Instituto de Conservación y Mejora
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Exploitation of *Solanum chilense* and *Solanum peruvianum* in tomato breeding for resistance to Tomato yellow leaf curl disease

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Por fin llegó la recta final…el final de un largo camino, un camino lleno de aprendizaje, el cual creo que merece esta reflexión.

Llegado este momento me planteo, ¿qué ha sido esta tesis para mí? ¿qué he aprendido de todo esto?

Primero de todo quiero decir que para mí esta tesis es algo que va más allá de un título o de una serie de publicaciones. A lo largo de este doctorado he aprendido muchas cosas, algunas de carácter científico, pero también cosas acerca de la vida.

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ABSTRACT

English version

Among viral diseases affecting cultivated tomato, Tomato yellow leaf curl disease (TYLCD) is one of the most devastating. This disease is caused by a complex of viruses of which *Tomato yellow leaf curl virus* (TYLCV) is regarded as the most important species. Current control strategies to fight viral diseases in tomato are mainly based on genetic resistance derived from wild relatives. In the present thesis, resistance derived from *S. chilense* and *S. peruvianum* has been exploited in breeding for resistance to TYLCD. In a previous study, TYLCV-resistant breeding lines derived from LA1932, LA1960 and LA1971 *S. chilense* accessions were developed. Therefore, the first objective of this thesis was to study the genetic control of the resistance derived from these accessions. With this aim, response to viral infection was assayed in segregating generations derived from the aforementioned resistant lines. The results obtained were compatible with a monogenic control of resistance. Resistance levels were higher in LA1960- and LA1971-derived F_2 generations, as shown by slighter symptoms in the resistant plants and a higher number of asymptomatic plants compared with the results obtained in the LA1932-derived F_2 generation. It is noteworthy that the level of resistance present in our materials is comparable to or even higher than the levels found in tomato lines homozygous for *Ty-1*. The response in plants heterozygous for the resistance gene was comparable to the response in homozygous plants for all three sources employed. This implies that the resistance genes derived from all three sources seem to be almost completely dominant. This effect was stronger for LA1971-derived resistance. The results were similar when comparing viral accumulation, as was expected, since a positive correlation was found in these families between viral accumulation and symptom scores. This has important implications in breeding, since the resistance will be used mostly for hybrid development.

Our second objective was to map the loci associated with the major resistance genes identified. A total of 263 markers were screened, 94 of them being polymorphic between both species. Recombinant analysis allowed the resistance loci to be localized on chromosome 6, in a marker interval of 25 cM. This interval includes the *Ty-1/Ty-3* region, where two *S. chilense*-derived TYLCD resistance loci were previously mapped. In order to test if the resistance genes identified in our populations were allelic to *Ty-1* and *Ty-3*, further fine mapping was carried out. A total of 13 additional molecular markers distributed on chromosome 6 allowed 66 recombinants to be identified, and the resistance

region to be shortened to a marker interval of approximately 950 kb, which overlaps with the *Ty-1/Ty-3* region described previously by other authors. Therefore, the results obtained indicate that closely linked genes or alleles of the same gene govern TYLCV resistance in several *S. chilense* accessions.

The third objective of the present thesis was to start the construction of a set of introgression lines (ILs) derived from *Solanum peruvianum* accession PI 126944 into the cultivated tomato genetic background. Once this collection of ILs is developed, it will represent a powerful tool for exploiting the resistance to different pathogens found in this particular accession in addition to other possible characters of interest. The starting plant material consisted of several segregating generations that were derived from two interspecific hybrids previously obtained by our group. Many crosses and embryo rescue were required to obtain subsequent generations due to the high sexual incompatibility that exists between tomato and PI 126944. Several mature fruits from the most advanced generations produced a few viable seeds, although embryo rescue was also employed to obtain progeny. As only a few plants were obtained by direct backcrossing, additional crosses were made in order to increase the number of descendants. A high degree of incompatibility was also found in crosses between sib plants. A total of 263 molecular markers were tested in some generations, 105 being polymorphic between tomato and PI 126944. Available generations were genotyped with these polymorphic markers in order to determine which alleles of *S. peruvianum* were already introgressed. On average, 79, 78 and 84 % of the *S. peruvianum* genome was represented in the pseudo-F_2, pseudo-F_4 and pseudo-F_5 generations, respectively, for the markers analyzed. A reduction in the *S. peruvianum* genome was observed in more advanced generations, such as BC_1 (56 %), pseudo-F_2-BC_1 (60 %) and pseudo-F_3-BC_1 (70 %). A greater reduction was observed in the pseudo-F_3-BC_2 generation (33 %). As a consequence of the reduction in the *S. peruvianum* genome, a loss of incompatibility was observed in some cases. The *S. peruvianum* genome was almost completely represented among the different plants of the most advanced generations. An evaluation for resistance to TYLCD and *Tomato spotted wilt virus* (TSWV) was carried out in some of the advanced generations, some of which were resistant to one or both viruses.

In conclusion, we have conducted a successful and deeper exploitation of two wild species with proved resistance to TYLCD, *S. chilense* and *S. peruvianum*, identifying and fine mapping new genes of resistance.

Versión en Español

De todas las enfermedades virales que afectan al tomate cultivado, la enfermedad del rizado amarillo del tomate (Tomato yellow leaf curl disease, TYLCD) es una de las más devastadoras. Esta enfermedad está causada por un complejo de virus de los cuales la especie *Tomato yellow leaf curl virus* (TYLCV) es considerado la más importante. Las actuales estrategias de control para luchar contra enfermedades virales están basadas principalmente en la resistencia genética derivada de especies silvestres. En la presente tesis, la resistencia derivada de *S. chilense* y *S. peruvianum* ha sido utilizada en mejora para la resistencia a TYLCD. En un estudio anterior, se desarrollaron líneas de mejora resistentes a TYLCV derivadas de las entradas de *S. chilense* LA1932, LA1960 y LA1971. Por tanto, el primer objetivo de esta tesis fue estudiar el control genético de la resistencia derivada de dichas entradas. Con este propósito, se evaluó la respuesta a la infección viral en generaciones segregantes derivadas de las líneas de mejora anteriormente citadas. Los resultados obtenidos fueron compatibles con un control monogénico de la resistencia. Los niveles de resistencia fueron mayores en las generaciones F_2 derivadas de las entradas LA1960 y LA1971. Cabe destacar que el nivel de resistencia presente en nuestros materiales es comparable o incluso mayor que los encontrados en líneas de tomate homocigotas para el gen *Ty-1*. La respuesta de plantas heterocigotas para el gen de resistencia fue comparable a la respuesta de las plantas homocigotas en las tres fuentes empleadas. Esto implica que los genes de resistencia derivados de estas fuentes parecen ser casi completamente dominantes. Este efecto fue más pronunciado en la resistencia derivada de LA1971. Los resultados fueron similares cuando se comparó la acumulación viral, tal y como se esperaba, ya que se encontró una correlación positiva entre acumulación viral y sintomatología en estas familias. Esto tiene importantes implicaciones en mejora, ya que la resistencia se utilizará principalmente para el desarrollo de híbridos.

El segundo objetivo fue mapear los loci asociados con los genes mayores de resistencia identificados. Se probaron un total de 263 marcadores, siendo 94 de ellos polimórficos entre tomate y *S. chilense*. El análisis de los recombinantes permitió localizar

los loci responsables de la resistencia en el cromosoma 6, en un intervalo de 25 cM. Este intervalo incluye la región *Ty-1/Ty-3*, donde habían sido mapeados previamente dos loci de resistencia a TYLCV derivados de distintas entradas *S. chilense*. Para determinar si los genes de resistencia identificados en nuestras poblaciones eran alélicos a *Ty-1* y *Ty-3*, se llevó a cabo posteriormente el mapeo con mayor precisión de los mismos. Un total de 13 marcadores moleculares adicionales distribuidos a lo largo del cromosoma 6 permitió identificar 66 recombinantes y acortar la región de la resistencia a un intervalo de aproximadamente 950 kb, el cual se solapa con la región *Ty-1/Ty-3* descrita previamente por otros autores. Por tanto, los resultados obtenidos indican que la resistencia a TYLCV en varias entradas de *S. chilense* está gobernada por genes estrechamente ligados o alelos del mismo gen.

El tercer objetivo de esta tesis fue el inicio de la construcción de un conjunto de líneas de introgresión (ILs) derivadas de la entrada de *S. peruvianum* PI 126944 en el fondo genético del tomate cultivado. El material vegetal inicial consistió en varias generaciones segregantes derivadas de dos híbridos interespecíficos obtenidos previamente por nuestro grupo. Se necesitaron numerosos cruces y rescate de embriones para obtener las distintas generaciones, debido a la incompatibilidad sexual existente entre el tomate y PI 126944. Fue posible obtener algunas semillas viables a partir de varios frutos maduros de las generaciones más avanzadas, aunque también se utilizó el rescate de embriones para obtener descendencia. Debido a que sólo se obtuvieron unas pocas plantasmediante retrocruce directo, se realizaron cruces adicionales para incrementar el número de descendientes. También se encontró un alto grado de incompatibilidad en cruces entre plantas hermanas. Se probaron un total de 263 marcadores moleculares en algunas generaciones, siendo 105 polimórficos entre tomate y PI 126944. Las generaciones disponibles fueron genotipadas con estos marcadores polimórficos para determinar qué alelos de *S. peruvianum* habían sido ya introgresados. Como media, el 79, 78 y 84% del genoma de *S. peruvianum* estuvo representado en las generaciones pseudo-F_2, pseudo-F_4 y pseudo-F_5, respectivamente, para los marcadores analizados. Se observó una reducción en el genoma de *S. peruvianum* en las generaciones más avanzadas, como BC_1 (56 %), pseudo-F_2-BC_1 (60 %) y pseudo-F_3-BC_1 (70 %). Una reducción todavía mayor fue observada en la generación pseudo-F_3-BC_2 (33 %). Como consecuencia de la reducción en el genoma de *S. peruvianum*, se observó una pérdida de incompatibilidad en algunos casos.

El genoma de *S. peruvianum* estuvo casi totalmente representado entre las distintas plantas de las generaciones más avanzadas. Se llevó a cabo la evaluación de la resistencia a TYLCV y *Tomato spotted wilt virus* (TSWV) en varias generaciones avanzadas, algunas de las cuales fueron resistentes a uno o ambos virus. Una vez que esta colección de ILs sea desarrollada, representará una poderosa herramienta para explotar la resistencia a diferentes patógenos encontrada en esta particular entrada además de otros posibles caracteres de interés.

En conclusión, hemos profundizado en la utilización de dos especies silvestres con demostrada resistencia a TYLCD y otros patógenos, *S. chilense* y *S. peruvianum*, identificando y mapeando de forma precisa nuevos genes de resistencia.

Versió en Valencià

De totes les malalties virals que afecten la tomaca cultivada, la malaltia de l'arrissat groc de la tomaca (Tomato yellow leaf curl disease, TYLCD) és una de les més devastadores. Aquesta malaltia està causada per un complex de virus dels quals l'espècie *Tomato yellow leaf curl virus* (TYLCV) és considerat l'espècie més important. Les actuals estratègies de control per a lluitar contra malalties virals estan basades principalment en la resistència genètica derivada d'espècies silvestres. En la present tesi, la resistència derivada de *S. chilense* i *S. peruvianum* ha sigut utilitzadaen millora per a la resistència a TYLCD. En un estudi anterior, es van desenvolupar línies de millora resistents a TYLCV derivades de les entrades de *S. chilense* LA1932, LA1960 i LA1971. Per tant, el primer objectiu d'aquesta tesi va ser estudiar el control genètic de la resistència derivada d'aquestes entrades. Amb aquest propòsit, es va avaluar la resposta a la infecció viral en generacions segregants derivades de les línies de millora anteriorment citades. Els resultats obtinguts van ser compatibles amb un control monogènic de la resistència . Els nivells de resistència foren majors en les generacions F_2 derivades de les entrades LA1960 i LA1971. Cal destacar que el nivell de resistència present en els nostres materials és comparable o fins i tot major que els trobats en línies de tomaca homozigotes pel gen *Ty-1*. La resposta de plantes heterozigotes pel gen de resistència va ser comparable a la resposta de les plantes homozigotes en les tres fonts emprades. Això implica que els gens de resistència derivats d'aquestes fonts semblen ser quasi completament dominants. Aquest efecte va ser més pronunciat en la resistència derivada de LA1971. Els resultats foren similars quan es va

comparar l'acumulació viral, tal com s'esperava, ja que es va trobar una correlació positiva entre acumulació viral i simptomatologia en aquestes famílies. Això té importants implicacions en millora, ja que la resistència s'utilitzarà principalment pel desenvolupament d'híbrids.

El segon objectiu va ser mapejar els loci associats amb els gens majors de resistència identificats. Es van provar un total de 263 marcadors, sent 94 d'ells polimòrfics entre la tomaca i *S. chilense*. L'anàlisi dels recombinants va permetre localitzar els loci responsables de la resistència al cromosoma 6, en un interval de 25 cM. Aquest interval inclou la regió *Ty-1/Ty-3*, on havien estat mapejats prèviament dos loci de resistència a TYLCV derivats de diferents entrades *S. chilense*. Per determinar si els gens de resistència identificats en les nostres poblacions eren al·lèlics a *Ty-1* i *Ty-3*, es va dur a terme posteriorment el mapatge amb major precisió dels mateixos. Un total de 13 marcadors moleculars addicionals distribuïts al llarg del cromosoma 6 va permetre identificar 66 recombinants i acurtar la regió de la resistència a un interval d'aproximadament 950 kb, el qual se solapa amb la regió *Ty-1/Ty-3* descrita prèviament per altres autors. Per tant, els resultats obtinguts indiquen que la resistència a TYLCV en diverses entrades de *S. chilense* està governada per gens estretament lligats o al·lels del mateix gen.

El tercer objectiu d'aquesta tesi va ser l'inici de la construcció d'un conjunt de línies d'introgressió (ILS) derivades de l'entrada de *S. peruvianum* PI 126944 al fons genètic de la tomaca cultivada. El material vegetal inicial va consistir en diverses generacions segregants derivades de dos híbrids interespecífics obtinguts prèviament pel nostre grup. Es van necessitar nombrosos encreuaments i rescat d'embrions per obtenir les diferents generacions, a causa de la incompatibilitat sexual existent entre la tomaca i PI 126944. Va ser possible obtenir algunes llavors viables a partir de diversos fruits madurs de les generacions més avançades, tot i que també es va utilitzar el rescat d'embrions per obtenir descendència. Com que només es van obtenir unes poques plantasmediante retroencreuament directe, es realitzaren creus addicionals per incrementar el nombre de descendents. També es va trobar un alt grau d'incompatibilitat en encreuaments entre plantes germanes. Es van provar un total de 263 marcadors moleculars en algunes generacions, sent 105 polimòrfics entre la tomaca i PI 126944. Les generacions disponibles van ser genotipades amb aquests marcadors polimòrfics per a veure què al·lels de *S. peruvianum* havien estat ja introgresats. Com a mitja, el 79, 78 i 84 % del genoma de *S.*

peruvianum va estar representat en les generacions pseudo-F_2, pseudo-F_4 i pseudo-F_5, respectivament, per als marcadors analitzats. Es va observar una reducció en el genoma de *S. peruvianum* a les generacions més avançades, com BC_1 (56%), pseudo-F_2-BC_1 (60%) i pseudo-F_3-BC_1 (70%). Una reducció encara més gran va ser observada en la generació pseudo-F_3-BC_2 (33%). Com a conseqüència de la reducció en el genoma de *S. peruvianum*, s'observà una pèrdua d'incompatibilitat en alguns casos. El genoma de *S. peruvianum* estigué quasi totalment representat entre les diferents plantes de les generacions més avançades. Es va dur a terme l'avaluació de la resistència a TYLCV i *Tomato spotted wilt virus* (TSWV) en diverses generacions avançades, algunes de les quals van ser resistents a un o als dos virus. Una vegada que aquesta col·lecció d'ILs sigui desenvolupada, representarà una poderosa eina per explotar la resistència a diferents patògens trobada en aquesta particular entrada a més d'altres possibles caràcters d'interès.

En conclusió, hem aprofundit en la utilització de dues espècies silvestres amb demostrada resistència a TYLCD i altres patògens, *S. chilense* i *S. peruvianum*, identificant i mapejant de forma precisa nous gens de resistència.

INTRODUCTION

1. Tomato taxonomy and related species

The location of tomato in the Solanaceae family has always been clear but its genus has not. Different and controversial classifications have been made based on morphological and biological concepts.

In 1694, Tournefort differentiated the *Lycopersicum* and *Solanum* genera. However, Linnaeus included *Lycopersicum* within *Solanum* in 1753, which resulted in cultivated tomato being called *Solanum lycopersicum*. One year later, both genera were separated again and Miller formally defined the cultivated tomato as *Lycopersicum esculentum*, based on differences in leaves and anther dehiscence.

In 1979, Rick made a classification based on biological concepts: the species were grouped according to their crossability with cultivated tomato. Nine species of *Lycopersicum* were identified and classified into two complexes: Esculentum and Peruvianum. The Esculentum complex comprised seven species (*L. esculentum*, *L. pimpinellifolium*, *L. cheesmaniae*, *L. pennellii*, *L. hirsutum*, *L. chmielewskii* and *L. parviflorum*), which are self-compatible and easily crossed with the cultivated tomato. The Peruvianum complex included the self-incompatible *L. chilense* and *L. peruvianum,* which were less compatible with cultivated tomato species.

In 1990, a new change was proposed: Child included tomatoes in the genus *Solanum* based on morphological characters. Further molecular studies have supported this classification, so placement of tomatoes within *Solanum* was also adopted by Peralta and Spooner (2000).

Peralta et al. (2008), summarising morphological and molecular studies, proposed a classification of tomatoes in the genus *Solanum* and recognized 17 species (Table 1). These species were divided into three sections: *Lycopersicon*, *Lycopersicoides* and *Juglandifolium*. Section *Lycopersicon* is divided into four groups: *Lycopersicon*, *Neolycopersicon*, *Eriopersicon* and *Arcanum*. *Solanum lycopersicum* is the only cultivated species and is included in the *Lycopersicon* group.

Table 1. Classification of tomato and related species according to Peralta et al. (2008)

Section	Group	Species
Lycopersicon	*Lycopersicon*	*S. cheesmaniae* (L. Riley) Fosberg *S. galapagense* S.Darwin & M.I. Peralta *S. lycopersicum* L. *S. pimpinellifolium* L.
	Neolycopersicon	*S. pennellii* Correll
	Eryopersicon	*S. chilense* (Dunal) Reich *S. corneliomuelleri* J.F. Macbr. *S. habrochaites* S. Knapp & D.M. Spooner *S. huaylasense* Peralta & S. Knapp *S. peruvianum* L.
	Arcanum	*S. arcanum* Peralta *S. chmielewskii* (C.M. Rick, Keisicki, Forbes & M.Holle) D.M. Spooner, G.J. Anderson & R.K. Jansen *S. neorickii* D.M. Spooner, G.J. Anderson & R.K. Jansen
Lycopersicoides		*S. lycopersicoides* Dunal *S. sitiens* I.M. Johnst
Juglandifolium		*S. juglandifolium* Dunal *S. ochrantum* Dunal

All tomatoes, including the cultivated species, are diploid (2n = 2x = 24) and have similar chromosome number and structure. Wild tomatoes are native to western South America and are distributed throughout Ecuador, Peru, northern Chile and the Galapagos Islands (Darwin et al. 2003). The wild ancestor of cultivated tomato, *S. lycopersicum* var. *cerasiforme*, is more widespread in other South American countries and all over the world (Rick 1976, 1978). Cultivated tomato has a narrow genetic base because of its domestication, so wild species are a useful source of genetic resources. The species with the greatest variability are *S. chilense*, *S. harochaites*, *S. peruvianum* and *S. pennellii,* whereas the least variable species are *S. cheesmanii* and *S. pimpinellifolium*.

All species within section *Lycopersicon* have hermaphrodite flowers and a complete range of mating systems. *S. cheesmanii* and *S. parviflorum* are autogamous, while most *S. chilense*, *S. habrochaites*, *S. peruvianum* and *S. pennellii* accessions are obligately outcrossed and self-incompatible. There are also self-compatible species with

various degrees of facultative outcrossing, like *S. chmielewskii*, *S. lycopersicum* and *S. pimpinellifolium* (Rick 1987).

Interspecific hybridization allows the exploitation of these wild species in tomato breeding. However, incompatibility barriers between some of them need to be overcome using special techniques, such as embryo rescue (Smith 1944).

Figure 1. Crossability relationships among tomato and its wild relatives

◼ Compatible crosses ◼ Compatible crosses with a lesser degree of crossability

◻ Crosses that can be retrieved by using special techniques ☐ Cross failures

(it must be taken into account that there are exceptions to this figure, such as cases of unilateral incompatibility or differences in crossability between accessions of the same species)

Crosses between cultivated tomato and species of section *Lycopersicon* are possible, although differences in crossability exist (Figure 1). Crosses between *S. lycopersicum*, *S. cheesmanii* and *S. pimpinellifolium* are the most compatible. Also, crosses between these species and *S. chmielewskii*, *S. pennellii*, *S. neorickii* or *S. habrochaites* are compatible but with a lower degree of crossability. *S. chilense* and *S. peruvianum* are the most incompatible species within the *Lycopersicon* section. Crosses between these two species and some species of this section, such as *S. lycopersicum*, *S. cheesmaniae* and *S. pimpinellifolium*, are possible using embryo rescue. However, hybridization fails when

they are crossed with other species such as *S. neorickii, S. habrochaites* or *S. chmielewskii* (Díez and Nuez 2008, Figure 1). For some species, like *S. peruvianum*, there are differences in incompatibility, which also depend on the accession considered; embryo rescue is required for most accessions, but it is not necessary in other cases. However, even in these favourable situations, the number of viable seeds in fruits is very low (Fulton et al. 1997a). In any case, crosses with species of sections *Lycopersicoides* and *Juglandifolium* are the most incompatible, as they all present strong crossability barriers (Peralta and Spooner 2005).

2. Exploitation of wild species in tomato breeding

Tomato breeding started in the same domestication process with the selection of the most useful genotypes among the existing germplasm. Initially, the hybridization of preexisting tomato types and further selection gave rise to new types that were adapted to different uses and environments. Evidence seems to indicate that no introgression from wild relatives took place before 1940 (Bai and Lindhout 2007). It was Dr. Rick who first observed the novel genetic variation revealed in the offspring of crosses between wild and cultivated species. Since then, wild relatives have been an important source of favourable traits introgressed into the cultivated tomato (Bai and Lindhout 2007).

2.1. Needs and breeding goals

Tomato breeding is basically focused on allowing the high-yield production of high-quality fruits while keeping costs as low as possible (Bai and Lindhout 2007). Despite these common objectives, breeding goals vary depending on the specific market and use, and have evolved over time.

One of the most important objectives in the 1970s was yield increase. The substitution of open-pollinated cultivars by hybrids allowed the exploitation of heterosis, with the consequent increase not only affecting productivity, but also uniformity. Nowadays, most of the varieties of fresh-market tomatoes, and an increasing number in the case of tomatoes for processing, are hybrids. In any case, yield increase is still a current breeding objective, currently developed by improving potential production through resistance or tolerance to biotic and abiotic stresses (Díez and Nuez 2008).

During the 1980s, one of the most important breeding objectives, especially for fresh-market tomatoes, was shelf life. Two genes derived from the cultivated species, *non-ripening* (*nor*) and *ripening inhibitor* (*rin*), were mainly used to obtain cultivars with long shelf-life.

Whereas quality has been one of the main breeding objectives for tomato for processing, this was not the case for tomato for fresh consumption. Moreover, both breeding for yield increase and breeding for long shelf-life positively affected external quality, even though they produced negative effects on internal fruit quality.

During the following decade, taste became the main breeding objective. Sugars, acids and more than 30 volatile compounds influence tomato flavour (Tieman et al. 2006). As a result, organoleptic quality is a complex trait and also relative, given that it depends on the market; however, significant improvement in tomato flavour is possible by increasing the sugar and acid contents and by modifying the balance between the two (Stevens et al. 1977).

In any case, the objectives regarding internal fruit quality have shifted to nutritional quality. Increasing health-related compounds is one of the current breeding objectives, given that certain consumer sectors are willing to pay higher prices for these products. As an example, this is the case of high-lycopene varieties. The antioxidant power of lycopene was long ago proven to be effective in reducing the risk of some types of cancer (Giovannucci 1999), along with other diseases. High-lycopene tomatoes for fresh consumption are widespread in several markets.

As previously stated, breeding for resistance or tolerance to biotic and abiotic stresses is one of the most important goals. This breeding objective contributes to increasing both yield and quality. On one hand, pest and pathogens cause important economic losses in tomato cultivation. Even though some can be controlled using chemical compounds, breeding for resistance is the most environmentally friendly and best long-term strategy. Wild relatives have been widely exploited for introgression genes against different diseases. Current tomato cultivars carry several wild-derived resistance genes (Díez and Nuez 2008). However, breeding for resistance to biotic stresses is still an important objective. The resistance conferred by available genes has in some cases been overcome by new races of the concrete pathogen. Moreover, for some pests and diseases, no resistance genes have been identified or the identified resistance has a complex

inheritance. On the other hand, breeding for abiotic stresses is an area that has not yielded important advances. The most important objectives include breeding varieties adapted to salinity and drought conditions, in addition to extending the thermal range appropriate for tomato cultivation. Variability sources are available from different wild tomato relatives, given that variability of habitats exists among different species. However, the complex genetic control of these traits makes their introgression into the cultivated species background quite difficult.

Biotechnological advances during the last few decades have provided breeders with important tools that can be incorporated into breeding programmes. The possibility of introducing genes from other organisms using transformation technologies is one of them. Even though transgenic tomatoes are routinely generated for research purposes, the release of transgenic tomato varieties has ceased due to the concerns of consumers. The development of DNA-based molecular markers has been another important tool that has contributed to increasing the efficiency of breeding programmes by marker-assisted selection. More recently, advances in genomics have led to a revolution in plant breeding, facilitating diversity studies, map construction, identification of candidate genes and the dissection of complex traits, among others (Pérez de Castro et al. 2012). All these tools will contribute to a better and easier exploitation of traits available in wild tomato relatives for breeding purposes.

2.2. Use of wild species in breeding

As stated before, the cultivated tomato has a narrow genetic basis as a consequence of its domestication process (Williams and St. Clair 1993). This is why tomato wild relatives have been extensively used in tomato breeding. In particular, they have been a valuable source of resistance genes to diseases and to characteristics related to fruit quality. Although some wild species inhabit saline, desert or cold areas, their exploitation for breeding for abiotic stresses has been considerably lower due to the complex genetic control of tolerance.

Regarding resistance to diseases, the main sources of resistance genes introgressed in commercial cultivars today are *S. pimpinellifolium, S. habrochaites, S. peruvianum* and to a lesser extent, *S. chilense* (Foolad 2007). This last species has been a valuable source of resistance genes to viruses, while the others have provided resistance genes to different

pathogens. Examples of the many genes introgressed into the cultivated species include the genes *Tm1* (Pelham 1966), identified in *S. habrochaites*, and *Tm2* (Laterrot and Pecaut, 1969) and *Tm2²* (Hall 1980), identified in *S. peruvianum*, which confer resistance to the *Tomato mosaic virus* (ToMV). These genes have been used commercially for more than 40 years and have remained effective until the present. Resistance to virus-transmitting vectors has been identified in *S. habrochaites, S. pennellii* and *S. pimpinellifolium*. However, its exploitation has not given rise to its commercial use (Lawson et al. 1997; Momotaz et al. 2005) due to the complicated genetic control, practical difficulties of phenotyping assays and linkage drag.

S. pennellii, S cheesmaniae and *S. pimpinellifolium* have been the most exploited species in the study of salinity tolerance (Cuartero and Fernández-Muñoz 1999). This has been by far the most studied abiotic stress, although it has not produced important practical results. This is because plant response to salinity, and in general to abiotic stresses, is modulated by many physiological and agronomical characteristics, which may be controlled by many genes. Stress tolerance is a developmentally regulated, stage-specific phenomenon; tolerance at one plant development stage is often not correlated with tolerance at another developmental stage. This complicates the process of breeding for abiotic stresses enormously. Many Quantitative Trait Loci (QTLs) have been identified, but the difficulties have prevented their introgression into commercial varieties. *S. pimpinellifolium* has also been studied for its tolerance to drought and cold, but it has produced even fewer practical results.

Regarding fruit quality, many QTLs related to different aspects of quality, like soluble solids, viscosity, colour, flavour and aroma have been identified, mapped and transferred into elite cultivars. *S. pimpinellifolium*, *S. pennellii*, *S. cheesmaniae* and *S. habrochaites* have been the most exploited species (Grajera et al. 2006; Roselló and Nuez 2006).

As stated above, *S. peruvianum* and *S. chilense* have been particularly exploited as sources of resistance genes to diseases. In fact, these species are considered the most variable tomato wild relatives with a great potential for crop improvement (Foolad 2007). Among the resistance genes identified and introgressed from these species are resistance genes to viruses (the *Sw-5* gene, which confers resistance to *Tomato spotted wilt virus*, TSWV (Stevens et al. 1992), and the *Tm-1*, *Tm-2*, and *Tm-2²* genes of resistance to *Tomato*

mosaic virus, ToMV), fungus (gene *Frl*, which confers resistance to *Fusarium oxysporum* f.sp. *radicis lycopersici* (Vakalounakis et al. 1997) and the recessive gene *py-1,* resistant to *Pyrenochaetam lycopersici*), nematodes (various *Mi* genes that confer resistance to some species of *Meloidogine*) and bacteria (several QTLs to *Clavibacter michiganensis* subsp. *michiganensis*). Within *S. chilense,* there are remarkably high levels of resistance to begomovirus, such as *Tomato yellow leaf curl virus*, TYLCV, and *Tomato mottle virus*, ToMoV (Zamir et al. 1994; Griffiths and Scott 2001; Ji et al. 2007a). The *Ty-1* and *Ty-3* genes have been identified in this species, the former of which has been widely used as a resistance source for TYLCV in breeding programmes all over the world. Other resistance genes that have also been introgressed from this species are the *Cmr* gene, conferring resistance to *Cucumber mosaic virus,* CMV (Stamova and Chetelat 2000), the *Sw-7* gene, which confers resistance to TSWV isolates that overcome the resistance conferred by the *Sw-5* gene, and the *Lv* gene, which confers resistance to the fungus *Leveillula taurica* (Stamova and Yordanov 1990). However, their self-incompatibility and the important crossability barriers between these two species and the cultivated tomato have conditioned their utilization. In fact, no breeding populations have been developed from *S. chilense,* and only a third backcross population has been constructed from *S. peruvianum*. No mapping populations have been constructed from *S. chilense* and tomato and a only few genes have been fine mapped and cloned, in contrast with the larger number of genes identified and cloned in other wild relatives (Foolad 2007). Until recently, the exploitation of these species has been done through backcross programmes for introgressing particular genes of interest.

More recently, the construction of prebreed populations and the availability of thousands of molecular markers have allowed the identification of hundreds of QTLs for a large number of characteristics related to vegetative traits, fruit, flower, etc., in all wild relatives. Despite the fact that marker-assisted selection is already being employed massively for qualitative traits, we are in a transitory phase and the advances and progress in the utilization of MAS for quantitative traits will allow a deeper exploitation of wild tomato relatives.

3. Prebreed populations

The exploitation of wild tomato relatives has traditionally been carried out through the initial development of the interspecific hybrid and subsequent backcrossing to the cultivated species. This approach is costly in both time and money. The construction of a prebreed population makes use of the effort made during this process. The development of these populations starts with the initial cross between a donor parent (the wild relative accession of interest) and a recurrent parent (the cultivated tomato accession). There are different types of prebreed populations depending on the crosses developed. They all have in common the fact that consist of a set of lines, each of which contains a fraction of the wild species genome in the cultivated species genetic background. Obtaining these populations is also a long process. However, they present many advantages that justify their development. On the one hand, these populations can be incorporated directly into breeding programmes, since they have a high proportion of the genetic background of the cultivated species. They also allow genome dissection, facilitating gene and QTL mapping as well as minor gene detection.

3.1. Types and description

The populations initially used for mapping in self-pollinated crops were F_2/F_3 or backcrosses. These early populations can be obtained easily. However, they have several limitations as regards the accurate identification and fine mapping of QTLs. These limitations include their low resolution power, the failure to identify QTLs with small effects and the possibility of interactions between two unlinked QTLs, which reduces the difference between the subgroups of the tested QTL. Additionally, these populations are not immediately applicable to breeding purposes, as each plant possesses a large fraction of the wild species genome. To avoid these problems, other types of populations have been derived, such as recombinant inbred lines (RILs), backcross recombinant inbred lines (BCRILs, Ramsay et al. 1996), also known as backcross inbred lines (BILs, Foolad 2007) and introgression lines (ILs, Eshed and Zamir 1995).

The more advanced prebreed populations (RILs, BCRILs and ILs) are described below:

Recombinant inbred lines, RILs

These populations are obtained through successive selfings of the initial interspecific cross until a set of homozygous plants is obtained (Figure 2). Each of the plants differs from the others in the combination of alleles. These sets of plants are self-perpetuating, which makes it possible to replicate experiments in different environments. One disadvantage of RILs is the fact that each plant includes a high proportion of the wild genome, which can cause fertility problems and make the evaluation of some traits related to yield or fruit quality difficult. Moreover, the development of RILs is not always possible, given that for some crosses the selfing of the interspecific hybrid and the first generations is not possible. In those cases, a pseudo-F_2 can be obtained by crossing two F_1 plants.
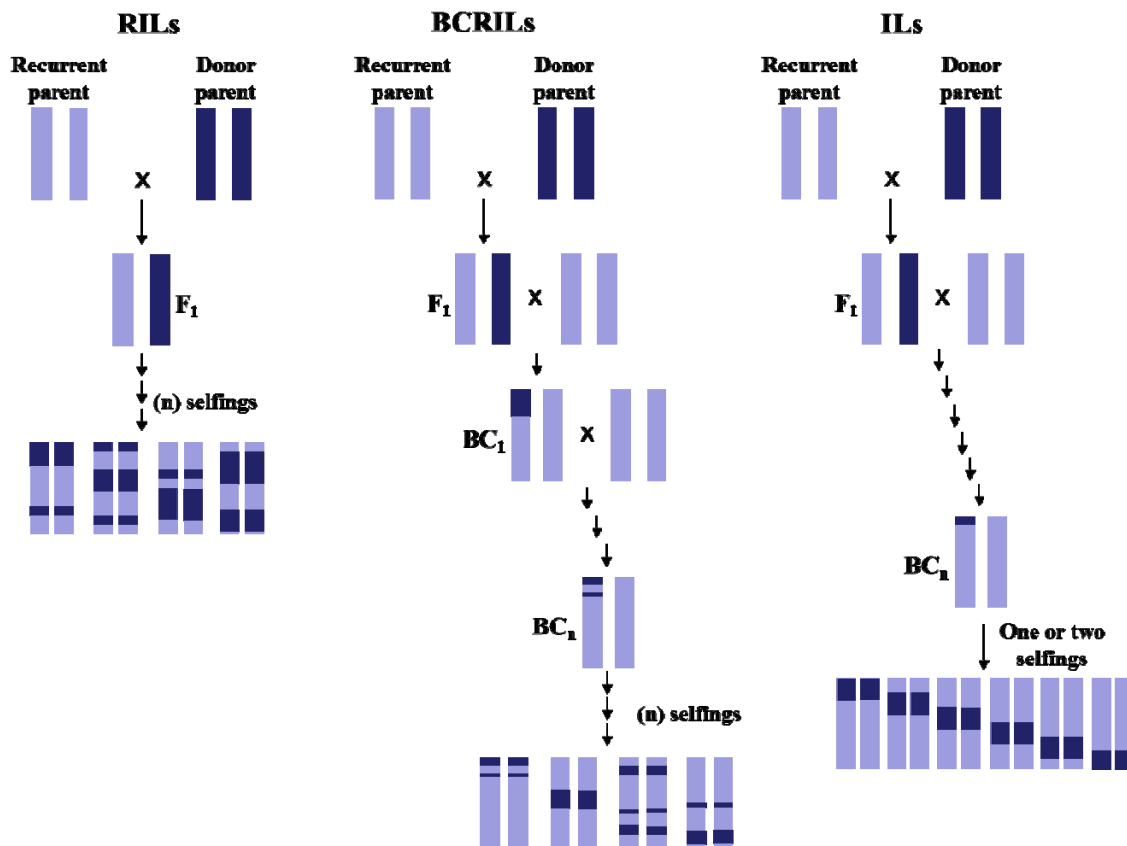
Figure 2. Diagram representing the development of Recombinant inbred lines (RILs), Backcross recombinant inbred lines (BCRILs) and Introgression lines (ILs) populations.

Backcross recombinant inbred lines, BCRILs

Starting with the interspecific hybrid, BCRILs are obtained by subsequent and repeated backcrosses and selfings. After several generations of backcrossing, the plant set obtained has the whole genome of the donor parent divided into small fragments in the cultivated species' genetic background. Moreover, the successive selfings have increasing homozygosity levels (Figure 2). Indeed, this kind of populations are more stable than the F_2/F_3 or backcrosses, presenting a repeatability similar to that of RILs. In addition, they have more uniform genetic backgrounds for trait evaluation, since they have a much smaller genome contribution from the wild parent. Therefore, with good genome coverage, they are efficient for gene mapping.

Introgression Lines, ILs

ILs consist of a set of lines, each of which contains only one fragment of the wild species genome in the cultivated species genetic background in such a way that, as a whole, all the wild species genome is represented in the introgressed fragments. These populations are more advanced than BCRILs since they have more backcross generations. Therefore, each line technically contains only a single fragment from the donor parent, and also the introgressed fragments are smaller than in BCRILs (Figure 2).

ILs are near isogenic lines (NILs) with respect to their recurrent parent, given that they differ only in a very small region of the genome. For that reason, phenotypic differences among each line and the recurrent parent can only be due to the introgressed fragment. In addition, these populations are highly repeatable since they are homozygous. Another advantage of ILs is the elimination of possible interference between genes or QTLs, as a consequence of the dissection of the donor parent genome into very small fragments. However, this also represents a disadvantage, as interactions cannot be detected. In these cases, crosses between ILs that contain the putative interacting QTLs would allow the study of the interaction in the progeny. All these characteristics make ILs a powerful tool for gene mapping, superior to the other types of populations. The main disadvantage is the long time required for their development.

3.2. Prebreed populations developed with wild tomato relatives

Different prebreed populations have been developed from different wild tomato relatives. Populations derived from *S. pennellii, S. pimpinellifolium* and *S. habrochaites* have been used most extensively.

The self-compatible *S. pennellii* accession, LA716, was used to develop a population of 50 ILs with 100% coverage of the wild genome (Eshed and Zamir 1995). A population of 500 subILs was generated from these ILs, which has allowed an increase of the mapping resolution. This population has been employed by many authors to study characters such as fruit weight (Alpert and Tanksley 1996; Frary et al. 2000) and colour (Ronen et al. 2000). A total of 2795 QTLs have been identified using this set of ILs (Lippman et al. 2007). Transgressive segregation has been reported in this population, manifested in characters such as fruit weight, shape and colour (Eshed and Zamir 1995; Ronen et al. 2000). In addition, via phenomic studies, the heterosis trait was dissected by crossing all ILs with the recurrent parent and localizing the genomic regions related to hybrid vigour (Semel et al. 2006). Another study carried out with this population was a large-scale association study aimed at the dissection of traits related to plant architecture and fruit metabolism (Schauer et al. 2006).

In the case of *S. pimpinellifolium,* a population of 196 BCRILs was obtained from accession LA1589; 100 lines were enough to represent the whole genome from the wild parent. This population allowed identifying several QTLs related to fruit and yield characters. Moreover, the wild allele represented an improvement over the cultivated species in approximately half of the characters analized (Doganlar et al. 2002). More recently, a population of RILs was developed from *S. pimpinellifolium* LA2093. This population was derived from F_2 plants selected for their resistance to diseases, to abiotic stresses and for their high lycopene content. A mid-density genetic map was also constructed using this population (Ashrafi et al. 2009).

With *S. habrochaites* LA1777, more than one hundred QTLs were identified from a BC_3 population. For some of the QTLs identified, the wild allele represented an improvement of the character despite the phenotypic inferiority of the wild parent (Bernachi et al. 1998). From these BC_3s, a set of ILs and BCRILs that covered 85% of the wild species genome was developed (Monforte and Tanksley 2000). Similarly, accession LA407 from this same species was employed to generate a population of BCRILs. This

accession had been described as resistant to *Clavibacter michiganensis* subsp. *michiganensis,* and the developed generations allowed the genetic control of the resistance to be determined. This, combined with the fact that most of the genome belongs to the cultivated species, makes this material directly usable in breeding for resistance to this disease (Francis et al. 2001).

Crossability barriers hamper the development of prebreed populations derived from other wild species. Even so, some progress has been achieved.

S. lycopersicoides is the most distant tomato relative used to develop prebreed populations, and therefore the one that presents the strongest crossability barriers. Due to unilateral incompatibility, *S. lycopersicoides* has to be the pollen donor. In addition, hybrids generally present low viability and sterility, as well as reduced recombination in posterior generations. In spite of these limitations, the development of an IL population from this species was achieved, covering up to 96% of the wild parent genome (Canady et al. 2005).

S. peruvianum is characterised as self-incompatible and also presents unilateral incompatibility that prevents its use as the female parent in crosses with tomato (Hogenboom 1972). However, it is more closely related to tomato than *S. lycopersicoides*. In any case, no advanced prebreed populations derived from this species have been developed. However, some other populations, such as BC_3 and BC_4, have, in fact, been obtained (Fulton et al. 1997a, 1997b). These populations were developed from *S. peruvianum* LA1706. From the first backcross, only a few seeds were viable due to the persistence of the incompatibility barriers. However, despite the distance from cultivated tomato, the percentage of recombination was higher than expected, which indicates similarity between homologue chromosomes (Fulton et al. 1997a).

With the BC_3, a linkage map was constructed and the 67% of the *S. peruvianum* genome was represented. Representation of the *S. peruvianum* genome was not achieved in those regions related to self-incompatibility, determinate growth habit or hybrid sterility, due to the selection against these characters. Other regions were randomly lost because BC_1 was composed of only a few plants (Fulton et al. 1997a). More than one hundred QTLs were detected using BC_4 generations for different traits such as yield, fruit weight or quality. For several characters, such as soluble solid content, yield, viscosity and fruit

weight, there was at least one QTL for which the wild allele represented an agronomic improvement over the allele from the cultivated tomato (Fulton et al. 1997b).

Before the development of these populations, *S. peruvianum* had only been used to introgress disease resistance genes. The study by Fulton et al. (1997b) was the first to be used for mapping quantitative genes. However, this study indicates that genes for improving agronomical traits can be found in this species even when positive effects would not be predicted by the parental phenotype. In this sense, the development of advanced prebreed populations from *S. peruvianum* could uncover this hidden variability and permit its use directly in tomato breeding. In addition, most of the QTLs found by Fulton et al. (1997b) were not allelic to those found in previous studies, which shows the large potential of this species for combining alleles with QTLs detected in other species. The availability of advanced prebreed populations would allow the screening for new genes/alleles, such as resistance genes, leading to their relatively easy combination with other sources and their subsequent introgression into elite varieties.

4. Tomato yellow leaf curl disease (TYLCD)

4.1 History

Tomato yellow leaf curl disease (TYLCD) is one of the most devastating diseases affecting cultivated tomato. This disease causes production losses of up to 100% in tropical and subtropical regions of the world (Cohen and Lapidot 2007). All the viral species causing TYLCD belong to the family *Geminiviridae*. The main viral species associated with this disease is *Tomato yellow leaf curl virus* (TYLCV), a monopartite begomovirus, transmitted by the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Czosnek 2008).

This disease was first described in Israel in 1939, associated with outbreaks of *B. tabaci*. Twenty years later, there was a heavy outbreak of *B. tabaci* in the Jordan Valley and the disease spread through every region of Israel, becoming the major limiting factor in tomato production (Cohen and Antignus 1994). Since then, it has had significant economic repercussions in many Middle Eastern, African and south-east Asian countries. It was later reported in the Mediterranean region, America and the Far East (Czosnek and Laterrot 1997). More recently, the disease has also been detected in California (Rojas et al. 2007), Hawaii (Melzer et al. 2010) and Australia (Brunschot et al. 2010).

In the last few years, whiteflies have expanded their range from tropical and subtropical regions to more temperate ones. This change has resulted in the rising economic impact of begomoviruses worldwide (Hanssen et al. 2010).

4.2. Virus biology

There are 11 species of geminiviruses currently described as causal agents of TYLCD (Fauquet et al. 2008). At least four of these species are associated with epidemics in the Mediterranean basin: *Tomato yellow leaf curl virus* (TYLCV), *Tomato yellow leaf curl Sardinia virus* (TYLCSV) and the recombinant species *Tomato yellow leaf curl Malaga virus* (TYLCMalV) and *Tomato yellow leaf curl Axarquia virus* (TYLCAxV) (Monci et al. 2002; García-Andrés et al. 2006).

Geminiviruses are a diverse family of plant-infecting viruses characterised by having a circular single-stranded DNA (ssDNA) genome covered by geminate particles which consist of two incomplete icosahedral virions. These viruses are transmitted by insects in field conditions. The insect vector, the genome organisation and the host range of the virus determine their classification in four genera: *Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus* (Fauquet and Stanley 2005).

Table 2. Proteins coded by the virus and their associated function

Protein (ORF)	Function
CP (ORF V1)	Genome encapsidation
	Virus movement
	Vector recognition
Precapside (ORF V2)	Symptom expression
	Virus movement
Rep (ORF C1)	Virus replication
TrAP (ORF C2)	Activation and transcription of V1 and V2
REn (ORF C3)	Interacts with C1 promoting viral accumulation
C4 (ORF C4)	Symptom expression
	Virus movement

Species belonging to the *Begomovirus* genus infect dicotyledonous plants, and most of them have a bipartite genome. In contrast, there are some species, such as the viruses

associated with TYLCD, that mostly have monopartite genomes. These species genome consists of a circular ssDNA of almost 2.8 Kb (Rochester et al. 1994). This molecule codes for six proteins (Table 2) with partially overlapping Open reading frames (ORFs) that are bidirectionally organised: two in viral sense (V1 and V2) and four in anti-sense (C1-C4). These ORFs are separated by a non-coding intergenic region of about 300 nucleotides. This region contains key elements for replication and transcription of the viral genome (Glick et al. 2009). In contrast, bipartite begomoviruses are the prevalent type causing TYLCD in the Western hemisphere.

4.3 Symptoms

The most typical symptoms of the disease are leaf curling and yellowing, although infected plants can also present a reduction of leaf area and rounded and thickened leaflets with marginal chlorosis. The leaf blade can be reduced until it almost disappears, leaving only a curved main leaf nerve. Other symptoms include severe stunting of the plant growth, erect shoots and abscission of flowers and fruits. Fruits set can be smaller and have a paler colour, reaching production losses of up to 100% in early infections (Picó et al. 1996; Cohen and Lapidot 2007).

In natural infections, the moment of symptom appearance and the kind of symptom developed depend on several factors, such as virus isolate, genetic background, environmental conditions and physiological state of the plant (Jordá 1995).

4. 4 Transmission

Viral species from the TYLCV complex are transmitted by the aleyrodid *B. tabaci*. *Trialeurodes vaporariorum* and *Myzus persicae* can acquire the virus in a non-specific manner, but they are not able to transmit it (Antignus et al. 1994).

B. tabaci is a vector of many plant viruses (Brown and Czosnek 2002). Its good efficiency for viral spread is due to its high rate of reproduction, its ability to disperse, and its obligate use of particular plants (Glick et al. 2009). Different biotypes of *B. tabaci* have been described, differing in their geographical distribution and in their biological and genetic characteristics. In Spain, the *B. tabaci* populations are composed mainly by biotype B, world-wide distributed, and biotype Q, specific from Spain and Portugal. These local populations differ in their ability to transmit the disease, being the biotype Q the most

efficient (Sánchez Campos et al. 1999). More recent studies state that the transmission efficiency of TYLCV by the whitefly is correlated with the presence of the specific symbiotic bacteria *Hamiltonella*. The GroEL protein produced by this bacteria (present in the B biotype, but absent in the Q biotype) facilitates TYLCV transmission (Gottlieb et al. 2010). On the other hand, it has been recently proposed to leave the typical *B. tabaci* classification in biotypes. According the new classification, biotypes B and Q belong to the same genetic group referred as Africa/Middle East/Asia Minor (De Barro et al. 2011).

Adults and first instar crawlers are the only stages during which *B. tabaci* is able to acquire and transmit TYLCV (Cohen and Nitzany 1966; Mehta et al. 1994). The insect acquires the virus while feeding and transmits it in a circulative and persistent manner (Cohen and Nitzany 1966). The virus travels from the digestive system to the hemolymph where it accumulates and is inoculated in the floema via salivary glands during feeding (Jiang et al. 2000; Czosnek et al. 2001). The acquisition and inoculation period are approximately 15 to 30 minutes each. The rate of transmission increases with longer acquisition and inoculation periods. The latent period is about 17 to 20 hours and infectivity in adults lasts for 7 to 20 days (Mehta et al. 1994). Nymphs are more effective than adults in virus acquisition, though they do not act in virus dispersion. Moreover, transmission efficiency is higher in females than in males (Cohen and Nitzany 1966; Caciagli et al. 1995).

Various studies have indicated that the virus can be transmitted sexually between insects, as well as to descendants for at least two generations, retaining its infectivity (Ghanim et al. 1998; Ghanim and Czosnek, 2000). However, it has been proven that the species (*Tomato yellow leaf curl Sardinia virus*, TYLCSV) is transmitted to progeny, but loses its infectivity (Bosco et al. 2004). In addition, the virus reduces the fecundity and longevity of the insects (Rubinstein and Czosnek 1997).

The virus can also be transmitted by graft, but transmission by seeds or soil has not been described (Cohen and Nitzany 1966; Makkouk et al. 1979; Credi et al. 1989). Moreover, the virus can be experimentally transmitted by agroinoculation or mechanical inoculation (Makkouk et al. 1979; Abdel-Sahun 1990).

Since *B. tabaci* currently constitutes an important vector in virus transmission with a significant agricultural impact, a functional genomics project was recently established. The cDNA libraries constructed in this project allowed the identification of genes involved

in cellular and developmental processes. Moreover, this *B. tabaci* database also constitutes an important tool for identifying genes involved in whitefly behaviour and its ability to transmit begomoviruses (Leshkowitz et al. 2006). In fact, genes encoding putative antimicrobial knottin proteins, *Btk-1* and *Btk-3*, were found to be upregulated in whiteflies infected with begomovirus (Shatters et al. 2008; Luan et al. 2011). Genes involved in the humoral response, such as complement-, coagulation-, and melanization-related genes, were also upregulated. In contrast, genes involved in signal transduction of the immune response and apoptosis were downregulated, suggesting that begomovirus inhibits the apoptosis pathway of the whitefly to ensure its replication and spread (Luan et al. 2011).

It has been demonstrated that the coat protein (CP) of the virus plays an important role in the begomivirus transmission cycle (Caciagli et al. 2009; Ohnesorge and Bejarano 2009). Three CP mutants of TYLCSV were found to be non-transmissible. One of these mutations affected virion stability, making them non-transmissible and hardly detectable in whiteflies. In contrast, the two other CP mutations did not affect virion stability, so they could be acquired and circulated in the whiteflies although they were non-transmissib. These results suggest that virion formation is necessary but not sufficient for begomovirus transmissibility. Other factors, such as the ability to cross gut epithelia, and the interaction with chaperones or molecular components within the salivary glands, may also influence transmissibility (Caciagli et al. 2009). In another study, the CP was used to screen a *Bemisia tabaci* cDNA library using the yeast two-hybrid system in a search for interacting partners. A member of the small heat-shock protein family was identified and its interaction with the CP was verified by an in-vitro pull-down assay, which indicates a putative role in transmission (Ohnesorge and Bejarano 2009).

4.5. Host range

Whitefly-transmitted geminiviruses generally have a narrow host range within dicotyledonous plants (Harrison 1985; Francki et al. 1991). In fact, TYLCD-associated species are only able to infect several species of certain families. However, virus inoculum is maintained in wild hosts, which provides a means for viruses to survive through the seasonal cycle and explains their predominance during epidemics. Therefore, the knowledge on the weed reservoirs of TYLCV can help to understand their epidemiology (Sánchez-Campos et al. 2000).

In the first experimental transmission studies using the *B. tabaci* vector, only 13 species belonging to six botanical families (Asclepidaceae, Compositae, Leguminoseae, Malvaceae, Solanaceae and Umbelliferae) were identified as TYLCV hosts. Some, such as tomato, *Datura stramonium* L. or *Nicotiana benthamiana* Domin. developed symptoms while others, including *Cynanchum acutum* L., *Phaseolus vulgaris* L. and *Nicotiana tabacum* L., were symptomless carriers of the virus (Cohen and Nitzany 1966; Cohen and Antignus 1994). More recently, up to 104 species belonging to 24 dicotiledonous families were described as TYLCV hosts (Dalmon and Marchoux 2000).

Under natural conditions, TYLCV can infect the species *Cynanchum acutum* L., *Datura stramonium* L., *Malva parviflora* L. (Cohen and Antignus 1994), *Plantago minor* Domin., *Mercurialis annua* L. (Abou-Jawdah et al. 1999), *Mercurialis ambigua* L. (Sánchez-Campos et al. 2000), *Cleome viscosa* L., *Croton lobatus* L., *Solanum nigrum* L., species from the genus *Malva*, *Macroptilium*, *Physalis*, *Polygonum*, *Sida* and *Wissadula*, non-identified species from the families *Acanthaceae*, *Cucurbitaceae* and *Nyctaginaceae* (Salati et al. 2002), and the species *Conyza sumatrensis* (Retz.) E.Walker, *Convolvulus* sp., *Cuscuta* sp. and *Chenopodium murale* L. (Jordá et al. 2001).

Among cultivated species, in addition to tomato, TYLCV has been detected in common bean (*Phaseolus vulgaris* L.) (Navas-Castillo et al. 1999), eggplant (*Solanum melongena* L.) (Abou-Jawdah et al. 1999), pepper (*Capsicum annuum* L., *C. chinense* Murray, *C. baccatum* L. and *C. frutescens* L.) (Reina et al. 1999; Roye et al. 1999; Salati et al. 2002; Morilla et al. 2005; Polston et al. 2006), lisianthus (*Eustoma grandiflorum* (Raf.) Shinners) (Cohen et al. 1995), zucchini (*Cucurbita pepo* L.) (Martínez-Zubiaur et al. 2004) and tobacco (*Nicotiana tabacum* L.) (Font et al. 2005).

Among cultivated species, TYLCSV species can infect tomato and pepper (Gorsane et al. 2004). It has also been found to infect *D. stramonium* L., *S. nigrum* L., *S. luteum* Mill., *Euphorbia* spp., *Malva parviflora* L. (Bosco et al. 1993; Davino et al. 1994; Bedford et al. 1998; Sánchez-Campos et al. 2000), *Convolvulus* sp., *Cuscuta* sp. and *Chenopodium murale* L. (Jordá et al. 2001) under natural conditions. Thus, differential host specificity exists within the TYLCV species (Sánchez-Campos et al. 1999). Moreover, the host range is also isolate-dependent (Monci et al. 2002; Salati et al. 2002; García-Andrés et al. 2006). On the other hand, recombinant species generally have a bigger host range than non-recombinant ones (Monci et al. 2002; García-Andrés et al. 2006).

4.6. TYLCD in Spain

The appearance of TYLCD in Spain was clearly associated with a progressive increase in the vector populations (Moriones et al. 1993). Currently, four of the 11 geminivirus species described as causal agents of the disease are present in Spain (Fauquet et al. 2008).

The first species detected in Spain in 1992 was the one coming from Sardinia (TYLCSV). It was detected in Murcia (Moriones et al. 1993) and Almería (Reina et al. 1994). Five years later, the species (TYLCV) was also detected in Almería (Navas-Castillo et al. 1997). Isolates found in these regions are closely related to Italian isolates, which suggests the existence of a geographical grouping of TYLCV isolates in the western Mediterranean basin. Nowadays, the disease is widespread throughout the Spanish Peninsula (Font et al. 2007) as well as the Canary (Font et al. 2000; Monci et al. 2000) and Balearic Islands (Font et al. 2002).

Although both species are present in Spain, TYLCSV has been progressively displaced by TYLCV mainly due to two ecological factors. Firstly, TYLCV is transmitted more efficiently by the local biotypes of *B. tabaci*. Secondly, the presence of alternative hosts of TYLCV and not of TYLCSV, such as some weeds and other crops like common bean, also contributes to this displacement (Sánchez-Campos et al. 1999). More recent studies demonstrate that the cultivation of resistant varieties is another factor involved in the displacement. It has been demonstrated that the resistance level and viral accumulation in resistant varieties depend on the type of infecting virus. In this sense, TYLCSV accumulates in lower levels and for less time than TYLCV in resistant plants (Lacasa et al. 2001; García-Andrés 2006, 2009).

Two natural recombinants of TYLCSV and TYLCV, recognised as new species (TYLCMalV and TYLCAxV), have been identified due to the coexistence of TYLCSV and TYLCV in mixed infections (Monci et al. 2002; García-Andrés et al. 2006). These recombinant species have new pathogenic properties, such as a broader host range or higher virulence (García-Andrés et al. 2006).

4.7. Methods for controlling the disease

The control strategies for TYLCV are mainly based in methods to prevent the disease and the development of materials with genetic resistance.

Some strategies are based on the reduction of inoculum quantity, like using virus-free material or removing infected plants and possible virus reservoirs (Ioannou et al. 1987; Cohen et al. 1988). Another strategy is based on controlling the insect vector by chemical, physical or biological methods. Chemical methods have little effect due to the low sensitivity of *B. tabaci* to insecticides, and because of the fast appearance of resistances (Horowitz et al. 2007). Moreover, the negative effects that pesticides have on the environment must be taken into account (Picó et al. 1996; Palumbo et al. 2001). On the other hand, physical methods, such as fine-mesh screens, present several disadvantages, such as problems with air circulation, which produces overheating and excessive shadow (Lapidot and Friedmann 2002). Natural enemies of whiteflies, such as parasitoids of the genera *Eretmocerus* (Urbaneja et al. 2007) or *Encarsia* (Gerling et al. 2001) are also commonly used. Nevertheless, those measures only delay the progress of the disease, since it has been reported that a threshold of only one or two insects per plant is needed for TYLCV transmission to occur (Caciagli et al. 1995).

There are also cultural practices, such as crop-free periods, altering planting dates or crop rotation, aimed at controlling the insect vector (Hilje et al. 2001). However, these are not completely efficient, either. Integrated management combining these methods with the use of varieties resistant to the virus or the vector represents the best strategy for fighting the disease (Stansly et al. 2004). Moreover, genetic resistance presents advantages, such as its respect for the environment (García-Arenal and Mc Donald 2003).

4.8. Breeding for resistance

Breeding for resistance to TYLCV started with the cultivated species. However, none of the genotypes showed sufficient resistance levels. Therefore, breeding for resistance has been focused on the introgression of resistance genes from wild tomato relatives (reviewed in Ji et al. 2007b).

Since *S. pimpinellifolium* crosses easily with cultivated tomato, it was the first species used to develop TYLCV-resistant lines. Concretely, the first breeding lines were derived from an accession coming from Israel, LA121. This accession presented absent or moderate symptomatology, even though it allowed viral replication. Lines derived from these materials presented moderate symptoms in the field but the growth and yield were drastically reduced (Pilowsky and Cohen 1974). Subsequently, new *S. pimpinellifolium*

materials, such as Hirsute-INRA, LA1478, PI407543 and PI407544 with varying resistance levels were found. A dominant gene (*Tylc*) was later proposed for the resistance gene in *S. pimpinellifolium* accessions Hirsute-INRA and LA1478 (Kasrawi 1989).

New breeding programmes were then initiated using *S. peruvianun*, which resulted in the development of the TY-20 hybrid, derived from accession PI 126935. This hybrid presented delayed symptom appearance and reduced viral accumulation, producing good yield despite the infection (Pilowsky et al. 1989; Pilowsky and Cohen 1990). In India, several TYLCV-resistant tomato lines were derived from *S. habrochaites* f. *glabratum* accession B6013. Among these lines, H24 performed best against TYLCV (Kalloo and Banerjee 1990).

Accessions of *S. chilense* and *S. peruvianum* have been reported as the most resistant sources. Concretely, *S. chilense* accession LA1969 was described by Zakay et al. (1991) as resistant and has been the most frequently employed in breeding programmes. Most of the commercial hybrids available nowadays were developed from this accession. Resistance in this accession is controlled by the partially dominant major gene *Ty-1*, located on chromosome 6, and by at least two modifier genes (Zamir et al. 1994).

More recently, partial resistance to TYLCD was also found in the L102 breeding material, derived from the *S. pimpinellifolium* accession UPV-16991 (Pérez de Castro et al. 2007a). However, although resistance germplasm have been found in *S. pimpinellifolium*, it is not the main resistance breeding resource in current breeding programmes as the resistance traits are not constant and the resistance does not work in certain areas (Chen et al. 2011).

In *S. peruvianum*, two more hybrids in addition to TY-20 were derived from other accessions (Pilowsky and Cohen 1995), as well as the breeding lines TY-172 and TY-197 (Lapidot et al. 1997; Friedman et al. 1998). Accession PI 126944 was also described as resistant to TYLCD (Picó et al. 1998; Pilowsky and Cohen 2000) and three interspecific hybrids were derived from it (Picó et al. 2002).

Since resistance derived from *S. habrochaites* f. *glabratum* accession B6013 is not effective against certain species causing TYLCD in several regions of the world (Mejía et al. 2005), other resistant sources have been used in breeding programmes. Several lines derived from accessions LA386 and LA1777, such as line 902, showed total immunity to whitefly-mediated inoculation (Vidavsky and Czosnek 1998). As a result, they were

employed in breeding programmes in the Middle East (Maruthi et al. 2003) and Guatemala (Mejía et al. 2005). Nevertheless, subsequent studies revealed that some breeding lines derived from line 902 carried not only introgressions from *S. habrochaites* but also an introgression from *S. chilense* (Martin et al. 2007; Menda et al. 2013). In a further study, two *S. habrochaites* TYLCD resistance sources, EELM-388 and EELM-889, were found after a wide germplasm screening. Moreover, this resistance was effective under field conditions with high TYLCD pressure (Tomás et al. 2011).

Several studies have confirmed the resistance of *S. chilense* accession LA1969 (Scott and Schuster 1991; Czosnek et al. 1993; Michelson et al. 1994; Picó et al. 1998; Pérez de Castro et al. 2005; Piñón et al. 2005). The resistance mechanism of the *Ty-1* gene is based on the interference of virus movement. However, when the inoculum concentration is high, antiviral factors that interfere with virus movement are not sufficient, as the virus can accumulate in low concentrations in the plant (Michelson et al. 1994; Zamir et al. 1994). For that reason, *Ty-1* carrier hybrids show resistance to TYLCV, although symptoms appear under high inoculum pressure.

Some breeding lines derived from the *S. chilense* accessions LA1932, LA2779 and LA1938 were initially reported as resistant to the Begomovirus ToMoV in the breeding programme carried out at the University of Florida. These lines were proved later to also be resistant to TYLCV in the Dominican Republic. These results suggested that the introgressed resistances were not virus-specific and are controlled multigenically (Scott et al. 1996; Scott 2001). Therefore, the selection of other resistance sources different from LA1969 represents an interesting approach to developing resistant lines. As a result, breeding programmes were initiated with accessions LA1932, LA1938, LA1960 and LA1971 (Picó et al. 1999). In further studies, accession UPV20306 and its hybrid also showed high resistance levels (Pérez de Castro et al. 2005).

Resistance to the disease is sometimes mediated by antibiosis or antixenosis mechanisms. This is the case of various *S. habrochaites* and *S. pennellii* accessions, in which resistance acts indirectly by preventing the vector feeding through physical interference by glandular trichomes and their secretions (Muniyappa et al. 1991; Channarayappa et al. 1992; Picó et al. 2001; Muigai et al. 2003). However, these sources have been underexploited because of the complex genetic control of the resistance. Moreover, non-desirable wild characters are linked to these resistance genes (Lawson et al.

1997; Momotaz et al. 2005). In *S. peruvianum* accession PI128657, resistance to whiteflies is controlled by the *Mi* gene. However, this resistance is overcome at high temperatures and depends on the plant stage, which makes it necessary to combine it with other resistance mechanisms (Nombela et al. 2003).

Inheritance of TYLCD resistance is very variable, ranging from a dominant monogenic to quantitative recessive control. Moreover, it varies depending on the species and accession considered (Lapidot et al. 2000). Therefore, the determination of the genetic control is a preliminary step in order to facilitate management of the resistance.

Genetic control of TYLCV resistance in *S. pimpinellifolium* appears to be contradictory. The first results obtained with accession LA121 suggested a monogenic control with incomplete dominance (Pilowsky and Cohen 1974). However, further studies with this accession indicated a quantitative genetic control with incomplete penetrance (Hassan et al. 1984). These authors attributed the differences found to differences in the virus isolate, environmental conditions, or inoculation and evaluation procedures. Resistance in *S. pimpinellifolium* accessions LA1478 and Hirsute-INRA was suggested as being controlled by the dominant gene *Tylc* (Kasrawi 1989; Vidavsky et al. 1998). However, results obtained by Chagué showed that this resistance was quantitative (Chagué et al. 1997). Further studies with *S. pimpinellifolium* accession UPV-16991 showed that TYLCD its resistance has a monogenic control with partial recessiveness and incomplete penetrance (Pérez de Castro et al. 2007a). TYLCV resistance in *S. peruvianum* is partially dominant and is controlled by several genes (Lapidot et al. 2000). Banerjee and Kalloo (1987) studied the inheritance of TYLCV resistance in *S. habrochaites* accession B6013 and concluded that two genes acting epistatically conditioned the resistance. An analysis of segregation of susceptibility, tolerance and resistance in breeding lines derived from an initial cross between *S. habrochaites* accessions LA386 and LA1777 indicated that tolerance is controlled by a dominant major gene and resistance by two to three additive recessive genes (Vidavsky and Czosnek 1998). Two independent loci, one dominant and one recessive, were associated with the TYLCD resistance in *S. habrochaites* EELM-889 (Tomás et al. 2011). In contrast, resistance in LA1969 was found to be conferred by one major gene, *Ty-1*, with at least two modifier genes (Zamir et al. 1994).

Despite the efforts made by different research groups to develop TYLCD-resistant varieties, the plant materials currently available do not present total resistance. In fact,

plants still develop symptoms under high inoculum pressure and early infection conditions. Therefore, it is of interest to incorporate resistance from the different available sources. With this aim, materials with high resistance levels have been obtained by combining the resistances of *S. pimpinellifolium*, *S. chilense*, *S. peruvianum* and *S. habrochaites* (Kasrawi and Mansour 1994;Vidavsky et al. 1998; Mejía et al. 2005; Pérez de Castro et al. 2008; Vidavski et al. 2008).

Another objective in breeding for resistance is to obtain broad spectrum resistance. For instance, in the aforementioned breeding programmes carried out in Florida, inoculations were performed independently with the begomoviruses ToMoV and TYLCV in order to obtain breeding lines with resistance to both viruses. Indeed, the ToMoV-resistant lines were usually resistant to TYLCV and often also to other geminiviruses (Scott et al. 1996; Scott 2001). Moreover, it has been reported in other studies that some materials resistant to TYLCV also present high resistance levels against *Tomato golden mosaic virus*, TGMV (Santana et al. 2001), *Tomato curly stunt virus*, ToCSV (Pietersen and Smith 2002) or *Tomato leaf curl virus*, ToLCV (Maruthi et al. 2003).

An alternative to the classical breeding programmes is to obtain protection against TYLCV by genetic engineering. For that aim, different strategies have been applied. One strategy is to transform the plant with pathogen-derived sequences, which involves the expression of functional as well as dysfunctional viral genes.

Initially, the gene that encodes for the capside protein (V1) was employed in transformed tomato plants, which allowed the obtaining of plants with variable levels of resistance. This resistance was associated with high levels of expressed CP. However, it was expressed as a delay in symptoms, rather than total immunity to the virus (Kunik et al. 1994).

Later, resistance against TYLCD in tomato and tobacco was achieved with a truncated version of the replicase gene (*C1*). However, this resistance was temporal and genetically unstable. Moreover, the transgene expression had deletereus effects in plants, such as curled leaves and sterile flowers (Noris et al. 1996; Brunetti et al. 1997; Brunetti et al. 2001). These deletereus effects caused by the *Rep* protein were avoided by reducing the size of the gene fragment used for the transformation. However, the resistance obtained by this strategy was isolate-specific (Antignus et al. 2004).

Pathogen-derived resistance has also been obtained by using gene-silencing technology. For instance, high resistance levels have been obtained by silencing the *V1* (Zrachya et al. 2007) and *C1* genes (Bendahmane and Gronenborn 1997; Yang et al. 2004). This was followed by another approach in which designed constructions contained non-coding sequences conserved among species belonging to the TYLCD complex, allowing broad-spectrum resistance to be obtained (Abhary et al. 2006).

Sequences from other origins have also been used. In this way, resistance has been obtained by transformation with the GroEL protein. Some plant viruses transmitted in a circulative manner by their insect vectors avoid destruction in the haemolymph by interacting with GroEL homologues, ensuring transmission. Transforming tomato plants with the gene that encodes for the GroEL protein allows TYLCV particles to be trapped by GroEL in the plant phloem, thereby inhibiting virus replication and movement (Akad et al. 2007).

Other studies indicate that it is possible to develop broad spectrum resistance by modifying genes which encode plant factors that are necessary for infection. Resistance against several begomovirus species has been obtained by transforming tomato plants with the recessive locus *tgr-1*, which encodes for a host factor necessary for virus movement (Bian et al. 2007).

4.9. Molecular marker identification and mapping of resistance genes

The identification of molecular markers associated with resistance genes is one of the most important objectives in breeding for resistance to TYLCD. Marker-assisted selection allows breeding programmes to be shortened and, in addition, is essential for resistance gene pyramiding.

Six resistance genes to TYLCD have been identified derived from different species. The first major TYLCV resistance gene mapped was *Ty-1*, located on chromosome 6 and derived from *S. chilense* accession LA1969 (Zamir et al. 1994). Another resistance gene, *Ty-3*, which also mapped on chromosome 6, was identified in advanced breeding lines derived from *S. chilense* accessions LA1932 and LA2779 (Ji et al. 2007a). The *Ty-4* minor resistance gene, mapped on the long arm of chromosome 3, was also identified in accession LA1932 (Ji et al. 2009a). The *Ty-2* gene, located on the short arm of chromosome 11, has been identified in lines derived from *S. habrochaites* (Hanson et al.

2006; Ji et al. 2009b). The *Ty-5* gene controls resistance in the breeding line TY172, initially thought to be originated from *S. peruvianum* (Anbinder et al. 2009). Recent studies suggest a mutation in the *S. lycopersicum* sequence as the origin of the resistance in TY172 (Levin et al. 2013). A recessive gene from the cultivar Tyking has been located in the same region as *Ty-5* and the symbol *ty-5* has been proposed (Hutton et al. 2012). Four recessive QTLs have been detected on chromosomes 4, 6, 10 and 11, respectively, in the tomato line FLA456, derived from *S. chilense* LA2779 and Royal Sluis tomato hybrid Tyking (Kardivel et al 2013). More recently, single marker analysis in breeding lines derived from LA2779 and LA1938 allowed the identification of a major resistance allele on chromosome 10, named *Ty-6*, which functions additively (Hutton and Scott 2013).

Different studies point to *S. chilense* species as the most effective TYLCV resistance source and, concretely, *S. chilense* accession LA1969 has been the most widely used accession in breeding programmes worldwide, as it exhibits the highest level of resistance (e.g. Ji et al. 2007a, 2007b). The major gene *Ty-1,* derived from *S. chilense* LA1969, was first mapped in the pericentromeric region of chromosome 6, near the Restriction Fragment Length Polymorphism (RFLP) markers TG297 and TG97. In addition, two modifier genes were mapped on chromosome 7, near TG61, and on chromosome 3, between makers TG66 and TG33, respectively (Zamir et al. 1994). Several PCR-based markers have been proposed for detecting the *Ty-1* locus. The first markers used were *Aps-1* (Rick and Forbes 1974; Williamson and Colwell 1991) and REX-1 (Williamson et al. 1994), both of which linked to the nematode resistance gene *Mi*. These markers were also used as markers for *Ty-1* due to the proximity between these two loci (Zhang et al. 2002). Nevertheless, the presence of the *Mi* gene was demonstrated to interfere, giving false-positive results. Therefore, a new Cleaved Amplified Polymorphic Sequence (CAPS) marker, JB-1, which is more tightly linked to the *Ty-1* locus, was developed in a further study (Perez de Castro et al. 2007b).

A more recent study has demonstrated that *Ty-1* is located on the long arm of chromosome 6, between markers MSc05732-4 and MSc05732-14, an interval of approximately 600 Kb that is nearly 5 Mb below the position reported by Zamir et al. (1994). This study attributes the imprecise location of *Ty-1* in previous works to low marker coverage in combination with severe recombination suppression in the *Ty-1* region. This suppression is partly caused by chromosomal rearrangements between *S. chilense*

LA1969 and *S. lycopersicum* in the pericentromere heterochromatic regions of both the short and long arms of chromosome 6 (Verlaan et al. 2011).

The *S. chilense* accessions LA1932, LA2779 and LA1938 that were found to be resistant to ToMoV in the breeding programme carried out at the University of Florida later proved to also be resistant to TYLCV (Scott et al. 1996). Inheritance studies and QTL mapping revealed three regions on chromosome 6 that contributed to resistance to both TYLCV and ToMoV in these accessions. The resistance was explained mainly by two QTLs in populations derived from LA1932 and LA1938 and one QTL in populations derived from LA 2779. The two QTLs introgressed from LA1932 and LA1938 mapped in the long arm of chromosome 6, in a different region from where the *Ty-1* gene was mapped. However, resistance derived from accession LA2779 mapped in a homologous region to this gene (Griffiths 1998; Griffiths and Scott 2001; Agrama and Scott 2006). RAPD markers linked to the potential resistance loci were identified (Ji and Scott 2005a). Some of these RAPDs were transformed in CAPS or in sequenced characterized amplified region markers (SCAR) (Ji and Scott 2005b).

More markers were used to better localize the resistance region in advanced breeding lines derived from LA1932 and LA2779. A major resistance locus, *Ty-3*, was mapped on the long arm of chromosome 6, between the markers cLEG-31-P-16 and T1079. In contrast to *Ty-1,* which is almost completely dominant, *Ty-3* has a nearly equal contribution of additive and dominance effects. This locus was reported to account for approximately 65% of the variance in the TYLCD resistance derived from LA2779. Similarly, the locus responsible for TYLCV resistance in the LA1932-derived breeding lines was mapped to the *Ty-3* region, but with a lesser dominance effect. On the other hand, the large introgression derived from LA2779 spans both the *Ty-1* and *Ty-3* regions and the introgression in LA1932 is much shorter, spanning only the *Ty-3* region. Evidence derived from this study supports the hypothesis that *Ty-1* and *Ty-3* are not allelic (Ji et al. 2007a).

More recent studies have better defined the genetic position of *Ty-3*. Preliminary data suggested that *Ty-3* was located between T0774 and cLEG-31-P16 (Hutton et al. 2010). Verlaan et al. (2011) showed that the introgression carrying *Ty-3* in a line derived from LA2779 partly overlapped with the chromosomal region where the *Ty-1* gene is located. Recombination was also severely suppressed in the pericentromeric region in the

Ty-3-carrying population, indicating similar rearrangements in LA2779 and in LA1969. Recently, *Ty-1* and *Ty-3* have been fine mapped to a region of approximately 70 kb and it has been shown by Virus-Induced Gene Silencing (VIGS) that they are allelic (Verlaan et al. 2012, 2013).

Using advanced breeding lines derived from *S. chilense* accession LA1932, a new TYLCV resistance locus, *Ty-4*, was mapped on the long arm of chromosome 3, within the marker interval between C2_At4g17300 and C2_At5g60160. While *Ty-3* accounted for about 60% of the variance in the TYLCV resistance in a segregating population, *Ty-4* accounted for only 16%, which suggests that it confers a lesser effect on TYLCV resistance (Ji et al. 2009a).

Three aditional TYLCV resistance genes have been identified in *S. habrochaites*, *S. peruvianum* and *S. chilense*. A dominant locus responsible for the resistance in line H24, derived from *S. habrochaites* f. *glabratum* accession B6013 (Kalloo and Banerjee 1990), was localized on the long arm of chromosome 11, delimited by the RFLP markers TG393 and TG36 (Hanson et al. 2000). This locus was further delimited to a smaller interval, a 4.5 cM interval between C2_At1g07960 and cLEN-11-F24, and was formally designated *Ty-2* (Hanson et al. 2006; Ji et al 2009b).

TYLCV resistance in the TY172 line is controlled by a major QTL and four additional minor QTLs. The major QTL, *Ty-5*, maps to chromosome 4 and accounts for 39.7 to 46.6% of the variation in symptom severity among segregating plants (Anbinder et al. 2009).

Breeding lines Fla. 8753 and Fla. 344 both have high levels of resistance to TYLCV derived from the cultivar Tyking and from the *S. chilense* accession LA1938, but none of their parent lines contain any of the known *Ty-1* to *Ty-4* genes. Appropriate segregating populations were analyzed using the *Ty-5* marker, SlNAC1. Results showed that SlNAC1 cosegregates with a recessive allele derived from Tyking, named *ty-5* (Hutton et al. 2012). More recently, marker analysis in a population derived from the breeding line Fla. 8383, coming from LA2779, allowed the identification of a major resistance allele on chromosome 10, named *Ty-6*, which functions additively (Hutton and Scott 2013).

In addition to the aforementioned *Ty* genes, several other regions associated with TYLCV resistance have been identified. In the *S. chilense* accession LA1969, two modifier genes on chromosomes 3 and 7 were identified in addition to *Ty-1* (Zamir et al. 1994). In

the *S. pimpinellifolium* accession Hirsute-INRA, a QTL which explains 27.7% of the resistance has been mapped to a marker interval between TG153 and CT83 on the long arm of chromosome 6 (Chague et al. 1997). This marker interval is close to the *Ty-3* position reported in *S. chilense* (Ji et al 2007a). Resistance in breeding lines derived from an initial cross between *S. habrochaites* accessions LA386 and LA1777 is controlled by a dominant major gene and two to three additive recessive genes (Vidavsky and Czosnek 1998). Agrama and Scott (2006) mapped three different QTLs (named TYLCV1, TYLCV2 and TYLCV) on chromosome 6 by analyzing three mapping populations derived from *S. chilense* accessions LA1932, LA2779 and LA1938. Anbinder et al. (2009) reported that TYLCV resistance in the TY172 line is controlled by the *Ty-5* gene and four additional minor QTLs. The minor QTLs were mapped to chromosomes 1, 7, 9 and 11. In *S. habrochaites* accession LA1777, four QTLs were found to be associated with resistance to *B. tabaci*. These QTLs were identified near the TG313 markers on chromosome 10, C2_At2g41680 on chromosome 9, TG523/T0408 on chromosome 11 and TG400/cLEG-37-G17 on chromosome 11 (Momotaz et al. 2010).

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OBJECTIVES

Viral diseases are an important limiting factor in tomato production worldwide. In temperate regions, Tomato yellow leaf curl disease is one of the main tomato viral diseases. Since genetic resistance has been proven to be the best way to face viral diseases, the identification of new resistance genes is an essential step in the development of highly resistant varieties with more durable resistance. Breeding programs were initiated by our group to exploit the resistance derived from the wild species *S. chilense* and *S. peruvianum*. Firstly, new TYLCD resistance sources were identified in *S. chilense* accessions LA1932, LA1960 and LA1971, from which resistant breeding lines were derived (Picó et al. 1999; Julián et al. 2008). Additionally, to make use of the resistance to different pathogens identified in *S. peruvianum* accession PI 126944, several hybrids between this accession and the cultivated tomato were obtained (Picó et al. 2002). In this context, the following specific objectives have been addressed by the present thesis:

1) Study of the genetic control of the resistance to TYLCV derived from *S. chilense* accessions LA1932, LA1960 and LA1971

2) Identification of polymorphic markers between *S. lycopersicum* and *S. chilense* accessions LA1932, LA1960 and LA1971

3) Mapping of the loci associated with the resistance derived from these sources

4) Identification of polymorphic markers between *S. lycopersicum* and *S. peruvianum* accession PI 126944

5) Starting the construction of a set of ILs derived from PI 126944 into the cultivated tomato genetic background

6) Testing of advanced generations for their resistance to TYLCD

Genetic control and mapping of *Solanum chilense* LA1932, LA1960 and LA1971-derived resistance to Tomato yellow leaf curl disease

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ABSTRACT

Tomato yellow leaf curl disease (TYLCD) is caused by a complex of begomovirus. Breeding for resistance to this disease has mainly been based on *Ty-1* gene, derived from *Solanum chilense* LA1969. Commercial varieties available to date still develop symptoms and suffer yield losses with high inoculum pressure and early infections. It is of interest to incorporate in breeding programs resistance from the different available sources. Lines with resistance to TYLCD derived from *S. chilense* accessions LA1932, LA1960 and LA1971 were previously developed. The objectives of this work were to study the genetic control of the resistance derived from these accessions and to map the resistance loci. Response to viral infection was assayed in segregating generations derived from these sources. Results obtained were compatible with a monogenic control of resistance. A total of 94 markers were used to locate the *S. chilense* introgressions in each of the lines. Only the presence of a large introgression in chromosome 6 was common to all lines. Analysis of recombinants allowed localizing the resistance loci in an interval of approximately 25 cM, also common to all five families. This interval includes the region to which two previously *S. chilense*-derived TYLCD resistance loci have been mapped, the *Ty-1/Ty-3* region. This is the first report of LA1960 and LA1971-derived TYLCV resistance loci to be located on chromosome 6. Further work will be done to fine map the loci found in the present work, in order to determine if they are indeed located in the *Ty-1/Ty-3* region.

Keywords: molecular markers, resistance, *Solanum chilense*, tomato, TYLCD

INTRODUCTION

Tomato yellow leaf curl disease (TYLCD) causes great economic losses in tomato (*Solanum lycopersicum* L.) crops in temperate, tropical, and subtropical areas worldwide (Cohen and Lapidot 2007). The disease is caused by a complex of viral species belonging to the family *Geminiviridae*, genus *Begomovirus*. All viral species causing TYLCD are whitefly-transmitted in field conditions, concretely by *Bemisia tabaci* Gennadius.

Strategies to fight this disease include those based on reducing the inoculum sources and controlling the insect vector by means of physical, chemical, biological or cultural methods. However, these measures are not enough on their own. Integrated management combining these methods with the use of resistant varieties can contribute to reduce the incidence of the disease (Stansly et al. 2004).

Resistance to TYLCD has not been found among cultivated tomato accessions. Searching for resistance has focused from the beginning on wild tomato relatives. Variable levels of resistance have been identified in different accession belonging to *S. galapagense* S. Darwin & Peralta, *S. pimpinellifolium* L., *S. peruvianum* L., *S. habrochaites* S. Knapp & D.M. Spooner and *S. chilense* (Dunal) Reiche (reviewed in Ji et al. 2007a). Inheritance of TYLCD resistance depends on the accession considered, ranging from dominant monogenic to quantitative recessive. In this sense, some sources have been underexploited because of the complexity of the genetic control.

Accessions of *S. chilense* and *S. peruvianum* have been reported as the most resistant sources. *S. chilense*-derived resistance has been the most widely used in breeding programmes around the world, concretely resistance from LA1969. Accession LA1969 was first reported as highly resistant in 1991 (Zakay et al. 1991) and since then resistance has been confirmed in several conditions (Scott and Schuster 1991; Czosnek et al. 1993; Michelson et al. 1994; Picó et al. 1998; Piñón et al. 2005). Resistance derived from this accession is controlled by one major gene, named *Ty-1*, which maps to chromosome 6; two minor modifier genes implicated in resistance from this accession have been identified in chromosomes 3 and 7 (Zamir et al. 1994). Some breeding lines, derived from other *S. chilense* accessions initially reported as resistant to the *Begomovirus Tomato mottle virus* (ToMoV) in the breeding program developed at the University of Florida, were later proved to be also resistant to TYLCV (Scott et al. 1996). The loci responsible for the resistance derived from some of these accessions have been identified. This is the case of resistance derived from accessions LA1932 and LA2779. A major resistance locus, *Ty-3*, was reported as accounting for approximately 65% of the variance in TYLCD resistance derived from LA2779 (Ji et al. 2007b). The major resistance gene derived from LA1932 seemed also to be located at the *Ty-3* locus, but with lower dominant effect (Ji et al. 2007b). Evidence derived from that study supported the hypothesis that *Ty-1* and *Ty-3* were not allelic. However, recent work developed with better marker coverage, suggested that *Ty-1* and *Ty-3* are likely allelic (Verlaan et al. 2011). Another TYLCV resistance locus, *Ty-4*, also derived from LA1932 but conferring a lesser effect on TYLCV resistance, has been mapped on chromosome 3 (Ji et al. 2009a). Other resistance loci have been identified from different wild tomato relatives: *Ty-2*, on chromosome 11 and derived from *S. habrochaites* (Hanson et al. 2006; Ji et al. 2009b), and *Ty-5*, originated from *S. peruvianum* and mapped on chromosome 4 (Anbinder et al. 2009). Recently, a recessive

gene from the tomato cultivar Tyking has been reported to be located in the same region as *Ty-5* and the symbol *ty-5* has been proposed (Hutton et al. 2012).

Several *S. chilense* accessions (some of them common to the breeding program carried out in the University of Florida) were also used in a breeding program designed from the beginning to the development of TYLCD resistant varieties (Picó et al. 1999). Different advanced generations selected for resistance to TYLCD were derived from accessions LA1932, LA1960 and LA1971 by backcross to tomato and selfing (Julián et al. 2008). Generations originated from LA1932, although sharing the source of resistance with lines derived at the University of Florida, could differ in the loci conferring resistance, given the different selection program followed. Genetic control or genome regions associated with resistance derived from LA1960 and LA1971 have not previously been studied.

Despite the efforts made by different research groups to develop TYLCD resistant varieties, the plant materials available are not a solution, as with high inoculum pressure conditions and early infections, plants still develop symptoms and yield losses are caused. It is of interest to incorporate in breeding programmes resistance from the different available sources. The determination of the genetic control is a preliminary step in order to facilitate management of the resistance.

The objective of the work here presented was the determination of the genetic control of TYLCD resistance derived from *S. chilense* LA1932, LA1960 and LA1971. Initial mapping of loci associated to resistance derived from these sources was also carried out.

MATERIALS AND METHODS

Plant material

Different populations with *S. chilense*-derived resistance to TYLCD were assayed. The breeding program leading to the obtaining of these populations started with the development of the interspecific hybrids between *S. lycopersicum* Fortuna C (FC) and *S. chilense* accessions LA1932, LA1960 and LA1971, respectively (Picó et al. 1999). These hybrids were backcrossed to FC and subsequently four selfing generations were carried out; in the case of LA1932-derived populations another backcross to FC followed by two more selfing generations were developed (Julián et al. 2008). Selection for resistance to

TYLCD was carried out at each of the generations developed. Plants for each of the populations obtained were selected as resistant parents: two different plants derived from LA1932, one derived from LA1960 and two derived from LA1971 (Julián et al. 2008). These resistant plants were crossed to FC as susceptible parent to obtain the generations used in this work (Table 1): four F_2 generations (two derived from LA1932, one derived from LA1960 and one derived from LA1971) and one complete family - including resistant parent (P_R), susceptible parent (P_S), F_1, F_2, backcross to the susceptible parent (BC_S) and a backcross to the resistant parent (BC_R) - with resistance derived from LA1971. The resistant and susceptible parents of each generation were also included in the inoculation assay, except for the resistant parent of LA1971-derived F_2 generation, for which seeds were not available (Table 1).

Additionally, different *S. chilense* accessions (kindly provided by the Tomato Genetics Resource Center, University of California, Davis, USA) were used in the molecular marker analysis to identify polymorphism between tomato and *S. chilense*. The corresponding F_1 generations obtained by crossing each of the parental lines with the tomato variety FC were also included in the marker analysis.

Inoculation method

Whitefly-mediated inoculation in muslin-covered cages inside a growth chamber was used. The conditions were: 25ºC temperature, 60 to 65% and 95 to 99% relative humidity (day/night), $34\mu Em^{-2}s^{-1}$ of irradiance and a 16/8 (light/dark) photoperiod. Whiteflies were biotype Q (supplied by F. Beitia, Instituto Valenciano de Investigaciones Agrarias, IVIA, Valencia, Spain) and viruliferous for the Spanish TYLCV isolate TYLCV-Mld[ES:72:97] (accession No. AF071228). Plants to be inoculated (Table 1) were grown in pots in an insect-proof greenhouse with controlled light and temperature. Plants at the three true leaf stage were caged with 20-50 whiteflies per plant for seven days. Plants were distributed in a completely randomized block design. During the inoculation period plants were shaken daily to ensure the uniform distribution of the whiteflies. After inoculation, plants were sprayed with the insecticides endosulfan (35% w/v) and bifentrin (10% w/v) and two days later plants were transplanted to bigger pots and transferred to an insect-proof greenhouse, where they remained until the end of the assay. Plants were grown on 12 liter pots with coconut fibre and fertirrigated with the usual doses and regularity for tomato in the cultivation area.

Table 1 Source of resistance, generations and number of plants analyzed for each family

Family	Source of resistance	Generations	Number of plants
1	LA1932	P_R	23
		P_S	25
		F_2	155
2	LA1932	P_R	18
		P_s	25
		F_2	113
3	LA1960	P_R	9
		P_S	25
		F_2	155
4	LA1971	P_R	na
		P_S	25
		F_2	116
5	LA1971	P_R	8
		P_S	25
		F_1	9
		F_2	143
		BC_S	89
		BC_R	73

na: not assayed

Disease assessment

Symptoms and viral accumulation were assessed at 15, 25, 35, 45 and 55 days postinoculation (dpi). Symptoms were evaluated following the scale developed by Friedmann et al. (1998) ranging from 0 (no visible symptoms, inoculated plants show same growth and development as uninoculated plants) to 4 (very severe plant stunting and yellowing, and pronounced cupping and curling; plants cease to grow). Intermediate scores (0.5, 1.5, 2.5 and 3.5) were also used to obtain accurate evaluations.

Molecular hybridization was used to assess viral accumulation. DNA extraction was carried out following the procedure developed by Crespi et al. (1991) with some modifications described by Pérez de Castro et al. (2007). After extraction, 2.5 μl of each sample and a ten-fold dilution were denatured with 30 mM NaOH and 1 mM EDTA for 30 min and blotted on to nylon membranes for hybridization. DNA was fixed by UV crosslinking. The probe (supplied by E.R. Bejarano, Universidad de Málaga, Spain) contained the intergenic region of the TYLCV-Mld[ES:72:97] isolate and was labelled by incorporation of digoxigenin-11-dUTP during PCR. Hybridization was carried out

following "The DIG system user's guide for filter hybridization" (Roche Molecular Biochemicals). Membranes were prehybridized in standard hybridization buffer plus 50% deionized formamide (50% formamide deionized, 5x SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, 2% blocking Reagent) for at least 1 h. Subsequent hybridization was done at 42ºC overnight in fresh prehybridization solution containing 20 ng of denatured probe per ml. Washing steps and incubation with antibody were developed according to manufacturer's instructions. Detection was carried out with CSPD and direct exposure to a CCD camera for approximately 1 h (Intelligent Dark Box-II, Fujifilm, Tokyo, Japan).

Viral ssDNA was quantified by comparison with a standard curve of TYLCV dotted on the same membrane (ranging from 10 ng to 1 pg). Total plant DNA extracted was also quantified by fluorimetry (Hoefer DyNA Quant 300 fluorometer, according to manufacturer's instructions) to relate viral amounts detected to plant DNA present at each sample.

Evaluation criteria

The limit to classify individual plants as resistant or susceptible was established at symptom score 2, based on previous studies (Pérez de Castro et al. 2007). Plants scored under 2 were considered resistant, given that no significant yield losses were expected as a consequence of infection, while plants scored 2 or higher were considered susceptible.

Molecular marker analysis

The 79 CAPS and 184 SSR markers were first used for polymorphism identification between the original *S. chilense* accessions and the tomato variety Fortuna C, used in the development of the populations. A total of 94 markers revealed polymorphism; for these markers, primer sequences and the basis of their design are listed in Supplementary Table 1. All markers were codominant with the exception of JB-1 marker, for which homozygotes for *S. chilense* allele and heterozygotes showed the same band pattern.

DNA extraction for molecular marker analysis was developed from 75 mg of fresh tissue, following the procedure described by Doyle and Doyle (1990) with some modifications.

PCR reactions for CAPS markers were carried out in a total volume of 12 μl containing: 1x buffer recommended by suppliers, 2 mM MgCl$_2$, 0.25 mM of each primer,

0.2 µM dNTPs, 1 U of *Taq* polymerase and 40-100 ng of template DNA. The amplification was carried out in an Eppendorf Martercycler Thermal Cycler with the following conditions: 35 cycles of 94ºC for 30 s, annealing (temperature depending on the marker, see Supplementary Table 1) for 1 min and 72ºC for 2 min, followed by an extension step of 10 min at 72ºC. Restrictions of 5 µl of the amplified products were performed overnight, in a total volume of 20 µl with 10 U of the corresponding enzyme, using buffers recommended by the suppliers at the recommended temperature. Digestion products were analized by agarose gel electrophoresis (2% agarose w/v with TBE 1X buffer) and visualized by GelRed (Biotium) staining.

PCR reaction conditions for SSR markers differed with respect to those described for CAPS markers regarding primer concentration and thermal profile. Primer concentrations were: direct primer 0.25 µM, M13 tailed reverse primer 0.05 µM and fluorescent-labelled (IRDye-700 or IRDye-800) M13 primer 0.2 µM. The thermal profile used was: 30 cycles of 94ºC for 30 s, annealing (temperature depending on the marker, see Supplementary Table 1) for 30 s and 72ºC for 1 min, followed by an extension step of 10 min at 72ºC. SSR fragments were separated on a LI-COR 4300 DNA Analyzer (Biosciences, Lincoln, Nebraska, USA) with 6% acrylamide denaturing gels.

RESULTS

Inheritance of the resistance to TYLCD derived from LA1932, LA1960 and LA1971

Five F_2 generations were analized to determine the genetic control of resistance to TYLCD derived from *S. chilense* accessions LA1932, LA1960 and LA1971. Plants of the susceptible parent FC started showing symptoms at 15 dpi, and at 25 dpi all of them showed severe symptoms (Supplementary Table 2). A high percentage of the plants of the resistant parents for each of the generations analyzed remained asymptomatic, the rest of them showing only slight symptoms in no case scored over 1 (Supplementary Table 2). The percentage of asymptomatic plants was higher for parents of LA1960 and LA1971-derived families, averaging a lower symptom score. In the case of family 3 (LA1960-derived) some plants of the resistant parent showed very slight symptoms (scored 0.5) in the first sampling dates, which disappeared on subsequent dates. As expected, segregation for symptom development was observed in the F_2 generations analyzed, with plant scores ranging from 0 to 4 for all of them (Supplementary Table 3). According to the criteria

chosen to classify plants as resistant or susceptible (see materials and methods section), plants in all F_2 segregated in a 3:1 ratio (resistant:susceptible) compatible with a monogenic control of resistance (chi-square values (and probability) were 0.0343 (0.853), 0.427 (0.513), 0.308 (0.579), 0.0460 (0.830) and 2.88 (0.0898) for families 1 to 5, respectively). However, some differences were observed among the five F_2 generations. Symptoms in resistant plants at the end of the assay were more pronounced for both LA1932-derived F_2 generations, as the percentage of resistant plants which remained asymptomatic or showed symptoms scored 0.5 was lower than for F_2 derived from LA1960 and LA1971 (Supplementary Table 3).

Viral accumulation was also measured in individual plants at all sampling dates. Availability of the complete family (family 5), allowed comparison of results in the three genotype homogeneous generations (both parents and the F_1 generation) (Fig. 1a). Viral accumulation was higher in the susceptible parent for all dates. Differences were significant at 25, 45 and 55 dpi. Viral accumulation did not statistically differ between the resistant parent and the F_1 plants. These results were consistent with those observed when separately analyzing resistant and susceptible plants for each of the generations, i.e., accumulation in susceptible plants from the F_2 was higher than in resistant plants (Fig. 1b), and the same was true for BC_S susceptible and resistant plants (Fig. 1c). Accumulation in BC_R plants (all resistant) was comparable to that observed in the other resistant genotypes (Fig. 1c).

Considering all plants included in the assay, there was a moderate positive correlation, although highly significant, between symptom development and viral accumulation for each sampling date, except for 15 dpi (value for correlation was 0.33, 0.41, 0.52 and 0.47, for 25, 35, 45 and 55 dpi, respectively, P<0.00001 in all cases).
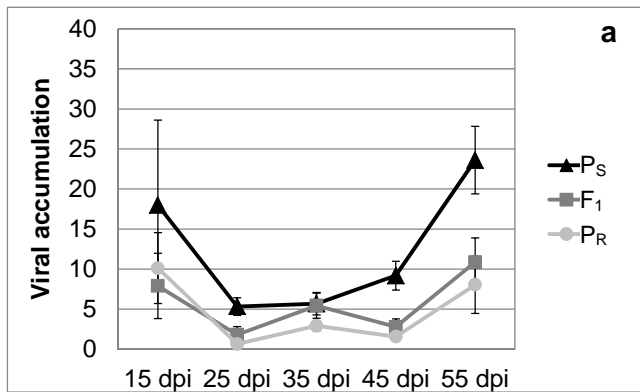
◄ **Fig. 1** Viral accumulation (ng viral DNA/μg total DNA extracted) after whitefly-mediated inoculation with *Tomato yellow leaf curl virus* in different generations of family 5, LA1971-derived: a) resistant parent (P_R), F_1 generation (F_1), and susceptible parent (P_S); b) F_2 generation susceptible (F_2-S) and resistant (F_2-R) plants; c) BC_S generation susceptible (BC_S-S) and resistant (BC_S-R) plants, and BC_R plants (all resistant, BC_R-R). Error bars represent standard error
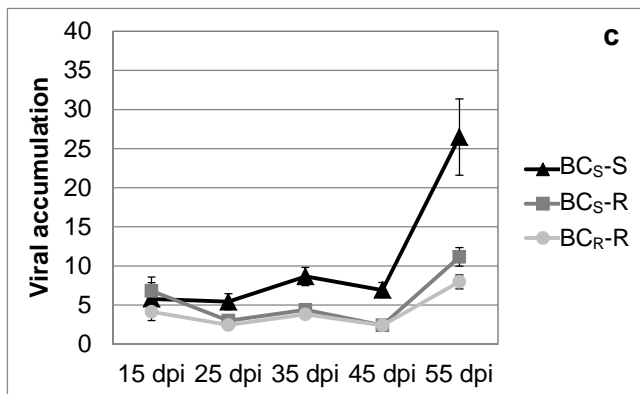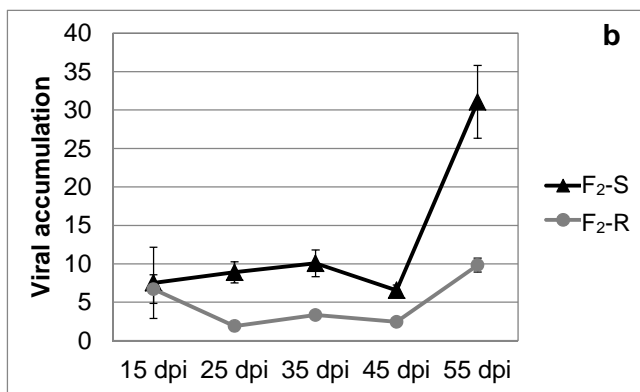
***Solanum chilense* introgressions in TYLCV-resistant breeding lines**

A total of 94 markers out of the 263 tested revealed polymorphism between tomato and *S. chilense*. These markers were used to screen the five TYLCV-resistant lines derived from *S. chilense* accessions LA1932, LA1960 and LA1971 to identify *S. chilense* introgressions. A large introgression in chromosome 6 was common to all breeding lines (Fig. 2). This introgression was variable in length. The introgression in LA1932-derived

line from family 1 encompassed the whole chromosome, being homozygous for the region between markers Aps-1 and C2_At1g21640. The other LA1932-derived line was homozygous for *S. chilense* introgression between the distal end of the short arm to the region above marker SSR578 and heterozygous from the fragment spanning from this marker to marker TAHINA-6-85. Introgression in LA1960 and LA1971-derived lines was shorter. LA1960-derived line was homozygous for the region from the distal end of the short arm to marker JB-1; for a small region below this marker, corresponding to marker TAHINA-6-85, this line was heterozygous. Introgressions were similar for both LA1971-derived lines, spanning from the distal end of the short arm to SSR128; additionally, parental line of family 5 carried *S. chilense* fragment corresponding to TAHINA-6-85 in heterozygous state.

It was also common to all parent lines the absence of *S. chilense* introgressions in chromosome 12 for the markers used.

Apart from introgression in chromosomes 6, very few *S. chilense* fragments were present in LA1932-derived lines. Both lines shared a small fragment at the long arm distal end of chromosome 5 (heterozygous in line 1 and homozygous in line 2). Line 2 also carried an introgression in heterozygous state corresponding to marker SSR85 in chromosome 10.

Introgressions in chromosomes 2, 3, 4, 7, 8, 9 and 10 were present in line 3. Both LA1971-derived lines shared introgressions (although variable in length in some cases) in chromosomes 5, 10 and 11. A small *S. chilense* fragment was also identified in chromosome 1 for line 4, while line 5 carried introgressions in chromosomes 3, 4, and 7.

Heterozygous introgressions were less promising candidate regions to hold resistance associated loci. However, for some of the introgressions present in heterozygous state, *S. chilense* allele was transmitted to the F_1 plant. All markers for which *S. chilense* allele was present in each of the F_1 plants were analized for the corresponding F_2 generation.
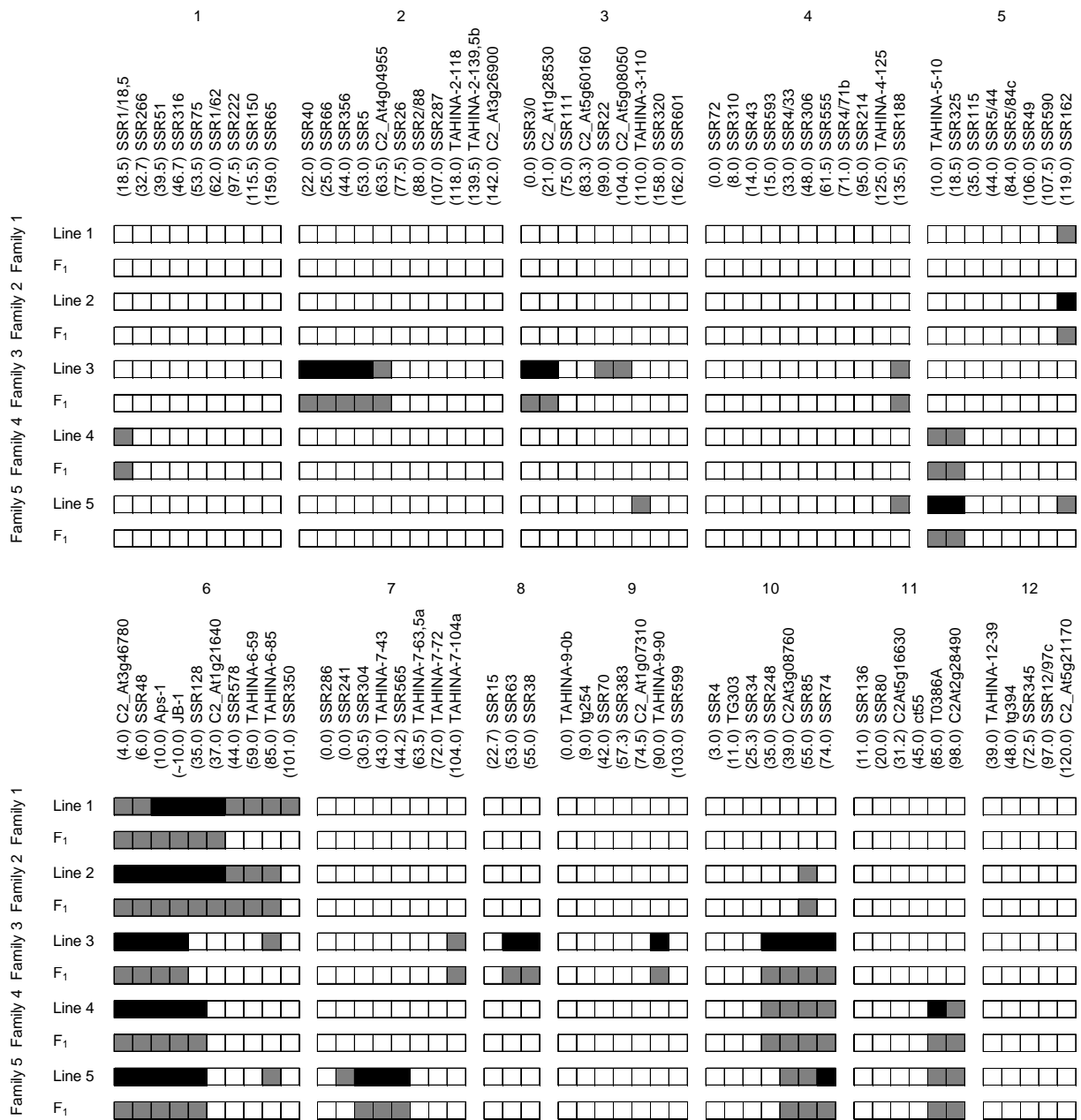
Fig. 2. *Solanum chilense* introgressions in each of the five lines developed and the corresponding F₁ generations. The top rows indicate chromosomes with markers (not to scale, map positions in brackets, according to Tomato-EXPEN2000 published in the Sol Genomics Network, http://solgenomics.net). Black represents homozygous for *S. chilense* allele, grey heterozygous and white homozygous for *S. lycopersicum* allele

Mapping of TYLCV-resistance loci

For all families there was complete association between the presence of *S. chilense* introgression for chromosome 6 (either in homozygous or heterozygous state) and TYLCV resistance. Those F_2 plants carrying the whole *S. chilense* introgression present in the corresponding F_1 plant were resistant while those homozygous for *S. lycopersicum* allele were susceptible.

A total of 61 informative recombinants were detected for introgression in chromosome 6 considering all plants analized (Table 2, Supplementary Table 4). These recombinants allowed delimiting the location of the resistance locus. In the case of LA1932-derived generations, all informative recombinants found in family 1 had a crossing over between markers JB-1 and SSR128 (Table 2). Four of them were homozygous for *S. lycopersicum* introgression for marker JB-1 and the region above it and heterozygous below this marker. Two of these recombinants were resistant (type 9-2) and the other two were susceptible (type 9-1 and 11), so the resistance locus should be located between markers JB-1 and SSR128. The other recombinant type found in this family was resistant and homozygous for tomato below JB-1 and heterozygous for this marker and the region above. This information combined with resistant type 9-2 recombinants confirmed the location of the resistance locus (which was also compatible with recombinants in family 2). The same type of recombinants was found in families 4 and 5. Thus, the resistant locus could also be delimited to the region between both markers in LA1971-derived plant materials.

Marker interval for LA1960-derived families included this region, although information provided by recombinants only allowed locating the resistant locus below C2_At3g46780 marker (and over SSR128 marker, given that introgression in this family did not include this marker). However, two non-recombinant plants were more informative: one of them was resistant and homozygous for tomato alleles for all markers, while the other was susceptible and heterozygous for the whole introgression. Both results showed that JB-1 is the upper limit to the region containing the resistance locus, coinciding then the location with the obtained for LA1932 and LA1971-derived resistance.

Table 2 Recombinants identified on chromosome 6 in the region flanked by markers C2_At3g46780 and TAHINA-6-85 in F_2 generation plants

			Markers[1]						Phenotype[2]	Recombinant type[3]	Recombinants identified in each family (source)[4]				
C2_At3g46780	SSR48	Aps-1	JB-1	SSR128	C2_At1g21640	SSR578	TAHINA-6-59	TAHINA-6-85			Family 1 (LA1932)	Family 2 (LA1932)	Family 3 (La1960)	Family 4 (LA1971)	Family 5 (LA1971)
a	h	h	c						R	1			1		
h	a	a	a						S	2		2			
h	h	a	a	nd					S	3				1	
a	h	h	c	b					R	4					1
a	h	h	c	h					R	5					1
h	a	a	a	h					S	6					1
h	a	a	a	a					S	7				1	
h	h	a	a	a					S	8					1
a	a	a	a	h	nd				S	9-1	1				2
a	a	a	a	h	nd				R	9-2	2				2
h	h	h	c	a					S	10-1				1	1
h	h	h	c	a					R	10-2				2	4
a	a	a	a	h	h				S	11	1				
h	h	h	c	a	a				R	12	1				
a	h	h	c	h	a	a	a	h	R	13		1			
a	h	h	c	h	h	h	a	a	R	14		1			
h	a	a	a	h	h	h	h	h	S	15		1			
b	h	h	c	h	h	h	h	a	R	16		1			
h	h	a	a	a	a	a	a	h	S	17		1			
a	a	a	a	h	h	h	h	a	R	18		1			
h	h	h	c	a	a	h	h	h	S	19		1			
b	b	b	c	a	a	a	a	h	R	20		1			
h	h	h	c	a	a	a	a	h	R	21		1			
h	h	h	c	a	a	a	a	a	R	22		1			
b	b	b	c	h	h	h	a	a	R	23		1			
a	a	a	a	a	a	h	h	a	S	24		1			
a	a	a	a	a	a	h	h	h	S	25		5			
h	h	h	c	h	h	a	a	h	R	26		1			
a	a	a	a	a	a	a	h	h	S	27		1			
h	h	h	c	h	h	h	a	a	R	28		5			
a	a	a	a	a	a	a	a	h	S	29		4			
h	h	h	c	h	h	h	h	a	R	30		5			
b	b	b	c	b	b	b	b	b	R	31		2			

[1] a: homozygous for *Solanum lycopersicum* allele; b: homozygous for *Solanum chilense* allele; h: heterozygous; c: either b or h; nd: not determined (in the case of recombinants type 9-1 and 9-2, C2_At1g21640 was not determined for family 1, and not introgresed for family 5)

[2] R/S: classification of each plant as resistant (R: symptom scoring < 2 in all evaluation dates) or susceptible (S: Symptom scoring ≥ 2 in one or more evaluation dates)

[3] code for recombinants: position of crossing-over - type of allele

[4] for more detailed information see Supplementary Table 4

When analyzing association of marker Aps-1 (which is very close to JB-1 and is codominant) with resistance in F_2 plants for all the families, a partially dominant effect was detected. Symptom severity was significantly higher at all dates in plants carrying tomato allele for this marker than in plants with *S. chilense* introgression (Table 3). Moreover, for all F_2 generations analyzed there were significant differences at some date between symptoms in plants homozygous and heterozygous for *S. chilense* allele: this was the case for the last three sampling dates (35, 45 and 55 dpi) for LA1932 and LA1960-derived F_2, while for LA1971-derived F_2 generations differences were only significant for one of the sampling dates. In all cases, symptom scores in heterozygotes were skewed towards scores in homozygotes for *S. chilense*. Similar results were obtained when comparing viral accumulation (data for the whole family are presented, Supplementary Table 5).

For family 1 no *S. chilense* introgressions were present in F_1 generation except for those in chromosome 6. For the rest of the families, *S. chilense* alleles were present in F_1 plants for some markers (Fig. 2). However, for none of them there was significant association between resistance and the presence of the wild introgression (data not shown).

Table 3 Average *Tomato yellow leaf curl virus* symptom score of plants for all five F_2 generations analyzed, according to the genotype for marker Aps-1 on chromosome 6

	Genotype[a]	N[b]	15 dpi[c]	25 dpi	35 dpi	45 dpi	55 dpi
Family 1	a	38	0.70 a	2.87 a	3.17 a	3.53 a	3.51 a
	h	78	0.12 b	0.41 b	0.71 b	0.65 b	0.76 b
	b	39	0.07 b	0.24 b	0.27 c	0.14 c	0.37 c
Family 2	a	31	0.45 a	2.08 a	2.77 a	3.16 a	3.60 a
	h	57	0.14 b	0.40 b	0.50 b	0.68 b	0.68 b
	b	25	0.04 b	0.06 b	0.10 c	0.32 c	0.22 c
Family 3	a	42	1.83 a	2.93 a	3.36 a	3.51 a	3.54 a
	h	77	0.28 b	0.51 b	0.49 b	0.51 b	0.51 b
	b	36	0.15 b	0.36 b	0.19 c	0.22 c	0.29 c
Family 4	a	30	0.16 a	1.67 a	2.37 a	2.83 a	3.07 a
	h	54	0.02 b	0.19 b	0.22 b	0.13 b	0.34 b
	b	32	0.00 b	0.06 b	0.00 c	0.00 b	0.19 b
Family 5	a	44	1.58 a	2.10 a	2.56 a	2.90 a	3.25 a
	h	76	0.14 b	0.33 b	0.25 b	0.26 b	0.33 b
	b	23	0.00 b	0.07 b	0.02 b	0.02 c	0.11 b

[a] Genotype: a, homozygous for *Solanum lycopersicum allele*; h, heterozygous; b, homozygous for *Solanum chilense* allele

[b] Number of plants

[c] dpi: days postinoculation. Symptom score from 0 (symptomless) to 4 (severe symptoms). See text for further description of the scale. Different letters in the same column and for each family, represent statistically significant differences (*P*<0.05) based on LSD test

DISCUSSION

The source of TYLCD resistance mainly used in the development of commercial varieties has been to date *S. chilense* LA1969. Resistance derived from this accession is controlled by the major gene *Ty-1*. The simple genetic control and the high level of resistance conferred by *Ty-1* are the reason for LA1969-derived resistance to be the most frequently incorporated in commercial hybrids. Nevertheless, symptoms are shown by these hybrids and yield losses are caused with high inoculum pressure and after early infections. It is interesting to make use of the different sources available, in order to increase the resistance levels.

Resistance in lines tested in this work, i.e. LA1932, LA1960 and LA1971-derived, is also controlled by major genes. Additionally, the level of resistance in these lines is comparable or even higher to the level found in tomato lines homozygous for *Ty-1*. The homozygous lines for the five families analyzed here have shown high percentages of asymptomatic plants, with very slight symptoms in symptomatic plants. The *Ty-1* gene, when analyzed in the cultivated tomato background in homozygous state has shown variable results, from symptomless in some studies (Michelson et al. 1994) to showing slight symptoms in most of them (Zamir et al. 1994; Hanson et al. 2000; Pérez de Castro et al. 2008). Differences could be due to the differences in the tomato line used as recurrent parent or in the percentage of tomato background present depending on the generations developed.

The response in plants heterozygous for the resistance gene is even more important, considering that it will mostly be used to breed hybrids. For all three sources studied here, heterozygotes analyzed in segregating generations showed average symptom scores only slightly higher with respect to homozygotes in the same generations; the resistance genes derived from LA1932, LA1960, and LA1971 seem to be almost completely dominant. The effect was stronger for LA1971-derived resistance, given that differences between homozygous and heterozygous plants in families 4 and 5 were significant only in one of the sampling dates. Results were similar when comparing viral accumulation, as expected given the positive correlation found in these families between viral accumulation and symptom scores. The *Ty-1* gene has been mainly reported as incompletely dominant (Zamir et al. 1994; Pérez de Castro et al. 2008), although in some genetic background heterozygous plants show high resistance levels (personal communication from breeders).

This background effect could be also exploited in resistance derived from the three sources tested here; thus, it would be interesting to find tomato backgrounds in which these genes were completely dominant.

The availability of markers linked to the resistance genes derived from these sources would facilitate their use in breeding programs. Moreover, it would be interesting to map the major loci responsible for the resistance and to identify putative minor loci affecting the resistance, in case they were present. In order to accomplish these aims, it was necessary to find polymorphic markers between cultivated tomato and *S. chilense*. A total of 263 markers were screened, with 94 of them being polymorphic between both species. These markers were analyzed in the *S. chilense*-derived lines available. This marker density has been proven useful to detect wild introgressions associated with resistance in other TYLCV-resistant lines. This is the case of *S. habrochaites*-derived line H24; the resistance locus *Ty-2* was first mapped to a 19 cM region on chromosome 11 using 90 RFLP markers (Hanson et al. 2000) and these results were later confirmed by fine mapping this gene to a marker interval of 4.5 cM (Ji et al. 2009b). The polymorphic markers used in the present study allowed determining the wild introgressions in each of the resistant lines derived from LA1932, LA1960 and 1971, respectively. These lines have been developed by backcrossing the initial interspecific hybrid to tomato, followed by four selfing generations; another backcross to the cultivated tomato and two additional selfing generations were also carried out in LA1932-derived lines. Selection for resistance to TYLCD was carried out in all generations. All five lines carried more than one *S. chilense* introgression, although the percentage of wild genome was variable and dependent upon the pedigree. In the case of LA1932-derived lines, very few fragments remained and (except for introgression in chromosome 6) they were short. Similar results were found for *S. habrochaites*-derived TYLCV-resistant line H24, although this line was selected after four backcrosses to cultivated tomato and two generations of inbreeding (Hanson et al. 2000). In the case of LA1960 and LA1971-derived lines, that are less advanced, the percentage of wild genome was higher.

Only the presence of introgression in chromosome 6 was common to all lines tested. This introgression was variable in length for the different lines, but all of them shared the region spanning from the distal region of the short arm to marker JB-1. Presence of *S. chilense* introgression for chromosome 6 was strongly associated with resistance in

segregating generations belonging to all families assayed. This is the first report of LA1960 and LA1971-derived TYLCV resistance loci to be located on chromosome 6.

The major loci responsible for the resistance to TYLCD derived from different *S. chilense* accessions have been mapped to chromosome 6, concretely the genes *Ty-1* (Zamir et al. 1994) and *Ty-3* (Ji et al. 2007b). Introgressions including the *Ty-3* locus in chromosome 6 have also been detected in advanced breeding lines derived from *S. peruvianum* and *S. habrochaites* resistant to bipartite begomovirus in Guatemala (Ji et al. 2007b).

Introgression in LA1969-derived generations obtained after two backcrosses to cultivated tomato was similar in length to those obtained here (Zamir et al. 1994). A recent study demonstrated that even commercial hybrids carrying *Ty-1* locus keep large wild introgressions and revealed chromosomal rearrangements between tomato and *S. chilense* as the cause of inhibition of recombination in this region (Verlaan et al. 2011). Nevertheless, in the present study, analysis of recombinants in this chromosome allowed localizing the resistance loci in a marker interval of 25 cM, between markers JB-1 and SSR128, common for all five families used in this work. This interval included the *Ty-1/Ty-3* region, previously reported as carrying TYLCV-resistance derived from LA1969, LA1932 and LA2779 (Zamir et al. 1994; Ji et al. 2007b; Verlaan et al. 2011). It has been suggested that both genes, *Ty-1* and *Ty-3* could be allelic (Verlaan et al. 2011). The fact that the interval containing the resistance loci tested here includes the *Ty-1/Ty-3* region raises the possibility of alleles derived from these sources being also allelic. Further screening with markers in this region will be done in order to fine map these genes to test this hypothesis.

As previously mentioned, lines tested in this work with LA1932-derived resistance had different origin with respect to lines developed in the University of Florida breeding program. However, results obtained here are compatible with the location for *Ty-3*, the resistance locus present in these lines (Ji et al. 2007b). An additional locus, *Ty-4*, conferring lesser effect on TYLCV resistance in these same breeding lines derived from LA1932 has been described on the long arm of chromosome 3 (Ji et al. 2009a). Lines developed in the present work do not show an *S. chilense* introgression for this region in chromosome 3. Thus, this minor locus has not been kept in LA1932-derived lines developed in our program. Introgression for this region was neither retained in LA1960 or

LA1971-derived lines, so it cannot be concluded if a minor locus similar to *Ty-4* was present in the original accessions.

In fact, no minor loci affecting resistance have been identified in this work. Fragments maintained in the breeding lines in chromosomes other than 6 were not associated with TYLCD resistance. In any case, from a practical point of view, it seems more efficient to make use of major genes identified so far, instead of searching for minor loci with less reproducible effects (Anbinder et al. 2009). On one hand, it will be interesting, when possible, to combine in the same hybrid the different genes identified from different sources, i.e., *Ty-2*, *Ty-4* and *Ty-5*. On the other hand, for the resistance loci identified in the work presented here, the first step would consist of confirming their location in the *Ty-1/Ty-3* region. If this is the case, they should be combined in heterozygous state, among them or with *Ty-1* and *Ty-3*. Independently of them being allelic or not to *Ty-1* and *Ty-3*, their close location in a region in which recombination is inhibited would greatly reduce the possibility to combine them in cis (Verlaan et al. 2011).

Differences have been reported regarding the dominance or the range of resistance to begomovirus for *Ty-1* and *Ty-3* (Ji et al. 2007a). Further work will be necessary to investigate the range of resistance conferred by the loci identified in this work. Also, the different possibilities combining the alleles available should be explored in order to find the most appropriate for each epidemiological situation.

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Supplementary Table 1 Polymorphic markers between *Solanum lycopersicum* and *S. chilense* used to genotype the parental line

Marker[1]	Ch[2]	cM[3]	Annealing temperature	Restriction enzyme	Fragment sizes[4]		Primers sequence (5'→3')	Reference[5]
					S. lycopersicum	*S. chilense*		
SSR1/18,5	1	18.5	55	-	182	180	AAAAGTGGGGAAGGAGCCTA / ACCTCAACGGGAAAGTACCA	Designed by S. Vilanova (COMAV)
SSR266	1	32.7	47	-	219	-	CAAGTTCACCTCATTTGACCC / TGTGTGAGCACTAAAGGACGA	SGN
SSR51	1	39.5	50	-	149	-	CTACCCTGGTCTTGGTGGAA / AAAGGATGCTCTAGCTTCTCCA	SGN
SSR316	1	46.7	55	-	235	-	CCACCGCAACAAACCTTATT / GGGTGGTGAGAAGGATCTGA	SGN
SSR75	1	53.5	55	-	175	-	CCATCTATTATCTTCTCTCCAACAC / GGTCCCAACTCGGTACACAC	SGN
SSR1/62	1	62	60	-	185	-	GGACCCACACACCATCTTTC / CCAAATGTGCACGTTCTAAGG	This work
SSR222	1	97.5	55	-	181	-	TCTCATCTGGTGCTGCTGTT / TTCTTGGAGGACCCAGAAAC	SGN
SSR150	1	115.5	50	-	235	-	ATGCCTCGCTACCTCCTCTT / AATCGTTCGTTCACAAACCC	SGN
SSR65	1	159	50	-	254	-	GGCAGGAGATTGGTTGCTTA / TTCCTCCTGTTTCATGCATTC	SGN
SSR40	2	22	55	-	160	191	TGCAGGTATGTCTCACACCA / TTGCAAGAACACCTCCCTTT	SGN
SSR66	2	25	50	-	204	192	TGCAACAACTGGATAGGTCG / TGGATGAAACGGATGTTGAA	SGN
SSR356	2	44	55	-	265	222	ACCATCGAGGCTGCATAAAG / AACCATCCACTGCCTCAATC	SGN
SSR5	2	53	45	-	213	204	TGGCCGGCTTCTAGAAATAA / TGAAATCACCCGTGACCTTT	SGN
C2_At4g04955	2	63.5	55	*Hinf*I	400	300	TTGCTGTGGGGAACCAAGCAGATATAG / TCCCAGAGAGTCTTGATCCCATGTATGC	SGN

Supplementary Table 1 (continuation)

Marker[1]	Ch[2]	cM[3]	Annealing temperature	Restriction enzyme	Fragment sizes[4] *S. lycopersicum*	*S. chilense*	Primers sequence (5'→3')	Reference[5]
SSR26	2	77.5	50	-	196	-	CGCCTATCGATACCACCACT ATTGATCCGTTTGGTTCTGC	SGN
SSR2/88	2	88	55	-	214	222	CCAAGGCATGACGTTAATTTG TCTTTTTCCATGTGTCAGTCAAC	This work
SSR287	2	107	45	-	186	-	GCATCCCAAACAATCCAATC TCCACTTTCAAGATCAGAGCAA	SGN
TAHINA-2-118	2	118	58	-	223	221	CAGAAGATTCGAAGGGTGCT AGGTGACTCCTCCACTGTCG	Trujillo-Moya et al. (2011)
TAHINA-2-139,5b	2	139.5	50	-	259	-	ATGTGCACACACGTGAACC ATGCAATTGCCATTGACCTA	Trujillo-Moya et al. (2011)
C2_At3g26900	2	142	55	*Hae*III	380/420	-	CCAAGGCATGACGTTAATTTG TCTTTTTCCATGTGTCAGTCAAC	SGN
SSR3/0	3	0	55	-	263	249	GGAAGTCGAGAGGTGGTGAG CCACTTTTCCAGCCACATTG	This work
C2_At1g28530	3	21	55	*Dra*I	600	400	ATTATGAAGATGTCTATACACTTCCCTAC AGAGATTGCTTTTGACATAGAAATGCTT	SGN
SSR111	3	75	55	-	200	-	TTCTTCCCTTCCATCAGTTCT TTTGCTGCTATACTGCTGACA	SGN
C2_At5g60160	3	83.3	50	*Nla*III	450	-	TTCTCGCGGCCTTTTCTCCTC TCGTGATCGCAAACATATACTCGC	Ji et al. (2009a)
SSR22	3	99	50	-	232	226	GATCGGCAGTAGGTGCTCTC CAAGAAACACCCATATCCGC	SGN
C2_At5g08050	3	104	55	-	650	700	TGCGATTTCACGTTTCTCTGCTTC TCCCCTACTGGAAATACAGTTGTTG	SGN
TAHINA-3-110	3	110	55	-	263	253	AGAAGCGTACCCAATCATGC GGCTGGATTGTGAGTGGATT	Trujillo-Moya et al. (2011)
SSR320	3	158	55	-	189	-	ATGAGGCAATCTTCACCTGG TTCAGCTGATAGTTCCTGCG	SGN

Supplementary Table 1 (continuation)

Marker[1]	Ch[2]	cM[3]	Annealing temperature	Restriction enzyme	Fragment sizes[4]		Primers sequence (5'→3')	Reference[5]
					S. lycopersicum	*S. chilense*		
SSR601	3	162	55	-	184	-	TCTGCATCTGGTGAAGCAAG CTGGATTGCCTGGTTGATTT	SGN
SSR72	4	0	52	-	198	-	GGTTCCCTTCTCTCTTTGTCC GCGTGTTCTTCGATTTGACA	SGN
SSR310	4	8	55	-	166	-	GCGATGAGGATGACATTGAG TTTACAGGCTGTCGCTTCCT	SGN
SSR43	4	14	50	-	237	-	CTCCAAATTGGGCAATAACA TTAGGAAGTTGCATTAGGCCA	SGN
SSR593	4	15	55	-	314		TGGCATGAACAACAACCAAT AGGAAGTTGCATTAGGCCAT	SGN
SSR4/33	4	33	53	-	265	-	CAAATTCATTCAGTGCTAAAAGG CAAAATTAAACTCTCCCATGAACA	Designed by S. Vilanova (COMAV)
SSR306	4	48	55	-	280	-	ACATGAGCCCAATGAACCTC AACCATTCCGCACGTACATA	SGN
SSR555	4	61.5	41	-	228	-	TTGATATTAACCATGGCAGCAG TTGATGGGATTGCACAGAAA	SGN
SSR4/71b	4	71	50	-	227	-	TGTAAACCCTTTATCCCCTTTT TTCAAATAAGGGCTTTCTCAACA	This work
SSR214	4	95	50	-	243	-	AAATTCCCAACACTTGCCAC CCCACCACTATCCAAACCC	SGN
TAHINA-4-125	4	125	55	-	253	-	AGGGCTATGCCCATAGTGTG GGAGGGCATGGGTAGAGATT	Trujillo-Moya et al. (2011)
SSR188	4	135.5	50	-	159	150, 161	TGCAGTGAGTCTCGATTTGC GGTCTCATTGCAGATAGGGC	SGN
TAHINA-5-10	5	10	55	-	220	185, 194	AAGCATAGTATCACAAAGCTGACC ATGGAAGCCACAACCAAATG	Trujillo-Moya et al. (2011)
SSR325	5	18.5	50	-	152	154	CCATTGACAGCCCATTATCC TGATGTGAAAGAGTTGATGAGG	SGN

Supplementary Table 1 (continuation)

Marker[1]	Ch[2]	cM[3]	Annealing temperature	Restriction enzyme	Fragment sizes[4]		Primers sequence (5'→3')	Reference[5]
					S. lycopersicum	*S. chilense*		
SSR115	5	35	50	-	233	-	CACCCTTTATTCAGATTCCTCT	SGN
							ATTGAGGGTATGCAACAGCC	
SSR5/44	5	44	55	-	230	-	TGATGCCTACAGGCACAAAG	Designed by S. Vilanova (COMAV)
							TGCAGTAATGAGAATATGTTGGATG	
SSR5/84c	5	84	54	-	268	-	ATTCGGTTTTGGGTTGATTG	This work
							TTGGTAATTCAGTAATTTGGTTCG	
SSR49	5	106	50	-	176	-	TCTCAAAGTCGTTCCTTCTTGA	SGN
							GGAAGAGAAACGCGGACATA	
SSR590	5	107.5	55	-	175	-	TCTCAAAGTCGTTCCTTCTTGA	SGN
							GGAAGAGAAACGCGGACATA	
SSR162	5	119	50	-	268	259, 261	GCTCTCTACAAGTGGAACTTTCTC	SGN
							CAACAGCCAGGAACAAGGAT	
C2_At3g46780	6	4	55	-	1200	1150, 1250	ATGGCTCCAACTCTTACTTCAAATTC	SGN
							TCTGCATCTTGAAATGATGATGCAAC	
SSR48	6	6	50	-	214	217	ATCTCCTTGGCCTCCTGTTT	SGN
							GTCATGGCCACATGAATACG	
Aps-1	6	10	53	*Taq*I	275	350	GGCAGGAGAATATGCCAAAA	Pérez de Castro et al. (2007)
							CGTTCCATTCTCAACCCATT	
JB-1	6	~ 10	53	*Taq*I	425	500	AACCATTATCCGGTTCACTC	Pérez de Castro et al. (2007)
							TTTCCATTCCTTGTTTCTCTG	
SSR128	6	35	50	-	137	122, 134	GGTCCAGTTCAATCAACCGA	SGN
							TGAAGTCGTCTCATGGTTCG	
C2_At1g21640	6	37	55	*Apo*I	200	400	AGAAAAGTCATCCATGGAAACAACAC	SGN
							TGGCCACAATGACACCATCACCTTG	
SSR578	6	44	55	-	312	306	ATTCCCAGCACAACCAGACT	SGN
							GTTGGTGGATGAAATTTGTG	
TAHINA-6-59	6	59	50	-	271	276	TTTTCCTGGGGTAAGCAGAA	Trujillo-Moya et al. (2011)
							TTCAACTTTTCACTTTGGAGCTT	

Supplementary Table 1 (continuation)

Marker[1]	Ch[2]	cM[3]	Annealing temperature	Restriction enzyme	Fragment sizes[4]		Primers sequence (5'→3')	Reference[5]
					S. lycopersicum	*S. chilense*		
TAHINA-6-85	6	85	55	-	250	239, 252, 264	CATGTTGGCCAAACAATCTG	Trujillo-Moya et al. (2011)
							GCAAGGGATGCTGTCTTCTT	
SSR350	6	101	55	-	289	284	GGAATAACCTCTAACTGCGGG	SGN
							CGATGCCTTCATTTGGACTT	
SSR286	7	0	48	-	214	202	AGCTATGGAGTTTCAGGACCA	SGN
							ATTCAGGTAGCATGGAACGC	
SSR241	7	0	55	-	126	-	TCAACAGCATAGTGGAGGAGG	SGN
							TCCTCGGTAATTGATCCACC	
SSR304	7	30.5	52	-	203	197	TCCTCCGGTTGTTACTCCAC	SGN
							TTAGCACTTCCACCGATTCC	
TAHINA-7-43	7	43	55	-	266	258	GCAGCCAAATAGAAATTGGAAG	Trujillo-Moya et al. (2011)
							CACATGTTAAAAGGTTGGTCAC	
SSR565	7	44.2	55	-	398	386, 400	GAGGATGATGAGAACTCGCC	SGN
							TCAGAGGCTTCTGGGTCAGT	
TAHINA-7-63,5a	7	63.5	56	-	271	-	AGATGTGGACCTCCTTCGAC	Trujillo-Moya et al. (2011)
							TTCTCACCTAACCCAGTACCAC	
TAHINA-7-72	7	72	50	-	178	-	TTATTTTTGTCTTCGCTTTATTTTT	Trujillo-Moya et al. (2011)
							AACTCCCAAAGCGTAATTTGA	
TAHINA-7-104a	7	104	52	-	264	287	TGCCCTTAATTATGCGAACAG	Trujillo-Moya et al. (2011)
							TGGCAATCTCTAGTGAAAATGTC	
SSR15	8	22.7	40-55	-	212	-	CACTTGCCATCTTCTAGCCC	SGN
							ATGGATGCCCAAATTGAAGA	
SSR63	8	53	55	-	229	238	CCACAAACAATTCCATCTCA	SGN
							GCTTCCGCCATACTGATACG	
SSR38	8	55	55	-	257	239	GTTTCTATAGCTGAAACTCAACCTG	SGN
							GGGTTCATCAAATCTACCATCA	
TAHINA-9-0b	9	0	52	-	242	-	AAAAGGTTCACGAAGGGAAAA	Trujillo-Moya et al. (2011)
							AATCAAAACACCTTCAACGACT	

Supplementary Table 1 (continuation)

Marker[1]	Ch[2]	cM[3]	Annealing temperature	Restriction enzyme	Fragment sizes[4]		Primers sequence (5'→3')	Reference[5]
					S. lycopersicum	*S. chilense*		
tg254	9	9	55	*Sau*3AI	410	-	TTGGGAATATAGTGTAGGAAG CTGGAAAGGGGAAAGAC	Bai et al. (2003)
SSR70	9	42	50	-	137	144	TTTAGGGTGTCTGTGGGTCC GGAGTGCGCAGAGGATAGAG	SGN
SSR383	9	57.3	55	-	261	207	ATTGTACAAAGACCCGTGGC GTTGCACACTGGATCAATGC	SGN
C2_At1g07310	9	74.5	55	*Hae*III	350	-	AGAAAACCTACGATCTCGAAATCACC AAACTGCCATAGCTAGATTGCCG	SGN
TAHINA-9-90	9	90	55	-	267	272	CTAGATAGGGCCCAGGGGTA TCAAGGCGAAATCAAGATCA	Trujillo-Moya et al. (2011)
SSR599	9	103	55	-	303	-	GGATTTCTCATGGAGAATCAGTC TCCCTTGATCTTGATGATGTTG	SGN
SSR4	10	3	50	-	184	-	TTCTTCGGAGACGAAGGGTA CCTTCAATCCTCCAGATCCA	SGN
TG303	10	11	55	*Alu*I	280	-	CGTAAAGGGTTGTTCTTGTGC TGTTTTCGAGTGGGGTTCAT	Canady et al. (2005)
SSR34	10	25.3	50	-	204	-	TTCGGATAAAGCAATCCACC TCGATTGTGTACCAACGTCC	SGN
SSR248	10	35	55	-	261	245, 276	GCATTCGCTGTAGCTCGTTT GGGAGCTTCATCATAGTAACG	SGN
C2At3g08760	10	39	55	*Alu*I	500/600	400	TCTCCAGAACGTTGTGTGTCAGAAGG TCCTCATGTAGAAATGTAAGACCTTG	SGN
SSR85	10	55	50	-	198	185, 192	ATCCGTTAGCTATTGTGCCG TTGCCATGCACTTATCTTCG	SGN
SSR74	10	74	55	-	221	218	ACTCACCATGGCTGCTTCTT TTTCTTGAAGGGTCTTTCCC	SGN
SSR136	11	11	50	-	165	-	GAAACCGCCTCTTTCACTTG CAGCAATGATTCCAGCGATA	SGN

Supplementary Table 1 (continuation)

Marker[1]	Ch[2]	cM[3]	Annealing temperature	Restriction enzyme	Fragment sizes[4] S. lycopersicum	Fragment sizes[4] S. chilense	Primers sequence (5'→3')	Reference[5]
SSR80	11	20	50	-	205	-	GGCAAATGTCAAAGGATTGG	SGN
							AGGGTCATGTTCTTGATTGTCA	
C2At5g16630	11	31.2	55	*Taq*I	700	-	TAAATGCAATCACTGATGGAGAGCA	SGN
							TGCCAATACTGCATCCCACCAAAT	
ct55	11	45	56	*Dde*I	200/350	-	CATCTGGTGAGGCGGTGAAGTA	Bai et al. (2003)
							TCCGCCCAAACAAAACAGTAATA	
T0386A	11	85	52	*Hinf*I	500	200/300	ATGCTGATGAAAGATTGGGCGCTG	Ji et al. (2009b)
							TTAGGCTTTGGCTTCTCGACCACT	
C2At2g28490	11	98	55	*Taq*I	380	350	ACGGAGTATTCTCCATTGAAACACTCTG	SGN
							ATTGAATTCTGACCCACCAAGAACTG	
TAHINA-12-39	12	39	52	-	230	-	ATTGCCACGTGGATTGACTC	Trujillo-Moya et al. (2011)
							TGCAAGCTGTTCTTTTCAGAC	
tg394	12	48	56	*Fok*I	280	-	AGCCTCATGAGACCTACAA	Bai et al. (2003)
							TACAGCACAATCTTCTACC	
SSR345	12	72.5	60	-	177	-	AAGCCAAGCTCGAACCTGTA	SGN
							ATCCATGCTGTCGCTTTCAT	
SSR12/97c	12	97	53	-	257	-	TTGCTTCACTTGTGTCGAATC	This work
							TCTGTTGCAGTCCCAAAAAG	
C2_At5g21170	12	120	55	*EcoR*I	400	-	TCCTTCCATCGGGTATATATCATTACAA	SGN
							ACTGCCACTGGCTCCTTTTCAAAATC	

Supplementary Table 1 (continuation)

[1] SSR: Simple Sequence Repeat; TAHINA are also SSR. The rest are Cleaved Amplified Polymorphic Sequence (CAPS) markers
[2] Chromosome
[3] cM according to Tomato- EXPEN2000
[4] Fragment sizes (pb) are provided for *Solanum lycopersicum* (Fortuna C) alleles for all markers. Fragment sizes are also provided for *S. chilense* alleles for markers corresponding to introgressions in parental lines.
Slash (/) separates bands of the same allele for CAPS markers; commas (,) separate different alleles
[5] SGN: Sol Genomics Network (solgenomics.net)

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Supplementary Table 2 Average *Tomato yellow leaf curl virus* symptom score (± standard error) and percentage of asymptomatic plants (in brackets) for parent generations and the F_1 generation analyzed

Genotype	15 dpi[a]	25 dpi	35 dpi	45 dpi	55 dpi
Fortuna C	1.98 ± 0.37 (30.43)	3.72 ± 0.11 (0.00)	3.80 ± 0.08 (0.00)	3.87± 0.08 (0.00)	3.89 ± 0.08 (0.00)
Parent (Family 1) LA1932	0.00 ± 0.00 (100.00)	0.11 ± 0.05 (77.27)	0.32 ± 0.07 (45.45)	0.36 ± 0.07 (36.36)	0.41 ± 0.07 (31.82)
Parent (Family 2) LA1932	0.00 ± 0.00 (100.00)	0.08 ± 0.05 (83.33)	0.17 ± 0.06 (66.67)	0.19± 0.06 (61.11)	0.25 ± 0.07 (55.56)
Parent (Family 3) LA1960	0.17 ± 0.08 (66.67)	0.17 ± 0.12 (77.78)	0.11 ± 0.11 (88.89)	0.06± 0.06 (88.89)	0.06± 0.06 (88.89)
Parent (Family 4) LA1971	na[b] na	na na	na na	na na	na na
Parent (Family 5) LA1971	0.00 ± 0.00 (100.00)	0.00 ± 0.00 (100.00)	0.00 ± 0.00 (100.00)	0.00 ± 0.00 (100.00)	0.13 ± 0.23 (75.00)
F_1 (Family 5) LA1971	0.00 ± 0.00 (100.00)	0.16 ± 0.08 (66.67)	0.22 ± 0.14 (77.78)	0.33 ± 0.16 (66.67)	0.38 ± 0.16 (55.56)

[a] dpi: days postinoculation

[b] na: not assayed

Supplementary Table 3 Percentage of plants in each score and mean score of *Tomato yellow leaf curl virus* symptoms in the five F$_2$ generations analyzed

Family[a]	dpi[b]	0	0.5	1	1.5	2	2.5	3	3.5	4	Mean Score
				Percentage of plants assigned score[c]							
	15	77.6	8.3	7.7	1.9	1.3	0.6	1.9	0.0	0.0	0.25
	25	32.7	35.9	8.3	1.9	0.6	3.8	5.8	3.8	6.4	0.97
1	35	25.0	27.6	14.7	9.6	0.6	1.9	7.7	3.2	9.0	1.20
(LA1932)	45	32.7	21.2	14.1	8.3	0.0	0.6	5.8	1.3	15.4	1.23
	55	17.9	28.8	18.6	9.6	0.0	3.8	3.8	4.5	12.2	1.34
	15	82.3	7.1	5.3	1.8	1.8	0.0	1.8	0.0	0.0	0.20
	25	45.1	26.5	7.1	4.4	2.7	1.8	5.3	4.4	2.7	0.79
2	35	33.6	31.0	9.7	2.7	0.9	4.4	8.8	0.9	8.0	1.04
(LA1932)	45	30.1	17.7	15.9	11.5	0.0	2.7	8.0	2.7	11.5	1.28
	55	25.7	22.1	17.7	7.1	0.0	2.7	6.2	1.8	16.8	1.38
	15	59.4	12.3	3.9	8.4	3.9	5.2	3.2	2.6	1.3	0.67
	25	32.9	24.5	11.6	7.7	1.3	1.9	7.7	3.9	8.4	1.13
3	35	38.1	20.0	9.0	7.1	1.3	3.2	4.5	2.6	14.2	1.20
(LA1960)	45	28.4	32.9	7.1	5.2	0.0	3.9	4.5	2.6	15.5	1.25
	55	31.6	25.8	8.4	7.1	0.6	2.6	6.5	1.9	15.5	1.28
	15	94.0	3.4	0.9	1.7	0.0	0.0	0.0	0.0	0.0	0.05
	25	59.5	22.4	3.4	3.4	1.7	4.3	0.9	0.0	4.3	0.54
4	35	58.6	14.7	6.0	1.7	5.2	2.6	6.0	0.0	5.2	0.72
(LA1971)	45	62.9	10.3	2.6	0.0	6.0	6.0	3.4	0.9	7.8	0.79
	55	37.1	31.9	5.2	0.0	5.2	6.0	4.3	0.9	9.5	1.00
	15	67,8	9,6	4,8	0,7	4,1	2,1	4,1	0,7	4,1	0,56
	25	54,1	11,0	9,6	4,1	0,7	4,8	7,5	0,7	5,5	0,83
5	35	51,4	12,3	4,8	4,8	3,4	6,2	9,6	1,4	4,1	0,92
(LA1971)	45	45,2	19,2	3,4	0,7	4,8	7,5	8,2	3,4	5,5	1,03
	55	41,1	17,1	8,2	1,4	4,1	4,8	5,5	3,4	12,3	1,19

[a] In brackets, source of resistance
[b] dpi: days postinoculation
[c] Symptom score from 0 (symptomless) to 4 (severe symptoms). See text for further description of the scale

Supplementary Table 4 Recombinants identified in F_2 generations in the region between markers C2_At3g46780 and TAHINA-6-85 in chromosome 6. Disease scores range from 0 (no symptoms) to 4 (severe symptoms)

Solanum chilense source of resistance	Family[1]	Type[2]	C2_At3g46780	SSR48	Aps-1	JB-1	SSR128	C2_At1g21640	SSR578	TAHINA-6-59	TAHINA-6-85	R/S	15 dpi	25 dpi	35 dpi	45 dpi	55 dpi
	1	9-2	a	a	a	a	h	nd				R	0	0.5	0	0	0.5
	1	9-1	a	a	a	a	h	nd				S	3	3	3	4	4
	1	9-2	a	a	a	a	h	nd				R	0	0	0.5	1	1
	1	11	a	a	a	a	h	h				S	2	1	2.5	3	4
	1	12	h	h	h	c	a	a				R	0	0	0	1	1.5
	2	13	a	h	h	c	h	a	a	a	h	R	0	0.5	0.5	0	1
	2	14	a	h	h	c	h	h	h	a	a	R	0.5	0.5	0.5	1	1
	2	15	h	a	a	a	h	h	h	h	h	S	0	2.5	2.5	4	4
	2	16	b	h	h	c	h	h	h	h	a	R	0.5	0.5	0.5	1	1
	2	17	h	h	a	a	a	a	a	a	h	S	0	0	0	0	3
	2	18	a	a	a	a	h	h	h	h	a	R	0	0	0.5	0.5	1.5
	2	19	h	h	h	c	a	a	h	h	h	S	0	1.5	3	3	2.5
	2	20	b	b	b	c	a	a	a	a	h	R	0	0	0	0	0
	2	21	h	h	h	c	a	a	a	a	h	R	0	0.5	1	0.5	0.5
	2	22	h	h	h	c	a	a	a	a	a	R	0	0.5	0	0.5	1
	2	23	b	b	b	c	h	h	h	a	a	R	0	0	0	0.5	0
	2	24	a	a	a	a	a	a	h	h	a	S	0	0.5	4	4	4
	2	25	a	a	a	a	a	a	h	h	h	S	3	2	3	3	4
	2	25	a	a	a	a	a	a	h	h	h	S	0	0.5	0.5	2.5	2.5
LA1932	2	25	a	a	a	a	a	a	h	h	h	S	0.5	3	3	3	3
	2	25	a	a	a	a	a	a	h	h	h	S	0	3	4	4	4
	2	25	a	a	a	a	a	a	h	h	h	S	0	3.5	3.5	4	4
	2	26	h	h	h	c	h	h	a	a	h	R	0	1	1	0.5	0.5
	2	27	a	a	a	a	a	a	a	h	h	S	0	3	2.5	2.5	3
	2	28	h	h	h	c	h	h	h	a	a	R	0	0.5	0.5	1	1
	2	28	h	h	h	c	h	h	h	a	a	R	0	0.5	1	1	0.5
	2	28	h	h	h	c	h	h	h	a	a	R	0	0	0.5	0.5	0
	2	28	h	h	h	c	h	h	h	a	a	R	0	0	0.5	1.5	0.5
	2	28	h	h	h	c	h	h	h	a	a	R	0	0.5	0.5	1	1
	2	29	a	a	a	a	a	a	a	a	h	S	2	3.5	4	4	4
	2	29	a	a	a	a	a	a	a	a	h	S	2	3	3	4	4
	2	29	a	a	a	a	a	a	a	a	h	S	0	0	2.5	4	4
	2	29	a	a	a	a	a	a	a	a	h	S	3	2	3	3.5	4
	2	30	h	h	h	c	h	h	h	h	a	R	0	0	0	0.5	1
	2	30	h	h	h	c	h	h	h	h	a	R	0	0	1	1.5	1
	2	30	h	h	h	c	h	h	h	h	a	R	1.5	1	1.5	1	1.5
	2	30	h	h	h	c	h	h	h	h	a	R	0.5	0.5	1.5	1.5	1.5
	2	30	h	h	h	c	h	h	h	h	a	R	0	0	0	0	0
	2	31	b	b	b	c	b	b	b	b	a	R	1	0	0.5	0	0.5
	2	31	b	b	b	c	b	b	b	b	a	R	0	0	0	1	0

Supplementary Table 4 (continuation)

Solanum chilense source of resistance	Family[1]	Type[2]	C2_At3g46780	SSR48	Aps-1	JB-1	SSR128	C2_At1g21640	SSR578	TAHINA-6-59	TAHINA-6-85	R/S	15 dpi	25 dpi	35 dpi	45 dpi	55 dpi
							Markers[3]							Disease scoring[4]			
LA1960	3	1	a	h	h	c						R	0.5	1.5	0.5	0.5	1
	3	2	h	a	a	a						S	3	4	4	4	3
	3	2	h	a	a	a						S	0	1	2.5	3	3
LA1971	4	7	h	a	a	a	a					S	0	2.5	4	4	4
	4	3	h	h	a	a	nd					S	0	4	4	4	4
	4	10-2	h	h	h	c	a					R	0	0	0	0.5	0.5
	4	10-1	h	h	h	c	a					S	0	1	2.5	3.5	3.5
	4	10-2	h	h	h	c	a					R	0	0	0	0	0
	5	4	a	h	h	c	b					R	0	0	0	0	0
	5	5	a	h	h	c	h					R	0.5	1	1.5	1.5	1.5
	5	6	h	a	a	a	h					S	0	0	2	3	4
	5	8	h	h	a	a	a					S	2	2.5	2.5	2.5	2.5
	5	9-2	a	a	a	a	h					R	0	0.5	0.5	0.5	0.5
	5	9-1	a	a	a	a	h					S	0	0	0	2	2
	5	9-1	a	a	a	a	h					S	2	4	3	3	3
	5	9-2	a	a	a	a	h					R	0	0	0	0	0
	5	10-2	h	h	h	c	a					R	0	0	0	0	0
	5	10-2	h	h	h	c	a					R	0	0	0	0.5	0.5
	5	10-2	h	h	h	c	a					R	0	0	0	0.5	0.5
	5	10-2	h	h	h	c	a					R	0	0	0	0.5	1
	5	10-1	h	h	h	c	a					S	0	1	1.5	3	4

[1] See Materials and methods section for further description of the families

[2] Recombinant type: position of crossing-over - type of allele (see Table 2)

[3] a: homozygous for *Solanum lycopersicum* allele; b: homozygous for *Solanum chilense* allele; h: heterozygous; c: either b or h; nd: not determined

[4] R/S: classification of each plant as resistant (R: symptom scoring < 2 in all evaluation dates) or susceptible (S: Symptom scoring $\geq$ 2 in one or more evaluation dates); dpi: days postinoculation

Supplementary Table 5 Viral accumulation (ng viral DNA/μg total DNA extracted) after whitefly-mediated inoculation with *Tomato yellow leaf curl virus* in different generations of family 5 (LA1971-derived), according to the genotype for marker Aps-1 on chromosome 6

Generation	Genotype[a]	N[b]	15 dpi[c]	25 dpi	35 dpi	45 dpi	55 dpi
F_2	a	44	2.00 a	6.53 a	8.66 a	6.17 a	20.27 a
	h	76	1.77 a	1.87 b	3.48 b	2.71 b	10.14 b
	b	23	1.74 a	0.99 b	2.88 b	1.26 c	6.49 b
BC_S	a	32	3.15 a	4.85 a	7.98 a	5.66 a	19.51 a
	h	57	4.18 a	2.39 b	4.40 b	2.41 b	11.16 b
BC_R	h	31	0.41 a	1.27 a	2.93 a	2.72 a	10.54 a
	b	42	0.88 a	1.95 a	4.53 a	2.11 a	6.00 b

[a] Genotype: a, homozygous for *Solanum lycopersicum allele*; h, heterozygous; b, homozygous for *Solanum chilense* allele

[b] Number of plants

[c] dpi: days postinoculation. Different letters in the same column and for each family, represent statistically significant differences (*P*<0.05) based on LSD test.

Fine mapping of *Tomato yellow leaf curl virus* resistance genes derived from *Solanum chilense* LA1932, LA1960 and LA1971 to the *Ty-1/Ty-3* region

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ABSTRACT

Tomato yellow leaf curl disease (TYLCD) is one of the most devastating diseases affecting cultivated tomato. This disease is caused by a complex of virus belonging to the family Geminiviridae, being the *Tomato yellow leaf curl virus* (TYLCV) the most important. The resistance gene *Ty-1*, derived from *Solanum chilense* LA1969, has been the most widely used in breeding. However, it is of interest to incorporate in breeding programs resistance from other sources. In a previous study, lines with resistance to TYLCV derived from *S. chilense* accessions LA1932, LA1960 and LA1971 were developed. Resistance in these lines is controlled by a major dominant gene located on chromosome 6, in an overlapping region of 25 cM including the *Ty-1/Ty-3* region. The objective of this work was to fine map these resistance genes by analyzing segregating generations derived from these sources. A total of 13 markers distributed on chromosome 6 allowed identifying 66 recombinants and located the resistance gene locus in our populations in the *Ty-1/Ty-3* region. These results indicate that closely linked genes or alleles of the same gene govern the TYLCV resistance in several *S. chilense* accessions. Alternatively, the resistance found in our populations could be conferred by new alleles/genes different from *Ty-1* and *Ty-3*, maybe with different specificities. Suppression of recombination was found for the chromosomal region near the *Ty-1/ Ty-3* region. This phenomenon was also observed in other studies in generations derived from interspecific crosses between cultivated tomato and *S. chilense*.

Key words: TYLCV, *Solanum chilense*, tomato, fine mapping, molecular markers

INTRODUCTION

Tomato yellow leaf curl disease (TYLCD) is one of the most devastating diseases in the cultivated tomato (*Solanum lycopersicum* L.) causing production losses up to 100% in tropical and subtropical regions in the world (Cohen and Lapidot 2007). The most typical symptoms are leaf yellowing and curling, stunting of the plant and abscission of flowers and fruits (Cohen and Lapidot 2007). All the viral species causing this disease belong to the family *Geminiviridae*, being the main viral species the *Tomato yellow leaf curl virus* (TYLCV), a monopartite begomovirus, transmitted by the whitefly *Bemisia tabaci* (Genn.) (Czosnek 2008).

Development of tomato TYLCV resistant cultivars is an important approach to cope with this disease. All *S. lycopersicum* germplasm has been described as susceptible. Therefore, breeding for resistance has been focused on introgression of resistance genes from wild tomato relatives like *S. habrochaites*, *S. pimpinellifolium*, *S. cheesmaniae*, *S. peruvianum* and *S. chilense* (Picó et al. 1996; Ji et al. 2007; Scott 2007).

Five resistance genes have been identified from *S. chilense*, *S. habrochaites* and *S. peruvianum*. The first major TYLCV resistance gene mapped was *Ty-1*, derived from *S. chilense* accession LA1969. This gene, located on chromosome 6, is dominant and with at least two modifier genes located on chromosomes 3 and 7 (Zamir et al. 1994). Another resistance gene, *Ty-3*, also mapped on chromosome 6, was identified in advanced breeding lines derived from *S. chilense* accessions LA1932 and LA2779. This gene, in contrast to *Ty-1* that is almost completely dominant has a nearly equal contribution of additive and dominance effects (Ji et al. 2007). The *Ty-4* minor resistance gene, mapped on the long arm of chromosome 3, was also identified in accession LA1932 (Ji et al. 2009a). Other resistance genes, *Ty-2* (located on the short arm of chromosome 11) and *Ty-5* (mapped on chromosome 4) have been identified in lines derived from *S. habrochaites* (Hanson et al. 2006; Ji et al. 2009b) and *S. peruvianum* (Anbinder et al. 2009), respectively. A recessive gene from the cultivar Tyking has been located in the same region as *Ty-5* and the symbol *ty-5* has been proposed (Hutton et al. 2012). Four recessive QTLs have been detected on chromosomes 4, 6, 10, and 11, respectively, in the tomato line FLA456, derived from *S. chilense* LA2779 and Royal Sluis tomato hybrid Tyking (Kardivel et al 2013).

Different studies point at *S. chilense* species as the most effective TYLCV resistance source and, concretely, *S. chilense* accession LA1969 has been the most widely used in breeding programs worldwide since it exhibits the highest level of resistance (e.g. Ji et al. 2007). The major gene *Ty-1* derived from *S. chilense* LA1969 was firstly mapped in the pericentromeric region of chromosome 6, near the RFLP markers TG297 and TG97 (Zamir et al. 1994). Further studies have contributed to a better defined genetic position of the *Ty-1* locus (Milo 2001; Pérez de Castro et al. 2007). A more recent study by Verlaan et al. (2011) demonstrated that *Ty-1* is located on the long arm of tomato chromosome 6, between markers MSc05732-4 and MSc05732-14, an interval of approximately 600 Kb that is nearly 5 Mb below the position reported by Zamir et al. (1994). This study attributes the imprecise location of *Ty-1* in previous works to the low marker coverage in

combination with a severe recombination suppression in the *Ty-1* region. This suppression is partly caused by chromosomal rearrangements between *S. chilense* LA1969 and *S. lycopersicum* in the pericentromere heterochromatic regions of both the short and long arms of chromosome 6 (Verlaan et al. 2011).

Ty-3, a begomovirus resistance gene derived from *S. chilense* accessions LA1932 and LA2779, was mapped on the long arm of tomato chromosome 6 (Ji et al. 2007; Hutton et al. 2010). Verlaan et al. (2011) showed that the introgression carrying *Ty-3* in a line derived from LA2779 partly overlapped with the chromosomal region where the *Ty-1* gene is located. Recombination was also severely suppressed in the pericentromeric region in the *Ty-3* carrying population, indicating similar rearrangements in LA2779 as in LA1969. Recently, *Ty-1* and *Ty-3* have been fine mapped to a region of approximately 70 kb and it has been shown by Virus Induced Gene Silencing (VIGS) that they are allelic (Verlaan et al. 2012, 2013).

At present, most of the commercial hybrids resistant to TYLCV are carriers of the *Ty-1* gene. However, this resistance can be overcome under a high inoculum presure. Therefore, it is of interest to select other resistance sources different from LA1969 to develop highly TYLCV resistant lines. With this aim, in a previous study, interspecific hybrids resistant to TYLCV were obtained from *S. chilense* accessions LA1932, LA1960 and LA1971 (Picó et al. 1999). Different breeding lines fixed for resistance to TYLCV were derived from these interspecific hybrids. Inheritance studies of the resistance in several families developed from these lines showed that the resistance to TYLCV is controlled by a major dominant gene on chromosome 6 in an overlapping region of 25 cM including the *Ty-1*/*Ty-3* region (Pérez de Castro et. al. 2012). These lines represent an interesting source of resistance.

The purpose of this study was to fine map the resistance identified in the breeding lines derived from *S. chilense* accessions LA1932, LA1960 and LA1971 on chromosome 6. Mapping was carried out by analyzing segregating generations derived from these lines. Our results show that the major resistance gene in our populations is located in the chromosomal region where *Ty-1* and *Ty-3* are mapped. Suppression of recombination was found and may be a general phenomenon for the chromosomal region proximal to the *Ty-1* and *Ty-3 loci* in interspecific crosses between cultivated tomato and *S. chilense* accessions.

MATERIALS AND METHODS

Plant material

In a previous study, interspecific hybrids between *S. lycopersicum* and *S. chilense* accessions LA1932, LA1960 and LA1971 were developed (Picó et al. 1999). Hybrids derived from LA1932 were backcrossed one time followed by four selfing generations. The BC_1S_4 generation was backcrossed again to *S. lycopersicum* and selfed two more times (Fig. 1). Hybrids derived from the accessions LA1960 and LA1971 were backcrossed one time and selfed four times. The breeding line Fortuna C (FC) was used as susceptible recurrent parent. In each generation, plants were selected based on absence of TYLCD symptoms, fertility and phenotypical similarity with cultivated tomato. As a result, several lines fixed for resistance to TYLCD were obtained. Four of these lines (two derived from LA1932, one derived from LA1960 and one derived from LA1971) were used to generate different F_2 populations. Another line, derived from accession LA1971, was used as resistant parent to develop a complete family: parentals, F_1, F_2, backcross to the susceptible parent (BC_{1S}) and a backcross to the resistant parent (BC_{1R}) (Table 1). The susceptible parent used for these families was FC (Pérez de Castro et al. 2012).

Disease test

Disease tests were carried out by whitefly-mediated inoculation as previously described by Pérez de Castro et al. (2012). Briefly, plants were inoculated at 3-4 true-leaf stage during seven days in a climatic chamber. After this period, plants were transplanted in a greenhouse until the end of the assay. Symptom severity was scored at 15, 25, 35, 45 and 55 days post inoculation using a scale (Friedmann et al. 1998) from 0 (no visible symptoms), 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 symptoms; plants cease to grow) (Fig. 2). Results obtained in previous works indicate a moderate to high level of resistance present in our material. This resistance results in a reduction of symptom severity, which is positively correlated with viral titer decrease (Pérez de Castro et al. 2012).
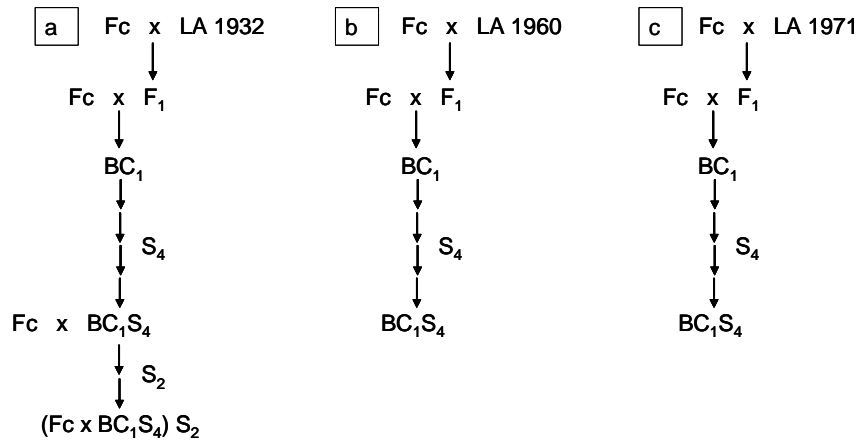
Fig. 1 Development of resistant parental lines derived from a cross between the tomato breeding line Fortuna C (FC, susceptible) and *Solanum chilense* accessions LA1932 (A), LA1960 (B) and LA1971 (C) (Picó et al., 1999). BC: backcross generation with selection; S: selfing generation with selection

| Family[1] | Susceptible parent | Resistant parent [2] | *Solanum chilense* accession | Number of plants per genotype used in the disease test[3] | | | | | |
				P_1	P_2	F_1	F_2	BC_{1S}	BC_{1R}
1	Fortuna C	(FC x BC_1S_4)S_2	LA1932	25	23		155		
2	Fortuna C	(FC x BC_1S_4)S_2		25	18		113		
3	Fortuna C	BC_1S_4	LA1960	25	9		155		
4	Fortuna C	BC_1S_4	LA1971	25	0		116		
5	Fortuna C	BC_1S_4		25	8	9	143	89	73

Table 1 Progenies derived from *Solanum chilense* LA1932, LA1960 and LA1971

[1] Families 1 and 2, as well as 4 and 5 were derived from different plants of the same population
[2] FC: susceptible parent; BC: backcross generation with selection; S: selfing generation with selection (see Figure 1 for details). Subscript numbers after BC and S indicate generations
[3] P_1: susceptible parent; P_2: resistant parent; BC_1S: backcross to the susceptible parent; BC_1R: backcross to the resistant parent

Fig. 2 Representative pictures of disease scores. Symptom severity scale (Friedmann et al., 1998) from 0 (no symptoms) to 4 (severe symptoms) (see text for description)

Markers

Leaf tissue samples were harvested and total DNA was extracted from fresh tissue following the protocol described by Doyle and Doyle (1990).

A total number of 13 PCR-based markers were used in this study for the initial genotyping (Table 2). This marker set consisted of two Simple Sequence Repeat (SSR) and 11 Cleaved Amplified Polymorphic Sequence (CAPS) markers. Polymerase chain reaction (PCR) amplification was performed according to standard protocols. For SSR markers consisted of an initial incubation at 94ºC 5 min, 30 cycles of denaturation at 94ºC 30 s, annealing at 50ºC 30 s, and elongation at 72ºC 1 min, with a final elongation step at 72ºC 10 min. PCR for CAPS markers consisted of an initial incubation at 95ºC 3 min, 35 cycles of denaturation at 95ºC 30s, annealing at temperatures between 50-60ºC 1 min, and elongation at 72ºC (either 1 or 2 min), with a final elongation step at 72ºC 7 min (Table 2). SSR markers were analyzed on a LICOR 4300 DNA sequencer. Digestion products of CAPS markers were analyzed by 1.5% agarose gel electrophoresis and visualized by GelRed (Biotium) staining.

Linkage analysis

Linkage maps were constructed using JoinMap4 and all mapped distances were calculated with the Kosambi mapping function (Kosambi 1944).

Table 2 Markers used in this study

Name[1]	Sequence 5'- 3'	Annealing temperature	Elongation time	Restriction enzyme	Reference[2]
C2_At3g46780	ATGGCTCCAACTCTTACTTCAAATTC TCTGCATCTTGAAATGATGATGCAAC	55°C	2'	-	SGN
SSR48	ATCTCCTTGGCCTCCTGTTT GTCATGGCCACATGAATACG	50°C	1'	-	SGN
C2_At5g61510	AGTTCCTACTGCGCCGCTGCTTC AGCATGAACAAGTACTGTGTGCCCACG	55°C	2'	*Hinf*I	SGN
M-H040F08	AATTACCGCTTCCTCCAGGT AATGTCTCCCCAAACAGCAC	60°C	1'	*Hpy*CH4IV	Verlaan et al. (2011)
T1563	ACTTCACCTACAAATCCTTCCAGA GCCCTTCCCAATCCAGCAGT	56°C	2'	*Taq*I	Ji et al. (2007)
M-M026P18	GCATGTGTGCAGCTCACTCTCCC TCAAGTCCGAATCGAAGCCCCA	60°C	1'	*Alu*I	Verlaan et al. (2011)
M-M082G10	GGCATCGCCATCATCTCTAAGTCCA GCCTCAACCTACTGCCTTGCAAAT	60°C	1'	*Fsp*B1	Verlaan et al. (2011)
SSR6/18	GCGATATTTTGCTTTTTCACTT AAAAATTATTATGAGATGCAAATCAAC	50°C	1'	-	Present
MSc05732-4	ACGAGATGGAGCGGTCTTCAAGCT GACAGATCTCCCGGTAGGAGAGCA	55°C	1'	*Dde*I	Verlaan et al. (2011)
PG3	ATGACTCCAACAAGCAAAGGCACGAG AAAGAGAAGCTGCAATGTGTCGCC	55°C	2'	*Hinf*I	Ji et al. (2007)
MSc05732-18	TTGAGTCTGGCCTGCTCTGAATCT CATTCTGCTCGTCTTCAGAACACCTC	55°C	1'	*Alu*I	Verlaan et al. (2011)
G8	CATCCCGTGCATCATCCAAAGTGAC CTAAGGGTGTACCCCAAGGGAAC	55°C	2'	*Taq*I	Maxwell et al. (2007)
M-M005H10	AAATCACCTTCCACAGTGCAG CTGGCCATAAAGTCTGGACAA	55°C	1'	*Rsa*I	Verlaan et al. (2011)

[1]SSR: Simple Sequence Repeat; the other markers used in this study were Cleaved Amplified Polymorphic Sequence (CAPS) markers
[2]SGN: Sol Genomics Network (solgenomics.net)

RESULTS

In our previous work, TYLCV resistance was found in *Solanum chilense* accessions LA1932, LA1960 and LA1971 (Picó et al., 1999). Resistance in each accession was controlled by a major gene located on the long arm of chromosome 6 in an overlapping region of 25 cM including the *Ty-1*/*Ty-3* region (Pérez de Castro et al. 2012). In this study, we used multiple markers on chromosome 6 to fine map the resistances identified in these accessions.

Suppression of recombination

The region analized corresponds to a physical distance of approximately 31 million base pairs (mbp) spanning between markers C2_At3g46780 and M-M005H10 (Tomato

WGS Chromosomes SL2.40). A total number of 66 recombinants were found in this study, corresponding to a genetic distance of 4.0, 4.0 and 5.2 cM in families derived from LA1932, LA1960 and LA1971, respectively (Fig. 3).

The genetic maps derived from these populations were compared with the tomato reference map (Tomato-EXPEN 2000) (Fulton et al. 2002). The linear order of common markers (C2_At3g46780, SSR48, C2_At5g61510 and T1563) was the same in all maps and markers positions in these maps were coherent with markers positions on the tomato physical map (Fig. 3, Supplementary Table 1).

Compared with the tomato reference map, certain marker regions were identified where suppression of recombination was obvious. Recombination rates for the *S. chilense* introgressed segment spanning from C2_At3g46780 to C2_At5g61510 markers were similar to the rates of the tomato reference map (Fig. 3). Genetic distance between C2_At3g46780 and SSR48 markers was estimated to be between 1 and 1.7 cM, comparable to the distance of 2 cM in the tomato reference map. As for genetic distance between SSR48 and C2_At5g61510 markers, it was estimated to be between 0.1 and 0.6 cM, being 0.2 cM in the reference map. Physical distance in these two intervals was around 2 mbp and 15 mbp, respectively. Within the analized region, no recombinants were identified between markers C2_At5g61510 and M-H040F08, corresponding to a physical distance of around 1.2 mbp. Reduction of recombination was observed for all populations in the region between markers C2_At5g61510 and M-M005H10. Genetic distance in our populations was estimated to be between 2.2 and 2.9cM, an eight and six fold reduction if compared with the distance of 18.3 cM from the tomato reference map.

Mapping of the TYLCV resistance *loci*

In families 1 and 2 (derived from LA1932), 22 recombinants were found. The shortest *S. chilense* introgression in the resistant plants was in recombinant type 5-1 (Table 3, Supplementary Table 1). This recombinant carries a heterozygous introgression starting below M-M026P18 to M-M005H10. Since the recombinant was resistant, the resistance *locus* must be located below the marker M-M026P18. This prediction is supported by recombinants of type 5-2, which are homozygous for *S. lycopersicum* in that region and were susceptible. Recombinant type 9-1, which was susceptible and has a heterozygous introgression below the marker MSc05732-18, pinpoints the resistance *locus* is proximal to

G8. Thus, the markers M-M026P18 and G8 flank the resistance *locus* identified in LA1932 (Fig. 3).

The resistance locus in family 3 (derived from LA1960), was fine mapped with the information provided by recombinants of type 5-2 and type 8-4 (Table 3, Supplementary Table 1). Recombinant of type 5-2 was susceptible and homozygous for *S. lycopersicum* in the region below marker M-M026P18, showing that this marker is the upper limit for the interval containing the resistance locus. These results coincide with those obtained for LA1932-derived resistance. The lower border was delimited by the resistant recombinant type 8-4. This recombinant was resistant and is heterozygous for the *S. chilense* introgression including MSc05732-4 and the region above this marker (genotype for marker PG3 was not determined in this recombinant). Thus, according to the results available, the resistance locus derived from LA1960 is located in the region bordered by markers M-M026P18 and MSc05732-18.

The shortest marker interval for the resistance gene was obtained for families 4 and 5 (both derived from LA1971) (Table 3, Supplementary Table 1). In family 4, one recombinant of type 8-3 was identified, being susceptible and homozygous for *S. lycopersicum* allele in the region situated below marker MSc05732-4. Moreover, recombinants of type 8-1 allowed the shortening of the interval for the resistance *locus*. Two out of the 20 recombinants found in family 5 were type 8-1. These recombinants were homozygous for *S. lycopersicum* in the region above marker PG3, and heterozygous for the region below. As one of these recombinants was susceptible and the other one was resistant, the resistance gene should thus be located between markers MSc05732-4 and PG3 (Fig. 3). This region is within the chromosomal interval for the resistance *loci* identified in LA1932 and LA1960 (Fig. 3).

In summary, in families derived from *S. chilense* accession LA1932 (family 1 and family 2), the proposed resistance gene location was between markers M-M026P18 and G8. The marker interval that delimits the resistance gene region in the family derived from accession LA1960 (family 3) was shorter, concretely between markers M-M026P18 and MSc05732-18. The shortest marker interval for the resistance gene was obtained with families derived from accession LA1971 (family 4 and family 5). Analysis in those families located the putative resistance *locus* between markers MSc05732-4 and PG3. The region for the resistance genes partially overlaps in all analized families (Fig. 3).
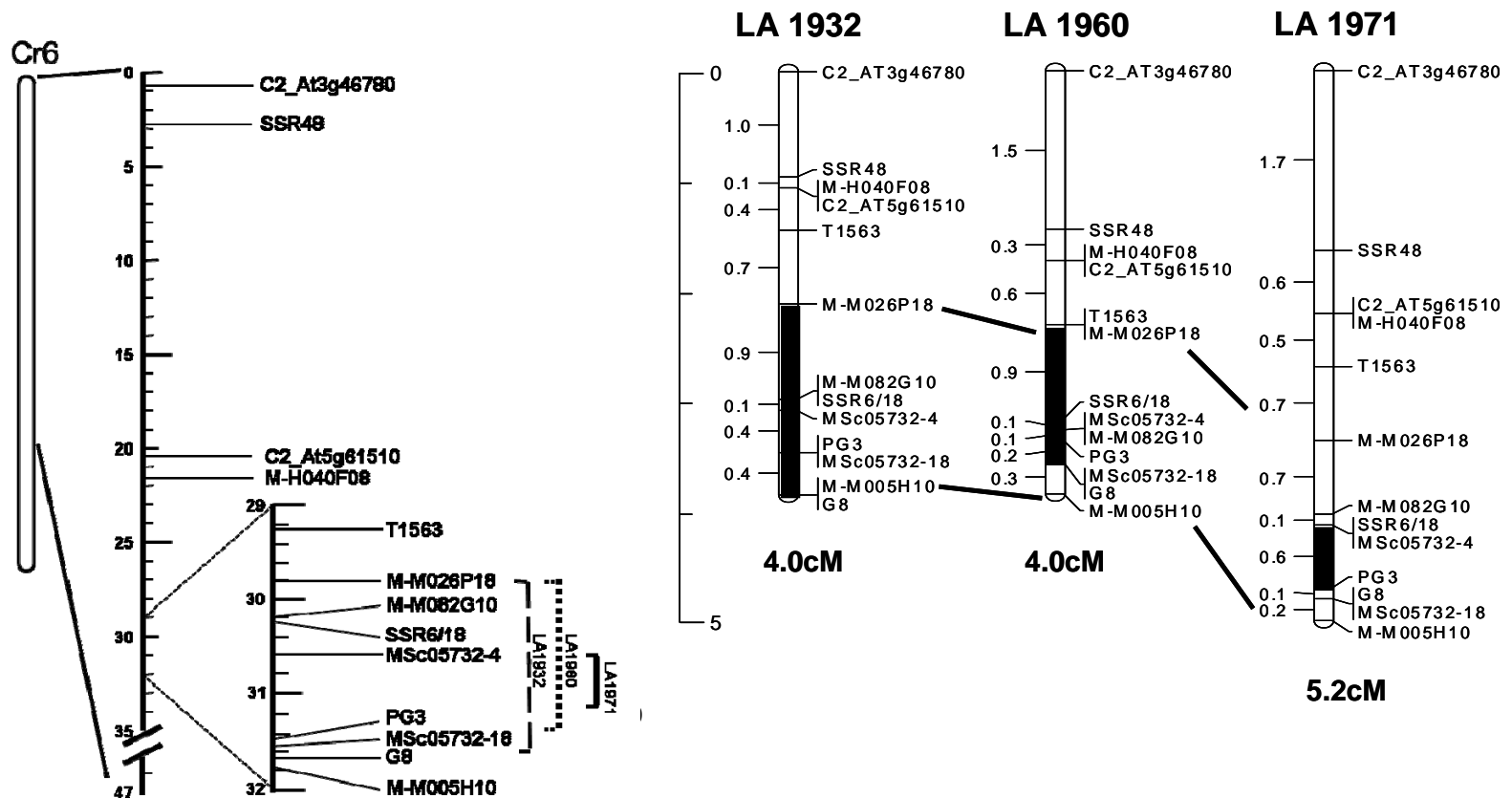
Fig. 3 Physical and genetic maps of the short arm, the centromere and a part of the long arm of chromosome 6 (Left) Physical map positions of the markers used in the present study, based on Tomato WGS Chromosomes (SL2.40) database. The numbers on the left of the map represent million base pairs (mbp). TYLCV major resistance gene location for *S. chilense* derived families is indicated with lines (▬ ▬ ▬ LA1932 derived families) (▪▪▪▪ LA1960 derived family) (▬▬▬ LA1971 derived families). (Right) Genetic map positions of the markers used in present study. The numbers on the left of the map represent cM. TYLCV major resistance gene location for *S. chilense* derived families is indicated by shaded regions.

Table 3 Recombinants identified on chromosome 6 in the region flanked by markers C2_At3g46780 and M-M005H10

C2_At3g46780	SSR48	C2_At5g61510	M-H040F08	T1563	M-M026P18	M-M082G10	SSR6/18	MSc05732-4	PG3	MSc05732-18	G8	M-M005H10	Phenotype[2]	Recombinant type[3]	LA 1932 Family 1 n=155 F2	LA 1932 Family 2 n=113 F2	LA 1960 Family 3 n=155 F2	LA 1971 Family 4 n=116 F2	LA1971 Family 5 n=143 F2	LA1971 Family 5 n=89 BC1S	LA1971 Family 5 n=73 BC1R
a	h	h	h	h	h	h	h	h	h	h	h	h	R	1-1	-	2	1	-	2	-	-
h	a	a	a	a	a	a	a	a	a	a	a	a	S	1-2	-	1	2	1	-	-	-
b	h	h	h	h	h	h	h	h	h	h	h	h	R	1-3	-	1	-	3	-	-	2
h	b	b	b	b	b	b	b	b	b	b	b	b	R	1-4	1	-	-	-	1	-	-
h	h	a	a	a	a	a	a	a	a	a	a	a	S	2-1	-	1	-	1	1	-	-
b	b	h	h	h	h	h	h	h	h	h	h	h	R	2-2	-	-	1	1	-	-	1
a	a	a	a	h	h	h	h	h	h	h	h	h	R	3-1	1	1	1	-	-	-	-
b	b	b	b	h	h	h	h	h	h	h	h	h	R	3-2	-	-	1	1	-	-	-
h	h	h	h	b	b	b	b	b	b	b	b	b	R	3-3	-	-	-	1	-	-	1
a	a	a	a	a	h	h	h	h	h	h	h	h	R	4-1	1	-	-	1	-	-	-
h	h	h	h	h	a	a	a	a	a	a	a	a	S	4-2	-	-	-	-	-	1	-
b	b	b	b	b	h	h	h	h	h	h	h	h	R	4-3	-	3	-	-	-	-	-
h	h	h	h	h	b	b	b	b	b	b	b	b	R	4-4	-	-	-	3	-	-	-
a	a	a	a	a	a	h	h	h	h	h	h	h	R	5-1	**1**	-	-	-	1	-	-
h	h	h	h	h	h	a	a	a	a	a	a	a	S	5-2	-	**2**	1	-	-	1	-
b	b	b	b	b	b	h	h	h	h	h	h	h	R	5-3	-	-	2	-	-	-	-
h	h	h	h	h	h	b	b	b	b	b	b	b	R	5-4	-	2	-	-	-	-	2
h	h	h	h	h	h	h	a	a	a	a	a	a	S	6-1	-	-	-	-	1	-	-
h	h	h	h	h	h	h	h	b	b	b	b	b	R	7-1	-	1	-	-	-	-	-
a	a	a	a	a	a	a	a	a	h	h	h	h	S	8-1	-	-	-	-	**1**	-	-
a	a	a	a	a	a	a	a	a	h	h	h	h	R	8-1	-	-	-	-	**1**	-	-
a	a	a	a	a	a	a	a	a	nd	h	h	h	S	8-2	-	-	-	-	1	-	-
h	h	h	h	h	h	h	h	h	a	a	a	a	S	8-3	-	-	-	1	-	-	-
h	h	h	h	h	h	h	h	h	nd	a	a	a	R	8-4	-	-	1	-	-	-	-
h	h	h	h	h	h	h	h	h	b	b	b	b	R	8-5	1	1	-	1	-	-	-
a	a	a	a	a	a	a	a	a	a	h	h	h	S	9-1	**1**	-	-	-	-	-	-
b	b	b	b	b	b	b	b	b	b	h	h	h	R	9-2	1	-	-	-	-	-	-
h	h	h	h	h	h	h	h	h	h	h	h	a	R	10-1	-	-	-	-	1	-	-
b	b	b	b	b	b	b	b	b	b	b	b	h	R	10-2	-	-	1	-	-	-	-
h	a	a	a	a	a	a	a	a	a	a	a	h	S	11-1	-	-	-	-	1	-	-

| 17 | 6 | 0 | 7 | 9 | 12 | 1 | 1 | 8 | 0 | 2 | 3 | number of recombinants between each pair of markers |
| 2,0 | 17,5 | 1,2 | 7,7 | 0,5 | 0,4 | 0,02 | 0,38 | 0,95 | 0,1 | 0,1 | 0,1 | mbp |

[1] a: homozygous for *Solanum lycopersicum* allele; b: homozygous for *Solanum chilense* allele; h: heterozygous; d: either a or h; nd: not determined

[2] R/S: classification of each plant as resistant (R: symptom scoring < 2 in all evaluation dates) or susceptible (S: Symptom scoring ≥ 2 in one or more evaluation dates)

[3] code for recombinants: position of crossing-over - type of allele

[4] BC1S: backcross to the susceptible parent; BC1R: backcross to the resistant parent; n: number of plants; numbers highlighted in black are the recombinants aforementioned in text (for more detailed information see Online Resource 1)
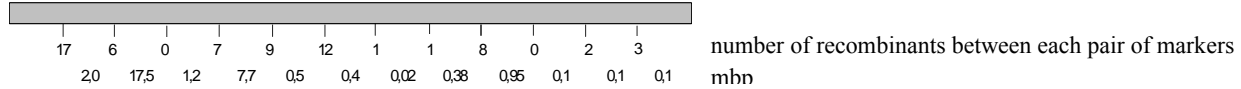
DISCUSSION

Several TYLCV resistance genes have been identified in various wild tomato relatives. Currently, most of the resistant commercial hybrids carry the *Ty-1* gene as it confers a high level of resistance. However, *Ty-1* resistant plants can show slight to moderate symptoms under a high inoculum pressure. Undesirable characteristics are also attributed to the presence of this gene due to linkage drag effects. In previous studies we found resistance to TYLCD in the *S. chilense* accessions LA1932, LA1960 and LA1971 (Picó et al. 1999) and showed that the resistance derived from these accessions is controlled by a major dominant gene on chromosome 6 in an overlapping region of 25 cM including the *Ty-1/Ty-3* region (Pérez de Castro et al. 2012). In this study, the genetic location of the resistance identified in each population is delimited to the *Ty-1/Ty-3* region.

Closely linked genes or alleles of the same gene govern the TYLCV resistance in several *S. chilense* accessions

In the present study, analysis of populations derived from accessions LA1932, LA1960 and LA1971 with several molecular markers located the putative major TYLCV resistance locus to the *Ty-1/Ty-3* region on chromosome 6 (Fig. 3). Previous work located the *Ty-1* gene in a region of approximately 600 kb flanked by markers MSc05732-4 and MSc05732-14 (Verlaan et al. 2011), partially overlapping with the reported *Ty-3* region (Hutton et al. 2010). Recently both genes were fine mapped to a region of approximately 70 kb and it has been shown by Virus Induced Gene Silencing (VIGS) that *Ty-1* and *Ty-3* are allelic (Verlaan et al. 2013). The region containing *Ty-1* and *Ty-3* is also included in the interval between markers M-M026P18 and G8, the longest interval for the resistance gene in the family derived from LA1932, and in the shorter interval between markers MSc05732-4 and PG3 for the family derived from LA1971. Together, these data suggest the possibility of the existence of a common TYLCV resistance locus in *S. chilense* accessions LA1969, LA1932, LA2779, LA1960 and LA1971. Future work will include the use of VIGS approach to determine if the resistance genes present in our lines are also allelic to *Ty-1* and *Ty-3*. Genes with multiple alleles controlling the resistance to different pathogens have been identified in previous studies by other authors. This is the case of the *L* locus of flax, which has several alleles controlling flax rust resistance. These alleles and their different specificities were created by intragenic crossover events (Luck et al. 2000).

The other possibility would be that the TYLCV resistance in these *S. chilense* accessions are controlled by tightly linked homologous genes of the same family. This situation is common in resistance genes such as the *Cf* genes (Thomas et al. 1998) or the *Mla* powdery mildew resistance locus (Wei et al. 1999).

Based on our data we cannot reject the hypothesis that the resistance found in the lines derived from accessions LA1932, LA1960 and LA1971 is conferred by new alleles/genes different from *Ty-1* and *Ty-3*. This is the first study in which genes for resistance to TYLCV have been fine mapped in lines derived from LA1960 and LA1971. The LA1932 advanced breeding lines generated by Scott et al. (1996) were derived from the screening for resistance to ToMoV. Later, they found that these lines were also resistant to TYLCV. In contrast, our LA1932-derived breeding lines were developed by direct selection for resistance to TYLCV and TYLCSV (Picó et al. 1999). Considering the different selection procedures and heterogeneity present in *S. chilense*, we cannot rule out the possibility that in our material alleles conferring TYLCV resistance are different from the one identified by Scott et al. (1996). Given the different resistance spectrum of *Ty-1* and *Ty-3*, it is possible that the resistance genes identified from accessions LA1960 and LA1971 confer resistance to other geminiviruses. These genes should be studied more deeply in order to know their specificity and effectiveness. Combination of different alleles in heterozygous state could lead to the increase of resistance levels and/or broadening the resistance to a wider range of begomoviruses.

Reduction of recombination

One of the main difficulties to fine map and clone these genes is the suppression of recombination. In the present study, recombination rates for the *S. chilense* introgressed segments spanning from C2_At3g46780 to C2_At5g61510 were similar to the rates from the tomato reference map (Tomato-EXPEN 2000 map) (Fulton et al. 2002). If compared with the tomato reference map the recombination is, in all populations analyzed, more than sixfold reduced in the *S. chilense* introgressed segment bordered by the markers M-H040F08 and M-M005H10 (Tomato-EXPEN 2000 map) (Fulton et al. 2002). This phenomenon probably occurred due to sequence divergence between *S. chilense* and the cultivated tomato, as was found in previous studies with this species (Ji et al. 2007; Ji et al. 2009a), and with other wild relatives (Alpert and Tanksley 1996; Monforte and Tanksley

2000; Canady et al. 2005). The most severe reduction of recombination was observed in the region between markers SSR48 and M-H040F08. It is due, at least in part, to a general repression of crossing over around tomato centromeres (Tanksley et al. 1992; Alpert and Tanksley 1996). The suppression of recombination at regions situated physically close to the centromeres was also observed at other *loci* associated with disease resistance in tomato (van Daelen et al. 1993; Ganal and Tanksley 1989). Suppression of recombination in populations derived from *S. chilense* LA1969 and LA2779 was reported, due to chromosomal rearrangements between homoeologous chromosomes (Verlaan et al. 2011). The same rearrangements may be present in *S. chilense* accessions used in this study.

Fine mapping of a gene can become difficult if there is severe recombination suppression because a huge number of plants need to be screened to find enough informative recombinants. Despite the high suppression of recombination observed in the resistance gene region, it was possible to find some recombinants in our populations that allowed fine mapping of these genes in all populations analized in the present study.

Implications for breeding

Results obtained in this study show the presence of TYLCV resistance genes on chromosome 6 in different accessions of *S. chilense*. These genes may be allelic to *Ty-1* and *Ty-3* or can be different homologous genes that are tightly linked. This has important implications in breeding. Firstly, the new genes/alleles should be studied more deeply in order to know their specificity and effectiveness. Later, if they are different genes, they can be combined in different ways with the aim of obtaining the most effective and durable combinations, although it would be difficult due to the close linkage. Alternatively, if they are new alleles of the *Ty-1/Ty-3* genes, they can be combined in heterozygosis in order to join their effects. It could lead to the increase of resistance levels and/or broadening the resistance to a wider range of begomoviruses.

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Supplementary Table 1 Recombinants identified in the region between markers C2_At3g46780 and M-M005H10 in chromosome 6. Disease scores range from 0 (no symptoms) to 4 (severe symptoms)

Solanum chilense source of resistance	Family[1]	Type[2]	C2_At3g46780	SSR48	C2_At5g61510	M-H040F08	T1563	M-M026P18	M-M082G10	SSR6/18	MSc05732-4	PG3	MSc05732-18	G8	M-M005H10	R/S	15 dpi	25 dpi	35 dpi	45 dpi	55 dpi
								Markers[3]										Disease scoring[4]			
LA1932	1	1-4	h	b	b	b	b	b	b	b	b	b	b	b	b	R	0	0	0.5	0	0
	1	3-1	a	a	a	a	h	h	h	h	h	h	h	h	h	R	0	0.5	1	0.5	0.5
	1	4-1	a	a	a	a	a	h	h	h	h	h	h	h	h	R	0	0	0.5	1	1
	1	5-1	a	a	a	a	a	a	h	h	h	h	h	h	h	R	0	0.5	0	0	0.5
	1	8-5	h	h	h	h	h	h	h	h	h	b	b	b	b	R	0	0.5	0.5	0.5	0
	1	9-1	a	a	a	a	a	a	a	a	a	a	a	h	h	S	3	3	3	4	4
	1	9-2	b	b	b	b	b	b	b	b	b	b	b	h	h	R	0	0	0	0	0.5
	2	1-1	a	h	h	h	h	h	h	h	h	h	h	h	h	R	0.5	0.5	0.5	1	1
	2	1-1	a	h	h	h	h	h	h	h	h	h	h	h	h	R	0	0.5	0.5	0	1
	2	1-2	h	a	a	a	a	a	a	a	a	a	a	a	a	S	0	2.5	2.5	4	4
	2	1-3	b	h	h	h	h	h	h	h	h	h	h	h	h	R	0.5	0.5	0.5	1	1
	2	2-1	h	h	a	a	a	a	a	a	a	a	a	a	a	S	0	0	0	0	3
	2	3-1	a	a	a	a	h	h	h	h	h	h	h	h	h	R	0	0	0.5	0.5	1.5
	2	4-3	b	b	b	b	b	h	h	h	h	h	h	h	h	R	0	0	0	0	0
	2	4-3	b	b	b	b	b	h	h	h	h	h	h	h	h	R	0	0.5	0.5	0.5	1
	2	4-3	b	b	b	b	b	h	h	h	h	h	h	h	h	R	0	0.5	0.5	0	0
	2	5-2	h	h	h	h	h	h	a	a	a	a	a	a	a	S	0	1.5	3	3	2.5
	2	5-2	h	h	h	h	h	h	a	a	a	a	a	a	a	S	0	1.5	1.5	3	4
	2	5-4	h	h	h	h	h	h	b	b	b	b	b	b	b	R	0	0	0	0	0
	2	5-4	h	h	h	h	h	h	b	b	b	b	b	b	b	R	0	0	0	0.5	1
	2	7-1	h	h	h	h	h	h	h	h	b	b	b	b	b	R	0	0	0.5	0.5	0.5
	2	8-5	h	h	h	h	h	h	h	h	h	b	b	b	b	R	0	1.5	0.5	0.5	0.5
	2	5-2	h	h	h	h	h	h	a	a	a	a	a	a	a	S	0	1.5	3	3	2.5
	2	5-2	h	h	h	h	h	h	a	a	a	a	a	a	a	S	0	1.5	1.5	3	4
	2	5-4	h	h	h	h	h	h	b	b	b	b	b	b	b	R	0	0	0	0	0
	2	5-4	h	h	h	h	h	h	b	b	b	b	b	b	b	R	0	0	0	0.5	1
	2	7-1	h	h	h	h	h	h	h	h	b	b	b	b	b	R	0	0	0.5	0.5	0.5
	2	8-5	h	h	h	h	h	h	h	h	h	b	b	b	b	R	0	1.5	0.5	0.5	0.5

Solanum chilense source of resistance	Family[1]	Type[2]	C2_At3g46780	SSR48	C2_At5g61510	M-H040F08	T1563	M-M026P18	M-M082G10	SSR6/18	MSc05732-4	PG3	MSc05732-18	G8	M-M005H10	R/S	15 dpi	25 dpi	35 dpi	45 dpi	55 dpi
LA1960	3	1-1	a	h	h	h	h	h	h	h	h	h	h	h	h	R	0.5	1.5	0.5	0.5	1
	3	1-2	h	a	a	a	a	a	a	a	a	a	a	a	a	S	0	1	2.5	3	3
	3	1-2	h	a	a	a	a	a	a	a	a	a	a	a	a	S	3	4	4	4	3
	3	2-2	b	b	h	h	h	h	h	h	h	h	h	h	h	R	1	0.5	0.5	0.5	0
	3	3-1	a	a	a	a	h	h	h	h	h	h	h	h	h	R	0	1	1.5	1	0.5
	3	3-2	b	b	b	b	h	h	h	h	h	h	h	h	h	R	0	0.5	1.5	1.5	1.5
	3	5-2	h	h	h	h	h	h	a	a	a	a	a	a	a	S	0	2	3	3	3
	3	5-3	b	b	b	b	b	b	h	h	h	h	h	h	h	R	0	0	0	0	0
	3	5-3	b	b	b	b	b	b	h	h	h	h	h	h	h	R	0.5	1.5	1	0.5	0
	3	8-4	h	h	h	h	h	h	h	h	h	nd	a	a	a	R	0	0	0.5	1	1
	3	10-2	b	b	b	b	b	b	b	b	b	b	b	b	h	R	0	0	0	0	0.5
LA1971	4	1-2	h	a	a	a	a	a	a	a	a	a	a	a	a	S	0	2.5	4	4	4
	4	1-3	b	h	h	h	h	h	h	h	h	h	h	h	h	R	0	0	0	0	0.5
	4	1-3	b	h	h	h	h	h	h	h	h	h	h	h	h	R	0	0.5	0.5	0.5	0.5
	4	1-3	b	h	h	h	h	h	h	h	h	h	h	h	h	R	0	0	0	0	0.5
	4	2-1	h	h	a	a	a	a	a	a	a	a	a	a	a	S	0	4	4	4	4
	4	2-2	b	b	h	h	h	h	h	h	h	h	h	h	h	R	0	0	0	0	0
	4	3-2	b	b	b	b	h	h	h	h	h	h	h	h	h	R	0	0	0	0	0.5
	4	3-3	h	h	h	h	b	b	b	b	b	b	b	b	b	R	0	0	0	0	0
	4	4-1	a	a	a	a	a	h	h	h	h	h	h	h	h	R	0	0.5	1	0.5	0.5
	4	4-4	h	h	h	h	h	b	b	b	b	b	b	b	b	R	0	0	0	0	0
	4	4-4	h	h	h	h	h	b	b	b	b	b	b	b	b	R	0	0	0	0	0
	4	4-4	h	h	h	h	h	b	b	b	b	b	b	b	b	R	0	0	0	0	0.5
	4	8-3	h	h	h	h	h	h	h	h	h	a	a	a	a	S	0	1	2.5	3.5	3.5
	4	8-5	h	h	h	h	h	h	h	h	h	b	b	b	b	R	0	0	0	0	0

Supplementary Table 1 (continuation)

Solanum chilense source of resistance	Family[1]	Type[2]	C2_At3g46780	SSR48	C2_At5g61510	M-H040F08	T1563	M-M026P18	M-M082G10	SSR6/18	MSc05732-4	PG3	MSc05732-18	G8	M-M005H10	R/S	15 dpi	25 dpi	35 dpi	45 dpi	55 dpi
	5a	5-1	a	a	a	a	a	a	h	h	h	h	h	h	h	R	0	0.5	0.5	0.5	0.5
	5a	6-1	h	h	h	h	h	h	h	a	a	a	a	a	a	S	0	1	1.5	3	4
	5a	8-1	a	a	a	a	a	a	a	a	a	h	h	h	h	S	2	4	3	3	3
	5a	8-1	a	a	a	a	a	a	a	a	a	h	h	h	h	R	0	0	0	0	0
	5a	8-2	a	a	a	a	a	a	a	a	a	nd	h	h	h	S	0	0	0	2	2
	5a	10-1	h	h	h	h	h	h	h	h	h	h	h	h	a	R	0	0	0	0.5	0.5
	5a	11-1	h	a	a	a	a	a	a	a	a	a	a	a	h	S	0	0	2	3	4
LA1971	5b	4-2	h	h	h	h	h	a	a	a	a	a	a	a	a	S	3	3	2	2	2
	5b	5-2	h	h	h	h	h	h	a	a	a	a	a	a	a	S	3	3	2.5	3	3
	5c	1-3	b	h	h	h	h	h	h	h	h	h	h	h	h	R	0	0	0.5	0	0.5
	5c	1-3	b	h	h	h	h	h	h	h	h	h	h	h	h	R	0	0	0	0.5	0.5
	5c	1-3	b	b	h	h	h	h	h	h	h	h	h	h	h	R	0	0	0	0	0
	5c	3-3	h	h	h	h	b	b	b	b	b	b	b	b	b	R	0	0	0	0	0
	5c	5-4	h	h	h	h	h	h	b	b	b	b	b	b	b	R	0	0	0	0	0
	5c	5-4	h	h	h	h	h	h	b	b	b	b	b	b	b	R	0	0	0	0.5	0.5

[1] Families 1 and 2: F_2 generations derived from LA1932; Family 3: F_2 generations derived from LA1960; Families 4 and 5a: F_2 generations derived from LA1971; 5b: F_1 x susceptible parent; 5c: F_1 x resistant parent

[2] Recombinant type (See Table 3 for details)

[3] a: homozygous for *Solanum lycopersicum* allele; b: homozygous for *Solanum chilense* allele; h: heterozygous; d: either a or h; nd: not determined

[4] R/S: classification of each plant as resistant (R: symptom scoring < 2 in all evaluation dates) or susceptible (S: Symptom scoring ≥ 2 in one or more evaluation dates); dpi days post inoculation

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Initial development of a set of introgression lines from *Solanum peruvianum* PI 126944 into tomato: Exploitation of resistance to viruses

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ABSTRACT

Resistance to *Tomato yellow leaf curl virus* (TYLCV) and *Tomato spotted wilt virus* (TSWV), among other diseases, has been reported in *S. peruvianum* PI 126944. Introgression lines (ILs) from *S. peruvianum* PI 126944 into the genetic background of cultivated tomato (*S. lycopersicum*) are being developed. Several generations were derived from three interspecific hybrids previously obtained. A lot of crosses and embryo rescue were required until the third backcross, due to the high degree of incompatibility existing between tomato and PI 126944. Crosses between F_1 plants were made to obtain a pseudo-F_2 generation. The same procedure was followed up to the pseudo-F_6 generation. Additional crosses between plants of different generations were made in order to increase progeny. Of 263 molecular markers tested, 105 were polymorphic between tomato and PI 126944. This set of polymorphic markers consisted of 90 Simple Sequence Repeats (SSRs) and 15 Cleaved Amplified Polymorphic Sequences (CAPs). The amount of the *S. peruvianum* genome was reduced in advancing generations and this was coupled in some cases with a reduction of incompatibility. However, the *S. peruvianum* genome was almost completely represented among the different plants of the most advanced generations. ILs will be basically developed from them. Some of the generations developed were resistant to *Tomato yellow leaf curl virus* (TYLCV) and *Tomato spotted wilt virus* (TSWV).

Key words: embryo rescue, molecular markers, *Solanum lycopersicum*, *Tomato spotted wilt virus*, *Tomato yellow leaf curl virus*

INTRODUCTION

Due to the narrow genetic base of tomatoes (Miller and Tanskley 1990), tomato breeding has been focused for decades on the exploitation of its wild relatives). The use of wild relatives has allowed the identification and introgression of many genes of interest, as well as the construction of mapping populations with sufficient DNA polymorphism. Populations initially used for mapping in self-pollinated crops were F_2/F_3, backcrosses or recombinant inbreds. However, these types of populations have several limitations in the accurate identification and fine mapping of Quantitative Trait Loci (QTLs). These limitations include their low resolution power, the failure to identify QTLs with small effects and the possibility of interactions between two unlinked QTLs, which reduces the difference between the subgroups of the tested QTL. Additionally, in these populations

each plant possesses a large fraction of the wild species genome, affecting their fertility and the expression of yield and some other characteristics (Eshed and Zamir 1995). To avoid these problems other types of populations have been derived, such as backcross recombinant inbred lines (BCRILs, Ramsay et al. 1996) or introgression lines (ILs, Eshed and Zamir 1995). These populations also circumvent the problem of self-incompatibility in the interspecific hybrids, which occurs in crosses with some wild relatives. Self-incompatibility prevents selfing and consequently the construction of populations like recombinant inbred lines (RILs).

In tomato several interspecific breeding populations have been developed: ILs from *S. pennellii* LA716 (Eshed and Zamir 1995), a BC_3 population from *S. peruvianum* LA1708 (Fulton et al. 1997), ILs and backcross inbred lines (BILs) from *S. habrochaites* LA1777 (Monforte and Tanksley 2000), BILs from *S. pimpinellifolium* LA1589 (Doganlar et al. 2002) and RILs from *S. pimpinellifolium* LA2093 (Ashrafi et al. 2009), and ILs from *S. lycopersicoides* LA2951 (Canady et al. 2005). These populations have been used for the identification of many QTLs (Foolad 2007).

S. peruvianum is considered the most variable tomato wild relative. This species is self-incompatible and its use as a female parent in crosses with tomato is prevented by the existence of unilateral incompatibility (Hogenboom 1972). However, this species has been extensively used in breeding due to the identification of accessions with resistance to abiotic and biotic stresses. Many genes have been introgressed into tomato: of interest in the current study is the *Sw-5* gene that confers resistance to *Tomato spotted wilt virus* (TSWV) (Stevens et al. 1992), and the *Ty-5* gene conferring resistance to the Tomato yellow leaf curl disease (TYLCD) (Anbinder et al. 2009). In particular, the accession *S. peruvianum* PI 126944 has been described as resistant to TSWV (Paterson et al. 1989), *Tobacco mosaic virus* (TMV) (Yamakawa and Nagata 1975), *Tomato leaf curl virus* (ToLCV) (Muniyappa et al. 1991), to some species belonging to the virus complex responsible for TYLCD (Picó et al. 1998; Pilowsky and Cohen 2000) and to *Fusarium oxysporum* f.sp. *radicis-lycopersici* (Rowe and Farley 1981). Our group constructed some interspecific hybrids between this accession and tomato, which were resistant to TSWV and TYLCD (Picó et al. 2002).

Diseases caused by TSWV and the complex of TYLCV-like viruses are two of the most devastating diseases that affect tomato cultivation in all tropical and subtropical areas worldwide (Picó et al. 1996; Roselló et al. 1996; Hanssen et al. 2010). Genetic resistance

has been identified for both viruses and transferred to tomato. The most used TSWV resistance gene is *Sw-5*, which was identified in the species *S. peruvianum* (Stevens et al. 1992). Some TYLCD resistance genes have been identified from different wild tomato relatives. *Ty-1* (Zamir et al. 1994), *Ty-3* (Ji et al. 2007) and *Ty-4* (Ji et al. 2009) come from *S. chilense*, *Ty-2* was identified in *S. habrochaites* (Hanson et al. 2006), and *Ty-5* in *S. peruvianum* (Anbinder et al. 2009). Quantitative resistance derived from *S. peruvianum* has also been reported (Pilowsky and Cohen, 1990; Vidavsky et al. 1998). However, these genes are not a definitive solution for both diseases. On one hand, the high variability found in these pathogens often results in the appearance of new isolates able to overcome existing resistance. This is the case for TSWV, for which isolates overcoming the resistance conferred by the *Sw-5* gene have been reported (Aramburu and Martí, 2003). On the other hand, resistance to TYLCD conferred by currently available genes is not completely effective. Moreover, the great variability reported for TYLCV-like species from different geographical areas threatens the durability of TYLCV resistance genes. The use of different resistance genes helps to prevent the development of epidemics. Besides, pyramiding different resistance genes has proven to increase the level of resistance to TYLCD (Vidavsky et al. 1998; 2008).

In order to better exploit the potential of PI 126944 in breeding for disease resistance we initiated a project to construct a set of introgression lines and evaluate advanced generations obtained from the available interspecific hybrids for resistance to TSWV and TYLCD.

MATERIALS AND METHODS

Populations development

Plant material

Plant material consisted of a collection of generations derived from *S. peruvianum* PI 126944 (Fig. 1). In previous work reported d by our group, the tomato line NE-1 was crossed as a female parent to some plants of *S. peruvianum* PI 126944 and three interspecific and self-incompatible hybrids (F_1-A, F_1-B and F_1-E) were obtained by embryo rescue (Picó et al. 2002). Three backcross generations to the old tomato variety Fortuna C (FC) were obtained. Due to strong incompatibility between tomato and *S. peruvianum*, the number of plants generated by backcrossing was limited and did not represent the whole *S.*

peruvianum genome, making it necessary to produce additional crosses. Self-incompatibility did not allow progenies by selfing to be obtained from the interspecific hybrids, so crosses between the hybrids F_1-B and F_1-E were made, obtaining a pseudo-F_2 generation (so called because it did not come from a selfed F_1 plant). The same procedure was carried out until the pseudo-F_6 generation, because of the persistence of self-incompatibility. From the pseudo-F_2 generation, a pseudo-F_2-BC_1 generation was obtained. One pseudo-F_3 generation was also backcrossed twice to tomato, obtaining five pseudo-F_3-BC_1 generations and one pseudo-F_3-BC_2 by embryo rescue. Six pseudo-F_3-BC_1 plants were intercrossed and abundant progeny were obtained. These progeny were backcrossed once to tomato and four plants were obtained from mature seeds. Several crosses between one BC_1 and one pseudo-F_3-BC_1 were also carried out.

Immature embryo rescue

Immature embryo rescue was carried out from fruits three weeks after pollination. Different media were selected depending on the embryo developmental stage (Supplementary Fig. 1). For globular embryos medium 1 was used (4.414 g/L Murashige & Skoog Medium (MS) + Gamborg Vitamins B5, 30 g/L sucrose, 1 g/L yeast extract, 0.8% agar, 2 mg/L 2,4-dichlorophenoxyacetic acid, 1 mg/L 6-benzilaminopurine. The pH was adjusted to 5.7). Globular embryos were cultured inside the opened immature seeds to protect embryos from dehydration. This was performed by making a small cut in the chalazal region and placing the cut side in contact with medium 1. To induce the organogenesis pathway, embryos were kept in the dark for seven days at 24-26ºC. Once the organogenesis pathway was induced, *calli* were transferred to medium 2 (4.414 g/L Murashige & Skoog Medium (MS) + Gamborg Vitamins B5, 20 g/L sucrose, 0.8% agar, 2 mg/L indolacetic acid, 1 mg/L N6-[2-isopentenyl]adenine. The pH was adjusted to 5.7) and were grown in a chamber with fluorescent light (50µmol photons/m^{2}s) for 16 h per day. Heart and abnormal torpedo embryos were found mostly from crosses of pseudo-F_3-BC generations to FC (Supplementary Fig. 1). For these embryos medium 3 was used (4.414 g/L Murashige & Skoog Medium (MS) + Gamborg Vitamins B5, 20 g/L sucrose, 0.8% agar, 0.1 mg/L indolacetic acid. The pH was adjusted to 5.7). Once the plants started to grow from the *callus*, they were transferred to a base medium without growth regulators, medium 4 (4.414 g/L Murashige & Skoog Medium (MS) + Gamborg Vitamins B5, 20 g/L sucrose, 0.8% agar. The pH was adjusted to 5.7).
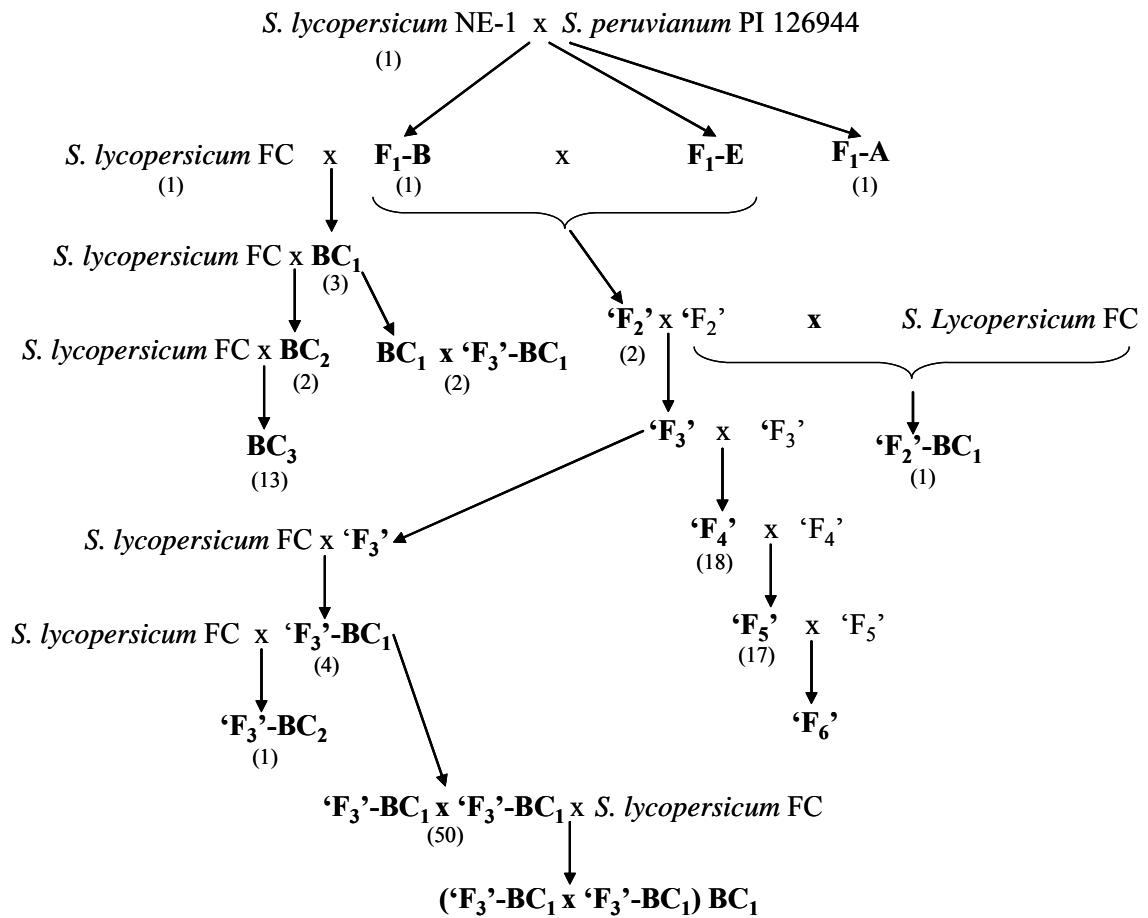
Fig. 1 Populations development. Generations written inside inverted commas are not true F_2, F_3, etc. as they were obtained by crossing different plants instead of self-pollinating. Numbers in brackets indicate the number of plants genotyped of each generation. See text for detailed information

Inoculation and disease assessment

Inoculation trials were conducted to test resistance to TSWV and TYLCV-like viruses.

Plant material

Clonal replicates of the three hybrids developed by Picó et al. (2002) were employed in a first inoculation trial (Inoculation trial 1, IT1, Table 1). Inoculation of pseudo-F_2, pseudo-F_3-BC_1 and intercrosses between pseudo-F_3-BC_1 plants (Inoculation trial 2, IT2) was also carried out (Table 1). In inoculation trials I and II, the tomato line NE-1 was used as susceptible control and accession PI 126944 as resistant control for both

viruses. The TY-197 line, with resistance to TYLCV derived from *S. peruvianum*, was also employed as resistant control. The tomato variety FC was included as susceptible control for both viruses in IT2. In this same trial, the RDD line, homozygous for the *Sw-5* gene, was also used as susceptible control for TSWV isolate GRAU, which overcomes the resistance conferred by *Sw-5* gene (Aramburu and Martí, 2003).

Inoculation and assessment for TSWV

Clonal replicates of each hybrid, pseudo-F_3-BC_1, pseudo-F_3-BC_1 x pseudo-F_3-BC_1 generations and controls were inoculated (Table 1). Two TSWV isolates were used, one not overcoming the resistance conferred by the *Sw-5* gene and the other one overcoming this resistance: HA-931100 (provided by Dr. C. Jordá, Universitat Politècnica de València) in IT1, and GRAU (provided by Dr. J. Aramburu, Institut de Recerca i Tecnologia Agroalimentàries, IRTA, Barcelona) in IT2, respectively. Mechanical inoculation was carried out in a climatic chamber with environmental conditions of 25/18ºC (day/night) temperature, 65/95% (day/night) relative humidity and 65-85 µmol/m^{2}s of irradiance from Sylvania Grolux fluorescent tubes with a wavelength interval between 400 and 700 nm. The photoperiod was 14 light hours. Inoculum was prepared by grinding infected leaves of the susceptible tomato line NE-1 in cold 0.1 M phosphate buffer, pH 7.0, containing 0.2% $Na_2S_2O_5$ and 0.2% sodium diethyldithiocarbamate in a proportion of 1:5 (wt/vol) and 1% 600 mesh Carborundum (Soler et al. 1998). Seven days after the first inoculation plants were inoculated again to avoid escapes. Symptoms were evaluated at 15, 30, 45 and 60 days after the second inoculation. At the same time, samples from inoculated and non-inoculated leaves were harvested and virus presence was detected using DAS-ELISA (Ding et al., 1995). Absorbance of serologic reaction was measured at a wavelength of 405 nm in a Titertek multiscan MCC/340 photometer. Samples with absorbance three times higher than the average absorbance of samples from non-inoculated plants were considered positive or TSWV infected.

Table 1 Inoculation trials. Generations and number of plants evaluated in each trial

	Generation	Procedence	[1]Number of plants tested TYLCV and TYLCSV	TSWV
Inoculation trial 1	F$_1$-A (NE-1 x PI 126944)	Picó et al. 2002	13	15
	F$_1$-B (NE-1 x PI 126944)	Picó et al. 2002	18	15
	F$_1$-E (NE-1 x PI 126944)	Picó et al. 2002	8	15
	Controls			
	Susceptible NE-1	COMAV	3	5
	Resistant TY-197	Lapidot et al. 1997	5	-
	PI 126944	USDA	5	5
Inoculation trial 2	pseudo-F$_2$ (F$_1$-B x F$_1$-E)	Present study	**TYLCV** 80	**TSWV** -
	pseudo-F$_3$-BC$_1$			
	71-1		10	9
	71-2		9	9
	71-3	Present study	9	8
	71-4		9	10
	71-5		9	10
	71-7		10	-
	pseudo-F$_3$-BC$_1$ x pseudo-F$_3$-BC$_1$			
	71-1 x 71-3		7	-
	71-4 x 71-2	Present study	21	19
	71-4 x 71-3		14	15
	Controls			
	Susceptible FC	COMAV	27	12
	NE-1	COMAV	27	11
	RDD	COMAV	-	11
	Resistant TY-197	Lapidot et al. 1997	27	-
	PI 126944	USDA	27	11

[1]Plants tested of each hybrid and of pseudo-F$_3$-BC$_1$ generation were clonal replicates. Pseudo-F$_2$, pseudo-F$_3$-BC$_1$ x pseudo-F$_3$-BC$_1$ generations and controls came from seeds

Inoculation and assessment for TYLCV

Clonal replicates of each hybrid, pseudo-F$_2$, pseudo-F$_3$-BC$_1$, pseudo-F$_3$-BC$_1$ x pseudo-F$_3$-BC$_1$ generations and controls were inoculated (Table 1). Both TYLCV and TYLCSV species were used in IT1, while TYLCV was the species used in IT2. Isolates used were TYLCV-Mld [ES:72:97] (accession L27708) and TYLCSV-ES[2] (accession L27708), kindly provided by Dr. E. Moriones (Estación Experimental "La Mayora", Málaga), and Dr. E. R. Bejarano (Universidad de Málaga), respectively. Agroinoculation at four true-leaves state was used in IT1, following the methodology described by Picó et al. (2002). Plants in IT2 were whitefly-inoculated with *Bemisia tabaci* Genn. biotype Q, (provided by Dr. F. Beitia, Instituto Valenciano de Investigaciones Agrarias, Valencia) inside muslin-covered cages for seven days. Symptom severity was scored at 15, 30, 45 and 60 days post inoculation (dpi). Moreover, virus DNA accumulation was measured on

each date. Leaf tissue samples were harvested and total DNA was extracted following the protocol described by Crespi et al. (1991). Viral DNA was detected by dot-blot and molecular hybridization with specific digoxigenin-labelled probes for TYLCSV and TYLCV provided by Dr. E.R. Bejarano (Universidad de Málaga) and chemiluminescent detection, following the protocol described in "The DIG system user's guide for filter hybridization" of Roche Molecular Biochemicals. Viral DNA was quantified according to a standard curve. Total plant DNA extracted was also quantified by agarose gel electrophoresis using the software Image Gauge V.4.0., to relate viral amounts detected to plant DNA present at each sample.

Genotyping

A total of 117 plants were genotyped, belonging to the following generations: one plant of each tomato parent (NE-1 and FC), F_1-A and F_1-B (F_1-E was not available), three BC_1, two BC_2, 13 BC_3, two pseudo-F_2, 18 pseudo-F_4, 17 pseudo-F_5, one pseudo-F_2-BC_1, four pseudo-F_3-BC_1, one pseudo-F_3-BC_2, two pseudo-F_3-BC_1 x BC_1 and 50 pseudo-F_3-BC_1 x pseudo-F_3-BC_1 generations (Fig. 1). Leaf tissue samples were harvested and total DNA was extracted following the protocol described by Doyle and Doyle (1990).

A total of 263 markers were analyzed. Polymorphism was revealed by 105 out of the marker set (Supplementary Table 1; only the 105 polymorphic ones are shown). The polymorphic marker set consisted of 61 Simple Sequence Repeat (SSR) and 12 Conserved Ortholog Set (COSII) described and mapped in the Sol Genomics Network (http://solgenomics.net/), 29 SSR designed from the sequences available in that database, using the free access programmes WebSat (http://wsmartins.net/websat/) and Primer 3 (http://frodo.wi.mit.edu/primer3/), and three Restriction Fragment Length Polymorphism (RFLP) converted in Cleaved Amplified Polymorphic Sequences (CAPS) (Bai et al. 2004).

For SSR markers polymerase chain reaction (PCR) consisted of an initial incubation at 94ºC 5 min, 30 cycles of denaturation at 94ºC 30 s, annealing at temperatures between 40-60ºC 30 s (see Supplementary Table 1), and elongation at 72ºC 1 min, with a final elongation step at 72ºC 10 min. PCR for COS II and CAPS markers consisted of an initial incubation at 94ºC 5 min, 35 cycles of denaturation at 95ºC 30 s, annealing at temperatures between 55-56ºC 1 min (see Supplementary Table 1), and elongation at 72ºC 2 min, with a final elongation step at 72ºC 10 min.

SSR markers were analyzed on a LICOR 4300 DNA sequencer. Digestion products of CAPS markers were analyzed by 1.5% agarose gel electrophoresis and visualized by GelRed (Biotium) or ethidium bromide staining.

RESULTS

Population development

In previous work by our group, crosses between tomato and some plants of *S. peruvianum* PI 126944 were made. From these crosses, three interspecific and self-incompatible hybrids (F_1-A, F_1-B and F_1-E) were obtained by embryo rescue (Picó et al. 2002). In the present work, several generations derived from these hybrids were developed (Fig. 1). Due to the high degree of incompatibility between *S. lycopersicum* and PI 126944, a lot of crosses and embryo rescue were required to obtain the backcross generations (Supplementary Table 2). In the first backcross, a total number of 129 embryos were obtained from 65 fruits and four of them developed into plants. Most of the embryos were rescued at globular stage; a few of them reached the heart or torpedo stages. From the second and third backcross (BC_2 and BC_3), some mature fruits produced a few viable seeds, although embryo rescue was also employed to obtain progeny. In the BC_2, most embryos developed up to the torpedo stage.

As only a few plants were obtained by direct backcrosses, additional crosses were made in order to increase the number of descendants. Self-incompatibility did not allow progeny to be obtained by selfing, so crosses between pseudo-F_2 pants were made (and this strategy was used until the pseudo-F_6 generations). A high degree of incompatibility was also found in crosses between pseudo-F_2 plants. From more than one hundred pseudo-F_2 plants, only 19 pseudo-F_3 generations were obtained from crosses involving 20 pseudo-F_2 plants. These 20 pseudo-F_2 plants showed different levels of incompatibility: some of them were compatible with several plants while others were only compatible with one or two of the plants. Plants of the 19 pseudo-F_3 generations obtained were intercrossed in many combinations to obtain pseudo-F_4 generations. Only three pseudo-F_3 plants were involved in crosses that produced the pseudo-F_4 progeny.

In an attempt to continue with the introgression of PI 126944 into the tomato genetic background, a lot of crosses between tomato and 10 pseudo-F_3 plants were also made but only one of them produced pseudo-F_3-BC_1 descendants by embryo rescue. A total of 136 embryos were obtained from 10 fruits derived from this cross, and 15 of them

developed into plants (Supplementary Table 1; Fig. 1). In these generations several embryos developed up to the torpedo stage, although some of them had an abnormal development consisting of irregular cotyledon growth (Supplementary Fig. 1). These abnormal embryos did not develop into plants. In pseudo-F_2-BC_1 and pseudo-F_3-BC_2 generations several backcrosses to tomato followed by embryo rescue were carried out, but only one plant of each generation was obtained. Most of the embryos found in pseudo-F_2-BC_1 generations were globular. Several plants of pseudo-F_5 and pseudo-F_6 generations were also backcrossed once to tomato. Some embryos were found, most of them at globular stage, but no plants were obtained from these embryos. Crosses between pseudo-F_3-BC_1 generations were more compatible and produced a lot of progeny. Several plants from these crosses were backcrossed to tomato (FC x [pseudo-F_3-BC_1 x pseudo-F_3-BC_1]), but no progeny were obtained from these crosses by embryo rescue. However, several fruits from this backcross were allowed to mature and produced a few viable seeds.

Inoculation response to TSWV

Inoculation trial 1

At 15 dpi plants of the susceptible control showed severe symptoms and high absorbance values. Plants of the resistant control PI 126944 remained symptomless for the duration of the trial and did not accumulate virus. Clonal replicates of the three hybrids behaved as the resistant control.

Inoculation trial 2

All susceptible controls (NE-1, FC and RDD) showed systemic infection, but symptom severity and absorbance values were higher in NE-1 than in FC and RDD. Systemic infection was detected only in one of the 11 plants of the resistant PI 126944 tested and this plant showed only slight symptoms. All clonal replicates of pseudo-F_3-BC_1 generations behaved as resistant PI 126944 plants, so symptoms were mild and systemic infection was not detected. The percentage of systemically infected plants in crosses between pseudo-F_3BC_1 generations ranged between 21% (71-4 x 71-2) and 28% (71-4 x 71-3). These results could suggest a monogenic control of the resistance. Symptoms in infected plants were more severe than the ones shown by the resistant control but markedly lower that the ones exhibited by the susceptible ones.

Inoculation response to TYLCV and TYLCSV

Inoculation trial 1

The susceptible control, NE-1, showed severe symptoms since 15 dpi. Viral DNA of both TYLCV and TYLCSV species was detected in all plants from this date. The resistant control, TY-197, remained symptomless although virus was detected since 25 dpi. PI 126944 showed mild symptoms and only accumulated TYLCV. Hybrids did not show a consistent behaviour. F_1-A hybrid plants were susceptible since 15 dpi. The number of infected plants was higher for TYLCV than for TYLCSV. Both F_1-B and F_1-E hybrids were resistant: plants were symptomless and viral DNA was detected only in three plants.

Inoculation trial 2

As expected, susceptible controls showed severe symptoms and high DNA accumulation. Most of the TY-197 and PI 126944 plants remained asymptomatic and the rest showed very mild symptoms. However, viral accumulation in PI 126944 was comparable to accumulation levels detected in the susceptible controls NE-1 and FC. TY-197 showed a marked reduction in viral DNA accumulation.

Some of the plants of pseudo-F_2 generation could not be evaluated because of their abnormal growth habit and ambiguous symptomology. Most of the tested plants remained asymptomatic or showed mild symptoms. Only two plants displayed severe symptoms 60 dpi. Viral accumulation was not detected in 14 plants. Average viral accumulation in plants in which virus was detected was similar to that of the resistant control TY-197 and lower than the amount of virus in NE-1 and FC 35 dpi, when accumulation was maximum.

Symptoms in the pseudo-F_3BC_1 plants were less severe than in the susceptible controls. The most severe symptoms were shown by replicates from plants 71-2 and 71-4. Clonal replicates from plants 71-1 and 71-7 displayed milder symptoms. Clone 71-1 had the best response to TYLCV and it was similar to that of PI 126944. Clonal replicates from 71-2 and 71-3 accumulated viral amounts similar to those detected in the most resistant control, TY-197.

Plants from the cross (71-1 x 71-3) remained asymptomatic or displayed mild symptoms. Percentage of asymptomatic plants from crosses (71-4 x 71-2) and (71-4 x 71-3) was lower when compared to plants from cross (71-1 x 71-3).

It was possible to select plants which remained asymptomatic and also accumulated low amounts of virus in all generations evaluated.

Genotyping

Among the 263 markers tested, 95 were monomorphic and 63 did not amplify. Polymorphism between tomato and *S. peruvianum* was revealed by 53% of the markers that amplified. Different plants of PI 126944 were used to produce the three interspecific hybrids. As a result several *S. peruvianum* alleles existed in the different generations for 39% of the polymorphic markers analyzed. Similarly, since two different tomato parents (NE-1 and FC) were used to develop the populations, two different tomato alleles were found for 10% of the polymorphic markers analyzed. Although various *S. peruvianum* alleles were found for several markers, these alleles were not present in all generations. In some cases all plants of the most advanced generations exhibited only one of the possible *S. peruvianum* alleles. The other allele was always present in less advanced generations such as the pseudo-F_4 or pseudo-F_5. Markers with different *S. peruvianum* alleles were mostly on chromosomes 1, 2, 3, 7 and 12. However, only one *S. peruvianum* allele was found for most of the markers analyzed on chromosomes 4, 5, 8 and 10. Similarly, the same tomato allele was found in both tomato parents for all markers analyzed on chromosomes 4, 5, 8, 10 and 11. For the rest of the chromosomes there was at least one marker in which the tomato allele was different in both tomato parents.

First plant set genotyping

Thirteen plants of different generations (two F_1, two pseudo-F_2, three BC_1, one pseudo-F_2-BC_1, four pseudo-F_3-BC_1 and one pseudo-F_3-BC_2) were genotyped with the 105 polymorphic markers (Fig. 1). Assuming an average spacing between markers of 10 cM (Table 2), almost 60% of the genome was covered.

On average, 79% of the *S. peruvianum* genome was represented in the pseudo-F_2 generations (20% in the homozygous state and 59% in the heterozygous state). Considering both pseudo-F_2 plants analyzed, most of the *S. peruvianum* genome was present (Fig. 2). Only regions covered by markers TAHINA-3-123, in chromosome 3, and TAHINA-8-2, in chromosome 8, were fixed for the tomato alleles in both plants.

A reduction in the *S. peruvianum* genome was observed in more advanced generations such as BC_1 (56%), pseudo-F_2-BC_1 (60%) and pseudo-F_3-BC_1 (70%). A

greater reduction was observed in the pseudo-F_3-BC_2 generation (33%). *S. peruvianum* alleles were represented in all regions analyzed for the group of plants belonging to BC_1, pseudo-F_2-BC_1 and pseudo-F_3-BC_1 generations. The pseudo-F_3-BC_2 generation was composed of only one plant. Several regions in all chromosomes were fixed for *S. lycopersicum* alleles in this generation.

In general, recombination was variable depending on the chromosome. For example, on average in the pseudo-F_2 and BC_1 generations, recombination was higher at chromosomes 1, 6, 7, 9, 11 and 12, and lower at chromosome 2.

Table 2 Percentages of genome coverage and *S. peruvianum* genome representation with the markers analyzed in all generations genotyped

Generation[1]	Number of plants	Markers analyzed	% genome coverage[2]	% genome *S. peruvianum*		
				Homozygous	Hererozygous	Total
F_1-A	1	105	60	0	100	100
F_1-B	1	105	60	0	100	100
'F_2'	2	105	60	20	59	79
BC_1	3	105	60	0	56	56
'F_2'-BC_1	1	105	60	0	60	60
'F_3'-BC_1	4	105	60	0	70	70
'F_3'-BC_2	1	105	60	0	33	33
'F_4'	18	64	40	39	39	78
BC_1 x 'F_3'-BC_1	2	64	40	19	44	63
'F_3'-BC_1 x 'F_3'-BC_1	50	64	40	11	36	47
'F_5'	17	43	28	50	34	84

[1]Generations written inside inverted commas are not true F_2, F_3, etc. as they were obtained by crossing different plants instead of self-pollinating
[2]Percentage of genome coverage, according an average space of 10cM

Second plant set genotyping

A set of 83 plants (18 pseudo-F_4 plants, 50 pseudo-F_3-BC_1 x pseudo-F_3-BC_1, two BC_1 x pseudo-F_3-BC_1 and 13 BC_3) (Fig. 1), was analyzed with 64 of the polymorphic markers, selected to be equally distributed over the genome. With this marker set, 40% of genome was covered (Table 2).

On average, the *S. peruvianum* genome was represented in 78% of the markers analyzed in these generations (39% in the homozygous state and 39% in the heterozygous state) (Table 2). Considering all pseudo-F_4 plants analyzed, most of *S. peruvianum* genome was present. In all pseudo-F_4 plants, wild species alleles predominated in some chromosomes while in other chromosomes a higher tomato genome proportion predominated (Fig. 3a). Most of chromosome 8 was covered by tomato alleles. However, *S. peruvianum* alleles were found for all markers analyzed for this chromosome in at least

one plant of this generation, with the exception of the distal end of the short arm, covered by the marker TAHINA-8-2.

In pseudo-F_3-BC_1 x pseudo-F_3-BC_1 generations, *S. peruvianum* alleles were present for 47% of markers analyzed (11% in the homozygous state and 36% in the heterozygous state) (Table 2). However, in some regions of chromosomes 1, 6, 7, 8, and 9 *S. peruvianum* alleles were not represented (Fig. 3b). Similarly to the pseudo-F_4 generations, tomato and *S. peruvianum* alleles were almost fixed for some regions.

In plants of BC_1 x pseudo-F_3-BC_1 generations, the average percentage of the *S. peruvianum* genome was 63% (19% in the homozygous state and 44% in the heterozygous state) for the markers analyzed (Table 2). *S. peruvianum* alleles were represented in most of the regions analyzed in one of the two plants. In only a few regions of chromosomes 2, 4, 5, 6, 7, 8, 9 and 12 were *S. peruvianum* alleles not represented. On the other hand, some regions of chromosome 3, 6 and 8 were fixed for *S. peruvianum* alleles in those generations (data not shown in Fig. 3).

S. peruvianum alleles were not found in BC_3 generations for the 64 markers analyzed. However, these plants produced small orange fruits, probably due to the presence of *S. peruvianum* alleles.

Third plant set genotyping

A total of 17 plants of pseudo-F_5 generation were analyzed with 43 polymorphic markers, covering 28% of genome. On average, the *S. peruvianum* genome was represented in 84% of markers analyzed (50% in the homozygous state and 34% in the heterozygous state). Considering all plants of this generation, *S. peruvianum* alleles were present in all regions analyzed, with the exception of the region covered by the marker SSRB105694 on chromosome 8 and marker C2_At1g07310 on chromosome 9 (Fig. 3c).
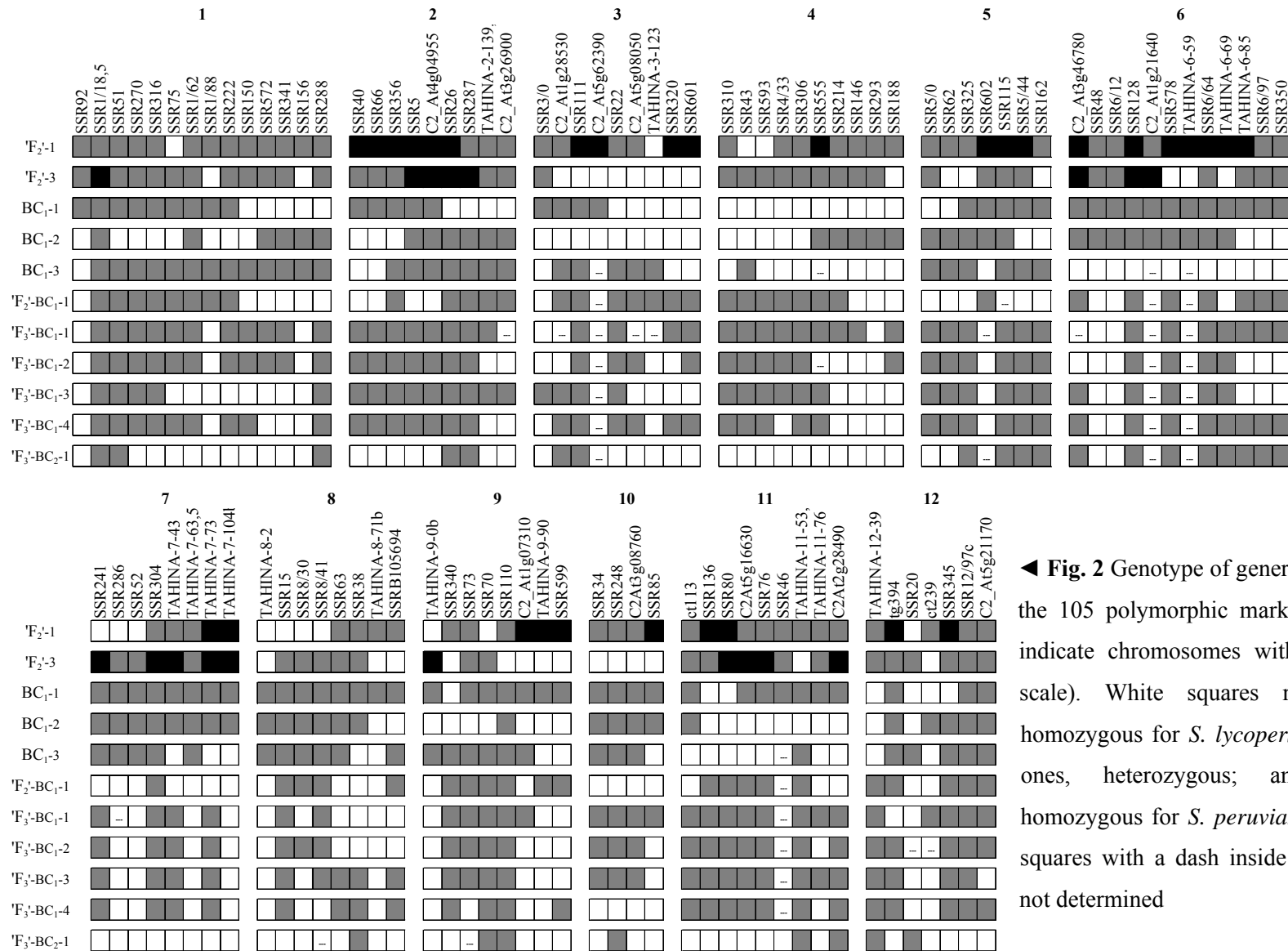
◀ **Fig. 2** Genotype of generations analyzed for the 105 polymorphic markers. The top rows indicate chromosomes with markers (not to scale). White squares represent markers homozygous for *S. lycopersicum* alleles; grey ones, heterozygous; and black ones, homozygous for *S. peruvianum* alleles. White squares with a dash inside represent markers not determined
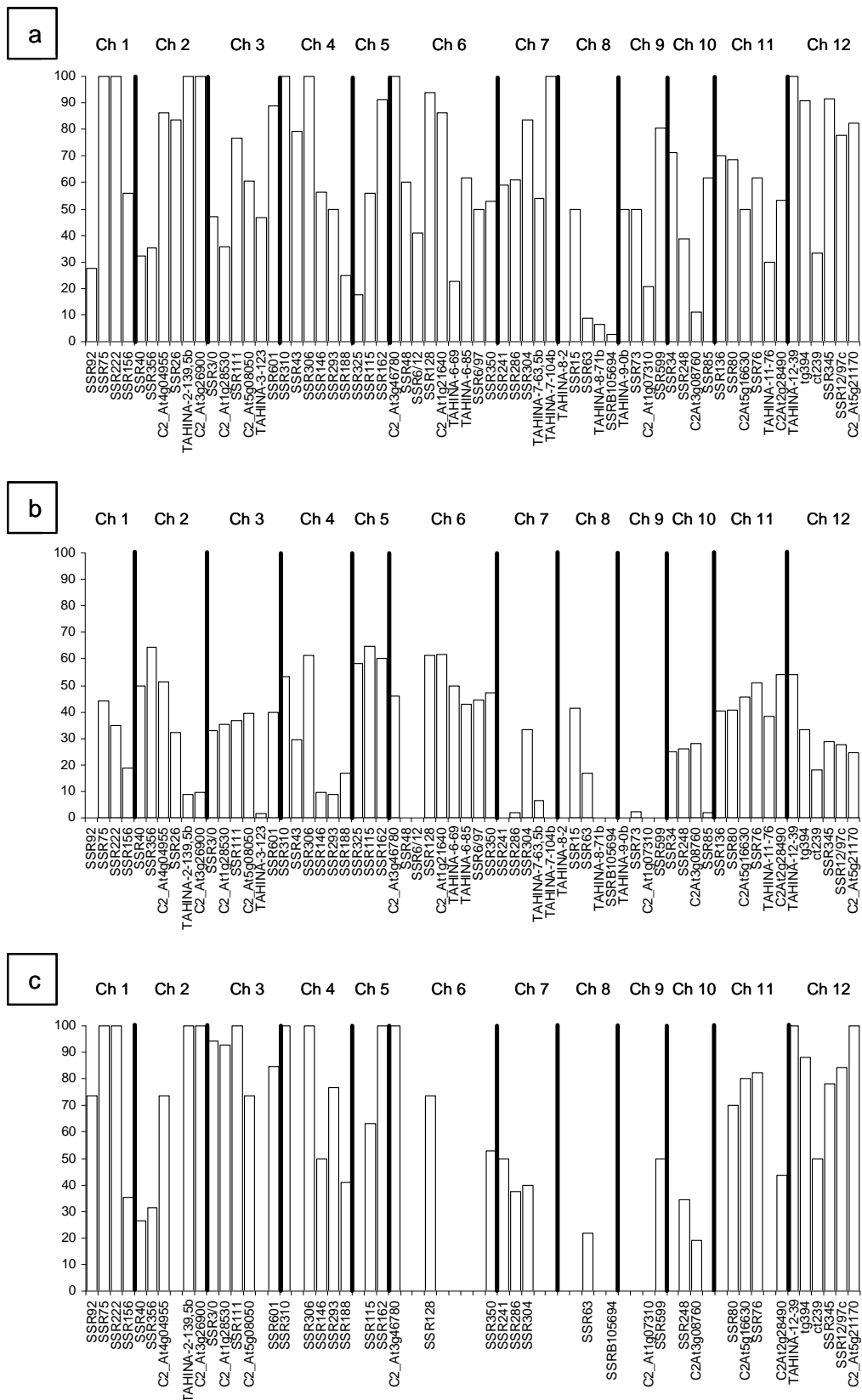
Fig. 3 Percentage of *S. peruvianum* genome considering all plants of pseudo-F$_4$ (a), pseudo-F$_3$-BC$_1$ x pseudo-F$_3$-BC$_1$ (b) and pseudo-F$_5$ (c) generations for each marker analyzed for each chromosome (Ch)

DISCUSSION

The aim of this work was to develop a set of ILs derived from PI 126944 due to the resistance to different pathogens reported in this accession. Several generations derived from crosses between this accession and cultivated tomato were obtained despite the high incompatibility existing between *S. peruvianum* and the cultivated species. Concretely, PI 126944 was strongly incompatible in the initial crosses made to obtain the interspecific hybrids (Picó et al. 2002). Using immature seed culture, some hybrid plants were obtained by Picó et al. (2002) and subsequent generations were derived from these hybrids in this study, also using immature seed culture. A 'genotype-dependent' efficiency was observed in the embryo rescue technique. As an example, descendants were obtained from only one of the ten pseudo-F_3 plants crossed to tomato: only this one cross was completely compatible. Additionally, variation among different genotypes of the recurrent parent has been reported in overcoming crossability barriers (Sacks et al. 1997). The study of these factors was not the main objective of this work; however, the feasibility of using other recurrent parents to facilitate the obtaining of more descendants could be explored in future studies. In any case, we have obtained abundant progeny from some crosses using embryo rescue. Moreover, the incompatibility is starting to be lost in the most advanced generations, thus progeny can be obtained by normal hybridizations. Embryo rescue in early generations, followed by reduction in incompatibility in later generations, has allowed us to construct a set of ILs from this particular accession.

Resistance to TSWV and to TYLCV derived from PI 126944 has been successfully introgressed into several of the generations obtained in the present work.

There was no systemic infection to TSWV in the three F_1 hybrids, as previously reported by Picó et al. (2002). On the other hand, approximately one third of the plants derived from crosses between pseudo-F_3-BC_1 generations were susceptible, suggesting a simple dominant gene controls resistance. This assumption will be confirmed in the future. Resistance to TSWV derived from *S. peruvianum* has been previously reported as controlled by the single dominant gene *Sw-5* (Stevens et al. 1992). The marker SSR599, located on chromosome 9, is the closest marker to *Sw-5* of all the polymorphic markers analyzed in the present work. Several TSWV resistant plants of generations pseudo-F_3-BC_1 and pseudo-F_3-BC_1 x pseudo-F_3-BC_1 displayed tomato alleles for this marker. Therefore,

these results suggest that the gene controlling TSWV resistance in PI 126944 may not be *Sw-5*.

Regarding the response to TYLCV, two F_1 hybrids were resistant while the third was susceptible. Variability was also observed among plants of the resistant parent PI 126944. Although Pilowsky and Cohen (2000) tested this accession for resistance to TYLCV and obtained a consistent resistant response in the 21 tested plants, our results suggest the existence of genetic variability for the genes of resistance in the set of plants used in this study. The different levels of resistance in all generations tested suggest resistance is of a quantitative nature, which agrees with the reported genetic control for the resistance in some *S. peruvianum* accessions (Pilowsky and Cohen, 1990).

Polymorphism between tomato and *S. peruvianum* was revealed by 53% of the markers analyzed. This result is slightly lower than the one obtained by Fulton et al. (1997), in which 65% of markers analyzed were polymorphic. This difference can be due in part to the different types of molecular markers used in both studies: RFLPs (assayed with different restriction enzymes) were used by Fulton et al. (1997) whereas we used mostly SSRs in our study. Interestingly, 39% of the polymorphic markers exhibited different *S. peruvianum* alleles in our set of genotyped plants. This will allow the development of different sets of ILs with each allele, capturing more of the variability existing in the original accession. In those cases when all the possible *S. peruvianum* alleles are not present in the most advanced generations, it will be necessary to make use of less advanced generations, such as pseudo-F_4 or pseudo-F_5, to have them all represented. The loss of *S. peruvianum* alleles in more advanced generations was probably random, as the number of plants involved in the construction of these generations was small. The proportion of markers with different *S. peruvianum* alleles varied among chromosomes, as did the proportion of markers with different tomato alleles. In general, chromosomes 4, 5, 8, and 10 had the lowest proportion of markers that exhibited different *S. peruvianum* or tomato alleles which may be due to a higher conservation of the genomic regions on these chromosomes.

A progressive reduction in the proportion of the *S. peruvianum* genome and also in the size of the introgressed fragments was observed in generations obtained with one or more backcrosses to tomato. As a whole, the percentage of the *S. peruvianum* genome in these generations was 55% compared to the 80% for pseudo-F_2, pseudo-F_4 and pseudo-F_5 generations (Table 2). Different authors observed that recombination rate, which

determines the fragment sizes, depends not only on the species but also on the region of the genome considered. Bonnema et al. (1997) compared recombination rates in an F_2 population obtained from a cross between tomato and *S. peruvianum*, with recombination rates of the F_2 population derived from *S. pennellii* LA716 (Tanksley et al. 1992). They observed that recombination was reduced at chromosome 2 and 5, while at chromosomes 1, 7, 9, 10 and 11 recombination rates were higher. We obtained similar results with our pseudo-F_2 and BC_1 generations. Recombination was lower at chromosome 2 and higher at chromosomes 1, 6, 7, 9, 11 and 12. Fragmentation of the introgressed segments after some selfing or backcross generations has been also demonstrated by Eshed et al. (1992) and Tanksley et al. (1996) respectively. Fulton et al. (1997) also found a progressive decrease of the size of the introgressed fragments of *S. peruvianum* in *S. lycopersicum*. Consequently, an efficient introgression of *S. peruvianum* genes into cultivated tomato was shown to be possible, in spite of the great distance between both species.

Deviation in the percentage of the *S. peruvianum* genome in the homozygous or heterozygous condition from expected values was observed in many generations. Thus, the percentage of *S. peruvianum* genome in the pseudo-F_4 generations (39% homozygous and 39% heterozygous) differed from the expected values for a F_4 generation (25% homozygous and 50% heterozygous). This percentage was higher than expected in the pseudo-F_5 generations (84% for the markers analyzed, 50% homozygous and 34% heterozygous). This is due to the specific genotype of the pseudo-F_4 plants involved in crosses to produce the pseudo-F_5 generation. Although hundreds of crosses between most pseudo-F_4 plants available were made in order to obtain the pseudo-F_5 generations, progeny were only obtained from two plants. These two pseudo-F_4 plants were the ones with the highest proportion of *S. peruvianum* genome. Maybe the success of these crosses was due to the higher wild species genome content and consequently the higher genetic similarity leading to compatibility between them. There is no information available about the genotype of the pseudo-F_3 plants from which these pseudo-F_4 generations were derived, but the reason for the percentage deviation could be the same as for the pseudo-F_5 generations.

Tomato and *S. peruvianum* alleles were not evenly distributed in the genome among the different generations. These deviations may be caused by chance, because of the small number of plants involved in the construction of the different generations due to the strong incompatibility. Additionally, it is also possible that for some loci a distortion of

segregation exists. This distortion of segregation has been observed by other authors working with materials derived from interspecific crosses between tomato and some wild species (Fulton et al. 1997, Canady et al. 2005).

BC_3 generations are potentially very useful. However, no *S. peruvianum* genome was found with the markers used. Despite this, these plants displayed some *S. peruvianum* characters, like small orange fruits, that could be related with the presence of *S. peruvianum* alleles. Therefore, introgression fragments in these plants must be very short. In the present work, three BC_1 generations were genotyped and very short *S. peruvianum* fragments were already found on them. So, it is possible that most *S. peruvianum* fragments have been lost by the BC_3 generations and that the ones conserved are very short. Future work will include the analysis of these plants with the rest of the markers available in order to identify these fragments.

CONCLUSION

In order to make efficient use of wild species, introgression of the genes of interest into the cultivated species is necessary. We have demonstrated that resistance to TSWV and TYLCD from *S. peruvianum* PI 126944 can be successfully introgressed and expressed into tomato background. This accession is also resistant to other diseases, so it will be interesting to test the final set of ILs for resistance to other pathogens.

The *S. peruvianum* genome is almost completely represented across different plants of the most advanced generations. Development of ILs will continue by backcrossing the most advanced generations available to tomato, for example, BC_1 x pseudo-F_3-BC_1 and pseudo-F_3-BC_1 x pseudo-F_3-BC_1. In any case, it will be necessary to make use of less advanced generations, such as pseudo-F_6 or BC_1 to introgress some fragments not present in these advanced generations. For this purpose it will be necessary to continue using embryo rescue due to the high incompatibility existent. In any case, a reduction of this incompatibility, as a consequence of a reduction in the *S. peruvianum* genome, has already been achieved.

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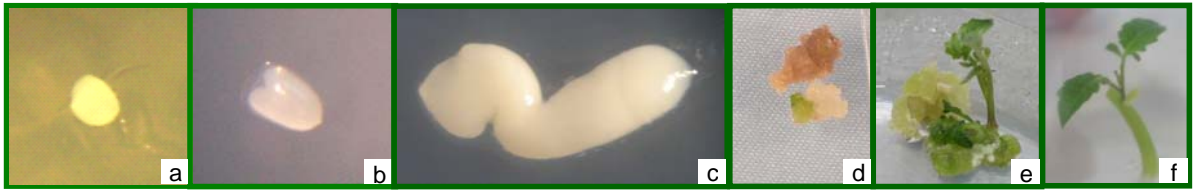
Supplementary Fig. 1 Developmental stage of some rescued embryos and subsequent phases of development. a) Globular embryo b) Heart embryo c) Abnormal torpedo embryo; this type of embryo was found in some backcrosses of pseudo-F$_3$ plants d) Heart embryo with *callus* formation e) Plant regenerated from a *callus* f) Clonal replicate from a regenerated plant

Supplementary Table 1 Polymorphic marker set used to genotype generations available

Marker[1]	Ch[2]	cM[3]	Annealing temperature	Restriction enzyme	Fragment sizes[4]		Primers sequence (5'→3')	Reference[5]
					S. lycopersicum	*S. chilense*		
SSR92	1	0	50	-	192, 190	186	AAGAAGAAGGATCGATCGAAGA TCATGACCACGATACTACATGTTTC	SGN
SSR1/18,5	1	18,5	55	-	178	216, 245	AAAAGTGGGGAAGGAGCCTA ACCTCAACGGGAAAGTACCA	Designed by S. Vilanova (COMAV)
SSR51	1	39,5	50	-	144	153, 160	CTACCCTGGTCTTGGTGGAA AAAGGATGCTCTAGCTTCTCCA	SGN
SSR270	1	46	50	-	206	212	AGCTCAAGGCTTCTGTTGGA AACCACCTCAGGCACTTCAT	SGN
SSR316	1	46,7	55	-	234	243, 248	CCACCGCAACAAACCTTATT GGGTGGTGAGAAGGATCTGA	SGN
SSR75	1	53,5	55	-	172	166	CCATCTATTATCTTCTCTCCAACAC GGTCCCAACTCGGTACACAC	SGN
SSR1/62	1	62	60	-	184	175, 181, 182	GGACCCACACACCATCTTTC CCAAATGTGCACGTTCTAAGG	Pérez-de-Castro et al. 2012
SSR1/88	1	88	55	-	168, 174	171	TGGTTGGAAGTCTCAAGAACC CCCTTCTTTCTCTTGGTTTCG	This work
SSR222	1	97,5	55	-	178	181, 190	TCTCATCTGGTGCTGCTGTT TTCTTGGAGGACCCAGAAAC	SGN
SSR150	1	115,5	50	-	236	228, 231	ATGCCTCGCTACCTCCTCTT AATCGTTCGTTCACAAACCC	SGN
SSR572	1	136,5	45	-	294	297, 299	AATTCACCTTTCTTCCGTCG TGCAAAGAACAAAGACCGTG	SGN
SSR341	1	137,5	48	-	311	316, 318	TTTCTCTTGTGGGTGGCAAT AAGCCCTCGAATCTGGTAGC	SGN
SSR156	1	146	50	-	175	182, 184/191	CACGCCTATGCACCTTTCTT CTTCAAGGCTAAACCTCCGA	SGN
SSR288	1	158,5	45	-	293	290	TCGTGGGAATTTGTTAACCC TCTTCATCGTCCTCCTCCTG	SGN

Supplementary Table 1 (continuation)

Marker[1]	Ch[2]	cM[3]	Annealing temperature	Restriction enzyme	Fragment sizes[4]		Primers sequence (5'→3')	Reference[5]
					S. lycopersicum	*S. chilense*		
SSR40	2	22	55	-	160	158,135	TGCAGGTATGTCTCACACCA	SGN
							TTGCAAGAACACCTCCCTTT	
SSR66	2	25	50	-	203	194	TGCAACAACTGGATAGGTCG	SGN
							TGGATGAAACGGATGTTGAA	
SSR356	2	44	55	-	269, 265	214	ACCATCGAGGCTGCATAAAG	SGN
							AACCATCCACTGCCTCAATC	
SSR5	2	53	45	-	211	199, 205	TGGCCGGCTTCTAGAAATAA	SGN
							TGAAATCACCCGTGACCTTT	
C2_At4g04955	2	63,5	55	HinfI	400	300	TTGCTGTGGGGAACCAAGCAGATATAG	SGN
							TCCCAGAGAGTCTTGATCCCATGTATGC	
SSR26	2	77,5	50	-	194	189	CGCCTATCGATACCACCACT	SGN
							ATTGATCCGTTTGGTTCTGC	
SSR287	2	107	45	-	183	188, 194	GCATCCCAAACAATCCAATC	SGN
							TCCACTTTCAAGATCAGAGCAA	
TAHINA-2-139,5b	2	139,5	50	-	260	254, 256	ATGTGCACACACGTGAACC	Trujillo-Moya et al. 2011
							ATGCAATTGCCATTGACCTA	
C2_At3g26900	2	142	55	HaeIII	380/420	800	CCAAGGCATGACGTTAATTTG	SGN
							TCTTTTTCCATGTGTCAGTCAAC	
SSR3/0	3	0	55	-	263	241	GGAAGTCGAGAGGTGGTGAG	Pérez-de-Castro et al. 2012
							CCACTTTTCCAGCCACATTG	
C2_At1g28530	3	21	55	DraI	600	400	ATTATGAAGATGTCTATACACTTCCCTAC	SGN
							AGAGATTGCTTTTGACATAGAAATGCTT	
SSR111	3	75	55	-	179, 183	173,175	TTCTTCCCTTCCATCAGTTCT	SGN
							TTTGCTGCTATACTGCTGACA	
C2_At5g62390	3	75,6	55	EcoRV	300/1000/1100	1120	TGCTACTAACTGTTGATGCCATTGAG	SGN
							TTGGGGGTCGATAACATCAAGC	
SSR22	3	99	50	-	228	222, 232	GATCGGCAGTAGGTGCTCTC	SGN
							CAAGAAACACCCATATCCGC	

Supplementary Table 1 (continuation)

Marker[1]	Ch[2]	cM[3]	Annealing temperature	Restriction enzyme	Fragment sizes[4]		Primers sequence (5'→3')	Reference[5]
					S. lycopersicum	*S. chilense*		
C2_At5g08 050	3	104	55	-	650	675	TGCGATTTCACGTTTCTCTGCTTC	SGN
							TCCCCTACTGGAAATACAGTTGTTG	
TAHINA-3-123	3	123	55	-	261, 276	286	GCGCTGAATCTCAAACTCG	Trujillo-Moya et al. 2011
							AGCAAAGGGGTTGTACATGG	
SSR320	3	158	55	-	190	178, 180	ATGAGGCAATCTTCACCTGG	SGN
							TTCAGCTGATAGTTCCTGCG	
SSR601	3	162	55	-	185	174, 179	TCTGCATCTGGTGAAGCAAG	SGN
							CTGGATTGCCTGGTTGATTT	
SSR310	4	8	55	-	164	148	GCGATGAGGATGACATTGAG	SGN
							TTTACAGGCTGTCGCTTCCT	
SSR43	4	14	50	-	236	229	CTCCAAATTGGGCAATAACA	SGN
							TTAGGAAGTTGCATTAGGCCA	
SSR593	4	15	55	-	313	306	TGGCATGAACAACAACCAAT	SGN
							AGGAAGTTGCATTAGGCCAT	
SSR4/33	4	33	53	-	265	224/237	CAAATTCATTCAGTGCTAAAAGG	Designed by S. Vilanova (COMAV)
							CAAAATTAAACTCTCCCATGAACA	
SSR306	4	48	55	-	280	269	ACATGAGCCCAATGAACCTC	SGN
							AACCATTCCGCACGTACATA	
SSR555	4	61,5	41	-	226	232	TTGATATTAACCATGGCAGCAG	SGN
							TTGATGGGATTGCACAGAAA	
SSR214	4	95	50	-	240	237	AAATTCCCAACACTTGCCAC	SGN
							CCCACCACTATCCAAACCC	
SSR146	4	103	50	-	257	251	TATGGCCATGGCTGAACC	SGN
							CGAACGCCACCACTATACCT	
SSR293	4	109	50	-	143	137	GCAAAGAGCTCGATCTCCAA	SGN
							TTCAGTTACTGGCCTTCGCT	
SSR188	4	135,5	50	-	160	172	TGCAGTGAGTCTCGATTTGC	SGN
							GGTCTCATTGCAGATAGGGC	

Marker1	Ch2	cM3	Annealing temperature	Restriction enzyme	Fragment sizes4		Primers sequence (5'→3')	Reference5
					S. lycopersicum	S. chilense		
SSR5/0	5	0	53	-	300	328	CAACATTTCGTCTGTTTTTGTAATG	Designed by S. Vilanova (COMAV)
							GCGATGAGATCGACTCAAAA	
SSR62	5	10,5	40	-	357	348	TGCAAATGAATGTCCAGGAT	SGN
							TCAGCAGAGTTATGCCATGC	
SSR325	5	18,5	50	-	151	148	CCATTGACAGCCCATTATCC	SGN
							TGATGTGAAAGAGTTGATGAGG	
SSR602	5	27	55	-	317	314, 315	GGGTCACATACACTCATACTAAGGA	SGN
							GGCAATCATAGCCACTTGGT	
SSR115	5	35	50	-	233	217, 224	CACCCTTTATTCAGATTCCTCT	SGN
							ATTGAGGGTATGCAACAGCC	
SSR5/44	5	44	55	-	229	118	TGATGCCTACAGGCACAAAG	Designed by S. Vilanova (COMAV)
							TGCAGTAATGAGAATATGTTGGATG	
SSR162	5	119	50	-	270	254, 259	GCTCTCTACAAGTGGAACTTTCTC	SGN
							CAACAGCCAGGAACAAGGAT	
C2_At3g46780	6	4	55	-	1200	1150	ATGGCTCCAACTCTTACTTCAAATTC	SGN
							TCTGCATCTTGAAATGATGATGCAAC	
SSR48	6	6	50	-	215	221	ATCTCCTTGGCCTCCTGTTT	SGN
							GTCATGGCCACATGAATACG	
SSR6/12	6	12	53	-	244/249, 247	256	TTGGAAGCAAATGAGTGTGG	Designed by S. Vilanova (COMAV)
							GAAAGATGCAGCCAACTTCAA	
SSR128	6	35	50	-	137	143, 144, 146, 147	GGTCCAGTTCAATCAACCGA	SGN
							TGAAGTCGTCTCATGGTTCG	
C2_At1g21640	6	37	55	DraI	500	400	AGAAAAGTCATCCATGGAAACAACAC	SGN
							TGGCCACAATGACACCATCACCTTG	
SSR578	6	44	55	-	310, 313	301	ATTCCCAGCACAACCAGACT	SGN
							GTTGGTGGATGAAATTTGTG	
TAHINA-6-59	6	59	50	-	268	306	TTTTCCTGGGGTAAGCAGAA	Trujillo-Moya et al. 2011
							TTCAACTTTTCACTTTGGAGCTT	

Supplementary Table 1 (continuation)

Marker[1]	Ch[2]	cM[3]	Annealing temperature	Restriction enzyme	Fragment sizes[4] S. lycopersicum	S. chilense	Primers sequence (5'→3')	Reference[5]
SSR6/64	6	64	55	-	245	236, 242	CAATGGGCAGGTACTCCATC GCACAGCAACATTACCAACAG	This work
TAHINA-6-69	6	69	55	-	186	314	CTTGCAAATGAAGGGTCTCC AGGATTGGACCAGTGTTTTCA	Trujillo-Moya et al. 2011
TAHINA-6-85	6	85	55	-	249	243, 251	TTTTCCTGGGGTAAGCAGAA GCAAGGGATGCTGTCTTCTT	Trujillo-Moya et al. 2011
SSR6/97	6	97	53	-	223, 220	187	TGCCCAGATCTCTTTCTCTTTC TTTTGTATGGGCAACTTTCAGA	Designed by S. Vilanova (COMAV)
SSR350	6	101	55	-	288	269, 277	GGAATAACCTCTAACTGCGGG CGATGCCTTCATTTGGACTT	SGN
SSR286	7	0	48	-	214	198, 202	AGCTATGGAGTTTCAGGACCA ATTCAGGTAGCATGGAACGC	SGN
SSR241	7	0	55	-	207	210	TCAACAGCATAGTGGAGGAGG TCCTCGGTAATTGATCCACC	SGN
SSR52	7	3	50	-	123	134, 138	TGATGGCAGCATCGTAGAAG GGTGCGAAGGGATTTACAGA	SGN
SSR304	7	30,5	52	-	205	181, 202	TCCTCCGGTTGTTACTCCAC TTAGCACTTCCACCGATTCC	SGN
TAHINA-7-43	7	43	55	-	258, 267	246, 296	GCAGCCAAATAGAAATTGGAAG CACATGTTAAAAGGTTGGTCAC	Trujillo-Moya et al. 2011
TAHINA-7-63,5b	7	63	53	-	204	195	GGCCAGCAATTATTAGAGTAGG TTACTAATTTGGCCGGCTTG	Trujillo-Moya et al. 2011
TAHINA-7-73	7	73	50	-	225	222, 228	GGAAGCTTGATTAGTGGAATGG TTTTTGGGAGAGCATGTTTG	Trujillo-Moya et al. 2011
TAHINA-7-104b	7	104	52	-	270, 263	223	TTGGACTAATCTAAAAGGCATTGAC TTTTGTAAATAATGGCAAATGTGAG	Trujillo-Moya et al. 2011
TAHINA-8-2	8	2	57	-	273	263	AAGCTAACAACATTAACTCAATTATCG TGGTTGGAGGTATTTATAGGGTTG	Trujillo-Moya et al. 2011

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Supplementary Table 1 (continuation)

Marker[1]	Ch[2]	cM[3]	Annealing temperature	Restriction enzyme	Fragment sizes[4]		Primers sequence (5'→3')	Reference[5]
					S. lycopersicum	*S. chilense*		
SSR15	8	22,7	40-55	-	211	202, 217	CACTTGCCATCTTCTAGCCC	SGN
							ATGGATGCCCAAATTGAAGA	
SSR8/30	8	30	55	-	253	139	TTGAATTTCGCCAACTCTATG	Designed by S. Vilanova (COMAV)
							AAATTTCTCACTCATTTTGTTAGTGAC	
SSR8/41	8	41	54	-	353	339	GGTGTCCAATCCATTTTGTG	This work
							TGATCACTTTCTCCATCTTAATTG	
SSR63	8	53	55	-	230	201, 205	CCACAAACAATTCCATCTCA	SGN
							GCTTCCGCCATACTGATACG	
SSR38	8	55	55	-	255	243, 247	GTTTCTATAGCTGAAACTCAACCTG	SGN
							GGGGTTCATCAAATCTACCATCA	
TAHINA-8-71b	8	71	53	-	215	208	TCCAGGATAGACCTTGAGGAAC	Trujillo-Moya et al. 2011
							TCCACCAGCTTTTGGATCTG	
SSRB105694	8	87	53	-	261	252	AAAGCCAAAGTGGAAGAACTCAAGG	SGN
							CTCGTAAAACGTTCATCAATCTCGC	
TAHINA-9-0b	9	0	52	-	245	267	AAAAGGTTCACGAAGGGAAAA	Trujillo-Moya et al. 2011
							AATCAAAACACCTTCAACGACT	
SSR340	9	18	50	-	283	299	TTCTCTCTGTCGCCATTGTG	SGN
							AAATCAACGCCAATGGTAGG	
SSR73	9	32	45	-	600	1000, 1200	TGGGAAGATCCTGATGATGG	SGN
							TTCCCTTTCCTCTGGACTCA	
SSR70	9	42	50	-	137, 143	109	TTTAGGGTGTCTGTGGGTCC	SGN
							GGAGTGCGCAGAGGATAGAG	
SSR110	9	55,7	42	-	186	173	TGTAACGTCAAACTTCAGGTG	SGN
							CTCCGCAATGTGTTGTATGG	
C2_At1g07310	9	74,5	55	HaeIII	350	700	AGAAAACCTACGATCTCGAAATCACC	SGN
							AAACTGCCATAGCTAGATTGCCG	
TAHINA-9-90	9	90	55	-	267	209	CTAGATAGGGCCCAGGGGTA	Trujillo-Moya et al. 2011
							TCAAGGCGAAATCAAGATCA	

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Supplementary Table 1 (continuation)

Marker[1]	Ch[2]	cM[3]	Annealing temperature	Restriction enzyme	Fragment sizes[4]		Primers sequence (5'→3')	Reference[5]
					S. lycopersicum	*S. chilense*		
SSR599	9	103	55	-	303	288, 300	GGATTTCTCATGGAGAATCAGTC TCCCTTGATCTTGATGATGTTG	SGN
SSR34	10	25,3	50	-	200	201	TTCGGATAAAGCAATCCACC TCGATTGTGTACCAACGTCC	SGN
SSR248	10	35	55	-	262	244	GCATTCGCTGTAGCTCGTTT GGGAGCTTCATCATAGTAACG	SGN
C2At3g08760	10	39	55	AluI	500/600	400	TCTCCAGAACGTTGTGTGTCAGAAGG TCCTCATGTAGAAATGTAAGACCTTG	SGN
SSR85	10	55	50	-	197	185	ATCCGTTAGCTATTGTGCCG TTGCCATGCACTTATCTTCG	SGN
ct113	10	0	56	MboI	100/250	150	ACAACGGGCAACAGACGCAACC AGCTCGAGGATGGCCGCACTTT	Bai et al. 2003
SSR136	11	11	50	-	166	160, 163	GAAACCGCCTCTTTCACTTG CAGCAATGATTCCAGCGATA	SGN
SSR80	11	20	50	-	203	200	GGCAAATGTCAAAGGATTGG AGGGTCATGTTCTTGATTGTCA	SGN
C2At5g16630	11	31,2	55	TaqI	700	800	TAAATGCAATCACTGATGGAGAGCA TGCCAATACTGCATCCCACCAAAT	SGN
SSR76	11	37,5	50	-	220	215, 217	ACGGGTCGTCTTTGAAACAA CCACCGGATTCTTCTTCGTA	SGN
SSR46	11	40	50	-	321	484	CCGAGGCGAATCTTGAATAC GCACCATCTCTTGTGCCTCT	SGN
TAHINA-11-53,5	11	53,5	53	-	211	215	CGATAGCATTTGATCTGATTTTG AATTTTAACTGCATCGCATGG	Trujillo-Moya et al. 2011
TAHINA-11-76	11	76	52	-	265	262	TAAACGAATCGGGACAGAAC CGTACACACATCAAACACTCACC	Trujillo-Moya et al. 2011
C2At2g28490	11	98	55	TaqI	380/420	350/450, 780	ACGGAGTATTCTCCATTGAAACACTCTG ATTGAATTCTGACCCACCAAGAACTG	SGN

Supplementary Table 1 (continuation)

Marker[1]	Ch[2]	cM[3]	Annealing temperature	Restriction enzyme	Fragment sizes[4] *S. lycopersicum*	Fragment sizes[4] *S. chilense*	Primers sequence (5'→3')	Reference[5]
TAHINA-12-39	12	39	52	-	225	154, 195	ATTGCCACGTGGATTGACTC TGCAAGCTGTTCTTTTCAGAC	Trujillo-Moya et al. 2011
tg394	12	48	56	FokI	280	320	AGCCTCATGAGACCTACAA TACAGCACAATCTTCTACC	Bai et al. 2003
SSR20	12	58,2	50	-	172, 166	163	GAGGACGACAACAACAACGA GACATGCCACTTAGATCCACAA	SGN
ct239	12	68	56	HinfI	550	650	TGGAACGGAGTACAAAACAGAAGA GAATGCCATCAGGGAAAGGTAACT	Bai et al. 2003
SSR345	12	72,5	60	-	175	173, 190	AAGCCAAGCTCGAACCTGTA ATCCATGCTGTCGCTTTCAT	SGN
SSR12/97c	12	97	53	-	257	242, 249	TTGCTTCACTTGTGTCGAATC TCTGTTGCAGTCCCAAAAAG	Pérez-de-Castro et al. 2012
C2_At5g21 170	12	120	55	EcoRI	400	120/250	TCCTTCCATCGGGTATATATCATTACAA ACTGCCACTGGCTCCTTTTCAAAATC	SGN

[1] Markers which start with SSR or TAHINA are Simple Sequence Repeat (SSR); ct113, tg394 and ct239 markers are Restriction Fragment Length Polymorphism (RFLP) converted in Cleaved Amplified Polymorphic Sequence (CAPS). The rest of the markers are Conserved Ortholog Set (COSII)

[2] For the COSII markers C2_At1g21640 and C2At5g16630, restriction enzymes used were not the same than the ones reported in Sol Genomics Network (http://solgenomics.net/)

3 Fragment sizes (bp): Numbers separated by bars correspond to those alleles with more than one fragment. Numbers separated by comma in *S. peruvianum* column indicates different *S. peruvianum* alleles. For markers which were polymorphic between NE-1 and FC, the first number in *S. lycopersicum* column indicates the fragment size for NE-1 allele and second number indicates the fragment size for FC allele.

[4] SGN: Sol Genomics Network (solgenomics.net)

Bai Y, Huang CC, van der Hulst RGM, Meijer-Dekens RG Bonnema AB, Lindhout WH (2003) QTLs for tomato powdery mildew resisance (*Oidium lycopersici*) in *Lycopersicon parviflorum* G1.1601 colocalize with two qualitative powdery mildew resistance genes. Mol Plant-Microbe Interact 16:169-176

Pérez-de-Castro A, Julián O, Díez MJ (2012) Genetic control and mapping of *Solanum chilense* LA1932, LA1960 and LA1971-derived resistance to Tomato yellow leaf curl disease. (submitted to be published)

Trujillo-Moya C, Gisbert C, Vilanova S, Nuez F (2011) Localization of QTLs for in vitro plant regeneration in tomato. BMC Plant Biol 11:140

Supplementary Table 2 Generations obtained by embryo rescue

Cross	Generation	Nombre of fruits	Number of embryos	Number of plants
Fc x F_1-B	BC_1	65	129	4
Fc x BC_1-1	BC_2	4	10	0
Fc x BC_1-2	BC_2	ND[1]	ND	2
Fc x BC_2-1	BC_3	20	180	2
Fc x BC_2-2	BC_3	26	276	0
Fc x 'F_2'-1	'F_2'-BC_1	129	141	0
Fc x 'F_2'-2	'F_2'-BC_1	ND	ND	1
Fc x 'F_3'-1	'F_3'-BC_1	10	136	15
Fc x 'F_3'-2	'F_3'-BC_1	5	7	0
Fc x 'F_3'-3	'F_3'-BC_1	5	11	0
Fc x 'F_3'-4	'F_3'-BC_1	2	9	0
Fc x 'F_3'-5	'F_3'-BC_1	8	83	0
Fc x 'F_3'-6	'F_3'-BC_1	2	2	0
Fc x 'F_3'-7	'F_3'-BC_1	6	24	0
Fc x 'F_3'-8	'F_3'-BC_1	1	5	0
Fc x 'F_3'-9	'F_3'-BC_1	1	2	0
Fc x 'F_3'-10	'F_3'-BC_1	3	24	0
Fc x 'F_3'-BC_1-1	'F_3'-BC_2	ND	ND	1
Fc x ('F_3'-BC_1 x 'F_3'-BC_1)1	('F_3' x 'F_3')-BC_2	7	0	0
Fc x ('F_3'-BC_1 x 'F_3'-BC_1)2	('F_3' x 'F_3')-BC_2	1	0	0
Fc x ('F_3'-BC_1 x 'F_3'-BC_1)3	('F_3' x 'F_3')-BC_2	1	0	0
Fc x 'F_5'-1	'F_5'-BC_1	16	66	0
Fc x 'F_5'-2	'F_5'-BC_1	3	6	0
Fc x 'F_5'-3	'F_5'-BC_1	7	18	0
Fc x 'F_5'-4	'F_5'-BC_1	18	12	0
Fc x 'F_5'-5	'F_5'-BC_1	2	0	0
Fc x 'F_5'-6	'F_5'-BC_1	2	0	0
Fc x 'F_5'-7	'F_5'-BC_1	1	0	0
Fc x 'F_6'-1	'F_6'-BC_1	6	0	0
Fc x 'F_6'-2	'F_6'-BC_1	3	0	0
Fc x 'F_6'-3	'F_6'-BC_1	1	6	0
	Total	**355**	**1147**	**25**

[1]ND: Not determined

GENERAL DISCUSSION

Viral diseases are an important limiting factor in tomato production worldwide. As there are no antiviral products available, control strategies are mainly based on genetic resistance derived from tomato wild relatives. Among the viral diseases affecting tomato, geminiviruses are one of the most devastating. Ever since the worldwide emergence of whiteflies, the complex of geminivirus species causing TYLCD has constituted a serious problem for tomato production in many tropical and subtropical regions. Many different species have been described within this group, with TYLCV being the main viral species associated with this disease.

The results presented in this thesis represent an important contribution to breeding for resistance to viral diseases in tomato, mainly to TYLCD. For that aim, resistance derived from the wild tomato relatives *S. chilense* and *S. peruvianum* was exploited. In spite of the incompatibility barriers that exist between these species and tomato, the behaviour of the accessions of *S. chilense* that were used behaved as compatible enough to permit the introgression of the resistance into tomato. Several resistant lines were obtained from various accessions of *S. chilense*. These lines were used to determine the genetic control of the resistance and fine map the resistance genes.

Contrarily, the strong incompatibility between the *S. peruvianum* accession PI 126944 and tomato prevented the introgression of the resistance and the obtaining of abundant progeny to be used for the elucidation of the genetic control of the resistance. Moreover, resistance in this species seems to be more complex than that of *S. chilense*. In order to circumvent these problems, and better exploit the potential of this accession in breeding for disease resistance, a set of ILs in the cultivated tomato genetic background was constructed.

Identification of new resistance genes in *S. chilense*: Determination of genetic control and mapping

Several TYLCV resistance genes have been identified in various wild tomato relatives. However, the resistance conferred by currently available genes is not completely effective, since resistant plants may show slight to moderate symptoms and yield losses under high inoculum pressures. The resistance derived from the *S. chilense* accession LA1969 has been the most employed in breeding programmes worldwide. This resistance is controlled by the *Ty-1* gene, which confers high but not complete resistance. Some other

S. chilense accessions have been reported to be resistant to TYLCV and other begomoviruses. The study of the genetic control of the resistance, including the mode of inheritance and expression level and its use in breeding are necessary in order to develop new cultivars with enhanced resistance.

Facing this situation, breeding programs were initiated by our group to develop resistant lines derived from the *S. chilense* accessions LA1932, LA1960 and LA1971 (Picó et al. 1999; Julián et al. 2008). As a result of the work carried out, genetic control of the resistance derived from these lines was determined to be controlled by major genes. This simple genetic control makes its use in breeding easy and useful. In fact, monogenic resistance is the one most commonly used in breeding for resistance to viruses, and current hybrids have accumulate monogenic resistances to more than a dozen pathogens. For instance, the *Sw5* gene, which provides resistance against TSWV (Stevens et al. 1992), and the *Tm* genes, which confer resistance to *Tomato mosaic virus* (Sobir et al. 2000; Scott 2007), have been widely used for hybrid development.

It is also important to mention that the level of resistance present in our materials is comparable to or even higher than that found in tomato lines that are homozygous for *Ty-1* (Michelson et al. 1994; Zamir et al. 1994; Hanson et al. 2000; Pérez de Castro et al. 2008). Moreover, the response in plants that are heterozygous for the resistance gene was comparable to the response in homozygous plants in the case of all three sources employed. This has important implications for breeding, as the resistance will be used mostly for hybrid development. In fact, in most cases, commercial hybrids are carriers of dominant resistance genes in heterozygous condition. Additionally, this represents an important advantage, as the introgression of genomic fragments from wild relatives always involves the drag of linked fragments which frequently carry unfavourable agronomic characteristics. The heterozygous condition usually hides the expression of these detrimental effects, thereby allowing the expression of the complete genetic potential of the hybrids.

On the other hand, the effect of the genetic background in the expression of the resistance should be taken into account. This effect has already been observed by breeders in the *Ty-1* gene, whose expression ranges from partially to completely dominant upon different backgrounds. In that sense, it would be of interest to our breeding programme to

identify tomato backgrounds in which heterozygous plants show even higher resistance levels.

In the present thesis, significant progress has been made regarding the identification of new molecular markers linked to the resistance genes derived from these sources. In the first work presented here (Pérez de Castro et al. 2012), 263 markers were screened in order to map the major loci responsible for resistance, 94 of which were polymorphic between both species. The polymorphic markers found allowed us to determine the wild introgressions in each of the resistant lines and map the putative major resistance gene on chromosome 6.

In previous studies carried out by other authors, the major loci responsible for the resistance to TYLCD derived from *S. chilense* accessions LA1969, LA1932 and LA2779, denominated as genes *Ty-1* and *Ty-3*, were also mapped to chromosome 6 (Zamir et al. 1994; Ji et al. 2007b). An additional minor gene, *Ty-4*, was described in chromosome 3 in breeding lines derived from LA1932 (Ji et al. 2009). Some years later, the *Ty-1* gene was fine mapped in a region of approximately 600 kb (Verlaan et al. 2011), partially overlapping with the reported *Ty-3* region (Hutton et al. 2010). More recently, the *Ty-1* and *Ty-3* genes were fine mapped to a region of approximately 70 kb, which was recently shown to be allelic using VIGS (Verlaan et al. 2013).

Our results show that the genetic location of the resistance identified in our populations is delimited to the *Ty-1*/*Ty-3* region described previously by other authors. Since Verlaan et al. (2013) demonstrated that *Ty-1* and *Ty-3* are allelic, the fact that the resistance loci in our populations are contained in this region indicates the possibility that the genes present in our sources are also allelic to *Ty-1* and *Ty-3*. On the other hand, no minor loci affecting resistance were identified in the other chromosomes in any of the families studied.

In order to test if the resistance genes identified in our populations were allelic to *Ty-1* and *Ty-3*, further fine mapping was carried out in the second work here presented. A screening with more molecular markers within the region where the putative resistance gene was present allowed us to shorten the resistance region to a marker interval of approximately 950 kb, which overlaps with the region described by Verlaan et al. (2011, 2013).

These data together suggest the possibility of the existence of a common TYLCV resistance locus in *S. chilense* accessions LA1969, LA1932, LA2779, LA1960 and LA1971. Genes with multiple alleles controlling the resistance to different pathogens have been identified in previous studies by other authors. This is the case of the *L* locus in flax, which has several alleles controlling flax rust resistance. These alleles and their different specificities were created by intragenic crossover events (Luck et al. 2000). The other possibility is that the TYLCV resistance in these *S. chilense* accessions is controlled by tightly linked homologous genes of the same family. This situation is common in resistance genes such as the *Cf* genes (Thomas et al. 1998) or the *Mla* powdery mildew resistance locus (Wei et al. 1999).

Based on our data we cannot reject the hypothesis that the genes/alleles responsible for TYLCD resistance in our populations are different from *Ty-1* and *Ty-3*. Firstly, the LA1932-derived breeding lines were developed by our group following selection procedures that differed from that of Scott et al. (1996). Considering this along with the heterogeneity present in *S. chilense*, it is possible that alleles identified in our materials are different from the one identified by Scott et al. (1996). On the other hand, LA1960 and LA1971 are *S. chilense* accessions that have to date gone unexploited, making this the first time that genes for resistance to TYLCV derived from these accessions have been identified. Therefore, the resistance genes/alleles present in these accessions may be different from *Ty-1* and *Ty-3* or may even have different resistance spectrums.

The fact that these genes may be allelic or may be different homologous genes that are tightly linked has important implications for breeding. Firstly, the specificity and effectiveness of the new genes/alleles identified should be studied more thoroughly. If they are found to be different genes, they may subsequently be combined in different ways with the aim of obtaining the most effective and durable combinations, although this would be difficult due to the close linkage. Alternatively, if they turn out to be new alleles of the *Ty-1/Ty-3* genes, they may be combined in heterozygosis in order to merge their effects. This could allow higher resistance levels and/or a broader resistance spectrum to be obtained.

The cloning and characterisation of *Ty-1* and *Ty-3* has shown that they code for an RNA-dependent RNA polymerase (RDR) for which no function has yet been described, which seems to suggest that *Ty-1/Ty-3* constitute a completely new class of resistance genes (Verlaan et al. 2013). The authors have speculated as to the resistance mechanism of

Ty-1/Ty-3 and their specificity towards TYLCV. They suggest that the resistance mechanism may be based on the amplification of the RNAi response and transcriptional silencing of different plant geminiviruses. Concretely, the transcriptional up-regulation of *Ty-1* seems to be the most likely explanation for the resistance.

The fact that *Ty-1/Ty-3* are not typical R genes results interesting, since resistance in this kind of genes is easily overcome by pathogens and, particularly, by viruses. However, resistance caused by transcriptional up-regulation of RDR genes seems to be not so easily overcome by viruses (Leibman et al. 2011). This is therefore an indication that these genes will probably confer broader resistance than the commonly known R genes.

Use of resistance derived from *S. peruvianum* PI 126944: construction of a set of ILs

Although some tomato wild relatives are cross-compatible with the cultivated species, in others the incompatibility barriers make hybridisation and gene introgression a laborious and time-consuming process. Indeed, the high incompatibility present in crosses between *S. peruvianum* and cultivated tomato makes the use of this species for breeding purposes difficult. Self-incompatibility and sterility frequently prevent the construction of selfing generations from interspecific crosses as well as from more advanced generations.

The development of ILs from wild species in a constant genetic background makes the use of these genetic sources more precise and efficient. These permanent breeding populations prevent the process of introgression for each trait from having to be repeated. In addition, since each IL carries only a small fraction of the wild species genome, most fertility problems are eliminated, thereby preventing the possible overshadowing effect on QTL (Eshed and Zamir 1995).

The aim of the work here presented is to exploit the resistance to different pathogens derived from the *S. peruvianum* accession PI 126944. Because of the high degree of incompatibility that exists between *S. lycopersicum* and PI 126944, and in order to better exploit the breeding potential of this accession, the strategy employed consisted in the development of a set of ILs in the cultivated tomato genetic background. Several other IL collections have been previously derived from other tomato relatives such as *S. pennellii* (Eshed and Zamir 1995) and *S. habrochaites* (Monforte and Tanksley 2000), and even from more distant species, such as *S. lycopersicoides* (Canady et al. 2005). In the case of *S. peruvianum*, a fourth backcross from accession LA1708 is the most advanced generation

available (Fulton et al. 1997b). In all cases, these breeding populations have proven to be very useful in the identification of many QTLs of interest.

The significance of the work here presented goes beyond the use of PI 126944 in breeding for disease resistance. The efforts made to overcome crossability barriers along with the generations that have been developed will facilitate the exploitation of this accession to different objectives. Indeed, a huge number of crosses and embryo rescues were required to obtain the hybrids and subsequent generations. The strongest level of incompatibility was found in the initial crosses made to obtain the interspecific hybrids (Picó et al. 2002). In subsequent generations derived from these hybrids, a 'genotype-dependent' response was observed; the percentage of fruit set, viable immature embryos per fruit and regenerated plants were variable depending on the genotype considered. This could be due to differences in pollen viability or in the intensity of the incompatibility barriers. Environmental factors, such as the moment when crosses were made, light and temperature, could also influence the fruit set (Sacks et al. 1997).

For the development of the first three interspecific hybrids, Picó et al. (2002) obtained the best results with stigma and pistil complementation using hormones followed by immature seed culture. In the present study, immature seed culture and embryo rescue were also required, since high incompatibility was still present in our materials. In contrast, in other studies carried out with other *S. peruvianum* accessions, embryo rescue was not necessary, and crossability barriers were overcome with less difficult techniques, such as pollen mixture (Frieddman et al. 1998) or normal crosses, but also with a low viable seed yield per fruit (Fulton et al. 1997a). Therefore, even though *S. peruvianum* is one of the most incompatible tomato wild relatives, the especially strong crossability barriers present in crosses with PI 126944 and the difficulty in obtaining progeny in subsequent generations are remarkable.

In spite of these difficulties, our data suggest that incompatibility is starting to be lost in the most advanced generations. In the study carried out by Picó et al. (2002), all seeds derived from mature fruits had a necrosed point, indicating embryo abortion. The embryos obtained in this study were globular, although some heart and torpedo embryos were found in more advanced generations. In addition, some viable seeds were obtained from the second backcross to tomato. This demonstrates a reduction in its incompatibility that will facilitate IL development from this accession. Reduced incompatibility in

advanced generations was also observed in other studies, like the one developed by Fulton et al. (1997a) with the *S. peruvianum* accession LA1708. In this study, the strongest incompatibility barriers were found in the BC$_1$, which had low viable seed yield and sterility problems, although the number of viable seeds increased considerably in subsequent generations.

There are several factors that influence embryo germination and callus development that could be modified in order to help overcome crossability barriers, such as the crossing technique employed, the most suitable moment to do the embryo rescue, the culture media or the recurrent parent used. The study of all these factors is an interesting approach that is currently being carried out by our group based on the framework of this thesis.

In other studies where BCRILs and ILs were developed, generations were derived from one single plant (Eshed and Zamir 1995; Fulton et al. 1997a; Doganlar et al. 2002; Canady et al. 2005). However, in this study, different PI 126944 plants were used to produce the first three interspecific hybrids. As a result, several different *S. peruvianum* alleles existed in the various generations, which is an interesting point because a different IL with each allele will be developed. This will add extra value to our final collection of ILs, since more of the variability existing in the original accession will be represented.

Results obtained in the study of Fulton et al. (1997a) indicate that relatively high levels of recombination occur between the *S. peruvianum* and tomato genomes, allowing a progressive reduction of the wild species genome in advanced generations. In the work here presented, this reduction is patent, thereby proving the efficient introgression of PI 126944 genes into cultivated tomato to be possible. This highlights the possibility that the genetic variation present in this accession may be efficiently used for many purposes in tomato breeding.

In the present work, we have demonstrated that resistance to TSWV and TYLCD from *S. peruvianum* PI 126944 can be successfully introgressed into and expressed in tomato background. The determination of the genetic control of the resistance was not possible due to the small number of available plants of each generation. However, the segregation observed in plants derived from certain generations suggests a quantitative genetic control for TYLCV. These results agree with previous studies carried out with other *S. peruvianum* accessions in which TYLCV resistance was also shown to be

controlled by various genes (Pilowsky and Cohen 1990, Friedmann et al. 1998; Anbinder et al. 2009). Concretely, TYLCV resistance derived from TY-20 was described as being controlled by at least three recessive genes (Pilowsky and Cohen 1990). On the other hand, resistance in breeding line TY-172 was described as being controlled by a major dominant QTL, *Ty-5*, and four minor recessive QTLs (Anbinder et al. 2009). It is therefore possible that other new recessive genes may control the resistance in our materials, so it would be interesting to map these genes and also determine their contribution to the variation in symptom severity. Indeed, this has occurred in *S. chilense*, in which several TYLCV resistance genes have been identified in different accessions (Zamir et al. 1994; Ji et al. 2007b; Ji et al. 2009).

The currently known major TYLCV resistance genes are not a definitive solution for fighting these viruses; these genes are not completely effective and the resistances based only on major genes can be more easily overcome by new virus isolates. Consequently, the use of different resistance genes and their pyramiding will confer a higher and more durable resistance. With this aim, Vidavsky et al. (2008) pyramided genes conferring resistance to TYLCV from different wild tomato species and observed that the combination between a resistant *S. habrochaites* line and a resistant *S. peruvianum* line exhibited the lowest yield loss and the mildest level of symptoms. Similarly, the resistance present in our materials could be combined with resistance from other sources to confer higher resistance levels.

It is important to note that the utility of this IL collection is not limited to the resistances found in PI 126944. Fulton et al. (1997b) already showed that *S. peruvianum* contains many QTLs related to yield and quality traits. Moreover, they found that for several characters, such as soluble solid content, yield, viscosity and fruit weight, there was at least one QTL for which the wild allele represented an agronomic improvement over the allele from the cultivated tomato (Fulton et al. 1997b). Similar findings occurred in studies carried out with other tomato relatives, such as *S. pennellii* (Eshed and Zamir 1995) and *S. pimpinellifolium* (Doganlar et al. 2002).

Therefore, genes for improving important agronomical traits other than resistances can be found in *S. peruvianum*. However, this variability is sometimes hidden, as the positive effects of these genes cannot be predicted by the parental phenotype. The development of a set of ILs derived from this particular *S. peruvianum* accession does not

just represent a contribution to breeding for resistance to diseases, but it would also allow this hidden variability to be revealed and used for other breeding purposes.

Future perspectives

In spite of the enormous efforts made by many research groups worldwide, the TYLCD problem remains unsolved. Several resistance genes have been identified, introduced in commercial varieties and then widely used in agriculture. Moreover, the most employed resistance gene, *Ty-1*, was recently cloned successfully, and important efforts are being carried out for the cloning of two other genes, *Ty-2* and *ty-5*. Availability of the sequence of these genes and knowledge of their function will facilitate a more suitable gene combination. It will ensure higher levels of resistance with a higher degree of durability in the cultivars in which they are introduced. In this context, the present thesis intends to provide modest progress in the resolution of the TYLCD problem.

Firstly, the results obtained in this thesis show the presence of possible new TYLCV resistance alleles on chromosome 6 in materials derived from *S. chilense* that were heretofore unexploited. Moreover, the identified molecular markers that were found to be tightly linked to these resistance genes will facilitate their use in breeding programmes. Since the *Ty-1* and *Ty-3* genes have different dominances and ranges of resistance to begomovirus (Ji et al. 2007a), the genes identified by our group should be studied more deeply in order to reveal more about their specificity and effectiveness. It will also be of interest, when possible, to combine these genes with other *Ty* genes identified in other sources, i.e., *Ty-2*, *Ty-4* and *Ty-5*, in the same hybrid. Another step would be to study the effect of these genes in different tomato genetic backgrounds in order to find the one in which they express higher levels of resistance in heterozygous state. Finally, the latest developments in TYLCV resistance mechanisms (Verlaan et al. 2013) offer very useful information for future research; it would be desirable to use the VIGS approach to determine if the genes found in this study are allelic to *Ty-1* and *Ty-3* and, in the case that they are new alleles, to test if the resistance mechanism is the same as that proposed by Verlaan et al. (2013).

Regarding *S. peruvianum*, further work will include obtaining new backcross progenies with the materials already available. In fact, our group recently modified several factors in order to improve the overcoming of crossability barriers. New culture media,

different recurrent parents and different fruit harvesting dates have been tried with very positive results, as abundant progeny have been obtained. Moreover, new molecular markers are currently being developed in order to cover the genome with a higher density in order to more precisely delimit the introgressed fragments. Another step would be the identification and characterisation of the resistances present in the set of ILs. For instance, the mapping of genes controlling TYLCV and TSWV resistance and the determination of their contributions to the variation in symptom severity will be one of the next objectives. The genes controlling TYLCV resistance in this accession seem to be minor recessive genes, which makes their contribution to the resistance lower. However, the combination of major and minor genes in the same breeding material is essential in order to confer higher and more durable resistance levels. In contrast, the TSWV resistance in our materials appears to be conferred by a single dominant gene, which could constitute a new resistance gene since it does not cosegregate with the well-known *Sw-5* gene. This assumption will be confirmed in further studies. In any case, the characterisation of these genes is essential for pyramiding them with genes derived from other sources. Finding a good combination of genes would lead to higher and more durable resistance levels. On the other hand, there are other resistances in this accession, such as *Tobacco mosaic virus* (TMV) and *Fusarium oxysporum* resistance that, with this set of ILs, could also be easily characterised and exploited in breeding programmes. In addition, when the definitive collection of ILs is set up, it will constitute a new and powerful tool for the QTL mapping of different characters of interest in tomato breeding, from disease resistances to all kinds of useful agronomic traits.

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CONCLUSIONS

1) The response to TYLCV infection assayed in segregating generations derived from *S. chilense* accessions LA1932, LA1960 and LA1971 is compatible with a monogenic control of resistance. Moreover, resistance levels in heterozygous genotypes were similar to those of homozygous genotypes in the same generations, indicating that resistance is almost completely dominant. The resistance found in these *S. chilense* accessions is based on a lower viral accumulation, which leads to a reduction in symptom development. The high level of resistance found in these sources and its simple and almost dominant genetic control make its use in breeding programs focused on the development of hybrids quite easy.

2) A total of 263 molecular markers were screened, 94 of them being polymorphic between tomato and *S. chilense*. These polymorphic markers allowed the putative major resistance gene to be mapped on chromosome 6, delimited to the *Ty-1/Ty-3* region. No minor loci affecting the resistance have been identified in any other chromosomes in any of the families studied. These data together suggest the possibility of the existence of a common TYLCV resistance locus in *S. chilense* accessions LA1932, LA1960 and LA1971. However, we cannot reject the hypothesis that the genes/alleles responsible for TYLCD resistance in our populations are different from *Ty-1* and *Ty-3* or even have different resistance spectra.

3) A large number of crosses and embryo rescues were required to obtain progeny from the *S. peruvianum* PI 126944 accession and the cultivated tomato due to the high incompatibility between these two species. The incompatibility started to diminish in the most advanced generations, and some progeny were obtained by normal hybridization. Thus, embryo rescue in early generations, followed by the reduction of incompatibility in later generations, allowed the construction the set of ILs to be initiated. The huge efforts made to overcome the crossability barriers and the generations developed were worth it, since this IL population will allow this accession to be exploited with many different objectives.

4) A total of 105 markers out of 263 were identified as polymorphic between tomato and PI 126944. Available generations were genotyped with these polymorphic markers to determine which alleles of *S. peruvianum* were already introgressed. A reduction in *S. peruvianum* genome fragments was observed in advanced generations, indicating the possibility of an efficient introgression of *S. peruvianum* genes into cultivated tomato. This reduction was accompanied by a loss of incompatibility in some cases.

5) The *S. peruvianum* genome was almost completely represented among the different plants of the most advanced generations. Development of ILs will continue by backcrossing the most advanced generations available to tomato. In any case, it will be necessary to make use of less advanced generations, such as pseudo-F_4 or BC_1 to introgress certain fragments or alleles lost during the introgression process.

6) Some of the advanced generations derived from *S. peruvianum* PI 126944 were resistant to TYLCD and/or TSWV. Therefore, resistance from this particular accession can be successfully introgressed and expressed into tomato background.