruvianum* to chromosome 4 (Ji et al., 2007b). Further, mapping *Ty-1* near TG97 did not consider the possibility that the introgression in the RIL might extend well below TG97. Better marker coverage would have shown this, as we demonstrate in the present study by revealing that TY52—developed from the original *Ty-1* mapping work—has

an introgression extending into the 600 Kb genic region. It appears that subsequent approaches to more precisely map *Ty-1* were based on the originally reported position. These attempts were unsuccessful in correctly locating the gene because they targeted recombination in a nearby but non-genic region.

The resistance gene *Ty-3*, derived from either *S. chilense* LA2779 or LA1932 accessions, was also recently mapped to the long arm of chromosome 6 between markers cLEG-31-P16 and T1079 (Ji et al., 2007a). However, as with the original *Ty-1* work, defining the location of *Ty-3* within this interval did not take into account how far the introgression might extend above cLEG-31-P16 or below T1079, into regions where there was previously no marker coverage. In fact, preliminary data suggests that *Ty-3* is located between T0774 and cLEG-31-P16 (Hutton et al., 2010). The introgression present in Su09E941-164-1 is shown to extend approximately 300 Kb above cLEG-31-P16, beginning below MSc05732-4 (Table 3). Together, these data indicate that *Ty-1* and *Ty-3* are likely located in an overlapping chromosomal region and may be allelic (Figure 4). Efforts are underway to fine-map both genes to determine if this is indeed the case.

BAC-FISH can be used as a diagnostic tool in introgression breeding

Our initial aim was to fine-map the *Ty-1* locus on tomato chromosome 6, but screening a large number of plants for recombinants revealed a strong suppression of recombination in the *S. chilense* LA1969 introgressed region. This was shown to be the case in two independent commercial *Ty-1* hybrids. With the use of FISH, we uncovered that this suppression was caused by two inversions between *S. chilense* LA1969 and *S. lycopersicum* in the pericentromere heterochromatic regions of both the short and long arms of chromosome 6 (Figure 3). Due to these inversions, chromosomal pairing during meiosis has been interrupted. The presence of these inversions has caused recombination suppression, which contributed to the failure to accurately map the *Ty-1* locus. In short, because recombination was suppressed in this region, the association of resistance with markers on the short arm (such as REX-1) (Milo, 2001) was no less accurate than the mapping of *Ty-1* near markers on the long arm (such as CT21) (Pérez de Castro et al., 2007). Furthermore, the large introgression present in *Ty-1* hybrids shows that inverted chromosomal regions are introgressed as a whole. It is not yet clear whether the same inversion is present in other *S. chilense* accessions, although the suppression of recombination in this region in P-3 indicates a likely inversion in *S. chilense* LA2779. Chromosomal rearrangements between two related species have previously been described, e.g. in the Mi-1 cluster between *S. lycopersicum* and *S. peruvianum*; the *sun* locus between *S. lycopersicum* and *S. pimpinellifolium*; and the short arm of chromosome

6 between *S. lycopersicum* and *S. tuberosum* (Seah et al., 2004; Van der Knaap et al., 2004; Tang et al., 2008). Recently, Szinay et al. (2010) described a comprehensive study on a large series of chromosomal rearrangements among *Solanum* species, which altogether suggests that rearrangements as described here are not unique.

For many traits the gene pool of *S. lycopersicum* is quite narrow, which has forced breeders to use related wild species in breeding programs. This so-called "introgression breeding" allows access to the variation present in numerous *Solanum* accessions. Our results suggest that this type of breeding can be hampered by chromosomal rearrangements between related species and that these rearrangements can thus influence the success of introgression breeding when interspecific crosses are used. Herein we present the concept on how to apply BAC-FISH as a diagnostic tool to investigate chromosomal rearrangements in genetic mapping and introgression breeding, which will have a potential impact on introgression breeding in many crops. Furthermore, the occurrence of chromosomal rearrangements stresses the importance of a physical map to order scaffolds if related Solanum species are being sequenced (current efforts of large sequencing consortia).

Impact of the presented results on plant breeding

Disease resistance genes in the tomato genome tend to be clustered as opposed to randomly distributed (Yang and Francis, 2007), and repulsion linkages can be problematic for tomato breeders who want to combine resistance genes that are closely linked. Doing this becomes extremely difficult when introgressions from wild species overlap and there is recombination suppression as has been shown in this study. The general genomic region where *Ty-1* and *Ty-3* reside has the most reported disease resistance genes in the entire genome. Besides the mentioned resistance to root knot nematode are genes for resistance to *Ralstonia solanacearum* (bacterial wilt), *Clavibacter michaginensis (bacterial canker), Oidium lycopersicum* (powdery mildew) and *Cladosporium fulvum* (leaf mold). It will be useful to fine map these resistance genes and develop lines with minimal introgression sizes to facilitate combining of the genes in cis and to eliminate any associated linkage drag. Once combined, the close linkages will be beneficial for keeping the genes together.

The present work shows that *Ty-1* and *Ty-3* are very close and are perhaps allelic. This finding has important breeding implications. It reduces the likelihood that the genes can be pyramided homozygously but instead points to the making of hybrids heterozygous for both genes / alleles by combining parents homozygous for *Ty-1* with parents homozygous for *Ty-3*. The *Ty-1* locus has shown dominance for TYLCV

resistance, but has been ineffective against some TYLCV strains (Scott, 2007) and against bipartite begomoviruses (Mejia et al., 2005). The *Ty-3* locus has generally shown less dominance but a wider range of resistance against TYLCV strains and bipartite begomoviruses. Hybrids with the heterozygous combination of both genes/alleles may prove to be effective and durable against a wide array of such viruses. Vidavsky (2007) showed that combining different begomovirus resistance genes can have unanticipated synergistic effects, and this combination should be tested in this regard.

Materials and methods

Plant material

Two commercial hybrids derived from different parental lines were used to produce two F2 populations (P-1 and P-2) in which Ty-1 was segregating. For both F2 populations multiple F1 plants were selfed to get enough F2 seeds. These two hybrids were provided by breeding companies within the cooperative framework of the Centre for BioSystems Genomics (CBSG). The *Ty-1* locus was introgressed from *S. chilense* LA1969 in the genetic background of cultivated tomato *S. lycopersicium*. These two F2 populations were used for recombinant screenings and selected recombinants were selfed to produce F3 families for further testing with TYLCV. As a susceptible control, *S. lycopersicum* cv Moneymaker was included.

Another population (P-3) was developed in a *Ty-3* fine-mapping effort at University of Florida, USA. F2 plants segregating for a large *Ty-3* introgression from *S. chilense* accession LA2779 (spanning approximately 20 cM from the *Mi* locus to M-M005H10) were screened for recombination events within this interval. Su09E941-164-1 is a recombinant inbred line (RIL) developed from this population; resistance to TYLCV in this line has been confirmed over multiple seasons and is conferred by an approximately 4 cM *Ty-3* introgression that does not overlap the previously reported *Ty-1* region by Zamir et al.(1994) (Hutton, unpublished). TY52 is an introgression line that contains *Ty-1* introgression from *S. chilense* LA1969 (Michelson et al., 1994).

Markers

All markers are PCR-based CAPS markers taken from publicly available data (Table S1). Tomato BACs in the region of *Ty-1* and *Ty-3* were selected according to a physical map of tomato chromosome 6 (Tang et al., 2008; Peters et al., 2009) (Figure 2) and sequences were downloaded from the SOL website (http://solgenomics.net/). For each BAC

multiple random primer pairs, which would result in products of approximately 800 bp, were designed using PrimerBLAST from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Since the parental lines of the F2 populations could not be obtained due to confidentiality, resistant and susceptible F2 plants were selected. Two DNA-pools were made containing DNA of either 10 resistant (R) or 10 susceptible (S) F2 plants. These pools (R-pool and S-pool) were used for marker development. In case PCR products obtained from both pools showed no length polymorphism, several restriction enzymes were used to search for polymorphisms. BAC-derived markers are named after the BAC name, e.g. M-H304P16 means marker (M) generated from the sequence of BAC H304P16 (Table S1). Scaffolds that covered the gaps between the BAC contigs were also selected and used for marker development using the same strategy as described.

Recombinant screening

For P-1 and P-2 leaf samples were collected from two-week-old seedlings and DNA was extracted according to the NaOH extraction method as described (Wang et al., 1993), with some slight modifications. In brief, small leaf samples were ground for 5 minutes in 20 µl 0.5 M NaOH using a tissue striker (Kisan Biotech™). After addition of 20 µl of 100 mM Tris (pH 7.5), 5 µl was added to 200 µl 100 mM Tris (pH 7.5) to prepare (crude) genomic DNA template for polymerase chain reaction (PCR). For P-3, tissue was collected according to the methods described above and frozen at -80°C. Frozen leaf samples were then ground for 2 minutes using a high throughput homogenizer (Talboys by Troemner), and DNA was extracted according to the method described by (Fulton et al., 1995). PCR amplification was performed according to standard protocols in either an Applied Biosystems GeneAmp 2700 system or an Eppendorf Mastercycler® pro.

TYLCV inoculation and disease evaluation

For P-1, two week old seedlings were put in a cage in the presence of whiteflies carrying TYLCV-Alm. After 4 days, whiteflies were killed by adding Admire (imidocloprid) (Bayer Cropscience) to the soil. After another two weeks plants were scored as R (no symptoms) or S (showing curling and yellowing of the leaves).

To test plants of recombinant families selected from P-1, agroinoculation was used. An infectious TYLCV clone (pTYCz40a, kindly provided by Dr. Eduardo Rodríguez Bejarano, Universidad de Malaga) was transformed to Agrobacterium tumefaciens strain LBA4404 and used to agroinoculate tomato seedlings. To this end, A. tumefaciens containing the TYLCV clone was grown overnight at 28 °C in 3 ml LB medium (10 gL-1 trypton, 5 gL-1 yeast, 4 gL-1 NaCl, 1 gL-1 KCl, 3 gL-1 MgSO4.2H2O). From the overnight

culture 600 µl was transferred to 3 ml induction medium (10.5 gL-1 K2HPO44, 4.5 gL-1 KH2PO4, 1 gL-1(NH4)2SO4, 0.5 gL-1 Sodium citrate.2H2O, 1 mM MgSO4.7H2O, 0,2% (w/v) glucose, 0,5% (v/v) glycerol; after autoclaving 50 µM acetosyringone and 10 mM MES (pH 5.6) are added) and grown overnight at 28°C. Bacteria were pelleted by centrifugation for 10 min at 2000 g and resuspended in MS medium (supplemented with 150 µM acetosyringone and 10 mM MES) at an OD600 of 0.5. The first pair of true leaves of three weeks old seedlings were agroinoculated with the TYLCV construct by pressure inoculation with a syringe.

Plants were kept under greenhouse conditions at a temperature of 23 °C and relative humidity of 60% during a 16-hour day / 8-hour night regime. Four weeks after agroinfiltration, plants were evaluated for TYLCV symptoms by using the disease severity index as described by (Lapidot and Friedmann, 2002).

Fluorescence in situ Hybridization (FISH)

Slide preparation: Young flower buds were collected in the greenhouse and fixed overnight in fresh Carnoy solution (1:3=acetic acid:ethanol), then transferred to 70 % ethanol for storage at 4 °C. Slides were prepared according to (Szinay et al., 2008) without post-fixation with 1 % formaldehyde.

BAC and COT 100 isolation and labelling: BAC DNA was isolated using High Pure Plasmid Isolation Kit (Roche 11754785001). Isolated DNA was labeled by nick translation according to the manufacturer's protocol of Roche (http://www.roche.com). Probes were labeled either directly with Cy3-dUTP (Amersham, http://www.amershambiosciences.com/), Cy3.5-dCTP (Amersham) and Diethylaminocoumarin-5-dUTP (DEAC) (Perkin Elmer, http://www.perkinelmer.com) or indirectly with biotin or digoxigenin. Cot 100 isolation, necessary to block repeats present in most of the BACs, was performed according to (Szinay et al., 2008).

Procedure, microscopy and data analysis: FISH experiments were performed according to (Szinay et al., 2008) with some slight modifications. Probes were hybridized for 48 hours at 37 °C (Rens et al., 2006). For the stringent washing, 64% formamide was used 3 times for 5 min at 42 °C (Schwarzacher and Heslop-Harrison, 2000). Biotin labeled probes were amplified using streptavidin - Cy5 and biotinylated anti-streptavidin. Digoxigenin labeled probes were amplified using antidig FITC and antisheep FITC. Microscopy and data analysis were performed according to (Szinay et al., 2008).

Acknowledgements

Much of this project was carried out within the research programme of the Centre of

BioSystems Genomics (CBSG), which is part of the Netherlands Genomics Initiative/ Netherlands Organization for Scientific Research. Another part of this research was supported by a USDA CREES-NRI (now AFRI) grant. The infectious TYLCV clone was kindly provided by Professor Eduardo Rodríguez Bejarano (Universidad de Málaga). We thank Dr. Ir. Dick Peters for his help with the whitefly transmission and Dick Lohuis for his help with agro-infiltrations. We thank Tingting Xiao, Xiaoxin Liu and Ada Lucia Angulo Fernandez for their help with marker development and recombinant screenings. Special thanks is also given to Dolly Cummings and Jose Diaz for help with DNA extractions and recombinant screenings.

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Supplementary table: CAPS markers on chromosome 6.

Name		Sequence 5′-3′	Annealing temperature	Restriction enzyme	Reference
Mi23	F	TGGAAAAATGTTGAATTTCTTTTG	57°C	BstNI	(Seah et al., 2007)
	R	GCATACTATATGGCTTGTTTACCC			
REX-1	F	TCGGAGCCTTGGTCTGAATT	55°C	TaqI	(Castro et al., 2007)
	R	ATGCCAGAGATGATTCGTGA			
Aps-1	F	GGCAGGAGAATATGCCAAAA	55°C	TaqI	(Castro et al., 2007)
	R	CGTTCCATTCTCAACCCATT			
C2_ At4g01900	F	TCATTTCAAAATCCAATTTCTCC	55°C	HinfI	SGN
	R	TGGGCGCCAAAACCACGAAC			
M-H304P16	F	AGCCCCCAGAAAGACTTGTT	60°C	HpyF3I	Present
	R	TTTTTAAGGGGTGTGCCAAG			
C2_ At5g61510	F	AGTTCCTACTGCGCCGCTGCTTC	55°C	HinfI	SGN
	R	AGCATGAACAAGTACTGTGTGCCCACG			
M-H309K01	F	ACCGGTGCATATAGAGGTCG	55°C	TaqI	Present
	R	TGAAGGGCAAGTCTCCCATA			
M-H271L05	F	GGAAAGCAAAGAAGGCAGTG	60°C	HpyF10VI	Present
	R	AGCCTCTACAAGCACCTCCA			
JB-1	F	AACCATTATCCGGTTCACTC	55°C	TaqI	(Castro et al., 2007)
	R	TTTCCATTCCTTGTTTCTCTG			
M-H040F08	F	AATTACCGCTTCCTCCAGGT	60°C	HpyCH4IV	Present
	R	AATGTCTCCCCAAACAGCAC			
MSc09983-6	F	GCTCCCCAACTCGCAACCTGC	60°C	BseDI	Present
	R	TGGCTCCATTCGAACCGCCA			
TG231	F	CCATCCTGATTGAAGGGAAACAAGC	55°C	TaqI	(Ji et al., 2007a)
	R	CTAGATGAAATGTACCATGCTGCCC			
TG97	F	CACCACATAATTGAGAAGGACAACAC	55°C	TasI	(Ji et al., 2007a)
	R	CATCATTGCTATTGAAGTCATCCG			
M-M067G18	F	CGACTCGTCATCTATCGCAA	55°C	RsaI	Present
	R	TTCTTGAAGGTGCTTGGCTT			
T1563	F	ACTTCACCTACAAATCCTTCCAGA	56°C	TaqI	(Ji et al., 2007a)
	R	GCCCTTCCCAATCCAGCAGT			
MSc01216-6	F	CGCTCGGCCTCGGCAAATGA	60°C	BspLI	Present
	R	CAGCCGGCGCTAAGGCATCA			

M-M026P18	F	GCATGTGTGCAGCTCACTCTCCC	60°C	AluI	Present
	R	TCAAGTCCGAATCGAAGCCCCA			
M-H302A23	F	TCCCGTCTCCTGCACCTACTTCT	60°C	HpyF10VI	Present
	R	AAAGGGGTGGTGCTCGCCCT			
M-M082G10	F	GGCATCGCCATCATCTCTAAGTCCA	60°C	FspBI	Present
	R	GCCTCAACCTACTGCCTTGCAAAT			
MSc05732-3	F	ATGCTTTTCGAGCACGAGCCT	55°C	RsaI	Present
	R	AGCCTAAAGAGAACTAGGCAGGGGA			
MSc05732-4	F	ACGAGATGGAGCGGTCTTCAAGCT	55°C	Dde	Present
	R	GACAGATCTCCCGGTAGGAGAGCA			
MSc05732-14	F	GTGGGGCCTCGATCCCAGTCA	55°C	NcoI	Present
	R	GGCCCTTTAGTGTGTTTCACACCT			
MSc05732-18	F	TTGAGTCTGGCCTGCTCTGAATCT	55°C	*Alu*I	Present
	R	CATTCTGCTCGTCTTCAGAACACCTC			
PG9	F	CAATACACAAGGCTAACGCAGGCA	55°C	*Hae*III	(Ji *et al.*, 2007a)
	R	AACATCCATCCCAGACAGCACCT			
C2_At3g11210	F	AGGCCTGTATAGAGCTATGCAAAGAG	60°C	*Hinf*I	SGN
	R	AATTCTGTTGCCATTGATTTCCAGTG			
M-M005H10	F	AAATCACCTTCCACAGTGCAG	55°C	*Rsa*I	Present
	R	CTGGCCATAAAGTCTGGACAA			

Chapter 3

The tomato yellow leaf curl virus resistance genes *Ty-1* and *Ty-3* are allelic and code for DFDGD-class RNA-dependent RNA polymerases

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Published as
Maarten G. Verlaan, Samuel F. Hutton, Ragy M. Ibrahem, Richard Kormelink, Richard GF Visser, John W. Scott, Jeremy D. Edwards, Yuling Bai (2013) The Tomato Yellow Leaf Curl Virus Resistance Genes *Ty-1* and *Ty-3* are Allelic and Code for DFDGD-class RNA-dependent RNA Polymerases, PLoS Genetics 9(3): e1003399

Summary

Tomato Yellow Leaf Curl Virus Disease incited by *Tomato yellow leaf curl virus* (TYLCV) causes huge losses in tomato production worldwide and is caused by different related begomovirus species. Breeding for TYLCV resistance has been based on the introgression of multiple resistance genes originating from several wild tomato species. In this study we have fine mapped the widely used *S. chilense* derived *Ty-1* and *Ty-3* genes by screening nearly 12,000 plants for recombination events and generating recombinant inbred lines. Multiple molecular markers were developed and used in combination with disease tests to fine map the genes to a small genomic region (approximately 70 kb). Using a Tobacco Rattle Virus-Virus Induced Gene Silencing approach, the resistance gene was identified. It is shown that *Ty-1* and *Ty-3* are allelic and that they code for a RNA-dependent RNA polymerase (RDR) belonging to the RDRγ type which has an atypical DFDGD motif in the catalytic domain. In contrast to the RDRα type, characterized by a catalytic DLDGD motif, no clear function has yet been described for the RDRγ type and thus the *Ty-1*/*Ty-3* gene unveils a completely new class of resistance gene. Although speculative, the resistance mechanism of *Ty-1*/*Ty-3* and its specificity towards TYLCV is discussed in light of the function of the related RDRα class in the amplification of the RNAi response in plants and transcriptional silencing of geminiviruses in plants.

Introduction

Plant pathogens are a major limiting factor for agricultural productivity worldwide. Viruses are among these and cause large yield losses in a variety of economically important crops. Although most viruses have small genomes and code for a very limited amount of proteins, they can cause a variety of disease symptoms, and the mechanisms underlying these are still mostly unknown. Plants utilize several lines of defense mechanisms to protect themselves from pathogen invasion. The mechanism that has been studied the most is resistance (R) gene-mediated resistance, which relies on the ability of a plant to recognize a pathogen and consequently trigger the hypersensitive cell death response (HR) (Jones and Dangl, 2006). Meanwhile, a large number of R genes have been identified, including ones responsible for the (in)direct recognition of viruses, such as *Sw-5* for tospoviruses in tomato (Brommonschenkel et al., 2000), *Rx2* for *Potato virus X* (Bendahmane et al., 2000) and the *I* locus for *Bean common mosaic virus* (Vallejos et al., 2006). In addition to these dominant R genes, a second type of resistance gene is inherited recessively, which is more common in resistances to viruses compared with resistance to fungi or bacteria (Kang et al., 2005; Truniger et al., 2009). Most of these genes are linked to the eukaryotic translation initiation complex and negatively affect the viral RNA replication cycle (Robaglia and Caranta, 2006).

RNA silencing (also called RNA interference, RNAi), is a conserved eukaryotic gene regulation mechanism that involves the biogenesis of small (s)RNA molecules of ~21-26 nucleotides in size from perfect or imperfect long double stranded (ds)RNA molecules by an enzyme designated Dicer (mammals, insects), or Dicer-like protein (DCL) (plants) (Bernstein et al., 2001). One strand of these sRNA molecules is incorporated into an RNA-induced silencing complex (RISC) and enables the latter to sense and target RNA molecules with sequence complementarity to the uploaded RNA strand for degradation or translational arrest by means of the core Argonaute (AGO) protein. In recent years, RNA silencing has become known as an antiviral defense mechanism in plants and insects in which viral double-stranded RNA replicative intermediates or secondary RNA folding structures are cleaved into primary, small-interfering (si)RNA molecules. In plants, the viral primary siRNA molecules also act as primers for the host RNA-dependent RNA polymerases (RDR) to convert (aberrant) RNA target sequences into new long dsRNAs. These in turn become processed into secondary siRNAs. This not only leads to an amplification of the siRNA signal, but also results in a distributional spread of siRNA molecules from the entire RNA target sequence, referred to as transitive silencing (Sijen et al., 2001). The amplification of siRNAs is required to mount a strong

antiviral RNAi response. Arabidopsis *RDR1*, *2* and *6*, and orthologs of these genes, have been demonstrated to be involved in this amplification and plants from which these genes have been knocked-out exhibit higher susceptibility to various plant viruses (Schwach et al., 2005; Diaz-Pendon et al., 2007; Pandey et al., 2008; Garcia-Ruiz et al., 2010; Wang et al., 2010).

The whitefly transmitted tomato yellow leaf curl disease (TYLCD) is one of the most devastating diseases of tomato (*Solanum lycopersicum*) and is caused by several species of the *Begomovirus* genus (*Geminiviridae*) (Fauquet et al., 2008). Tomato yellow leaf curl viruses (TYLCV) are the most widespread and currently rank 3rd among the economically and scientifically most important plant viruses worldwide (Scholthof et al., 2011). They have a single-stranded circular bi-directionally organized DNA genome with six partially-overlapping open reading frames (Gronenborn, 2007). Because of their limited coding capacity they rely, like most viruses, not only on their own proteins but also on the host cell machinery for their infection cycle (Hanley-Bowdoin et al., 2004). Since the whitefly insect vector is hard to control, breeding TYLCV resistant tomato cultivars provides an attractive strategy to manage TYLCV. All domesticated tomatoes are susceptible to TYLCV, but high levels of resistance were found in several related wild tomato species. Genetic studies have led to the mapping of five TYLCV resistance/tolerance genes which are being exploited for resistance breeding. These genes have different origins: *Ty-2* was introgressed from *S. habrochaites*, *Ty-5* (*ty-5*) was introgressed from *S. peruvianum* while *Ty-1*, *Ty-3* and *Ty-4* all originated from different *S. chilense* accessions (Zamir et al., 1994; Hanson et al., 2006; Ji et al., 2007; Anbinder et al., 2009; Ji et al., 2009; Hutton et al., 2012). So far, none of these genes have been cloned and the underlying resistance mechanisms are still unknown. In contrast with classical R-genes none of the resistances to TYLCV described so far are associated with a HR. Moreover, in almost all TYLCV resistant materials, viral replication occurs (Pico et al., 1999; Pico et al., 2000; Narasegowda Maruthi et al., 2003; Perez de Castro et al., 2005). This also holds true for *Ty-1*/*Ty-3*, where in the donors (*S. chilense* LA1969/LA1932) as well as in a commercial line with a *Ty-1* introgression (3761, A.B. Seeds, Ness Ziona, Israel) TYLCV is replicating and detectable (Pérez de Castro et al.; Fargette et al., 1996; Lapidot et al., 1997), although the level does not exceed more than 10% of that in susceptible tomato cultivars.

Though many loci (i.e. *Ty-1* to *Ty-5*) for TYLCV resistance have been described, the genes conferring resistance have not been identified. Recently, several papers have reported on host genes in a gene network contributing to the resistance originating from *S. habrochaites* (Eybishtz et al., 2009b; Eybishtz et al., 2009a; Sade et al., 2012). By

differential cDNA library comparisons of susceptible and resistant tomato lines before and after TYLCV inoculation, approximately 70 genes were found to be preferentially expressed in a tomato line with a resistance introgressed from *S. habrochaites*. For three of those, a lipocalin-like protein (*SlVRSLip*), a *Permease I-like protein* and a hexose transporter *LeHT1*, it was shown that their silencing (partly) compromised resistance.

In our previous study we found that *Ty-1* and *Ty-3* map closer than previously reported and that they might be allelic (Verlaan et al., 2011). In the present study *Ty-1* and *Ty-3* are fine mapped, and using a Tobacco Rattle Virus (TRV) induced silencing approach, the genes have been identified and found to be allelic. They code for an RNA-dependent RNA polymerase (RDR) of the γ class, a class of RDRs for which no function is yet described. The role of this new class of resistance genes will be discussed in light of the TYLCV infection cycle.

Results

Fine-mapping of *Ty-1* and *Ty-3*

Previously, we mapped *Ty-1* in the interval between MSc05732-4 and MSc05732-14 (Verlaan et al., 2011). To fine-map *Ty-1*, markers T0774 and SL_2.40ch06_30.891, which flank this interval, were used to screen an F_2 population derived from a cross between the susceptible Fla. 7776 and a recombinant inbred line (RIL) carrying the *S. chilense Ty-1* introgression. Approximately 2,000 F_2 plants were screened, 13 recombinants were identified, and RILs were developed for each of these (designated R1 to R13). Four RILs (R1, 4, 12 and 5) containing the *S. chilense* introgression between markers Hba0161K22 and WU_M31 were resistant, while eight RILs that lacked this interval were susceptible (Figure 1A). R7, which resulted from a recombination event between markers WU-M27 and UF_TY3-P19, showed an intermediate response. These results were confirmed for the three most informative recombinants (R7, R8 and R11) (Table S1) using agroinoculation and show that *Ty-1* is located between HBa0161K22 and WU_M31, an interval of approximately 70 kb.

The *Ty-3* gene was previously mapped between T0774 and T1079 (Ji et al., 2007). By screening an F_2 population (n=717) from a cross between the susceptible line Fla. 7781 with the resistant line Fla. 8680 (carrying the *Ty-3* introgression from *S. chilense* LA2779), 30 recombinants were identified. RILs of these recombinants were generated and tested with TYLCV. Results mapped *Ty-3* to the interval between T0774 and P6-25 (Table S2). To further narrow down the *Ty-3* interval, RILs of two key recombinants

were used to generate three F_2 sub-populations, A, B and C. Screening more than 10,500 individuals of these sub-populations with markers Mi23 and P6-25 (sub-population A and B) and markers T0774 and T0834 (sub-population C) identified 309 recombinants (Table S3). Cuttings of these recombinants were evaluated for TYLCV disease severity (Table S3; control experiments, Table S4) and interval QTL mapping confirmed the location of *Ty-3* between markers T0774 and P6-25, with a LOD of over 50 in an interval between markers SL_2.40ch06_30.696 and cLEG-31-P16 (Figure S1). Recombinants in this interval were further analysed by testing their RILs with TYLCV and by saturating this region with additional molecular markers (Figure 1B). RILs carrying the *S. chilense* LA2779 introgression between markers UF_TY3_P1 and UF_TY3_P23 were resistant (recombinant class C to I, Figure 1B), while RILs with introgressions that did not span this region were susceptible; these results map *Ty-3* to a region of approximately 71kb that overlaps the region containing *Ty-1* (Figure 2).

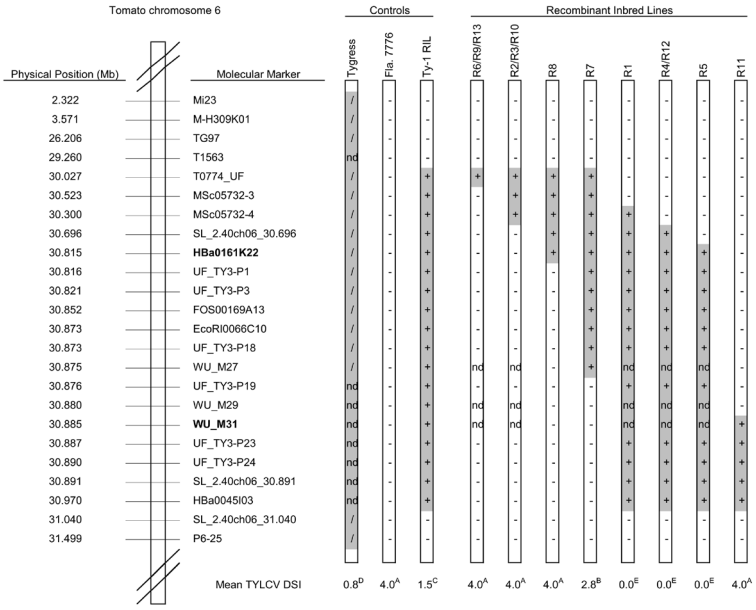
Figure 1. Physical maps showing control lines and introgressed fragments in the RILs used to map *Ty-1* and *Ty-3*.

Introgressed segments of the S. chilense genome are shaded grey; genotype for each line at each marker is indicated (+ = homozygous *S. chilense*; / = heterozygous; - = homozygous *S. lycopersicum*; nd = not determined). Approximate physical positions are based on the tomato genome assembly SL_2.40, available through the Sol Genomics Network (SGN; http://solgenomics.net/). DSI = mean disease severity index as described in the Materials and Methods; within either population, different superscript letters represent statistically significant differences at $P < 0.05$ based on Duncan's multiple range test.

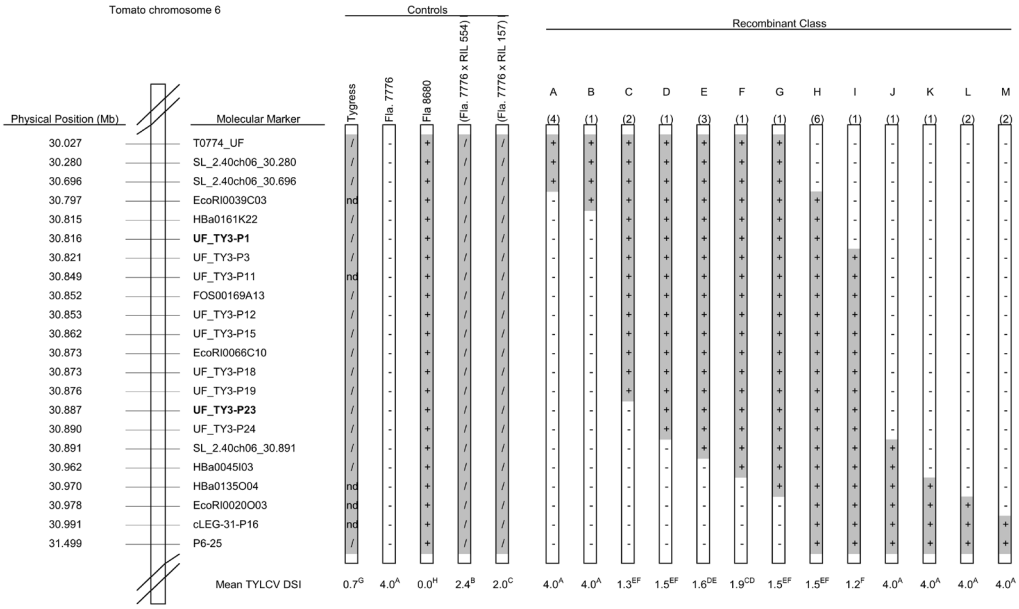
A: Control lines and RILs used for mapping of *Ty-1*. Flanking markers of the *Ty-1* region, HBa0161K22 and WU_M31, are depicted in bold.

B: Control lines and RILs used for mapping of *Ty-3*. The number of recombinants recovered in each class is given in parentheses above each recombinant chromosome. Flanking markers, UF_TY3-P1 and UF_TY3-P23 of the *Ty-3* region are depicted in bold.

A


Tomato chromosome 6
Controls
Recombinant Inbred Lines
Physical Position (Mb)
Molecular Marker
Tygress
Fla. 7776
Ty-1 RIL
R6/R9/R13
R2/R3/R10
R8
R7
R1
R4/R12
R5
R11
2.322 Mi23
3.571 M-H309K01
26.206 TG97
29.260 T1563
30.027 T0774_UF
30.523 MSc05732-3
30.300 MSc05732-4
30.696 SL_2.40ch06_30.696
30.815 HBa0161K22
30.816 UF_TY3-P1
30.821 UF_TY3-P3
30.852 FOS00169A13
30.873 EcoRI0066C10
30.873 UF_TY3-P18
30.875 WU_M27
30.876 UF_TY3-P19
30.880 WU_M29
30.885 WU_M31
30.887 UF_TY3-P23
30.890 UF_TY3-P24
30.891 SL_2.40ch06_30.891
30.970 HBa0045I03
31.040 SL_2.40ch06_31.040
31.499 P6-25
Mean TYLCV DSI
0.8D 4.0A 1.5C 4.0A 4.0A 4.0A 2.8B 0.0E 0.0E 0.0E 4.0A

B


Tomato chromosome 6
Controls
Recombinant Class
Physical Position (Mb)
Molecular Marker
Tygress
Fla. 7776
Fla 8680
(Fla. 7776 x RIL 554)
(Fla. 7776 x RIL 157)
A (4) B (1) C (2) D (1) E (3) F (1) G (1) H (6) I (1) J (1) K (1) L (2) M (2)
30.027 T0774_UF
30.280 SL_2.40ch06_30.280
30.696 SL_2.40ch06_30.696
30.797 EcoRI0039C03
30.815 HBa0161K22
30.816 UF_TY3-P1
30.821 UF_TY3-P3
30.849 UF_TY3-P11
30.852 FOS00169A13
30.853 UF_TY3-P12
30.862 UF_TY3-P15
30.873 EcoRI0066C10
30.873 UF_TY3-P18
30.876 UF_TY3-P19
30.887 UF_TY3-P23
30.890 UF_TY3-P24
30.891 SL_2.40ch06_30.891
30.962 HBa0045I03
30.970 HBa0135O04
30.978 EcoRI0020O03
30.991 cLEG-31-P16
31.499 P6-25
Mean TYLCV DSI
0.7G 4.0A 0.0H 2.4B 2.0C 4.0A 4.0A 1.3EF 1.5EF 1.6DE 1.9CD 1.5EF 1.5EF 1.2F 4.0A 4.0A 4.0A 4.0A

Candidate genes for *Ty-1* and *Ty-3*

According to the ITAG2.3 release of the tomato genome, the region to which *Ty-1*/*Ty-3* mapped was predicted to contain five genes; Solyc06g051160 (408 bp), Solyc06g051170 (1728 bp), Solyc06g051180 (438 bp), Solyc06g051190 (957 bp) and Solyc06g051200 (843 bp) (Bombarely et al., 2011) (Figure 2). While gene Solyc06g051160 has an unknown function and Solyc06g051200 encodes a predicted ribosomal protein, the other three genes are each predicted to encode (parts of) an RNA-dependent RNA polymerase (RDR). *Arabidopsis thaliana* RDRs in general are approximately 3kb in size, but these three predicted genes are all much shorter. Since the genes only share low homology they likely are not paralogous. Interestingly, the crossing-over event in the intermediate resistant R7 occurred within the candidate gene Solyc06g051190. After amplification and sequence analysis of this gene from R7 and subsequent alignment to the corresponding regions of a *Ty-1* line and a *ty-1* line, the recombination site in R7 could be pinpointed between two SNPs. This region covered less than 100 base pairs in which the recombination point mapped to the last part of predicted exon number 4 (Figure S2 and S3). Plants of R7 thus contained a chimeric predicted gene Solyc06g051190.

Silencing of Solyc06g051180 and Solyc06g051190 compromises resistance

To identify the *Ty-1* gene from the five candidate genes predicted in the *Ty-1* interval, a Tobacco Rattle Virus (TRV) based Virus Induced Gene Silencing (VIGS) approach was applied. For three out of five genes a VIGS construct could be made; TRV2-160 for Solyc06g051160; TRV2-180 for Solyc06g051180 and TRV2-190 for Solyc06g051190. The two VIGS vectors, TRV2-180 and TR2-190, are specific and both are assumed to target an individual RDR, due to low homology between Solyc06g051180 and Solyc06g051190. Several attempts to make a VIGS construct for Solyc06g051170 and Solyc06g051200 failed so experiments were done with the available constructs. When plants containing *Ty-1* were agroinfiltrated with empty vector control (EV, TRV2 without an insert) or TRV2-160, and two weeks later superimposed with a TYLCV challenge, the plants maintained resistance to TYLCV. However, when either TRV2-180 or TRV2-190 was used, the resistance was compromised as observed by the appearance of TYLCV disease symptoms (Figure 3). Repeated analysis confirmed these results, which, together with the fact that both Solyc06g051180 and Solyc06g051190 are predicted RDRs located in close proximity to one another within the *Ty-1*/*Ty-3* region, suggest that Solyc06g051180 and Solyc06g051190 might belong to one and the same gene.

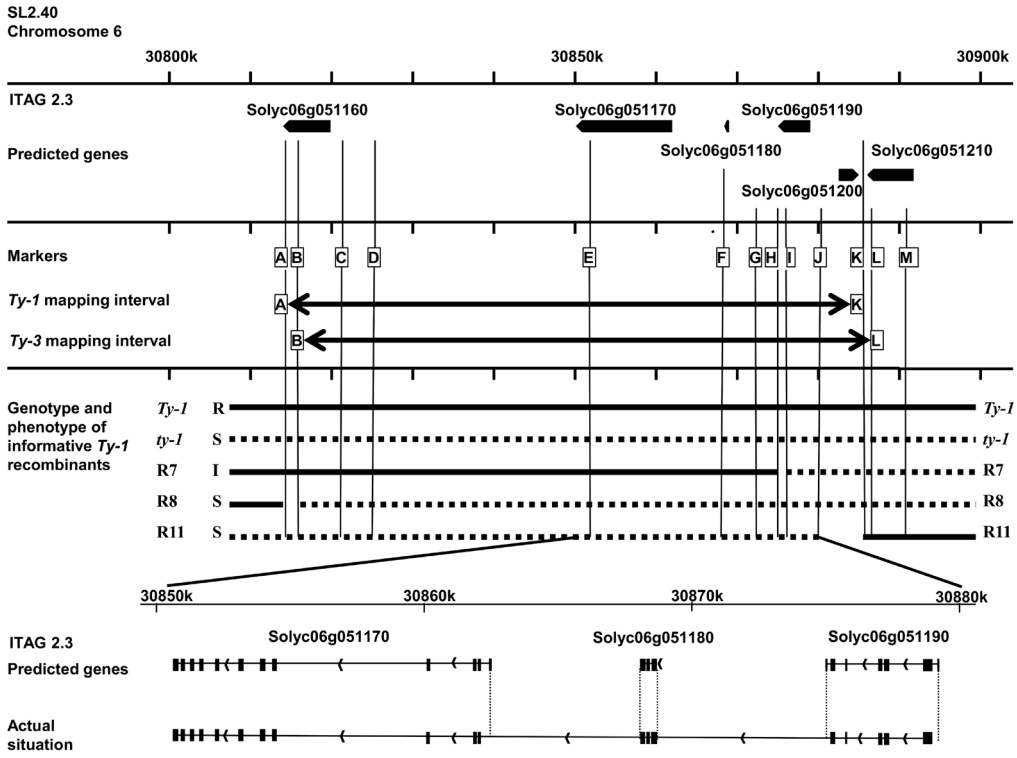


Figure 2. Schematic representation of the region of interest of chromosome 6.

Depicted is the region 30,800,000 to 30,900,000 of chromosome 6 with the genomic annotations of the ITAG2.3 release (Bombarely et al., 2011). In the first frame the six predicted genes are represented by arrows. In the next frame the markers used to genotype the recombinants in this study are shown (A=HBa0161K22, B=UF_TY3-P1, C=UF_TY3-P3, D=WU_M17, E=FOS00169A13, F=WUR_M25, G=UF_TY3-P18, H=WU_M27, I=UF_TY3-P19, J=WU_M29, K=WU_M31, L=UF_TY3-P23, M=UF_TY3-P24). In this frame also the *Ty-1* and *Ty-3* intervals with their flanking markers are depicted. The third frame shows the genotype of the informative recombinants used to fine map *Ty-1*, R7, R8 and R11, note that only for R7 the precise recombination point is known (Figure S2). Also their phenotype upon TYLCV challenge inoculation is shown (Resistant (R), Susceptible (S) and Intermediate (I)). The last frame shows the predicted splicing of gene Solyc06g051170, Solyc06g051180 and Solyc06g051190 compared with the actual situation; differences are indicated with dotted lines.

***Ty-1* and *Ty-3* are allelic**

Our initial mapping studies indicated that *Ty-1* and *Ty-3* could be alleles of the same gene (Verlaan et al., 2011), and the fine mapping of both genes to a similar marker interval strengthened this hypothesis. To test this, the *Ty-1* VIGS approach was again applied to compromise TYLCV resistance in plants carrying the *Ty-3*; as a control, plants with resistance based on *Ty-2* were included. As in the *Ty-1* plants, resistance in the *Ty-3* lines was compromised by TRV2-180 and TRV2-190, but not by TRV-160 (Figure 3). On the other hand, plants containing *Ty-2* remained fully resistant against TYLCV after silencing with all three constructs. Altogether these data indicate that *Ty-1* and *Ty-3* indeed are allelic, while *Ty-2* belongs to another class of resistance genes.

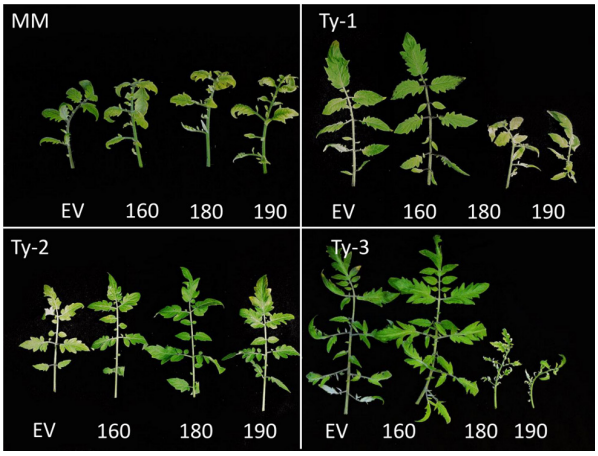


Figure 3. Silencing with constructs TRV2-180 and TRV2-190 compromises TYLCV resistance in *Ty-1* and *Ty-3* lines.

Depicted are leaves of plants 6 weeks after inoculation of the TRV silencing constructs and 4 weeks after TYLCV challenge inoculation. EV, empty vector control; 160, TRV2-160; 180, TRV2-180; 190, TRV2-190. All Moneymaker (MM) plants are susceptible, constructs TRV2-180 and TRV2-190 compromise resistance in *Ty-1* and *Ty-3* carrying lines but not in a line with a *Ty-2* introgression.

Solyc06g051170, Solyc06g051180 and Solyc06g051190 together code for *Ty-1* and *Ty-3*

To test the hypothesis that Solyc06g051170, Solyc06g051180 and Solyc06g051190 were part of the same gene, and to clone the entire *Ty-1* gene, several primer pairs were designed to enable RT-PCR amplification of the exons from the three predicted genes, and tested on cDNA of *Ty-1* lines and TYLCV susceptible cv. Moneymaker. Primers designed on the start and stop codons of the three predicted genes did not amplify any products. However, when primers were used that were located a bit downstream of the start codon or upstream of the stop codon products were amplified, indicating that the predicted start and stop codons were wrong. To test whether the initially predicted genes were all part of one RDR-encoding ORF other primer pairs were tested. When primers targeting Solyc06g051170 were combined with Solyc06g051190 (Figure S4, F6-R4) surprisingly a product of approximately 700 bp was amplified indicating that all three predicted genes were indeed not paralogous but part of one and the same RDR

gene. This was confirmed by sequence analysis of all overlapping PCR fragments obtained (Figure S4). Using a GeneRacer (Invitrogen) approach the genuine start and stop codons of the RDR gene were identified. Based on these sequences new primers (Table S6, Ty-F7-CACC and Ty-R5) were designed that supported the amplification of a product of approximately 3.1 kb from cDNA of a *Ty-1* line, a *Ty-3* line and from cv. Moneymaker.

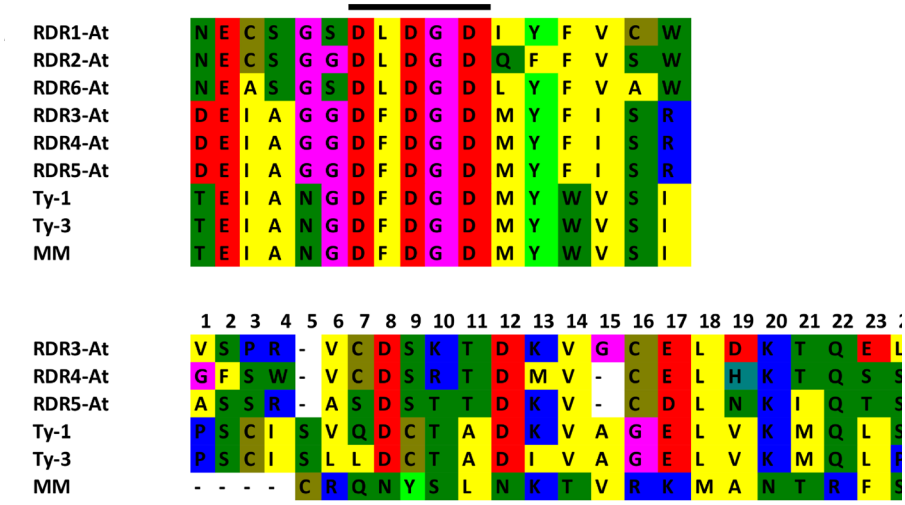


Figure 4. Catalytic domain and polymorphism of *Ty-1* and *Ty-3*.

A: Alignment of the catalytic domain and 6 amino acids up- and downstream of the *A. thaliana* RDR1-6, *Ty-1*, *Ty-3* and Moneymaker (MM) alleles. The catalytic domain is indicated with a black bar above the alignment.

B: All 24 polymorphisms between Moneymaker and *S. chilense* are shown. The RDR3-5 *A. thaliana* amino acids depicted are taken from a Clustel W alignment (Figure S5 and S6).

***Ty-1* and *Ty-3* are RDR3/4/5 homologues**

Sequence analysis of the amplified *Ty-1*/*Ty-3* gene products revealed that the gene contained 19 exons. Compared with the three predicted genes the first predicted exon of Solyc06g051190 was not expressed, nor was the last exon containing the stop codon (Figure 2). For Solyc06g051180 the first exon started earlier than predicted, the last exon was shorter than predicted, again losing the stop codon. Finally for Solyc06g051170 the first predicted exon was not expressed. Alignment of the amino acid (aa) sequences of *Ty-1*, *Ty-3* and *ty-1* (the susceptible allele from tomato cv. Moneymaker) revealed high sequence identity between all alleles, with only small differences. The most significant difference was a four aa deletion in the N-terminal domain of the susceptible allele. In addition, 20 aa changes were observed, with only small differences between *Ty-1* and *Ty-3*.

Multiple sequence alignment with the six RDRs identified in *A. thaliana* (Figure S5 and S6) showed a high sequence homology to RDR3, RDR4, and RDR5 and the presence of the atypical DFDGD catalytic motif of these genes in both *Ty-1* and *Ty-3* alleles (Figure 4 panel A). This homology was supported by a phylogenetic analysis using an unrooted neighbor joining tree, in which *Ty-1* and *Ty-3* grouped in the clade containing RDR3, 4 and 5 (Figure 5). Interestingly, although the *ty-1* allele (Moneymaker) appeared in the same clade, it showed less similarity to RDR3/4/5 then the *Ty-1*/*Ty-3* allele (Figure 4 panel B).

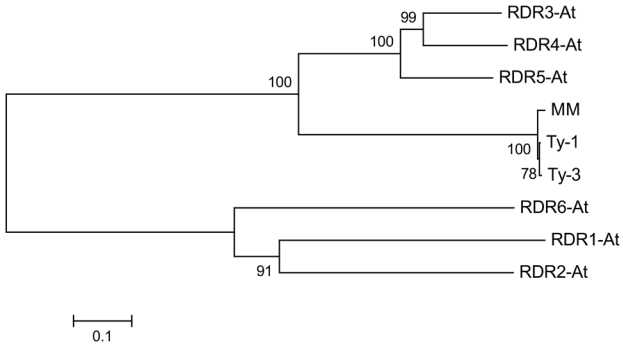


Figure 5. Neighbour joining tree of protein sequences of *A. thaliana* RDR1-6, Ty-1, Ty-3 and the susceptible Moneymaker allele (MM).

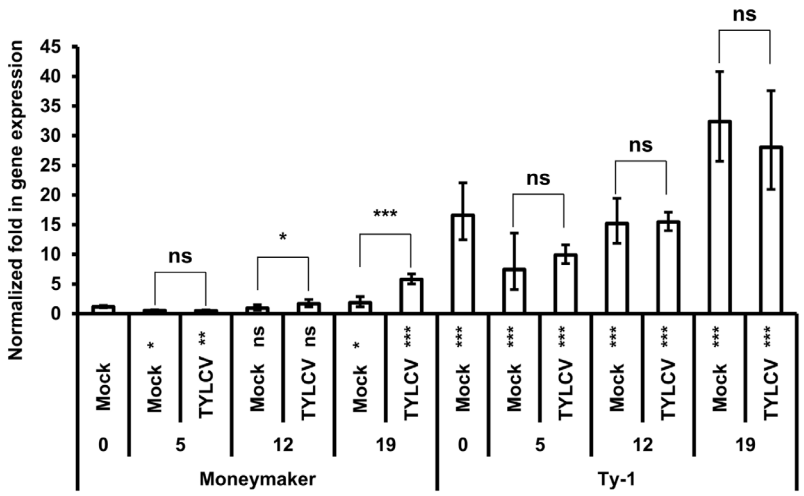


Figure 6. *Ty-1* expression is elevated in resistant lines.

Normalized fold in gene expression of the *ty-1* (susceptible) and *Ty-1* (resistant) allele. Numbers below x-axis indicate days after inoculation. Values are normalized against the Moneymaker day 0 sample, bars represent means and standard deviation of five biological replicas. Asterisks under the x-axis represent significant differences to the Moneymaker day 0 sample, asterisks above the bars represent significant differences between Mock or TYLCV treatment. (*=P<0.05, **=P<0.01, ***=P<0.001, ns=not significant).

Ty-1 is relatively high expressed

Considering a potential role of the *Ty-1* encoded RDR in mounting a strong antiviral RNAi response, its transcriptional expression level was analyzed. To this end, a time-series experiment was performed during which expression of the resistant *Ty-1* and the susceptible *ty-1* allele was quantified upon TYLCV-challenge via agroinoculation in both tomato lines. The expression level of the specific allele was measured by qPCR at several time points (Figure 6). The results showed that at all time points the basic transcription level of the *Ty-1* allele was significantly higher compared to the *ty-1* allele. In the resistance line, no significant difference was observed for the *Ty-1* expression between mock and TYLCV inoculated plants at all time points. However, in the susceptible Moneymaker line, the expression of the *ty-1* allele was induced upon TYLCV inoculation at 12 and 19 days. Compared with day 0 of resistant and susceptible lines, the respective expression of *Ty-1* and *ty-1* was decreased at day 5 and increased at day 19.

Discussion

Nowadays many dominant and recessive virus resistance genes are well characterized and used in breeding of various crops. Most of these genes either do not allow/prevent viral replication or limit this to the first cells of entry in the host. The TYLCV resistance genes *Ty-1* and *Ty-3* are different from these because they lead to a level of virus tolerance (rather than immunity). Plants carrying these genes and challenged by the virus still show low levels of viral replication and systemic spread but with moderate (as with *Ty-3*) or no (as with *Ty-1*) visual symptoms. Recently we observed that the *S. chilense* LA1969 derived *Ty-1* and the *S. chilense* LA2779 derived *Ty-3* map close to each other and that they might be allelic (Verlaan et al., 2011). Here we show by fine mapping and functional analysis that *Ty-1* and *Ty-3* are alleles of the same gene and code for RNA-dependent RNA polymerases from a class of functionally unknown RDR genes.

Sequence data shows that most of the SNPs that are present in *Ty-1* are also present in *Ty-3*, which is logical since both alleles originate from *S. chilense* accessions. The most striking difference between *Ty-1*/*Ty-3* and the *ty-1* allele is a deletion of 4 amino acids in the first amino-terminal part of the protein. However, it is not likely that this deletion solely causes a functional loss, since recombinant R7 contains a chimeric RDR – with the N-terminal part of *ty-1*, and still confers partial resistance to TYLCV.

Recently, three genes have been reported which are involved in different networks related to TYLCV resistance introgressed from *S. habrochaites* (Eybishtz et al., 2009b; Eybishtz et al., 2009a; Sade et al., 2012). Of the three identified genes, *SlVRSLip* functions downstream *LeHT1* within the same network, while *Permease I-like protein* functions in a different network (Eybishtz et al., 2009b; Eybishtz et al., 2009a; Sade et al., 2012). In another study, 18 host genes with a potential role in Tomato Yellow Leaf Curl Sardinia Virus (TYLCSV) infection were identified. Interestingly, almost half of these genes had a role in posttranslational modifications (Lozano-Durán et al., 2011). Whether RDRs encoded by *Ty-1* and *Ty-3* play a role in one any of these networks remains to be analysed.

RDRs are defined by a conserved catalytic domain and are found in RNA viruses and multicellular organisms (plants, fungi and invertebrate animals), but so far are not described in vertebrates and insects. For RNA viruses, the RDR is required to enable replication of its RNA genome to render viral progeny (Poch et al., 1990). In multicellular organisms, three major classes of eukaryotic RDRs have been described and some of their functions have been unraveled. The first class is presented by RDRα and members of these are found in plants, animals and fungi. The class of RDRβ genes has been found

only in animals and fungi while RDRγ members are only found in plants and fungi (Zong et al., 2009). In the model plant *A. thaliana* a total of six RDRs have been identified (Wassenegger and Krczal, 2006). Three of them belong to the RDRα type, i.e. *RDR1*, *RDR2* and *RDR6*, and are characterized by a catalytic DLDGD motif. The other three belong to the RDRγ class of genes and are denoted *RDR3*, *RDR4* and *RDR5* (also referred to as *RDR3a*, *RDR3b* and *RDR3c*, respectively). Members of this class have an atypical DFDGD motif in the catalytic domain (Wassenegger and Krczal, 2006).

The RDRα genes are all known to be involved in RNA silencing, specifically in the amplification of the siRNA signal and resulting in transitive silencing. RNA silencing is generally accepted as a defense system against viral invasion, and is induced by viral dsRNA replicative intermediates or folding structures (Ding and Voinnet, 2007). Geminiviruses are also targeted by RNAi, as observed by the synthesis of geminivirus-specific siRNAs, (small-RNA directed) viral DNA methylation and post-transcriptional gene silencing of the protein-coding genes (Chellappan et al., 2004; Ribeiro et al., 2007; Yadav and Chattopadhyay, 2011; Yang et al., 2011). Although geminiviruses contain a single stranded DNA genome, siRNAs have been observed to originate from the entire virus genome although their distribution was not always equal. The siRNAs are postulated to originate in two ways; 1) as a result of DCL processing from dsRNA molecules that are generated by RDR from bidirectional geminivirus transcripts with overlapping 3′ ends, and 2) mRNA folding structures (Chellappan et al., 2004; Vanitharani et al., 2005; Ribeiro et al., 2007; Yang et al., 2011).

It is proposed that plants employ silencing of DNA by RNA-directed methylation as a strategy to repress geminivirus replication/transcription (Raja et al., 2008). This is supported by two major observations; methylation of geminivirus DNA greatly reduces its ability to replicate in protoplasts (Brough et al., 1992), and the identification of geminivirus RNA silencing suppressor proteins (RSS) C2, C4 and V2 that exert their activity by interference in the process of DNA methylation and transcriptional gene silencing (Voinnet et al., 1999; van Wezel et al., 2002; Wang et al., 2003; Vanitharani et al., 2004; Wang et al., 2005; Buchmann et al., 2009; Zhang et al., 2011; Luna et al., 2012). Antiviral RNAi defense against geminiviruses thus seems to mostly rely on a methylation-based defence, a process that involves the action of siRNA-directed methylation pathway component Ago4. Although several studies have pointed towards the involvement of RDR1 and RDR6 in the biogenesis of geminivirus-specific siRNAs, the involvement of other antiviral RDRs in this cannot yet be excluded (Muangsan et al., 2004; Wang et al., 2010).

Besides their role in RNAi, several studies have described other (endogenous)

functions of the RDRα (1, 2 and 6) genes (Voinnet, 2008), e.g. being involved in herbivore resistance (RDR1) (Pandey and Baldwin, 2007), female gamete formation (RDR2 and 6) (Olmedo-Monfil et al., 2010) or in developmental timing (RDR6) (Peragine et al., 2004). While a knockdown of RDR from the RDR1/2/6 class renders plants highly susceptible to many different viruses (Garcia-Ruiz et al., 2010), their transcriptional up-regulation has been observed to lead to (elevated) resistance levels against different plant viruses (Leibman et al., 2011). Viruses are able to counteract RNAi by coding for viral RSS proteins, and many of these have been shown to sequester siRNAs and prevent their uploading into RISC (Wu et al., 2010). The presence of a viral RSS, however, does not seem to enable viruses to overcome elevated levels of resistance caused by transcriptional up-regulation of the RDR1/2/6 class of genes.

For RDR3, RDR4, and RDR5 a function has not yet been described (Willmann et al., 2011). How to explain the resistance mechanism of the *Ty-1*/*Ty-3* encoded RDRs remains speculative at present. The resistance spectrum of these alleles is not well studied; *Ty-3* also provides resistance to the bipartite *Tomato mottle virus* (ToMoV), but studies describing disease tests with other geminiviruses on *Ty-1*/*Ty-3* carrying lines are not available (Ji et al., 2007). These genes act specifically on geminiviruses; what then is the identity of the (conserved?) Avr protein, and what are the characteristics of resistance breaking isolates? Considering the role of the DLDGD type of RDRs (1,2 and 6) in the generation of secondary siRNAs, irrespective of the RNA virus involved, it is tempting to propose a role of the DFDGD type of RDRs (3,4 and 5), and thus of *Ty-1*/*Ty-3*, in the formation of dsRNA too. Since *Ty-1*/*Ty-3* lines are resistant to TYLCV, but still allow for a symptomless (*Ty-1*) or an almost symptomless (*Ty-3*) infection with low titres of TYLCV, a resistance strategy as earlier described for the RDRα (1,2 and 6) genes could be possible, where transcriptional up regulation provides (elevated) resistance levels against different plant viruses.

In light of this, transcriptional expression analysis of *Ty-1* showed elevated expression levels in resistant lines compared to those in susceptible lines, even without TYLCV challenging. Whether differences in the *Ty-1* vs. *ty-1* protein or just those in transcriptional expression levels, or even a combination of both, are the cause of resistance remains to be investigated. However, since we did not observe hyper-susceptibility in tomato Moneymaker after silencing of the susceptible allele, as what is observed for Potato Virus X (PVX) and potato potyvirus Y (PVY) after silencing of *Nicotiana benthamina RDR6* (Schwach et al., 2005)2005, a function of *ty-1* in resistance is highly unlikely. The functionality and transcriptional upregulation of *Ty-1* thus seems the most plausible reason to explain the resistance. To solve this issue, transgenic tomato

lines (over)expressing either the resistant allele or the susceptible allele will be made. Analysis of the expression level and protein sequence of *Ty-1*/*ty-1* in other resistant/susceptible tomato varieties and wild species will additionally be informative and experiments for these are currently being prepared.

The observed resistance specificity of *Ty-1*/*Ty-3* against TYLCV does seem to contradict the idea that its transcriptional up regulation provides (elevated) resistance levels against other geminiviruses unless people have somehow overlooked a partial resistance to other, distinct geminiviruses. Furthermore, it is possible that the RDRγ (3, 4 and 5) class of genes may be involved in the generation of siRNAs that will mainly direct methylation of DNA and thereby support transcriptional silencing of geminivirus DNA genomes. If this hypothesis is true, this could explain why these genes will not confer (partial) resistance to most other plant viruses, of which ~75% harbours an RNA genome and thus cannot be transcriptionally silenced by the siRNA-directed DNA methylation pathway.

The possibility of an alternate route for dsRNA formation during geminivirus infections, besides the one involving RDR1/2/6, is being supported by the observations that mutants lacking RDR1, RDR2 and RDR6 still revealed basal levels of RNA silencing and siRNA biogenesis, and plants infected with TYLCV only showed a moderate increase in susceptibility to geminiviruses in plants deficient in RDR2 and 6 (Raja et al., 2008; Garcia-Ruiz et al., 2010). Whether the *Ty-1*/*Ty-3* encoded RDR represents a player in this, and how the resistance mechanism acts, will be a challenge to investigate in the near future.

Materials and methods

Plant material

For fine-mapping *Ty-1* from *S. chilense* accession LA1969, a TYLCV-resistant commercial hybrid Tygress with an introgression between markers Mi23 and P6-25, reflecting the same interval as described by Verlaan et al. (2011), was used. This *Ty-1* introgression was done by Jaap Hoogstraten of the Royal Sluis Seed Company, and it is different from the LA1969 *Ty-1* introgression that was done in Israel (Zamir et al., 1994). This hybrid was self-pollinated to produce F_2 progeny. Through two cycles of selection for recombination in this F_2 population, two recombinants were identified and used to generate RILs by selfing and selection with marker genotyping for homozygous introgressions. The first recombination event resulted in a resistant RIL containing a *S. chilense* introgression

flanked by markers Mi23 and HBa0045I03 and was used as a control (named as *Ty-1* RIL, Table 1) in all *Ty-1* experiments. Another recombination event resulted in a resistant RIL containing a *S. chilense* introgression between markers T0774 and HBa0045I03. The susceptible Fla. 7776 was crossed to this inbred and an F_2 population was generated. Approximately 2000 F_2 plants were screened for recombination between the markers T0774 and SL_2.40ch06_30.891 and 13 recombinants were identified. These recombinants were selfed to develop F_4 RILs as described before. RILs were evaluated, along with the controls Fla. 7776, Tygress and the *Ty-1* RIL in fall 2011. Four week-old seedlings were inoculated with TYLCV for 11 days then transplanted to the field on 4 October in a non-randomized trial with two replications of 4-plant plots. TYLCV disease severity was evaluated on each plant 6 weeks after exposure to whiteflies. For the three most informative recombinants (R7, R8 and R11) results were confirmed in the greenhouse using agroinoculation as described below.

Fla. 8680, which contains *Ty-3* within an approximately 27 cM introgression from the *S. chilense* accession LA2779, was crossed to the susceptible breeding line Fla. 7781 to produce an F_2 population. F_2 plants (n=717) were individually screened in fall 2006 for recombination between the molecular markers C2_At2g39590 and T0834, located near the distal ends of the introgression. Recombinants selected from this F_2 population were used to develop RILs as described above. The F_4 and F_5 RILs were evaluated for resistance in fall 2007 and spring 2008, respectively, in a randomized complete block design with three blocks and 12-plant plots. To further fine-map the *Ty-3* locus, three F_2 sub-populations were developed using two key recombinants, i.e. 554 and 157 (Table S3). Sub-population A was an F_4 generated by self-pollinating F_3 progeny of recombinant 554 which were heterozygous for the introgression; sub-population B was an F_2 derived from a cross between the susceptible breeding line Fla. 7776 and the F_5 RIL of recombinant 554 (RIL 554). Sub-population C was also an F_2 developed from a cross of Fla. 7776 and the F_5 RIL of recombinant 157 (RIL 157). Seeds of all three sub-populations were sown and leaf tissue was collected from each plant at approximately 5 weeks after sowing. Sub-populations A and B were screened with the markers Mi23 and P6-25, and the markers T0774 and T0834 were used to screen sub-population C. Recombinants were transplanted to the field, along with controls, in early to mid-March, 2009. Controls included the TYLCV resistant commercial hybrids Tygress and SecuriTY 28, the resistant inbreds Fla. 8680 and Fla. 8602, the susceptible inbreds Horizon and Fla. 7776, RILs 554 and 157 and their F_1 hybrids with Fla. 7776. One month after transplanting to the field, 6-8 cuttings were taken from each plant, rooted in a 1:1 perlite, fine vermiculite media under mist for 2 weeks, then inoculated with whiteflies viruliferous for TYLCV for 11

days. Inoculated cuttings were transplanted to the field on 11 May in a non-randomized design with 3 replications of 2-plant plots, with the exception that only 2 replications were planted for recombinants having cross-overs outside the T0774 to P6-25 interval. TYLCV disease severity was evaluated on each plant at 5-6 weeks after exposure to whiteflies.

Self-pollinated seed was harvested from all original recombinant plants, and progeny were grown out in summer 2009 from 26 individuals with recombination between markers SL_2.40ch06_30.696 and cLEG-31-P16. Plants homozygous for the recombined introgression were selected for producing RILs. These RILs were grown in spring 2010, along with the controls Fla. 7776, Fla. 8680, the F_1 hybrids between Fla. 7776 and each of RILs 554 and 157, and the commercial hybrid Tygress. Three week-old seedlings were inoculated with TYLCV for two weeks then transplanted to the field on 23 March in a randomized complete block design with three blocks and six-plant plots. TYLCV disease severity was evaluated on each plant at seven weeks after exposure to whiteflies.

TYLCV inoculation and disease evaluation

Whitefly mediated inoculation: Plants were inoculated with whiteflies viruliferous for the TYLCV-IL strain according to the method of (Griffiths and Scott, 2001) with some modifications. Briefly, plants were exposed to viruliferous whiteflies in growth chambers for the specified period of time. After inoculation, the whiteflies were killed by treating plants with an insecticidal soap and with Admire (imidacloprid), and the plants were then transplanted to the field. Plants were rated for disease severity on a 0 to 4 disease severity index scale as described by Scott et al. (1996), where 0 = no symptoms and 4 = severe symptoms and stunting. Intermediate scores such as 1.5, 2.5, etc. were incorporated to allow for more precise disease severity ratings.

Agrobacterium mediated inoculation: An infectious TYLCV-IL clone (pTYCz40a) was used for agroinoculation using the method as described in (Verlaan et al., 2011). Briefly, *A. tumefaciens* LBA4404 was transformed, cultured in LB, pelleted and resuspended in infiltration medium at an OD_{600} of 0.5. Three week old seedlings were infiltrated by pressure inoculation in the leaves with a needle-less syringe. For the VIGS experiments the agro infiltration was done two weeks after TRV inoculation.

DNA Extraction, Molecular Marker Design and Testing, and Statistical Analysis

DNA was extracted from young leaves using the cetyltrimethyl ammonium bromide (CTAB) protocol of (Doyle and Doyle, 1987) with minor modifications as described by

(Fulton et al., 1995). Molecular markers used in this study were either publicly available, or were designed using the software Primer3 (Rozen and Skaletsky, 2000) from *Ty-3*-region BAC-end sequences, FOS-end sequences, the draft tomato genome available through the Sol Genomics Network (SGN; http://solgenomics.net/) (Bombarely et al., 2011), or from a private database of *S. lycopersicum* sequences. Polymerase chain reaction (PCR) parameters, primer sequences, restriction enzymes, and detection methods were described by (Hutton and Scott, 2011) or (Verlaan et al., 2011). Additional molecular markers designed are described in Table S5 and Figure S3, and used the same PCR parameters described by (Hutton and Scott, 2011). Analyses of variance, SE calculations, and Duncan's multiple range tests were performed in SAS (Version 9.1; SAS Institute, Cary, NC). Mapping and interval analysis of *Ty-3* was performed in Windows QTL Cartographer 2.0 (2007, N.C. State University) using mean disease severity of the cuttings for each recombinant and a subset of molecular markers specific to the *Ty-3* region.

Generation of TRV vectors for silencing

For gene silencing, the TRV based VIGS system as described in (Liu et al., 2002) was used. Briefly, fragments of approximately 350 base pairs of Solyc06g051160, Solyc06g051180 and Solyc06g051190 were amplified from *Ty-1* cDNA using primers compatible with the Gateway® system (Table S6). After cloning to pENTR the inserts were sequenced to confirm their identity. Positive clones were selected for further processing of the inserts into the TRV2 vector and subsequently transformed to *Agrobacterium tumefaciens* strain GV3101.

TRV infection by agrobacterium-mediated infiltration

A 3 ml culture of *A. tumefaciens* strain GV3101 containing the TRV replicons was grown overnight at 28°C, 200 RPM in appropriate selective LB medium. Cultures were transferred to 20 mL LB containing proper selection pressure, 10 mM MES and 200 μM acetosyringone, and further grown overnight in a 28°C shaker. *A. tumefaciens* cells were pelleted, and resuspended in infiltration buffer (20 g/L sucrose, 5g/L MS salts (no vitamins), 10 mM MES) to a final OD_{600} of 1. Agro infiltration was performed on cotyledons of 10 day old seedlings using pressure inoculation with a 2, 5 mL syringe without a needle.

Phylogenetic Analysis

A neighbour joining tree with a bootstrap value of 1000 was generated using *MEGA* version 5 (Tamura et al., 2011). Arabidopsis RDR sequences were downloaded from The

Arabidopsis Information Resource (www.arabidopsis.org) (Lamesch et al., 2011).

Quantitative RT-PCR

For gene expression analysis, 17 day old seedlings were agroinoculated as described above. For the mock treatment infiltration buffer without bacteria was used. Top leaves of plants were harvested 0, 5, 12 and 19 days after TYLCV inoculation and grinded in liquid nitrogen using mortar and pestle. Total RNA was extracted by using the RNeasy Plant Mini Kit (Quiagen) as described by the manufacturer. One μg RNA was digested using DNase I (Amp. Grade) following the manufacturers protocol (Invitrogen) and cDNA was synthesized using the iScript cDNA Synthesis Kit following the protocol (Bio-Rad). Quantitative Real-Time PCR was performed in 10 μl reactions in a Bio-Rad iCycler iQ5 using SYBR Green Supermix (Bio-Rad) according to the protocol provided by the manufacturer.

For quantitative RT-PCR of *Ty-1*/*ty-1* the forward primer 180-F1 (5'-GGCAAAATATGCAGCCAGGCTTTCC-3') and the reverse primer 180-R1 (5'-TCAGTATGTATACGAGGTTCGCCGT-3') were used. As a reference the ACT gene was used as described by (Løvdal and Lillo, 2009) with primers: ACT-F (GAAATAGCATAAGATGGCAGACG)andACT-R(ATACCCACCATCACACCAGTAT). Gene expression levels were calculated using the DDCt method as described by (Livak and Schmittgen, 2001).

Acknowledgements

The infectious TYLCV clone was kindly provided by Professor Eduardo Rodríguez Bejarano (Universidad de Málaga). We thank Drs. Greg Martin and Jim Giovannoni for their advice and help in identifying sequences specific to the *Ty-3* region which was useful in marker development. We thank Dave Schuster for his help with whitefly inoculations and Dick Lohuis for his help with agro-infiltrations. We thank Luis Alonso Rios Zertuche for his help with making figure 3. We also credit The International Tomato Genome Sequencing Consortium for the draft assembly of the tomato genome that was used in this project.

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Supplemental figures

Figure S1. Interval mapping for TYLCV resistance on tomato chromosome 6.

Maximal logarithm of odds (LOD) score for disease severity on cuttings from approximately 300 recombinant plants from the Ty-3 fine mapping population. Approximate physical positions are based on the tomato genome assembly SL_2.40, available through the Sol Genomics Network (SGN; http://solgenomics.net/).

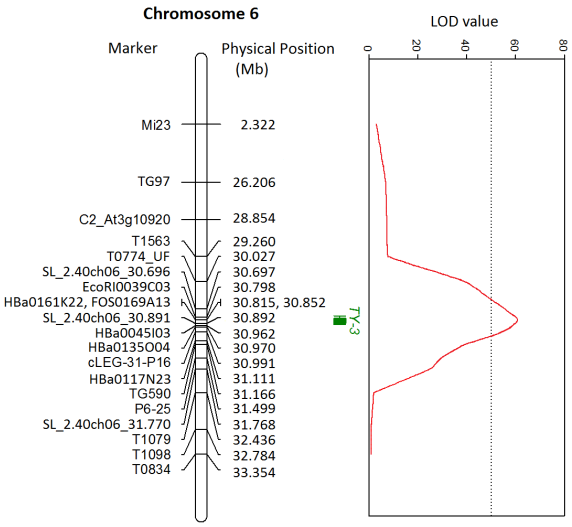
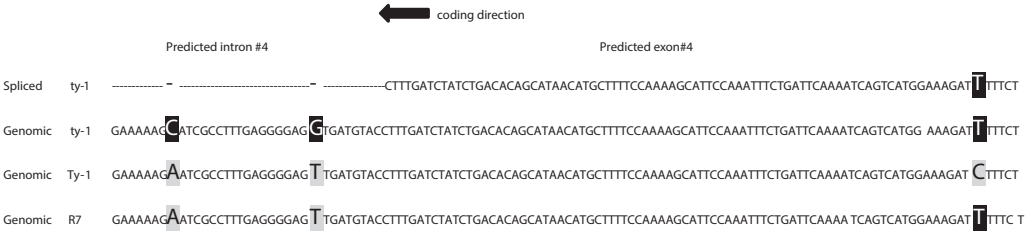


Figure S2. Determining the exact point of recombination in R7.

Depicted is a part of the sequence of predicted gene Solyc06g051190, the first lines shows the spliced sequence, the second, third and fourth line show the genomic sequence of ty-1, Ty-1 and R7 respectively. Based on the three SNPs that are present in this region the recombination point in R7 could be located in between the second and third SNP shown here.



3

Figure S3. SNPs of markers M17, M25, M27, M29, M31 in R7, R8 and R11.

SNPs shown in black and grey were used to genotype R7, R8 and R11.

M17
ty - 1 TTCTCTCAATCGGACAATCC
Ty - 1 TTCTCCTAATCAAACAATCC
R7 TTCTCCTAATCAAACAATCC
R8 TTCTCTCAATCGGACAATCC
R11 TTCTCTCAATCGGACAATCC

M25
ty - 1 TACACAACATTGAAACTGTAAATCCGTAC
Ty - 1 TACACGACATTGAAACTGTAAATCTGTAC
R7 TACACGACATTGAAACTGTAAATCTGTAC
R8 TACACAACATTGAAACTGTAAATCCGTAC
R11 TACACAACATTGAAACTGTAAATCCGTAC

M27
ty - 1 TAATGTCAAAATTGATGGTCA
Ty - 1 TAATGCCAAAATTGACGGTCA
R7 TAATGCCAAAATTGACGGTCA
R8 TAATGTCAAAATTGATGGTCA
R11 TAATGTCAAAATTGATGGTCA

M29
ty - 1 TAGTTCAATGACCTCTTATGAT
Ty - 1 TAGTTCACTGACCTCTTAAGAT
R7 TAGTTCAATGACCTCTTATGAT
R8 TAGTTCAATGACCTCTTATGAT
R11 TAGTTCAATGACCTCTTATGAT

M31
ty - 1 GAGAAAGGTAACAGCAACATT
Ty - 1 GAGAAAGGGAACAGTAACATT
R7 GAGAAAGGTAACAGCAACATT
R8 GAGAAAGGTAACAGCAACATT
R11 GAGAAAGGGAACAGTAACATT

Figure S4. PCR strategy to prove that predicted Solyc06g051170, Solyc06g051180 and Solyc06g051190 are one gene.

Primers used are indicated with their name (for primer sequences: Table S6), F4-R5 proved the connection between Solyc06g051180 and Solyc06g051190 and F6-R4 showed all three predicted genes are connected. F3-R10 was 1069 bp and F7-R7 was 786 bp, both as expected. F6-R4 had an expected size of 695 bp but the obtained fragment was 668 bp, for F4-R5 the expected size was 889 bp but the obtained fragment had a size of 925 bp. These size differences could be explained because the last predicted exon of Solyc06g051190 was not expressed and for Solyc06g051180 the first exon started earlier than predicted, the last exon was shorter than predicted. Finally for Solyc06g051170 the first predicted exon was not expressed.

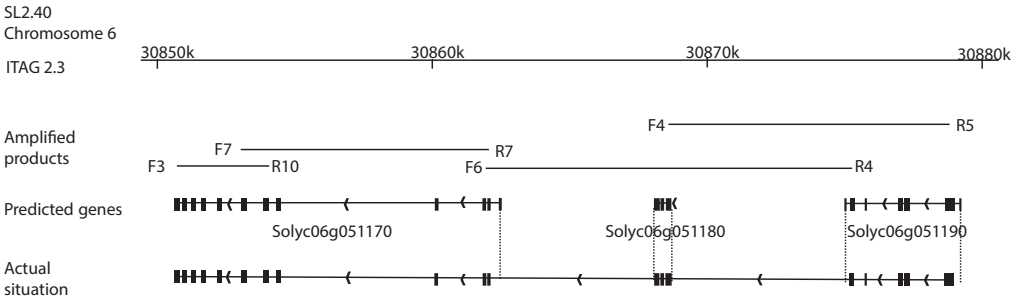


Figure S5. Clustal W alignment of *A. thaliana* RDR1 to RDR6, Ty-1, Ty-3 and ty-1.

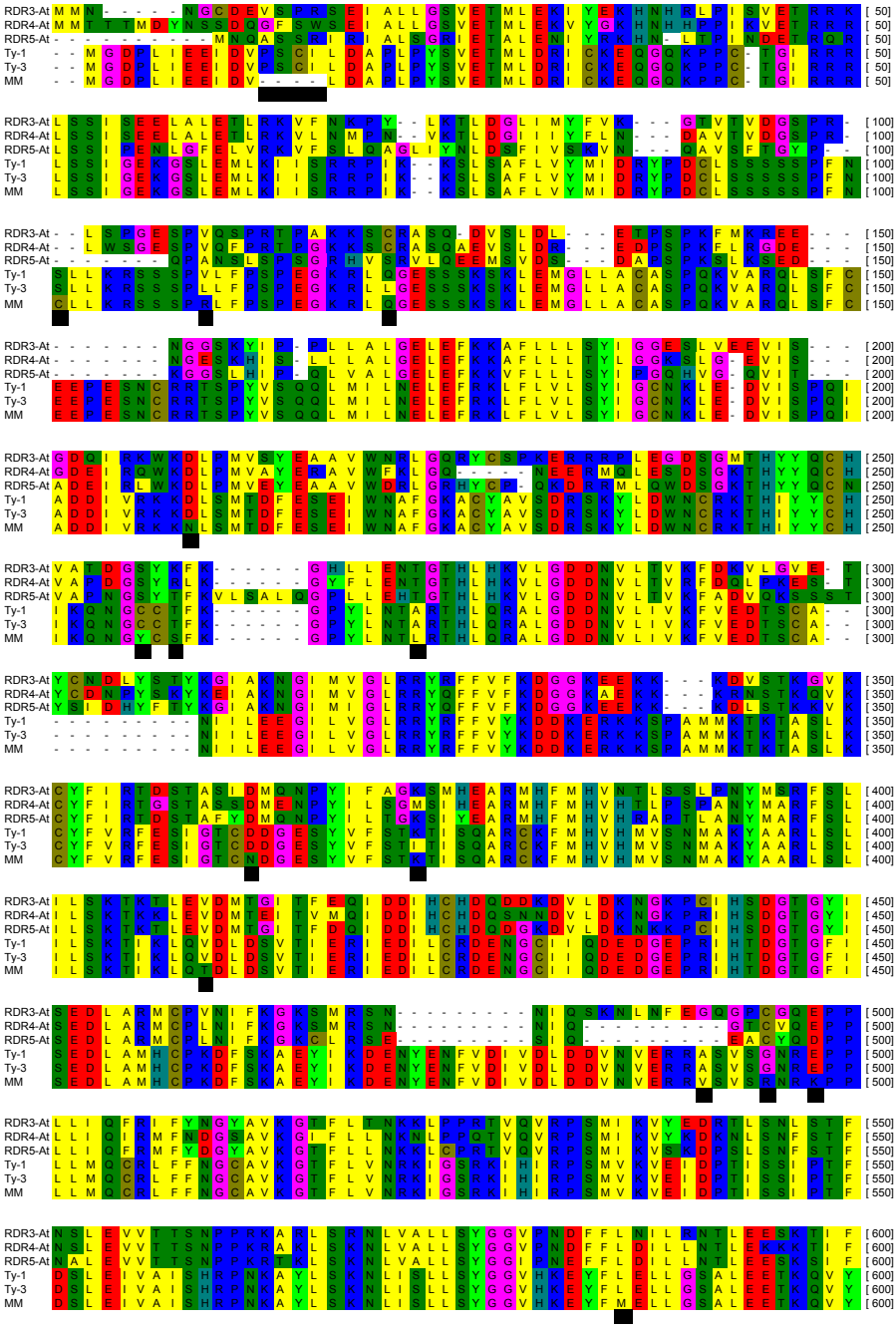
Differences between Ty-1, Ty-3 and MM are indicated with black boxes beneath the alignment.


RDR1-At [500]
RDR2-At [500]
RDR6-At [500]
RDR3-At [500]
RDR4-At [500]
RDR5-At [500]
Ty-1 [500]
Ty-3 [500]
MM [500]
RDR1-At [550]
RDR2-At [550]
RDR6-At [550]
RDR3-At [550]
RDR4-At [550]
RDR5-At [550]
Ty-1 [550]
Ty-3 [550]
MM [550]
RDR1-At [600]
RDR2-At [600]
RDR6-At [600]
RDR3-At [600]
RDR4-At [600]
RDR5-At [600]
Ty-1 [600]
Ty-3 [600]
MM [600]
RDR1-At [650]
RDR2-At [650]
RDR6-At [650]
RDR3-At [650]
RDR4-At [650]
RDR5-At [650]
Ty-1 [650]
Ty-3 [650]
MM [650]
RDR1-At [700]
RDR2-At [700]
RDR6-At [700]
RDR3-At [700]
RDR4-At [700]
RDR5-At [700]
Ty-1 [700]
Ty-3 [700]
MM [700]
RDR1-At [750]
RDR2-At [750]
RDR6-At [750]
RDR3-At [750]
RDR4-At [750]
RDR5-At [750]
Ty-1 [750]
Ty-3 [750]
MM [750]
RDR1-At [800]
RDR2-At [800]
RDR6-At [800]
RDR3-At [800]
RDR4-At [800]
RDR5-At [800]
Ty-1 [800]
Ty-3 [800]
MM [800]
RDR1-At [850]
RDR2-At [850]
RDR6-At [850]
RDR3-At [850]
RDR4-At [850]
RDR5-At [850]
Ty-1 [850]
Ty-3 [850]
MM [850]
RDR1-At [900]
RDR2-At [900]
RDR6-At [900]
RDR3-At [900]
RDR4-At [900]
RDR5-At [900]
Ty-1 [900]
Ty-3 [900]
MM [900]


RDR1-At
RDR2-At
RDR3-At
RDR4-At
RDR5-At
Ty-1
Ty-3
MM

Figure S6. Clustal W alignment of *A. thaliana* RDR3, RDR4, RDR5, Ty-1, Ty-3 and ty-1.

Differences between Ty-1, Ty-3 and MM are indicated with black boxes beneath the alignment.




RDR3-At
RDR4-At
RDR5-At
Ty-1
Ty-3
MM

Supplemental tables

Table S1. Agroinoculation disease test on the three most informative *Ty-1* RILS.

			Markers[3]									
Line	**Average DI[1]**	**N[2]**	M-H304P16	M-H309K01	T1563	T0774	MSc05732-3	MSc05732-4	SL_2.40ch06_30.696	HBa0161K22	FOS169A13	SL_2.40ch06_30.891
R7	1.3 ± 0.5	7	-	-	-	+	+	+	+	+	+	-
	0	7	-	-	-	+	+	+	+	+	+	/
	0	3	-	-	-	+	+	+	+	+	+	+
R8	4	6	-	-	-	+	+	+	+	+	-	-
	4	10	-	-	-	/	/	/	/	/	-	-
	4	6	-	-	-	-	-	-	-	-	-	-
R11	4	3	-	-	-	-	-	-	-	-	-	+
	4	6	-	-	-	-	-	-	-	-	-	/
	4	1	-	-	-	-	-	-	-	-	-	-
R12	0	4	-	-	-	-	-	-	+	+	+	+
	0	5	-	-	-	-	-	-	/	/	/	/
	4	2	-	-	-	-	-	-	-	-	-	-

[1] Average Disease Index (DI)

[2] Numbers of plant tested

[3] + = homozygous *Solanum chilense* , / = heterozygous, - = homozygous *S. lycopersicum*

Table S2. Recombinant inbred lines (RILs) derived from the cross between tomato inbreds Fla. 7781 and Fla. 8680, their genotypes for the *Ty-3* region of chromosome 6, and their phenotypes across two growing seasons.

	Chromosome 6 Introgressed Segment[z]												Mean RIL Rating[y]	
Recombinant	C2_At2g39590	Mi23	TG436	TG178	T0892	T1563	**T0774**	cLEG-31-P16	**P6-25**	T1079	T1098	T0834	Fall 2007	Spring 2008
683	+	+	-	-	-	-	-	-	-	-	-	-		3.4 ^{ab}
343	+	+	+	-	-	-	-	-	-	-	-	-	1.8 $^{f-i}$	2.5 $^{c-e}$
463	+	+	+	-	-	-	-	-	-	-	-	-		3.3 ^{ab}
11	+	+	+	+	-	-	-	-	-	-	-	-	3.0 ^{b}	3.7 ^{ab}
359	+	+	+	+	-	-	-	-	-	-	-	-	3.2 ^{ab}	3.9 ^{ab}
503	+	+	+	+	-	-	-	-	-	-	-	-	1.2 $^{k-n}$	3.0 ^{bc}
506	+	+	+	+	+	+	-	-	-	-	-	-	2.4 ^{cd}	3.0 ^{bc}
724	+	+	+	+	+	+	-	-	-	-	-	-	2.3 ^{de}	2.7 $^{c-e}$
186	+	+	+	+	+	+	+	-	-	-	-	-	3.0 ^{b}	3.7 ^{ab}
403	+	+	+	+	+	+	+	-	-	-	-	-	2.0 $^{d-g}$	2.1 $^{e-h}$
554	+	+	+	+	+	+	+	+	+	-	-	-	2.1 $^{d-f}$	0.9 $^{o-r}$
78	+	+	+	+	+	+	+	+	+	+	-	-	1.3 $^{j-m}$	1.0 $^{m-r}$
705	+	+	+	+	+	+	+	+	+	+	-	-	1.1 $^{k-n}$	2.1 $^{e-h}$
432	+	+	+	+	+	+	+	+	+	+	+	-	0.8 $^{n-p}$	1.6 $^{h-n}$
362	-	+	+	+	+	+	+	+	+	+	+	+	0.6 $^{o-q}$	1.6 $^{h-m}$
116	-	+	+	+	+	+	+	+	+	+	+	+	0.6 $^{o-r}$	0.9 $^{o-r}$
718	-	-	-	-	+	+	+	+	+	+	+	+	1.3 $^{j-l}$	1.9 $^{f-j}$
688	-	-	-	-	+	+	+	+	+	+	+	+	1.1 $^{l-m}$	0.9 $^{o-r}$
552	-	-	-	-	+	+	+	+	+	+	+	+	1.2 $^{k-n}$	2.0 $^{f-i}$
71	-	-	-	-	+	+	+	+	+	+	+	+		1.5 $^{h-o}$
719	-	-	-	-	-	+	+	+	+	+	+	+	1.0 $^{l-o}$	1.1 $^{l-r}$
157	-	-	-	-	-	-	+	+	+	+	+	+	1.8 $^{f-i}$	2.4 $^{d-g}$
553	-	-	-	-	-	-	-	+	+	+	+	+	0.9 $^{m-o}$	1.5 $^{h-o}$
367	-	-	-	-	-	-	-	+	+	+	+	+	0.9 $^{m-o}$	2.0 $^{f-h}$
18	-	-	-	-	-	-	-	+	+	+	+	+	1.0 $^{l-o}$	1.6 $^{h-m}$
6[x]	-	-	-	-	-	-	-	-	+	+	+	+		
616	-	-	-	-	-	-	-	-	-	+	+	+	3.1 ^{b}	3.7 ^{a}
517	-	-	-	-	-	-	-	-	-	+	+	+	2.8 ^{bc}	
390	-	-	-	-	-	-	-	-	-	-	-	+	3.5 ^{a}	2.9 $^{b-d}$
405	-	-	-	-	-	-	-	-	-	-	-	+	1.9 $^{e-h}$	2.7 $^{c-e}$
Horizon	-	-	-	-	-	-	-	-	-	-	-	-	2.8 ^{bc}	2.9 $^{b-d}$
Fla. 8602	+	+	+	+	+	+	+	+	+	+	+	+	1.7 ^{gj}	
Tygress	-	/	/	/	/	/	/	/	/	-	-	-		1.9 $^{g-k}$
HA3074	-	/	/	/	/	/	/	/	/	-	-	-	0.4 $^{p-s}$	
Security 28	-	/	/	/	/	/	/	/	/	-	-	-		1.5 $^{h-o}$

[z] + = homozygous *Solanum chilense* , / = heterozygous, - = homozygous *S. lycopersicum*

[y] Different superscript letters represent statistically significant differences at $P < 0.05$ based on Duncan's multiple range test.

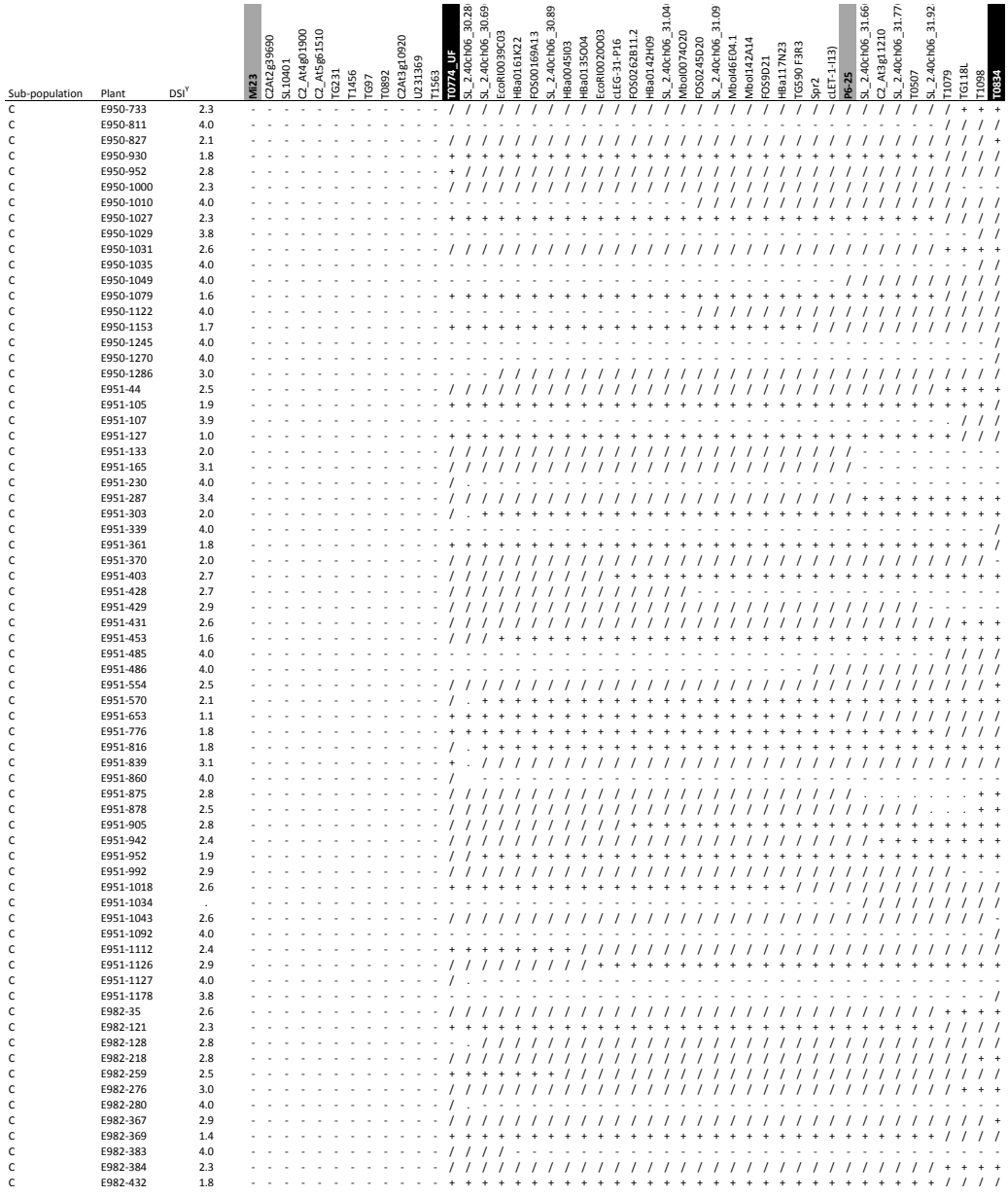
[x] RIL was not tested in Fall 2007 or Spring 2008; evaluation in Fall 2008 indicated susceptibility (data not shown).

Table S3. Tomato plants from the *Ty-3* fine-mapping population selected for recombination in the *Ty-3* region of chromosome 6, mean Tomato Yellow Leaf Curl Virus (TYLCV) disease severity of their cuttings, and their genotype throughout the region.

			Chromosome 6 Introgressed Segment[Z]																																												
Sub-population	Plant	DSI[Y]	Mi23	C2At2g39690	SL10401	C2_At4g01900	C2_At5g61510	TG231	T1456	TG97	T0892	C2At3g10920	U231369	T1563	T0774_UF	SL_2.40ch06_30.280	SL_2.40ch06_30.696	EcoRI0039C03	HBa0161K22	FOS00169A13	SL_2.40ch06_30.891	HBa0045I03	HBa0135O04	EcoRI0020O03	cLEG-31-P16	FOS0262B11.2	HBa0142H09	SL_2.40ch06_31.040	MboI0074O20	FOS0245D20	SL_2.40ch06_31.097	MboI46E04.1	MboI142A14	FOS9D21	HBa117N23	TG590 F3R3	Spr2	cLET-1-I13)	P6-25	SL_2.40ch06_31.660	C2_At3g11210	SL_2.40ch06_31.770	T0507	SL_2.40ch06_31.928	T1079	TG118L	T1098	T0834
A	E944-22	1.1	/	/	/	/	/	/	/	/	/	/	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
A	E944-288	1.3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E944-355	2.2	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E944-399	2.3	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E944-510	1.6	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
A	E944-604	1.4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E945-20	1.9	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E945-25	2.8	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E945-102	2.4	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E945-221	1.9	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E945-247	1.3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E945-254	2.1	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E945-355	1.8	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E945-368	1.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E945-374	2.0	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E945-554	2.4	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E945-597	2.3	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E945-612	2.4	-	-	-	-	-	-	-	-	-	-	-	-	-	.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E945-644	3.9	/	/	/	/	/	/	/	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A	E945-651	2.3	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E946-153	1.8	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E946-163	1.0	/	/	/	/	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
A	E946-171	1.1	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
A	E946-250	4.0	/	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A	E946-251	2.4	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E946-285	2.4	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E946-304	2.1	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E946-451	1.6	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E946-485	1.8	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E946-511	1.1	/	/	/	/	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
A	E947-14	1.2	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E947-53	1.8	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E947-317	1.0	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
A	E947-356	4.0	/	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A	E947-370	1.6	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E947-383	1.8	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E947-387	4.0	/	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A	E947-558	4.0	/	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A	E947-588	3.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E947-589	1.8	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
A	E947-602	1.8	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E947-617	1.3	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
A	E947-625	1.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E947-633	1.0	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E947-713	1.6	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A	E947-735	1.4	-	-	-	-	-	-	-	-	-	-	-	-	-	.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E947-757	1.0	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
A	E947-809	3.5	/	/	/	/	/	/	/	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	E940-37	2.5	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
B	E940-61	1.8	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
B	E940-81	1.9	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
B	E940-238	3.0	+	+	+	+	+	+	+	+	.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
B	E940-254	2.5	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
B	E940-302	2.4	-	-	-	-	-	-	-	-	-	-	-	-	-	.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
B	E940-326	1.3	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
B	E940-397	2.1	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
B	E940-406	3.6	-	-	-	-	-	-	-	-	.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
B	E940-523	3.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	-	-	-	-
B	E940-528	1.8	+	+	+	+	+	+	+	+	.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
B	E940-541	3.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	-	-	-	-
B	E940-561	2.4	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
B	E940-765	2.4	/	/	/	/	/	/	/	/	/	/	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
B	E940-770	2.4	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
B	E940-777	2.3	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
B	E940-800	2.0	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
B	E940-837	2.3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
B	E940-855	2.2	/	/	/	/	/	/	/	/	/	/	/	.	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	E940-881	2.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
B	E940-918	2.3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-
B	E940-1068	2.1	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-

Sub-population	Plant	DSI[Y]		Mi23	C2At2g39690	SL10401	C2_At4g01900	C2_At5g61510	TG231	T1456	TG97	T0892	C2At3g10920	U231369	T1563	T0774_UF	SL_2.40ch06_30.28(	SL_2.40ch06_30.69(	EcoRI0039C03	HBa0161K22	FOS00169A13	SL_2.40ch06_30.891	HBa0045I03	HBa0135O04	EcoRI0020O03	cLEG-31-P16	FOS0262B11.2	HBa0142H09	SL_2.40ch06_31.04(	Mbol0074O20	FOS0245D20	SL_2.40ch06_31.097	Mbol46E04.1	Mbol142A14	FOS9D21	HBa117N23
B	E941-33		2.5	/	/	/	/	/	/	/	/	.	.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E941-42		2.8	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E941-110		3.1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E941-164		2.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E941-174		3.3	/	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	E941-175		1.6	/	/	/	/	/	/	/	/	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E941-194		2.0	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E941-209		2.5	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E941-249		2.1	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E941-255		2.1	/	/	/	/	/	/	/	/	.	.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E941-285		3.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E941-310		4.0	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	E941-383		3.4	+	+	+	+	+	+	+	+	+	+	+	+	+	.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E941-398		2.9	/	/	/	/	/	/	/	/	/	/	.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E941-450		4.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	E941-459		3.5	+	.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E941-515		2.9	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E941-555		3.3	/	/	/	/	/	/	/	/	.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E941-558		3.5	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	+	+	+	+	+	+	+	+	+	+
B	E941-573		2.4	/	/	/	/	/	/	/	/	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E941-602		3.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E941-812		2.1	+	.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E941-816		2.1	/	/	/	/	/	/	/	/	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E941-899		2.3	+	.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E942-10		2.4	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E942-19		1.0	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E942-20		2.8	/	/	/	/	/	/	/	/	/	/	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E942-103		3.0	/	/	/	/	/	/	/	/	.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E942-127		3.9	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E942-162		2.4	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E942-165		2.3	/	/	/	.	.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E942-202		2.9	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E942-242		3.0	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E942-396		3.0	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E942-402		2.9	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E942-411		2.8	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E942-425		2.8	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E942-482		2.3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E942-601		3.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E942-603		2.8	-	-	-	-	-	-	-	-	.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E942-665		4.0	/	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	E942-717		3.1	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E942-719		3.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	E942-773		2.5	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E942-979		1.1	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E942-1017		1.6	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E942-1019		2.0	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E942-1078		1.6	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E942-1204		2.3	/	/	/	/	/	/	/	/	/	/	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E942-1205		1.5	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E943-51		2.9	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E943-111		3.2	/	/	/	/	/	/	/	/	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E943-118		1.5	/	/	/	/	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	E943-140		2.0	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Sub-population	Plant	DSI[Y]	Mi23	C2At2g39690	SL10401	C2_At4g01900	C2_At5g61510	TG231	T1456	TG97	T0892	C2At3g10920	U231369	T1563	10774_UF	SL_2.40ch06_30.28(	SL_2.40ch06_30.69(	EcoRI0039C03	HBa0161K22	FOS00169A13	SL_2.40ch06_30.891	HBa0045I03	HBa0135O04	EcoRI0020O03	cLEG-31-P16	FOS0262B11.2	HBa0142H09	SL_2.40ch06_31.04(	Mbol0074O20	FOS0245D20	SL_2.40ch06_31.097	Mbol46E04.1	Mbol142A14	FOS9D21	HBa117N23
B	E943-1043	4.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	E943-1291	2.8	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
B	E943-1302	1.1	/	/	/	/	/	/	/	/	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E948-14	1.8	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E948-39	2.9	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E948-50	2.2	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C	E948-120	1.6	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E948-168	1.4	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E948-251	2.3	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E948-280	3.1	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E948-281	2.0	-	-	-	-	-	-	-	-	-	-	-	-	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E948-292	1.9	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E948-321	2.5	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E948-341	2.9	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E948-351	2.0	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E948-375	1.8	-	-	-	-	-	-	-	-	-	-	-	-	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E948-647	2.3	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E948-693	2.1	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E948-697	2.3	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E948-721	2.5	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E948-725	3.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E948-739	2.3	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E948-770	2.0	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E948-811	1.8	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E948-874	1.8	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-	-	-
C	E948-882	1.4	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E948-944	2.1	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E948-950	1.0	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E948-1002	2.8	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E948-1057	1.5	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E948-1066	1.6	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E948-1076	1.6	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E948-1146	2.8	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E948-1243	2.5	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E949-95	2.1	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E949-148	2.1	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E949-403	4.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C	E949-481	2.4	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E949-678	2.4	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E949-686	2.8	-	-	-	-	-	-	-	-	-	-	-	-	.	.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E949-782	3.0	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E949-787	4.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C	E949-806	2.8	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E949-885	2.2	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/	/	/	/
C	E949-1023	2.6	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E949-1026	3.0	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+
C	E949-1266	2.1	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E949-1283	2.8	-	-	-	-	-	-	-	-	-	-	-	-	-	.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E949-1284	4.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C	E949-1332	2.0	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-	-	-	-	-
C	E949-1338	1.6	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E949-1351	2.4	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E949-1393	4.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-


Sub-population
Plant
DSI[Y]
M23
C2At2g39690
SL10401
C2_At4g01900
C2_At5g61510
TG231
T1456
TG97
T0892
C2At3g10920
U231369
T1563
T0774 UF
SL_2.40ch06_30.28
SL_2.40ch06_30.69
EcoR10039C03
HBa0161K22
FOS00169A13
SL_2.40ch06_30.89
HBa0045I03
HBa0135O04
EcoR10020O03
cLEG-31-P16
FOS0262B11.2
HBa0142H09
SL_2.40ch06_31.04
Mbol0074O20
FOS0245D20
SL_2.40ch06_31.09
Mbol46E04.1
Mbol142A14
FOS9D21
HBa1117N23
TGS90 F3R3
Spr2
cLET-1-I13)
P6-25
SL_2.40ch06_31.66
C2_At3g11210
SL_2.40ch06_31.77
T0507
SL_2.40ch06_31.92
T1079
TG118L
T1098
T0834

Sub-population	Plant	DSI[Y]	M123	C2At2g39690	SL10401	C2_At4g01900	C2_At5g61510	TG231	T1456	TG97	T0892	C2At3g10920	U231369	T1563	T0774_UF	SL_2.40ch06_30.28(	SL_2.40ch06_30.69(	EcoRI0039C03	HBa0161K22	FOS00169A13	SL_2.40ch06_30.89)	HBa0045I03	HBa0135O04	EcoRI0020O03	cLEG-31-P16	FOS0262B11.2	HBa0142H09	SL_2.40ch06_31.04(	Mbol0074O20	FOS0245D20	SL_2.40ch06_31.09)	Mbol46E04.1	Mbol142A14	FOS9D21	HBa117N23	TG590 F3R3	Shr2	cLET-1-I13)	P6-25	SL_2.40ch06_31.66(	C2_At3g11210	SL_2.40ch06_31.77(	T0507	SL_2.40ch06_31.92(	T1079	TG118L	T1098	T0834
C	E982-446	2.8	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	+	+	+
C	E982-468	2.0	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/
C	E982-533	4.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/
C	E982-584	2.5	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E982-626	2.4	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	+
C	E982-690	4.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/
C	E982-693	2.6	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C	E982-699	2.1	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	+	+	+	+	+	+
C	E982-721	2.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E982-803	2.8	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-
C	E982-816	2.1	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/
C	E982-818	4.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/
C	E982-823	4.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/
C	E982-892	2.6	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/
C	E982-915	2.4	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-	-	-	-	-	-	-	-	-	-
C	E982-927	2.1	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/
C	E982-966	2.1	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	.	+	+	+
C	E982-1034	2.5	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	E982-1055	3.0	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-
C	E982-1069	2.6	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/
C	E982-1097	1.5	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/
C	E982-1098	3.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
C	E982-1110	1.6	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/	/
C	E982-1132	2.8	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	-	-
C	E982-1148	1.9	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/
C	E982-1170	2.0	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	/	/
C	E982-1212	4.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/
C	E982-1245	2.9	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	+	+	+	+

[Z] + = homozygous *Solanum chilense* , / = heterozygous, - = homozygous *S. lycopersicum*, "." = *missing data*

[Y] DSI = mean disease severity index as described in the "Materials and Methods"

Table S4. Average Tomato yellow leaf curl virus disease severity index (DSI) for tomato cuttings evaluated in spring 2009.

Line	Description	DSI			
Fla. 7776	susceptible parent	4.0 ^{A}	±	0.0	(n=6)
(Fla. 7776 x RIL 554) F_1	heterozygous control	3.3 ^{B}	±	0.1	(n=5)
(Fla. 7776 x RIL 157) F_1	heterozygous control	3.3 ^{B}	±	0.1	(n=6)
RIL 554	resistant parent	1.3 $^{D-E}$	±	0.2	(n=6)
RIL 157	resistant parent	1.4 ^{D}	±	0.2	(n=5)
Fla. 8680	resistant donor	1.0 ^{E}	±	0.0	(n=6)
Tygress	commercial hybrid	1.1 $^{D-E}$	±	0.1	(n=6)
Security28	commercial hybrid	1.8 ^{C}	±	0.1	(n=6)
Fla. 8602	resistant control	1.0 ^{E}	±	0.0	(n=4)
Horizon	susceptible control	4.0 ^{A}	±	0.0	(n=4)

Results are displayes as mean ± SE; n = number of cuttings evaluated for each line; DSI = disease severity index as described in the "Materials and Methods" where higher numbers indicate more disease; different superscript letters represent statistically significant differences at $P < 0.05$ based on Duncan's multiple range test.

Table S5. Molecular markers on chromosome 6 of tomato.

Marker name	Marker type		Primer sequence (5'-3')	Annealing temperature	Restriction enzyme	Reference	Location on Tomato WGS Chromosomes (SL2.40)	Letter used in figure 2
M-H309K01	CAPS	F	AGCCCCCAGAAAGACTTGTT	60°C	HpyF3I	Verlaan 2011	SL2.40ch06:3571844..3571863	
		R	TTTTTAAGGGGTGTGCCAAG				SL2.40ch06:3571219..3571238	
T1563	CAPS	F	ACTTCACCTACAAATCCTTCCAGA	56°C	Taq I	Ji 2007	SL2.40ch06:29260641..29260664	
		R	GCCCTTCCCAATCCAGCAGT				SL2.40ch06:29259047..29259066	
T0774	CAPS	F	CTCCTCAACTGGCTAATTCTCAGG	55°C	MnI I	Ji 2007	SL2.40ch06:30027683..30027706	
		R	GCAAACCAAATACATCGCGTATCAC				SL2.40ch06:30027028..30027052	
MSc05732-3	CAPS	F	ATGCTTTTCGAGCACGAGCCT	55°C	Rsa I	Verlaan 2011	SL2.40ch06:30523528..30523548	
		R	AGCCTAAAGAGAACTAGGCAGGGGA				SL2.40ch06:30524212..30524236	
MSc05732-4	CAPS	F	ACGAGATGGAGCGGTCTTCAAGCT	55°C	Dde	Verlaan 2011	SL2.40ch06:30600857..30600880	
		R	GACAGATCTCCCGGTAGGAGAGCA				SL2.40ch06:30601501..30601524	
SL_2.40ch06_30.696 (Ty-3-M1)	CAPS	F	TCTGAATGCAGGGTAATCATGT	60°C	Msp I	Hutton 2011	SL2.40ch06:30696326..30696347	
		R	TGATTTCAGGCCTCATAGAACC				SL2.40ch06:30696915..30696936	
HBa0161K22 (Ty-3-M3)	CAPS	F	AGCTCTTCCAGGAGCAGTTTG	58°C	Mse I	Hutton 2011	SL2.40ch06:30814757..30814777	A
		R	CCCTCCAAATAGTTTCACAAATACG				SL2.40ch06:30815261..30815285	
UF_TY3-P1	CAPS	F	AAGGAGGATCTGGCTGCTTT	55 °C	Alu I	Present	SL2.40ch06:30816304..30816323	B
		R	GTTGGTTTGAATCGCCTTCT				SL2.40ch06:30816698..30816717	
UF_TY3-P3	CAPS	F	TCAATTTTCACTGTTCCTGACT	55 °C	BstN I	Present	SL2.40ch06:30821240..30821261	C
		R	CAAGAGTGAGGTTGTTCTTGATG				SL2.40ch06:30821517..30821539	
WU_M17	Sequence based	F	CCCCCTTAGGAACATTCGTCCTCA	55°C	n/a	Present	SL2.40ch06:30824901-30826100	D
		R	AGGGTAGGGAACAAGCCAAGGCA				SL2.40ch06:30826113..30826135	
FOS00169A13 (Ty-3-M4)	CAPS	F	AGCTATCAGCTGCCAGAGACAT	56°C	Mse I	Hutton 2011	SL2.40ch06:30852074..30852095	E
		R	CACCATCATTGTATCCAGAGAGC				SL2.40ch06:30852469..30852492	
WUR_M25	Sequence based	F	TGCCAGACTCAGCATTAGTTTGGGG	55°C	n/a	Present	SL2.40ch06:30868214..30868238	F
		R	TGTCCCCATCATGCCACACTTCCA				SL2.40ch06:30869157..30869180	
UF_TY3-P18	CAPS	F	AAGGTGCGGAGTGAAATTCT	55 °C	Hinf I	Present	SL2.40ch06:30872101-30873200	G
		R	TTGGTGAGGCGTTGATACAC				SL2.40ch06:30873067..30873086	
WU_M27	Sequence based	F	TGTCATCTCCCAGGGCTCTCTGT	55°C	n/a	Present	SL2.40ch06:30875560..30875582	H
		R	ACCTGTGGTGAAGGTAGTGCGGA				SL2.40ch06:30876226..30876248	
UF_TY3-P19	SCAR	F	CGTTCTGCTTAATGTGGCAAT	55 °C	n/a	Present	SL2.40ch06:30876061..30876081	I
		R	CAACGGAGGGAGCATATCAT				SL2.40ch06:30876451..30876470	
WU_M29	Sequence based	F	TCTACACTATGAGCCACTGCTCGT	55°C	n/a	Present	SL2.40ch06:30880703..30880726	J
		R	TCCTGAATCGGCCTCTGATTTGGA				SL2.40ch06:30881414..30881437	
WU_M31	Sequence based	F	GCCTGGACGAATGGGAGGCAC	55°C	n/a	Present	SL2.40ch06:30885455..30885475	K
		R	ATGGGCATCGGTCACTCGCG				SL2.40ch06:30886383..30886402	
UF_TY3-P23	CAPS	F	CAGAAAGGGCCGATGAATAA	55 °C	Alu I	Present	SL2.40ch06:30886934..30886953	L
		R	GCGCAAATGGAGGTAAAAAG				SL2.40ch06:30887313..30887332	
UF_TY3-P24	CAPS	F	TGGTCCTCCCTTAACCATTG	55 °C	Dpn II	Present	SL2.40ch06:30890519..30890538	M
		R	TGGAACCGTGAGGAAGAAAC				SL2.40ch06:30890878..30890897	
SL_2.40ch06_30.891 (Ty-3-M5)	CAPS	F	CCGAAGGTGATAACCACGAC	56°C	BstN I	Hutton 2011	SL2.40ch06:30891965..30891984	
		R	GCGACCCTAACCAACAATAAAC				SL2.40ch06:30891379..30891400	
SL_2.40ch06_31.040	CAPS	F	TTCCTCCAAGTCATCCACCTAC	56°C	HpyCH4 IV	Hutton 2011	SL2.40ch06:3104393..31040415	
		R	GAGTCGTTTATTCGTGCAGATG				SL2.40ch06:31039807..31039825	

Table S6. Primers used in this study.

Primers used to make the TRV2 VIGS constructs

		Sequence 5'-3'
TRV2-160	F	CACCATGGGAGCGATAACATTGAG
	R	CCCACTTCCACCACAACTCT
TRV2-180	F	CACCATGAAGACAAAAACTGCTTCTTTG
	R	ACCATTTTCATCCCGACAAA
TRV2-190	F	CACCGCTTGAGATGGGCTTATTGG
	R	TCTCCCAGGGCTCTCTGTAA
TRV2-PDS	F	CGGTCTAGAGGCACTCAACTTTATAAACC
	R	CGGGGATCCCTTCAGTTTTCTGTCAAACC

Primers used to amplify products that show Solyc06g051170, Solyc06g051180 and Solyc06g051190 together code for one gene (See supplemental figure 4)

	Sequence 5'-3'
F3	GCGTGGATCCTGCAAAGCGCA
R10	GGGAACAGCTGACCCCACTGGT
F7	CAGTAGCAGCTGACCTCGGGC
R7	TCGTGGACCTTGATGACGTGAATG
F6	CGGCACTGCATCAACAAAGGCG
R4	ACAGAGAGCCCTGGGAGATGACA
F4	CCATCATTGCAGGTTCCAATGGACT
R5	AGCAGGGGCAAAAACCACCG

Primers used for GeneRacer

	Sequence 5'-3'
F5	CTGAGGGCTTGCACAGGCCAAT
GeneRacer 5'	CGACTGGAGCACGAGGACACTGA
R3	GCAGCTGATAGCTGGCTGGC
GeneRacer 3'	GCTGTCAACGATACGCTACGTAACG

Primers used to amplify and TOPO clone the full length *Ty-1* and *Ty-3* . Start and stop codon are in bold

	Sequence 5'-3'
Ty-F7-CACC	CACCTTCAAGTATATACAGGAAAA**ATG**GGTGATCCG
Ty-R5	**CTA**GAGTATTTCCTGCAAAACCGATG

Chapter 4

Allelic variation of the *Ty-1* and *Ty-3* genes conferring resistance to Tomato Yellow Leaf Curl Virus

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Summary

Tomato Yellow Leaf Curl Virus (TYLCV) hampers tomato production worldwide. Our previous studies have focussed on mapping and ultimately cloning of the TYLCV resistance genes *Ty-1* and *Ty-3*. Both genes are derived from *Solanum chilense* and were shown to be allelic. The *Ty-1*/*Ty-3* allele is characterized by a 4 amino acid insertion at the 5-prime part of the protein and by a catalytic DFDGD motif. In this study the allelic variation of this gene is examined using cDNA from five *S. chilense* derived lines and using draft assemblies of whole genome sequences from more than 50 tomato cultivars, landraces and related wild species. Tobacco Rattle Virus induced gene silencing is used to silence *Ty-1*/*Ty-3* and shows resistance is compromised in three out of five *S. chilense* derived lines tested. One line with resistance derived from *S. chilense* LA1971 remained resistance after silencing of *Ty-1*/*Ty-3*. For another line (8783) only 4 out of 13 plants showed symptoms after silencing. Comparison of the two typical features of the *Ty-1*/*Ty-3* gene showed that there is no sequence variation amongst the *S. chilense* derived lines. The catalytic domain was conserved among all tomato lines and species analysed. The characteristic 4 amino acid insertion is also present in three species closely related to *S. chilense,* e.g. *Solanum corneliomulleri, Solanum peruvianum* and *Solanum huaylasense*. In conclusion this study showed that probably most *S. chilense* accessions carry a TYLCV resistance/tolerance locus on chromosome 6, allelic to *Ty-1*/*Ty-3* and that *Solanum* species related to *S. chilense* could possibly be useful for future TYLCV resistance breeding.

Introduction

Tomato Yellow Leaf Curl Virus (TYLCV), a *begomovirus* of the *geminiviridae* family, is a phloem limited single stranded DNA virus that is vectored by the whitefly (*Bemisia tabaci*). TYLCV is one of the causing viruses of Tomato Yellow Leaf Curl Disease (TYLCD). In the last two decades TYLCD was a major constraint on tomato production in many warm and (sub) tropical regions worldwide and nowadays it is still a huge problem for many farmers. Tomato plants affected by TYLCD show yellowing and curling of apical leaves and when plants are severely affected flowers are abscised and plants completely stop growing. Controlling vector whitefly populations is expensive and labour intensive, resistant tomato cultivars are thus a good solution to control TYLCV. In cultivated tomato no resistance has been described and breeders have screened related species to find resistance sources (Vidavski, 2007). Until now five TYLCV resistance/tolerance genes have been described, e.g. *Ty-1* to *Ty-5* (Zamir et al., 1994; Hanson et al., 2006; Ji et al., 2007; Anbinder et al., 2009; Ji et al., 2009). *Ty-1*, *Ty-3* and *Ty-4* all originate from different *S. chilense* accessions. *Ty-1* is derived from LA1969, *Ty-3* from LA1932 and LA2779, and *Ty-4* also from LA1932. In a recent study from Spain *S. chilense* LA1932, LA1960 and LA1971 were used to introgress TYLCV tolerance. All the resistant lines developed in this study had *S. chilense* introgressions in the *Ty-1* region and the authors suggest that the resistances they describe are possibly also allelic to *Ty-1*/*Ty-3* (Pérez de Castro et al.). Multiple other *S. chilense* accessions have also been described as symptomless after TYLCV inoculation, like LA1938, LA1959, LA1961 and LA1968, the causal genes have however not been mapped (Pico et al., 1999). Two other known TYLCV resistance genes are not originating from *S. chilense*. *Ty-2* is introgressed from *Solanum habrochaites* and *Ty-5* has been first described in TY172, a breeding line derived from crosses of four *Solanum peruvianum* accessions. Recently the recessive resistance in the cultivar Tyking was shown to be co-localized with the resistance in TY172 and because of its recessive nature should be renamed to *ty-5* (Hutton et al., 2012).

Recently we fine mapped and cloned the *S. chilense* derived *Ty-1* and *Ty-3* genes (Verlaan et al., 2011; Verlaan et al., 2013). The genes were found to be allelic and code for RNA-dependent RNA polymerases (RDR) belonging to the RDRγ type. RDRs are defined by a conserved catalytic domain, DFDGD for the RDRγ, and DLDGD for the RDRα type. For RDRα many functions have been described. These studies have mainly been done in *Arabidopsis thaliana* and RDRα were found to be involved in stress response, pathogen resistance, female gamete formation, transgene silencing amongst many other functions (excellently reviewed in (Willmann et al., 2011)). In contrast with the RDRα

type no functions have been described in literature for RDRγ. The recently cloned *Ty-1* / *Ty-3* code for RDRs of the RDRγ type and thus provide a first insight into a possible new class of resistance genes.

Geminiviruses are assumed to be targeted by post transcriptional gene silencing and methylation based transcriptional gene silencing (PTGS and TGS) because of three major findings. The first is the presence of geminivirus-specific small interfering (si) RNAs in infected plants, the second is the reduction of replication and transcription of methylated geminiviruses. The third is that all geminiviruses encode (multiple) RNA silencing suppressors (RSS), which suppress PTGS and TGS in different ways (Brough et al., 1992; Voinnet et al., 1999; Wang et al., 2003; Chellappan et al., 2004; Vanitharani et al., 2004; Wang et al., 2005; Ribeiro et al., 2007; Raja et al., 2008; Buchmann et al., 2009; Yadav and Chattopadhyay, 2011; Yang et al., 2011; Luna et al., 2012). The mechanism of *Ty-1* / *Ty-3* based resistance is unknown, but because the RDRα type are known to be involved in the amplification of the siRNA signal it could be possible that the *Ty-1* / *Ty-3* encoded RDRγ has a similar function in siRNA amplification. However this remains speculative and further research has to be done to discover the key players, pathways and mechanisms involved.

In a previous study we showed the susceptible *ty-1* allele differs from the resistant *Ty-1* / *Ty-3* allele on multiple amino acid positions (Verlaan et al., 2013). The most striking difference was a 4 amino acid deletion almost at the start of the protein, while in the catalytic domain there were no differences. In this study we compare the deletion and catalytic domain among 9 different tomato lines that have *S. chilense* derived TYLCV resistance to see whether the deletion and catalytic domain are conserved. The same regions are also analyzed for approximately 80 cultivated tomato lines and 30 related wild tomato species to see if the *S. chilense* allele is unique. We also silence the alleles of the *Ty-1* / *Ty-3* gene in in several TYLCV resistant tomato lines to check if this silencing causes symptoms in these normally symptomless plants and if thus the same gene is responsible for TYLCV resistance.

Results

Silencing of *Ty-1*/*Ty-3* compromises resistance in multiple *S. chilense* derived lines

To prove whether TYLCV resistance in advanced breeding lines containing different *S. chilense* introgressions was based on *Ty-1* / *Ty-3* alleles the TRV2-180 and TRV2-190 silencing constructs for a VIGS approach as described in (Verlaan et al., 2013) were used. In the first experiment (Table 1, experiment 1) a tomato line (1538) with resistance from

S. chilense LA1932 and a plant line with resistance from LA1971 (1594) were tested. Both lines remained symptom free after inoculation of TYLCV two weeks after inoculation with the empty vector (EV) control or with a construct targeting a gene close to *Ty-1*/*Ty-3* (TRV2-160). In line 1538 symptoms appeared in all plants after silencing with TRV2-180 (n=3) or TRV2-190 (n=3), both targeting *Ty-1*/*Ty-3*. Plants (n=8) of line 1594, with resistance derived from LA1971, remained symptomless after silencing with the same two constructs targeting *Ty-1*/*Ty-3* (Table 1). VIGS was working in 1594 as all PDS control plants were showing photo bleaching.

Table 1: Virus Induced Gene Silencing of *Ty-1* followed by agroinoculation of TYLCV.

Tomato line	Resistance introgressed from	VIGS construct	Number of plants tested	Resistant (DI<2)	Susceptible (DI≥2)
Experiment 1					
1538	*S. chilense* LA1932	-	3	3	-
		TRV2-EV	2	2	-
		TRV2-160	3	3	-
		TRV2-180	3	-	3
		TRV2-190	3	-	3
1594	*S. chilense* LA1971	-	3	3	-
		TRV2-EV	3	3	-
		TRV2-160	4	4	-
		TRV2-180	4	4	-
		TRV2-190	4	4	-
Experiment 2					
B26	*S. chilense* LA1932	TRV2-EV	2	2	-
		TRV2-180	4	-	4
8783	*S. chilense* LA1932	TRV2-EV	4	4	-
		TRV2-180	13	9	4
F11E976-BK	*S. chilense* LA1938	TRV2-EV	4	4	-
		TRV2-180	8	-	8
1594	*S. chilense* LA1971	TRV2-EV	1	1	-
		TRV2-180	2	2	-
G1.1554 (wild accession)	*S. pimpinellifolium*	TRV2-EV	4	4	-
		TRV2-180	18	18	-

A second experiment was done to test whether in other TYLCV resistant lines resistance could also be compromised if *Ty-1*/*Ty-3* was silenced (Table 1, experiment 2). As control TRV2-EV was used and TRV2-190 was used to silence *Ty-1*/*Ty-3*. Line 1594 was tested again and the results were similar as in the first experiment; the two tested plants remained symptomless after silencing of *Ty-1*/*Ty-3*. In two other lines (B26 and F11E976-BK) with *S. chilense* derived resistance all plants showed symptoms after silencing with TRV2-180 (B26 and F11E976-BK), these lines have introgressions from LA1932 and LA1938 respectively. For tomato line 8783 with an LA1932 introgression 4 out of 13 plants showed symptoms after *Ty-1*/*Ty-3* silencing, it should be noted that from the three PDS control plants of this line only 1 showed photo bleaching, indicating silencing efficiency is low in this line. In this second experiment also a wild TYLCV resistant *S. pimpinellifolium* was included. Silencing of *Ty-1*/*Ty-3* did not result in TYLCV symptoms in this line.

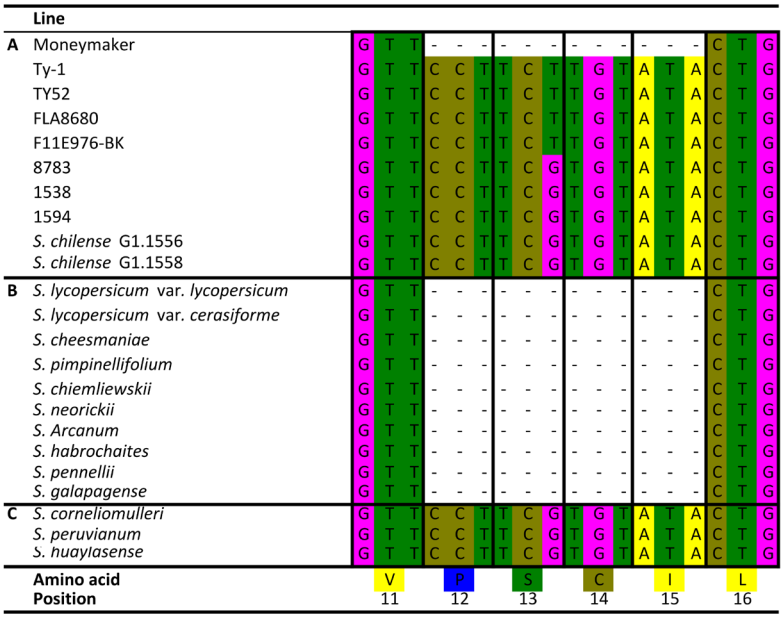

Figure 1. Alignment of sequences of the region containing the 5 prime deletion.

All *S. chilense* derived lines have the 12 base pair insertion (A). There is one synonymous SNP. Of the multiple species tested, three had the insertion, e.g. *S. corneliomulleri*, *S. peruvianum* and *S. huaylasense* (B and C). Sequences from (A) have been obtained from cDNA, sequences from (B) and (C) have been obtained from whole genome sequencing

Analysis of the 5′ prime deletion and the catalytic domain in *S. chilense* derived lines

In the TYLCV susceptible control Moneymaker a 4 amino acid deletion from position 12 to 15 is present in the *ty-1* allele if compared with the *Ty-1*/*Ty-3* alleles that were cloned (Verlaan et al., 2013). To check whether this deletion is unique for susceptible Moneymaker and not present in a variety of *S. chilense* derived TYLCV resistant lines, cDNA of these lines was made. Primers were designed to amplify the region of interest and sequence analysis showed that these 4 amino acids (Proline, Serine, Cysteine, Isoleucine) are present in all lines, however there is one synonymous SNP (T-G) among the *S. chilense* derived lines (Figure 1, A).

The catalytic domain of the *Ty-1*/*Ty-3* allele is characterized by a 5 amino acid long DFDGD motif (position 723-727). SNPs in this domain could potentially have an effect on the functioning of this protein. Sequence analysis of an amplified cDNA fragment among all tested *S. chilense* derived lines showed there were no SNPs present in the catalytic domain, also 4 amino acids up- or downstream of the catalytic domain no differences were found. A *Ty-2* carrying line and a wild *S. pimpinellifolium* were also included in this cDNA analysis but also here no differences were found (Figure 2).

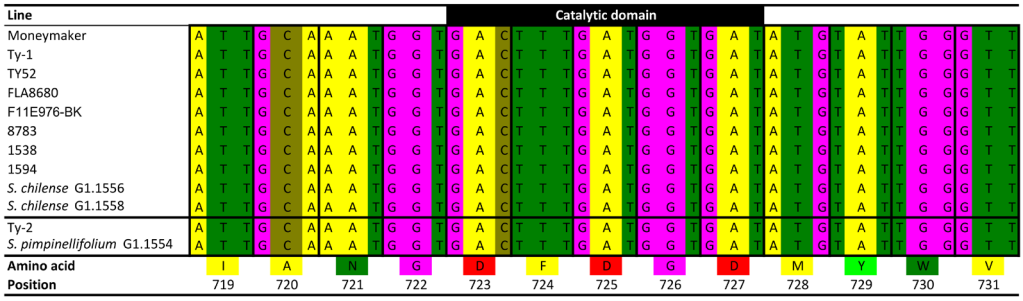

Figure 2. Alignment of cDNA sequences of the region containing the catalytic domain.

All *S. chilense* derived lines have a similar sequence in this region. Also a *Ty-2* line and *S. pimpinellifolium* G1.1554 had a similar sequence. All species in the full genome data set were also analysed but no SNPs were observed (data not shown).

Sequence variation of *Ty-1*/*Ty-3* amongst multiple tomato lines

To check the abundance of the 4 amino acid indel amongst cultivated tomato and wild tomato species the draft genome assemblies of all available sequenced lines were compared for this region. Of all species depicted in the phylogenetic tree (Figure 3) at least one accession was included in the dataset. It was found that all cultivated tomato lines in the test panel do not have these 4 amino acids (e.g. *S. lycopersicum* var.

lycopersicum and *S. lycopersicum* var. *cerasiforme* (cherry tomato)) (Figure 1, B). Also the majority of the wild species do not have the insertion (Figure 1, B). Several related wild species in the test panel do however have the insertion, these are *Solanum corneliomulleri*, *Solanum peruvianum* and two accessions of *Solanum huaylasense* respectively (Figure 1, C). The sequence coding for the catalytic DFDGD motif was also compared among all available sequences. This region was found to be highly conserved and in none of the susceptible and resistant lines analysed a SNP was detected (data not shown).

Discussion

Recently we fine mapped and cloned the *S. chilense* derived TYLCV resistance genes *Ty-1* and *Ty-3* and found that they were allelic and coding for RNA dependent RNA polymerases of the DFDGD class (Verlaan et al., 2013). Because *Ty-1* and *Ty-3* were always thought to be two different genes and were both introgressed from *S. chilense* we speculated that maybe all TYLCV tolerance introgressed from *S. chilense* is based on different alleles of the same gene. In this study we show, using VIGS, that this hypothesis holds true for the majority of *S. chilense* derived TYLCV resistant lines. We also show the catalytic domain of the gene is conserved among cultivated tomato and several species in the tomato clade. An insertion of 12 base pairs at the 5 prime part of the coding sequence is however only found in *S. chilense* derived alleles and in a few related wild *Solanum* species.

When *Ty*-1/*Ty*-3 was silenced in three out of six lines tested resistance was broken in all plants tested (e.g. 1538, B26 and F11E976-BK). Lines 1538 and B26 both have resistance introgressed from LA1932, an accession which has been described as a *Ty-3* carrying line (Ji et al., 2007). In these two lines the same gene (or allele of the same gene) as described in (Verlaan et al., 2013) is thus probably causing the resistance. The same conclusion can be made for line F11E976-B, derived from *S. chilense* LA1938. For tomato line 8783, with an LA1932 introgression (just as 1538), similar results were expected, but only some plants showed symptoms after *Ty-1*/*Ty-3* silencing. Because only one out of three control PDS plants showed photo bleaching it is possible that also *Ty-1*/*Ty-3* silencing failed in some plants. If this is the case this could be the reason that some plants did not show symptoms. Another possibility is the presence of a second segregating resistance gene which causes resistance even when *Ty-1*/*Ty-3* is silenced. The presence of a second resistance allele in *S. chilense* could also explain why in both experiments line 1594, with S. *chilense* LA1971 derived resistance, remained symptomless after

silencing of *Ty-1*/*Ty-3* followed by TYLCV challenging. Two families with *S. chilense* LA1971 derived resistance are described in (Pérez de Castro et al., 2012). Both of the families have an introgression on chromosome 6, but they also share an introgression on chromosome 10. Interestingly the recently described *S. chilense* LA2779 derived *Ty-6* gene is also mapped to chromosome 10 (Hutton, 2013). In line 1594 a similar gene to *Ty-6* is thus maybe causing resistance. Future cloning of *Ty-6* would allow silencing of this gene in line 1594 and possibly in combination with a mapping approach the puzzle can be solved.

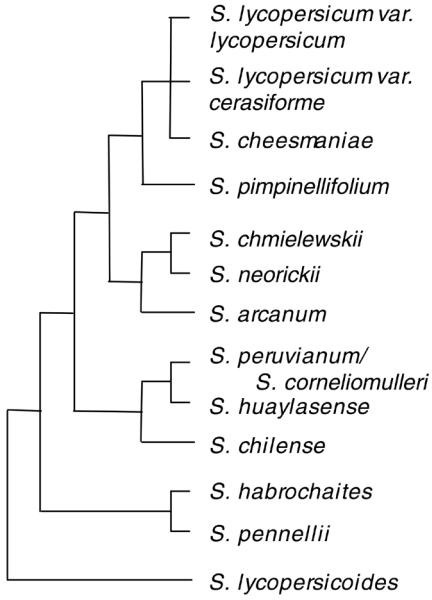

Figure 3. Phylogenetic tree of the plant clade *Solanum*, section *Lycopersicon* (adapted from Moyle, 2008).

Close species out group *Solanum lycopersicoides* is included for reference

The most striking difference between the *Ty-1*/*Ty-3* and *ty-1* alleles found was a deletion of 12 base pairs in the 5 prime part of the coding sequence in susceptible line Moneymaker (Verlaan et al., 2013). Our results show that a set of 9 different *S. chilense* (derived) lines don't have this deletion. In a large set of cultivated and wild tomato lines of which genome draft sequences were available, this deletion was present and the 15 base pair insertion was only detected in the related wild species *Solanum corneliomulleri*, *Solanum peruvianum* and *Solanum huaylasense*. These species all cluster together in a phylogenetic tree of tomato and are closely related to *S. chilense* (Anderson et al., 2010) (Figure 3). It will be interesting to test whether these lines also have TYLCV resistance. In a previous study we found that *Ty-1* was higher expressed then *ty-1* (Verlaan et al., 2013). Expression level experiments of the *Ty* allele in these lines could possibly also give

more insight into the resistance mechanism of *Ty-1*. In the same study we described a recombinant (R7) that has a chimeric *Ty-1* allele, the 5 prime region of the gene is from a susceptible cultivar and the 3 prime region is from *S. chilense* LA1969 (Chapter 3). This line has intermediate TYLCV resistance showing only slight symptoms upon TYLCV inoculation. The insertion is thus not solely responsible for the resistance, future studies with other (man-made) chimeric *Ty* alleles could possibly answer which parts of the protein are essential.

The same set of tomato lines was also used to compare the typical DFDGD catalytic domain. No SNPs were found in the domain and also 12 base pairs up- or downstream of this domain no differences were found among a *Ty-2* carrying line, a wild *S. pimpinellifolium* and the same 9 *S. chilense* lines as described before. The region was also compared amongst the same set of cultivated and wild tomato lines. It was found that the catalytic domain was conserved and no SNPs were found in any of the lines tested. This could indicate this gene is important for the plant and SNPs in the catalytic domain have possibly an effect on plant fitness.

In conclusion this study showed that probably most *S. chilense* accessions carry a TYLCV resistance/tolerance locus on chromosome 6, allelic to *Ty-1*/*Ty-3*. Fine mapping and/or more VIGS experiments could prove whether this is really true. The catalytic domain of this gene is conserved among *Solanum* species. The typical 15 base pair insertion of *Ty-1*/*Ty-3* is only present in *S. chilense* and in some related wild *Solanum* species. These related species could possibly be useful for future TYLCV resistance introgression breeding.

Materials and methods

Plant materials

Selected lines with different *S. chilense* introgressions derived from LA1932, LA1938 and LA1971 were collected. Seeds were sown and plants were kept under greenhouse conditions at a temperature of 23 °C and relative humidity of 60% during a 16-hour day / 8-hour night regime.

TYLCV inoculation

An infectious TYLCV-IL clone (pTYCz40a) was used for agroinoculation using the method as described in (Verlaan et al., 2011). Briefly, *A. tumefaciens* LBA4404 was transformed, cultured in LB, pelleted and resuspended in infiltration medium at an OD_{600} of 0.5. Three week old seedlings were infiltrated by pressure inoculation in the

leaves with a needle-less syringe. For the VIGS experiments the agro infiltration was done two weeks after TRV inoculation.

TRV based Virus Induced Gene Silencing

For the silencing experiments TRV constructs and procedures as described in (Verlaan et al., 2013) were used. Briefly, *A. tumefaciens* strain GV3101 containing the TRV replicons were cultured, pelleted and resuspended in infiltration medium. Agro infiltration was performed on cotyledons of 10 day old seedlings using pressure inoculation.

RNA isolation and cDNA synthesis

For sequence analysis 3 week old seedlings were agro inoculated as described above. Three weeks after agroinoculation top leaves of plants were harvested and grinded in liquid nitrogen using mortar and pestle. Total RNA was extracted by using the RNeasy Plant Mini Kit (Qiagen) as described by the manufacturer. One μg RNA was digested using DNase I (Amp. Grade) following the manufacturers protocol (Invitrogen) and cDNA was synthesized using the iScript cDNA Synthesis Kit following the protocol (Bio-Rad).

Sequence analysis of the *S. chilense* derived lines

For amplifying the region containing the 5 prime deletion primers Del-F1 (5′-TTCAAGTATATACAGGAAAAATGGGTGATCCG-3′) and Del-R1 (5′-CTGAGGGCTTGCACAGGCCAAT-3′) were used. For amplifying the region containing the catalytic domain, primers DFDGD-F4 (5′-GGGCGTGTTTTGGTCTACAG-3′) and DFDG-R4 (5′-GCTATCAGCTGCCAGAGACAT-3′) were used. PCR amplification was performed according to standard protocols in an Eppendorf Mastercycler Pro. Amplified fragments were sequenced and analyzed using SeqMan Pro 9 (DNA Star). Alignments were made with MEGA version 5 (Tamura et al., 2011).

Sequence analysis of the deletion and the catalytic domain

84 tomato and related wild species were re-sequenced with a read depth of approximately 30x (Aflitos *et al*, in preparation). In short, reads were mapped to the reference genome of *S. lycopersicon* cv. Heinz (Consortium, 2012) using BWA (Li and Durbin, 2009), SNP and INDELS were called using samtools (Li et al., 2009) and saved in the variant call format (VCF) (Danecek et al., 2011). Variants were visually inspected using the Integrative Genomics Viewer (IGV) (Robinson et al., 2011). For a list of sequenced species we refer to http://www.tomatogenome.net/accessions.html.

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Chapter 5

The TYLCV resistance gene *Ty-1* confers resistance in tomato through enhanced transcriptional gene silencing

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Summary

Tomato Yellow Leaf Curl virus and related begomoviruses are a major threat to tomato production worldwide. Genes from different wild tomato species are introgressed to confer against the virus. Recently the *Ty-1* resistance gene was identified and shown to be allelic with *Ty-3*. The genes were found to code for an RNA-dependent RNA Polymerase and for this reason most likely conferred resistance involving amplification of the siRNA signal. Here we show that upon TYLCV-challenging of resistant lines with *Ty-1* or *-3* low virus titers were detected concomitant with the production of relatively high levels of siRNAs. In contrast to the situation in susceptible tomato Moneymaker where high virus titers were observed and the amount of siRNAs produced lower compared to those in *Ty-1* and *-3*. Comparative analysis of the spatial genomic siRNA distribution showed a consistent and subtle enrichment for siRNAs derived from the CP (V1) and C3 gene in *Ty-1* and *Ty-3* lines. In tomato lines containing the *Ty-2* resistance gene, included as a control, the virus was hardly detectable but the siRNA profile similar to the one observed in TYLCV-challenged susceptible tomato Moneymaker. Furthermore, genome methylation analysis did reveal a relative hypermethylation of the TYLCV CP (V1) promoter region in genomic DNA collected from *Ty-1* in comparison to susceptible tomato Moneymaker. The results indicate that *Ty-1*, representative for a novel class of *R*-genes, confers resistance through enhanced transcriptional gene silencing.

Introduction

Tomato Yellow Leaf Curl Virus Disease causes enormous yield losses in tomato production worldwide and is caused by different related begomoviruses with tomato yellow leaf curl virus (TYLCV) as the most important one. The virus, like all begomoviruses, is transmitted in a persistent manner by the whitefly *Bemisia tabaci*. TYLCV contains a monopartite single-stranded DNA genome that is bi-directionally transcribed for the expression of six partially-overlapping open reading frames (ORFs). The genes responsible and involved in the regulation of genome replication and transcription (C1-4) locate to the left-hand side of the genome, while those involved in packaging, movement and transmission (V1 and V2) are located on the right hand side. From the genes involved in replication, the replication-associated protein (Rep; C1), transcription activator protein TrAP (C2) and replication-enhancer protein (REn; C3) have been relatively well studied and described. The right-handed side genes code for the coat protein (CP; V1) and precoat protein (PCP; V2). Since geminiviruses do not code for their own DNA polymerase, they re-activate the S phase in differentiated cells, to support and enable replication of their DNA genome (Jeske, 2009).

Besides control of the insect vector populations, breeding for resistance against TYLCV has been employed as the main strategy in disease management. Currently, six resistance/tolerance loci have been mapped and described in tomato. *Ty-1, -3 , -4* and *-6* are introgressed from different *S. chilense* accessions, *Ty-2* derives from *S. habrochaites* and *ty-5* from *S. peruvianum* (Zamir et al., 1994; Hanson et al., 2006; Ji et al., 2007; Anbinder et al., 2009; Ji et al., 2009; Hutton et al., 2012). Interestingly, low levels of virus replication have been reported with all these resistance genes. Instead of the classical R-gene mediated HR response, plants containing *Ty* genes thus exhibit more of a tolerance phenotype. Recently, the *Ty-1* and *Ty-3* resistance genes have been identified and cloned (Verlaan et al., 2011; Verlaan et al., 2013). The genes were found to be allelic, although both genes derive from different *S. chilense* accessions. They code for an RNA-dependent RNA polymerase (RDR) with sequence similarity to the Arabidopsis RDR 3, 4 and 5 type of genes, and distinct from the well-known and characterized RDR1, 2 and 6 type of genes. For RDR3, 4 and 5, no functions have been described so far, and their involvement in geminivirus resistance has unveiled a new class of resistance genes.

RNA silencing (also referred to as RNA interference, or RNAi) is a conserved eukaryotic gene regulation mechanism that plays a key role in various developmental processes. This pathway involves small (s)RNA molecules of ~21-26 nucleotides in size generated from perfect or imperfect long double stranded (ds)RNA molecules by

an enzyme designated Dicer (mammals, insects), or Dicer-like protein (DCL) (plants) (Ding and Voinnet, 2007). After unwinding of the sRNA molecules one strand, the so-called guide strand, is incorporated into an RNA-induced silencing complex (RISC). This complex senses RNA molecules with sequence complementary to the guide strand and targets these for degradation or translational arrest by means of the core Argonaute (AGO) protein. In plants and insects RNAi also plays an important role as antiviral defense mechanism, triggered by viral dsRNA replicative intermediates or RNA folding structures. In plants the RNAi signal is amplified with the involvement of RDRs. During this process generated viral siRNAs, instead of becoming uploaded into RISC, alternatively prime dsRNA synthesis by RDR on aberrant or functional viral RNA transcripts. This not only leads to the amplification of viral siRNAs but also to spreading of the siRNA signal to other parts of the viral genome, referred to as transitive silencing (Sijen et al., 2001). From the RDRs described only RDR1, 2 and 6 are so far known to be involved in the amplification of the siRNA signal and resulting in transitive silencing.

While geminiviruses harbor a single stranded (ss)DNA genome, they also are subject to transcriptional and post-transcriptional RNA silencing as observed by the synthesis of geminivirus-specific siRNAs, (small-RNA directed) viral DNA methylation and post-transcriptional gene silencing of the protein-coding genes (Chellappan et al., 2004; Ribeiro et al., 2007; Yadav and Chattopadhyay, 2011; Yang et al., 2011). However, antiviral RNAi defense against geminiviruses is suggested to mostly rely on a methylation-based defense, a process that involves the action of siRNA-directed methylation pathway component Ago4 (Hamilton et al., 2002; Zilberman et al., 2003).

During geminivirus infection RNA silencing is triggered by dsRNA that arises from overlapping and complementary 3'- ends of bi-directionally transcribed genes, followed by transitive silencing of the other left- and right-hand sided transcripts from the viral genome. As a result siRNAs are observed to originate from the entire virus genome although their distribution is not always equal (Rodriguez-Negrete et al., 2009; Yadav and Chattopadhyay, 2011). Geminiviruses code for RNA silencing suppressor (RSS) proteins that either sequester the single-stranded siRNA and prevent their loading into RISC, or interact and thereby inhibit adenosine kinase (ADK), an essential factor required in the process of DNA methylation and transcriptional gene silencing (Voinnet et al., 1999; van Wezel et al., 2002; Wang et al., 2003; Vanitharani et al., 2004; Wang et al., 2005; Buchmann et al., 2009; Zhang et al., 2011; Luna et al., 2012). For geminiviruses, V2 and C2 have been identified as silencing suppressors involved both in transcriptional gene silencing (TGS) and post transcriptional gene silencing (PTGS) (Wang et al., 2005; Yang et al., 2007; Buchmann et al., 2009), while C4 has been identified to suppress PTGS

(Vanitharani et al., 2004).

Considering the role of RDRs 1,2 and 6 in RNAi, a role of the RDRs 3,4 and 5, and thus of *Ty-1*/*Ty-3*, in the amplification of the siRNA signal is tempting. This idea is being supported by the observation that mutants lacking RDR1, RDR2 and RDR6 still revealed basal levels of RNA silencing and siRNA biogenesis, and plants deficient in RDR2 and 6 only showed a moderate increase in susceptibility to geminiviruses (Raja et al., 2008; Garcia-Ruiz et al., 2010). Although RDR1 and RDR6 have been demonstrated to be involved in the biogenesis of geminivirus-specific siRNAs, those of other RDRs thus cannot be excluded (Muangsan et al., 2004; Wang et al., 2010).

Here we show that in *Ty-1*/*Ty-3* lines, but not in *Ty-2* lines nor susceptible tomato Moneymaker (MM), siRNA levels are relatively increased, and coincide with hypermethylation of the TYLCV V1 (CP) promoter region, indicating that *Ty-1* based resistance against TYLCV involves enhanced TGS.

Results

Ty-1/*Ty-3* lines support higher levels of siRNA amplification and slight siRNA enrichment for V1 and C3

Considering the function of *Ty-1* and its relation to the RDR1, 2 and 6 class of genes, it was hypothesized that resistance to TYLCV conferred by *Ty-1* likely involves enhanced (post-) transcriptional gene silencing, mediated by higher levels of siRNA amplification relative to those amplified in susceptible tomato MM (*ty-1*). To test this hypothesis, the amounts of siRNAs produced and their spatial distribution on the viral DNA genome were comparatively analyzed in *Ty-1*/*Ty-3* lines versus MM challenged with TYLCV. As another control, a *Ty-2* line containing a resistance gene against TYLCV that is mapped on chromosome 11, was included. Small RNAs were purified from TYLCV challenged plants as described in Materials and Methods. These experiments were done with 3 replicates, and sRNA samples ranged in concentrations from 7 to 130 ng per gram leaf material.

Purified siRNAs from TYLCV infected MM and *Ty-1*, *-2* and *-3* plants were radiolabelled and hybridized to PCR fragments spanning the entire TYLCV genome (Fig. 1). While, as expected, no signal was observed when small RNA from mock inoculated plants was used as a probe (data not shown), all TYLCV-challenged, susceptible and resistant, tomato plants revealed the presence of TYLCV-specific siRNAs reminiscent of an ongoing viral infection. A closer look at the results from 3 replicates revealed overall

higher amounts of siRNAs purified from *Ty-1* line compared to those from MM and *Ty-2*. The amount of siRNAs from *Ty-3* was seemingly lower, however the results not yet corrected for an internal control. Furthermore, a subtle but consistent difference in the spatial distribution and enrichment of siRNAs on the TYLCV genome was observed between the population of siRNAs from TYLCV-challenged MM, *Ty-1*, *-2* and *-3* lines. While siRNAs collected from susceptible MM and resistant *Ty-2* lines showed a similar siRNA distribution on the viral DNA genome (Fig. 1, panels MM and *Ty-2*), the siRNA profile from *Ty-1* and *Ty-3* consistently showed a relative increase of C3 and CP (V1)-derived siRNAs.

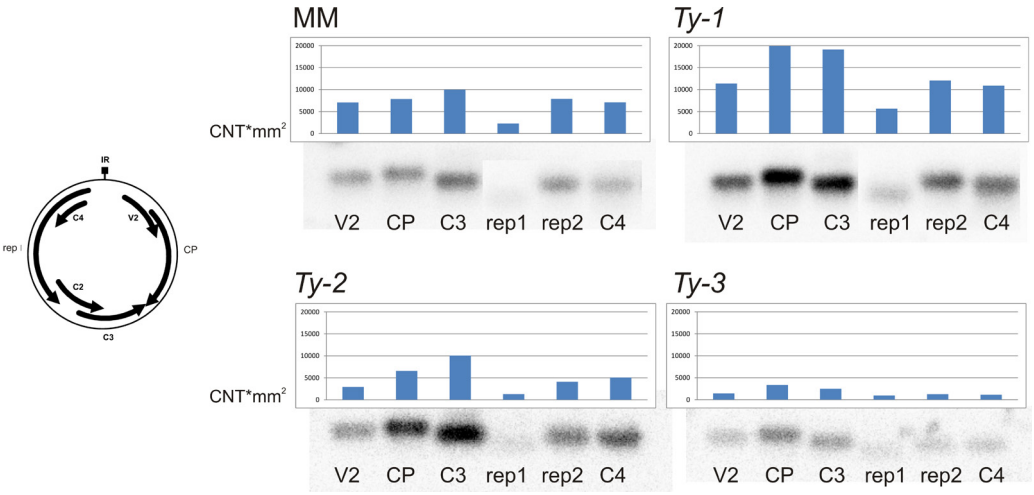


Figure 1: Relative abundance of TYLCV-derived siRNAs determined by Southern blot.

siRNAs isolated from TYLCV infected susceptible Moneymaker (MM) and resistant Ty-1, Ty-2 and Ty-3 lines were radiolabelled and hybridized to PCR fragments presenting the entire TYLCV genome. siRNA concentrations were quantified by Blot intensities.

To allow a quantitative comparison of siRNAs, a repetition of the experiment was performed in which siRNAs were purified from a fixed amount of leave material from *Ty-1* and MM. As an internal control for siRNA purification, and to assess for relative levels of TYLCV-derived siRNAs from different plants, a standardized amount of leaf material from a GFP-silenced *Nicotiana benthamiana* 16C line (Ruiz et al., 1998) was added during the purifications Radiolabelled siRNAs were probed on PCR fragments spanning the entire TYLCV genome, and TYLCV-specific siRNAs were quantified relative to the internal GFP-specific siRNAs (Fig.2). The results were in agreement with the first experiments and confirmed the production of higher amounts of TYLCV-

specific siRNAs in *Ty-1* lines in comparison to those from MM (a 3 fold increase), and in specific of those from the C3 and CP (V1) region. While siRNAs corresponding to the CP (V1) region were up to 8 times more abundant in *Ty-1* compared to MM (Fig.2), for those corresponding to V2, C3, C4, rep1 and rep2, an increase of 1.7, 2.5, 4, 4 and 3-fold was observed, respectively. Furthermore and interestingly, a subtle increase in the siRNA ratio of CP/C3 (from 0.77, MM to 1.43, *Ty-1*) was observed that already and consistently showed up during the first experimental replicates (Fig. 2).

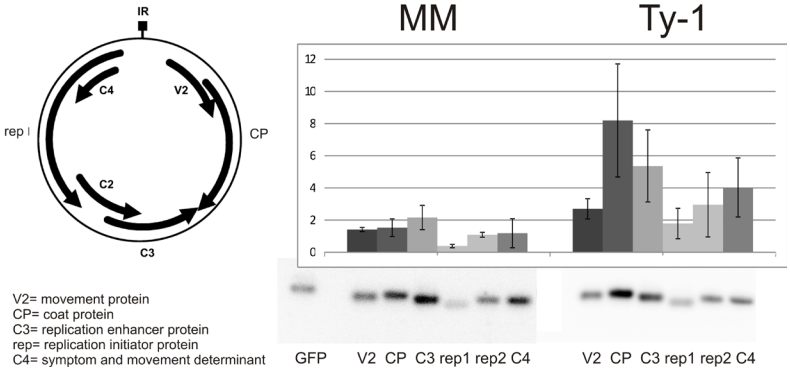


Figure 2: Quantification of TYLCV-derived siRNA levels relative to an internal GFP-siRNA control.

siRNAs isolated from TYLCV-infected susceptible Moneymaker (MM) and resistant Ty-1 plants were radiolabelled and hybridized to PCR fragments presenting the entire TYLCV genome. Y-axis indicates the expression levels relative to the internal GFP control as shown on the southern blot (ratio).

Simultaneous to the analysis of TYLCV-specific siRNAs and their genomic distribution in TYLCV-challenged *Ty-1*/*Ty-3* versus MM (*ty-1*), TYLCV virus titers were determined by TaqMan PCR. Whereas TYLCV-infected MM showed clear signs of infection by their stunted phenotype, systemic leaf chlorosis and curling, no clear visual symptoms were observed in *Ty-1*, *-2* and *-3* lines. As expected, TYLCV was easily detected by Taqman analysis in tomato MM, yielding on average (based on 5 plants) a Ct value of ~19.1 with a COX value of ~21.4. TYLCV was hardly detected in the resistant *Ty-1* and *-3* lines, with 3 plants for *Ty-1* and 2 plants for *Ty-3* showing Ct values that resembled those from the mock inoculated negative control plants. For the other *Ty-1* and *Ty-3* plants Ct values of ~ 22.9 and ~21.8 with a COX value of ~22.3 and ~21.7 respectively were detected, which is a Ct value increase of ~3 towards MM, and pointed up to a 10-fold reduction in TYLCV titers (Klerks et al., 2006; Table 1).

Table 1: Results of Taqman PCR for TYLCV virus and COX, and description of virus diseasesymptoms on tomato leaves 21 dpi.

Tomato line	virus	Ct value	COX	symptoms
MM	TYLCV	18.35	21.07	plant is small and yellow
MM	TYLCV	18.76	21.88	plant is small and yellow
MM	TYLCV	19.16	21.41	plant is small and yellow
MM	TYLCV	19.43	21.08	plant is small and yellow
MM	TYLCV	19.71	21.49	plant is small and yellow
Ty-1	TYLCV	37.60	21.82	no symptoms, size as negative control
Ty-1	TYLCV	undetermined	21.98	no symptoms, size larger than negative control
Ty-1	TYLCV	22.50	22.44	no symptoms, size larger than negative control
Ty-1	TYLCV	23.30	22.20	no symptoms, size larger than negative control
Ty-1	TYLCV	36.03	22.24	no symptoms, size as negative control
Ty-3	TYLCV	21.59	21.55	yellowing in lower leaves, sizs as negative control
Ty-3	TYLCV	21.92	21.93	yellowing in lower leaves, sizs as negative control
Ty-3	TYLCV	38.11	22.36	no symptoms, size larger than negative control
Ty-3	TYLCV	undetermined	21.95	no symptoms, size larger than negative control
Ty-3	TYLCV	21.85	21.72	yellowing in lower leaves, sizs as negative control
MM	mock	undetermined	21.85	
Ty-1	mock	39.17	22.36	
Ty-3	mock	39.52	22.19	
MM	mock	undetermined	21.85	

***Ty-1* lines show higher levels of TYLCV DNA methylation**

Since antiviral RNAi defense against geminiviruses is suggested to mostly rely on a methylation-based defense (Brough et al., 1992; Raja et al., 2008), we next tested whether *Ty-1* mediated siRNA amplification led to increased geminivirus DNA methylation and thereby caused transcriptional silencing and an overall TYLCV resistance/tolerance. If true, and considering the relative enrichment for CP (V1) and C3 derived siRNAs in *Ty-1* plants, we expected to see a difference in methylation of the corresponding genome regions between TYLCV DNA collected from *Ty-1* and MM plants. To test for this, a bisulfate DNA treatment strategy was employed to chemically convert unmethylated cytosines into uracils. During a subsequent PCR amplification and sequence analysis

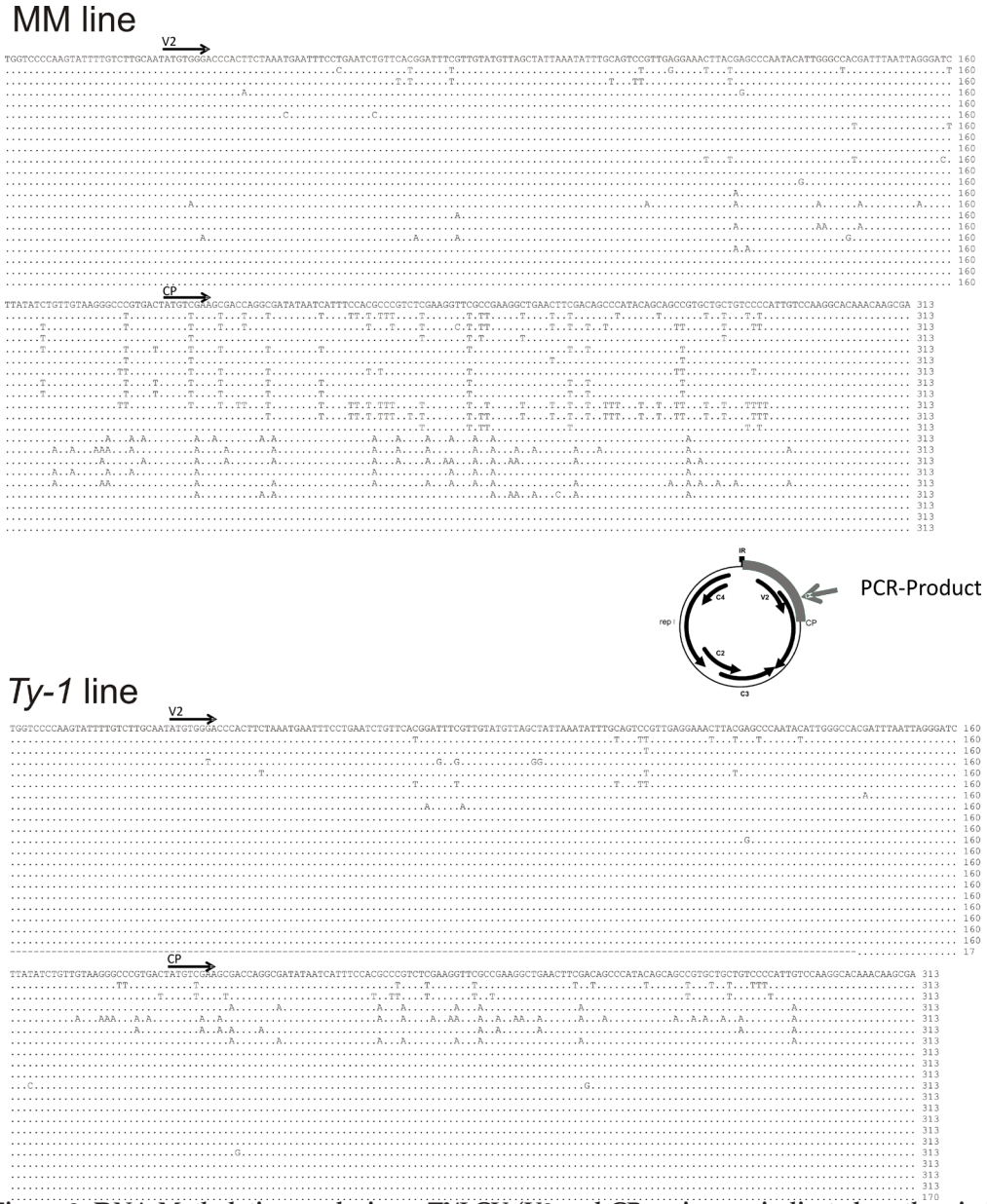
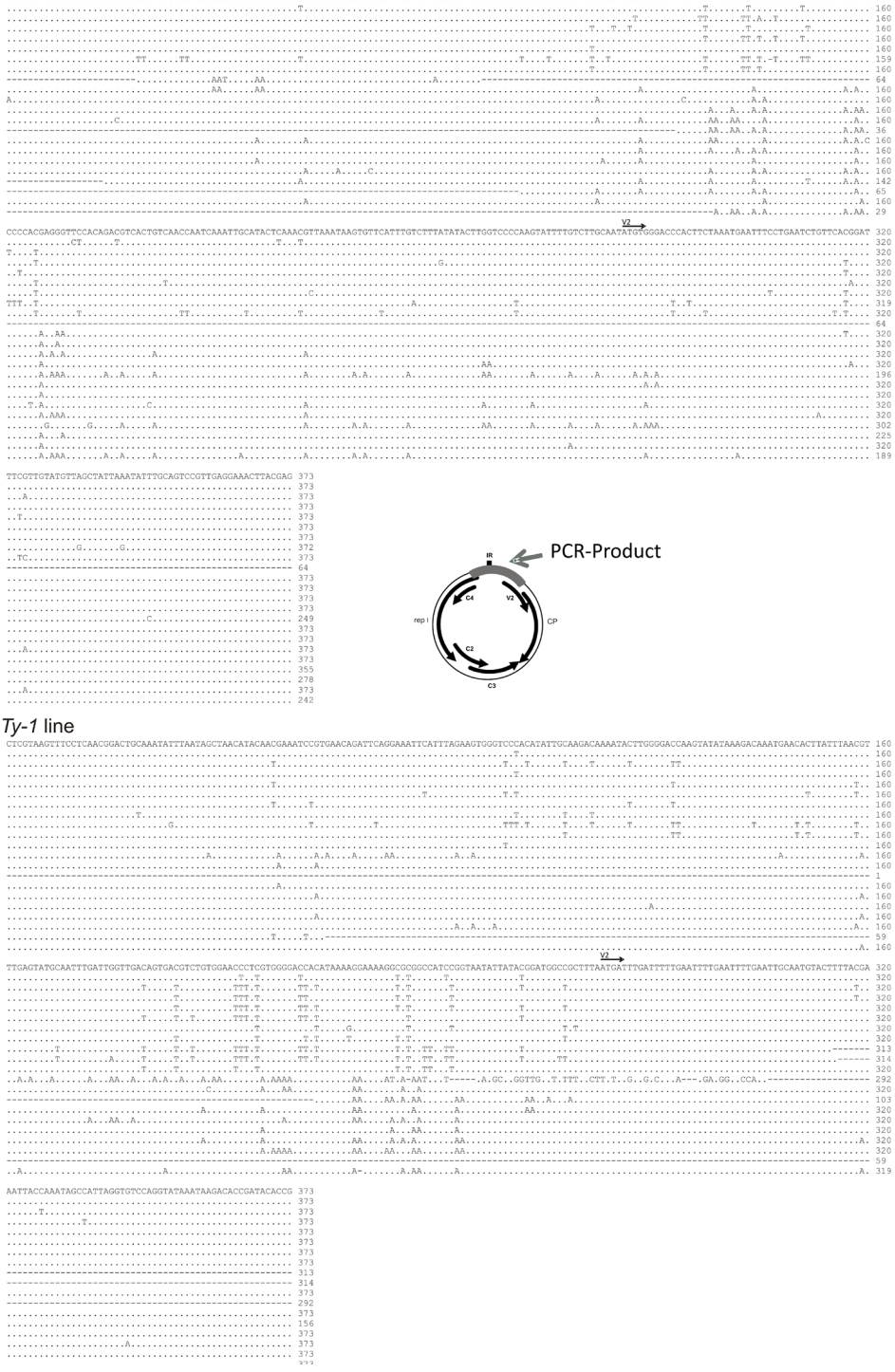


Figure 3: DNA Methylation analysis on TYLCV (V2 and CP region, as indicated on the viral map) in MM and *Ty-1*.

Multiple sequence alignment of cloned PCR products obtained from Bisulfite-treated DNA genome templates, and spanning the coding region of V2 and CP. Nucleotides identical to the reference sequence from TYLCV (ALM) are shown as dots. Thymidines (T) indicated, reveal the position of unmethylated cytosines in the TYLCV genome. Start of open reading frames are indicated by arrows.



Ty-1 line

these would then be identified as thymidines and distinguish from the original viral genome sequence. Two primer sets were designed to enable amplification of the 5′-UTRs and coding sequence of V2 and CP (V1). DNA was isolated from the same TYLCV infected *Ty-1* and MM lines that were earlier used for sRNA purifications. After bisulfite treatment of the DNA PCR amplification of the V2 and CP (V1) regions was performed. DNA fragments corresponding in size to the expected V2 and CP (V1)-fragments were cloned and individual clones selected for sequence analysis (20 clones per sample). The status of DNA-methylation in TYLCV collected from infected MM and *Ty-1* plants was determined by sequence comparison to the original sequence of TYLCV (Fig. 3, 4). Multiple sequence alignment covering the entire V2-V1 (CP) region of TYLCV collected from *Ty-1*, revealed that only 2 clones exhibited a cytosine conversion into thymidine, and indicating that all other cytosines were methylated (Fig. 3). In contrast to the situation in MM where this was observed for 10 out of 20 clones. The hypermethylation of the start region of CP (V1) in *Ty-1* lines was supported by the absence of a clear difference in the methylation profile of the upstream 5′-UTR and start coding region of V2 between the same clones collected from *Ty-1* and MM. Out of 20 clones, 9 respectively 8 contained unmethylated cytosine derived thymidines in that region for *Ty-1* and MM (Fig. 4).

Figure 4. DNA Methylation analysis on TYLCV (V2 region as indicated in the TYLCV map) in MM and *Ty-1*.

Alignment of clones from cloned PCR products encompassing the intergenic- and coding region of V2 and obtained from Bisulfite-treated DNA genome templates. Nucleotides identical to the reference sequence from TYLCV (ALM) are shown as dots. Thymidines (T) indicated, reveal the position of unmethylated cytosines in the TYLCV genome. Start of open reading frames are indicated by arrows.

Discussion

Based on the identification and cloning of the *Ty-1*/*Ty-3* resistance genes and their similarity to RDR1, 2 and 6, with well described roles in the amplification of the siRNA signal, we earlier hypothesized on a resistance mechanism involving enhanced TGS (Verlaan et al., 2013). In this study we have shown that upon challenging with TYLCV, *Ty-1*/*Ty-3* lines support the production of high levels of TYLCV-specific siRNAs with a consistent enrichment for siRNAs derived from C3 and CP (V1), relative to low concentrations of virus titer and in comparison to well infected and susceptible MM (*ty-1*). The high levels of siRNA production coincided with events of hypermethylation in corresponding regions of the TYLCV DNA genome, as tested and demonstrated for the promoter region of CP (V1) for which elevated levels of siRNAs were found. These data altogether support the idea that resistance, or rather the tolerance for TYLCV conferred by *Ty-1*/*Ty-3* most likely is based on enhanced TGS. The observed enhanced levels of siRNA amplification in *Ty-1*/*Ty-3* lines in comparison to those in MM (*ty-1*) also provides the first experimental proof that the DFDGD-class of RDR genes, to which *Ty-1* belongs, are involved in the biogenesis and amplification of siRNAs. RDRs with a catalytic DLDGD motif (RDR1, -2 and-6) have earlier been demonstrated to be involved in the biogenesis of geminivirus-specific siRNAs (Muangsan et al., 2004; Wang et al., 2010), but mutants lacking RDR1, RDR2 and RDR6 still revealed basal levels of RNA silencing and siRNA biogenesis. The additional observation that plants deficient in RDR2 and 6 only showed a moderate increase in susceptibility to geminiviruses (Raja et al., 2008; Garcia-Ruiz et al., 2010), agrees to the involvement of other RDRs, *Ty-1* as shown here, in RNAi amplification. While transcriptional upregulation of RDR2 and 6, both involved in siRNA amplification required for PTGS of plant RNA viruses, was shown earlier to confer levels of resistance to various plant RNA viruses, the *Ty-1*-mediated siRNA amplification does not provide resistance to plant RNA viruses (Schwach et al., 2005)2005, and here also tested and confirmed for CMV and TSWV (data not shown). This would suggest that *Ty-1* mediated siRNA amplification is not needed to support PTGS, and makes it tempting to hypothesize that *Ty-1,* and the other members from the DFDGD-class of RDR genes, are only involved in the biogenesis and amplification of siRNAs required for TGS by Ago4 mediated siRNA-directed DNA methylation (RdDM). This also makes sense in light of a primary role for a methylation-based defense against geminivirus infections, as demonstrated by several observations: 1) plant lines deficient for different TGS pathway components (e.g. AGO4) exhibit hypersusceptibility to geminiviruses, combined with

low levels of DNA genome methylation (20%) (Raja et al., 2008); 2) geminivirus genome copies collected from infection-recovery plant tissues show a hypermethylation (80% in the intergenic region) (Raja et al., 2008; Rodriguez-Negrete et al., 2009), and 3) infections with TGS suppression-deficient mutant viruses lead to plant recovery (Wang et al., 2003; Raja et al., 2008).

One of the most intriguing and puzzling questions on the outcome of this research is how to explain for the relative enrichment and change in the siRNA ratio of V1 (CP)/C3 concomitant with a hypermethylation at the promoter region of CP (V1) in TYLCV collected from *Ty-1* in comparison to susceptible MM. Although the intergenic region upstream of V2 also showed an siRNA level increase by 1.7 fold, no differences in its methylation profile from *Ty-1* versus MM were observed. Considering that RDRs do not seem to have a bias for a specific viral RNA transcript templates, the siRNA enrichment for V1 (CP)/C3 could only make sense in light of both gene transcripts being involved in the bi-directional transcript overlap (CP-C3, Fig. 1) and leading to the generation of primary siRNAs. Whether in addition, V1 and C3 transcripts are relatively abundant and play part in this as well, remains unknown.

Considering the function of *Ty-1* and its involvement in siRNA amplification, it is difficult to explain the earlier reported resistance specificity against TYLCV. A study on *Ty-1* hybrids, however, indicated that the gene also seems to control a tolerance response to distinct (bi-partite) Begomovirus species (Boiteux et al., 2007) It indeed seems most likely that *Ty-1* also confers resistance to other geminiviruses, and not only to TYLCV. A pilot to test for this using the related TYLCV Sardinian strain, and the bipartite viruses *Tomato golden mosaic virus* yellow vain strain (TGMV-yv) and *Tomato severe rugose virus* (ToSRV), failed due to low infection scores obtained after agroinoculation of the respective strain clones on MM and *Ty-1* plants. Experiments will be repeated in near future to answer this question.

An additional interesting outcome of this study was the observation that *Ty-2* lines challenged with the virus also revealed the presence of TYLCV-specific sRNAs. Although these plants never showed any symptoms and the presence of virus has not been reported so far, the presence of TYLCV-specific siRNAs is clearly indicative for an ongoing viral infection. The additional observation that the siRNA profile obtained from *Ty-2* lines revealed similarities to the one from susceptible MM (*ty-1*), and not to those from *Ty-1*/*Ty-3*, not only strengthens all observations made, but supports the idea that resistance based on *Ty-2* also relates to tolerance and does not seem to involve enhanced TGS. In summary, here we have provided the first evidence that *Ty-1*/*Ty-3*, coding for a member of the DFDGD-class of RDR genes and presenting a new class of resistance

genes, provides a tolerance against TYLCV and most likely other geminiviruses by enhanced TGS. Elucidating the details of this mechanism and exploiting its potential in plant resistance breeding will be one of the challenges for the future.

Materials and methods

Plant material

Tomato lines MM, *Ty-1*, *Ty-2* and *Ty-3* were grown in the greenhouse at 22°C. Agrobacterium mediated inoculation of the infectious TYLCV-IL clone (pTYCz40a) was performed as described in (Verlaan et al., 2013). Three week old seedlings were infiltrated by pressure inoculation in the leaves with a 30 needle-less syringe.
Leaf tissues were sampled after 37 and 41 dpi, frozen immediately in liquid nitrogen, and stored at -80°C until use.

For GFP silencing assays, *N. benthamiana* line 16C stably expressing a functional copy of GFP (kindly provided by prof. David Baulcombe) were grown and infiltrated with *A. tumefaciens* containing construct pBinGFP (Tsien, 1998). Fourteen days post agroinfiltration (dpa) leaf tissue was harvested from top leaves exhibiting strong silencing of GFP, as visually monitored by UV light exposure.

Nucleic acid isolation

Total DNA and RNA was isolated from frozen tissue in xx extractionbuffer, followed by phenol-chloroform extractions and ethanol precipitation (ref?). Nucleic acid content was checked by gel electrophoresis and quantified using Nanodrop (ThermoFisher Scientific, Breda, The Netherlands). Subsequently Low-molecular-weight RNA molecules were separated from total DNA/RNA preparations by PEG precipitation. The DNA/RNA fraction pellet was resolved in 50µl TE and stored at -20°C until further use. For the purification of siRNAs, total LMW RNA was resolved on a 15% denaturing polyacrylamide gel containing 8M urea. After ethidium bromide staining, the small RNA fraction co-migrating with a purified siRNA marker reference, was excised from the gel, ground to small pieces and incubated overnight at 4°C in 3M NaCl for diffusion extraction. After centrifugation, the supernatant was collected and the siRNAs precipitated by ethanol.

For Taqman PCR assays DNA was extracted from young leaves using the cetyltrimethyl ammonium bromide (CTAB) (Doyle and Doyle, 1987). DNA concentration was estimated by Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Breda, The Netherlands).

Taqman PCR assay

The Taqman PCR assay was performed according to (Powell et al., 2012). The primers Fw: TYLCV-SAR 1669F: GTCGAGATATTCTTTAAATGATGATTGTG, Rv: TYLCV-cons 1756R: GGCAAGCCCATTCAAATTAAAGG were used in combination with the probe TYLCV-cons 1701T: HEX-CCTGGATTGCAGAGGAAGATAGTGGGAATTC-Black Hole Quencher 1 to detect TYLCV. A Taqman assay on COX (cytochrome oxidase 1) was used as a control according to (van Gent-Pelzer et al., 2010). For this primers COX F: CGTCGCATTCCAGATTATCCA and COX R: CAACTACGGATATATAAGRRCC RRAACTG were used together with the probe COX-Sol 1511T: Yakima-Yellow-AGGGCATTCCATCCAGCGTAAGCA-Black Hole Quencher 1. Taqman-PCR was performed in the ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, USA) with Taqman Universal Master Mix (Applied Biosystems, Foster City, USA) using the following PCR conditions: 2 min at 50°C, 10 min 95°C, and followed by 40 cycles of 15 sec 95°C and 1 min 60°C.

Southern Blotting

siRNAs were dephosphorylated with calf intestinal alkaline phosphatase (Promega, Leiden, The Netherlands) and subsequently radiolabelled with γ-32P ATP by T4 polynucleotide kinase according to the manufacturer's instructions (Invitrogen, Breda, The Netherlands).

Six sequence fragments representing the entire genome of TYLCV were amplified from the TYLCV amplicon as template using primer sets TAATATTACCGGATGG and TCAGGGCTTCGATACATTC (V2), TTACGCCTTATTGG and CCATGGAGACCTAATAG (rep1), TCTCGTGGAGTTCTCTGC and TACGGATGGCCGCTTTAATG (rep2), TGTTCCCCGTGGATGTG and TTAATTTGATATTGAATC (coat protein, CP), TAAAATTTATATTTTATATCATG and GCGTGTAGACCTAGAC (C3), CCGCGCAGCGGAAGA and ATGGGGAACCACATCTC (C4). Primer sets rep1 and rep2 amplified the replicase sequence from the start codon of C2 to the start codon of C3, and from stop codon of C4 to the start codon of C2, respectively. Equimolar amounts of purified PCR products were resolved on a 1% agarose gel and subsequently blotted to Hybond-N membrane (Amersham Bioscience Limited, UK) by top-down blotting. Filters were subsequently hybridized overnight to γ-32P-labelled siRNAs in Church Buffer at 48°C (Sambrook et al, 1989). Filters were washed in SSC 2x with 0.1% SDS at room temperature, and subsequently exposed to a phosphor screen (Biorad, Veenendaal, The Netherlands). Radioactive hybridization signals were visualized by phosphoimaging

(Molecular Dynamics Typhoon Phosphoimager, Amersham Biosciences Bioscience Limited, UK).

Bisulfite reaction, PCR and Cloning

Primer sets encompassing specified TYLCV genome regions were designed using the BiSearch program and resulted in 2 primer sets: TGGTCCCCAAGTATTTTGTC and TCGCTTGTTTGTGCCTTGGA (coat protein); CGGTGTATCGGTGTCTTATT and CTCGTAAGTTTCCTCAACGG (V2).

Bisulfite modification was carried out on DNA isolates using the Qiagen Bisulfite kit (Qiagen, Venlo, The Netherlands) according to the manual. PCR amplification was carried out using GoTaq (Promega, Leiden, The Netherlands). PCR products were gel purified and cloned into the pGEMt-easy vector system (Promega, Leiden, The Netherlands) followed by transformation into E.coli DH5α (Invitrogen, Breda, The Netherlands).

Sequence analysis

Sequence analysis was done according to Sänger-dideoxy sequencing (EUROFINS, MWG Operon, Ebersberg, Germany). Sequences derived from complementary strand TYLCV templates, and exhibiting thymidines in place of guanidines, were discarded from analysis.

The retrieved sequences were aligned to the TYLCV(alm) (genbank accession number AJ489258) reference sequence using BioEdit software (Hall, 1999) for the identification of transformed cytosines.

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Chapter 6

General discussion

Plants are the basis of most food humans consume. To keep up with food demand of the increasing world population farmers worldwide need to produce more and more. At the same time the amount of constraints are constantly increasing. The constraints vary from water availability to water logging, soil salinization, availability of suitable land and a multitude of other abiotic and biotic stresses. Research on all aspects of agricultural production can help to enable future food security for all people on earth.

The research described in this thesis was done within the framework of the Centre for BioSystems Genomics (CBSG). CBSG is a consortium of major Dutch and international companies and plant scientists working on the food crops potato, tomato and Brassica. Fundamental research on the model plant *Arabidopsis thaliana* is also within the scope of the consortium. The goal of CBSG is described as follows: '*CBSG2012 aims to exploit the full potential of a broad range of genomics approaches in order to create new opportunities for sustainable agro-production systems for potato, tomato and Brassica which shall have socio-economic implications for producer, processor and consumer alike, through crop production, enhanced food quality and reduced environmental impact*.' (www.cbsg.nl)

The research described within this PhD thesis fell within the pathogenomics cluster. A collection of research projects that focused on the interaction between plants and their pathogens. A pathogen that is causing major losses in tomato production during recent years is Tomato Yellow Leaf Curl Virus, spread by the whitefly *Bemisia tabaci*. The research described in this thesis focused on the mapping and characterization of the widely used TYLCV resistance genes *Ty-1* and *Ty-3*.

_Ty-1_ and _Ty-3_: a long way from mapping to cloning

Already in 1994 the first paper describing a TYLCV tolerance allele (*Ty-1*) introgressed from *S. chilense* LA1969 was published (Zamir et al., 1994). This study used 58 RFLP markers which spanned the complete tomato genome. The authors described a strong linkage between *Ty*-1 and markers TG97 and TG297. For more than 10 years this location was the most accurate location described for *Ty-1* and many tomato cultivars harboring *Ty-1* were developed by breeders worldwide using markers described in this paper. The introgression of *Ty-1* was however accompanied by severe linkage drag and a more accurate map position was required to facilitate breeding efforts. A number of other important resistance genes like *Cf* for *Cladosporum fulvum*, the *Ol*-genes for *Oidum lycopersicum* and *Mi* for root knot nematodes were also mapped to this region on chromosome 6 (Dickinson et al., 1993; Kaloshian et al., 1998; Bai et al., 2005). This stressed the importance of accurate map positions, so these genes could be more easily combined into one breeding line or commercial variety. In 2007 a study aiming to find a

marker closely linked to *Ty-1* was published (Pérez de Castro et al., 2007). In this study multiple lines having *Ty-1* and/or *Mi* were being used to unravel the order of markers and genes in this region. They concluded that the newly developed CAPS marker JB-1 was more useful for marker-assisted selection (MAS) of *Ty-1* then the previously used *Aps-1* marker because JB-1 did not interfere with *Mi* introgressions. The exact location of *Ty-1* was still not clear but they further concluded that most *Ty-1* carrying lines have large *S. chilense* introgressions. Meanwhile a second TYLCV tolerance gene (*Ty-3*) originating from *S. chilense* accessions LA2779 and LA1932 was mapped to a region distal from the *Ty-1* region on chromosome 6 (Ji et al., 2007).

By the end of 2009, and at the start of the research described in this thesis, the draft genome sequence of tomato (inbred cultivar Heinz 1706) became available. For the first time the order of markers on genetic maps could be compared with a physical map, which was very useful for further mapping of *Ty-1*. The order of markers as described by (Pérez de Castro et al., 2007) was found not to be accurate. For TG97, TG231 and JB-1 the order on the physical map was exactly opposite as proposed in their paper. Using the available tomato genome sequence we developed new markers and in combination with large recombinant screenings this allowed a more detailed mapping of *Ty-1*. The gene was found to be located approximately 10 million base pairs from the previously described location. The failure in accurate mapping was caused by recombination suppression in the *S. chilense* introgression. This suppression was caused by chromosomal rearrangements as shown by Fluorescent *in situ* Hybridization (FISH) experiments (outlined in detail in **Chapter 2**).

FISH as a tool for introgression breeding

The rearrangements found in the *S. chilense* introgressed region were not unique events. Many other rearrangements have been described in related species, when compared with *S. lycopersicum* (excellently reviewed in (Szinay et al., 2012)). Because the genetic basis of cultivated tomato is quite narrow breeders often use related wild species to introgress traits of interest like disease resistance or certain abiotic stress tolerances (Tanksley and McCouch, 1997). Together with the trait of interest, genes that negatively affect plant performance are introgressed, a phenomenon known as linkage drag. To loose these traits multiple backcrossings with the recipient crop plant need to be done. This approach has also been used by breeders for *Ty-1* but large *S. chilense* segments remained present in the lines carrying the gene and recombination suppression was observed. By FISH we showed that this was caused by chromosomal rearrangements and we showed the gene to be located just downstream of the region with these

rearrangements (**Chapter 2**). In this case it was a matter of luck that the gene was not located inside the region with rearrangements. If this was the case it would have been impossible to develop *Ty-1* tomato lines without linkage drag, thus with very short *S. chilense* introgressions. A situation like observed for *Ty-1* is something that breeders may encounter more often when wild species are used in breeding programs. In case large recombinant screenings indicate that it seems impossible to get rid of large wild introgressions (in combination with the trait of interest), a BAC-FISH approach might show whether the lack of crossovers are due to chromosomal rearrangements. If this appears to be the case, and the trait of interest is located inside the rearrangement, more recombinant screenings will not solve this issue and linkage drag will remain during introgression of this trait. A possible solution is to screen other related species for the same trait and check whether these species do not have the rearrangement. Another possibility is to apply a GMO approach, but this is only possible when the gene is known and subject regulations are clear and there is a societal acceptance. A strategy to finemap and clone genes in rearrangements is to use intraspecific crosses (e.g. between plants of the same accession but with contrasting phenotypes of the same trait) or interspecific crosses between two species of which both have the rearrangement compared to the cultivated crop (Kaloshian et al., 1998).

Future breeding with *Ty-1* and *Ty-3*

The rearrangements discovered by the FISH experiments are probably the reason the order of markers as proposed by (Pérez de Castro et al., 2007)) was not accurate. The *Ty-1* gene was located downstream of the rearrangements which allowed further fine mapping by recombinant screening and Recombinant Inbred Line (RIL) development. *Ty-1* and *Ty-3* both mapped to a similar 70 kb region and Virus Induced Gene Silencing (VIGS) was used to identify the genes and show that they are allelic and code for an RNA-Dependent RNA Polymerase (RDR) of the DFDGD class (**Chapter 3**). Because the first mapping of *Ty-1* was very inaccurate, markers used to select for *Ty-1* were actually not really close to the gene. Using these markers was no problem because the chromosomal rearrangements hindered recombination, thus a big piece of *S. chilense* including the *Ty-1* gene was introgressed when these markers were used. This inaccurate mapping position of *Ty-1* was also one of the main reasons that most likely lead to a false conclusion that *Ty-3* was a new gene instead of an allele of *Ty-1* from a different *S. chilense* accession (Ji et al., 2007).

By identifying the gene and showing the genes are allelic a major step has been made in breeding for TYLCV tolerant/resistant tomato cultivars. For many years

breeders have tried to combine *Ty-1* and *Ty-3* in cis because they assumed that they were located on different locations on chromosome 6. Breeders can now stop trying because this is impossible. Instead in-gene markers can now be used in combination with large populations to produce parental lines with a really short *S. chilense Ty-1* or *Ty-3* introgression and limited or no linkage drag. These lines can be the basis for further breeding and used in combination with other lines that have other chromosome 6 located resistance genes (like the *Cf*, *Ol* or *Mi* genes).

With *S. chilense* as a source of resistance, accessions LA1969 (*Ty-1*), LA1932 (*Ty-3*) and LA2779 (*Ty-3*) have been mainly used. It is however known that other *S. chilense* accessions also show TYLCV tolerance (Pilowsky and Cohen, 2000). Recently two other *S. chilense* accessions (LA1960 and LA1971) were used to introgress TYLCV resistance (Pérez de Castro et al., 2012). In that study multiple lines were developed and all shown to share an introgression on chromosome 6 that covers the *Ty-1*/*Ty-3* region. Taken all results together it now seems plausible that all *S. chilense* accessions have a *Ty-1*/*Ty-3* allele. This hypothesis is supported by results described in **Chapter 4** in which silencing *Ty-1*/*Ty-3* in most *S. chilense* derived lines compromised resistance against TYLCV. *S. chilense* tomato lines that did not show symptoms likely carry a second resistance allele derived from *S. chilense*. This gene, named *Ty-6*, has recently been described and maps to chromosome 10 (Hutton, 2013).

For TYLCV resistance breeding in total six genes are now described (e.g. *Ty-1* to *Ty-6*) (**Chapter 1** and Hutton, 2013). All these genes still allow viral replication and thus by definition present tolerance genes. The resistance spectrum of these genes still has not been well studied. In light of its function it is likely that *Ty-1*/*Ty-3* has a broad spectrum, i.e. not only confers resistance to (multiple) TYLCV strains and to geminiviruses from other genera but, although speculative, also to other DNA viruses. A tolerance to *Tomato rugose mosaic virus* and *Tomato yellow vein streak virus* has been described for hybrid lines carrying *Ty-1* (Boiteux et al., 2007). For *Ty-3* resistance to the geminivirus *Tomato mottle virus* has been reported but disease tests on *Ty-1*/*Ty-3* plants with other geminiviruses are scarce (Ji et al., 2007). *Nanoviridae* and *Caulimoviridae* are two other groups of DNA viruses and it would be interesting to test whether the resistance also holds to viruses classified in these families. Unfortunately tomato infecting virus species of these families are not known. A possible strategy to test the spectrum of *Ty-1*/*Ty-3* based resistance would be to transform the gene to *Nicotiana benthamiana.* This species is susceptible to many viruses including *Cauliflower mosaic virus* from the *Caulimoviridae* family and the *Faba bean necrotic yellows virus* from the *Nanoviridae* family (Grigoras et al., 2008). These experiments could give further insight into the resistance spectrum and mechanism.

More knowledge on the resistance mechanism could also lead the way to search for DNA virus tolerance/resistance genes like *Ty-1* in other crops.

At this moment, three out of the six TYLCV resistance genes have been cloned, i.e. *Ty-1* and *Ty-3* (this thesis) and recently *ty-5* (Levin, 2013). The t*y-5* gene is coding for PELOTA, which is presumably involved in ribosome recycling (Levin, 2013). *ty-5* based resistance is caused by the loss-of-function of PELOTA and thus relies on a completely different mechanism compared with *Ty-1*/*Ty-3*. For *Ty-2* the gene is not known yet, but the resistance mechanism is probably not based on enhanced TGS as is the case for *Ty-1*/*Ty-3* (**Chapter 5**). From a breeding perspective it thus might be useful to combine *Ty-1*/*Ty-3* with *Ty-2* and/or *ty-5* because this might lead to enhanced and/or more broad spectrum resistance. This combined resistance is probably also more durable although predicting durability is always hard (Lecoq et al., 2004). Combining should be feasible from a breeding point of view because *ty-5* is located on chromosome 4, *Ty-2* on chromosome 10 and *Ty-1*/*Ty-3* on chromosome 6.

***Ty-1* and *Ty-3* code for an RNA-dependent RNA polymerase**

Ty-1 and *Ty-3* were found to code for and RDR of the DFDGD class, for which no function was described before. As such we have unveiled a new class of resistance genes. To check for the presence of additional RDRs in the tomato genome the ITAG2.3 release of the annotated tomato genome was searched with the term 'RNA-dependent RNA polymerase' (Bombarely et al., 2011). In total 16 genes were predicted to present an RDR (Table 1). Three of these (number 6, 7 and 8) together were shown to code for *ty-1* (Verlaan et al., 2013). Number 13 and 14 are located next to each other and probably may code for one gene as well, while the same applies for 15 and 16. In total there are three genes predicted with a DLDGD motif (number 3, 4 and 10), these are thus of the RDRα type, also two genes of the RDRγ are predicted (number 6 and 15). In *A. thaliana* 6 RDRs are described. Three of the RDRα type (RDR1, RDR2 and RDR6) and three of the RDRγ type (RDR3, RDR4 and RDR5, also called RDR3a, -3b and -3c). Based on this annotation, tomato seems to have no copy of RDR1, a single copy of RDR2 and two copies of RDR6, and thus, similar to just like Arabidopsis three of the RDRα type. In contrast only two of the RDRγ type are predicted (named RDR3a and RDR3b in the ITAG2.3 database) instead of three like Arabidopsis. The other RDRs predicted are al really short and their identity thus remains disputable.

The identification and characterization of *Ty-1* allowed further exploration by allele mining. The RDRγ genes all share a typical catalytic DFDGD domain. The *Ty-1*/*Ty-3* allele is characterized by a 12 base pair insertion in the 5 prime part, compared to

Table 1. RNA-dependent RNA polymerase in *Solanum lycopersicum.*

The tomato annotation ITAG2.3 was searched with the term 'RNA-dependent RNA polymerase'. In total 16 hits were found in the database. Number 6, 7 and 8 are known together to code for *Ty-1*. Based on size and location probably number 13 and 14 and number 15 and 16 also together code for one gene. Not that the number after 'Solyc' or 'AT' indicates the chromosome number.

		Lenght predicted amino acid	RDRγ (DFDGD motif)	RDRα (DLDGD motif)
	S. lycopersicum			
1	Solyc00g142170.2.1	306		
2	Solyc03g063470.1.1	245		
3	Solyc03g114140.2.1 (RDR2)	1120		824-828
4	Solyc04g014870.2.1 (RDR6a)	1198		864-868
5	Solyc05g008740.1.1	134		
6	Solyc06g051170.2.1 (RDR3b)	576	280-284	
7	Solyc06g051180.1.1	146		
8	Solyc06g051190.1.1	319		
9	Solyc07g008830.1.1	140		
10	Solyc08g075820.2.1 (RDR6b)	1180		843-847
11	Solyc09g056290.1.1	133		
12	Solyc11g030860.1.1	162		
13	Solyc11g063530.1.1	141		
14	Solyc11g063540.1.1	302		
15	Solyc12g008410.1.1 (RDR3a)	831	745-749	
16	Solyc12g008420.1.1	262		
	A. thaliana			
1	AT1G14790 (RDR1)	1107		797-801
2	AT4G11130 (RDR2)	1133		830-834
3	AT2G19910 (RDR3)	992	693-697	
4	AT2G19920 (RDR4)	927	682-686	
5	AT2G19930 (RDR5)	977	682-686	
6	AT3G49500 (RDR6)	1196		863-867

the copy present in susceptible cultivated tomato. Allele mining in multiple *S. chilense* accessions showed that the catalytic domain is conserved amongst many wild tomato species (**Chapter 4**). The insertion, however, is only and consistently found in related species *S. peruvianum*, *S. corneliomulleri* and *S. huaylasense*. For some accessions of *S. peruvianum* TYLCV resistance has already been described (Pilowsky and Cohen, 2000). In light of future TYLCV resistance breeding it now will be interesting to test whether accessions of *S. corneliomulleri* and *S. huaylasense* are exhibit TYLCV resistance. As nicely shown in this thesis, VIGS experiments in combination with mapping studies provide nice tools to determine whether in the accessions also *Ty-1*/*Ty-3* is responsible for the resistant phenotype. Transcriptional expression of *Ty-1*in resistant lines was found to be higher compared to *ty-1* (**Chapter 3**) and whether this up-regulation and/or differences in protein sequences contribute to the resistance still remains to be investigated.

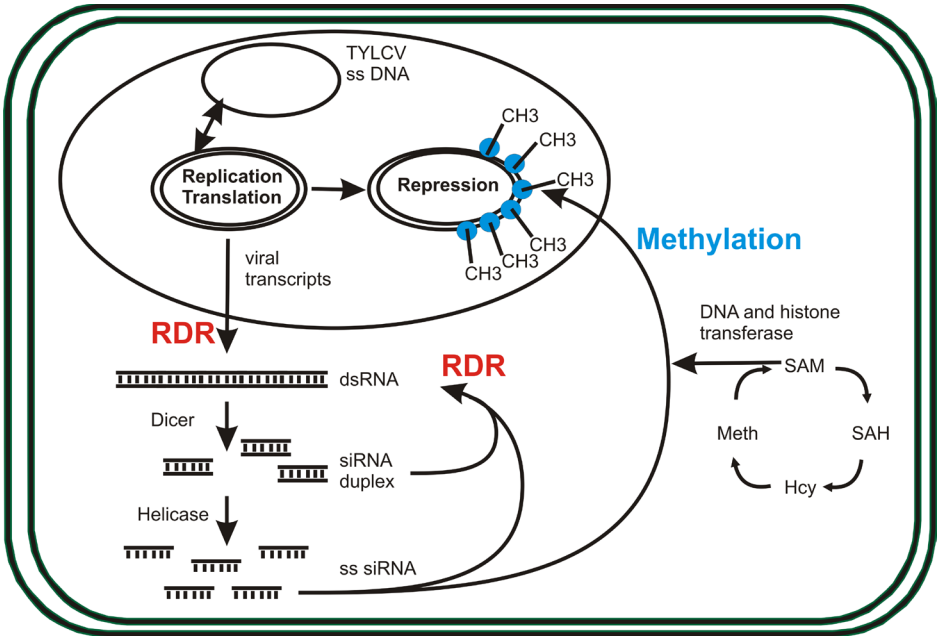

Figure 1. A simplified model for *Ty-1*/*Ty-3* based resistance.

TYLCV replicates in the nucleus, from where transcripts are transported to the cytoplasma. *Ty-1*/*Ty-3* (RDR) converts single stranded (ss)RNA into double stranded (ds)RNA, which in turn is processed by DICER into siRNA duplexes. These are unwound into single stranded siRNAs that either become uploaded into RISC or alternatively are used to prime dsRNA synthesis on ssRNA target molecules. siRNAs uploaded into a RISC-Ago4 complex will direct DNA methylation of the corresponding TYLCV genomic region. Upon genome methylation, transcription of these TYLCV genomic sequences is suppressed and this eventually results in low virus titers and tolerance of the plant to TYLCV.

***Ty-1*/*Ty-3* are involved in transcriptional gene silencing**

Considering the role of RDR1, RDR2 and RDR6 in RNAi we speculated *Ty-1*/*Ty-3* was also involved in siRNA amplification. By TYLCV inoculation of *Ty-1*/*Ty-2*/*Ty-3* and MM plants we showed that *Ty-1* and *Ty-3* plants produced enhanced levels of TYLCV specific siRNAs with an enrichment for the C3 en V1 genes. These high levels coincided with hypermethylation in the corresponding V1 regions of the TYLCV genomes (**Chapter 5**). A correlation between methylation of viral DNA and suppression of transcription and replication of geminiviruses has earlier been described in multiple studies using a variety of plants and virus species, e.g. Arabidopsis with *Beet curly top virus* (BCTV) (Raja et al., 2010), watermelon with *Cucurbit leaf crumple virus* (Hagen et al., 2008) and pepper with *Pepper golden mosaic virus* (Rodriguez-Negrete et al., 2009). The presence of relatively low levels of methylation in the same region of TYLCV collected from susceptible MM compared to *Ty-1* plants, may additionally have been a consequence of (higher levels of) RNA silencing suppressor (RSS) activity of TYLCV. Almost all plant viruses encode proteins that interfere with the host RNA silencing pathway (Wang et al., 2012). Geminiviruses, including TYLCV, also encode RSS proteins that besides post transcriptional gene silencing (PTGS) suppress transcriptional gene silencing (TGS) (Yang et al., 2011; Luna et al., 2012). While in *Ty-1* plants TGS is probably also suppressed, the higher levels of siRNA amplification by *Ty-1* and, as a consequence, of transcriptional silencing results in a stronger reduction in TYLCV levels, including a weaker RSS response.

A model for *Ty-1*/*Ty-3* based resistance

Our data provide a first glimpse on the mechanistic mode of *Ty-1*/*Ty-3* based resistance. It is shown that in *Ty-1*/*Ty-3* plants TYLCV is targeted by enhanced transcriptional gene silencing (TGS) (Figure 1). This enhanced TGS is a result of higher levels of TYLCV derived siRNAs that sequentially lead to enhanced TYLCV genome methylation (**Chapter 5**). The elevated level of siRNAs is a direct consequence of the presence and action of *Ty-1*/*Ty-3*. Based on the results shown we postulate that *Ty-1* and similar genes from the DFDGD class of RDRs (RDR γ), for which no function has been described sofar, have a similar role as members of the DLDGD class of RDRs (RDR α). In other words, they are involved in the amplification and biogenesis of siRNAs. Since *Ty-1* plants are not resistant to RNA viruses it is tempting to speculate that members of the DFDGD class of RDRs are only involved in the amplification of siRNAs that are required for TGS. From the practical point of view, the research described in this thesis will aid in breeding for TYLCV tolerant/resistant tomato cultivars and contribute to the search for other

tolerance genes to DNA viruses. If farmers can combine cultivars containing these genes with good agricultural practices that reduce insect vector populations this could turn into an effective and sustainable strategy for tomato cultivation in TYLCV prone areas.

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Summary

Summary

Tomato yellow leaf curl disease, a devastating disease of tomato, is caused by a complex of begomoviruses generally referred to as *Tomato yellow leaf curl virus* (TYLCV). Almost all breeding for TYLCV resistance has been based on the introgression of the *Ty-1* and *Ty-3* resistance loci derived from *Solanum chilense* LA1969 and LA1932/LA2779 respectively. The aim of this thesis was to fine map, clone and characterize these two TYLCV resistance genes.

The *Ty-1* gene has been used in tomato breeding already for almost 20 years. Its exact genetic location was however unknown which made precise marker assisted breeding difficult. Here we have analysed the recombination behavior of the chromosomal region where *Ty-1* is introgressed by applying newly developed molecular markers in two F_2 populations obtained from two commercial *Ty-1* carrying hybrids. A big *S. chilense* introgression was detected in both populations that cover almost the whole short arm and a part of the long arm of chromosome 6. In this introgression recombination suppression was detected and Fluorescence *in situ* Hybridization (FISH) analysis revealed two chromosomal rearrangements between *S. lycopersicum* and *S. chilense* LA1969. These rearrangements are most likely the cause of the observed recombination suppression. Using disease tests on progeny of informative recombinants *Ty-1* was mapped to a region of approximately 600 kb which partly overlapped with the mapped region for *Ty-3*, which led to the indication that *Ty-1* and *Ty-3* could be allelic. Altogether these results nicely demonstrate the usefulness of FISH as a powerful tool to aid in the accurate mapping of genes that are introgressed from wild species into cultivated tomato (**Chapter 2**).

To further fine map and ultimately clone *Ty-1* and *Ty-3* more plants were screened for recombination events and consequently recombinant inbred lines were generated. By developing new markers in combination with disease tests both genes were fine mapped to a very small, almost similar genomic region (approximately 70 kb). Using a Tobacco Rattle Virus-Virus Induced Gene Silencing approach, the resistance genes were finally identified. It was shown that *Ty-1* and *Ty-3* are allelic and that they code for a RNA-dependent RNA polymerase (RDR) belonging to the RDRγ type which has an atypical DFDGD motif in the catalytic domain. In contrast to the RDRα type, characterized by a catalytic DLDGD motif, no clear function has yet been described for the RDRγ type. With the identification of *Ty-1*/*Ty-3*, a completely new class of resistance genes was unveiled (**Chapter 3**).

The *Ty-1*/*Ty-3* allele is characterized by a 4 amino acid insertion at the 5-prime

part of the protein and by a catalytic DFDGD motif. The allelic variation of this gene was examined using cDNA from five *S. chilense* derived lines and using draft assemblies of whole genome sequences from more than 50 tomato cultivars, landraces and related wild species. Tobacco Rattle Virus induced gene silencing was used to silence *Ty-1*/*Ty-3* and altogether showed that resistance was compromised in three out of five *S. chilense* derived lines tested. One line with resistance derived from *S. chilense* LA1971 remained resistance after silencing of *Ty-1*/*Ty-3*. For another line (8783, derived from LA1932) only 4 out of 13 plants showed symptoms after silencing, but silencing in this line was inefficient because only one out of three PDS controls showed photobleaching. Comparison of the two typical features of the *Ty-1*/*Ty-3* gene showed no sequence variation amongst *S. chilense* derived lines. The catalytic domain was found to be conserved among all tomato lines and species analysed, while the characteristic 4 amino acid insertion was also observed in three species closely related to *S. chilense*, e.g. *Solanum corneliomulleri*, *Solanum peruvianum* and *Solanum huaylasense*. This indicated that most *S. chilense* accessions most likely carry a functional TYLCV resistance locus on chromosome 6, allelic to *Ty-1*/*Ty-3*, and *Solanum* species related to *S. chilense* could possibly be useful for future TYLCV resistance breeding (**Chapter 4**).

The *Ty*-1 gene encoded an RDR and for this reason most likely conferred resistance involving amplification of the siRNA signal. In the last experimental chapter (**Chapter 5**) this hypothesis was tested. It was shown that upon TYLCV challenging of resistant *Ty-1* and *Ty-3* lines low virus titers were detected concomitant with the production of relatively high levels of siRNAs. In contrast to the situation in susceptible tomato Moneymaker where high virus titers were observed, but the amount of siRNAs produced lower compared to those in *Ty-1* and *Ty*-3. Analysis of the spatial genomic siRNA distribution showed a consistent and subtle enrichment for siRNAs derived from the CP (V1) and C3 gene in *Ty-1* and *Ty-3* lines compared with Moneymaker. In tomato plants containing the *Ty*-2 resistance gene, included as a control and not an RDR, the virus was hardly detectable but the siRNA profile similar to the one observed in TYLCV-challenged susceptible tomato Moneymaker. Furthermore, genome methylation analysis revealed a relative hypermethylation of the TYLCV CP (V1) promoter region in genomic DNA collected from *Ty-1* in comparison to susceptible tomato Moneymaker.

Altogether this thesis describes the mapping, cloning and characterization of the TYLCV resistance genes *Ty*-1 and *Ty*-3. Future breeding efforts for TYLCV resistance can now exploit in-gene markers and the insights obtained can possibly direct future research and breeding efforts on plant virus resistance (**Chapter 6**).

Samenvatting

Samenvatting

Tomaten geelbladkrulziekte is een vernietigende ziekte die wordt veroorzaakt door verschillende begomovirussen, meestal wordt de term Tomato yellow leaf curl virus (TYLCV) gebruikt. TYLCV resistentie veredeling is tot nu toe bijna altijd gebaseerd op de introgressie van de resistentie genen *Ty-1* en *Ty-3*. Deze genen zijn afkomstig uit de *Solanum chilense* accessies LA1969 (*Ty-1*) en LA1932/LA2779 (*Ty-3*). Het doel van het onderzoek beschreven in deze thesis was om deze twee genen te fijn karteren en te karakteriseren.

Het *Ty-1* gen wordt nu al bijna 20 jaar gebruikt in de tomaten veredeling maar de exacte locatie was onbekend, wat het merker geassisteerde veredelen bemoeilijkte. Het recombinatie 'gedrag' van de chromosomale regio waar *Ty-1* is ingekruist werd geanalyseerd door nieuw ontwikkelde merkers toe te passen op twee F2 populaties afkomstig van twee commerciële tomaten hybriden die het *Ty-1* gen dragen. In beide populaties werd een grote *S. chilense* introgressie gevonden die bijna de hele korte arm en een gedeelte van de lange arm van chromosoom 6 overspande. In deze introgressie was de recombinatie onderdrukt en Fluorescence in situ Hybridization (FISH) toonde aan dat er twee chromosomale herschikkingen zijn als *S. lycopersicon* en *S. chilense* worden vergeleken. Deze herschikkingen zijn waarschijnlijk de oorzaak van de geobserveerde recombinatie suppressie. Door middel van ziektetoetsen op nakomelingen van informatieve recombinanten kon *Ty-1* worden gekarteerd tot een regio van ongeveer 600 kb. Deze regio overlapte gedeeltelijk met de gekarteerde *Ty-3* regio, wat het vermoedde opwekte dat *Ty-1* en *Ty-3* misschien allelisch zijn. Samengevat laten deze resultaten zien dat FISH een krachtig middel is dat kan helpen bij het fijn karteren van genen die ingekruist zijn uit wilde soorten in gecultiveerde tomaat (Hoofdstuk 2).

Om *Ty-1* en *Ty-3* verder fijn te karteren en uiteindelijk te kloneren werden meer planten gescreend voor recombinatie en vervolgens werden 'recombinant inbred lines' gegenereerd. Ontwikkelen van nieuwe merkers in combinatie met ziektetoetsen resulteerde in een korte overlappende gekarteerde regio voor beide genen (ongeveer 70 kb). Met een tabak mozaïek virus geïnduceerde gene silencing werden de resistentie genen geïdentificeerd. Er werd aangetoond dat *Ty-1* en *Ty-3* allelisch zijn en dat ze coderen voor RNA-dependent RNA polymerases (RDR) van de RDRγ klasse, dus met een atypisch DFDGD katalytisch domein. In tegenstelling tot de RDRα klasse, met een DLDGD katalytisch domein, is er voor de RDRγ klasse nog geen duidelijke functie beschreven. Met het identificeren van *Ty-1*/*Ty-3* is er dus een nieuwe klasse resistentie genen beschreven (Hoofdstuk 3).

Het *Ty-1* / *Ty-3* allel is herkenbaar aan een 4 aminozuur deletie in de 5-prime van het eiwit en aan het DFDGD motief. De allelische variatie van dit gen werd bestudeerd door cDNA van vijf verschillende *S. chilense* accessies te bestuderen alsmede 'draft genome assemblies' van meer dan 50 tomaten cultivars, landrassen en gerelateerde wilde soorten. Tabak mozaïek virus geïnduceerde gene silencing werd gebruikt om *Ty-1* / *Ty-3* expressie te verlagen. Samengevat werd in 3 van de 5 geteste *S. chilense* lijnen de resistentie doorbroken. Een lijn met resistentie afkomstig uit LA1971 bleef resistent na 'silencing' van *Ty-1* / *Ty-3*. Van een andere lijn (8783, met resistentie van LA1932) toonde een gedeelte van de planten symptomen na silencing (4 van de 13). Silencing was echter niet efficiënt aangezien bij maar 1 van de 3 controle PDS planten "photobleaching" te zien was. Vergelijking van de twee typische kenmerken van het *Ty-1* / *Ty-3* gen toonde geen sequentievariatie binnen alle *S. chilense* lijnen. Het katalytische domein bleek geconserveerd te zijn tussen alle geanalyseerde tomatenlijnen en soorten, terwijl de karakteristieke 4 aminozuur insertie ook werd gevonden in drie soorten die nauw verwant zijn aan *S. chilense*; *Solanum corneliomulleri*, *Solanum peruvianum* en *Solanum huaylasense*. Dit wijst erop dat de meeste *S. chilense* accessies hoogstwaarschijnlijk een functioneel TYLCV resistentie locus op chromosoom 6 dragen dat allelisch is aan *Ty-1* / *Ty-3*. De *Solanum* soorten verwant aan *S. chilense* kunnen eventueel nuttig zijn voor toekomstige TYLCV resistentieveredeling (Hoofdstuk 4).

Het *Ty-1* gen codeert voor een RDR en de verleende resistentie is daarom waarschijnlijk gebaseerd op amplificatie van het siRNA signaal. In het laatste experimentele hoofdstuk (Hoofdstuk 5) werd deze hypothese getest. Er werd aangetoond dat er lage TYLCV concentraties aanwezig zijn als resistente *Ty-1* en *Ty-3* lijnen worden geïnfecteerd met het virus, deze lage concentraties gaan samen met een relatief hoog gehalte siRNAs. Dit contrasteert met het resultaat in de vatbare Moneymaker waar hoge virus concentraties en lage hoeveelheden siRNAs werden gevonden. Analyse van de verdeling van de siRNAs op het genoom van TYLCV liet een consistente en subtiele verrijking van siRNAs afgeleid van het CP (V1) en C3-gen zien in *Ty-1* en *Ty-3* in vergelijking met Moneymaker. In tomatenplanten met het *Ty-2* resistentie gen, meegenomen als controle, was het virus nauwelijks waarneembaar en het siRNA profiel was vergelijkbaar met dat van Moneymaker. Genoom methylatie analyse toonde aan dat de promotor regio van TYLCV CP (V1) meer was gemythyleerd in vergelijking met deze regio van TYLCV afkomstig uit Moneymaker.

Samengevat beschrijft dit proefschrift de kartering, klonering en karakterisering van de TYLCV resistentiegenen *Ty-1* en *Ty-3*. Toekomstige veredeling voor TYLCV resistentie kan nu gebruik maken van in-gen merkers. De verkregen inzichten kunnen

mogelijk richting geven aan toekomstig onderzoek en veredelings inspanningen voor plant virus resistentie (hoofdstuk 6).

Dankwoord

Onderzoek doe je natuurlijk niet alleen, en ik wil dan ook graag een aantal mensen bedanken. Ten eerste natuurlijk Yuling, bedankt voor het vertrouwen dat je vanaf het begin in mij hebt gehad. Vooral de eerste maanden heb je me een paar keer moeten overtuigen dat we dit project succesvol zouden kunnen afsluiten en dat is uiteindelijk zeker gelukt! De stap om samen te gaan werken met Sam en Jay bleek ook een hele goede te zijn en ik hoop dat dit project een begin is van nog meer succesvolle projecten voor jou. Ten tweede wil ik graag Richard Kormelink bedanken. Na het trieste overlijden van Rob en de verhuizing van virologie naar Radix duurde het even voordat we waren opgestart maar dat is helemaal goed gekomen. De werkdiscussies op maandag heb ik altijd erg gewaardeerd en ik vond het erg leuk om te merken dat je steeds enthousiaster werd richting het einde van het project. Natuurlijk wil ik ook mijn promoter Richard Visser bedanken. We hebben niet veel contact gehad de eerste drie jaar maar de snelheid en efficiëntie waarmee je in de afrondende fase hielp met schrijven heb ik erg gewaardeerd. Natuurlijk ook dank aan alle anderen die hebben meegeholpen de resultaten te produceren die te vinden zijn in dit boekje. Ten eerste Patrick, bedankt voor hoofdstuk 5, in een relatief korte tijd zijn we veel te weten gekomen mede dankzij de perfecte hulp van Annette en Dick. Natuurlijk ook Dora en Hans, dankzij jullie gevis hebben we van hoofdstuk twee een mooie publicatie weten te maken. Richard Finkers, bedankt voor de sequenties. Je bent toch mooi de eerste Richard van de drie Richards van hoofdstuk 4.
I would also like to thank Sam and Jay, I think our collaboration was extremely beneficial for both of our teams! The excellent plant material you developed allowed me to speed up the finemapping and cloning and I'm extremely thankful for that.

During these four years I had many different office mates. I would specifically like to thank the boys from the first two years. Luigi, Ali and Zheng, you were the best! We had so much fun although working was hard sometimes while watching youtube movies, the Tour de France and the world cup. I wish you all the best in your future lives all around the world. I hope one day we can all have a beer together somewhere. Also thanks to Dong Li, it was really nice seeing you change from a very shy girl to somebody that was making fun of me! Myluska, a BIG thanks to you, you were the best student I had and you contributed to many parts of this book. I really appreciated all the discussions we had and now you are continuing some of the research that I started. I wish you all the best for the coming years. I'm sure you'll be successful!

Also thanks to my other students that produced valuable data; Frank, Ting Ting and Ada. I think you can all find some of your hard work somewhere in this book, a big

thanks for that!

In these four years we also had a small but very nice collaboration with the University from Malaga, Olga and Nuria thanks for your work. Also thanks to your supervisor Maria. I hope soon our first publication together will be published!

De data wordt natuurlijk geproduceerd in het lab en daar voelde ik me altijd erg thuis vooral dankzij Danny, Doret, Johan, Gert en Hanneke. Super bedankt voor al het geklets en gegrap. Some moest ik wel even uithuilen op kantoor als jullie (of eigenlijk alleen Doret en Danny) me weer teveel geplaagd hadden, maar ik heb het altijd enorm naar mijn zin gehad op het leukste lab van de vakgroep! Natuurlijk ook dank aan alle anderen bij wie ik terecht kon met vragen over enzymen, zaden, gelletjes, protocollen, centrifuges, pipetten, kitjes etc.

Van het Unifarm team wil ik in het bijzonder Henk, Bert en Teus bedanken voor de goede zorg voor mijn planten en zaden.

Dan hebben we nog Peter en Thijs, dank voor alle koffiepauzes, slechte grappen, het lunchen, nog een koffiepauze, nog slechtere grappen en dan nog maar een koffiepauze. Succes met schrijven en veel plezier met opblaasmaarten. Also thanks to Andres, I really enjoyed having you around as a friend and colleague, good luck with your last year and stay in touch!

Plant Breeding is een enorme groep mensen en een aantal personen wil ik in het bijzonder bedanken voor hun gevarieerde bijdragen. Herman, Henk, Chris, Gerard, Sjaak, Collette, Tim en Paul bedankt! Natuurlijk ook dank aan alle dames van het secretariaat.

Ook een bedankje voor Adriaan, we hebben het toch mooi gedaan allebei, dat mag ook wel na zo veel succesvolle experimenten in de eerste drie jaar van onze studie!

Naaste de wetenschap is er natuurlijk meer, veel meer zelfs. Daarom een bedankje aan Ferdinand en de fantastische groep mensen die hij om zich heen heeft verzameld bij Diveplace. De vele uren onder water (en nog meer uren boven water) zijn altijd gezellig, ontspannend en vaak ook leerzaam en zijn altijd een perfecte afleiding.

Lieve vriendjes (jullie weten heel goed wie jullie zijn), dank voor die ontelbare avonden

en weekenden die we nu al meer dan tien jaar (!) samen doorbrengen. Het is fijn om te weten dat we er altijd voor elkaar zijn, jullie zijn me allemaal enorm dierbaar! Een extra bedankje voor Bram, dank voor het delen van de promotie perikelen en de hulp bij het vormgeven van dit boekje. Roel, bedankt voor de mooie cover. Ook een extra bedankje voor Rutger, dank voor de vele uurtjes voor, tijdens en na het sporten, en voor het paranimfen natuurlijk.

Jasper, wat vind ik het leuk dat jij tijdens de verdediging naast mij zit. Je bent dan wel (bijna) altijd ver weg en we zien elkaar dus niet heel vaak, maar het is elke keer weer heel leuk jou en Kate te spreken en te zien, bedankt!

Maaike, wat ben ik blij met jou, de schrijffase was in een flits voorbij en dat kwam door jou, bedankt!

Als laatste natuurlijk pap en mam. Bedankt voor het vertrouwen en het steunen in alle keuzes die ik maak. Het is elke keer weer zo fijn jullie te zien en ik leer elke keer als ik jullie zie nog zo veel. Dank voor alles!

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Maarten Verlaan
Date: 19 June 2013
Group: Plant Breeding, Wageningen University & Research Centre

1) Start-up phase	date
► **First presentation of your project**	
Characterization of major resistance genes to tomato yellow leaf curl virus	Mar 31, 2009
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*1,5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD Student Day 2009, Leiden	Feb 26, 2009
EPS PhD Student Day 2010, Utrecht	Jun 01, 2010
► **EPS theme symposia**	
EPS Theme 2 meeting 'Interactions between Plants and Biotic Agents, Utrecht	Jan 23, 2009
EPS Theme 2 meeting 'Interactions between Plants and Biotic Agents, Utrecht	Jan 15, 2010
EPS Theme 2 meeting 'Interactions between Plants and Biotic Agents, Amsterdam	Feb 3, 2011
► **NWO Lunteren days and other National Platforms**	
NWO-ALW meeting 'Experimental Plant Sciences, Lunteren	Apr 06-07, 2009
NWO-ALW meeting 'Experimental Plant Sciences, Lunteren	Apr 19-20, 2010
NWO-ALW meeting 'Experimental Plant Sciences, Lunteren	Apr 04-05, 2011
NWO-ALW meeting 'Experimental Plant Sciences, Lunteren	Apr 02-03, 2012
► **Seminars (series), workshops and symposia**	
CBSG summit 2009	Mar 16-17, 2009
CBSG clustermeeting pathogenomics 2009	Sep 30, 2009
CBSG summit 2010	Mar 15-16, 2010
CBSG clustermeeting pathogenomics 2010	Oct 21, 2010
CBSG summit 2011	Jan 31-Feb 01, 2011
CBSG summit 2012	Feb 29-Mar 01, 2012
CBSG clustermeeting pathogenomics 2012	Oct 25, 2012
CBSG summit 2013	Feb 11-12, 2013
Seminar Dr. Cyril Zipfel, Receptor kinase signalling in plant innate immunity	Feb 05, 2009
Plant Breeding Research Day 2009	Mar 03, 2009
Molecular markers (internal Plant Breeding course)	Mar 26, 2009
Seminar Isgouhi Kaloshian, Tomato innate immunity to root-knot nematodes and aphids	May 14, 2009
Seminar J.D.H. Keatinge, AVRDC and WUR need to fight the battle against poverty & malnutrition together	Jun 18, 2009
Seminar Wallace A. Cowling, Linkage disequilibrium and association mapping, helping to overcome the paradox of modern plant breeding	Jun 26, 2009
Plant Science Seminar, Harro Bouwmeester (Plant Physiology) & Ton Bisseling (Molecular Biology)	Sep 08, 2009
Seminar Valerie Williamson, Connecting genetics and genomics of pathogenicity and behavior in root-knot nematodes	Oct 23, 2009
Plant Science Seminar, Fred van Eeuwijk (Biometris) & Pierre de Wit (Phytopathology)	Nov 10, 2009
Seminar Julie Scholes, Bewitched: Physiological and genomic approaches to understanding the Striga/cereal interaction	Dec 03, 2009
Plant Science Seminar, Ken Giller (Plant Production Systems) & Richard Visser (Plant Breeding)	Dec 08, 2009
Plant Science Seminar, Marcel Dicke (Entomology) & Marcel Janson (Plant Cell Biology)	Jan 12, 2010
Seminar Frontiers in Plant Microbe Interactions: Christiane Gebhardt & Peter Moffett	Feb 05, 2010
Plant Breeding Research Day 2010	Feb 08,2010
CBSG Workshop - Intellectual Property Rights: basic principles and applications	Feb 11, 2010
Illumina Agrigenomics Seminar	Mar 03, 2010
Plant Science Seminar, Louise Vet (Entomology) & Just Vlak (Virology)	May 11, 2010
Seminar Paul Birch, Trying to understand susceptibility and exploit resistance in potato-Phytophthora infestans interactions	May 20, 2010
Seminar Dr. Brigitte Mauch-Mani, Grapevine and downy mildew - Wine is not the only difference between grapevine and Arabidopsis	May 31, 2010
Seminar Prof. Felix Mauch, Old fashioned secondary metabolites save Arabidopsis from Phytophthora brassicae	May 31, 2010
Seminar Sir David C. Baulcombe, Mobile RNA silencing in plants	Sep 27, 2010
CBSG Workshop - Genome Mining Tomato	Oct 26, 2010
CBSG Technology Symposium: Advances in life-science Technologies	Nov 25, 2010
Plant Science Seminar: Martin van Itersum & Gerard van der Linden	Feb 08, 2011
CBSG Workshop - IP in a PPP context	Mar 31, 2011
CBSG Workshop - Open Source Biotech with Richard Jefferson	Oct 04, 2011
Symposium: Plant Breeding in the Genomics Era	Nov 25, 2011
Plant Breeding Research Day 2012	Feb 29, 2012
CBSG Workshop - IPR in a Breeding Context	Apr 17, 2012
Seminar Dr. Daniella Ribeiro, Peroxisomal and Mitochondrial-mediated Antiviral Immunity	Jun 29, 2012
Seminar Sir David C. Baulcombe, Plant versus virus: defense, counter defense and counter counter defense	Oct 10, 2012
► **Seminar plus**	
► **International symposia and congresses**	
SOL 2010 (Dundee, UK)	Sep 05-08, 2010
Eucarpia Tomato 2011 (Malaga, Spain)	Apr 12-13 2011
SOL 2011 (Kobe, Japan)	Nov 28-Dec 02 2011
Conference Next Generation Plant Breeding (Ede, the Netherlands)	Nov 12-14, 2012
PAG Asia (Singapore)	Mar 17-19, 2013
► **Presentations**	
CBSG summit 2009 (oral)	Mar 17, 2009
CBSG clustermeeting pathogenomics 2009 (oral)	Sep 30, 2009
CBSG summit 2010 (poster)	Mar 15, 2010
CBSG clustermeeting pathogenomics 2010 (oral)	Oct 21, 2010

	CBSG summit 2011 (poster & oral)	Jan 31-Feb 01, 2011
	NWO-ALW meeting 'Experimental Plant Sciences, Lunteren (poster)	Apr 04-05, 2011
	Eucarpia Tomato 2011 Malaga, Spain (oral)	Apr 12, 2011
	SOL 2011 Kobe, Japan (oral)	Nov 29, 2011
	CBSG summit 2012 (poster)	Feb 29-Mar 01, 2012
	CBSG clustermeeting pathogenomics 2012 (oral)	Oct 25, 2012
	Conference Next Generation Plant Breeding (oral)	Nov 12, 2012
	CBSG summit 2013 (poster)	Feb 11-12, 2013
	PAG Asia, Singapore (oral)	Mar 17-19, 2013
►	**IAB interview**	Feb 17, 2011
►	**Excursions**	
	CBSG Matchmaking Event, Visit to Rijk-Zwaan and Monsanto	Oct 18, 2012
	Subtotal Scientific Exposure	*33,8 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	EPS Summer School on Environmental Signaling	Aug 24-26, 2009
	Bioinformatics: A User's Approach	Aug 30-Sep 03, 2010
►	**Journal club**	
►	**Member of literature discussion group**	2011-2012
►	**Individual research training**	
	Subtotal In-Depth Studies	*3,4 credits**

4) Personal development		*date*
►	**Skill training courses**	
	PhD Competence Assessment	May 12, 2009
	Information Literacy	Jun 09-10, 2009
	Mobilising your Scientific Network	Jun 02 & 10, 2010
	Moral dilemmas in your daily scientific practices	Feb 15, 16 & 18 2011
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*3,1 credits**

TOTAL NUMBER OF CREDIT POINTS*	**41,8**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*