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Molecular dissection of *Tomato leaf curl virus* resistance in tomato line TY172 derived from *Solanum peruvianum*

**Ilana Anbinder · Moshe Reuveni · Raviv Azari ·
Ilan Paran · Sahadia Nahon · Haviva Shlomo ·
Lea Chen · Moshe Lapidot · Ilan Levin**

Abstract *Tomato yellow leaf curl virus* (TYLCV) is devastating to tomato (*Solanum lycopersicum*) crops and resistant cultivars are highly effective in controlling the disease. The breeding line TY172, originating from *Solanum peruvianum*, is highly resistant to TYLCV. To map quantitative trait loci (QTLs) controlling TYLCV resistance in TY172, appropriate segregating populations were analyzed using 69 polymorphic DNA markers spanning the entire tomato genome. Results show that TYLCV resistance in TY172 is controlled by a previously unknown major QTL, originating from the resistant line, and four additional minor QTLs. The major QTL, we term *Ty-5*, maps to chromosome 4 and accounts for 39.7–46.6% of the variation in symptom severity among segregating plants (LOD score 33–35). The minor QTLs, originated either from the resistant or susceptible parents, were mapped to chromosomes 1, 7, 9 and 11, and contributed 12% to the variation in symptom severity in addition to *Ty-5*.

Introduction

Tomato yellow leaf curl virus (TYLCV) is currently considered as one of the most devastating viruses of cultivated tomatoes [*Solanum* (*S.*) *lycopersicum*] in tropical and subtropical regions. Although first identified in the eastern Mediterranean (Cohen and Harpaz 1964), it has spread and

I. Anbinder · M. Reuveni · R. Azari · I. Paran · S. Nahon ·
H. Shlomo · L. Chen · M. Lapidot · I. Levin (✉)
Institute of Plant Sciences, Agricultural Research Organization,
The Volcani Center, P.O. Box 6, 50250 Bet Dagan, Israel
e-mail: vclevini@volcani.agri.gov.il

reached worldwide distribution (Czosnek and Laterrot 1997; Polston and Anderson 1997; Moriones and Navas-Castillo 2000). TYLCV induces a severe tomato disease characterized by yellowing and cupping of apex leaves as well as stunted plant growth coupled with significant yield losses. In many tomato-growing areas, TYLCV has become a major limiting factor in tomato production (Lapidot and Friedmann 2002).

TYLCV belongs to the family *Geminiviridae*, genus *Begomovirus*, and transmitted by the whitefly *Bemisia tabaci* in a circulative and persistent manner. The virus genome is composed of a single (monopartite) circular single-stranded DNA molecule of about 2,800 nucleotides. In the past, there has been some confusion regarding the taxonomy of TYLCV. Several begomoviruses, inducing similar symptoms in tomato, were all named TYLCV. Further analyses of these viruses showed that the tomato yellow leaf curl disease (TYLCD) is induced by a heterogeneous complex of begomoviruses (Moriones and Navas-Castillo 2000). Most of the isolates have a monopartite genome, and recently a TYLCV isolate containing a DNA-β satellite was identified (Khan et al. 2008).

The management of TYLCV is difficult because its whitefly vector populations can reach high numbers. Chemical control methods have been only partially effective, while raising concerns that the vector may develop pesticide resistance and that intense application of pesticides may have deleterious environmental consequences (Palumbo et al. 2001). Physical barriers such as fine-mesh screens and UV-absorbing plastic sheets or screens are used in the Mediterranean region to protect crops (Cohen and Antignus 1994; Antignus et al. 2001). However, such physical barriers add to production costs and may also result in suboptimal light conditions, overheating, and increased humidity, which can hamper appropriate plant growth and

development. Genetic resistance of the host plant, on the other hand, requires no chemical application or plant seclusion and is potentially stable and long lasting. Therefore, breeding crops which are resistant or tolerant to the virus is considered highly effective in reducing yield losses due to TYLCV (Morales 2001; Lapidot and Friedmann 2002).

There have been extensive efforts to breed tomato cultivars resistant to TYLCV. Since all cultivated tomato accessions are considered susceptible to the disease, wild tomato species were screened to identify and introgress resistance genes (reviewed in Nakhla and Maxwell 1998; Lapidot and Friedmann 2002; Ji et al. 2007b). Genes controlling resistance to TYLCV have been identified and introgressed from several wild tomato species, including: *Solanum pimpinellifolium*, *S. peruvianum*, *S. chilense*, and *S. habrochaites*. The inheritance of genes controlling TYLCV resistance originating from nearly all of these wild species has been characterized using classical genetic methodologies. However, only a few were scrupulously characterized or mapped to the tomato genome using molecular DNA markers (Ji et al. 2007b).

Breeding for TYLCV resistance was initiated in Israel in the late 1960s (Pilowsky and Cohen 1974). The first commercial resistant hybrid, TY20, was released in 1988 (Pilowsky and Cohen 1990). TY20 carries resistance derived from *S. peruvianum* (accession PI 126935) that is inherited as five recessive genes. The resistance in TY20 induces a delay in the development of disease symptoms following infection, and infected plants are able to produce an acceptable yield.

Resistance introgressed from *S. chilense* accession LA1969 was found to be controlled by a major partially dominant gene, termed *Ty-1*, and at least two additional modifier genes (Zamir et al. 1994). *Ty-1* was mapped to the top of chromosome 6 at the vicinity of marker TG97, while the two modifiers were mapped to chromosomes 3 and 7 (Zamir et al. 1994). TYLCV resistance derived from *S. pimpinellifolium* Hirsute-INRA was found to be mediated by a single dominant gene (Kasrawi 1989). DNA marker analysis identified a quantitative trait locus (QTL), accounting for up to 27.7% of the variation in symptom severity, on chromosome 6. However, its map location is different from that of *Ty-1* (between markers TG153 and CT83) (Chague et al. 1997).

Hanson et al. (2000) analyzed the resistant line H24, which contains resistance introgressed from accession B 6013 of *S. habrochaites* (Kalloo and Banerjee 1990). The authors screened resistant plants using what at the time they thought were three different isolates of TYLCV. It was, however, later found that those viral isolates were in fact isolates of *Tomato leaf curl virus* (ToLCV), not TYLCV. This resistance was mapped to the short arm of chromosome 11, between the markers TG393 and TG36, and was

found to be dominant (Hanson et al. 2000). In a recent study, it was shown that the resistance is located closer to marker TG36 and was termed *Ty-2* (Hanson et al. 2006). H24 response to TYLCV inoculation varied, susceptibility depending upon the strain (Ji et al. 2007b).

Three accessions from *S. chilense*, LA1932, LA2779 and LA1938, were found to be resistant to TYLCV as well as to *Tomato mottle virus* (ToMoV) (Agrama and Scott 2006). Introgression into susceptible lines, inheritance studies and QTL mapping revealed three regions on chromosome 6 which contribute to both TYLCV and ToMoV resistance (Agrama and Scott 2006). In a recent study, more markers were used to localize the introgression in an advanced breeding line derived from LA2779. A major partially dominant gene, termed *Ty-3*, was mapped to chromosome 6 between the markers cLEG-31-P-16 and T1079 (Ji and Scott 2006; Ji et al. 2007a). The introgression derived from LA2779 was found to contain *Ty-1* as well, suggesting a genetic linkage between *Ty-1* and *Ty-3* (Ji et al. 2007a).

Recently, using advanced breeding lines derived from the above three *S. chilense* accessions, a new TYLCV-resistance locus, termed *Ty-4*, was mapped between the markers C2_At4g17300 and C2_At5g60610 on the long arm of Chromosome 3 (Ji et al. 2008). While approximately 60% of the variance in the TYLCV resistance in a segregating population was explained by the *Ty-3* locus, *Ty-4* accounted for only 16%. It was therefore concluded that *Ty-3* has a major effect on resistance, while *Ty-4* has a lesser effect (Ji et al. 2008).

Resistance to TYLCV was also introgressed from two accessions of *S. habrochaites* (LA1777 and LA0386). Two BC_1F_4 lines, termed 902 and 908, were derived from this introgression (Vidavsky and Czosnek 1998). Segregation analysis indicated that two to three additive recessive genes control resistance to TYLCV in line 902, while in Line 908 resistance is controlled by a single dominant major gene (Vidavsky and Czosnek 1998). Preliminary results indicate the presence of *Ty-3* in line 902, however, its effect on resistance is this line remains to be evaluated (Ji et al. 2007b).

Line TY172 had been derived from four different accessions of *S. peruvianum* (Friedmann et al. 1998). These four accessions were crossed with *S. lycopersicum*, and the resulting interspecific hybrids were backcrossed to the susceptible parent until a BC_3F_3 generation was secured. At this stage, crosses were made between the four different lines, and F_2 and F_3 generations were produced and screened for resistance. A highly resistant F_3 line was selected, and its F_4 offspring were bulked and designated TY172 (Friedmann et al. 1998). Progeny testing for two consecutive generations showed that this line is fixed for resistance and the resulting F_6 plants were used in the

experiments described here. TY172 is highly resistant to TYLCV: it shows minimal symptoms following infection and contains low levels of viral DNA (Lapidot et al. 1997; Friedmann et al. 1998). Classical genetic studies have suggested that resistance in TY172 is controlled by three genes exerting a partially dominant effect (Friedmann et al. 1998). TY172 exhibited the highest level of resistance in a field trial designed to compare yield components of various resistant accessions following inoculation with TYLCV (Lapidot et al. 1997). It was also found that TY172, probably due to its high level of TYLCV resistance, is a poor source for viral acquisition and transmission by whiteflies (Lapidot et al. 2001).

Cumulatively these results underline the importance of TY172 for tomato breeding and suggest that its resistance is controlled by a different genetic mechanism than the previously characterized resistance sources. This study was therefore designed to map genes or QTLs controlling TYLCV resistance in TY172. To accomplish this task, populations segregating for the resistance were established and DNA markers were screened using a map based approach.

Materials and methods

Plant material, crosses, populations and experimental layout

To increase the polymorphism content between parental lines in this study, a wild tomato species was initially selected as a susceptible line. To accomplish this task, ten *S. pimpinellifolium* accessions were inoculated with TYLCV. All of these accessions were found highly susceptible to the virus. From these ten accessions, LA1589 was chosen as a susceptible paternal line and was crossed to TY172 to generate F_1 plants. These F_1 plants were allowed to self pollinate to produce F_2 seeds. A total of 247 F_2 plants resulting from this cross were planted during the winter season of 2006/2007. F_3 seeds and DNA samples were individually extracted from each one of these F_2 plants. The inoculation experiments were carried out on the F_3 seedlings (progeny test) in 8 row × 16 column sowing trays with 40 ml planting soil (Givat Ada Ltd, Israel) for each seedling. The seedlings were fertilized on a weekly basis. A total of 48 F_3 seedlings, representing each one of the 247 F_2 plants (F_3 families), were inoculated with TYLCV during the summer season of 2007 (a total of 11,856 F_3 seedlings). Twenty-four seedlings of each of the 247 F_3 families were inoculated on May 10, 2007 and 24 additional seedlings of the same F_3 families were inoculated on September 2, 2007. Eight seedlings of the two parental lines were included as controls in each sowing tray. In addition, five of the sowing trays in each inocula-

tion experiment also included eight F_1 hybrid seedlings which were inoculated and scored together with their parental counterparts. TYLCV-induced symptoms were evaluated using the procedure described below in disease severity scoring.

To confirm results obtained from the TY172 × LA1589 segregating population, 311 F_2 seedlings, originating from a cross between TY172 and a susceptible processing line M-82 (LA3475), were inoculated on April 15, 2008 together with their parental lines and F_1 hybrid seedlings. These seedlings were scored, as we describe below, and transplanted to the field. The parental lines and their F_1 hybrid were planted in a randomized block design with three blocks while their F_2 counterparts were planted at random. Planting was in single rows allowing 50 cM space between plants. Plants were grown under drip irrigation and cultivated according a procedure commonly used for processing tomatoes in Israel. At the end of the growing season, all fruits were harvested at the mature red stage from each plant separately, counted, and plant as well as total fruit weight were recorded.

Whitefly maintenance, virus acquisition and plant inoculation

Whitefly colonies (*B. tabaci*, biotype B) were reared on cotton plants (*Gossypium hirsutum* L.) in muslin-covered cages held in a growth chamber at 23–30°C. Cultures of the Israeli isolate of TYLCV (Genbank Accession number X15656) (Navot et al. 1991) were maintained in susceptible tomato (cv. Rehovot 13) in an insect-proof greenhouse. For TYLCV inoculation, the whiteflies were given a 48-h acquisition period on TYLCV-infected tomato source plants, followed by a 48-h inoculation access period on the tomato experimental plants (first leaf stage) with about 50 whiteflies per plant as described previously (Lapidot et al. 1997). Thereafter, the plants were sprayed with the systemic pesticide Imidacloprid (Confidor, Bayer, Leverkusen, Germany) and held in an insect-proof greenhouse at 26–32°C for 30 days until symptoms could be clearly scored.

Disease severity scoring

TYLCV-induced symptoms of TY172 × LA1589 F_3 as well as the TY172 × M-82 F_2 seedlings were evaluated 30 days following inoculation in the greenhouse. Symptoms were evaluated according to the disease severity index (DSI) described before (Friedmann et al. 1998; Lapidot and Friedmann 2002): (0) no visible symptoms, inoculated plants show same growth and development as non-inoculated plants; (1) very slight yellowing of leaflet margins on apical leaf; (2) some yellowing and minor curling of leaflet ends; (3) a wide range of leaf yellowing, curling and cupping, with

some reduction in size, yet plants continue to develop and (4) very severe plant stunting and yellowing, pronounced cupping and curling of leaves, plants growth is stopped.

Genomic DNA extraction, DNA markers, polymerase chain reaction and polymorphism identification

Genomic DNA was extracted from individual plants according to Fulton et al. (1995). A total of 215 polymerase chain reaction (PCR) markers scattered along the 12 tomato chromosomes, representing an average of 17.9 markers per chromosome, were used in this study to genotype the parental lines TY172 and LA1589. Sequences for designing these PCR markers were obtained from the Solanaceae Genomics Network (SGN at http://www.sgn.cornell.edu), the National Center for Biotechnology Information (NCBI at http://www.ncbi.nlm.nih.gov/) and the Dana-Farber Cancer Institute tomato EST database (DFCI at http://compbio. dfci.harvard.edu/tgi/tgipage.html). These markers were based on candidate gene, tomato genomic (TG), simple sequence repeat (SSR), conserved ortholog set (COS) and bacterial artificial chromosome (BAC) clone sequences deposited in these sites. The candidate genes selected were host genes that have been experimentally implicated in geminivirus infectivity in tomato and other species such as *Arabidopsis thaliana*, *Glycine max* and *Nicotiana benthamiana*. These genes included the tomato gene homologs encoding NAC-domain proteins, SUMO-conjugating enzymes, Ubiquitin-like modifiers and others. The genes were identified either directly, using keywords, or through BLAST analysis against sequences deposited in SGN, NCBI and DFCI.

DNA primers used in the course of this study were purchased from the Molecular Biology Center, Ness-Ziona, Israel. Sequence analysis and locus-specific primer design were carried out with the DNAMAN sequence analysis software v4.1 (Lynnon BioSoft, Quebec, Canada). Primer sequences for markers which are specifically discussed in this manuscript are presented in Table 1.

Polymerase chain reaction was performed using the T-GRADIENT thermal cycler (Biometra biomedizinische Analytik GmbH, Gottingen, Germany) in a volume of 15 µl containing 15 ng of template DNA, 10 pmol of each of two primers, 0.2 mM of each of the dNTPs, 2 mM of $MgCl_2$, 0.5 U of Taq DNA polymerase and 1× PCR buffer. The PCR profile included: an initial incubation at 94°C 3 min, followed by 35 cycles of denaturation at 94°C 30 s, annealing at 50–60°C (depending upon the specific melting temperature of the primers) 30 s, and 72°C 60 s. A final elongation step at 72°C was carried out for 5 min following the completion of the above cycles.

Following amplification, PCR products were digested, according to the manufacturer recommendations, with ten restriction endonuclease enzymes for polymorphism discovery. The enzymes used, *Alu*I, *Dpn*I, *Dra*I, *Eco*105I, *Hae*III, *Hha*I, *Hin*fI, *Hpa*II, *Rsa*I, and *Taq*I were all purchased from Fermentas Inc. (Hanover, MD, USA). In some cases, PCR products were directly sequenced for nucleotide polymorphism discovery between parental lines. In these cases, sequencing was carried out with an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Polymerase chain reaction products were visualized by electrophoresis in 1.5–2.0% agarose gels stained with ethidium bromide. The DNA size marker used throughout this study is a 2-log DNA size ladder (New England BioLabs, Ipswich, MA, USA).

Statistical analyses

Analyses of variance, correlation and chi-square (χ^2) were carried out with the JMP statistical discovery software (SAS Institute Inc., Cary, NC, USA). Differences among means are presented as different superscript letters that represent statistically significant mean values ($P < 0.05$) based on Tukey–Kramer honestly significant difference (HSD) test (Kramer 1956). MAPMAKER Macintosh V2.0 (Lander et al. 1987) was used to establish the linkage map with map distances calculated using the Kosambi mapping function (Kosambi 1944). QTL interval analysis was carried out using QGene software Version 3.04 (Nelson 1997).

Identification of DNA markers associated with TYLCV resistance and their analysis

The 215 DNA markers used in this study were first utilized to genotype the parental lines TY172 and LA1589, at indicated above, for polymorphism identification. This screen identified 69 polymorphic DNA markers that were used to genotype the two extreme tails of the TY172 × LA1589 F_2 population: 18 plants with the highest (>2.5) and 18 plants with the lowest (<0.5) DSI as determined by their F_3 progenies (individual tail analysis). Fourteen DNA markers showing a statistically significant χ^2 values between the allele frequencies in the two tails were used to genotype all 247 TY172 × LA1589 F_2 plants. The genotype information of each of these DNA markers was then used as independent variables in three types of association analyses in which average DSI obtained from F_3 descendents of these TY172 × LA1589 F_2 plants served as a dependent variable: (1) one-way analysis of variance for each marker, (2) all possible two-way analyses of variance for each pair of markers, including the interaction between the two markers analyzed, and finally (3) a model which included all markers showing either a significant additive effect or a significant interaction with any of the other markers. In addition,

Table 1 Tail analysis by χ^2 displaying markers which were found statistically significant and specifically discussed in this manuscript

Marker	Primer sequence	Ch.	R.E.	Tail analysis				χ^2	$P(\chi^2)$
				Resistant tail		Susceptible tail			
				R alleles	S alleles	R alleles	S alleles		
C2_at4g34700	F = TGAAGCATCCTACACAATAAGTGGCG R = TCGTTGAACTTGGAACCACCAGG	1	*RsaI*	13	23	23	13	5.5	0.018
J04-1	F = TATTCTAATTTTGGAACAAGCTGCC R = TACAATGTGCGACAGCCGAC	4	*TaqI*	28	8	14	22	11.2	0.001
SlNAC1	F = TGCCTGGTTTCTGCTGTCA R = TAAAGCTGAAGAAGGACTTACCCT	4	*TaqI*	34	2	7	29	41.3	1.3×10^{-10}
TG182	F = CTCGGGCAACAGTGAACTA F = TCCTTCTCTCACTTGTTGGTG'	4	*RsaI*	32	4	12	24	23.4	1.3×10^{-6}
M13-1	F = GCCCTCGATAAAGGAAGTACAGATA R = CTACGAGTGTCAAAAATGAATCCA	4	*HpaII*	30	6	11	25	20.4	6.1×10^{-6}
C2_at5g37360	F = TGCAGCATTTGATCTTTCAAATGG R = TGATTTTTGAGAGCCTGTCAATGAG	4	*TaqI*	30	6	11	25	20.4	6.1×10^{-6}
O09-2	F = AATAGGACATCAGGAAGGCTGT R = ACAACCACCAACCAAACGAC	4	*RsaI*	30	6	12	24	18.5	1.7×10^{-5}
K13-3	F = ACATTTCCTCACATCACGGTG R = AAAATAAAGTGTAGAAGCCGGCAT	4	*HinfI*	28	8	15	21	9.8	0.002
TG443	F = TTGGGTGGGCAATGGGTCAAT R = GCCGCCGTCACAAGTAAAGT	4	*RsaI*	28	8	16	20	8.4	0.004
TG174	F = TTCCAAGATCTTTTAGCGTCTC R = CTGTTGCGGATGTGATCATT	7	*RsaI*	11	25	23	13	8.0	0.005
SlSUMO	F = AGCTCATGAATGCTTATTGTGAC R = ACCATATTAAGAGTAAACTTTTACAAATG	9	*HinfI*	21	15	12	24	4.5	0.034
C2_at4g22260	F = TCCTCTAACGGTCTAGAGAAATGGG R = AGGAACTCTTGCAATTGTTTCCAGAAC	11	*HinfI*	14	22	27	9	9.6	0.002

Forward (F) and reverse (R) primer sequences are presented 5'–3'

Ch. chromosome, *R.E.* restriction endonuclease used to obtain polymorphism, *R* and *S* alleles originating from the resistant (TY172) and susceptible (LA1589) lines, respectively, χ^2 chi-square value

MAPMAKER analysis was used to establish the linkage map of the major QTL identified in this study and QTL interval analysis by QGene software was carried out to detect its most probable location.

Association analyses were also carried out on the TY172 × M-82 F_2 population in which only two of the above markers were found polymorphic and therefore segregated. These two markers were therefore used as independent variable in one- and two-way analyses of variance in which DSI scores of individual F_2 plants served as a dependent variable.

Results

Disease severity index of the parental lines and their hybrids

Thirty days following inoculation with TYLCV, the tested plants were assayed for TYLCV-induced symptom severity. Average DSI of the parental lines participating in this study as well as their hybrids is presented in Table 2. These results show that the average DSI of the hybrid plants of both populations is significantly lower than the susceptible lines and significantly higher than the resistant line, indicating a partial dominant effect of the gene or genes controlling TYLCV resistance. Interestingly, while the average DSI of the TY172 × M-82 F_1 line was essentially in-between the average DSI scores of its parental lines, the average DSI of TY172 × LA1589 was a slightly skewed toward the susceptible line LA1589. Because in both crosses TY172 was used as the resistant line, these results show that the genetic background of the susceptible line may impact or modulate the degree of resistance expressed in F_1.

Association between the two inoculation experiments in TY172 × LA1589 F_3 families

Each of the 247 TY172 × LA1589 F_3 families was subjected to two separate inoculation experiments, one at the beginning and the other at the end of the summer of 2007. The linear association between these two inoculation experiments was characterized by a coefficient of determination (R^2) of 50%, representing a linear correlation of about 0.71 with a highly significant slope [$P(t) = 8 \times 10^{-39}$]. These values show that the reproducibility of our experimental procedure is quite high. Indeed, the genetic effects obtained in this study were highly similar when analyzed based on each of the inoculations separately or based on the average DSI scores of the two inoculations (see for example Fig. 1). For simplicity reasons, we will mainly present data based on the average DSI scores of the two inoculations. It should

Table 2 Average disease severity index (DSI) of parental lines and their F_1 hybrids

Line	DSI first inoculation	DSI second inoculation
LA1589	$3.3^A \pm 0.0$ ($n = 40$)	$3.3^A \pm 0.1$ ($n = 40$)
TY172 × LA1589	$2.1^B \pm 0.1$ ($n = 40$)	$2.3^B \pm 0.1$ ($n = 40$)
TY172	$0.1^C \pm 0.0$ ($n = 40$)	$0.1^C \pm 0.0$ ($n = 40$)
M-82	$3.3^A \pm 0.0$ ($n = 28$)	
TY172 × M-82	$1.7^B \pm 0.1$ ($n = 31$)	
TY172	$0.0^C \pm 0.0$ ($n = 19$)	

Results are displayed as mean ± SE; *n* number of plants in each accession; *DSI* disease severity index as described in the "Materials and methods" and previously in Friedmann et al. (1998) and in Lapidot and Friedmann (2002); different superscript letters represent statistically significant mean values ($P < 0.05$) based on Tukey–Kramer HSD test (Kramer 1956)

be noted that these average DSI scores are identical to the combined average DSI scores because the same number of seedlings were inculated in each experiment.

Association analysis between DNA markers and TYLCV DSI

As already indicated, 215 PCR markers were used in this study. Of these markers, 69 (32%) displayed polymorphic patterns between TY172 and LA1589 following endonuclease digestion with one or more of the ten restriction enzymes used. The polymorphic DNA markers where initially used to genotype plants from the two extreme tails of the F_2 population displaying either the lowest or highest DSI as determined by their F_3 progenies. DNA markers showing a significant chi-square (χ^2) value between allelic frequencies in these tails were later used to genotype the entire F_2 population and also subjected to association analyses with DSI scores. Tail analyses, by χ^2, displaying markers which were found statistically significant throughout the course of all of our analyses and specifically discussed in this manuscript are presented in Table 1.

TYLCV resistance in TY172 is controlled by a major QTL on chromosome 4

Association analysis between the genotyping information collected from individual F_2 plants and the average DSI obtained from their F_3 offspring showed that TYLCV resistance in TY172 is controlled by a major QTL residing on chromosome 4. This QTL accounted for about 46.6% of the variation in DSI, characterized by 34 < LOD score < 35 and was mapped at the vicinity of *SlNAC1* marker (Fig. 1). The average DSI of the F_3 families according to the *SlNAC1* genotype indicate that the effect

Fig. 1 Mapping and interval analysis of the major TYLCV-resistance QTL identified on chromosome 4. **a** Map distance in centiMorgan among the markers analyzed on chromosome 4, **b** interval analysis displaying LOD scores along chromosome 4 for DSI obtained in the first and second inoculations separately as well as average DSI of the two inoculations
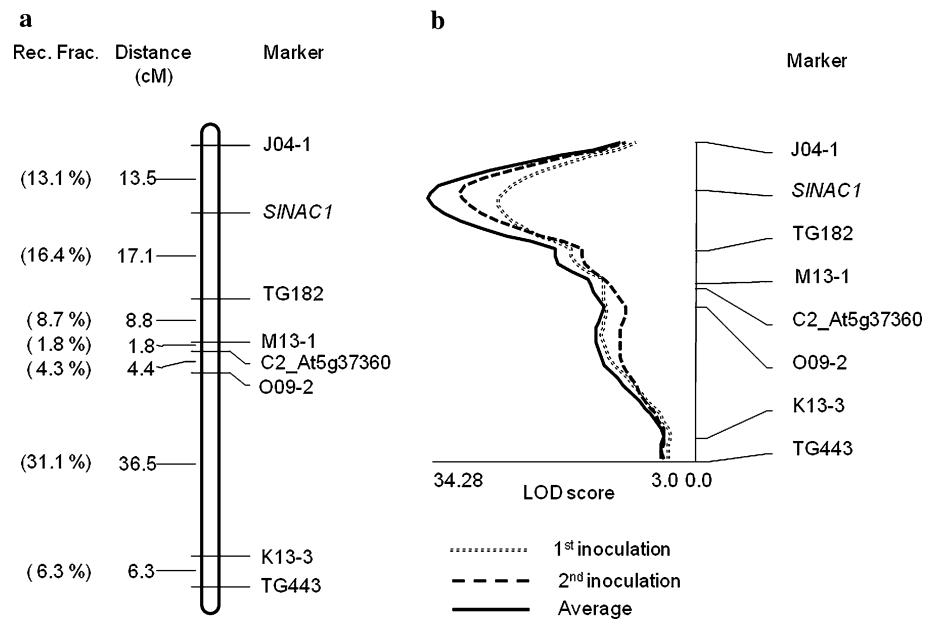

Table 3 Major and minor additive QTLs affecting TYLCV DSI among TY172 × LA1589 F$_3$ families according to the genotype of their parental F$_2$ plants

Genotype	Marker		
	SlNAC1	C2_at4g22260	*SlSUMO*
SS	2.1^A ± 0.0 (*n* = 72)	1.4^B ± 0.1 (*n* = 63)	1.7^A ± 0.1 (*n* = 37)
SR	1.7^B ± 0.0 (*n* = 110)	1.5^B ± 0.1 (*n* = 117)	1.6^{AB} ± 0.1 (*n* = 130)
RR	0.9^C ± 0.1 (*n* = 65)	1.8^A ± 0.1 (*n* = 67)	1.4^B ± 0.1 (*n* = 80)

Results are displayed as average DSI ± SE based on the average of the two inoculations; *n* number of F$_3$ families in each genotypic group; *DSI* disease severity index as described in the "Materials and methods" and previously in Friedmann et al. (1998) and in Lapidot and Friedmann (2002); *R* and *S* alleles originating from the resistant (TY172) and susceptible (LA1589) lines, respectively; different superscript letters represent statistically significant mean values (*P* < 0.05) based on Tukey–Kramer HSD test (Kramer 1956)

of the *SlNAC1* locus is, from a statistical point of view, partially dominant (Table 3), similar to the results obtained in the comparison of the average DSI of the parental lines and their hybrids (Table 2). However, in this analysis (Table 3), the average DSI of heterozygous plants is much more skewed toward average DSI scores of homozygous susceptible plants.

To confirm these results, we have inoculated 311 F$_2$ plants originating from the cross between TY172 and M-82, together with their parental lines and F$_1$ hybrid, scored their DSI, and transplanted them to the field. Throughout the season all fruits were harvested at the red stage from each plant separately. Fruit number, total fruit weight and plant weight were recorded. At the end of the season, harvest index (total fruit weight/plant weight) and total mass (total fruit weight + plant weight) were calculated. Results presented in Table 4 show a highly significant effect of the *SlNAC1* marker on DSI which was characterized by R^2 = 39.7% (33 < LOD score < 34). These results are highly similar to the results we have obtained from the segregating TY172 × LA1589 population. However, in this population the *SlNAC1* QTL exhibited a recessive effect unlike the partially dominant effect it displayed in the TY172 × LA1589 segregating population. As can be concluded from Table 4, the DSI was highly and negatively correlated with fruit number, total fruit weight, plant weight and total mass. Neither average fruit weight nor harvest index were significantly associated with the *SlNAC1* marker or with DSI.

TYLCV resistance in TY172 is modified by minor QTLs on chromosomes 1, 7, 9 and 11

In addition to the major QTL discovered on chromosome 4, four minor QTLs were found which affect resistance level. These minor QTLs can currently be detected by markers C2_at4g34700 on chromosome 1, TG174 on chromosome 7, *SlSUMO* on chromosome 9 and C2_at4g22260 on chromosome 11. Two of these minor QTLs, marked by C2_at4g22260 and *SlSUMO*, displayed a minor additive effect (Table 3). The QTL marked by *SlSUMO* displayed a partial dominance effect, the resistance allele originating from the resistant TY172 line, similar to *SlNAC1*. Interestingly, however, the QTL marked by C2_at4g22260

Table 4 Number of plants (n), DSI, fruit number, total fruit weight, plant weight, fruit weight, harvest index and total mass of TY172 × M-82 F$_2$ plants according to their *SlNAC1* genotype

Genotype	n	DSI	Fruit number	Total fruit weight (kg)	Plant weight (kg)	Average fruit weight (gr)	Harvest index	Total mass (kg)
SS	91	2.0[A] ± 0.1	55[B] ± 3	2.2[B] ± 0.2	1.09[B] ± 0.06	38.0[A] ± 1.9	2.1[A] ± 0.1	3.3[B] ± 0.2
SR	150	1.9[A] ± 0.0	62[B] ± 3	2.2[B] ± 0.1	1.17[B] ± 0.05	36.0[A] ± 1.7	2.0[A] ± 0.1	3.3[B] ± 0.1
RR	70	0.8[B] ± 0.0	84[A] ± 6	3.1[A] ± 0.3	2.09[A] ± 0.14	37.7[A] ± 2.3	1.7[A] ± 0.1	5.2[A] ± 0.3

Results are displayed as mean ± SE; *DSI* disease severity index as described in the "Materials and methods" and previously in Friedmann et al. (1998) and in Lapidot and Friedmann (2002); *R* and *S* alleles originating from the resistant (TY172) and susceptible (M-82) lines, respectively; different superscript letters represent statistically significant mean values ($P < 0.05$) based on Tukey–Kramer HSD test (Kramer 1956)

Table 5 Average DSI of TY172 × LA1589 F$_3$ families according to the *SlNAC1*, *SlSUMO*, C2_at4g34700 and TG174 genotype of their parental F$_2$ plants

SlNAC1 × *SlSUMO*		*SlNAC1* × C2_at4g22260		*SlNAC1* × C2_at4g34700		*SlNAC1* × TG174	
Genotype	DSI	Genotype	DSI	Genotype	DSI	Genotype	DSI
SS, SS	2.2[A] ± 0.1 (n = 31)	SS, RR	2.2[A] ± 0.1 (n = 17)	SS, RR	2.4[A] ± 0.1 (n = 13)	SS, RR	2.3[A] ± 0.1 (n = 14)
SS, SR	2.0[AB] ± 0.1 (n = 29)	SS, SR	2.0[A] ± 0.1 (n = 37)	SS, SR	2.0[AB] ± 0.1 (n = 37)	SS, SR	2.1[A] ± 0.1 (n = 41)
SR, SS	1.8[BC] ± 0.1 (n = 28)	SS, SS	2.0[AB] ± 0.1 (n = 18)	SS, SS	2.0[AB] ± 0.1 (n = 22)	SR, RR	1.8[AB] ± 0.1 (n = 22)
SR, SR	1.7[BC] ± 0.1 (n = 65)	SR, RR	1.9[AB] ± 0.1 (n = 32)	SR, RR	1.8[B] ± 0.1 (n = 24)	SR, SS	1.8[B] ± 0.1 (n = 32)
SS, RR	1.7[ABC] ± 0.2 (n = 12)	SR, SS	1.6[B] ± 0.1 (n = 29)	SR, SR	1.7[B] ± 0.1 (n = 64)	SR, SR	1.7[B] ± 0.1 (n = 56)
SR, RR	1.5[C] ± 0.1 (n = 17)	SR, SR	1.6[B] ± 0.1 (n = 49)	SR, SS	1.7[B] ± 0.1 (n = 22)	SS, SS	1.6[B] ± 0.2 (n = 17)
RR, SS	0.9[D] ± 0.1 (n = 22)	RR, RR	1.1[C] ± 0.1 (n = 14)	RR, SR	1.0[C] ± 0.1 (n = 37)	RR, RR	0.9[C] ± 0.1 (n = 10)
RR, SR	0.9[D] ± 0.1 (36)	RR, SR	0.9[C] ± 0.1 (n = 31)	RR, RR	0.8[C] ± 0.1 (n = 14)	RR, SR	0.9[C] ± 0.1 (n = 37)
RR, RR	0.5[D] ± 0.2 (n = 7)	RR, SS	0.7[C] ± 0.1 (n = 20)	RR, SS	0.6[C] ± 0.1 (n = 14)	RR, SS	0.8[C] ± 0.1 (n = 18)

Results are displayed as mean ± SE based on the average DSI of the two inoculations; *n* number of F$_3$ families in each genotypic group; *DSI* disease severity index as described in the "Materials and methods" and previously in Friedmann et al. (1998) and in Lapidot and Friedmann (2002); *R* and *S* alleles originating from the resistant (TY172) and susceptible (LA1589) lines, respectively; different superscript letters represent statistically significant mean values ($P < 0.05$) based on Tukey–Kramer HSD test (Kramer 1956)

displayed a dominant effect in which the resistance allele originated from the susceptible line LA1589.

When analyzed together with the *SlNAC1* marker, *SlSUMO* displayed a statistically significant effect (3 < LOD score < 4), but its interaction with *SlNAC1* was not statistically significant (R^2 = 50.3). Highly similar results were obtained for marker C2_at4g22260 when analyzed together with *SlNAC1* (3 < LOD score < 4, R^2 = 50.4 with no significant interaction). Despite the lack of a statistically significant interaction between *SlNAC1* and *SlSUMO*, the allelic combination *SlNAC1*^{S}/*SlNAC1*^{S} *SlSUMO*^{S}/*SlSUMO*^{S} showed a high average DSI whereas the allelic combination *SlNAC1*^{R}/*SlNAC1*^{R} *SlSUMO*^{R}/*SlSUMO*^{R} showed an exceptionally low average DSI; the latter being statistically different from the former (Table 5). In addition, the allelic combination *SlNAC1*^{S}/*SlNAC1*^{S} C2_at4g22260^R/C2_at4g22260^R displayed a high average DSI which was statistically different from the very low average DSI obtained for the allelic combination *SlNAC1*^{R}/*SlNAC1*^{R} C2_at4g22260^S/C2_at4g22260^S.

The two additional minor QTLs identified by the tails analysis, C2_at4g34700 and TG174, did not display a significant additive effect when analyzed separately in the entire population. However, when analyzed together with *SlNAC1*, both displayed a low statistically significant additive effect as well as a statistically significant interaction with *SlNAC1*. In these analyses C2_at4g34700 displayed a semi-dominant effect (1 < LOD score < 2) and a statistically significant interaction [$P(F)$ = 0.037, R^2 = 50.2%]. TG174, on the other hand had a higher effect of recessive nature (2 < LOD score < 3) and a more significant interaction effect [$P(F)$ = 0.0032, R^2 = 51.4%]. Interestingly, both markers were found associated in repulsion with average DSI; for both markers, the alleles originating from the susceptible line LA1589 were associated with lower DSI, similar to marker C2_at4g22260 (Table 5). As we have presented, the coefficient of determination, R^2, increased only slightly when incorporating *SlNAC1* marker and each one of the above four markers in the analysis model: from 46.6% using *SlNAC1* alone, to 51.4% when analyzing

TG174 and its interaction with *SlNAC1*. R^2 increased to only 58.6% when analyzing a model including *SlNAC1*, *SlSUMO*, C2_at4g34700, TG174, C2_at4g22260, the interaction between *SlNAC1* and C2_at4g34700 as well as the interaction between *SlNAC1* and TG174 (data not presented). More complex models were not attempted due to population size.

While attempting to validate these results on the segregating TY172 × M-82 F_2 population, we could not identify polymorphisms for markers C2_at4g34700, TG174 and C2_at4g22260 or markers residing at the vicinity of these markers. The only markers segregating in this population were *SlNAC1* and *SlSUMO*. Analysis of DSI, using these two markers in addition to their interaction as independent variables, revealed a highly significant effect for *SlNAC1* and no statistically significant effect for *SlSUMO* as well as its interaction with *SlNAC1*. Despite the lack of a statistically significant interaction between *SlNAC1* and *SlSUMO*, the allelic combination *SlNAC1*^{S}/*SlNAC1*^{S} *SlSUMO*^{S}/*SlSUMO*^{S} showed the highest average DSI whereas the allelic combination *SlNAC1*^{R}/*SlNAC1*^{R} *SlSUMO*^{R}/*SlSUMO*^{R} showed the lowest average DSI; the latter being statistically different from the former (Table 6). These results correspond nicely with the results obtained when analyzing these same markers in TY172 × LA1589 segregating population (Table 5). The correlation between the average DSI obtained in TY172 × LA1589 and those obtained in TY172 × M-82 population, according to the genotypic groups, was extremely high and highly significant ($r = 0.94$, $P = 0.0001$).

Table 6 Average DSI of TY172 × M-82 F_2 plants according to the *SlNAC1* and *SlSUMO* genotypes

SlNAC1 × *SlSUMO* genotype	n	DSI
SS, SS	31	$2.1^A \pm 0.1$
SR, SS	50	$2.0^A \pm 0.1$
SS, RR	13	$1.9^A \pm 0.2$
SS, SR	41	$1.9^A \pm 0.1$
SR, RR	28	$1.9^A \pm 0.1$
SR, SR	65	$1.9^A \pm 0.1$
RR, SR	26	$0.9^B \pm 0.1$
RR, SS	25	$0.8^B \pm 0.1$
RR, RR	13	$0.7^B \pm 0.1$

Results are displayed as mean ± SE; *n* number of F_2 plants in each genotypic group; *DSI* disease severity index as described in the "Materials and methods" and previously in Friedmann et al. (1998) and in Lapidot and Friedmann (2002); *R* and *S* alleles originating from the resistant (TY172) and susceptible (M-82) lines, respectively; different superscript letters represent statistically significant mean values ($P < 0.05$) based on Tukey–Kramer HSD test (Kramer 1956)

Discussion

TY172 is an open pollinated tomato line expressing a high level of resistance to TYLCV. This line was developed at the Volcani Center from a cross between *S. lycopersicum* line and a combination of four *S. peruvianum* accessions: PI 126926, PI 126930, PI 390681 and LA0441 (Friedmann et al. 1998). F_2 populations resulting from crosses between TY172 and susceptible lines usually produce about 10% of highly resistant plants characterized by DSI ranging between 0 and 0.5. Based on the average DSI of F_1 plants and its segregation ratios among F_2 plants, it was previously suggested that resistance in TY172 is controlled by three genes. Two of these genes were regarded additive, one partially dominant and the other recessive; both controlled epistatically by a third recessive gene (Friedmann et al. 1998). Such predictions are valuable, but their validation utilizing DNA markers is more informative. Such DNA markers can further serve as a tool for gene mapping contributing toward gene identification.

DNA markers associated with TYLCV resistance were previously identified in wild tomato species other than *S. peruvianum*. Major TYLCV resistance QTLs, *Ty-1* and *Ty-3*, were mapped, in close association, to chromosome 6 (Zamir et al. 1994; Agrama and Scott 2006; Ji and Scott 2006; Ji et al. 2007a), and *Ty-2* to chromosome 11 (Hanson et al. 2000; Hanson et al. 2006). A QTL, *Ty-4*, with a lesser effect on TYLCV resistance was mapped to chromosome 3 (Ji et al., 2008); in addition, two minor QTLs were identified that map to chromosomes 3 and 7 (Zamir et al. 1994).

Our results show that TYLCV resistance in TY172 is controlled by a reproducible major QTL allele originating from the resistant line and at least four minor QTLs. The major QTL we identified is novel because it maps to chromosome 4 and not to any of the aforementioned chromosomes thus far shown to harbor major or minor QTLs that control TYLCV resistance. The major QTL identified is highly associated with the *SlNAC1* marker ($34 < LOD$ score < 35), and accounts for 46.6% of the variation in DSI, measured based on average DSI of two inoculation experiments of a large population of F_3 families. This QTL displays a statistically significant partially dominant effect based on F_1 plants in comparison to their parental lines and also based on the segregation of marker *SlNAC1* in the TY172 × LA1589 population (Tables 2, 3). In both cases, however, the average DSI of heterozygous plants is skewed toward average DSI of the susceptible genotypes, but more notably in the TY172 × LA1589 segregating population (Table 3). This suggests the involvement of additional, most probably recessive, QTLs which can modulate TYLCV resistance attributed by *SlNAC1* QTL.

The average DSI of the *SlNAC1*[R]/*SlNAC1*[R] seedlings in our two segregating populations was low and close to 0.5, the upper level of a DSI characterizing TY172 resistant plants: 0.9 in the TY172 × LA1589 population and 0.8 in the TY172 × M-82 population (Tables 3, 4, respectively). Interestingly, genotyping additional cultivated TYLCV-susceptible genotypes and also resistant tomato genotypes originating from other wild sources revealed that *SlNAC1* banding pattern obtained for TY172 is unique. Moreover, the banding pattern of TY172 was identical to PI 126930, PI 390681 and LA0441, 3 of the resistant *S. peruvianum* accessions which are among the four resistant progenitors of TY172 (data not presented). The banding pattern of the fourth progenitor accession PI 126926 remains to be analyzed because we could not thus far obtain seeds or DNA samples from this accession. Together with the high reproducibility we have obtained for *SlNAC1* in the two segregating populations, our results suggest that the QTL we have identified on chromosome 4 can be highly effective in breeding TYLCV resistant cultivars and that the *SlNAC1* marker is of high utility. Due to the apparent strong effect of this QTL and in order to follow the terminology offered thus far for other major QTLs controlling TYLCV resistance, the term *Ty-5* is suggested for this QTL.

The four minor QTLs we have identified originate either from the resistant line (in coupling, the marker allele originating from the resistant line is associated with lower symptom severity) or from the susceptible line (in repulsion, the marker allele originating from the resistant line is associated with higher symptom severity). It can therefore be suggested that the cultivated tomato can serve as a source of genes which can increase TYLCV resistance attributed by major QTLs such as the one we have identified in this study or possibly major QTLs identified by others. We have further shown that together with the *SlNAC1* QTL, these minor QTLs can account for 58.6% of the variation in DSI in the TY172 × LA1589 population, representing only a small contribution to the resistance over the *SlNAC1* QTL alone.

To confirm our initial results and show that our markers can be utilized directly on F_2 populations in which a cultivated line replaces a wild accession as the susceptible line, we have generated a large F_2 population based on a cross between TY172 and M-82, a commonly used processing line. Our results show that the effect of the *SlNAC1* marker is highly reproducible ($R^2 = 39.7\%$, 33 < LOD score < 34), however with one exception: its effect among the F_2 plants is clearly recessive (Table 4), rather than partially dominant as was found in our previous analyses (Tables 2, 3). This discrepancy can be explained in at least two ways: (1) the less accurate resistance scoring of individual F_2 plants in comparison to the resistance scoring based on progeny testing as was carried out in our TY172 × LA1589 population,

or scoring of multiple plants belonging to the same pure-bred line as was done while scoring parental lines, and (2) the involvement of additional QTLs for resistance originating from the susceptible lines, as was found for three of our minor QTLs, and therefore their inheritance as well as effect is dependent upon the specific susceptible genetic background into which resistance genes are introgressed. Although the first explanation seems more acceptable, the second explanation can not be completely excluded because tomato breeders often report on much better TYLCV resistance performances in segregating populations obtained when the same genetic resistance is introgressed into certain cultivated lines over others. In any case, the highly negative correlation we obtained between DSI and components of yield and plant development in our TY-172 × M-82 F_2 population suggests that our scoring method is biologically relevant, in agreement with a former study (Lapidot et al. 2006).

The minor QTL, we discovered on chromosome 7, is probably not related to the minor QTL discovered by Zamir et al. (1994) on the same chromosome near TG61, because TG174, showing the highest R^2 when analyzed together with *SlNAC1*, resides about 31.5 cM away from TG61. Likewise, the minor QTL we identified on chromosome 11 is not related to the major QTL previously identified on the same chromosome near TG36 (Hanson et al. 2006), because C2_at4g22260, displaying the highest effect, resides about 47 cM away from TG36.

As we have already noted, the major QTL identified in the course of this study, *Ty-5*, maps to chromosome 4 and not to any of the other chromosomes thus far shown to contain QTLs controlling TYLCV resistance (Zamir et al. 1994; Chague et al. 1997; Hanson et al. 2000; Agrama and Scott 2006; Hanson et al. 2006; Ji and Scott 2006; Ji et al. 2007a, 2008). Due to lack of possible linkage, DNA markers associated *Ty-5* can be therefore effectively used in conjunction with DNA markers linked to any one of the major QTLs identified by others for efficient QTL pyramiding to breed highly resistant cultivars. In practical terms, this approach may be much more productive than identifying and utilizing QTLs with minor, less reproducible, effects in order to reach very high levels of TYLCV resistance. In this respect, it would be extremely interesting to analyze the general and specific combining ability of the five most major TYLCV resistance QTLs we and others identified thus far (*Ty-1*, *Ty-2*, *Ty-3*, *Ty-4* and *Ty-5*), at all possible combinations and against a range of begomoviruses inducing TYLCD.

Interestingly, two of the DNA markers which were identified in the course of this study as associated with TYLCV resistance are based on gene sequences. Our major QTL, *Ty-5* residing on chromosome 4, showed thus far the greatest association with *SlNAC1* (Fig. 1). *SlNAC1* was previously identified as a gene that encodes a member of the

NAC-domain protein family implicated in the replication of another tomato-infecting begomovirus, *Tomato leaf curl virus* (TLCV), by interacting with the viral replication enhancer protein (REn) (Selth et al. 2005). Selth et al. (2005) further showed that TLCV induces *SlNAC1* expression specifically in infected cells, and that this up-regulation requires REn. Also, in a transient TLCV replication system, over-expression of *SlNAC1* resulted in a substantial increase in viral DNA accumulation. Together, these results imply that *SlNAC1* plays an important role in the process by which REn enhances TLCV and possibly TYLCV replication.

One of our minor QTLs is based on *SlSUMO*, a gene encoding *Solanum* Small Ubiquitin-like Modifier (Hay 2005). A member of the *SUMO* gene family from *N. Benthamiana* has been shown to interact with a begomovirus AC1 protein, the protein which is essential for viral replication (Castillo et al. 2004).

Both genes, *SlNAC1* and *SlSUMO*, seem to be excellent candidate genes for TYLCV resistance because: (1) one of the main events that was thus far clearly associated with TYLCV infection is a reduced TYLCV single strand DNA accumulation at the site of inoculation in resistant TY172 tomato plants in comparison to their susceptible counterparts (Segev et al. 2004), and (2) Both *SlNAC1* and *SlSUMO* may be implicated in viral DNA replication and accumulation. To validate whether *SlNAC1* and *SlSUMO* are truly the genes which encode a certain major fraction of the resistance in TY172, more fine-tune mapping coupled with transgenic modulation of these genes in resistance and susceptible plants are required.

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