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INTROGRESSION OF RESISTANCE TO TWO MEDITERRANEAN VIRUS SPECIES CAUSING TOMATO YELLOW LEAF CURL INTO A VALUABLE TRADITIONAL TOMATO VARIETY

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SUMMARY

Tomato yellow leaf curl disease (TYLCD) causes devastation worldwide and in Italy is associated with *Tomato yellow leaf curl virus* (TYLCV) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV). Starting from the valuable traditional tomato cv. Eraldo, which is susceptible to TYLCD, crosses were made with accessions carrying *Ty-1* and *Ty-2* resistance loci. The F2 progenies were selected using both known and newly developed *Ty*-associated markers. F3 families derived from marker-selected F2 plants were challenged with isolates of TYLCV and TYLCSV present in Italy, under controlled greenhouse conditions. Genotypes carrying marker alleles associated with the *Ty-1* gene showed mild symptoms in all cases (tolerance), but the number of plants infected by TYLCV was much higher than those infected by TYLCSV. The majority of plants carrying the marker alleles associated to *Ty-2* did not allow any TYLCV replication but, when challenged with TYLCSV, most of them were infected and showed severe yellow leaf curl symptoms. To our knowledge this is the first report of *Ty-1* and *Ty-2*-carrying tomato plants challenged with the type-strain of TYLCSV.

Key words: TYLCV, TYLCSV, *Ty-1*, *Ty-2*, marker-assisted selection, resistance.

INTRODUCTION

Tomato yellow leaf curl disease (TYLCD) is a devastating viral disease of tomato (*Solanum lycopersicum*) worldwide, long known in the Middle East, north and central Africa and southern Asia. In the early nineties there were severe outbreaks in southern Europe. More recently, the disease was found in Mexico, the Caribbean and the USA (Lapidot *et al.,* 2000). In Mediterranean countries, two viruses (with several isolates) in the genus *Begomovirus*, family *Geminiviridae*, have been associated with the epidemics: *Tomato yellow leaf curl virus* (TYLCV) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV) (Louro *et al.*, 1996; Navas-Castillo *et al.,* 1997; Accotto *et al.,* 2000, 2003). The use of resistant hybrids is the most economic and sustainable way to control virus diseases, therefore increasing the level of resistance to TYLCD is an important objective for tomato breeders, especially for southern Italy where high quality tomatoes are produced in winter and spring, in unheated greenhouses, where conditions are particularly suited for infestation by the vector *Bemisia tabaci*.

Classical breeding for resistance first involves the identification of resistance sources, which are often found in wild germplasm unadapted to agriculture; this is followed by the introgression into cultivars via phenotypic selection of resistant progeny. Sources of resistance to TYLCD have been discovered in wild tomato species, including *Solanum pimpinellifolium*, *S. peruvianum, S. chilense, S. habrochaites* and *S. cheesmaniae* (Ji *et al.*, 2007a). Thus, breeding efforts to develop TYLCD resistance have been largely based on transfer of genes from these wild *Solanum* species.

The type of resistance, the availability of the infected vectors and the presence of different viruses causing TYLCD make classical selection slow and difficult because phenotypic analyses of partial resistance or tolerance traits are often of limited utility. Marker-assisted breeding overcomes such problems focusing on the direct selection of genomic loci underlying the trait.

Some of the TYLCD resistance loci reported have been placed on linkage maps with molecular markers. The first mapping study of a begomovirus resistance gene was reported by Zamir *et al.* (1994). The resistance locus, designated *Ty-1*, originated from *S. chilense* accession LA1969. The *Ty-1* gene confers partially dominant resistance and has been mapped to the region comprising markers TG297 and TG97 on chromosome 6. The resistance found in LA1969 has been introgressed into cultivated tomato, and several lines such as LA3473, partially resistant to TYLCV, have been developed (Laterrot, 1995; Michelson *et al.*, 1994; Zamir *et al.,* 1994). The *Ty-2* gene is derived from *S. habrochaites* accession B6013 (Hanson *et al.,* 2000) and in India Kalloo and

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Banerjee (1990) developed several TYLCV-resistant tomato lines derived from this source. One of these lines, H24, showed excellent TYLCV resistance in Taiwan and southern India. Investigation of these lines led to the identification of this novel locus on the long arm of chromosome 11 mapped at approximately 5 cM from markers TG105A and T0302, tightly linked one to another (Ji *et al.,* 2007a). Another begomovirus resistance locus described is *Ty-3,* in the same genomic region carrying *Ty-1* on chromosome 6 (Ji and Scott, 2006). Ji *et al.* (2007b) identified a large *S. chilense* introgression carrying *Ty-3* in LA2779-derived advanced breeding lines resistant to both TYLCV and *Tomato mottle virus* (ToMoV). More recently, two additional loci linked to resistance, *Ty-4* (Ji *et al.*, 2009) on chromosome 3, and *Ty-5* (Anbinder *et al.*, 2009) on chromosome 4, have been described. Vidavski *et al.* (2008) reported a pyramiding strategy, by classical breeding, of different sources of resistance to TYLCV coming from different wild species.

The preferential use of local varieties is a central theme of European agriculture; in the last few years many breeders have started programmes on some traditional Italian cultivars. Since they are susceptible to TYLCD, it would be of great interest to improve them through marker-assisted breeding and introgression of resistance loci. In a given region, it is very important to use the local viruses and strains for the assays. In several cases different levels of resistance have been attributed to the same genetic sources, mainly because of variability in assay conditions and in virus strains (Lapidot *et al.*, 2006). Moreover, while several reports have tested the resistance against TYLCV, there is very little available on responses to TYLCSV.

In Italy, two begomoviruses cause TYLCD: *Tomato yellow leaf curl Sardinia virus* (TYLCSV) and *Tomato yellow leaf curl virus* (TYLCV). Two strains of TYLCSV (TYLCSV-Sar-[IT:Sar:88] and TYLCSV-Sic-[IT:Sic]) and one of TYLCV (TYLCV-IL- [IT:Sic:04] have been described (Kheyr-Pour *et al.*, 1991; Davino *et al.*, 2006). Their spread in Italy is well documented and in recent years both species have frequently been found in mixed infections in single tomato plants (Davino *et al.*, 2006). TYLCSV and TYLCV have monopartite ssDNA genomes of about 2.8 kb, and share about 70% sequence similarity.

In the present work we have tested, under controlled conditions, the performance of lines carrying *Ty-1* and *Ty-2* genes against Italian isolates of TYLCV and TYLCSV, and we have developed two new CAPS (Cleaved Amplified Polymorphic Sequence) markers linked to *Ty-1* and *Ty-2*. The developed markers, together with two previously reported ones, were validated for introgression of the two loci linked with resistance into a traditional Italian cultivar by phenotypic tests of F3 marker-selected families.

MATERIALS AND METHODS

Plant materials. 'Eraldo', a selection of cv. Cuor di Bue di Albenga, was used as TYLCD-susceptible parent. It originated from Liguria (northern Italy), and is characterized by indeterminate growth, pear shaped fruits with marked ribs and an average weight of 200 g, with distinctive flavour appreciated by consumers. Two well characterized breeding lines, LA3473 and H24, were used as resistant parents. LA3473 carries *Ty-1* locus introgressed from *S. chilense* accession LA1969, while H24 bears the *Ty-2* gene derived from the original resistance source *S. habrochaites* accession B6013 (Kalloo and Banjeree, 1990). Four F2 populations (two segregating for *Ty-1*, and two for *Ty-2*) of 84 plants each were generated by controlled self-pollination of the F1 plants derived from the reciprocal crosses between the susceptible line 'Eraldo' and the two resistant lines LA3473 and H24. For marker analyses, three other accessions were also used: the *S. habrochaites* LA0094, the *S. chilense* accession LA1969, and the *S. lycopersicum* LA3474 (a susceptible control line of LA3473, not carrying *Ty-1*).

DNA isolation and marker analyses. Genomic DNA was extracted from young leaves using the Wizard Magnetic 96 DNA Plant System (Promega, USA) following manufacturer's instructions. DNA quality and quantity was determined by agarose gel electrophoresis and UV-spectrophotometry (NanoDrop 1000, Thermo Fisher Scientific, USA). All DNA for PCR reactions was used at 50 ng/µl. Four molecular markers were used (Table 1).

For marker TG178, primers were designed using Primer 3 software (Center for Genome Research, Whitehead Institute, USA); for the remaining three markers, TG436, TG105A and C2_At5g25760, the source of primers is indicated in Table 1. PCRs were performed in 25 µl reaction volumes containing 1 µl of 50 ng/µl genomic DNA, 1x buffer, 0.5 µM of each primer, 0.6 mM dNTPs, 1.4 mM MgCl2 and 1 unit of *Taq* polymerase (Promega, USA). The following conditions were used: an initial denaturation step at 95°C for 2 min, followed by 35 cycles of 95°C for 40 sec, annealing temperature (Table 1) for 40 sec, 72°C for 60 sec and a final extension at 72°C for 5 min. The PCR products were electrophoresed through a 1% agarose gel and visualized under UV light after staining with ethidium bromide. For each marker, the PCR product of each parent was then purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA) and sequenced using BigDye version 3.1 sequencing system (Applied Biosystems, USA) on a ABI3130xl sequencer, according to the manufacturer's instruction. The sequences from resistant and susceptible parental lines were then compared using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/). Restriction maps of the PCR product sequences from re-

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Table 1. Primer sequences, source, annealing temperature (Ann. T), restriction enzyme (RE) revealing a polymorphism and genome position of CAPS markers used in this study.

Marker	Gene	Primer sequence (5'-3')	Ann. T(°C)	Primer source	RE	SGN map position Chromosome, cM (map)
TG178	*Ty-1*	F: ggtactcctggaagggttaagg R: cacgctggttctgttgtatctc	56	Designed on Unigene U143258	*TaqI*	Chromosome 6, cM 8.60 (EXPEN 1992)
TG436	*Ty-1*	F: tctgcaagtcgcatcggaaggtatcg R: gtatgggccacctggcatgcacctcg	56	Maxwell *et al.* (2006)	*AdeI*	Chromosome 6, cM 10.0 (EXPEN 1992)
TG105A	*Ty-2*	F: cttcagaattcctgtttagtcagttgaacc R: atgtcacatttgttgcttggaccatcc	62	Maxwell *et al.* (2006)	*TaqI*	Chromosome 11, cM 90.00 (EXPEN 2000)
C2_At5g25760	*Ty-2*	F: tccttatgatggtggagttttccag R: aaagcaattatagctcgacaaacag	52	http://www.sgn.cornell.edu	*StyI*	Chromosome 11, cM 89.50 (EXPEN 2000)

sistant and susceptible parental lines were compared using WebCutter software (http://www.firstmarket.com/cutter). CAPS-based markers were digested with the enzymes listed in Table 1. Ten µl of PCR product were incubated with 10 U of restriction enzyme according to the manufacturer's instructions and then separated on a standard 1.5% agarose gel.

Selection of F3 progeny. The four F2 populations from a cross between susceptible and resistant parents were scored for polymorphisms. A total of 336 F2 seedlings, 84 for each breeding population studied, were grown in greenhouse and their DNA was isolated 40 days after sowing, and then screened by CAPS markers. Marker-selected F2 plants, homozygous for the alleles of the resistant parent carrying the *Ty* gene, were transplanted into large pots and self-pollinated to produce F3 seeds. Resistance tests were performed on F3 seedlings.

Virus resistance tests. Plants were challenged, under controlled conditions, with one TYLCSV and one TYLCV isolate, both widespread in tomato cultivations in Sicily and Sardinia (Davino *et al.*, 2006), as follows. Each virus was tested on 15 lines (six *Ty-1* - and six *Ty-2* - marker-selected F3 families derived from single F2 plants, and three parental lines), using 10 plants per line per virus, with a total of 300 plants. The experiment was replicated twice, in two different times of the year. Tomato seedlings were grown in 22-place vessels, containing two randomly chosen lines each (ten inoculated plus one non-inoculated control plants), that were randomly put on benches of an insect-proof glasshouse at average temperatures of 23°C (summer experiment) or 19°C (winter experiment). They were inoculated at the 4-true leaf stage, one month after sowing. Challenge was through agroinoculation, which avoids the use of insect vectors (Grimsley, 1987). For TYLCSV, we used *Agrobacterium tumefaciens* strain LBA4404/pBin19/TYLCV-Sr1.8 (Kheyr-Pour *et al.*, 1991) containing a redundant copy of TYLCSV DNA from Sardinia (GenBank accession No. X61153). For TYLCV, we used a similar construct, prepared from TYLCV isolated in Sicily [Davino *et al.* (2006), accession No. DQ144621]. Plants were analyzed for the presence of viral DNA at different times after inoculation by printing a small piece of the youngest expanding leaf near the apex on a positively charged nylon membrane which was then hybridized with digoxigenin-labelled virus-specific probes. Symptoms were scored using the Disease Severity Index (DSI) ranging from 0 (no visible symptoms) to 4 (very severe plant stunting and yellowing, pronounced leaf cupping and curling, and cessation of plant growth), according to Lapidot and Friedman (2002).

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RESULTS

Marker analysis. Marker-assisted selection of the *S. chilense* introgression in LA3473 carrying the *Ty-1* gene was performed with TG178 and TG436. For marker TG178, based on the sequence of tomato Unigene U143258 matched by the RFLP probe sequence, new primers were designed converting the RFLP marker into a PCR-based marker. The primer pair produced a single fragment of approximately 1000 bp. Its nucleotide sequence was 500 bp longer than the sequence deduced from Unigene because of the presence of four intron sequences in the genomic DNA. Sequencing of the PCR products amplified from genomic templates of LA3473, LA3474, 'Eraldo', and *S. chilense* (LA1969) revealed two genotype groups based on five SNPs. LA3473 and LA1969 carried the *chilense*-type allele whereas LA3474 and 'Eraldo' carried the *lycopersicum*-type allele, indicating that this marker can be located within the *chilense* introgression in LA3473. A co-dominant CAPS marker was developed by using restriction enzyme *TaqI* (Fig. 1a).

For the TG436 marker, a single product of approximately 350 bp was obtained by PCR. Sequencing of the PCR products from the different accessions confirmed the presence of two different alleles based on two InDels between LA3474 and LA3473, as already described by Maxwell *et al.* (2006). The 'Eraldo' sequence was identical to that from LA3474. The sequences were then analyzed for restriction enzyme sites and *AdeI* was selected in order to produce polymorphic digestion patterns between the different lines (Fig. 1b). This new enzyme was chosen, instead of *Taq*I suggested by Ji *et al.* (2007b), for the better resolution achieved. CAPS markers TG178 and TG436 were then scored in the two segregating F2 populations derived from 'Eraldo' x LA3473 and LA3473 x 'Eraldo'. For each population, three F2 plants homozygous for the *S. chilense* alleles at loci TG178 and TG436 were selected and F3 families were developed by self-pollination. A total of 120 F3 plants, 20 per F3 family, were used for phenotypic analysis of resistance against the two viruses.

Markers TG105A and C2_At5g25760 were used to select the *S. habrochaites* introgression in H24 carrying *Ty-2*. For the TG105A marker, a single PCR product of approximately 500 bp was obtained. Sequencing of the PCR products from the different parental lines confirmed the presence of a 14 bp insertion in H24 with respect to the *S. lycopersicum* accessions. To achieve a better separation of contrasting alleles after electrophoresis, their sequences were then analyzed for restriction sites (Fig. 2a), and a CAPS marker (*Taq*I) was developed, in accordance with the description of Maxwell (2006).

Marker C2_At5g25760 produced a single band of approximately 1300 bp. Analysis of the nucleotide sequence of the PCR products revealed many SNPs and InDels between *S. lycopersicum* susceptible lines and H24, indicating that this marker could be located inside the *S. habrochaites* introgression in H24. The sequences were analyzed for restriction site polymorphism and a CAPS marker (*Sty*I) was developed (Fig. 2b). After de-

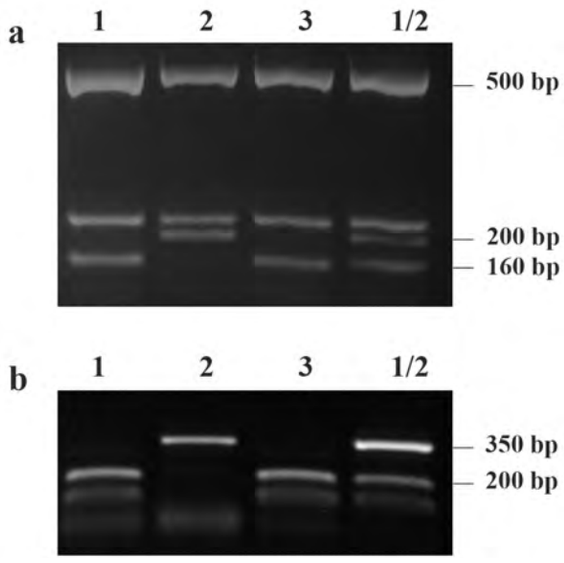

Fig. 1. Alleles for CAPS markers (*Ty-1*) TG178 (a) and TG436 (b) after enzymatic digestion of lines 'Eraldo' (1), LA3473 (2), LA3474 (3) and a heterozygous F2 plant (1/2). Size of DNA fragments following digestion are shown on the right.

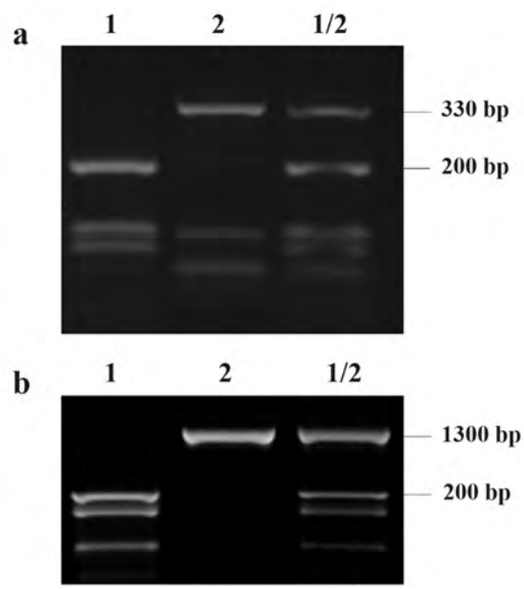

Fig. 2. Alleles for CAPS markers (*Ty-2*) TG105A (a) and C2_At5g25760 (b) after enzymatic digestion of lines 'Eraldo' (1), H24 (2), and a heterozygous F2 plant (1/2). Size of DNA fragments following digestion are shown on the right.

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Table 2. Evaluation of TYLCSV and TYLCV symptom severity at the end of the second experiment (see Fig. 3B, 8 wpi). Lines: F3 families derived from F2 plants selected for *Ty-1* (lines 731 to 736) or *Ty-2* (lines 699 to 713); 'Eraldo' susceptible parental line; LA3473 parental line carrying *Ty-1*; H24 parental line carrying *Ty-2*. DSI= Disease Severity Index (average value for each line), calculated only on plants (number in parenthesis) that were infected as detected by molecular hybridization; ND = not determined (no plants were infected).

Line	DSI TYLCSV	DSI TYLCV
'Eraldo'	4 (9)	4 (10)
LA3473	0 (1)	0.4 (7)
731	0 (1)	0 (3)
732	0 (1)	1.1 (7)
733	2 (1)	0.2 (5)
734	ND	0.3 (7)
735	1 (1)	0.1 (10)
736	0 (1)	0.1 (8)
H24	2.2 (6)	0 (3)
699	1.8 (5)	1 (2)
705	1.2 (6)	0 (1)
706	2.7 (7)	0.8 (4)
707	1.9 (9)	1.3 (3)
708	3.5 (9)	ND
713	3.7 (7)	1 (1)

tecting polymorphism between H24 and 'Eraldo' at the two marker loci, the two F2 populations derived from a reciprocal cross between H24 and 'Eraldo' were analysed for marker segregation. For each population, three F2 plants homozygous for the *S. habrochaites* alleles at TG105A and C2_At5g25760 loci were selected and self-pollinated in the greenhouse to produce a total of 120 F3 plants for the phenotypic analysis.

Resistance to virus challenge and marker validation. All parental lines used and F3 families selected by marker analysis were challenged with TYLCSV and TYLCV in two different experiments, one conducted during summer 2008 (Fig. 3A) and the second during winter 2008-2009 (Fig. 3B). Each experiment included, for each virus, 10 plants for each parental line and 10 for each F3 family studied. Due to different growth rates of plants in the two seasons, analyses were performed up to 4 and 8 weeks post inoculation (wpi) for the summer and winter experiment, respectively. Fig. 3 shows the results of molecular hybridization assays at two time points for each experiment.

In the summer experiment, at 2 wpi, when the susceptible parental line 'Eraldo' was not yet completely infected by the two viruses, lines carrying *Ty-1* appeared to partially contain infections, however at 4 wpi, with 100% infection in 'Eraldo', a partial resistance was observed only to TYLCSV, in both the parental LA3473 and its F3 families.

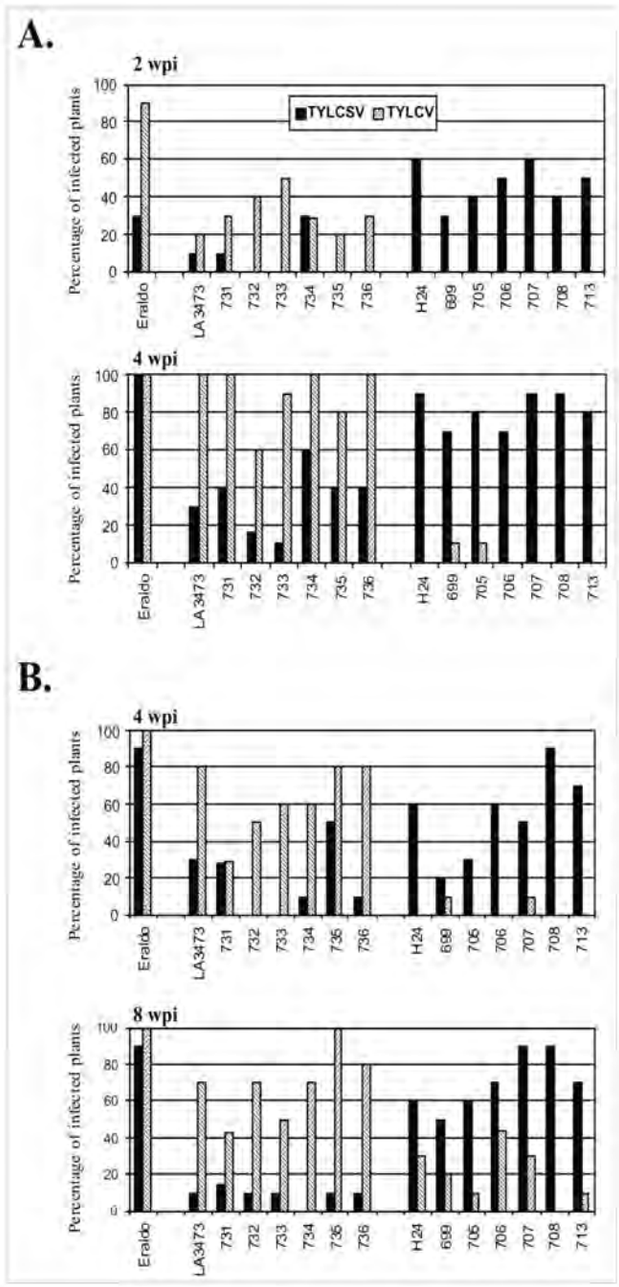

Fig. 3. Evaluation of infection by TYLCSV and TYLCV in two greenhouse experiments conducted in summer (A, average temperature 23°C) or in winter (B, average temperature 19°C) on F3 families derived from F2 plants selected for *Ty-1* (lines 731 to 736) or *Ty-2* (lines 699 to 713). 'Eraldo': susceptible parental line; LA3473: parental line carrying *Ty-1*; H24: parental line carrying *Ty-2*. The percentage of plants containing viral DNA in young tissues was determined by molecular hybridization with virus-specific probes. wpi: weeks post inoculation.

The H24 plants, carrying *Ty-2*, were not infected by the Italian isolate of TYLCV, even at 4 wpi. This resistance was confirmed in the F3 'Eraldo' x H24 plants (six families) selected for being homozygous for the resistant allele of markers TG105A and C2_At5g25760: most

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plants did not contain any detectable TYLCV DNA. As regards the challenge of the same parental line and derived F3 with TYLCSV, a very limited level of resistance was observed: 70 to 90% of the plants were infected at 4 wpi. No significant differences were detected between the families derived from reciprocal crosses.

The winter experiments yielded similar results, but with some peculiar features. Comparing the reaction of lines carrying *Ty-1* to TYLCSV challenge in the two time points, a recovery was observed in some cases (the parental LA3473, and the 731, 734 and 735 families). A second peculiarity was that H24 plants and its derivatives, carrying *Ty-2*, showed less resistance to TYLCV (an average of 20% infected plants), but a limited resistance to TYLCSV (an average of 70% infected plants) not observed in the summer experiment.

A semiquantitative measure of the viral load in infected plants was obtained by comparing the intensity of tissue prints between the 'Eraldo' susceptible line and the other lines (Fig. 4). Lines carrying *Ty-1* showed a marked reduction of viral load in the case of TYLCSV, and a limited reduction in the case of TYLCV. In plants carrying *Ty-2*, when infected by TYLCSV, no reduction of viral load was detected, while in the very few cases of TYLCV infection in the same lines a reduction was observed.

When symptom severity was measured (Table 2), the susceptible 'Eraldo' had the maximum score (DSI=4) for both viruses, while the lines carrying *Ty-1* were almost symptomless, with very low scores for both viruses (average DSI=0.6 for TYLCSV and 0.4 for TYLCV). On the other hand, the lines carrying *Ty-2* were almost symptomless for TYLCV (DSI=0 for H24, and average of 0.9 for the derived families), but showed some yellowing and curling of leaflets for TYLCSV (DSI=2.2 for H24, and average of 2.6 for the derived families).

DISCUSSION

The effectiveness of different resistance sources in different regions, and consequently against different begomoviruses, has been the main object of several tomato breeding programs. Differential responses of resistance genes to virus isolates suggested the necessity of developing tomato cultivars with resistance to TYLCD isolates from different geographic areas (Picó *et al.*, 1999).

Our first goal was to investigate the usefulness of *Ty-1* and *Ty-2* in Italian breeding programs developing tomato cultivars with resistance to TYLCD. The results obtained by using the tissue print assays indicated that *Ty-1* significantly reduced the number of infected plants in the case of TYLCSV, but had limited effect on TYLCV, whereas *Ty-2* was effective against TYLCV, but acted poorly on TYLCSV (Fig. 3 and 4). These results indicate that *Ty-1* and *Ty-2* respond differently if challenged with two apparently similar viruses. In fact, until some years ago, TYLCV and TYLCSV were considered strains of the same virus named TYLCV, resulting in a literature that is sometimes confusing. Most of the reports deal with TYLCV, and only a few with TYLCSV. Moreover, several reports of experiments performed outdoors in different regions of the world do not state which TYLCD-inducing virus was present (Ji *et al.*, 2007a). Greenhouse-controlled experiments allowed us to challenge plants with two well characterized isolates. The agroinoculation technique that we used is a more precise alternative to whitefly inoculation, delivering the same dose of inoculum to each plant, particularly suited when testing several lines under controlled conditions, and has been successfully applied to resistance assays for begomoviruses (Picó *et al.*, 2001; de Castro *et al.*, 2005).

To our knowledge, this is the first report of *Ty-1*- and *Ty-2*-carrying tomato plants challenged with the type strain of TYLCSV. A Spanish isolate of TYLCSV (accession No. L27708), taxonomically distinct from the type-strain employed in the present work, was used by Picó *et al.* (1999, 2001) and more recently by de Castro *et al.* (2005) on *S. chilense* accessions and derived breeding lines. These two genomic sequences share a similarity of 88%, a value that, considering the new criteria for classification of begomoviruses (Fauquet *et al.,* 2008), could even place them in distinct species. In our experiments inoculation of the type-strain of TYLCSV resulted in partial resistance in lines LA3473 and derivates carrying *Ty-1*, reflecting the results of Picó *et al.* (2001), who obtained 40-60% infection on line LA1969 (from which LA3473 was derived) when inoculated with a Spanish isolate.

Zamir *et al.* (1994) indicated in *Ty-1* a good source of resistance to TYLCV, because it confers tolerance to infection. In our experiments we classified *Ty-1*-carrying plants as susceptible to TYLCV on the basis of the presence of viral DNA (Fig. 3). The limited semi-quantitative analyses that we performed indicated that the tolerance observed with *Ty-1* can be related to a lower amount of viral DNA, and, for *Ty-2* plants infected by TYLCSV, the high DSI score correlates with levels of viral DNA similar to those observed in the 'Eraldo' susceptible plants (Fig. 4).

When we compared the performance simply in terms of disease symptoms, *Ty-1* clearly showed a very good tolerance to both TYLCV and TYLCSV, reducing the viral load, while *Ty-2* showed a very good tolerance to TYLCV, but only a limited reduction of symptoms when infected by TYLCSV, with no reduction of viral load. If the presence of viral DNA is considered, however, a consistent reduction in the number of plants infected by TYLCSV was conferred only by gene *Ty-1*, while TYLCV infection was controlled only by *Ty-2*. Although a tolerant phenotype is doubtless very useful for field applications, the presence of viral DNA can

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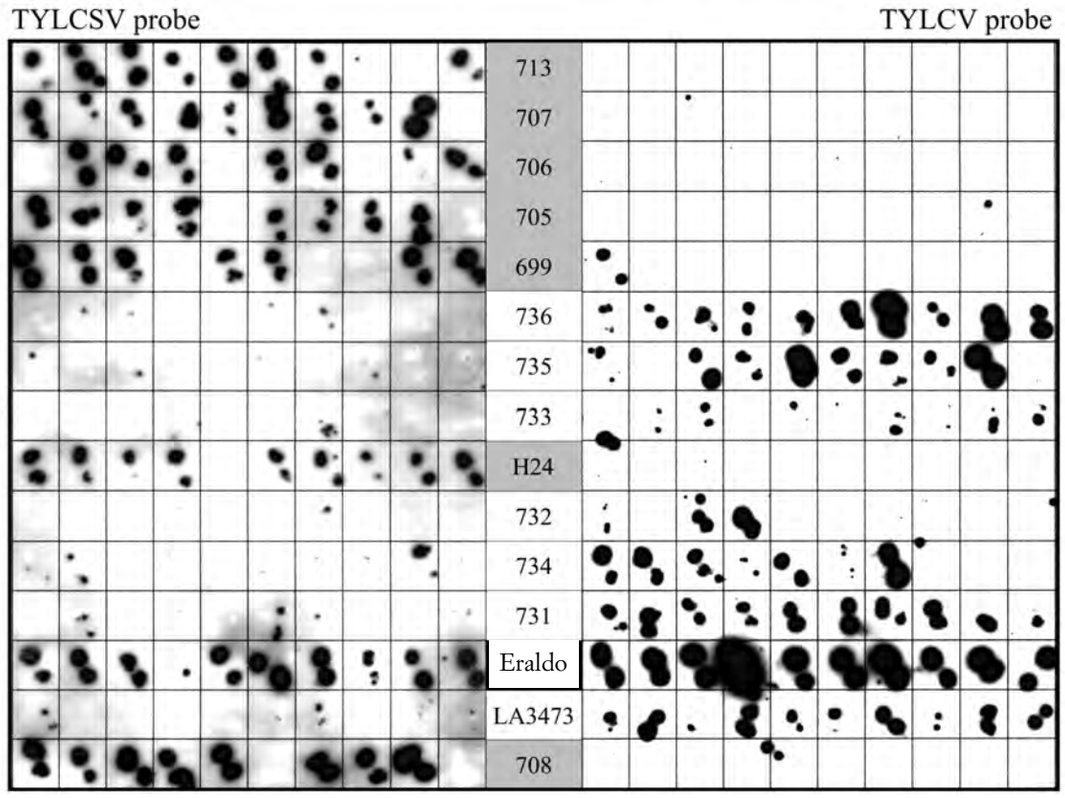

Fig. 4. Tissue print hybridization assay on plants of lines carrying *Ty-1* (white) or *Ty-2* (grey background) compared with parental lines LA3473 (*Ty-1*) H24 (*Ty-2*) and 'Eraldo' (susceptible). TYLCSV- and TYLCV-specific probes were used in the left and right panel, respectively.

constitute a dangerous reservoir of virus, from which whitefly vectors can acquire and transmit the disease to susceptible tomato cultivars and to wild species. The development of cultivars that prevent the invasion of both TYLCSV and TYLCV, by pyramiding resistance genes, is therefore an important goal to reduce the levels of these viruses in cultivation districts.

The second objective of this work was to identify molecular markers suitable for the introgression of begomovirus resistance loci into traditional Italian cultivars such as "Cuor di Bue di Albenga" extremely susceptible to TYLCD. Four CAPS markers were used, TG105A after Maxwell *et al.* (2006) and TG436 from the same author, although with an improved protocol (*Ade*I *vs*. *Taq*I), and two (C2_At5g25760 and TG178) developed *de novo*, thus increasing the choice of segregating markers in different crosses, and providing new co-dominant PCR-based markers suitable for an easy genotype screening.

By selecting for TG436 and TG178 markers (Fig. 1) a similar level of resistance to TYLCSV was obtained in LA3473 and in F3 plants, indicating that these markers can provide an efficient screen for *Ty-1*-mediated resistance in marker assisted programs against TYLCSV. Based on an updated genetic map of chromosome 6 described by Ji e*t al.* (2007b), *Ty-1* gene and TG178 mapped to the same locus, while TG436 was placed 0.2 cM distally from them. This would keep very low or insignificant the risk of marker/gene recombination in practical assisted breeding.

Similarly, the two *Ty-2*-linked markers TG105A and C2_At5g25760 (Fig. 2) were successfully used to introgress resistance to the Italian TYLCV isolate from the H24 resistance source. However, even if marker analyses provided us with good resistant F3 progenies for all the selected plants, it is still possible, in the case of *Ty-2* tagging markers (TG105A and C2_At5g25760), that a recombination event occurs between *Ty-2* and the two markers (among them tightly associated at 0.5 cM), with an expected frequency of about 5% [from a 5cM reported association (Ji *et al.*, 2007a)].

The recent discovery and mapping of *Ty-3*, *Ty-4* and *Ty-5* resistance loci rises a first question about linkage or allelism of *Ty-1* and *Ty-3* loci on chromosome 6, that would be interesting to investigate. Moreover, it should be worth trying to pyramid *Ty-4* and *Ty-5* genes into the background of the lines carrying two homozygous loci (*Ty-1* and *Ty-2*), to investigate the extent of protection achieved against begomoviruses.

Based on the results of the present work, the two

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new CAPS markers identified, in combination with previously released ones, will be useful in obtaining tomato genotypes of cv. Cuor di Bue di Albenga type selections where *Ty-1* and *Ty-2* genes are pyramided in homozygosis, possibly yielding a broader spectrum of resistance to the TYLCV and TYLCSV isolates present in tomato-growing regions of Italy and other Mediterranean areas. Further breeding steps consisting in rounds of marker-assisted backcross are therefore necessary before the two pyramided resistance genes are introgressed in tomato lines with the genomic background of cv. Eraldo and thus showing the desired "Cuore di Bue" phenotype. Extended field experiments will then be necessary to evaluate not only resistance to TYLCD in field conditions, but also agronomic performance.

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REFERENCES

Accotto G.P., Navas-Castillo J., Noris E., Moriones E., Luoro D., 2000. Typing of tomato yellow leaf curl viruses in Europe. *European Journal of Plant Pathology* **106**: 179-186.

Accotto G.P., Bragaloni M., Luison D., Davino S., Davino M., 2003. First report of *Tomato yellow leaf curl virus* (TYLCV) in Italy. *Plant Pathology* **52**: 799.

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

Davino S., Napoli C., Davino M., Accotto G.P., 2006. Spread of *Tomato yellow leaf curl virus* in Sicily: partial displacement of another geminivirus originally present. *European Journal of Plant Pathology* **114**: 293-299.

de Castro A.P., Díez M.J., Nuez F., 2005. Evaluation of breeding tomato lines partially resistant to *Tomato yellow leaf curl Sardinia virus* and *Tomato yellow leaf curl virus* derived from *Lycopersicon chilense*. *Canadian Journal of Plant Pathology* **27**: 268-275.

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

Grimsley N., Hohn T., Davis J.W., Hohn B., 1987. Agrobacterium-mediated delivery of infectious *Maize streak virus* into maize plants. *Nature* **325**: 177-179.

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

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

Ji Y., Scott J.W., Hanson P., Graham E., Maxwell D.P., 2007a. Sources of resistance, inheritance, and location of genetic loci conferring resistance to members of the tomato-infecting begomoviruses. In: Czosnek H. (ed.), Tomato Yellow Leaf Curl Virus Disease: Management, Molecular biology, Breeding for resistance, pp. 343-362. Springer, Dordrecht, The Netherlands.

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

Ji Y., Scott J.W., Schuster D.J., Maxwell D.P., 2009. Molecular mapping of *Ty-4*, a new Tomato yellow leaf curl virus resistance locus on chromosome 3 of tomato. *Journal of the American Society for Horticultural Science* **134**: 281-288.

Kalloo G., Banerjee M.K., 1990. Transfer of tomato leaf curl virus resistance from *Lycopersicon hirsutum* f. *glabratum* to *L. esculentum*. *Plant Breeding* **105**: 156-159.

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

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

Lapidot M., Goldray O., Ben-Joseph R., Cohen S., Friedmann M., Shlomo A., Nahon S., Chen L., Pilowsky M., 2000. Breeding tomatoes for resistance to tomato yellow leaf curl virus begomovirus. *Bulletin OEPP/EPPO Bulletin* **30**: 317-321.

Lapidot M., Friedman M., 2002. Breeding for resistance to whitefly-transmitted geminiviruses. *Annals of Applied Biology* **140**: 109-127.

Lapidot M., Ben Joseph R., Cohen L., Machbash Z., Levy D., 2006. Development of a scale for evaluation of *Tomato yellow leaf curl virus* resistance level in tomato plants. *Phytopathology* **96**: 1404-1408.

Laterrot H., 1995. Breeding network to create tomato varieties resistant to *Tomato yellow leaf curl virus* (TYLCV). *Fruits* **50**: 439-444.

Louro D., Noris E., Veratti F., Accotto G.P., 1996. First report of *Tomato yellow leaf curl virus* in Portugal. *Plant Disease* **80**: 1079.

Maxwell D.P., Martin C., Salus M.S., Montes L., Mejía L., 2006. Breeding tomatoes for resistance to tomato-infecting begomoviruses. International Plant Virology Laboratory,

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University of Wisconsin, Madison. http://www.plantpath.wisc.edu/ GeminivirusResistantTomatoes/index.htm.

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

Navas-Castillo J., Sánchez-Campos S., Díaz J.A., Sáez-Alonso E., Moriones E., 1997. First report of *Tomato yellow leaf curl virus-Is* in Spain: coexistence of two different geminiviruses in the same epidemic outbreak. *Plant Disease* **81**: 1461.

Picó B., Ferriol M., Díez M., Nuez F., 1999. Developing tomato breeding lines resistant to Tomato yellow leaf curl virus. *Plant Breeding* **118**: 537-542.

Picó B., Ferriol M., Díez M., Viñals F.N., 2001. Agroinoculation methods to screen wild *Lycopersicon* for resistance to *Tomato yellow leaf curl virus*. *Journal of Plant Pathology* **83**: 215-220.

Vidavski F., Czosnek H., Gazit S., Levy D., Lapidot M., 2008. Pyramiding of genes conferring resistance to *Tomato yellow leaf curl virus* from different wild tomato species. *Plant Breeding* **127**: 625-631.

Zamir D., Ekstein-Michelson I., Zakay Y., Navot N., Zeidan M., Sarfatti M., Eshed Y., Harel E., Pleben T., Van-Oss H., Jedar N., Rabinowitch H. D., Czosnek H., 1994. Mapping and introgression of a *Tomato yellow leaf curl virus* tolerance gene, *Ty*-1. *Theoretical and Applied Genetics* **88**: 141-146.

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