UNIVERSIDAD POLITÉCNICA DE VALENCIA

TESIS DOCTORAL

Mejora genética para la resistencia a los geminivirus *Tomato yellow leaf curl virus* (TYLCV) y *Tomato yellow leaf curl Sardinia virus* (TYLCSV) en tomate

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RESUMEN

La enfermedad del rizado amarillo del tomate (Tomato yellow leaf curl disease, TYLCD) causa graves daños en los cultivos de tomate (*Solanum lycopersicum* L.) del sudeste español y de la mayor parte de las zonas tropicales y subtropicales de todo el mundo. La enfermedad está causada por un complejo de virus pertenecientes al género *Begomovirus*, familia *Geminiviridae*. Se han descrito nueve especies causantes de TYLCD y otras seis han sido propuestas. En España están presentes cuatro de las especies virales asociadas a TYLCD. Las medidas preventivas de lucha contra la enfermedad, así como las basadas en el control del insecto vector transmisor (*Bemisia tabaci* Gen.) no resultan efectivas por si mismas, de forma que el desarrollo de materiales resistentes supone la mejor estrategia de lucha a largo plazo. Dado que en la especie cultivada no se han descrito entradas resistentes, la búsqueda de fuentes de resistencia se ha centrado en las especies silvestres del género relacionadas con el tomate. Se ha encontrado resistencia en distintas entradas de algunas de estas especies y se han desarrollado líneas de mejora y materiales comerciales con resistencia procedente de algunas de ellas. El gen *Ty-1*, derivado de la entrada LA1969 de *S. chilense*, ha sido el más empleado para la obtención de líneas e híbridos comerciales resistentes. Por otra parte, haciendo uso de los avances en las técnicas de ingeniería genética, también se han desarrollado materiales con resistencia derivada del patógeno.

Sin embargo, los materiales resistentes disponibles hasta el momento no suponen una solución definitiva al problema, ya que, con presiones fuertes de inóculo o infecciones tempranas, las plantas desarrollan síntomas de la enfermedad, produciéndose pérdidas de producción. Por este motivo, numerosos grupos de investigación continúan trabajando a nivel mundial con la finalidad de obtener materiales con niveles elevados de resistencia a TYLCD. Entre los objetivos actuales de mejora se incluyen el desarrollo de resistencia de amplio espectro a varios begomovirus, la combinación de genes de distinta procedencia para conseguir mayores niveles de resistencia y la identificación de marcadores moleculares ligados a los genes de resistencia que permitan acortar los programas de mejora y acumular en un mismo material genes de resistencia de distintas fuentes.

El grupo de “Mejora para la resistencia a la enfermedad del rizado amarillo del tomate”, del Instituto de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV), en el que se ha realizado la presente tesis doctoral, disponía al inicio de la misma de distintos materiales con resistencia a TYLCD, desarrollados en trabajos previos.

Entre estos materiales se encontraban 12 líneas de mejora derivadas de la entradas LA1932 y LA1938 de *S. chilense*, seleccionadas por su resistencia a la especie *Tomato yellow leaf curl Sardinia virus* (TYLCSV), la primera especie viral causante de TYLCD detectada en España. Resultaba de interés evaluar la respuesta de estos materiales a la especie *Tomato yellow leaf curl virus* (TYLCV), introducida posteriormente en España y más extendida a nivel mundial. Seis de las líneas evaluadas han mostrado niveles elevados de resistencia parcial a TYLCSV y TYLCV, consistente en la atenuación en la manifestación de síntomas y retraso en el momento de aparición de los mismos, además de en la reducción de la acumulación viral. Además, no se han observado en estas líneas pérdidas significativas en el rendimiento como consecuencia de la infección por TYLCV. Las características agronómicas las hacen apropiadas como parentales para el desarrollo de híbridos con elevada resistencia a TYLCSV y TYLCV.

Por otra parte, se habían identificado niveles elevados de resistencia en la entrada UPV16991 de *S. pimpinellifolium*. Una vez fijada la resistencia en el fondo genético de *S. lycopersicum*, y como paso previo al empleo en programas de mejora, era conveniente determinar el control genético de la misma, así como conocer su expresión en el fondo genético de la especie cultivada. Para ello se ha empleado la línea L102, que corresponde a la quinta generación de autofecundación a partir del cruce inicial *S. lycopersicum* NE-1 x *S. pimpinellifolium* UPV16991. Se ha comprobado que la resistencia parcial a TYLCV de la línea L102 está controlada por un gen con recesividad parcial y penetración incompleta. Además, la expresión de la misma depende considerablemente del fondo genético de *S. lycopersicum* en el que se introgresa, obteniéndose los mayores niveles de resistencia en cruces con líneas vigorosas. Por todo esto, se recomienda el uso de esta resistencia bien en homocigosis en el desarrollo de híbridos vigorosos, bien en combinación con resistencia procedente de otras fuentes.

En este sentido, se decidió evaluar la resistencia en materiales que combinaban el gen *Ty-1* y el gen derivado de UPV16991, ambos en heterocigosis. El nivel de resistencia en estos materiales ha sido superior al mostrado por los heterocigotos para cada uno de los genes, e incluso en algún caso se ha superado la resistencia de los homocigotos para cada uno de los genes. Esto indica que la combinación de la resistencia derivada de UPV16991 con el gen *Ty-1* es la aproximación más práctica para la utilización de esta resistencia, ya que evita la necesidad de fijar el gen de resistencia en ambos parentales.

Por último, para la acumulación de resistencia de distinta procedencia en un mismo material, resulta imprescindible disponer de marcadores moleculares ligados a los genes de

resistencia. Se han descrito algunos marcadores ligados al gen *Ty-1*, sin embargo, la presencia del gen *Mi*, derivado de *S. peruvianum*, interfiere con los resultados para estos marcadores, obteniéndose falsos positivos. En este trabajo se ha identificado un marcador molecular, JB-1, tipo CAPS (*Cleaved Amplified Polymorphic DNA*) ligado al gen de resistencia *Ty-1*. La presencia del gen *Mi*, así como introgresiones de otras especies como *S. lycopersicum* (antes *Lycopersicon esculentum* var. *cerasiforme*), *S. habrochaites* y *S. pimpinellifolium*, no interfieren con los resultados para este marcador. Además, el análisis de materiales con introgresiones de distintas especies silvestres relacionadas con el tomate para varios marcadores de la región del *Ty-1* ha permitido localizar el marcador CT21, el RFLP (*Restriction Fragment Length Polymorphism*) a partir del cual se desarrolló JB-1.

ABSTRACT

Tomato yellow leaf curl disease (TYLCD) causes great damage in tomato (*Solanum lycopersicum* L.) crops in south-eastern Spain and in many tropical and subtropical areas in the world. The disease is caused by a complex of viruses, all belonging to the genus *Begomovirus*, family *Geminiviridae*. Nine species have been reported causing TYLCD and six more have been proposed as tentative species. Four viral species associated with TYLCD are present in Spain. Preventive measures to fight the disease, as well as measures based on controlling the insect vector (*Bemisia tabaci* Gen.) are not effective, so the development of resistant varieties seems the best long term strategy. Given that resistance has not been reported in the cultivated species, screening for resistance has been focused on wild tomato relatives. Resistance has been found in different wild species and some resistant breeding lines and commercial varieties have been developed. *Ty-1*, derived from *S. chilense* LA1969, is the most frequently used gene. Advances in genetic engineering techniques have also been exploited in developing plant material with pathogen derived resistance.

However, resistant varieties currently available are not a solution, as with high inoculum pressure conditions and early infections, plants still develop symptoms and yield losses are caused. For that reason, many research groups continue working worldwide to obtain plants with high levels of resistance to TYLCD. Current breeding objectives are the development of broad spectrum resistance to several begomovirus, the accumulation of resistance genes from different sources to increase the levels of resistance and the identification of molecular markers tightly linked to resistance genes, which allows shortening breeding programmes and accumulating different resistance genes in the same plant material.

This work has been developed in the research group ‘Breeding for resistance to Tomato yellow leaf curl disease’ of the Institute for Conservation and Improvement of Agrodiversity (COMAV). When this project was initiated, several resistant plant materials had been developed from previous works of the group.

Twelve breeding lines derived from *S. chilense* LA1932 and LA1938 were available. These lines were resistant to *Tomato yellow leaf curl Sardinia virus* (TYLCSV), the first viral species described in Spain causing TYLCD. It was of interest the evaluation of resistance in these lines to *Tomato yellow leaf curl virus* (TYLCV), introduced later in Spain and spread worldwide. Six breeding lines showed high levels of partial resistance to

TYLCSV and TYLCV. The resistance consisted on attenuation and delay in symptom development, as well as reduction in viral accumulation. Significant yield losses due to viral infection were not observed in these lines. These lines also show good horticultural traits which make them appropriate to be base material for developing commercial hybrids resistant to both, TYLCSV and TYLCV.

High levels of resistance have also been identified in *S. pimpinellifolium* UPV16991. The resistance has already been fixed in the genetic background of *S. lycopersicum*. It was convenient to determine the genetic control of the resistance and the expression in tomato background, before using it in breeding programmes. For these purposes L102 was selected. L102 belongs to the F_6 generation, after the initial cross *S. lycopersicum* NE-1 x *S. pimpinellifolium* UPV16991. Resistance to TYLCV in L102 is controlled by one gene, with partial recessiveness and incomplete penetrance. Moreover, the expression of resistance strongly depends on *S. lycopersicum* background in which it is introgressed. The highest levels of resistance are obtained when crossing L102 with vigorous lines. So, we recommend to use UPV16991-derived resistance in the development of vigorous hybrids in homozygosis or combined with resistance from other sources.

To exploit resistance derived from UPV16991, L102 and some other lines with the same origin were crossed with a breeding line homozygous for *Ty-1*. Resistance was then evaluated in several plant material heterozygous for both, *Ty-1* and the resistance gene from UPV16991. These plant materials showed higher levels of resistance than heterozygotes for each of the genes. Resistance in one of the hybrids was even higher than in homozygotes for each of the genes. These results show that combining resistance from UPV16991 with *Ty-1* is the most practical approach to exploiting this resistance, since it allows the development of hybrids without the need of fixing the resistance gene in both parents.

Finally, availability of molecular markers tightly linked to the resistance genes is essential to accumulate resistance from different sources. Some molecular markers tightly linked to *Ty-1* have been reported. However, in all cases, *S. peruvianum*-derived *Mi* gene interferes with these markers, causing false positive results. In this work, a molecular marker, JB-1, tightly linked to *Ty-1* has been identified. This is a CAPS (*Cleaved Amplified Polymorphic DNA*) marker. The presence of *Mi*, as well as introgressions from other wild tomato relatives such as *S. lycopersicum* (formerly *Lycopersicon esculentum* var. *cerasiforme*), *S. habrochaites* and *S. pimpinellifolium* do not interfere with the results

for this marker. In addition, the analysis of several plant material with introgressions from different wild tomato relatives has allowed the location of CT21, the RFLP (*Restriction Fragment Length Polymorphism*) marker from which JB-1 was designed.

RESUM

La malaltia de l'arrissat groc de la tomaca (Tomato yellow leaf curl disease, TYLCD) causa greus danys als cultius de tomaca (*Solanum lycopersicum* L.) del sud-est espanyol i de la major part de les zones tropicals i subtropicals de tot el món. La malaltia està causada per un complex de virus pertanyents al gènere *Begomovirus*, família *Geminiviridae*. S'han descrit nou espècies causants de TYLCD i altres sis han sigut proposades. A Espanya estan presents quatre de les espècies virals associades a TYLCD. Les mesures preventives de lluita contra la malaltia, així com les basades en el control de l'insecte vector transmissor (*Bemisia tabaci* Gen.) no resulten efectives per si mateixes, de manera que el desenvolupament de materials resistents suposa la millor estratègia de lluita a llarg termini. Atés que en l'espècie cultivada no s'han descrit entrades resistents, la recerca de fonts de resistència s'ha centrat en les espècies silvestres del gènere relacionades amb la tomaca. S'ha trobat resistència en distintes entrades d'algunes d'estes espècies i s'han desenvolupat línies de millora i materials comercials amb resistència procedent d'algunes d'elles. El gen *Ty-1*, derivat de l'entrada LA1969 de *S. chilense*, ha sigut el més utilitzat per a l'obtenció de línies i híbrids comercials resistents. D'altra banda, fent ús dels avanços en les tècniques d'enginyeria genètica, també s'han desenvolupat materials amb resistència derivada del patogen.

No obstant, els materials resistents disponibles fins al moment no suposen una solució definitiva al problema, ja que, amb pressions fortes d'inòcul o infeccions primerenques, les plantes desenvolupen símptomes de la malaltia, produint-se pèrdues de producció. Per este motiu, nombrosos grups d'investigació continuen treballant a nivell mundial amb la finalitat d'obtindre materials amb nivells elevats de resistència a TYLCD. Entre els objectius actuals de millora s'inclouen el desenvolupament de resistència d'ampli espectre a diversos begomovirus, la combinació de gens de distinta procedència per a aconseguir majors nivells de resistència i la identificació de marcadors moleculars lligats als gens de resistència que permeten acurtar els programes de millora i acumular en un mateix material gens de resistència de distintes fonts.

El grup de "Millora per a la resistència a la malaltia de l'arrissat groc de la tomaca", de l'Institut de Conservació i Millora de l'Agrodiversitat Valenciana (COMAV), en el que s'ha realitzat la present tesi doctoral, disposava a l'inici de la mateixa, de distints materials amb resistència a TYLCD, desenvolupats en treballs previs.

Entre estos materials es trobaven 12 línies de millora derivades de l'entrades LA1932 i LA1938 de *S. chilense*, seleccionades per la seua resistència a l'espècie *Tomato yellow leaf curl Sardinia virus* (TYLCSV), la primera espècie viral causant de TYLCD detectada a Espanya. Resultava d'interés avaluar la resposta d'estos materials a l'espècie *Tomato yellow leaf curl virus* (TYLCV), introduïda posteriorment a Espanya i més estesa a nivell mundial. Sis de les línies avaluades han mostrat nivells elevats de resistència parcial a TYLCSV i TYLCV, consistent en l'atenuació en la manifestació de símptomes, retard en el moment d'aparició dels mateixos i reducció de l'acumulació viral. A més, no s'han observat en estes línies pèrdues significatives en el rendiment com a conseqüència de la infecció per TYLCV. Les característiques agronòmiques les fan apropiades com a parentals pel desenvolupament d'híbrids amb elevada resistència a TYLCSV i TYLCV.

D'altra banda, s'havien identificat nivells elevats de resistència en l'entrada UPV16991 de *S. pimpinellifolium*. Una vegada fixada la resistència en el fons genètic de *S. lycopersicum*, i com a pas previ a l'ús en programes de millora, era convenient determinar el control genètic de la mateixa, així com conéixer la seua expressió en el fons genètic de l'espècie cultivada. Per a això s'ha emprat la línia L102, que correspon a la quinta generació d'autofecundació a partir del creuament inicial *S. lycopersicum* NE-1 x *S. pimpinellifolium* UPV16991. S'ha comprovat que la resistència parcial a TYLCV de la línia L102 està controlada per un gen amb recessivitat parcial i penetració incompleta. A més, l'expressió de la mateixa depén considerablement del fons genètic de *S. lycopersicum* en el que s'introgresa, obtenint-se els majors nivells de resistència en creuaments amb línies vigoroses. Per tot açò, es recomana l'ús d'esta resistència bé en homozigosis en el desenvolupament d'híbrids vigorosos, bé en combinació amb resistència procedent d'altres fonts.

En este sentit, es va decidir avaluar la resistència en materials que combinaven el gen *Ty-1* i el gen derivat d'UPV16991, ambdós en heterozigosis. El nivell de resistència en estos materials ha sigut superior al mostrat pels heterozigots per a cada un dels gens, i fins i tot en algun cas s'ha superat la resistència dels homozigots per a cada un dels gens. Açò indica que la combinació de la resistència derivada d'UPV16991 amb el gen *Ty-1* és l'aproximació més pràctica per a la utilització d'esta resistència, ja que evita la necessitat de fixar el gen de resistència en ambdós parentals.

Finalment, per a l'acumulació de resistència de distinta procedència en un mateix material, resulta imprescindible disposar de marcadors moleculars lligats als gens de resistència. S'han descrit alguns marcadors lligats al gen *Ty-1*, no obstant, la presència del

gen *Mi*, derivat de *S. peruvianum*, interferix amb els resultats per a estos marcadors, obtenint-se falsos positius. En este treball s'ha identificat un marcador molecular, JB-1, tipus CAPS (*Cleaved Amplified Polymorphic DNA*) lligat al gen de resistència *Ty-1*. La presència del gen *Mi*, així com introgresions d'altres espècies com *S. lycopersicum* (abans *Lycopersicon esculentum* var. *cerasiforme*), *S. habrochaites* i *S. pimpinellifolium*, no interferixen amb els resultats per a este marcador. A més, l'anàlisi de materials amb introgresions de distintes espècies silvestres relacionades amb la tomaca per a diversos marcadors de la regió del *Ty-1* ha permés localitzar el marcador CT21, el RFLP (*Restriction Fragment Length Polymorphism*) a partir del qual es va desenvolupar JB-1.

ÍNDICE

1. INTRODUCCIÓN

1. INTRODUCCIÓN

1.1. Historia de la enfermedad del rizado amarillo del tomate

La enfermedad del rizado amarillo del tomate (Tomato yellow leaf curl disease, TYLCD) fue descrita por primera vez en Israel en 1939 (Avidov, 1944, citado por Cohen y Antignus, 1994). En 1959, todos los campos de cultivo de tomate en el Valle del Jordán se vieron afectados por una enfermedad que provocaba los mismos síntomas, perdiéndose por completo la producción. No fue hasta 1964 cuando se publicó por primera vez la naturaleza vírica de esta enfermedad y su asociación con el vector natural, la mosca blanca *Bemisia tabaci* Gennadius (Cohen y Harpaz, 1964). Años más tarde se determinó que el agente causal era un geminivirus (Cherif y Ruso, 1983) y se aisló la partícula viral (Czosnek *et al*., 1988). En 1991 se publicó la primera secuencia completa del virus, siendo el primer caso de un geminivirus transmitido por mosca blanca cuyo genoma es monopartito (Navot *et al*., 1991). Actualmente se sabe que TYLCD está causada por un complejo de virus pertenecientes al género *Begomovirus*, familia *Geminiviridae*.

Hasta el momento, la enfermedad se ha identificado en la mayor parte de las zonas tropicales y subtropicales del mundo. Desde Israel la enfermedad se extendió a Oriente Medio, al norte y centro de África y al sudeste de Asia (Cohen y Antignus, 1994; Czosnek y Laterrot, 1997). Más tarde, fue detectada en el sur de Europa, en países como Italia (Credi *et al*., 1989; Gallitelli *et al*., 1991), España (Moriones *et al*., 1993), Portugal (Louro *et al*., 1996), Francia (Dalmon *et al*., 2000) y Grecia (Avgelis *et al*., 2001; Papayiannis *et al*., 2007). También procedente de Israel, la introducción de la enfermedad en el Nuevo Mundo se produjo probablemente a partir de plantas infectadas de tomate exportadas a la República Dominicana (Polston *et al*., 1994; Morales y Anderson, 2001). Bien fuese a partir de esta primera introducción o como consecuencia de introducciones independientes, la enfermedad ha sido descrita en otras regiones de la islas del mar Caribe (Morales y Anderson, 2001), así como en México (Brown *et al*., 1986), Estados Unidos (Momol *et al*., 1999; Polston *et al*., 1999, 2002; Valverde *et al*., 2001; Ling *et al*., 2006; Akad *et al*., 2007a), Argentina (Czosnek y Laterrot, 1997) y Venezuela (Zambrano *et al*., 2007). La enfermedad también se ha detectado en Australia (Nahkla *et al*., 1993), Japón (Kato *et al*., 1998), China (Zeidan *et al*., 1998) y otras regiones de Extremo Oriente (Green *et al*., 2001), Marruecos (Peterschmit *et al*., 1999a), Isla Reunión (Peterschmit *et al*., 1999b),

Madagascar (Delatte *et al*., 2002), Etiopía (Shih *et al*., 2006), Indonesia (Tsai *et al*., 2006), etc.

En la actualidad la enfermedad se encuentra ampliamente extendida, ocasionando en muchos casos pérdidas del 100% de la producción en los cultivos de tomate (Picó *et al*., 1996; Czosnek y Laterrot, 1997; Moriones y Navas-Castillo, 2000).

1.2. Agente causal de TYLCD

TYLCD está causada por un complejo de virus pertenecientes al género *Begomovirus,* familia *Geminiviridae*. Los virus de esta familia se caracterizan por presentar partículas geminadas, formadas por dos icosaedros incompletos unidos por una de sus caras. El tamaño de la partícula es de 18 x 30 nm. La cápsida está constituida por un único tipo de proteína y en su interior se encuentra el DNA viral. El genoma de los geminivirus está compuesto por una o dos moléculas de DNA circular monocatenario, de entre 2,5 y 3,0 kb. El número de moléculas depende de la especie considerada, denominándose la especie monopartita en el caso de presentar una única molécula de DNA y bipartita si presenta dos.

La familia *Geminiviridae* incluye cuatro géneros: *Mastrevirus*, *Curtovirus*, *Begomovirus* y *Topocuvirus* (Fauquet y Stanley, 2005). Estos cuatro géneros vienen definidos por la organización del genoma, el tipo de insecto que lo transmite y la gama de hospedantes. El género begomovirus es el más numeroso de la familia, incluyendo más de 80 especies reconocidas. Los virus pertenecientes a este género infectan dicotiledóneas, principalmente solanáceas. En condiciones naturales son transmitidos por la mosca blanca *Bemisia tabaci*, si bien se ha descrito la transmisión de alguna especie de begomovirus por la mosca blanca *Trialeurodes ricini* Misra (Idriss *et al*., 1997).

En general, las especies del género begomovirus poseen genoma bipartito, aunque algunas presentan genoma monopartito. En concreto, la mayor parte de las especies asociadas a TYLCD, y entre ellas todas las detectadas hasta el momento en España, son monopartitas. Estas especies poseen una molécula de DNA circular de aproximadamente 2,8 kb. Esta molécula codifica seis proteínas, dos en el sentido viral y cuatro en el complementario. Los ORFs (*Open Reading Frames*, marcos abiertos de lectura) se solapan entre sí y están organizados de forma bidireccional en dos estructuras de transcripción, separados por una región intergénica, no codificante (Rybicki *et al*., 2000). En el sentido viral se encuentran dos ORFs que se solapan entre sí, V1 y V2. El ORF V1 codifica la

proteína de la cápsida, que es responsable de la encapsidación del genoma viral (Townsend *et al*., 1985) y que media el movimiento del virus (Noris *et al*., 1998) y el reconocimiento por parte del vector (Briddon *et al*., 1990). La proteína codificada por el ORF V2 está también implicada en el movimiento viral (Rojas *et al*., 2001), así como en la manifestación de síntomas (Wartig *et al*., 1997). En el sentido complementario se encuentran los ORFs C1, C2 y C3, parcialmente solapados, y el C4, contenido en el ORF C1. El ORF C1 codifica la replicasa viral (Rep), responsable de la iniciación de la replicación del virus (Desbiez *et al*., 1995). La proteína codificada por el ORF C2 se denomina proteína activadora de la transcripción (TrAp) y está implicada en la activación de la transcripción de los genes en el sentido viral, así como en el movimiento sistémico del virus (Wartig *et al*., 1997). Recientemente se ha relacionado también con la supresión del silenciamiento génico post-transcripcional (Dong *et al*., 2003). El ORF C3 codifica la proteína REn, proteína potenciadota de la replicación (Sunter *et al*., 1990). La proteína codificada por el ORF C4 está relacionada con el movimiento intracelular, la infección sistémica y la manifestación de síntomas (Jupin *et al*., 1994; Rojas *et al*., 2001; Gafni y Epel, 2002). También se ha descrito la capacidad de C4 de suprimir el silenciamiento génico post-transcripcional (Bisarro, 2006). La región intergénica, no codificante, contiene elementos esenciales para la replicación y la transcripción (Fontes *et al*., 1994; Orozco y Hanley-Bowdoin, 1996).

Dentro del género begomovirus, se han descrito nueve especies causantes de TYLCD (Fauquet y Stanley, 2005): *Tomato yellow leaf curl Axarquia virus* (TYLCAxV), *Tomato yellow leaf curl China virus* (TYLCCNV), *Tomato yellow leaf curl Guandong virus* (TYLCGuV), *Tomato yellow leaf curl Kanchanaburi virus* (TYLCKaV), *Tomato yellow leaf curl Malaga virus* (TYLCMalV), *Tomato yellow leaf curl Mali virus* (TYLCMLV), *Tomato yellow leaf curl Sardinia virus* (TYLCSV), *Tomato yellow leaf curl Thailand virus* (TYLCTHV) y *Tomato yellow leaf curl virus* (TYLCV). Además, otras cinco especies han sido propuestas: *Tomato yellow leaf curl Nigeria virus* (TYLCNV), *Tomato yellow leaf curl Kuwait virus* (TYLCKWV), *Tomato yellow leaf curl Saudi Arabia virus* (TYLCSAV), *Tomato yellow leaf curl Tanzania virus* (TYLCTZV) y *Tomato yellow leaf curl Yemen virus* (TYLCYV). Recientemente, el aislado detectado en Indonesia ha sido también propuesto como una nueva especie, *Tomato yellow leaf curl Indonesia virus* (TYLCIDV), según los criterios establecidos por el Comité Internacional de Taxonomía de Virus (*International Committee on Taxonomy of Viruses*, ICTV) (Tsai *et al*., 2006).

Dos de las especies virales causantes de TYLCD descritas hasta el momento provienen de sucesos de recombinación a partir de las especies TYLCSV y TYLCV. Así, la especie TYLCMalV es el resultado de recombinación entre los aislados TYLCV-[ES72/97] y TYLCSV-ES[1] (Monci *et al*., 2002) y la especie TYLCAxV proviene de la recombinación entre los aislados TYLCV-[Alm] y TYLCSV-ES[1] (García-Andrés *et al*., 2006). De hecho, la recombinación desempeña un papel fundamental en la diversidad genética y la evolución de los begomovirus (Padidam *et al*., 1999) y es un mecanismo adaptativo importante (García-Arenal y McDonald, 2003). Recientemente se ha comprobado empleando como modelo infecciones mixtas con las especies TYLCV y TYLCSV sobre tomate, que, principalmente tras períodos prolongados de coinfección, se produce con frecuencia la emergencia de recombinantes (García-Andrés *et al*., 2007a). Además, estos recombinantes son capaces de competir sobre las plantas con las especies originales y pueden ser transmitidos por el vector *B. tabaci* (García-Andrés *et al*., 2007a). Resulta de interés la evaluación de la diversidad de estos recombinantes así como de su capacidad adaptativa, con objeto de diseñar de forma eficiente y durable las estrategias de control de la enfermedad (García-Arenal y McDonald, 2003).

1.3. Sintomatología producida en tomate por los begomovirus asociados a TYLCD

Los síntomas provocados en tomate como consecuencia de la infección por las especies virales causantes de TYLCD comienzan a manifestarse entre dos y cuatro semanas después de la inoculación y aparecen perfectamente desarrollados al cabo de dos meses (Jordá, 1995). Los síntomas que se observan habitualmente son amarilleo marginal e internervial, rizado, fruncido y acucharado de los foliolos. Este último síntoma da el nombre coloquial con que se conocen estos virus, “virus de la hoja de cuchara”. En estados avanzados de la enfermedad, se observa una reducción en el tamaño de la lámina foliar de las hojas jóvenes, que unido al acortamiento de los entrenudos y la detención del crecimiento, dan a la planta un aspecto arbustivo (Figura 1). Si la infección se produce de forma temprana, el virus produce aborto floral y una importante reducción del cuajado de los frutos. Además, los tomates cuajados presentan un tamaño inferior al comercial, lo que provoca importantes pérdidas económicas. El tipo de síntomas manifestados, así como la intensidad de los mismos, dependen fundamentalmente de tres factores: la especie viral causante de la infección, las características de la planta infectada y las condiciones ambientales.

Figura 1. Síntomas de TYLCD en plantas de tomate.

1.4. Transmisión de los begomovirus asociados a TYLCD

La transmisión de los begomovirus en condiciones naturales se produce mediante la mosca blanca *Bemisia tabaci* Gennadius. Hasta el momento se han caracterizado aproximadamente 20 biotipos de esta especie (Perring, 2001), morfológicamente idénticos, pero cuyas características biológicas o genéticas son distintas (Brown *et al*., 1995). En la Península Ibérica se han identificado los biotipos B, Q y S. El biotipo B es el más extendido a nivel mundial (Bedford *et al*., 1994), mientras que en España es mayoritario el Q. Este biotipo había sido localizado inicialmente sólo en la cuenca mediterránea (Guirao *et al*., 1997), pero más recientemente ha sido detectado en otras áreas como China (Dong *et al*., 2005), Japón (Ueda y Brown, 2006) o Arizona (Dennehy *et al*., 2006). El biotipo S ha sido descrito en Málaga sobre *Ipomoea indica* (Burm.) Merr. (Banks *et al*., 1999).

La adquisición del virus por parte del insecto tiene lugar mediante la alimentación de plantas infectadas. Los frutos de tomate infectados pueden también actuar como reservorio, ya que el virus puede ser adquirido y transmitido por *B. tabaci* a partir de ellos (Delatte *et al*., 2003). La transmisión se produce de forma persistente y circulativa. Una vez ingerido por el vector, el virus debe pasar del tracto digestivo a las glándulas salivares para poder ser transmitido mediante la alimentación. Para evitar que se produzca la destrucción en la hemolinfa del insecto, el virus interacciona con homólogos de la proteína GroEL, una proteína de la familia de las chaperonas, producida por bacterias endosimbiontes (Morin *et al*., 1999). En estudios realizados con las especies TYLCV y TYLCSV se ha comprobado que los períodos mínimos tanto de adquisición como de transmisión son de 15 a 30 minutos, aunque la transmisión es más eficiente cuanto más se prolongan estos tiempos (Antignus y Cohen, 1994; Caciagli *et al*., 1995). El período de latencia hasta que el virus puede ser transmitido es de 17 a 20 horas y los insectos permanecen infectivos de 7 a 20 días (Mehta *et al*., 1994). Los estudios parecen indicar que el insecto inocula los viriones directamente en los tubos cribosos (Jiang *et al*., 2000). Las hembras transmiten el virus de forma más eficiente que los machos (Cohen y Nitzany, 1966; Caciagli *et al*., 1995). Las larvas también pueden ser portadoras del virus, pero al ser sésiles no dispersan la enfermedad. Según estudios realizados con el aislado israelí de la especie TYLCV es posible la transmisión sexual entre insectos (Ghanim y Czosnek, 2000), los cuales a su vez pueden transmitir el virus a un mínimo de dos generaciones de descendientes (Ghanim *et al.*, 1998), manteniéndose en ambos casos la capacidad infectiva. Sin embargo, parece ser que esto no es general entre los begomovirus causantes

de TYLCD. El aislado de Cerdeña de TYLCSV es transmitido a una generación de descendientes, pero pierde la infectividad (Bosco *et al*., 2004). Incluso existen diferencias entre aislados de la misma especie viral, ya que en estudios realizados con un aislado portugués de TYLCV no se observó la transmisión del virus a la primera generación de descendientes a partir de hembras infectivas (Bosco *et al*., 2004).

Por otra parte se ha observado que el virus puede tener efectos negativos sobre el vector; estudios realizados con la especie TYLCV han revelado que puede causar insectos menos longevos y con una tasa de fecundidad más baja (Rubinstein y Czosnek, 1997). Esto se han relacionado con la capacidad del virus para replicarse en el interior del insecto (Czosnek *et al*., 2001), ya que, otro begomovirus, el *Tomato mottle virus* (ToMoV), para el que no se produce transcripción en el vector no provoca efectos negativos en el mismo (Sinisterra *et al*., 2005)

En condiciones de laboratorio el virus puede ser transmitido por agroinoculación (Kheyr-Pour *et al*., 1991). También se ha conseguido experimentalmente la transmisión mecánica, aunque en condiciones naturales no tiene importancia en la diseminación de la enfermedad (Makkouk *et al*., 1979; Abdel-Sahun, 1990). Se transmite fácilmente por injerto, sin embargo hasta el momento no se ha citado la transmisión por suelo o por semilla (Cohen y Nitzany, 1966; Makkouk *et al*., 1979; Credi *et al*., 1989).

1.5. Gama de hospedantes de los begomovirus asociados a TYLCD

Los begomovirus no suelen tener una gama muy amplia de hospedantes (Harrison, 1985). En principio, las especies virales causantes de TYLCD no son una excepción, ya que se ha descrito su capacidad para infectar únicamente a algunas especies de varias familias. Sin embargo, sólo existen estudios de gama de hospedantes con las especies TYLCV y TYLCSV. Entre las especies cultivadas, TYLCV ha sido detectado infectando tomate, berenjena (*Solanum melongena* L.) (Abou-Jawdah *et al*., 1999), judía (*Phaseolus vulgaris* L.) (Navas-Castillo *et al*., 1999), pimiento (*Capsicum annuum* L. *C. chinense* Jacq., *C. baccatum* L. y *C. frutescens* L.) (Reina *et al*., 1999; Roye *et al*., 1999; Salati *et al*., 2002; Morilla *et al*., 2005; Polston *et al*., 2006), lisiantus (*Eustoma grandiflorum* L.) (Cohen *et al*., 1995), calabacín (*Cucurbita pepo* L.) (Martínez-Zubiaur *et al*., 2004) y tabaco (*Nicotiana tabacum* L.) (Font *et al*., 2005). La especie TYLCSV ha sido identificada infectando de forma natural tomate y pimiento (Gorsane *et al*., 2004).

El conocimiento acerca de las especies silvestres capaces de actuar como reservorio de estos virus puede aportar información sobre la epidemiología de la enfermedad y puede ayudar a planear las estrategias de control de la misma. Por este motivo, resulta de interés conocer qué malas hierbas son hospedantes naturales del virus. En este sentido, en los primeros estudios experimentales de transmisión, realizados empleando el vector *B. tabaci*, sólo encontraron plantas hospedantes de TYLCV en las siguientes familias: *Asclepiadaceae*, *Compositae*, *Leguminosae*, *Malvaceae*, *Solanaceae* y *Umbelliferae* (Cohen y Nitzani, 1966; Cohen y Antignus, 1994). En los últimos años, hasta 104 especies pertenecientes a 24 familias de dicotiledóneas aparecen citadas como hospedantes naturales o experimentales de TYLCV (Dalmon y Marchoux, 2000).

La especie TYLCV se ha encontrado infectando de forma natural las siguientes especies de plantas: *Cynanchum acutum* L., *Datura stramonium* L., *Malva parviflora* L. (Cohen y Antignus, 1994), *Plantago minor* L., *Mercurialis annua* L. (Abou-Jawdah *et al*., 1999), *Mercurialis ambigua* L. fil. (Sánchez-Campos *et al*., 2000), *Cleome viscosa* L., *Croton lobatus* L., *Solanum nigrum* L. y especies de los géneros *Malva*, *Macroptilium*, *Physalis*, *Polygonum*, *Sida* y *Wissadula*, especies sin identificar de las familias *Acanthaceae*, *Cucurbitaceae* y *Nyctaginaceae* (Salati *et al*., 2002), y las especies *Conyza sumatrensis* (Retz.) E. Walker, *Convolvulus* sp., *Cuscuta* sp., *Chenopodium murale* L. (Jordá *et al*., 2001).

La especie TYLCSV sólo ha sido detectada de forma natural infectando *D. stramonium*, *S. nigrum*, *S. luteum* L., *Euphorbia* spp., *Malva parviflora* (Bosco *et al*., 1993; Davino *et al*., 1994; Bedford *et al*., 1998; Sánchez-Campos *et al*., 2000), *Convolvulus* sp., *Cuscuta* sp. y *Chenopodium murale* L (Jordá *et al*., 2001).

La gama de hospedantes depende no sólo de la especie, sino también del aislado. Así, *S. nigrum*, ha sido detectada infectada por el aislado de TYLCV presente en la República Dominicana (Salati *et al*., 2002) y el aislado de la cepa "tipo" de Israel detectado en España (TYLCV-[Alm]) (García-Andrés *et al*., 2006), pero no es hospedante del aislado TYLCV-[ES72/97] (Monci *et al*., 2002). Por otra parte, se ha comprobado que las nuevas especies recombinantes descritas tienen una gama de hospedantes mayor que cada una de las especies de las que provienen. Es el caso de la especie TYLCMalV, resultado de recombinación entre aislados de TYLCV y TYLCSV presentes en España. El aislado correspondiente de TYLCV es capaz de infectar tomate y judía, pero no *S. nigrum* o *S. luteum*, mientras que el aislado de TYLCSV infecta tomate, *S. nigrum* y *S. luteum*, pero no judía. TYLCMalV puede infectar tomate, judía, *S. nigrum* y *S. luteum* (Monci *et*

al., 2002). Resultados muy similares se han obtenido con la nueva especie recombinante identificada en España, TYLCAxV (García-Andrés *et al*., 2006).

1.6. La enfermedad del rizado amarillo del tomate en España

La presencia de TYLCD fue detectada por primera vez en España en 1992, sobre plantas de tomate en invernaderos de Murcia (Moriones *et al*., 1993) y Almería (Reina *et al*., 1994). Los dos aislados identificados, el de Murcia y Almería, pertenecían a la especie procedente de Cerdeña (*Tomato yellow leaf curl Sardinia virus*, TYLCSV). Años más tarde, en 1997, asociada a epidemias más graves de la enfermedad, se detectó la presencia de la especie *Tomato yellow leaf curl virus* (Navas-Castillo *et al*., 1997). Se aisló a partir de plantas de tomate recogidas en Almería y se comprobó que se trataba de la cepa Mild de TYLCV (TYLCV-Mld) (Navas-Castillo *et al*., 1997). Esta especie fue identificada también infectando plantas de judía en Málaga y Almería (Navas-Castillo *et al*., 1999) y en pimiento en invernaderos de Almería (Reina *et al*., 1999). Actualmente, la enfermedad se encuentra ampliamente extendida en España en cultivos de tomate localizados tanto en la península (Málaga, Granada, Almería, Murcia, Alicante, Valencia, Castellón y Barcelona) (Font *et al*., 2007), como en las Islas Canarias (Font *et al*., 2000; Monci *et al*., 2000) y las Islas Baleares (Font *et al*., 2002). Diversos aislados de la especie TYLCV han sido detectados en España, entre ellos la cepa tipo descrita en Israel (Morilla *et al*., 2003), presente en España desde 1998 (García-Andrés *et al*., 2007b) y que además de en tomate ha sido identificada infectando plantas de pimiento (Morilla *et al*., 2005).

Desde la aparición en los cultivos españoles de la especie TYLCV, ambas especies, TYLCSV y TYLCV, coexisten en infecciones sobre tomate. Sin embargo, se ha constatado en muchas zonas el progresivo desplazamiento de TYLCSV por la especie TYLCV (Sánchez-Campos *et al*., 1999). Ambas especies presentan la misma adaptación sobre tomate, sin embargo TYLCV es transmitida de forma más eficiente por los biotipos de mosca blanca predominantes en España. Además, los cultivos de judía son reservorio de TYLCV, pero no de TYLCSV, entre los ciclos de otoño y primavera de tomate. Estos dos factores explican en parte el desplazamiento de TYLCSV por TYLCV (Sánchez-Campos *et al*., 1999). Más recientemente se ha puesto de manifiesto el hecho de que el cultivo generalizado de variedades resistentes a la enfermedad es otro de los factores implicados en este desplazamiento, ya que la especie TYLCV está mejor adaptada para la supervivencia en estas condiciones (Lacasa *et al*., 2001; García-Andrés, 2006).

Se ha demostrado que TYLCV y TYLCSV coexisten en infecciones mixtas en la misma planta, no sólo en tomate (Morilla *et al*., 2004), sino también en otras especies como *Solanum nigrum* (García-Andrés *et al*., 2006). Esta situación ha propiciado la generación de recombinantes entre ambas especies. Concretamente, se han detectado dos recombinantes naturales, cuyas características genéticas y patogénicas, así como los criterios para la denominación de geminivirus propuesta por el ICTV han determinado que sean considerados nuevas especies: *Tomato yellow leaf curl Malaga virus* (Monci *et al*., 2002) y *Tomato yellow leaf curl Axarquia virus* (García-Andrés *et al*., 2006). Ambos presentan una gama de hospedantes mayor que el de las especies de las que provienen y provocan síntomas más graves en algunos de estos hospedantes. Además, estos recombinantes se han dispersado con las epidemias en los cultivos comerciales de tomate (Monci *et al*., 2002; García-Andrés *et al*., 2006, 2007b). Dado que es frecuente la aparición de recombinantes como consecuencia de la coinfección (García-Andrés *et al*., 2007a), no se puede descartar la presencia de otros recombinantes en los cultivos españoles.

En España se han realizado tres de los escasos estudios disponibles en cuanto a la estructura y diversidad genética de las poblaciones de begomovirus asociadas a TYLCD. El primero de estos estudios se centró en la especie TYLCSV, durante los ocho años iniciales desde la aparición del virus en España, concluyendo la elevada estabilidad genética y la baja diversidad genética de la población durante este período (Sánchez-Campos *et al*., 2002). En un estudio posterior se analizaron las poblaciones de TYLCSV y TYLCV tanto en la costa mediterránea como en las Islas Baleares y Canarias (Font *et al*., 2007). En todas las regiones analizadas la población estaba compuesta por un haplotipo predominante, siendo la diversidad genética baja; la única excepción se observó en la región de Murcia, en la que la población era más heterogénea, con tres haplotipos predominantes. Además, se comprobó que se produce tráfico de begomovirus a larga distancia, ya que parte de los aislados de TYLCV presentes en la península son similares a aislados descritos en Portugal y Japón, y los hallados en Lanzarote se asimilan a los aislados detectados en el Caribe (Font *et al*., 2007). Más recientemente se ha realizado un estudio más amplio, en base a muestreos realizados en tomate y judía entre 1997 y 2003, en Italia y España (García-Andrés *et al*., 2007b). Se ha comprobado que las poblaciones analizadas están estructuradas según el hospedante y la zona geográfica, además de presentar variaciones con el tiempo. Por otra parte, la diversidad genética encontrada en estas poblaciones es baja, siendo mayor en regiones no codificantes. Este tipo de estudios resultan de interés, no sólo para profundizar en el conocimiento de la evolución de las

poblaciones de virus y su interacción con el hospedante, sino también para la planificación de las estrategias de control de la enfermedad. En este sentido, los estudios realizados ponen de manifiesto la adaptación ecológica de la cepa tipo de TYLCV (TYLCV-[Alm]) en los cultivos de tomate de la región del sudeste español; esta cepa está presente en las epidemias en España desde 1998 y está produciendo un desplazamiento de otros begomovirus asociados a TYLCD. Esta circunstancia debe considerarse a la hora de desarrollar materiales resistentes a la enfermedad (García-Andrés *et al*., 2007b).

1.7. Estrategias de control de la enfermedad

Los métodos de lucha contra la enfermedad se basan fundamentalmente en la disminución de la cantidad de inóculo, en el control del vector por métodos físicos, químicos, biológicos o mediante prácticas culturales y en el empleo de variedades resistentes (Picó *et al*., 1996).

Las medidas más frecuentemente adoptadas para disminuir la cantidad de inóculo consisten en la protección de los semilleros con el objeto de realizar los transplantes con material sano. Asimismo, es conveniente eliminar, antes del transplante, los restos del cultivo anterior y las malas hierbas que pueden actuar como reservorios del virus.

Por otra parte, se ha comprobado que la dispersión del virus está correlacionada con el tamaño de la población del vector en campo (Cohen *et al*., 1988), por lo que las medidas encaminadas a eliminar el vector contribuyen a controlar la enfermedad. En este sentido los tratamientos insecticidas no suelen resultar efectivos si no se combinan con otras medidas, dado que la eficacia de la transmisión es elevada y un número pequeño de individuos es capaz de iniciar una epidemia. Otras alternativas útiles consisten en la utilización de trampas cromotrópicas (Chu *et al*., 2000) o la protección de los cultivos con redes de malla densa (Arsénio *et al*., 2002). El control biológico con enemigos naturales es otra de las medidas empleadas, fundamentalmente con parasitoides de los géneros *Eretmocerus* (Urbaneja *et al*., 2007) o *Encarsia* (Gerling *et al*., 2001).

También se ha demostrado la utilidad del empleo de plásticos que filtran la luz ultravioleta, ya que de esta forma se consigue interferir en la visión de los insectos. En distintos estudios realizados en cultivos protegidos con este tipo de plásticos se ha observado una reducción importante de la incidencia de la enfermedad (Antignus *et al*., 1996; Monci *et al*., 2004; Velasco *et al*., 2005; Rapisarda *et al*., 2006). Sin embargo, no resulta aconsejable el empleo de estos plásticos combinado con el control biológico

mediante *Eretmocerus mundus*, ya que en estas condiciones el parasitoide no es capaz de localizar a la mosca (Chiel *et al*., 2006). En la práctica totalidad de las zonas de cultivo de tomate para consumo en fresco en Israel se están empleando pantallas repelentes de insectos, habiéndose comprobado que se trata de una medida efectiva y económicamente viable (Berlinger *et al*., 2002). El empleo de cultivos trampa está también proporcionando resultados positivos; se ha comprobado que se produce una disminución en el porcentaje de plantas con síntomas en los cultivos de tomate rodeados de pepino (Al-Hitty y Sharif, 1987) o calabaza (Schuster, 2004).

Algunas de estas medidas pueden ser útiles en cultivo protegido, mientras que en cultivo al aire libre para que resulten eficaces es necesario que se adopten de forma generalizada en toda el área productora. En cualquier caso, estas estrategias no resultan suficientes por sí mismas en condiciones de elevada presión de inóculo o infecciones tempranas.

Por lo tanto, resulta fundamental el desarrollo de variedades resistentes a TYLCD. El manejo integrado del cultivo, combinando el empleo de mallas anti-mosca, la lucha biológica y la utilización de variedades resistentes, puede contribuir a reducir los porcentajes de plantas infectadas y, consecuentemente, la incidencia de la enfermedad (Stansly *et al*., 2004).

1.8. Mejora genética para la resistencia a TYLCD

1.8.1. Fuentes de resistencia y materiales desarrollados

La búsqueda de fuentes de resistencia a TYLCD se inició en la especie cultivada. Algunos cultivares fueron clasificados como ligeramente tolerantes, sin embargo ninguno mostró niveles aceptables de resistencia o tolerancia (Picó *et al*., 1996). Por este motivo, la búsqueda se ha centrado en las especies silvestres relacionadas con el tomate. Se ha identificado resistencia en distintas entradas de cinco de estas especies: *Solanum galapagense* S.Darwin & Peralta (Hassan *et al*., 1982; 1984a), *S. pimpinellifolium* L. (Zakay *et al*., 1991; Hassan y Abdel-Ati 1999; Picó *et al*., 2000), *S. peruvianum* L. (Hassan *et al*., 1982; Hassan *et al*., 1987; Kasrawi *et al*., 1988; Pilowsky y Cohen 1990; Zakay *et al*., 1991), *S. habrochaites* S. Knapp & D.M. Spooner (Hassan *et al*., 1982, 1984a; Kasrawi *et al*., 1988; Zakay *et al*. 1991; Channarayappa *et al*., 1992; Czosnek *et al*., 1993) y *S. chilense* (Dunal) Reiche (Zakay *et al*., 1991; Czosnek *et al*., 1993; Picó *et al*., 1998).

No todas las entradas en las que se ha identificado resistencia han sido empleadas en el desarrollo de líneas de mejora o cultivares comerciales. Los programas de mejora dirigidos al desarrollo de materiales resistentes a TYLCD comenzaron en Israel, empleándose como fuente de resistencia la entrada LA121 de *S. pimpinellifolium* (Pilowsky y Cohen, 1974). Sin embargo, las líneas derivadas, a pesar de manifestar únicamente síntomas ligeros, mostraban una reducción importante del crecimiento y del rendimiento a causa de la enfermedad (Pilowsky y Cohen, 1974).

Como consecuencia, se inició un programa destinado al desarrollo de híbridos basados en la resistencia de *S. peruvianum*. El primer híbrido comercial con resistencia a TYLCD, denominado 'TY20', fue obtenido a partir de la entrada PI126935 de esta especie. En este híbrido se observaba un retraso en la aparición de los síntomas y la acumulación viral, siendo los síntomas leves; además el rendimiento era aceptable en condiciones de infección (Pilowsky *et al*., 1989; Pilowsky y Cohen, 1990). A partir de esta misma fuente se derivaron posteriormente otras series de híbridos de crecimiento determinado (Pilowsky y Cohen, 1995). Distintas entradas de *S. peruvianum* (PI126926, PI126930, PI390681 y LA441) se han empleado para el desarrollo de otras líneas con mayores niveles de resistencia, tales como "TY172" y "TY197" (Lapidot *et al*., 1997; Friedmann *et al*., 1998). También la entrada PI126944 ha sido descrita como resistente a TYLCD (Picó *et al*., 1998; Pilowsky y Cohen, 2000). El interés de esta entrada radica en que presenta además resistencia al virus del bronceado del tomate (*Tomato spotted wilt virus*, TSWV) (Paterson *et al*., 1989), al virus del mosaico del tabaco (*Tobacco mosaic virus*, TMV) (Yamakawa y Nagata, 1975), al virus del rizado del tomate (*Tomato leaf curl virus*, ToLCV) (Muniyappa *et al*., 1991) y a *Fusarium oxysporum* f.sp. *radicis-lycopersici* (Rowe y Farley, 1981). A partir de tres plantas distintas de esta entrada se han obtenido tres híbridos interespecíficos con la especie cultivada (Picó *et al*., 2000). La evaluación de estos híbridos ha puesto de manifiesto la heterogeneidad de la entrada original en cuanto a la resistencia a TYLCD, ya que dos de los híbridos han mostrado resistencia a TYLCSV y TYLCV, mientras que el tercero ha sido susceptible (Díez *et al*., 2003). Actualmente se está evaluando la resistencia en las generaciones F_2 y BC_1.

A partir de *S. habrochaites* también se han obtenido materiales resistentes a TYLCD. Destaca la línea H24, derivada de la entrada B6013 perteneciente a *S. habrochaites* f. *glabratum* (Kalloo y Banerjee, 1990) y que ha respondido eficazmente a los aislados de Taiwan y del sur de India (Hanson *et al*., 2000). Otra línea interesante desarrollada a partir de *S. habrochaites* es la línea 902, procedente las entradas LA386 y

LA1777 (Vidavsky y Czosnek, 1998). Esta línea se caracteriza por impedir la replicación del virus y no mostrar síntomas, incluso tras ser inoculada con poblaciones masivas de mosca blanca. La línea 902 está siendo empleada en los programas de mejora para la resistencia a begomovirus en Oriente Medio (Maruthi *et al*., 2003) y Guatemala (Mejía *et al*., 2005).

Sin embargo, las mejores fuentes de resistencia a TYLCD identificadas hasta el momento pertenecen a la especie *S. chilense*. La entrada LA1969 fue inicialmente descrita por Zakay *et al*. (1991) como altamente resistente. Estudios realizados por otros autores con esta misma entrada han confirmado estos resultados (Scott y Schuster, 1991; Czosnek *et al*., 1993; Michelson *et al*., 1994; Picó *et al*., 1998; Pérez de Castro *et al*., 2005; Piñón *et al*., 2005). La resistencia derivada de LA1969 está controlada por un gen mayor, el gen *Ty-1*, y dos genes modificadores (Zamir *et al*., 1994). El nivel elevado de resistencia encontrado en esta entrada, así como el control genético sencillo de la misma, han determinado que sea empleada en programas de mejora para la resistencia a begomovirus a nivel mundial (Chiang *et al*., 1994; Laterrot y Moretti, 1994; Scott *et al*., 1996; Gómez *et al*., 2004; Mejía *et al*., 2005; Piñón *et al*., 2005). Muchos de los híbridos comerciales incorporan resistencia a TYLCD derivada de LA1969. A pesar de ello, en condiciones de fuerte infección la mayor parte de estos híbridos terminan por mostrar síntomas de la enfermedad (Figura 2).

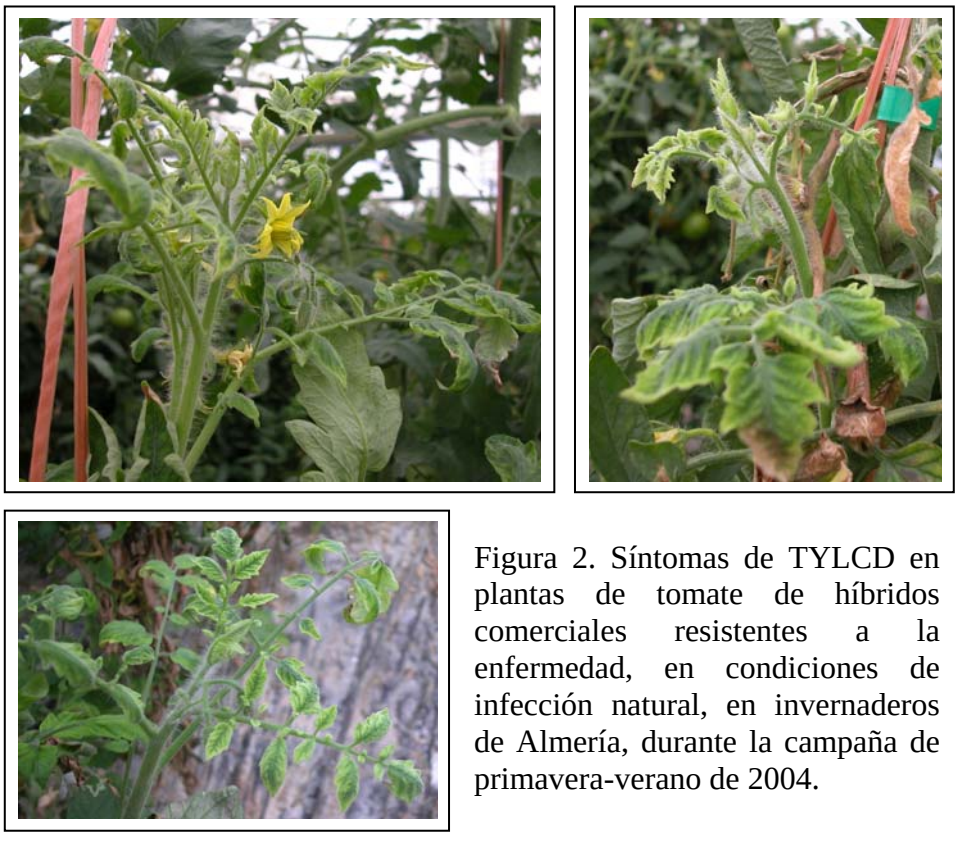

Figura 2. Síntomas de TYLCD en plantas de tomate de híbridos comerciales resistentes a la enfermedad, en condiciones de infección natural, en invernaderos de Almería, durante la campaña de primavera-verano de 2004.

En cualquier caso, esta no es la única entrada de la especie *S. chilense* que muestra niveles elevados de resistencia a la enfermedad. En ensayos de cribado recientes se han identificado distintas entradas con niveles de resistencia comparables a los detectados en LA1969 (Pérez de Castro *et al*., 2004, 2005). Existen entradas de esta especie que fueron descritas inicialmente como resistentes a otros begomovirus y que han resultado ser también resistentes a alguna de las especies virales causantes de TYLCD. Entre estas entradas se encuentran LA1932 y LA1938 (Picó *et al*., 1999). Con algunas de ellas se han iniciado programas de mejora para la introgresión de la resistencia en la especie cultivada (Pérez de Castro *et al*., 2004).

A partir de la entrada LA1401 de *S. galapagense* se desarrolló en Egipto una línea de mejora (línea 44) con resistencia moderada a TYLCD (Moustafa y Nakhla, 1990).

En determinadas entradas de algunas especies silvestres relacionadas con el tomate la resistencia se debe a que se ve impedida la transmisión por el vector natural *B. tabaci*, generalmente por fenómenos de antibiosis o antixenosis, y no a la restricción a la multiplicación o al movimiento del virus dentro de la planta (Channarayappa *et al*., 1992; Picó *et al*., 2001; Muigai *et al*., 2003). Es el caso de algunas entradas pertenecientes a *S. habrochaites*, en las que la resistencia a la mosca blanca se ha relacionado con la presencia de distintos aleloquímicos presentes en los tricomas glandulares de tipo IV en las hojas de esta especie (Muigai *et al*., 2002). También en *S. pennellii* Correll se ha identificado resistencia a *Bemisia*, mediada por acilazúcares presentes en los tricomas glandulares tipo IV (Lawson *et al*., 1997; Blauth *et al*., 1998). Para la entrada de *S. pimpinellifolium* Hirsute-INRA, que ha sido descrita como resistente (Kasrawi, 1989; Picó *et al*., 2001), estudios recientes ponen de manifiesto que la resistencia se debe en este caso, no a fenómenos de no preferencia, sino a modificaciones en el comportamiento alimenticio de la mosca blanca que impiden la transmisión del virus (Delatte *et al*., 2006). En cualquier caso, los intentos de transferir la resistencia a *B. tabaci* desde *S. habrochaites* o *S. pennellii* no han tenido éxito, debido a la complejidad del control genético de la misma y la dificultad de eliminar las características indeseables del parental donante ligadas a los genes de resistencia (Lawson *et al*., 1997; Momotaz *et al*., 2005).

También se han observado mecanismos de antibiosis y antixenosis frente a la mosca blanca en plantas de tomate portadoras del gen *Mi*, introgresado en la especie cultivada a partir de *S. peruvianum* y que confiere resistencia a nematodos del género *Meloidogyne* y a algunos aislados del áfido *Macrosiphum euphorbiae* (Thomas). En este caso la resistencia no está relacionada con la presencia de tricomas ni exudados (Nombela

et al., 2000, 2001). Se ha comprobado que el gen *Mi* es el responsable de la resistencia a *Bemisia* (Nombela *et al*., 2003). Sin embargo, la resistencia es superada a temperaturas elevadas y es dependiente del desarrollo de la planta, de forma que comienza a expresarse cuando la planta tiene dos meses de edad (Nombela *et al*., 2003). Además, la eficacia difiere en función del biotipo de *Bemisia* considerado, habiéndose comprobado que la resistencia es mayor frente al biotipo Q que frente al biotipo B (Nombela *et al*., 2001). Por sus características, la resistencia a *Bemisia* conferida por el gen *Mi* no es suficiente por si misma para evitar la infección por TYLCD en campo; sin embargo, puede resultar útil para complementar otros mecanismos de resistencia.

1.8.2. Control genético de la resistencia

En algunas de las entradas de especies silvestres relacionadas con el tomate, así como en algunas de las líneas de mejora desarrolladas, se ha estudiado el control genético de la resistencia a TYLCD.

Los estudios llevados a cabo con la especie *S. pimpinellifolium* son en muchos casos contradictorios. Se ha propuesto un control monogénico para la resistencia derivada de LA121 (Pilowsky y Cohen, 1974), Hirsute-INRA (Kasrawi, 1989; Vidavsky *et al*., 1998) y LA1478 (Kasrawi, 1989). Sin embargo, estudios posteriores describen un control poligénico de la resistencia en las entradas LA121 (Hassan *et al*., 1984b), Hirsute-INRA (Chagué *et al*., 1997) y en otras entradas de esta especie (Hassan *et al*., 1984b; Kasrawi y Mansour, 1994; Hassan y Abdel-Ati, 1999). Las discrepancias pueden atribuirse bien a diferencias en las especies virales o aislados empleados, bien a variaciones en las condiciones ambientales o en los métodos de inoculación empleados (Hassan *et al*., 1984b).

Se ha estudiado el control genético de la resistencia derivada de distintas entradas de *S. peruvianum* y en todos los casos parece estar conferida por varios genes (Pilowsky y Cohen, 1974; Vidavsky *et al*., 1998; Lapidot *et al*., 2000).

La resistencia derivada de la entrada LA386 de *S. habrochaites* ha sido estudiada por distintos autores. Hassan *et al*. (1984a) propusieron un control poligénico. Sin embargo, Vidavsky y Czosnek (1998) ensayaron líneas resistentes y tolerantes derivadas de un cruce inicial entre las entradas LA386 y LA1777 y concluyeron que la tolerancia estaba controlada por un gen y la resistencia por dos o tres genes aditivos. La resistencia a begomovirus derivada de la entrada B6013 parece estar controlada por dos genes

epistáticos (Banerjee y Kalloo, 1987), si bien en la línea de mejora H24, derivada de esta fuente, la resistencia está controlada por un gen mayor, *Ty-2* (Hanson *et al*., 2000, 2006).

En cuanto a la resistencia derivada de *S. chilense*, en el caso de la entrada LA1969 está controlada por un gen mayor parcialmente dominante, *Ty-1*, con dos genes modificadores (Zamir *et al*., 1994) y en el caso de la entrada LA2779, por un gen mayor recesivo, denominado *Ty-3* (Ji *et al*., 2007).

1.8.3. Identificación de marcadores moleculares ligados a la resistencia

La identificación de marcadores moleculares estrechamente ligados a las regiones del genoma que confieren resistencia a distintos patógenos es otro de los objetivos prioritarios de la mejora para la resistencia a enfermedades en general y a TYLCD en particular. La posibilidad de realizar selección asistida por marcadores permite reducir considerablemente el tiempo necesario para completar un programa de mejora, resultando además una herramienta indispensable para la acumulación en un mismo material de genes de resistencia de distinta procedencia. En la actualidad se dispone en tomate de una gran cantidad de marcadores moleculares, muchos de ellos cartografiados, y este número irá en aumento a medida que avance el proyecto de secuenciación de esta especie (Foolad y Sharma, 2005).

En cuanto a genes o QTLs (*Quantitative Trait Loci*) asociados a la resistencia a TYLCD, se dispone de marcadores moleculares asociados a algunos de ellos.

El primer gen de resistencia a TYLCD cartografiado fue el gen mayor *Ty-1*, localizado en el cromosoma 6, entre los marcadores RFLP TG297 y TG97 (Zamir *et al*., 1994). Otros dos *loci* con efectos menores sobre la resistencia se localizaron en los cromosomas 3 y 7. Se han empleado como marcadores para el gen *Ty-1* dos CAPS (*Cleaved Amplified Polymorphic Sequence*), uno basado en la secuencia del gen de la fosfatasa ácida (*Aps*) (García-Martínez *et al*., 2004) y el marcador REX-1 asociado al gen de resistencia a nematodos *Mi* (Williamson *et al*., 1994; Milo, 2001). Sin embargo, para poder emplear estos marcadores es necesario conocer la genealogía de los materiales con los que se trabaja, ya que la presencia del gen *Mi* interfiere con los resultados, obteniéndose falsos positivos. En estudios recientes se ha realizado una búsqueda de marcadores ligados al gen *Ty-1*, que podrían resultar útiles en futuros programas de mejora (González-Cabezuelo *et al*., 2007).

También se han identificado marcadores RAPD (*Random Amplified Polymorphic DNA*) asociados a la resistencia derivada de otras tres entradas de *S. chilense*, LA1932, LA1938 y LA2779 (Ji y Scott, 2005a). Algunos de estos marcadores RAPD han sido transformados en marcadores CAPS o SCAR (*Sequence Characterized Amplified Region*) (Ji y Scott, 2005b). La resistencia frente a TYLCV está fundamentalmente explicada por dos QTL en el caso de las entrada LA1932 y LA2779, y por uno en la entrada LA1938, todos ellos en el cromosoma 6 (Agrama y Scott, 2006). El gen mayor recesivo asociado a la resistencia derivada de LA2779 ha sido denominado *Ty-3* y se ha comprobado que está muy ligado al *Ty-1*, aunque no son alélicos (Ji y Scott, 2006; Ji *et al*., 2007).

Resulta interesante que la resistencia derivada de la entrada Hirsute INRA de *S. pimpinellifolium* ha sido cartografiada también en el cromosoma 6. Chagué *et al*. (1997), mediante el análisis de bloques segregantes, localizaron cuatro marcadores RAPD en el mismo grupo de ligamiento, asociados a un QTL que explicaba el 27,7% de la resistencia derivada de esta entrada.

La introgresión de *S. habrochaites* asociada a la resistencia en la línea H24 ha sido cartografiada en el brazo largo del cromosoma 11 (Hanson *et al*., 2000). Estudios posteriores han permitido comprobar que se trata de un solo gen, que ha sido denominado *Ty-2* (Hanson *et al*., 2006). Actualmente se está trabajando en el desarrollo de marcadores basados en la PCR más estrechamente ligados a este gen. En cuanto a los materiales derivados de la entrada LA1777 de esta misma especie, se ha asociado la resistencia a regiones en los cromosomas 1 y 7, si bien sólo parecen tener efectos menores sobre la misma (Momotaz *et al*., 2005).

1.8.4. Resistencia obtenida mediante ingeniería genética

El desarrollo de las técnicas de ingeniería genética ha permitido obtener plantas con distintos niveles de resistencia a TYLCD basadas en la transformación de plantas con secuencias de DNA procedentes del patógeno. En este sentido se han utilizado diversas estrategias. Inicialmente, se empleó el gen que codifica la proteína de la cápsida (*V1*) para transformar plantas de tomate, comprobándose que los transformantes que expresaban el gen sólo a nivel de RNA se mostraban susceptibles a la enfermedad, mientras que en aquellos que expresaban la proteína de la cápsida, la aparición de los síntomas se veía retrasada y las plantas se recuperaban de la enfermedad (Kunik *et al*., 1994). Posteriormente, se utilizó una versión truncada del gen que codifica la replicasa (*C1*) de

TYLCSV para transformar *Nicotiana benthamiana* (Noris *et al*., 1996; Brunetti *et al*., 2001) y tomate (Brunetti *et al*., 1997); en ambos casos se detectó que la acumulación de la proteína Rep truncada inhibía la transcripción y replicación del virus, interfiriendo de esa forma con la infección viral. Sin embargo, la resistencia obtenida mediante esta estrategia resultó ser muy específica de aislado (Brunetti *et al*., 1997, 2001), si bien estudios posteriores demostraron que la resistencia era también efectiva frente al aislado TYLCV-Mld[PT] (Lucioli *et al*., 2003). Con esta misma estrategia, Antignus *et al*. (2004) obtuvieron resistencia en tomate, siendo en este caso muy específica de aislado y dependiente del método de inoculación: las plantas mostraban resistencia cuando se inoculaban mediante mosca blanca, mientras que si se empleaba agroinoculación la respuesta variaba desde la susceptibilidad (33% de la plantas) a la inmunidad (21%). Por otra parte, mediante la transformación con el gen *Rep* completo en antisentido se han obtenido plantas de tabaco parcialmente resistentes al TYLCV, estando la resistencia mediada, también en este caso, por una inhibición de la replicación del virus (Bendahmane y Gronenborn, 1997). Sin embargo, la transformación de plantas de tomate con el gen *C4* (Krake *et al*., 1998) no ha resultado una estrategia eficaz, ya que los transformantes muestran fenotipo anormal, presentando un aspecto similar al de las plantas infectadas con el virus. Estos resultados confirman el hecho de que este gen codifica una proteína relacionada con el desarrollo de los síntomas en el proceso de infección. En cualquier caso, la resistencia obtenida mediante estas estrategias no es total y ninguno de los materiales de tomate desarrollados muestra resultados satisfactorios.

Más recientemente se han empleado construcciones que incluyen fragmentos del gen *C1* y de la región intergénica del virus, que, a través de mecanismos de silenciamiento génico post-transcripcional, confieren niveles elevados de resistencia frente al TYLCV en tomate y en tabaco (Yang *et al*., 2004). Incluso se ha descrito inmunidad a TYLCV tras inoculación artificial con poblaciones muy elevadas de *B. tabaci*, en plantas de tomate transformadas con una construcción sentido-intrón-antisentido basada en la secuencia del gen *C1* (Fuentes *et al*., 2006). Sin embargo, en ensayos similares, también en tomate, pero con la especie TYLCSV se ha observado la superación del silenciamiento, ya que, si bien se produce un retraso en la infección en las plantas transformadas, estas resultan susceptibles a la enfermedad (Noris *et al*., 2004).

Empleando una estrategia similar, pero dirigida al silenciamiento del gen que codifica la proteína de la cápsida *(V1*), se han obtenido plantas de tomate que permanecen asintomáticas hasta siete semanas después de la inoculación con TYLCV, mostrando

posteriormente síntomas ligeros de la enfermedad que no interfieren con la producción de frutos y semillas (Zrachya *et al*., 2007). Por otra parte, se ha comprobado que mediante construcciones de este mismo tipo que contienen secuencias no codificantes conservadas entre las distintas especies de virus causantes de TYLCD, es posible conseguir resistencia de amplio espectro en plantas de tabaco, estando también implicados en este caso mecanismos de silenciamiento génico post-transcripcional (Abhary *et al*., 2006).

La aproximación más innovadora para generar resistencia a TYLCV se basa en el conocimiento del mecanismo de unión del virus a GroEL en el interior del vector para evitar ser degradado. Se ha comprobado que plantas de tomate que expresan en el floema GroEL procedente de bacterias endosimbiontes de *Bemisia* permanecen asintomáticas tras ser inoculadas con TYLCV (Akad *et al*., 2007b). La hipótesis propuesta es que tras la infección las partículas virales deben quedar retenidas por GroEL, viéndose limitados la invasión de las células asociadas al floema por parte de las partículas virales, así como el movimiento a larga distancia. La cantidad de DNA viral en estas plantas es menor a la detectada en plantas no transformadas hasta 2-3 semanas después de la inoculación; sin embargo, seis semanas después de la inoculación la acumulación viral es similar en las plantas transformadas y las no transformadas. Esto indica que el virus es capaz de replicarse, aunque el hecho de que las plantas no muestren síntomas pone de manifiesto que el retraso en la acumulación del virus es suficiente para generar resistencia (Akad *et al*., 2007b). Este tipo de estrategia podría resultar útil para muchos de los virus transmitidos de forma circulativa por un insecto vector.

1.8.5. Aumento de los niveles de resistencia

Hasta el momento los materiales con resistencia a TYLCD desarrollados no poseen resistencia total a la enfermedad. El empleo de estas variedades combinado con medidas de tipo preventivo permiten reducir las pérdidas de producción. Sin embargo, en condiciones de infección temprana o de fuerte presión de inóculo las pérdidas económicas siguen siendo importantes. Una estrategia encaminada a obtener niveles de resistencia más elevados, y además aumentar la durabilidad de la misma, consiste en la incorporación en un mismo material de genes de resistencia procedentes de distintas fuentes.

En este sentido, se han realizado diversos ensayos encaminados a evaluar la resistencia en progenies derivadas de líneas de mejora con resistencia procedente de distintas especies silvestres relacionadas con el tomate. Kasrawi y Mansour (1994)

obtuvieron niveles de resistencia que excedían a los de los parentales en cruces a partir de líneas derivadas de *S. pimpinellifolium*, *S. chilense* y *S. habrochaites*. Resultados similares se obtuvieron a partir de cruces entre líneas con resistencia procedente de *S. peruvianum*, *S. chilense* y *S. pimpinellifolium*, siendo la combinación de la resistencia de estas dos últimas la que mayor resistencia proporcionó (Vidavsky *et al*., 1998). Del mismo modo, en el programa de mejora desarrollado en Guatemala, se han obtenido incrementos en el nivel de resistencia a distintos begomovirus, incluyendo TYLCV, combinando la resistencia derivada de *S. peruvianum* con la de *S. pimpinellifolium* y *S. habrochaites* (Mejía *et al*., 2005).

La combinación de resistencia procedente de distintas fuentes no sólo resulta útil para aumentar los niveles de resistencia en un mismo material, sino también para conseguir que esta sea más durable (Harrison, 2002; Pink, 2002; Lecoq *et al*., 2004). Por otra parte, los avances en la obtención de resistencia a begomovirus mediante técnicas de ingeniería genética, permitirán en un futuro combinar la resistencia procedente de la planta y la resistencia derivada del patógeno. Esta estrategia ha sido empleada anteriormente con éxito en el desarrollo de materiales resistentes a TSWV portadores del gen *Sw-5*, derivado de *S. peruvianum*, que han sido trasformados para expresar la proteína de la nucleocápsida (Gubba *et al*., 2002).

1.8.6. Resistencia de amplio espectro

Uno de los objetivos actuales de mejora es el de conseguir materiales con resistencia a un amplio espectro de begomovirus y que por tanto resulten de interés en muchas zonas de cultivo. En los programas de mejora desarrollados en Florida (EE.UU.) los ensayos se realizan mediante inoculaciones independientes con TYLCV y con el begomovirus bipartito ToMoV, habiéndose comprobado que los materiales resistentes a uno de los virus lo son también al otro (Scott *et al*., 1996). Por otra parte, distintos autores han evaluado el comportamiento frente a una especie de begomovirus en materiales descritos como resistentes frente a otro begomovirus distinto. Esta aproximación permite la utilización directa de materiales con buenas características agronómicas, reduciendo el esfuerzo y la inversión necesarios en el desarrollo de un programa de mejora completo.

En este sentido, materiales resistentes a distintos aislados de TYLCV han mostrado buen comportamiento frente a la cepa de TYLCV presente en el Líbano (Abou-Jawdah *et al*., 1999). También se ha identificado resistencia al begomovirus bipartito *Tomato golden*

mosaic virus (TGMV) presente en Brasil en algunos de los materiales previamente descritos como resistentes a TYLCV (Santana *et al*., 2001). Pietersen y Smith (2002) evaluaron el comportamiento frente al begomovirus *Tomato curly stunt virus* (ToCSV) de materiales resistentes a TYLCV derivados de *S. habrochaites* y *S. chilense*, comprobando que, con algunas excepciones, los síntomas eran menos graves y las pérdidas de rendimiento menores en los materiales resistentes a TYLCV que en los susceptibles. Un estudio similar fue desarrollado en Bangalore (India) y Rehovot (Israel), con objeto de identificar materiales resistentes al *Tomato leaf curl virus* (ToLCV), presente en India, y al TYLCV, presente en Israel. Se probaron frente al ToLCV 34 genotipos resistentes a TYLCV y frente a TYLCV, 16 genotipos resistentes a ToLCV. Algunos de los genotipos resultaron resistentes o tolerantes a ambas especies (Maruthi *et al*., 2003). Mejía *et al*. (2005) evaluaron en condiciones de infección natural en Guatemala, materiales de distinta procedencia con resistencia a TYLCV o a ToMoV. Algunos de estos materiales, como la línea H24, portadora del gen *Ty-2* de *S. habrochaites* (Hanson *et al*., 2006) y la línea TY-52, portadora del gen *Ty-1* de *S. chilense* (Zamir *et al*., 1994) resultaron ser susceptibles al complejo de begomovirus monopartitos presentes en Guatemala. Sin embargo, las líneas lh902, derivada de *S. habrochaites* (Vidavsky y Czosnek, 1998), TY172 y TY198, derivadas de *S. peruvianum* (Friedmann *et al*., 1998) y Fla595, derivada de la entrada LA2779 de *S. chilense* (Scott *et al*., 1996), mostraron niveles elevados de resistencia en las condiciones de infección de Guatemala.

Todos estos estudios ponen de manifiesto la complejidad de la resistencia a begomovirus, pero indican que es posible identificar genes que confieren resistencia a una amplia gama de virus de este género. Además, como se ha comentado previamente, mediante transformación genética con secuencias conservadas en varias especies de begomovirus ya se ha conseguido en tabaco plantas con resistencia de amplio espectro (Abhary *et al*., 2006).

1.8.7. Estudio del interactoma virus-planta

Otra estrategia actual dirigida a la obtención de resistencia a geminivirus se basa en el estudio del interactoma virus-planta. El conocimiento de las interacciones entre proteínas del virus y proteínas de la planta permite identificar las proteínas de la planta que resultan indispensables para que se produzca la infección. De esta forma es posible modificar la expresión de los genes que codifican estas proteínas con objeto de generar

resistencia (Colinet *et al*., 2001). Las proteínas Rep y REn son necesarias para la replicación eficiente del virus, aunque sólo Rep es esencial. Se sabe que la presencia del virus en la célula vegetal induce la producción de las proteínas necesarias para la replicación (Gutiérrez, 2000; Hanley-Bowdoin *et al*., 2004). En este sentido, se han identificado interacciones de la Rep del begomovirus TGMV con distintas proteínas implicadas en la replicación, como el antígeno nuclear de proliferación celular (*proliferating cell nuclear antigen*, PCNA) (Nagar *et al*., 1995) y retinoblastoma (pRb) (Kong *et al*., 2000). En estudios posteriores se ha comprobado que con la especie TYLCSV se produce interacción entre PCNA y las proteínas virales Rep y REn (Castillo *et al*., 2003). Además, se ha identificado interacción entre la Rep de TGMV y la proteína NbSCE1 (*N. benthamiana* SUMO conjugating enzyme) (Castillo *et al*., 2004). Esta proteína forma parte del sistema de sumoilación, un proceso de modificación post-traduccional mediante la unión covalente del polipéptido SUMO (*small ubiquitin-like modifier*) No se conocen muy bien los efectos de la sumoilación, si bien en plantas transgénicas de tabaco se ha comprobado que el aumento o la disminución de los niveles de SUMO interfieren con la infección por TGMV, reduciendo la replicación del virus (Castillo *et al*., 2004).

Por otra parte, para que se produzca infección sistémica, Rep y CP son las proteínas esenciales. Resulta por tanto de interés identificar también las proteínas de la planta que interaccionan con la proteína CP.

Parece probable que las estrategias basadas en la modificación de genes del hospedante proporcionen resistencia de amplio espectro. De hecho, recientemente se ha identificado en una línea de mejora de tomate un *locus*, *tgr-1*, que parece codificar un factor del hospedante indispensable para que se produzca la traslocación del virus en la planta (Bian *et al*., 2007). Esta línea deriva de distintas entradas de *S. chilense*, pero el control genético de la resistencia no se explica a partir de su genealogía; por este motivo los autores han planteado la hipótesis de que se haya producido una mutación en un *locus* del hospedante durante el apareamiento cromosómico. La resistencia en esta línea es efectiva frente a los begomovirus monopartitos TYLCV y ToLCV y al bipartito *Tomato leaf curl New Delhi virus* (ToLCNDV) (Bian *et al*., 2007).

Por último, citar la existencia de una herramienta que puede facilitar la identificación de las proteínas del hospedante necesarias para la replicación del virus (Morilla *et al*., 2006). Se trata de un casete de expresión que contiene la GFP (*Green Fluorescent Protein*) flanqueada por la región intergénica de TYLCSV. Se ha comprobado

que plantas de tabaco transformadas con esta construcción expresan la GFP en las regiones en las que se produce la replicación del virus, dado que se produce la interacción entre la Rep y la región intergénica. La utilidad de esta herramienta se ha demostrado silenciando de forma transitoria la expresión de la proteína PCNA, necesaria para la replicación del virus, ya que en las plantas en las que se produce el silenciamiento no se observa la expresión de GFP como consecuencia de la ausencia de la replicación del virus (Morilla *et al*., 2006).

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2. OBJETIVOS

2. OBJETIVOS

La enfermedad del rizado amarillo del tomate (Tomato yellow leaf curl disease, TYLCD) causa importantes pérdidas económicas en muchas zonas de cultivo de tomate de todo el mundo. De hecho, en condiciones de cultivo intensivo es uno de los principales factores limitantes para el cultivo del tomate. Dado que ni las medidas preventivas ni las medidas encaminadas al control de *Bemisia tabaci*, vector natural de los virus asociados a TYLCD, resultan efectivas por si mismas, la resistencia genética es la mejor estrategia de lucha contra la enfermedad.

Sin embargo, los cultivares resistentes desarrollados hasta el momento no suponen una solución definitiva en condiciones de elevada presión de inóculo o tras infecciones tempranas. Por este motivo, numerosos grupos de investigación continúan trabajando a nivel mundial con la finalidad de obtener materiales con niveles elevados de resistencia a TYLCD. Los objetivos actuales de mejora incluyen el desarrollo de resistencia de amplio espectro a distintos begomovirus, la combinación de genes de distinta procedencia para conseguir mayores niveles de resistencia y la identificación de marcadores moleculares ligados a la resistencia que permitan acortar los programas de mejora y acumular en un mismo material genes de resistencia de distintas fuentes.

La presente tesis doctoral se enmarca dentro de la línea de investigación “Mejora para la resistencia a la enfermedad del rizado amarillo del tomate”, del Instituto de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV). Como resultado de trabajos previos realizados por el grupo, al inicio de la tesis se disponía de distintos materiales con resistencia a la especie *Tomato yellow leaf curl Sardinia virus*, la primera especie viral causante de TYLCD detectada en España. Entre los materiales disponibles se encontraban líneas de mejora con resistencia a la enfermedad derivada de distintas entradas de *Solanum chilense*. Por otra parte, se habían identificado niveles elevados de resistencia en la entrada UPV16991 de *Solanum pimpinellifolium* y se habían iniciado el programa encaminado a la fijación de la resistencia encontrada en esta entrada.

En este contexto, considerando las necesidades actuales de mejora para la resistencia a TYLCD, así como los materiales desarrollados por nuestro grupo, los objetivos de la presente tesis doctoral son los siguientes:

- Evaluación de la resistencia de líneas de mejora derivadas de las entradas LA1932 y LA1938 de *S. chilense* a la enfermedad del rizado amarillo del tomate causada por las especies *Tomato yellow leaf curl Sardinia virus* (TYLCSV) y *Tomato yellow leaf curl virus* (TYLCV).
- Determinación del control genético de la resistencia a la enfermedad del rizado amarillo del tomate procedente de la entrada UPV16991 de *S. pimpinellifolium.*
- Caracterización del nivel de resistencia a TYLCD en materiales que combinan el gen *Ty-1*, derivado de la entrada LA1969 de *S. chilense*, con resistencia derivada de la entrada UPV16991 de *S. pimpinellifolium*.
- Identificación de un marcador molecular ligado al gen *Ty-1*.

3. MATERIALES Y MÉTODOS, RESULTADOS Y DISCUSIÓN

3.1. Evaluation of breeding tomato lines partially resistant to *Tomato yellow leaf curl Sardinia virus* and *Tomato yellow leaf curl virus* derived from *Lycopersicon chilense*

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Evaluation of breeding tomato lines partially resistant to *Tomato yellow leaf curl Sardinia virus* and *Tomato yellow leaf curl virus* derived from *Lycopersicon chilense*

Ana Pérez de Castro, María José Díez, and Fernando Nuez

Abstract: Twelve tomato (*Lycopersicon esculentum* Mill.) advanced breeding lines derived from *L. chilense* and partially resistant to *Tomato yellow leaf curl Sardinia virus* (TYLCSV) were evaluated for their resistance to the species from Israel (*Tomato yellow leaf curl virus*, TYLCV). Two assays were carried out in two consecutive years, using agroinoculation and whitefly-mediated inoculation, respectively. Symptom severity, percentage of infection, and viral DNA accumulation (using molecular hybridization) were measured. In the first assay, the 12 breeding lines were agroinoculated with both virus species. Resistance to TYLCSV was confirmed for the 12 breeding lines, but only 6 of them showed resistance to TYLCV. During the second assay these six breeding lines were whitefly-inoculated with TYLCV. All lines showed high levels of partial resistance to TYLCV consisting in attenuation and delay in time of symptom development and reduction in virus accumulation when compared with the susceptible control. Three of these lines even accumulated significantly lower amounts of viral DNA than the resistant controls 'Anastasia' and 'Boludo' hybrids. These lines also display good horticultural traits, appropriate for the protected growing system and for the fresh market requirements. These advanced breeding lines are base material for developing commercial hybrids highly resistant to TYLCSV and TYLCV.

Key words: TYLCD, breeding lines, *Lycopersicon chilense*.

Résumé : Douze lignées généalogiques avancées de tomate (*Lycopersicon esculentum* Mill.) issues du *L. chilense* et partiellement résistantes au virus sarde de l'enroulement des feuilles de la tomate (*Tomato yellow leaf curl Sardinia virus*; TYLCSV) ont été examinées pour leur résistance envers l'espèce virale provenant d'Israël (virus de l'enroulement des feuilles de la tomate; *Tomato yellow leaf curl virus*; TYLCV). Deux tests ont été réalisés lors de deux années consécutives par, respectivement, agroinoculation et inoculation par l'intermédiaire d'aleurodes. L'intensité des symptômes, le pourcentage d'infection et l'accumulation d'ADN viral (à l'aide de l'hybridation moléculaire) ont été déterminés. Lors du premier test, les 12 lignées généalogiques ont été agroinoculées avec les deux espèces virales. La résistance au TYLCSV a été confirmée pour les 12 lignées généalogiques, mais seulement 6 d'entre elles se sont avérées résistantes au TYLCV. Au cours du deuxième test, ces six lignées généalogiques ont été inoculées avec le TYLCV à l'aide d'aleurodes. Toutes les lignées ont fortement exprimé une résistance partielle au TYLCV qui consistait en une atténuation et un retard de développement des symptômes de même qu'une diminution de l'accumulation des virus par rapport à un témoin sensible. Trois de ces lignées ont même significativement accumulé moins d'ADN viral que les témoins résistants, les hybrides 'Anastasia' et 'Boludo'. Ces lignées possédaient aussi de bonnes caractéristiques horticoles qui les destinent à des systèmes de culture abritée et à la commercialisation à l'état frais. Ces lignées généalogiques avancées constituent le matériau de base pour le développement d'hybrides commerciaux très résistants au TYLCSV et au TYLCV.

Mots clés : TYLCD, lignées généalogiques, *Lycopersicon chilense*.

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Introduction

Tomato yellow leaf curl disease (TYLCD) has caused devastating damage in tomato (*Lycopersicon esculentum* Mill.) crops over the last 30 years in many tropical, subtropical, and temperate areas (Picó et al. 1996). Six species of the virus causal agent of this disease from different geographical areas have been described, and five more are considered tentative species (Fauquet et al. 2003).

Among them, the species *Tomato yellow leaf curl virus* (TYLCV) has been reported to be infecting tomato all over the world. This species is present in mixed epidemics in some geographical areas; TYLCV has been reported to coexist with TYLCSV in Portugal (Louro et al. 1996), Spain (Navas-Castillo et al. 1997), and Italy (Accotto et al. 2003).

The epidemiological situation has been thoroughly studied in Spain, providing useful information about the dynamics of TYLCV epidemics (Sánchez-Campos et al. 1999). *Tomato yellow leaf curl Sardinia virus* (TYLCSV) has been present in southern Spain since 1992 (Moriones et al. 1993). Later, in 1997, the species native to Israel (TYLCV) was also detected to be causing more severe outbreaks in tomato (Navas-Castillo et al. 1997). TYLCV has also been reported to be infecting common bean (*Phaseolus vulgaris* L.) (Navas-Castillo et al. 1999). These two species coexist in epidemics, and currently TYLCSV is being displaced by TYLCV in tomato crops (Sánchez-Campos et al. 1999). Although both virus species have the same fitness on tomato crops, TYLCV is more efficiently transmitted by the natural vector *Bemisia tabaci* (Genn.). Furthermore, common bean served as a reservoir for TYLCV but not TYLCSV between autumn and spring growing cycles in southern Spain. These two last factors could be responsible for the displacement of TYLCSV by TYLCV (Sánchez-Campos et al. 1999). The epidemiological situation became even more complicated because of the occurrence in Spanish fields of a natural recombinant between TYLCV and TYLCSV (Monci et al. 2002), which has been considered a new species (*Tomato yellow leaf curl Malaga virus*) (Fauquet et al. 2003). This recombinant has a wider host range than did TYLCV and TYLCSV. Moreover, the occurrence of other natural recombinants in tomato crops cannot be ruled out.

Some cultural practices could lead to a reduction in the incidence of this disease; however, genetic resistance seems to be the best long-term strategy to control the damage caused by this viral disease. Given that all tomato cultivars are susceptible to TYLCD, screening for resistance has focused on wild *Lycopersicon* species. Resistance was identified in some of these species such as *Lycopersicon pimpinellifolium* (Jusl.) Mill., *Lycopersicon hirsutum* Humb. & Bonpl., *Lycopersicon peruvianum* (L.) Mill., and *Lycopersicon chilense* Dun. (reviewed in Laterrot 1992; Picó et al. 1996, 1999; Pilowsky and Cohen 2000). The first commercial hybrids resistant to TYLCD were derived from *L. peruvianum* (Friedmann et al. 1998; Lapidot et al. 1997, 2000; Pilowsky et al. 1989). However, to date, *L. chilense* was reported as the most effective source of resistance to TYLCD. Mainly one accession, *L. chilense* LA 1969, was employed to develop lines resistant to TYLCD (Laterrot 1995; Michelson et al. 1994; Zakay et al. 1991). Some other accessions from *L. chilense* have been reported to be resistant to TYLCD and have also been used to develop breeding lines highly resistant to TYLCSV (Picó et al. 1999). Advanced breeding lines with high levels of resistance have been developed from *L. peruvianum* (Lapidot et al. 1997; Friedmann et al. 1998; Vidavsky et al. 1998), *L. chilense* (Zamir et al. 1994; Vidavsky et al. 1998; Picó et al. 1999), *L. pimpinellifolium* (Vidavsky et al. 1998), and *L. hirsutum* (Vidavsky and Czosnek 1998). However, these materials show different behaviour in response to different virus species (Laterrot 1995; Navas-Castillo et al. 1999; Picó et al. 1999). Therefore, given that more than one virus species coexists in the same geographical area, it is necessary to aim breeding programs to the development of materials resistant to different virus species.

In this work, some breeding lines partially resistant to TYLCSV were tested for their resistance to TYLCV. These advanced breeding lines could be used to develop commercial hybrids, given that their horticultural (i.e., plant and fruit) characteristics are appropriate for the protected growing system and for fresh market tomato requirements.

Materials and methods

Plant material

Twelve tomato breeding lines were assayed against TYLCV and TYLCSV. These 12 lines descend from 2 breeding lines (Ty 1 and Ty 6), which were derived from *L. chilense* by our team. Lines Ty 1 and Ty 6 were selected because of their resistance to TYLCSV and for their horticultural characteristics (Picó et al. 1999). Lines assayed were coded as follows: 432, 436, 439, 441, 442, and 446 were derived from Ty 1, and 114, 115, 123, 127, 140, and 148 were derived from Ty 6. The tomato line NE-1 was used as susceptible control. Commercial hybrids 'Anastasia' and 'Boludo' (Seminis Vegetable Seeds), with resistance to TYLCV derived from *L. chilense*, and the line TY-197 (supplied by Dr. M. Pilowsky, the Volcani Center, Israel), with resistance derived from *L. peruvianum* (Friedmann et al. 1998), were used as the resistant controls. All the resistant controls employed exhibit slight symptoms and accumulate very low amounts of viral DNA.

Two assays were performed over two consecutive years. The susceptible and tolerant controls described previously were employed in both assays. The 12 lines aforementioned were tested in the first assay, carried out during the autumn–winter in 2001–2002. Those that showed a better response were selected to be tested in the second assay, in the autumn–winter in 2002–2003.

For each assay, 24 plants of each genotype were grown in a greenhouse, in four independent blocks of 6 plants each. Two of the blocks of each genotype were inoculated and the other two were grown uninoculated in the same greenhouse.

Inoculation techniques

Two different inoculation methods were used: agroinoculation and whitefly-mediated inoculation. Several authors agree that both methods are better than natural field conditions for selecting resistant plants (reviewed in Lapidot and Friedmann 2002). Picó et al. (1998) found that infection after natural field inoculation was milder when

compared to artificial inoculation, probably because of late and unsynchronized infection. Vidavsky et al. (1998) has shown that natural field exposure infection is largely inefficient, as even with heavy inoculation pressure, 90 days after transplanting 10% of the susceptible plants still escaped infection.

Agroinoculation was employed for the first assay, as described in Picó et al. (2001). The inoculation was carried out with TYLCSV and TYLCV. Lines Ty 1 and Ty 6, from which the assayed lines descend, have been evaluated previously for their resistance to TYLCSV (Picó et al. 1999). Our purpose was to confirm the response to inoculation to TYLCSV in these breeding lines and to evaluate their resistance to TYLCV. The bacterial strain employed for agroinoculation was LBA 4404 of *Agrobacterium tumefaciens* (Smith & Townsend) Conn, transformed with a partially dimeric copy of the viral genome of TYLCV (TYLCV-[ES7297], accession No. AF071228, supplied by E. Moriones, Estación Experimental La Mayora, Málaga, Spain) and TYLCSV (TYLCSV-ES[2], accession No. L27708, supplied by E.R. Bejarano, University of Málaga, Spain), respectively. The bacteria were injected into the axillary buds of the three youngest leaves of tomato plants at their four true leaf stage. Each plant was inoculated with both virus species: 6 of the 12 plants of each genotype tested were first inoculated with one of the virus species, and 7 days later each plant was infected with the other species not used in the first inoculation. One week after agroinoculation plants were transplanted to an insect-proof greenhouse until the end of the assay.

Whitefly-mediated inoculation in cages was used for the second assay, following the procedure employed by Picó et al. (1998), with some modifications. The same Spanish TYLCV isolate employed for agroinoculation was maintained in plants of the susceptible tomato line FC by whitefly transmission. Whiteflies employed were biotype Q (supplied by F. Beitia, Instituto Valenciano de Investigaciones Agrarias, IVIA, Valencia, Spain). Infected plants and viruliferous whiteflies were held in muslin-covered cages. Twelve plants per genotype, grown in pots, were caged at the four true leaf stage for 2 days with 15–20 viruliferous whiteflies per plant. After this inoculation period, plants were sprayed with imidacloprid (Confidor®) and transferred to a new insect-proof cage, where they remained for 2 days to avoid the spread of whiteflies. Subsequently, plants were sprayed again with the insecticide and then transplanted to an insect-proof greenhouse, until the end of the assay.

Disease assessment

Symptom severity was assessed for each plant at 15, 30, 45, and 60 days postinoculation (dpi) and graded according to the scale described by Friedmann et al. (1998): 0 (no visible symptoms, inoculated plants show same growth and development as uninoculated plants), 1 (very slight yellowing of leaflet margins on apical leaf), 2 (some yellowing and minor curling of leaflet ends), 3 (a wide range of leaf yellowing, curling, and cupping, with some reduction in size, yet plants continue to develop), and 4 (very severe plant stunting and yellowing and pronounced cupping and curling; plants cease to grow). Molecular hybridization was used to test individual plants for the presence of TYLCV and TYLCSV viral DNA at these same dates in the first assay and at 15, 30, and 45 dpi in the second assay. The leaf tissue was taken from the upper canopy of the plant at each date. DNA extraction was carried out following the procedure described by Crespi et al. (1991), with modifications: 150 mg of frozen tissue was crushed in 500 mL of extraction buffer (100 mmol/L Tris–HCl (pH 8), 50 mmol/L EDTA, 500 mmol/L NaCl, 10 mmol/L 2-β-mercaptoethanol, and 1% sodium dodecyl sulfate) and incubated at 65 °C for 5 min. Then, 150 mL of 5 mol/L potassium acetate was added, and samples were incubated on ice for 10 min. After centrifugation for 10 min, DNA was precipitated from the supernatants with isopropanol and resuspended in 77 mL of distilled water. One microlitre of each sample, corresponding to about 1.5 mg of fresh tissue, and a 10-fold dilution of the sample were denatured with 30 mmol/L NaOH and 1 mmol/L EDTA for 30 min, and then blotted on nylon positively charged membranes for hybridization. DNA was fixed on the membrane by UV cross-linking. Hybridization was carried out according to the *DIG System User's Guide for Filter Hybridization* (Roche Molecular Biochemicals 2000) using digoxigenin-11-dUTP and chemiluminescent detection. Membranes were prehybridized in standard hybridization buffer plus 50% deionized formamide for at least 1 h. Subsequent hybridization was done at 42 °C overnight in fresh prehybridization solution containing 20 ng denatured probe per millilitre. The probes employed (supplied by E.R. Bejarano, Universidad de Málaga, Spain) represented the intergenic region of the Spanish isolates previously cited, belonging to TYLCV and TYLCSV species. The probes were labelled by incorporation of digoxigenin-11-dUTP during polymerase chain reaction (PCR). One replicate of each membrane was hybridized with each probe. Washing steps and incubation with antibody were done according to manufacturer's instructions. Detection was carried out with CSPD and direct exposition to a charge-coupled device camera (Intelligent Dark Box-II, Fujifilm, Tokyo, Japan).

The amount of viral ssDNA was quantified according to a standard curve of TYLCV or TYLCSV DNA, respectively, dotted on the same membrane (ranging from 25 ng to 1 pg).

Plant DNA extracted was also quantified to relate virus concentration to plant DNA present in each sample. Fluorimetry was employed as the method to quantify dsDNA (Hoefer DyNA Quant 200 fluorimeter, San Francisco, California, USA), according to manufacturer's instructions.

To evaluate the effect of TYLCV on yield losses, fruits of the eight first trusses of each plant were collected and weighed individually. The parameters assayed were number of fruits per plant and fruit mass.

Results

Twelve breeding lines as well as resistant and susceptible controls were tested during the first assay, in the autumn–winter season in 2001–2002. All plants of the susceptible control NE-1 developed severe symptoms by 15 dpi (Table 1). None of the resistant genotypes employed as con-

Table 1. Evaluation of symptom development in the breeding lines and the susceptible and resistant controls during assay 1 (autumn–winter 2001–2002) and assay 2 (autumn–winter 2002–2003).

	Assay 1				Assay 2			
Genotype	15 dpi	30 dpi	45 dpi	60 dpi	15 dpi	30 dpi	45 dpi	60 dpi
Ty 6 derived								
114	0.00	0.00	0.00	0.00	0.04	0.17	0.15	0.71
115	0.00	0.00	0.60	0.80	0.00	0.00	0.92	1.00
123	0.00	0.00	0.00	0.00	0.02	0.67	0.67	0.67
127	0.00	0.00	0.00	0.00	0.00	0.02	0.38	0.44
140	0.00	0.00	0.00	0.00	0.10	0.02	0.25	0.63
148	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ty 1 derived								
432	0.08	0.33	2.00	3.08	nt	nt	nt	nt
436	0.75	1.30	3.50	3.50	nt	nt	nt	nt
439	0.00	0.96	3.00	3.00	nt	nt	nt	nt
441	0.00	0.80	2.30	2.30	nt	nt	nt	nt
442	2.00	1.50	3.50	3.79	nt	nt	nt	nt
446	2.10	2.55	3.33	3.22	nt	nt	nt	nt
Controls								
'Anastasia'	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00
'Boludo'	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TY-197	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00
NE-1	4.00	4.00	4.00	4.00	1.29	4.00	4.00	4.00

Note: Values are mean scores of symptom severity for 12 plants of each genotype from 0 (symptomless) to 4 (severe symptoms) at 15, 30, 45, and 60 dpi. See text for the description of symptoms. dpi, days post inoculation; nt, not tested.

trols developed symptoms in the course of the assay. The breeding lines showed a variable response. Lines derived from Ty 1 developed symptoms consisting of yellowing and upward curling of apical shoots, delayed in time with respect to NE-1, but at 60 dpi, they were in most cases similar to those exhibited by this susceptible control. However, the lines derived from Ty 6 remained symptomless or showed mild symptoms at the end of the assay.

According to viral DNA detection, differences in infection percentages were not discriminatory among accessions, since at 60 dpi almost all reached 100% infection.

However, the level of virus accumulation varied depending on virus species. For all the lines and controls assayed, TYLCSV accumulation peaked at 30 dpi (Fig. 1A). At 15, 45, and 60 dpi, viral DNA amounts and differences among genotypes were significantly lower. At 30 dpi, virus accumulation in the susceptible line, NE-1, was statistically higher than that observed in the resistant controls. All the *L. chilense* derived breeding lines accumulated low amounts of TYLCSV.

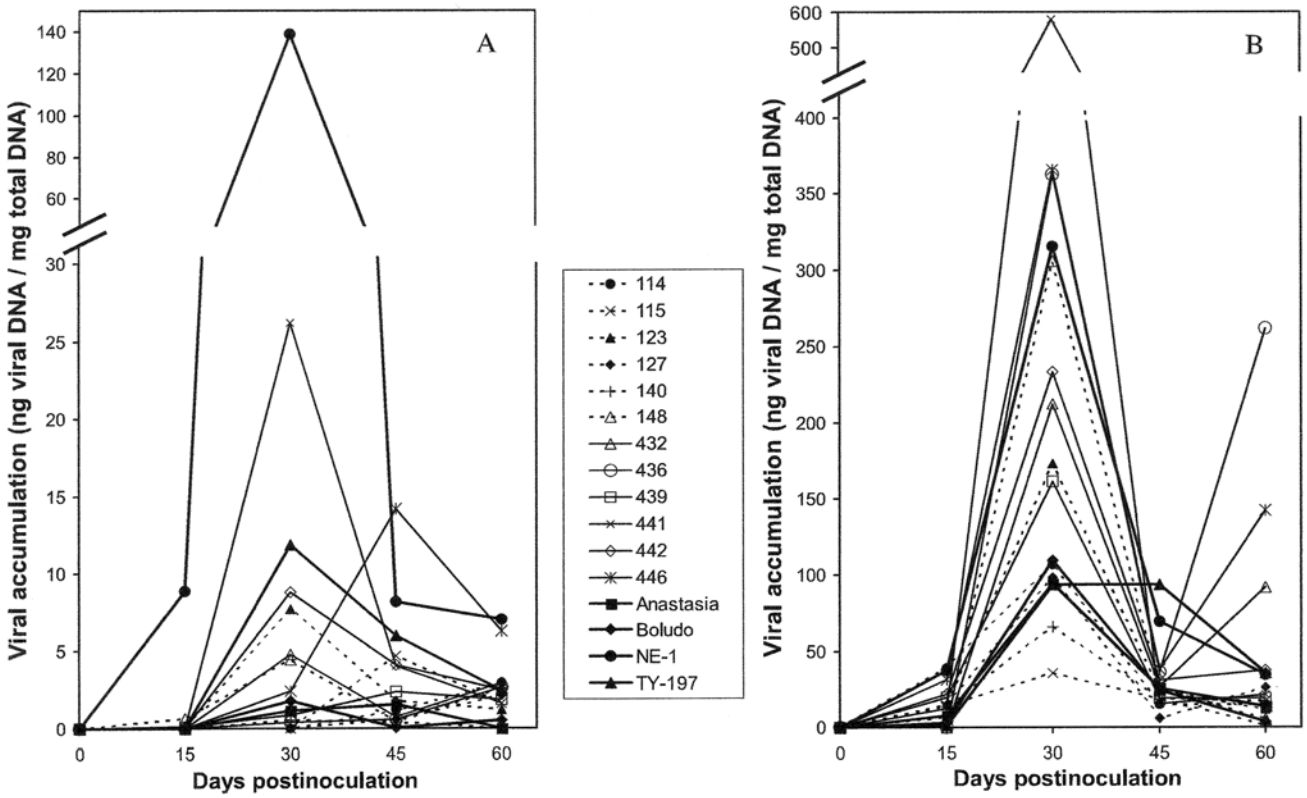
TYLCV accumulation also peaked at 30 dpi for all lines, except for TY-197, which maintained high values until 45 dpi (Fig. 1B). However, accumulation of TYLCV was higher than that of TYLCSV for all genotypes (Fig. 1A and 1B). TYLCV accumulation in the susceptible line NE-1 was significantly higher than that observed in the resistant controls. The response of the lines assayed regarding accumulation of TYLCV was variable. Those lines that exhibited severe symptoms accumulated viral DNA amounts similar to those of the susceptible line NE-1; the ones that remained symptomless contained significantly lower amounts of virus than NE-1 and, in some cases, even lower than the resistant controls.

This first assay allowed a resistant or susceptible behaviour among the 12 breeding lines tested to be distinguished. The lines derived from Ty 6 showed responses similar to those exhibited by the resistant controls. Hence, they were selected for the subsequent analysis. These six lines, as well as the susceptible and resistant controls, were tested during the autumn–winter season in 2002–2003. All plants of NE-1 showed symptoms by 15 dpi, although at this stage symptoms were milder than those observed at the same time period in the previous assay (Table 1). However, as observed during the first assay, plants were stunted at 60 dpi. The resistant controls remained symptomless. Symptoms observed in the breeding lines were slightly more severe in the whitefly-mediated inoculation assay than those exhibited in the agroinoculation assay. Symptoms consisted on yellowing, slight upward curling of leaflets and, in some cases, reduction of leave size. Despite showing these symptoms, plants developed normally, with flowering or fruit appearing to remain unaffected by viral infection.

Infection reached almost 100% for all genotypes at the end of the assay.

The amounts of virus detected were higher for all genotypes at 30 dpi, as shown in the first assay. NE-1 accumulated significantly more viral DNA than did the resistant controls and the lines tested. Levels of virus measured at 30 dpi in 'Anastasia' and 'Boludo' were, respectively, 12% and 12.5% of that of the susceptible control NE-1. Lines

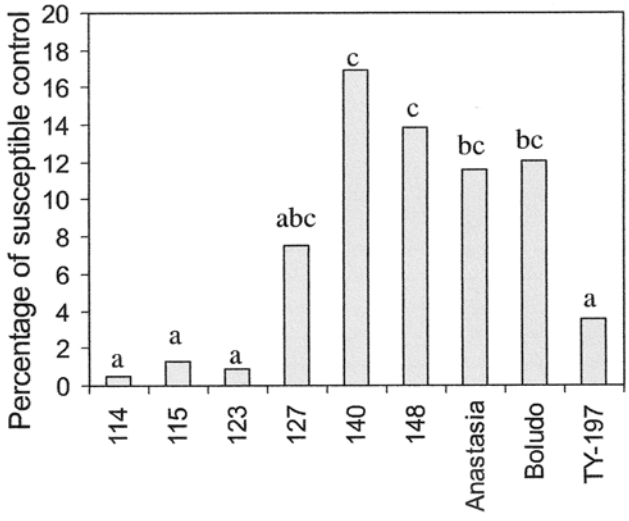
Fig. 1. Mean virus accumulation during the first assay for (A) TYLCSV (B) and TYLCV. Lines derived from Ty 1 are represented with continuous lines, and lines derived from Ty 6 with discontinuous lines.



114, 115, 123, and 127 accumulated lower amounts of virus than did 'Anastasia' and 'Boludo', having 0.5%, 1.4%, 0.9%, and 7.8%, respectively, of that in the susceptible line. For three of these lines (114, 115, and 123), accumulation was statistically lower (least significant difference multiple range test at $\alpha = 0.05$) than accumulation in the resistant controls 'Anastasia' and 'Boludo' (Fig. 2).

The inoculated plants of the susceptible control NE-1 yielded 18% of the number of fruits per plant with respect to the uninoculated plants (Table 2). Moreover, average fruit mass of the inoculated plants of this genotype was less than 50% of the average fruit mass in the uninoculated plants. For the resistant controls, there were no statistical differences between inoculated and uninoculated plants with respect to both number of fruits per plant and average fruit mass. For the breeding lines, only lines 127 and 140 showed a statistical difference in the number of fruits per plant; yield for the inoculated plants was 64.7% and 53.8%, respectively, of that of the uninoculated plants. There were no statistical differences for fruit mass among any of the breeding lines.

Fig. 2. Viral accumulation of TYLCV at 30 days postinoculation, during assay 2, expressed as percentage of susceptible control NE-1. Different letters denote statistical differences (least significant difference multiple range test at $\alpha = 0.05$).



Discussion

Frequently, the efforts for breeding resistance to TYLCD have focused on certain virus species. However, it has been shown that resistant materials respond differently to different TYLCV species (Laterrot 1995; Navas-Castillo et al. 1999; Picó et al. 1999). Furthermore, coexistence of different virus species in the same geographical area and the knowledge of the existence of a displacement between spe-

Table 2. Effect of *Tomato yellow leaf curl virus* on fruit number and fruit mass in the Ty 6 derived lines and the susceptible and resistant controls during assay 2.

	No. fruits/plant*		Fruit mass (g)†	
Genotype	Uninoculated	Inoculated	Uninoculated	Inoculated
114	34.6 a	24.8 a	88.7 a	82.8 a
115	31.7 a	21.5 a	79.2 a	93.5 a
123	42.9 a	30.2 a	85.5 a	100.7 a
127	56.3 a	36.4 b	75.1 a	87.1 a
140	58.2 a	31.3 b	84.4 a	87.7 a
148	25.0 a	22.2 a	96.7 a	87.0 a
'Anastasia'	41.7 a	40.1 a	125.7 a	129.8 a
'Boludo'	50.3 a	51.6 a	125.2 a	129.1 a
TY-197	45.3 a	41.4 a	39.6 a	46.8 a
NE-1	31.8 a	5.7 b	93.0 a	44.7 b

Note: Different letters within the same row and for the same parameter denote statistical differences (least significant difference multiple range test at $\alpha = 0.05$).

*Mean number of fruits per plant from the first eight trusses for the 12 plants in each condition.

†Mean mass of fruits from the first eight trusses for the 12 plants of each condition.

cies makes the epidemiological situation more complicated. The dynamics of TYLCV epidemics have been studied in Spain, revealing that TYLCV can displace TYLCSV. TYLCV is distributed worldwide, and therefore it is necessary to focus breeding programs on the development of materials resistant to different virus species causing TYLCD. In this work, response to TYLCV was tested in breeding lines derived from materials reported as being resistant to TYLCSV.

We used parameters traditionally used to differentiate levels of resistance against TYLCD: percentage of infection, symptom severity, and virus accumulation. The evaluations were carried out for two consecutive years.

Percentage of infection was not a discriminatory parameter, given that almost 100% of infection was detected for all lines and controls tested at the end of the two assays. It has already been reported that materials resistant to TYLCV support virus replication (Vidavsky et al. 1998).

Symptom severity allowed us to make a distinction between susceptible and resistant materials, but this was not a good parameter to differentiate between similar levels of resistance. During the first assay, six of the lines tested showed symptoms similar to those exhibited by the susceptible control NE-1; after subsequent analysis it was revealed that accumulation of TYLCV viral DNA in these lines was also similar or even higher to that in the susceptible control, although these six lines accumulated low amounts of TYLCSV, as reported in Picó et al. (1999). These lines were discarded because of their susceptibility to TYLCV. During the second assay, our objective was to further characterize the resistance of the six lines that remained symptomless and accumulated lower amounts of TYLCSV and TYLCV in the first assay. Symptom severity was not a discriminatory parameter to establish levels of resistance, given that all resistant material tested in this assay remained symptomless or developed only slight symptoms at the end of the assay. Differences observed among the lines tested were not significant. Despite showing some symptoms, plants developed normally, and neither flowering nor fruit set were affected by viral infection. The commercial hybrids employed as resistant controls did not show symptoms. In any case, the symptoms that developed were slightly higher in the second assay in which inoculation was carried out using *Bemisia tabaci*.

As stated in previous experiments, virus accumulation was the most discriminatory parameter to assess the level of resistance to TYLCV (Rom et al. 1993; Lapidot et al. 1997; Picó et al. 1999). In the first study, the maximum differences in viral DNA accumulation among resistant and susceptible genotypes were observed at 30 dpi, corresponding to the time when the highest amounts of viral DNA were detected for both TYLCSV and TYLCV. On previous dates, susceptible accesions tended to accumulate higher amounts of virus too, but differences among cultivars were less discriminatory. Later, at 45 dpi, the accumulation of viral DNA decreased and, for most of the lines tested, remained stable until 60 dpi. The results obtained during the second assay were similar regarding evolution in virus accumulation, so it was determined that evaluation of samples at 60 dpi did not give more relevant information on plant response. Rom et al. (1993) found similar results when studying accumulation of TYLCV DNA in some resistant cultivars, mainly derived from *L. peruvianum*. They observed a peak accumulation about 2 weeks after inoculation, with a subsequent reduction in levels of viral DNA that was stable until the end of their experiment. Similar results were obtained by Lapidot et al. (1997). Comparing the level of resistance to TYLCV in different breeding lines derived from *L. peruvianum* and some commercial cultivars, they found that accumulation peaked twice, at 21 and also at 63 dpi, except for the breeding lines with higher resistance, in which accumulation of viral DNA was practically constant and very low from 21 dpi on. Differences in the date of peak accumulation could be due to differences in environmental conditions in which the experiments were developed or to the different wild species from which resistance was derived. Indeed, TY-197, with resistance derived from *L. peruvianum*, and which was tested in the present study, showed a different re-

sponse regarding peak accumulation when compared with materials with *L. chilense* derived resistance, that is, virus accumulation remained high until 45 dpi.

The results of this work regarding evaluation of different materials for their resistance to TYLCSV and TYLCV have allowed us to select six breeding lines partially resistant to both virus species. These breeding lines have already been evaluated against TYLCSV in previous studies (Picó et al. 1999) and shown to have resistance to this virus species. However, when inoculated with TYLCV in the present study, some of the breeding lines were not resistant; they developed symptoms similar to those exhibited by the susceptible control and also accumulated similar amounts of virus. The differential response of some materials when assayed with different virus species was also observed by Picó et al. (1999). They found that backcrosses derived from *L. chilense* LA 1961 were highly susceptible to TYLCSV, while the original accession was reported to be highly resistant to TYLCV (Scott et al. 1996). In our work, six breeding lines were selected for their resistance to both virus species, TYLCSV and TYLCV. Three of these lines (114, 115, and 123) even accumulated statistically lower amounts of viral DNA than did the resistant controls 'Anastasia' and 'Boludo'. The line TY-197 also accumulated lower amounts of viral DNA. This line has *L. peruvianum* derived resistance, and its use in breeding programs is difficult because of the complexity of the genetic control of this resistance.

Our materials did not show total resistance, given that all lines supported virus replication. However, we found a high level of partial resistance consisting of attenuation of symptoms, delay in time of symptom development, and reduction in virus accumulation. Furthermore, effects of TYLCV on number of fruits per plant and fruit mass were not detected in most of the breeding lines when comparing inoculated and uninoculated plants.

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3.2. Inheritance of *Tomato yellow leaf curl virus* resistance derived from *Solanum pimpinellifolium* UPV16991

Plant Disease

Inheritance of *Tomato yellow leaf curl virus* Resistance Derived from *Solanum pimpinellifolium* UPV16991

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ABSTRACT

Pérez de Castro, A., Díez, M. J., and Nuez, F. 2007. Inheritance of *Tomato yellow leaf curl virus* resistance derived from *Solanum pimpinellifolium* UPV16991. Plant Dis. 91:879-885.

Resistance to tomato yellow leaf curl disease (TYLCD) in accession UPV16991 *Solanum pimpinellifolium* has been previously reported by our group. A breeding program was developed from an initial *S. lycopersicum* × *S. pimpinellifolium* UPV16991 cross. This first cross was followed by several selfing generations. Selection for resistance to *Tomato yellow leaf curl virus* (TYLCV) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV) was carried out for plants of each generation. One partially resistant F_6 plant (L102) was chosen to form the family to study the genetic control of resistance to TYLCV. Crosses between four breeding lines susceptible to TYLCD and L102 were also performed to study the dominance of the resistance in *S. lycopersicum* genetic backgrounds. Response to TYLCV infection of P_1, P_2, F_1, F_2, BC_1, and BC_2 generations fitted, for this line, a monogenic control with partial recessiveness and incomplete penetrance. The percentage of homozygotic plants with partial resistance was 72.75. Among the four hybrids developed, the highest levels of resistance were found in the hybrid formed from the most vigorous *S. lycopersicum* line. These results must be considered for breeding purposes. Partial resistance derived from UPV16991 will be useful in homozygosis or combined with resistance genes from other sources.

Tomato yellow leaf curl disease (TYLCD) causes great losses in tomato (*Solanum lycopersicum* L.) crops worldwide (24,27). Nine viral species have been reported as causal agents of this disease and five more are considered as tentative species (5). All of them belong to the genus *Begomovirus* (family *Geminiviridae*). One of these, *Tomato yellow leaf curl virus* (TYLCV), has been reported affecting tomato worldwide. In some geographical areas, this species is present in mixed epidemics (1,22,26).

Cultural practices to control the disease rely on management of the insect vector, *Bemisia tabaci* Genn. Measures such as the use of UV-absorbing high-density mesh can reduce infection levels (2,34). However, these methods are only effective if combined within an integrated management strategy (14,33). Consequently, breeding for resistance to TYLCD is the most advantageous approach to control the damage caused by this viral disease.

Cultivated tomato shows susceptibility to this disease, but variable levels of resistance have been found in numerous accessions of wild-tomato relatives such as *S. peruvianum* L., *S. chilense* (Dunal) Reiche, *S. habrochaites* S. Knapp & D.M. Spooner, and *S. pimpinellifolium* L. (17,20,27,28). Several of these resistant sources have been employed to develop resistant advanced breeding lines and commercial hybrids (6,18,28,36,37). However, in most cases, resistance sources have been underexploited because of the complex genetics of the resistance (19).

Genetic control of resistance to TYLCD has been studied in accessions of different wild-tomato relatives. Results found in *S. peruvianum* accessions suggest that resistance is conferred by several genetic factors (19,31,36). Resistance derived from *S. habrochaites* LA386 is dominant and controlled by more than one gene (12). Vidavsky and Czosnek (35) derived resistant and tolerant lines from an initial cross between resistant *S. habrochaites* LA1777 and LA386 and concluded that tolerance from these sources was conferred by a dominant major gene while resistance was controlled by two to three additive resistance genes. Resistance found in other sources from this species seems to be controlled by two genes acting epistatically (10). On the contrary, resistance derived from *S. chilense* LA1969 has been reported to be conferred by one major gene, *Ty-1*, with two minor modifier genes (37).

S. pimpinellifolium results regarding genetic control of the resistance to TYLCV are sometimes contradictory. Pilowsky and Cohen (30) concluded that resistance derived from LA121 was monogenic with incomplete dominance. Subsequent studies developed by Hassan et al. (13) with LA121 suggested that resistance was quantitatively inherited with partially recessive gene action and incomplete penetrance. These authors concluded that inheritance of the resistance found in *S. pimpinellifolium* LA373 followed this same model. The authors attributed discrepancies to differences in viral species or isolates, environmental conditions or inoculation, and evaluation procedures. Other *S. pimpinellifolium* accessions have also been investigated. Kasrawi (15) concluded that resistance found in accessions Hirsute-INRA and LA1478 was controlled by a single dominant gene; the proposed name was *Tylc*. Vidavsky et al. (36) agreed later with these results for Hirsute-INRA. However, segregation analysis developed by Chagué et al. (3) showed that resistance was inherited following a quantitative pattern. Studies developed with lines derived from different resistant sources of *S. pimpinellifolium* revealed that the gene action for TYLCD resistance varied with the source and was a quantitative trait with some dominance (16). Similar results were found when evaluating *S. pimpinellifolium* accessions PI407543, PI407544, and PI407555 (11). Three pairs of genes were estimated to be controlling the resistance, with complete dominance in PI407543 and PI407544 and partial dominance in PI407555. Gene action was additive in PI407543, while dominance and nonallelic interactions were important in accessions PI407544 and PI407555.

S. chilense and *S. peruvianum* are the wild species mainly used to develop hybrids or cultivars resistant to TYLCD. Commercial materials with resistance derived from *S. pimpinellifolium* are not available. In any case, the materials developed from different sources are not immune to TYLCD. Consequently, the efforts of different research groups are still aimed at controlling this disease. One possible way of developing a more durable resistance and increasing its level could be the accumulation of resistance genes from different sources. The study of progenies obtained from interspecific crosses involving *S. pimpinellifolium*, *S. peruvianum*, and *S. habrochaites* revealed that they showed higher levels of resistance than either parent (16). Similar results were found with crosses from *S. peruvianum*, *S. pimpinellifolium*, and *S. chilense*, suggest-

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ing that the highest levels of resistance could be obtained combining partly dominant genes from *S. pimpinellifolium* and *S. chilense* (36). More recently, high levels of resistance were also obtained in hybrids that combined resistance from *S. habrochaites* and *S. pimpinellifolium* with *S. peruvianum*-derived resistance (23).

Consequently, it is of interest to exploit resistance derived from *S. pimpinellifolium*, if not on its own, then combined with resistance derived from other wild-tomato relatives.

This study was conducted to characterize the resistance to TYLCV derived from *S. pimpinellifolium* UPV16991, determine the genetic basis of resistance, and study the dominance of this resistance in different *S. lycopersicum* backgrounds.

MATERIALS AND METHODS

Plant material. Partial resistance to *Tomato yellow leaf curl Sardinia virus* (TYLCSV) was detected by our group in *S. pimpinellifolium* accession UPV16991 (29). A breeding program was initiated to introgress resistance found in this accession into cultivated species. The program was developed from an initial cross between the susceptible *S. lycopersicum* NE-1, a breeding line developed at the Institute for the Conservation and Improvement of Agrodiversity (COMAV, Spain), and *S. pimpinellifolium* UPV16991. The first cross was followed by several selfing generations. Selection for resistance to TYLCV and TYLCSV was carried out for plants at each generation. In the F_6 generation, one plant of line L102 was selected to form the family in which to study the genetic control of resistance derived from UPV16991. The old Fortuna C cultivar (FC), used as a female parent (P_1), is highly susceptible to TYLCD and was crossed to L102 (P_2). The F_1 generation was selfed to provide an F_2 generation and also backcrossed as male parent to FC and L102 to produce the backcross generations BC_1 and BC_2, respectively. L102 was additionally crossed as male parent to three other susceptible breeding lines developed at the COMAV (NE-1, P-73, and V-5-7). The Pimhir population with partial resistance to TYLCV, developed by Laterrot (21) from *S. pimpinellifolium* Hirsute-INRA, was used as the resistant control.

In this study, 20 plants of each parent, of each of the four F_1 generations developed, as well as those of Pimhir, 150 plants of the F_2 generation, and 100 plants of each of the backcrosses were tested for their resistance to TYLCV.

Inoculation techniques. Inoculation was carried out with TYLCV. The Spanish TYLCV isolate TYLCV-Mld[ES7297] (GenBank Accession No. AF071228) was maintained in FC tomato plants by whitefly transmission inside muslin-covered cages in a growth chamber. The environmental conditions were: temperature at 25°C; 60 to 65% and 95 to 99% relative humidity (day/night); 34 $\mu E \cdot m^{-2} \cdot s^{-1}$ of irradiance; and a 16/8 (light/dark) photoperiod. The whiteflies used were biotype Q (supplied by F. Beitia, Instituto Valenciano de Investigaciones Agrarias, IVIA, Valencia, Spain). Plants to be inoculated were grown in pots in a greenhouse with controlled light and temperature. Plants (the number of plants for each generation is specified in the plant material section) were caged with at least 20 whiteflies per plant at the three true-leaf stage for 3 days. Plants were shaken daily to ensure that the distribution of the whiteflies was uniform. After the inoculation period, plants were sprayed with insecticide (piproxifen [10%], bifentrin [10%], imidacloprid [20%], teflubenzuron [15%], and endosulfan [35%], percentage of each component referred to the total volume of the mixture) and transferred to a new insect-proof cage for 2 days to avoid the spreading of whiteflies. Subsequently, plants were sprayed again with the insecticide, transplanted to bigger pots, and transferred to the greenhouse.

Experimental design. Plants for inoculation were divided among 18 trays. In each of the trays, plants belonging to each of the generations were included. The number of plants of each generation per tray was proportional to total number of plants used in the generation. Plants were distributed randomly in each tray. After inoculation, plants were transplanted to bigger pots in two adjacent greenhouses to the end of the assay. Each greenhouse was in turn divided into two plots. Plants of each generation were distributed in blocks of five plants and distributed randomly in the different plots, again maintaining the proportion of sample size of the generation in each block. Conditions in the greenhouses were: temperatures at 25 to 18°C (day/night); 80% relative humidity; and a 16/8 (light/dark) photoperiod.

Disease assessment. Symptom severity and viral DNA presence were assessed to evaluate the response of each plant at 15, 25, 35, and 45 days postinoculation (dpi). Additionally, a final evaluation of symptom scores was done at 60 dpi for the six generations used to study the genetic control of resistance.

At each date, the presence of the following symptoms was recorded: yellowing; crumpling; upward curling; reduction in leaf size; and stunting. Additionally, symptom severity was graded according to a scale described by Friedmann et al. (6): 0 = no visible symptoms, inoculated plants show same growth and development as uninoculated plants; 1 = very slight yellowing of leaflet margins on apical leaf; 2 = some yellowing and minor curling of leaflet ends; 3 = a wide range of leaf yellowing, curling and cupping, with some reduction in size, yet plants continue to develop; and 4 = very severe plant stunting and yellowing 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 of symptom severity.

The presence of TYLCV viral DNA was assessed by molecular hybridization. Approximately 150 mg of tissue from the upper canopy of the plant were taken at each date and DNA was extracted following the procedure described by Crespi et al. (4) with some modifications. The frozen tissue was crushed in 500 µL of extraction buffer (100 mM Tris-HCl [pH 8], 50 mM EDTA, 500 mM NaCl, 10 mM 2-β-mercaptoethanol, and 1% sodium dodecyl sulfate) and incubated at 65°C for 5 min. Afterward, 150 µl of 5 M potassium acetate were added. Following incubation on ice for 10 min, samples were centrifuged (16,110 × *g* for 10 min). The supernatant was recovered and DNA was precipitated with isopropanol by centrifugation (16,110 × *g* for 5 min) and then the DNA was resuspended in 77 µl of distilled water. One microliter of each sample, corresponding to approximately 1.5 mg of fresh tissue, and a 10-fold dilution of the sample were denatured with 30 mM NaOH and 1 mM EDTA for 30 min and then blotted onto positively charged nylon membranes for hybridization. DNA was fixed on the membrane by UV crosslinking. Hybridization was carried out according to "The DIG system user's guide for filter hybridization" (Roche Molecular Biochemicals, Mannheim, Germany) using digoxigenin-11-dUTP and chemiluminescent detection. Membranes were prehybridized in standard hybridization buffer plus 50% deionized formamide for at least 1 h. Subsequent hybridization was done at 42°C overnight in fresh prehybridization solution containing 20 ng of denatured probe per milliliter. The probe employed (supplied by E. R. Bejarano, Universidad de Málaga, Spain) represented the intergenic region of the Spanish TYLCV isolate previously cited. The probe was labeled by incorporation of digoxigenin-11-dUTP during polymerase chain reaction (PCR). Washing steps and incubation with antibody were done according to the manufacturer's instructions. Detection was carried out with CSPD and direct exposure to a CCD camera (Intelligent Dark Box-II; Fujifilm, Tokyo, Japan).

The amount of viral ssDNA was quantified according to a standard curve of TYLCV dotted on the same membrane (ranging from 25 ng to 1 pg).

Extracted plant DNA was also quantified to relate virus concentration to plant DNA present in each sample. Fluorimetry was employed as the method of quantifying doubled-stranded DNA (Hoefer DyNA Quant 200 fluorimeter, according to manufacturer's instructions, San Francisco, CA).

Those plants in which viral accumulation was not detected at any sampling date

were considered to have escaped infection, and were therefore, discarded and not included in the analysis. Plants that became infected by other viral diseases were also eliminated from the assay.

Evaluation criteria. To classify individual plants as partially resistant or susceptible, the limit was established at symptom score 2: plants that scored under 2 were considered partially resistant while plants that scored 2 or higher were considered susceptible. These criteria were set based on the scale described to evaluate symptom development. Slight symptoms (<2) do not affect a plant enough to cause yield losses, and it can therefore, be classified as partially resistant.

Genetic analyses. The observed percentages of partially resistant and susceptible plants at 60 dpi for each generation were fitted to a model appropriate for describing the response observed in the different generations. Parameters of the model were estimated by the least square regression method, considering as weights the number of plants at each generation. The joint fit of all generations to the proposed model was evaluated by the χ^2 weighted statistic obtained by the expression: χ^2 weighted = Σ [(observed relative frequency-expected relative frequency)^2 × weight].

RESULTS

Characterization of the resistance to TYLCD derived from *S. pimpinellifolium* UPV16991. Accession UPV16991 was previously selected from 40 *S. pimpinellifolium* accessions by our group (29) because it displayed a good response against TYLCD. UPV16991 showed a low percentage of infection, accumulated significantly lower viral amounts than what were found in susceptible genotypes, and exhibited mild symptoms. In the first screening, some plants of the accession were completely symptomless and virus-free when analyzed by squash-blot hybridization, and therefore, were selected to initiate the breeding program. Following a cross with the cultivated species, five selfing generations were developed in which selection for resistance to TYLCSV and TYLCV was carried out. In each generation, the same variation seen in the original source was found. Most of the plants remained symptomless or exhibited mild symptoms, whereas a variable percentage of the plants (7 to 30%) continued showing susceptibility to the disease with medium to severe symptoms (data not shown). Regarding viral accumulation, it was always lower than the accumulation in the susceptible controls. This response to infection observed in the successive selfing generations was homogeneous, indicating that the gene or genes controlling resistance were fixed.

Thus, assuming that resistance was fixed, one partially resistant F_6 plant was selected to form the family to study the genetic control and the dominance in different *S. lycopersicum* genetic backgrounds of the resistance derived from *S. pimpinellifolium* UPV16991. Response to infection in the selected line, L102, was compared with that of the susceptible and resistant controls, FC and Pimhir, respectively. A high percentage of plants of the susceptible FC control showed severe symptoms at 15 dpi (Table 1). At this time, symptoms consisted of yellowing, upward curling, and reduction in leaf size. The disease progressed rapidly and 100% of susceptible plants ceased to grow at 25 dpi. The first severe symptoms (symptom score of ≥2) appeared at 25 dpi in the resistance source, L102. Furthermore, some plants recovered, and at 45 dpi, 86.7% of the plants showed only slight symptoms and were considered partially resistant. Moreover, the susceptible plants of this generation in no case scored more than 2.5. Symptom appearance was also delayed in plants of the resistant Pimhir control, but at the end of the assay, 47.1% of the plants were classified as susceptible.

Viral accumulation in L102 and Pimhir was lower than in the susceptible control for all sampling dates. Accumulation peaked for all genotypes at 35 dpi and so this was the most appropriate date to discriminate responses. At this date, viral accumulation detected in Pimhir and in L102 did not differ significantly (Fig. 1).

Resistance was thus expressed in L102 as a delay in time and a recovery of symptom development as well as a lower percentage of plants showing severe symptoms at the end of the assay. Additionally, viral accumulation was always significantly lower than in susceptible controls. The appearance of susceptible plants in L102 (corresponding to the response observed in the previous generations studied) supports that the gene or genes controlling the resistance must have incomplete penetrance.

Inheritance of the resistance to TYLCD derived from *S. pimpinellifolium* UPV16991. Six generations (P_1, P_2, F_1, F_2, BC_1, and BC_2) were used to study the genetic control of the resistance in L102. Plants of the susceptible FC parent began showing symptoms of the disease at 15 dpi, and as of 25 dpi, all plants showed severe symptoms (Table 2). As previously described, the partially resistant L102 parent did not show complete resistance, given that as of 25 dpi some of the plants scored 2 or higher and so were classified as susceptible. However, some of the plants recovered. The partially resistant plants of L102 displayed variable levels of symptoms. The mean symptom score of the F_1 generation was lower than the mid-parent value for all the sampling dates. Even though 77.8% of the plants were susceptible at 60 dpi, none of them showed symptoms that scored over 2.5 (Fig. 2). The F_2 generation segregated continuously, and at 60 dpi, it was skewed toward susceptibility. The BC_1 generation mean was between the F_1 and P_1 means throughout the assay. All plants of this generation showed symptoms that scored over 2 at the end of the assay, so resistance was not detected in this generation at 60 dpi. The BC_2 generation mean was between the P_2 and F_1 means for all sampling dates. Some plants of this generation also recovered from severe symptoms. This generation was very similar to the partially resistant parent, although the percentage of susceptible plants was higher in the BC_2. With regard to the maximum symptom score, in none of these generations was it over 2.5 at 60 dpi.

Table 1. Percentage of partially resistant tomato plants for L102, the controls, and hybrids at different days postinoculation (dpi) with *Tomato yellow leaf curl virus*[a]

Genotype	15 dpi	25 dpi	35 dpi	45 dpi
Fortuna C[b]	10.5	0.0	0.0	0.0
L102	100.0	60.0	66.7	86.7
Pimhir[c]	94.1	100.0	52.9	52.9
V-5-7 × L102	76.5	70.6	47.1	47.1
NE-1 × L102	85.0	40.0	25.0	10.0
FC × L102	83.3	72.2	50.0	22.2
P-73 × L102	78.9	47.4	31.6	15.8

[a] A plant was considered partially resistant if the symptom score was less than 2 in a scale from 0 (symptomless) to 4 (severe symptoms).
[b] Susceptible control.
[c] Resistant control.

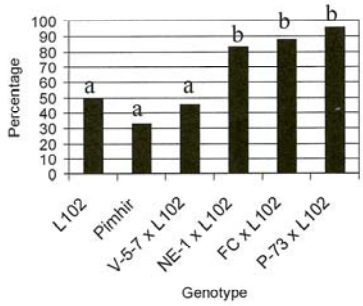

Fig. 1. Viral accumulation of *Tomato yellow leaf curl virus* at 35 days postinoculation expressed as percentage of susceptible control Fortuna C. Different letters denote statistical differences (LSD multiple range test at the 0.05 protection level).

Viral accumulation was also measured in individual plants at 15, 25, 35, and 45 dpi. The amount of virus detected peaked at 35 dpi. The maximum and minimum accumulation levels were found, respectively, in FC and L102. For the rest of the generations, viral accumulation was intermediate, following a similar pattern with respect to symptom development. There was a low positive correlation, though highly significant, between symptom development and viral accumulation (r = 0.127, $P = 2.37 \times 10^{-6}$) for the whole set of data. This correlation was higher (r = 0.211, $P = 1.37 \times 10^{-6}$) if only partially resistant plants were considered. So symptom score was the criterion chosen to classify plants as susceptible or partially resistant (see materials and methods). According to agronomic significance, data chosen to study the genetic control of the resistance were those recorded at 60 dpi.

The appearance of susceptible plants in L102 supports that the gene or genes controlling resistance must have incomplete penetrance. Moreover, the high percentage of susceptible plants in the F_1 indicated that resistance was not dominant. Thus, the proposed model needed to consider incomplete penetration and partial dominance of resistance. The observed percentages of partially resistant and susceptible plants for each generation were fitted to a monogenic model with two parameters to consider these effects (Table 3). The parameters, p and q, indicated the probability of susceptibility for, respectively, a homozygous or heterozygous plant for the resistance gene. The parameters were estimated by weighted least square regression (Table 4). Both parameters were highly significant. The estimate for parameter p indicates incomplete penetrance of resistance since approximately 27% of homozygous plants for the resistance gene are susceptible. The estimate for parameter q exceeds p, thus indicating partial recessiveness. The fit of each generation to the model was confirmed by comparing the observed and expected values. Data from all generations together also fit the proposed model and thus explained the observed data.

Characterization of dominance of resistance to TYLCD derived from *S. pimpinellifolium* in different *S. lycopersicum* genetic backgrounds. Symptom development and viral accumulation were evaluated in hybrids among four breeding lines susceptible to TYLCD and L102. The response of the four hybrids was intermediate between resistant and susceptible controls. Symptom development was delayed compared with the susceptible control (Table 1). The percentage of plants showing severe symptoms was lower than for FC, but in all cases, higher than for the resistant controls. Important differences were observed among the four hybrids, with the percentage of partially resistant plants at 45 dpi ranging from 10 to 47.1. The most severe symptoms and lowest percentage of partially resistant plants appeared in the hybrid involving NE-1, followed by plants of P-73 × L102. Plants of the hybrid with FC showed a delay in symptom development with respect to plants of the other three hybrids; however, at the end of the assay, most of the plants of this hybrid behaved as susceptible. The best response among the four hybrids was exhibited by V-5-7 × L102, which at the end of the assay showed a percentage of partially resistant plants similar to that displayed by Pimhir.

Viral accumulation was also measured. Levels of virus detected increased along with dpi and the maximum was reached at 35 dpi. At this date, viral accumulation detected in the hybrids P-73 × L102, FC × L102, and NE-1 × L102 did not significantly differ from that showed by the susceptible control FC (Fig. 1). However, the amount of virus accumulated in the hybrid

Table 2. Number of plants in each score of *Tomato yellow leaf curl virus* symptoms in the parents, F_1, F_2, and BC generations from the cross Fortuna C × L102

Generation	No. plants	dpi[b]	No. of plants assigned score[a]									Mean score
			0	0.5	1	1.5	2	2.5	3	3.5	4	
Fortuna C	19	15	0	0	0	2	1	2	3	6	5	3.16
		25	0	0	0	0	0	0	0	0	19	4.00
		35	0	0	0	0	0	0	0	0	19	4.00
		45	0	0	0	0	0	0	0	0	19	4.00
		60	0	0	0	0	0	0	0	0	19	4.00
L102	15	15	9	2	3	1	0	0	0	0	0	0.37
		25	3	3	0	3	5	1	0	0	0	1.23
		35	0	1	6	3	5	0	0	0	0	1.40
		45	0	3	7	3	1	1	0	0	0	1.17
		60	0	2	6	3	3	1	0	0	0	1.33
F_1	18	15	8	2	1	4	0	3	0	0	0	0.86
		25	2	1	7	3	3	0	2	0	0	1.33
		35	1	1	2	5	6	0	3	0	0	1.72
		45	0	1	3	0	5	9	0	0	0	2.00
		60	0	1	3	0	5	9	0	0	0	2.00
F_2	124	15	34	14	25	6	13	19	8	2	3	1.27
		25	1	12	13	10	26	14	26	15	7	2.25
		35	1	3	11	4	22	35	31	11	6	2.46
		45	0	2	1	5	22	40	33	16	5	2.65
		60	0	2	1	5	23	37	35	16	5	2.65
BC_1	96	15	14	6	10	13	10	15	18	4	6	1.90
		25	1	2	4	3	3	7	26	14	36	3.17
		35	1	0	6	3	4	8	28	23	23	3.07
		45	0	0	0	0	0	10	30	30	26	3.38
		60	0	0	0	0	0	10	30	30	26	3.38
BC_2	84	15	31	14	19	7	11	2	0	0	0	0.76
		25	11	17	17	16	10	5	6	2	0	1.27
		35	3	14	20	11	22	12	2	0	0	1.47
		45	0	12	19	16	24	13	0	0	0	1.54
		60	0	9	14	15	38	8	0	0	0	1.63

[a] Symptom score from 0 (symptomless) to 4 (severe symptoms). See text for further description of the scale.
[b] dpi = Days postinoculation.

V-5-7 × L102 was similar to that in the partially resistant parent L102 and in the resistant Pimhir control.

There was a positive correlation (r = 0.276, $P = 1.17 \times 10^{-6}$) between symptom development and viral accumulation for the four hybrids assayed.

DISCUSSION

Commercial materials resistant to TYLCD have been developed mainly on the basis of resistance derived from *S. chilense* and *S. peruvianum*. It is of interest to make use of the resistance found in other wild-tomato relatives. Variable levels of resistance have been found in different *S. pimpinellifolium* accessions, but currently, resistance derived from this source has not been exploited in developing commercial resistant materials. This study shows the usefulness of *S. pimpinellifolium* UPV16991 in breeding for resistance to TYLCD.

The response observed in the successive selfing generations from UPV16991 was homogeneous. Therefore, it could be assumed that the gene or genes controlling the partial resistance are fixed. The resistance derived from this accession was characterized by a restriction in viral accumulation and a delay in time of symptom development. However, at each selfing generation, a variable percentage of susceptible plants appeared, indicating the incomplete penetrance of the resistance.

The incomplete penetrance of resistance derived from *S. pimpinellifolium* seems to be the general pattern, as is also suggested by similar results found by other authors when studying different accessions of this species (13). Moreover, some authors have reported a complex genetics of the resistance derived from different *S. pimpinellifolium* accessions (3,11,13,16). These two facts would explain that, to date, resistance derived from this wild species has not been exploited to develop commercial materials resistant to TYLCD. However, some studies revealed that combining resistance derived from other wild-tomato relatives with S. *pimpinellifolium*-derived resistance an increase in the level of resistance was obtained (16,23,36). Thus, the resistance to TYLCD derived from *S. pimpinellifolium* could be useful if it was controlled by one or a few genes and expressed in *S. lycopersicum* background.

Inheritance studies were carried out that considered symptom development and viral accumulation for the six generations. There was a significant positive correlation between both parameters. Thus, the criteria ultimately established to classify each individual plant as susceptible or partially resistant were on the basis of symptom

Table 3. Monogenic model proposed to explain the inheritance of tomato yellow leaf curl disease (TYLCD) resistance derived from *Solanum pimpinellifolium* UPV16991. Incomplete penetrance and partial recessiveness are considered on the basis of the proportion of partially resistant and susceptible plants obtained in the studied generations

Generations		Model	Number of plants observed	Number of plants predicted[a]
P_2 (L102)	S[b]	p[c]	4	4
	R[d]	$1-p$	11	11
F_1 (FC × L102)	S	q[e]	14	18
	R	$1-q$	4	0
F_2 (selfed F_1)	S	$0.25 + 0.25p + 0.5q$	116	101
	R	$0.75 - (0.25p + 0.5q)$	8	23
BC_1 (FC × F_1)	S	$0.5 + 0.5q$	96	96
	R	$0.5 - 0.5q$	0	0
BC_2 (L102 × F_1)	S	$0.5p + 0.5q$	46	53
	R	$1 - (0.5p + 0.5q)$	38	31

[a] Prediction based on estimates for p and q by least square regression method.
[b] Susceptible plants.
[c] Probability of susceptibility of a homozygous plant for the resistance gene.
[d] Partially resistant plants.
[e] Probability of susceptibility of a heterozygous plant for the resistance gene.

Table 4. Estimates by the least square regression method of the proportion of susceptible plants in homozygous (p) and heterozygous (q) for the resistance gene derived from *Solanum pimpinellifolium* UPV16991

	P	*q*
Estimate	0.272	0.995
P	0.048	0.000
Standard error	0.117	0.080
Confidence intervals (95%)	0.002-0.543	0.810-1.179
R-squared (adjusted for degrees of freedom)	97.27	
χ^2 weighted	3.255	
P	0.354	

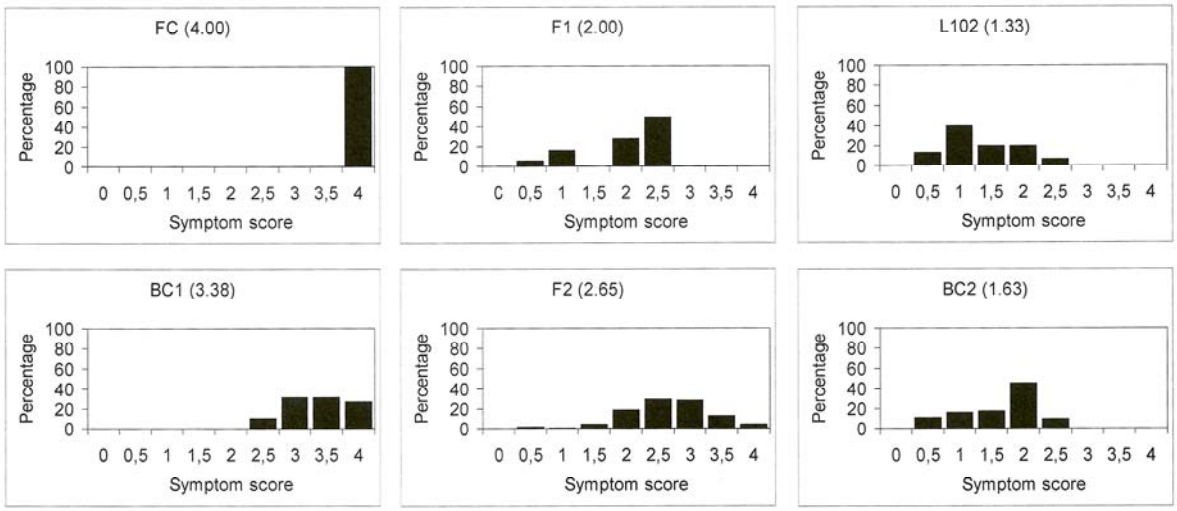

Fig. 2. Distribution of *Tomato yellow leaf curl virus* disease ratio 60 days postinoculation for P_1, P_2, F_1, F_2, BC_1, and BC_2 generations from the cross FC × L102. Mean score for each generation is indicated in brackets.

score at the end of the assay. In fact, most of the inheritance studies developed by different authors for resistance to TYLCD also used criteria based on symptom development. However, in most cases, plants were considered resistant only if they remained symptomless throughout the assay period (12,13,15,19,31). Our criteria considered that a plant was partially resistant if it was either symptomless or showed only slight symptoms that would not cause yield losses. Kasrawi and Mansour (16) established similar criteria in their study of the genetics of resistance to TYLCV derived from *S. pimpinellifolium* Hirsute-INRA where plants rated 0 were considered resistant, plants rated 4 were classified as susceptible, and plants in the remaining scores were considered to show variable levels of resistance. Other authors have established similar criteria when studying inheritance of resistance to different viral diseases such as *Tomato leaf curl virus* (ToLCV; 7), *Cucumber mosaic virus* (CMV; 32) or *Tomato chlorotic mottle virus* (ToCMoV; 8). These criteria turn out to be more useful in breeding programs, given that, as previously stated by Lapidot and Friedmann (17), the most relevant factor in evaluation for resistance is the effect of infection on total yield and yield components, but tests to compare yield in infected and noninfected plants can only be carried out for advanced materials of breeding programs.

It was under these premises that the results for the six generations were analyzed. The distribution of plants for each generation according to their symptom score suggested that the gene(s) controlling resistance had incomplete penetrance and partial dominance. Similar distributions were obtained by Kasrawi and Mansour (16) in families with *S. pimpinellifolium*-derived resistance and they inferred that the resistance was quantitatively inherited. The same conclusion was obtained from a very similar distribution when evaluating resistance to another geminivirus, *Tomato mottle virus* (ToMoV), derived from LA1932 (9). However, our data fit a monogenic model including partial recessiveness and incomplete penetrance. These results have implications for breeding purposes. Monogenic control of resistance facilitates introgression into the cultivated species. However, others have proposed a monogenic control of TYLCD resistance for other *S. pimpinellifolium* accessions that have not been used to develop commercial materials (15,36). On the other hand, the effects of partial recessiveness and incomplete penetrance must be considered. In fact, given the high dependence of resistance on gene dosage (measured by parameter q), UPV16991-derived resistance should not be used in heterozygosis. As a consequence of incomplete penetrance, a percentage of homozygous plants for the resistance gene will be susceptible to the disease. However, symptoms developed by the susceptible plants of the resistance source were only moderate, even under the high inoculum pressure reached in this assay. Thus, in natural field conditions, a better response would be expected for homozygous plants. Currently, even recessive genes are useful in breeding programs, given that it is possible to develop molecular markers tightly linked to the gene of interest. This prospect would make introgression programs much easier and allow incorporating the resistance gene in both parental lines of hybrids.

The results obtained here suggest that the expression of resistance derived from *S. pimpinellifolium* UPV16991 strongly depends on the *S. lycopersicum* genetic background in which it is introgressed. The percentage of partially resistant plants ranged from 10.0 to 47.1 among the four hybrids assayed. The lowest percentage of susceptible plants and lowest levels of viral accumulation were detected in the hybrid V-5-7 × L102. This hybrid also showed the mildest symptoms. The V-5-7 line is the most vigorous of the four tomato lines used to develop the hybrids. This fact could explain the better response found in the cross involving this line. Similar results were found when studying lines derived from crosses between susceptible tomato cultivars and *S. pimpinellifolium* LA121 and LA373 where generations developed from the most vigorous materials showed less severe symptoms (25). The influence of the genetic background in the expression of resistance to TYLCD has also been reported for *S. chilense* (28) and *S. habrochaites*-derived resistance (23).

The influence of the genetic background on the expression of resistance derived from *S. pimpinellifolium* UPV16991 conditions its use in breeding programs. Parental lines must be selected by taking into account not only the heterosis of the hybrid but also the level of expression of the resistance.

In conclusion, we recommend the use of the resistance gene introgressed from UPV16991 in homozygosis in the development of vigorous hybrids. Another approach would be the combination of resistance reported here with resistance derived from different sources in an attempt to achieve a more durable resistance and increase its level.

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3.3. Exploiting partial resistance to *Tomato yellow leaf curl virus* derived from *Solanum pimpinellifolium* UPV16991

Plant Disease

(aceptado con revisión)

Exploiting partial resistance to *Tomato yellow leaf curl virus* derived from *Solanum pimpinellifolium* UPV16991

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ABSTRACT

Tomato yellow leaf curl disease (TYLCD) causes great economic losses in tomato crops worldwide. Despite efforts undertaken by different research groups, there are no immune commercial plant materials available. Recently, our group reported partial resistance to TYLCD in line L102, derived from *Solanum pimpinellifolium* UPV16991. Resistance in this line is monogenic with partial recessiveness and incomplete penetrance. Even though the resistance gene in L102 is not dominant, we also proved that levels of resistance were high in hybrids with different tomato lines. The objective of this work was to evaluate the level of resistance in plants which combined UPV16991-derived resistance and the *Ty-1* gene, both in heterozygosis. Most of the hybrids between S. *pimpinellifolium* and *S. chilense*-derived resistant lines exhibited milder symptoms than heterozygotes for either *S. pimpinellifolium* or *S. chilense*-derived resistance. In some of the hybrids, viral accumulation was also lower than in respective heterozygotes. Our results support the utility of resistance derived from UPV16991 combined with the *Ty-1* gene in increasing levels of resistance to TYLCD in tomato hybrids. This is the most practical approach to exploiting resistance derived from UPV16991, since it allows the development of hybrids without the need of fixing the resistance gene in both parents.

INTERPRETIVE SUMMARY

Tomato yellow leaf curl disease (TYLCD) affects tomato crops worldwide. There are currently no totally resistant commercial varieties. We recently described partial resistance to TYLCD in one line derived from *Solanum pimpinellifolium*, a wild tomato relative very similar to the cultivated species. Resistance in this line was controlled by one gene, but the level of resistance conferred and the mode of action would not allow commercial plant materials to be based exclusively on this gene. It has been reported that combining resistance genes from different wild tomato relatives allows high levels of resistance to be achieved, and makes the resistance more durable. The objective of our work was to join the resistance gene derived from *S. pimpinellifolium* with the most effective gene described to date against TYLCD, the *Ty-1* gene, derived from *S. chilense*. Our results show that by combining *Ty-1* and the resistance gene from *S. pimpinellifolium*, both in heterozygosis, it is possible to increase the level of resistance when compared with each of the genes alone in heterozygosis. The importance of this finding is that it allows the exploitation of this resistance in the development of hybrids, without the need of incorporating the resistance gene in both parents of the hybrid.

INTRODUCTION

Tomato yellow leaf curl disease (TYLCD) produces great economic losses in tomato (*Solanum lycopersicum* L., syn. *Lycopersicon esculentum* Miller) crops in many temperate areas (13,17). TYLCD is caused by a complex of viruses belonging to the genus *Begomovirus*, family *Geminiviridae* (3). Among viral species causing TYLCD, *Tomato yellow leaf curl virus* (TYLCV) has been detected infecting tomato worldwide.

Variable levels of resistance to TYLCD have been reported in several wild tomato relatives, such as *S. peruvianum* L. (syn. *Lycopersicon peruvianum* (L.) Miller), *S. chilense* (Dunal) Reiche (syn. *Lycopersicon chilense* Dunal), *S. habrochaites* S. Knapp & D.M. Spooner (syn. *Lycopersicon hirsutum* Humb. & Bonpl.) and *S. pimpinellifolium* L. (syn. *Lycopersicon pimpinellifolium* (L.) Miller) (8). Some of these resistance sources have been used to develop commercial cultivars, mainly several accessions of *S. chilense* and *S. peruvianum* (8). *S. habrochaites*-derived resistance has also been exploited, mostly in Asia (10) and Guatemala (11). In any case, no immune commercial materials have been made available to date, so it is of interest to make use of all existing resistance sources that show potential.

S. pimpinellifolium accessions have not been an important source in breeding programs for resistance to TYLCD (9). Recently, our research group derived an F_6 line from *S. pimpinellifolium* UPV16991 and characterized resistance in this line (15). It was determined that resistance is monogenic with partial recessiveness and incomplete penetrance. Considering these results, we proposed the use of this resistance in homozygosis for the development of vigorous hybrids or in combination with resistance genes from other sources. If successful, the second approach would be of greater interest, given that it would allow the development of hybrids, thereby avoiding the need to fix the resistance gene in both parents.

Several attempts to increase resistance to TYLCD have been made based on the accumulation of resistance genes from different sources. Kasrawi and Mansour (7) developed crosses from lines with resistance derived from *S. pimpinellifolium*, *S. peruvianum* and *S. habrochaites*. Their results revealed that the level of resistance in progenies of interspecific crosses exceeded that of both parents. It has also been reported that the levels of resistance, not only to TYLCV but also to other begomoviruses infecting tomatoes in Guatemala, can be increased by combining *S. peruvianum*-derived resistance with resistance derived from *S. pimpinellifolium* and *S. habrochaites* (11). Similar results

were obtained in a study which included crosses between advanced breeding lines with variable levels of resistance derived from *S. peruvianum*, *S. pimpinellifolium* and *S. chilense* (23). In all cases, F_1 combinations which involved highly resistant parents derived from different wild species, showed higher resistance than that of any of the parents. Furthermore, even descendants of crosses involving one highly resistant line and another one with intermediate levels of resistance turned out to be highly resistant. The best results were obtained by combining *S. chilense* and *S. pimpinellifolium*-derived resistance.

Consequently, even though the line derived from UPV16991 shows only partial resistance, it would be of interest to combine it with genes from other sources that confer higher levels of resistance. Among the reported TYLCV resistance sources, *S. chilense* LA1969 shows the highest levels (2,18,24). The resistance mechanism has been characterized in plant materials derived from LA1969 (12). Resistance in this accession is controlled by one major incompletely dominant gene, *Ty-1*, with at least two modifier genes (25). The *Ty-1* gene has been mapped on chromosome 6 (25). A CAPS marker tightly linked to this gene has also been reported (16), which is essential in breeding programs aimed at the introgression of multiple resistance genes in the same plant material.

We have developed different crosses involving lines with *S. pimpinellifolium* UPV16991 and *S. chilense* LA1969-derived resistance. The objective of the work here reported was to evaluate the level of resistance in these plant materials, which included hybrids combining resistance from both sources.

MATERIALS AND METHODS

Plant material. The resistant plant materials used were seven lines with resistance derived from either *S. chilense* LA1969 or *S. pimpinellifolium* UPV16991 (Table 1). The two *S. chilense*-derived resistant lines were the BC_7S_1 generations from an initial NE- 1 x LA3473 cross; NE-1 is a susceptible breeding line developed at the Institute for the Conservation and Improvement of Agrodiversity (COMAV, Spain) and LA3473 is homozygous for the *Ty-1* allele, and was kindly provided by the Tomato Genetics Resource Center (TGRC, USA). Out of the BC_7S_1 plants, those that were homozygous for *Ty-1* were selected. Partial resistance to *Tomato yellow leaf curl Sardinia virus* (TYLCSV) was first described by our group in *S. pimpinellifolium* UPV16991 (19). This accession was collected in Piura (Peru) in an expedition of the Institute for the Conservation and Improvement of Agrodiversity. A breeding program for resistance to TYLCSV and

TYLCV was then initiated. The five *S. pimpinellifolium*-derived resistant lines had been previously developed from an initial cross between the susceptible *S. lycopersicum* NE-1 and *S. pimpinellifolium* UPV16991. The F_1 generation was selfed, and descendants were evaluated for resistance to TYLCV and TYLCSV. The breeding program was continued with three plants (L102, L54 and L48) selected from the F_2 generation. After 5-6 selfing generations, several advanced lines were obtained from each of these plants. Five of these lines were used in this assay. The susceptible parents used were the old Fortuna C variety (hereafter FC) and three susceptible breeding lines developed at the COMAV: NE-1, P-73 and V-5-7. The crosses developed include: *Ty-1* lines x susceptible, UPV16991 lines x susceptible (or reciprocal), *Ty-1* lines x UPV16991 lines, and UPV16991 lines x UPV16991 lines. The crosses are listed in Table 1. Eight plants of each of these crosses were included in the assay, as well as eight plants of each of the resistant lines and the susceptible control.

Inoculation techniques. Whitefly-mediated inoculation was used. The Spanish TYLCV isolate TYLCV-Mld[ES7297] (accession No. AF071228) was maintained in FC tomato plants by whitefly transmission. Infected plants and viruliferous whiteflies remained in muslin-covered cages inside a growth chamber. 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). Plants to be inoculated were grown in pots in an insect-proof greenhouse with controlled light and temperature. Eight plants per genotype were caged with at least 20 whiteflies per plant at the three true leaf stage for seven days. Plants were shaken daily to ensure that the distribution of the whiteflies was uniform. After the inoculation period, plants were sprayed with the insecticide imidacloprid and transferred to a new insect-proof cage for two days to avoid the spreading of whiteflies. Subsequently, plants were sprayed again with the insecticide, then 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 (14).

Experimental design. Plants for inoculation were divided into 8 trays. One plant of each of the genotypes assayed was included in each of the trays. Plants were distributed randomly in each tray. After inoculation, plants were transplanted to bigger pots in a greenhouse to the end of the assay. The greenhouse was divided into two plots. Plants of each genotype were grouped in blocks of two plants and distributed randomly in the

different plots. Conditions in the greenhouse were: 25-18ºC temperature (day/night), 80% relative humidity and 16/8 (light/dark) photoperiod.

Disease assessment. Symptoms and viral accumulation were assessed at 15, 25, 35, 45 and 60 days post-inoculation (dpi). Symptoms were evaluated following the scale developed by Friedmann et al. (4): 0 (no visible symptoms, inoculated plants show same growth and development as uninoculated plants), 1 (very slight yellowing of leaflet margins on apical leaf), 2 (some yellowing and minor curling of leaflet ends), 3 (a wide range of leaf yellowing, curling and cupping, with some reduction in size, yet plants continue to develop) and 4 (very severe plant stunting and yellowing, 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. Viral accumulation was assessed by molecular hybridization. DNA was extracted following the procedure described by Crespi et al. (1) with some modifications. Approximately 150 mg of tissue from the upper canopy of the plant were taken at each date. The frozen tissue was crushed in 500 µL of extraction buffer (100 mM Tris-HCl [pH 8], 50 mM EDTA, 500 mM NaCl, 10 mM 2-β-mercaptoethanol and 1% sodium dodecyl sulfate) and incubated at 65ºC for 5 min. After addition of 150 µl of 5 M potassium acetate, samples were incubated on ice for 10 min. Later samples were centrifuged at 16110 x *g* and the supernatant was recovered. Precipitation was carried out with isopropanol by centrifugation at 16110 x *g* for 5 min, and DNA was then resuspended in 77 µl of distilled water. One µl of each sample and a ten-fold dilution of the sample were denatured with 30 mM NaOH and 1 mM EDTA for 30 min and then blotted on to positively charged nylon membranes for hybridization. DNA was fixed by UV crosslinking. The probe used (kindly supplied by E.R. Bejarano, Universidad de Málaga, Spain) contained the intergenic region of the TYLCV-Mld[ES7297] isolate and was digoxygenin labelled by 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 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 30 min (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 25 ng to 1 pg). Total plant DNA extracted was also quantified to relate viral amounts detected to plant DNA present at each sample.

Extracted plant DNA was quantified by fluorimetry (Hoefer DyNA Quant 200 fluorimeter, according to manufacturer's instructions) in order to relate virus concentration to plant DNA present in each sample.

RESULTS

Parental lines

Plants of the susceptible control showed the first symptoms at 15 dpi. At 60 dpi, 100% of the plants exhibited severe symptoms (Fig. 1). For lines with *S. chilense*-derived resistance, only 40% of the plants showed some symptoms at some sampling dates. These symptoms were never scored over 1, and in almost all cases the plants recovered completely by 60 dpi. Variable responses were observed for *S. pimpinellifolium*-derived resistant lines. Generally, plants descendant from the L102 and L48-1 lines either remained asymptomatic or had slight symptoms. Only plants of one of the lines descendant from L102 displayed symptoms scored over 1.5 at 35 dpi, but these recovered by the end of the assay. One plant from a line descendant from L48 had symptoms scored higher than 2 from 35 dpi until the end of the assay. L54 derived lines behaved differently. Plants descendant from L54-2 remained symptomless. However, plants derived from L54-1 had symptom severity scores greater than 2 at 45 dpi. Symptoms in lines descendant from L54-1 were more severe later, although they were systematically lower than in the susceptible control FC and in no case did plants cease to grow.

Viral DNA was detected in all plants assayed (Fig. 1). Viral accumulation followed a similar pattern in most of the resistant genotypes studied with maximum accumulation occurring at 25 dpi. The susceptible FC control in which the maximum viral accumulation was detected at 35 dpi.

Both *S. chilense*-derived lines accumulated similar low amounts of virus. Viral accumulation in lines derived from *S. pimpinellifolium* was variable depending on the genotype. Lines descendant from L102 and L48 showed similar levels of viral accumulation, higher than those found in *S. chilense*-derived lines. Differences were found in lines descendant from L54. L54-2 registered the lowest viral accumulation among these lines, not only those *S. pimpinellifolium*-derived but also those derived from *S. chilense*. In

addition, viral accumulation was delayed in L54-2, since the virus was first detected at 35 dpi. However, L54-1, which showed the most severe symptoms, accumulated a significantly higher amount of virus at 25 dpi, although from 35 dpi viral accumulation was similar to that detected in the resistant lines.

***Ty-1* line x susceptible crosses**

Descendants from both crosses involving resistant *S. chilense*-derived lines with FC responded in a similar way. Approximately 30% of the plants remained symptomless throughout the entire assay. Some of the other plants started showing symptoms at 15 dpi (Fig. 2). At 35 dpi the most severe symptoms were observed, ranging from 1 to 3 on the scale used. From that date on, plants recovered and symptoms decreased. At the end of the assay, 70% and 60% of the plants from crosses with Ch-1 and Ch-2, respectively, showed no symptoms, and only 10% of plants showed symptoms scored over 2. For both genotypes and for all sampling dates, the average symptom score was considerably lower than the score for the susceptible parent and the mid-parent value and higher than the score for the resistant parent.

The timing of viral accumulation in heterozygote plants involving *S. chilense*-derived lines followed the aforementioned prevalent tendency (Fig. 2). Viral accumulation in the FC x Ch-1 cross was similar to the accumulation found in the resistant parent, Ch-1. However, the amount of viral DNA detected in plants of the FC x Ch-2 cross was higher than the viral accumulation in Ch-2.

UPV16991 line x susceptible crosses

Responses in crosses between susceptible genotypes and lines with *S. pimpinellifolium*-derived resistance depended on both the susceptible and resistant parents, as well as the interaction between them. The highest symptoms were observed at 60 dpi. The percentage of symptomatic plants at 60 dpi varied depending on the hybrid considered, ranging from 40 to 100%. In all hybrids, some of the plants showed severe symptoms, with percentages that were also variable. The only tendency to be highlighted was the fact that in crosses with FC, the percentage of plants displaying severe symptoms was always higher with respect to mild symptoms (<2). The average symptom score in the hybrids with L102-1, and L102-3 was always similar or higher than in the resistant parent, (Fig. 3). In the hybrids with L54-2 symptoms were higher than in the resistant parent. Otherwise, symptoms were lower in the hybrids than the mid-parent value, except for the FC x L54-1 cross. The mid-parent value was calculated based on symptoms in FC, which was the susceptible control included in the assay. Our experience in previous work showed that

symptom development in NE-1, V-5-7 and P-73 is comparable to the symptoms in FC. Unexpectedly, the best response was obtained in three of the hybrids involving the parent line which showed the lowest levels of resistance (L54-1).

Viral accumulation in crosses involving L102-1 was similar or higher than viral accumulation in the resistant parent (Fig. 3). Hybrids with L102-3 accumulated similar amounts of virus than the resistant parent. In a way similar to symptom expression, viral accumulation in hybrids with L54-1 was significantly lower than that found in the *S. pimpinellifolium*-derived parent. However, in crosses with L54-2, viral accumulation was higher than accumulation in the resistant parent.

UPV16991 line x UPV16991 line

The average symptom score was similar in all the crosses involving two lines with resistance derived from *S. pimpinellifolium* (Fig. 4) but the response was different in the three hybrids. Most of the plants from the L48-1 x L54-1 cross showed symptoms, but they showed mild symptoms. However, only some of the plants from L102-1 x L48-1 and L102-1 x L54-1 crosses showed more severe symptoms. For all the hybrids, there was a delay in time of symptom development with respect to both parents; the first symptoms in plants of the three hybrids appeared at 35 dpi. In both crosses with L54-1, the hybrids showed a response similar to that of the other, more resistant parent. Apart from that, the average symptom score was significantly lower than the mid-parent value. Plants from the hybrid L102-1 x L48-1 showed a response similar to that of both parents.

Response of the hybrids with respect to viral accumulation was comparable to that described for symptom development (Fig. 4).

***Ty-1* line x UPV16991 line crosses**

The average symptom score for all hybrids between *S. chilense*-derived and *S. pimpinellifolium*-derived resistant lines was under 1 for all sampling dates (Fig. 5). The first symptoms appeared at 25 or 35 dpi. At the end of the assay, the average was under 1 in all cases. Individual scores were also no higher than 1.5 for most of the hybrid plants. The exceptions were hybrids involving L54-1 and hybrid Ch-2 x L102-3, in which between 10 and 20% of the plants were scored 2 or 2.5 at some date. The average symptom score in hybrids was lower than the score for plants heterozygous for the resistance derived from *S. pimpinellifolium*. It was also lower than the symptom score in *S. pimpinellifolium* homozygotes, with the only exception being crosses with L54-2, which did not exceed the resistance found in the *S. pimpinellifolium*-derived parent. Compared with response found in *S. chilense*-derived resistance in heterozygous, in the hybrids, there was a delay in time

of symptom development. In crosses Ch-1 x L102-1, Ch-1 x L102-2, Ch-1 x L102-3 and Ch-2 x L102-2, symptom score in the hybrids was significantly lower than in those heterozygous for *Ty-1* at all sampling dates. For the rest of the hybrids, the symptom score was also lower than in *Ty-1* heterozygotes throughout the assay, although at 60 dpi, the symptom score was either very similar or higher in some cases. Plants of the Ch-2 x L102-2 hybrids, which remained symptomless throughout the assay, were even better than both lines homozygous for *Ty-1*.

Viral accumulation peaked for most of the crosses at 25 dpi (Fig. 5). Levels of virus accumulated in both crosses with L54-1 were high, similar to the amounts detected in L54-1. Viral accumulation was also high in crosses between Ch-1 and L102-2 and L102-3. Accumulation in the rest of the hybrids was significantly lower. In most of the hybrids, viral accumulation was lower than viral accumulation in respective heterozygotes for the resistance genes derived from *S. chilense* and *S. pimpinellifolium*.

DISCUSSION

Despite the efforts undertaken by different research groups since TYLCD was first reported, no immune commercial materials are available to date. Therefore, it is of interest to make use of all promising sources of resistance. Moreover, strategies such as combining resistance genes from different sources have already been used to achieve higher levels of resistance and make it more durable. Our work shows that *S. pimpinellifolium* UPV16991-derived resistance is useful when combined with the *Ty-1* gene.

S. chilense has been reported as the best source of resistance to TYLCD when compared with resistance found in other wild tomato relatives (20,24). Accordingly, accession LA1969 was reported as highly resistant since plants remained symptomless and viral DNA was rarely detected (24). The *Ty-1* gene is the major gene controlling resistance in this accession (25). This gene, when studied in a tomato background, showed incomplete dominance, and even homozygous plants developed mild disease symptoms (6,25). In other works, plants homozygous for *Ty-1* have been described as asymptomatic (5,12) and not as carriers of viral DNA (12) when inoculated with different viral species causing TYLCD. In our work, 40% of the plants in both lines tested showed slight symptoms at some sampling date, though at 60 dpi plants recovered and were symptomless. Also, viral DNA was detected systematically in plants of these lines, but at very low levels. Discrepancies found in different assays must be due to differences either in inoculation or

detection procedures, or in the background in which the resistance gene is expressed. In any case, in our work, resistance found in lines homozygous for *Ty-1* was higher than that found in most of the *S. pimpinellifolium*-derived lines, with respect to symptom development and levels of viral accumulation. Only L54-2 displayed milder symptoms and accumulated lower amounts of viral DNA than did Ch-1 and Ch-2.

The response in lines with *S. pimpinellifolium* derived resistance was variable. Lines descendant from L102 and L48 showed similar results with respect to symptom development and viral accumulation: most of the plants showed slight symptoms and viral accumulation was approximately half that detected in the susceptible control. These results are also consistent with the mode of gene action previously proposed for resistance in line L102 (15). Progeny from L54 behaved differently. L54-1, showed only low levels of resistance under the inoculum presure of this assay. On the other hand, L54-2 displayed a higher level of resistance than *S. chilense*-derived lines. It must be highlighted that all L54-2 plants were resistant, so the gene or genes controlling resistance in this line must have complete penetrance, unlike what was stated previously regarding the resistance gene in L102 (15). These results suggest that the gene or genes controlling resistance in L54-2 could differ from the reported gene in L102. Kasrawi and Mansour (7) also found variability when comparing several lines derived from *S. pimpinellifolium* LA121, in which the average symptom score ranged from 0.25 to 2.07 on a scale from 0 (symptomless) to 4 (severe symptoms).

The *Ty-1* gene was first described as incompletely dominant since resistance in heterozygotes was slightly lower than in homozygotes (25). Again, differences were observed in different assays, since some authors described *Ty-1* as dominant (5). In addition, resistance in some commercial hybrids is based on plants heterozygous for *Ty-1*, and most of these hybrids show high resistance to TYLCD (personal communication from breeders). Heterozygotes for *Ty-1* tested in our assay displayed more severe symptoms than homozygotes, thus supporting the incomplete dominance of the *Ty-1* gene. However, differences were observed in both hybrids. Plants of FC x Ch-1 accumulated amounts of virus comparable to the accumulation in the resistant parent, Ch-1. However, in the FC x Ch-2 hybrid, accumulation was significantly higher than in the resistant parent. These results highlight the importance of the genetic background in gene expression.

This was also true in heterozygous plants for *S. pimpinellifolium*-derived resistance. Values for symptom score and viral accumulation were, with exceptions, between those of the *S. pimpinellifolium* and *S. lycopersicum* parents, but there was variability depending on both

the susceptible and the resistant parent. There was also an important effect caused by the interaction between both parents, given that the highest levels of resistance were not obtained for hybrids involving the most resistant parent. Kasrawi and Mansour (7) found similar results when evaluating hybrids between lines with resistance derived from different *S. pimpinellifolium* accessions and the susceptible cultivar 'Special Back': the response in the hybrids did not always correlate to the response in the resistant parent. In our work, the most unexpected result was the response in hybrids involving L54-1, the least resistant line derived from *S. pimpinellifolium*. Three of these hybrids showed high levels of resistance, despite the fact that L54-1 showed only low levels of resistance.. One possible explanation is that resistance is better expressed in the hybrids due to genetic background effects.

Crosses between different *S. pimpinellifolium*-derived resistant lines did not show higher levels of resistance than each parent, as would be expected if different genes controlled resistance in the three lines. The response in UPV16991-derived lines suggested that the genetic control of the resistance in L54 differed from the model proposed for L102. Unfortunately, the line descendant from L54 that was crossed with the L48 and L102 lines showed only low levels of resistance, so no conclusions could be made from these crosses. Plants from the L102-1xL48-1 cross showed a response similar to that of both parents, supporting the hypothesis of a genetic control of the resistance common to both lines.

The most relevant results of this work involve the response in the hybrids between *S. pimpinellifolium* and *S. chilense*-derived resistant lines. As expected, these hybrids exhibited higher levels of resistance than heterozygotes for *S. pimpinellifolium*-derived resistance, given that they displayed significantly lower symptoms and accumulated either similar or lower viral DNA amounts. The only exceptions were hybrids with L54-1, in which viral accumulation was higher than in heterozygotes for *S. pimpinellifolium*-derived resistance. When compared with heterozygotes for *Ty-1*, the hybrids showed a delay in time of symptom development. In addition, the symptom score in the hybrids was generally lower than in heterozygotes for *Ty-1*, with some exceptions at 60 dpi. It is also remarkable that symptoms were mild in all of the hybrid plants. These results contrast with results in heterozygotes for both *S. pimpinellifolium* and *S. chilense*-derived resistance, in which, at 60 dpi or beforehand, some of the plants showed severe symptoms (≥2). Moreover, all plants of the hybrid Ch-2 x L102-2 remained symptomless and accumulated lower amounts of viral DNA, thus exceeding even the resistance level in lines homozygous for the *Ty-1* gene. The possibility of achieving high levels of resistance by combining *S.*

chilense and *S. pimpinellifolium*-derived resistance has already been reported by Vidavsky et al. (23). Similarly to our results, they found that even if the *S. pimpinellifolium* parent had intermediate levels of resistance, when crossed with a highly resistant line derived from *S. chilense*, highly resistant descendants resulted.

So, resistance derived from *S. pimpinellifolium* UPV16991 is useful when combined with the *Ty-1* gene in the development of tomato hybrids. This is the most practical approach to exploiting the resistance derived from UPV16991. Currently, the tomato market is mainly based on hybrid varieties (22). Furthermore, preferred resistance genes are those which there is no need to fix in both parents (21).

The *S. pimpinellifolium*-derived lines available are consecutive selfings from the initial *S. lycopersicum* NE-1 x *S. pimpinellifolium* UPV16991 cross and they have still too much *S. pimpinellifolium* background. Future work will include the introgression of the resistance gene into *S. lycopersicum* backgrounds. For this purpose, it would be advisable to identify a molecular marker linked to the resistance gene derived from UPV16991, in order to accelerate the breeding program. Once advanced lines which incorporate the UPV16991-derived resistance are achieved, crosses of these lines with homozygous lines for *Ty-1* should be developed in order to select those with higher levels of resistance. Finally, it would be of interest to further characterize the high resistance found in L54-2, in order to confirm whether it differs from the resistance previously reported in L102.

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Table 1. Plant materials analyzed, including the crosses developed.

Susceptible *S. lycopersicum* variety
Fortuna C (FC)
Ty-1 lines[1] (*S. chilense*-derived)
Ch-1
Ch-2
UPV16991 lines[2] (*S. pimpinellifolium*-derived)
L48
L54-1
L54-2
L 102-1
L 102-2
Ty-1 line x UPV16991 line crosses
Ch-1 x L102-1
Ch-1 x L102-2
Ch-1 x L102-3
Ch-1 x L54-1
Ch-1 x L54-2
Ch-2 x L102-2
Ch-2 x L54-1
Ch-2 x L54-2
Ch-2 x L102-3
UPV16991 line x UPV16991 line crosses
L 48 x L54-1
L 102-1 x L48
L 102-1 x L54-1

Susceptible x *Ty-1* line crosses
FC x Ch-1
FC x Ch-2
UPV16991 line x susceptible (or reciprocal) crosses
L102-1 x NE-1[3]
L102-1 x V-5-7[3]
L102-1 x P-73[3]
L102-1 x FC
L102-3 x NE-1
V-5-7 x L102-3
P- 73 x L102-3
FC x L102-3
L54-1 x NE-1
L54-1 x V-5-7
P- 73 x L54-1
FC x L54-1
L54-2 x NE-1
L54-2 x V-5-7
L54-2 x P- 73
FC x L54-2

[1] Lines homozygous for the *Ty-1* gene, selected among plants of the BC_7S_1 generation from the cross NE-1 x LA3473

[2] F_5-F_6 generations from the initial NE-1 x *S. pimpinellifolium* UPV16991 cross

[3] Susceptible breeding lines developed at the Institute for the Conservation and Improvement of Agrodiversity (COMAV)

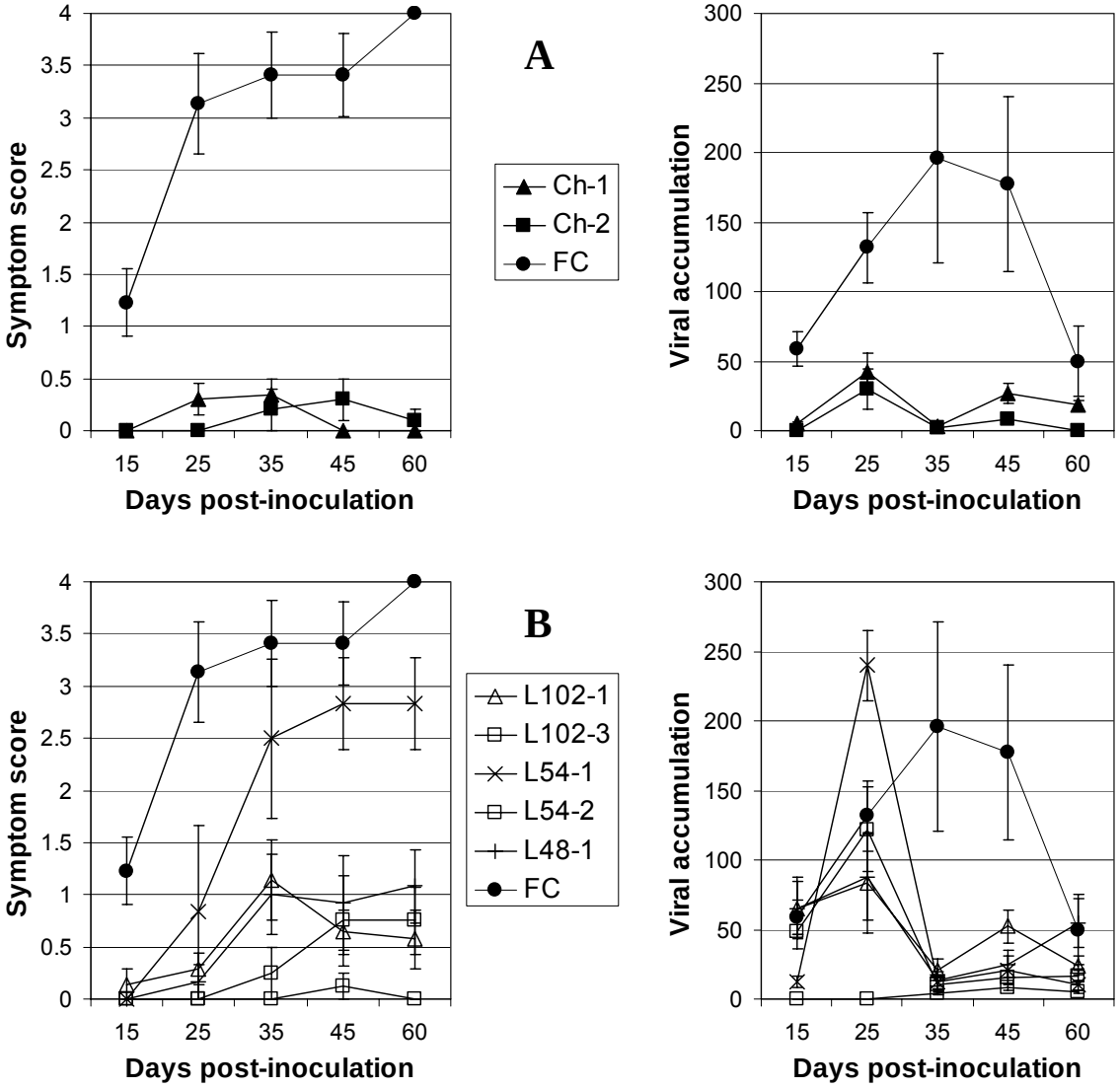

Fig.1. Average symptom score (left) and viral accumulation (right) in the resistant lines derived from *Solanum chilense* (A) and *Solanum pimpinellifolium* (B) and the susceptible control (FC), after whitefly-mediated inoculation with *Tomato yellow leaf curl virus*. Symptom score was evaluated using a scale from 0 (symptomless) to 4 (severe symptoms). Viral accumulation is expressed in ng viral DNA/μg total DNA extracted. Error bars represent standard error.

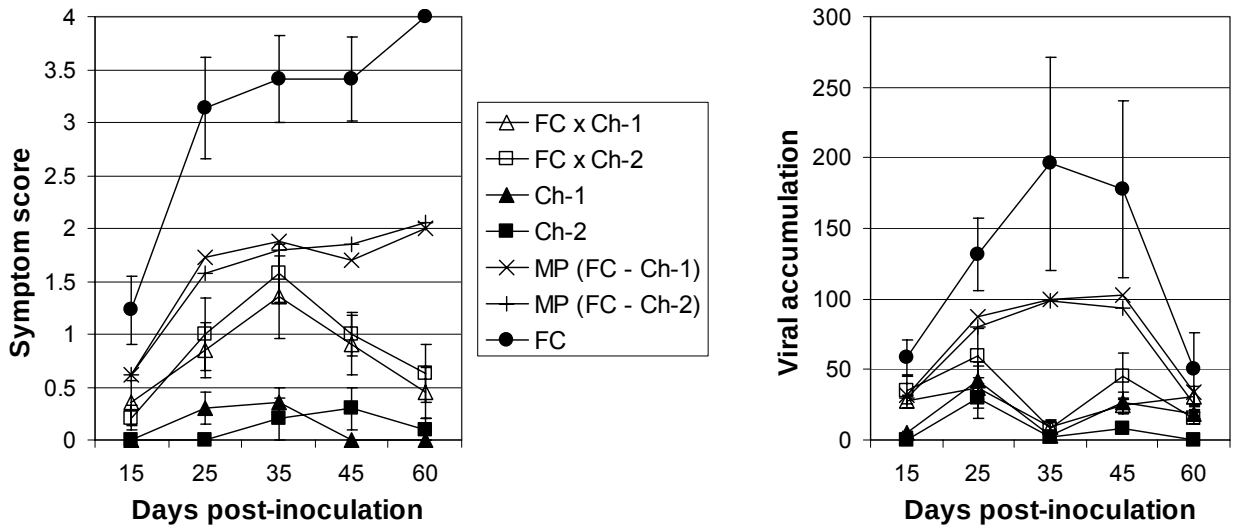

Fig.2. Average symptom score (left) and viral accumulation (right) in *Ty-1* line x susceptible crosses, after whitefly-mediated inoculation with *Tomato yellow leaf curl virus*. *Ty-1* lines, the susceptible control and the mid-parent (MP) value of each cross are included to facilitate comparisons. Symptom score was evaluated using a scale from 0 (symptomless) to 4 (severe symptoms). Viral accumulation is expressed in ng viral DNA/μg total DNA extracted. Error bars represent standard error.

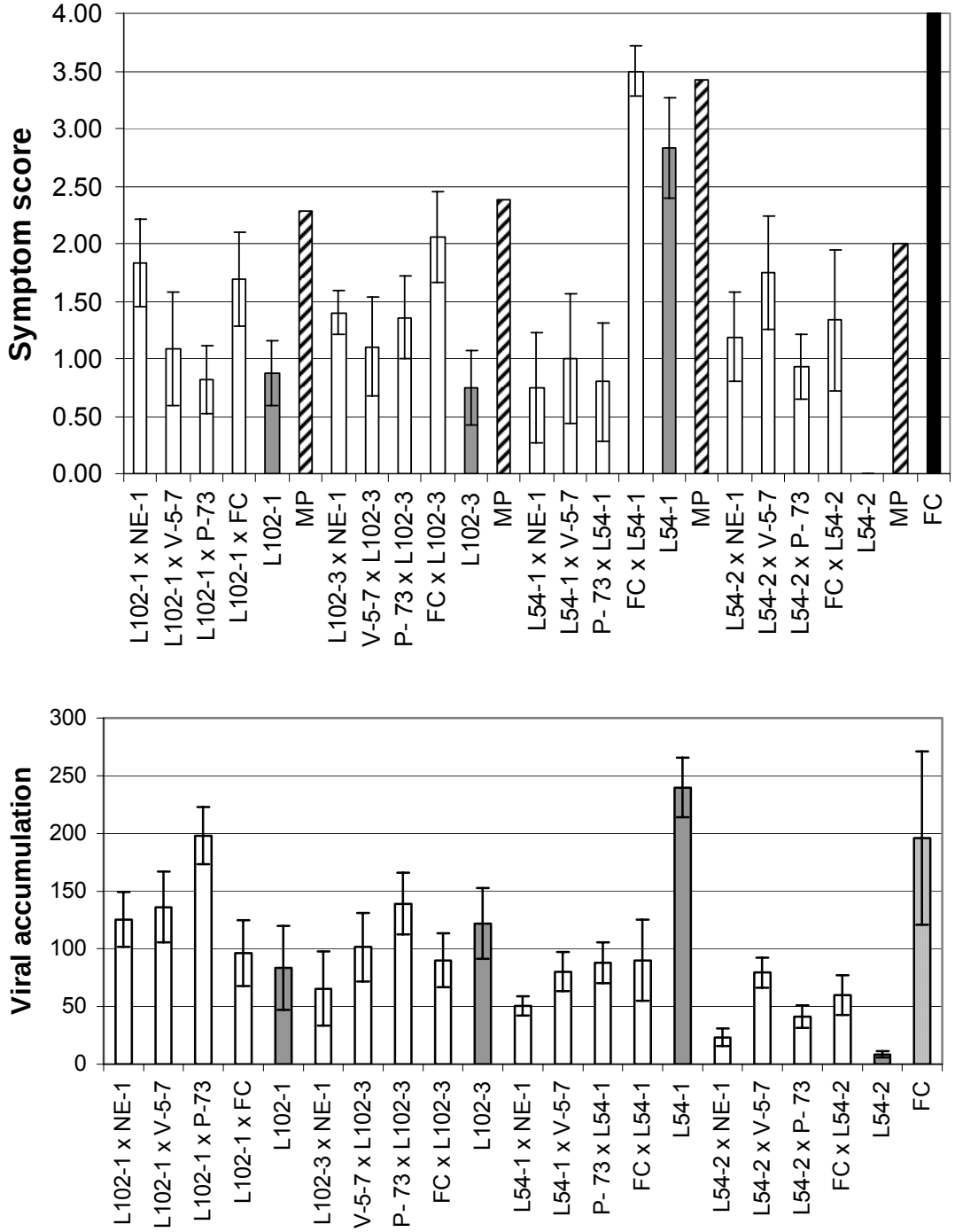

Fig.3. Average symptom score at 60 days post-inoculation (upper) and maximum viral accumulation (lower) in UPV16991 line x susceptible crosses, after whitefly-mediated inoculation with *Tomato yellow leaf curl virus*. UPV16991 lines, the susceptible control and the mid-parent (MP) value of each cross (for symptom score, and based on symptom development in the susceptible control, FC) are included to facilitate comparisons. Symptom score was evaluated using a scale from 0 (symptomless) to 4 (severe symptoms). Viral accumulation is expressed in ng viral DNA/μg total DNA extracted. Error bars represent standard error.

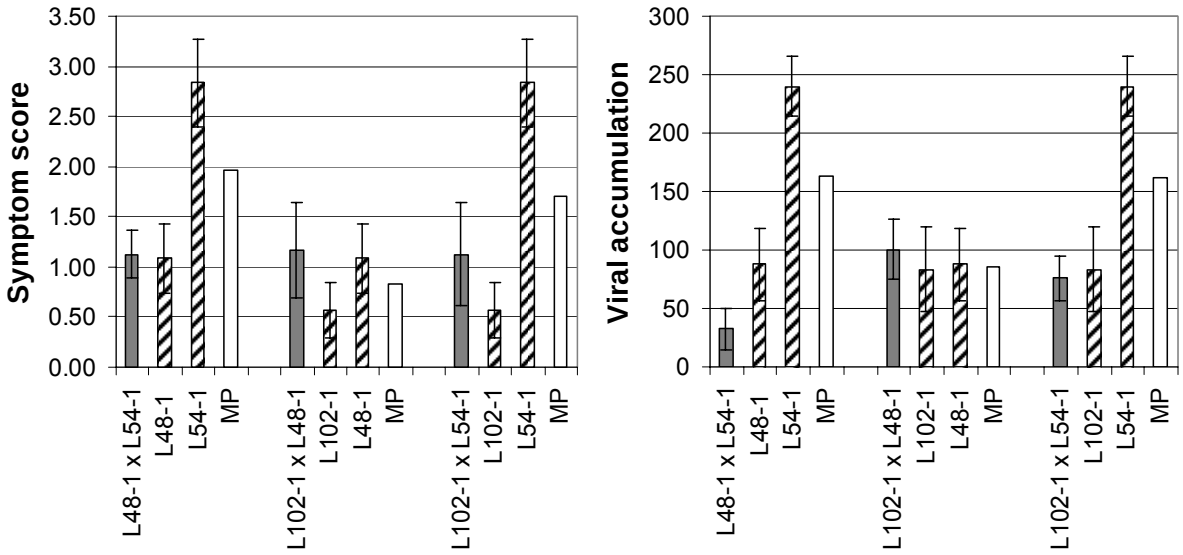

Fig.4. Average symptom score at 60 days post-inoculation (left) and maximum viral accumulation (right) in UPV16991 line x UPV16991 line crosses, after whitefly-mediated inoculation with *Tomato yellow leaf curl virus*. UPV16991 lines, and the mid-parent (MP) value of each cross are included to facilitate comparisons. Symptom score was evaluated using a scale from 0 (symptomless) to 4 (severe symptoms). Viral accumulation is expressed in ng viral DNA/µg total DNA extracted. Error bars represent standard error.

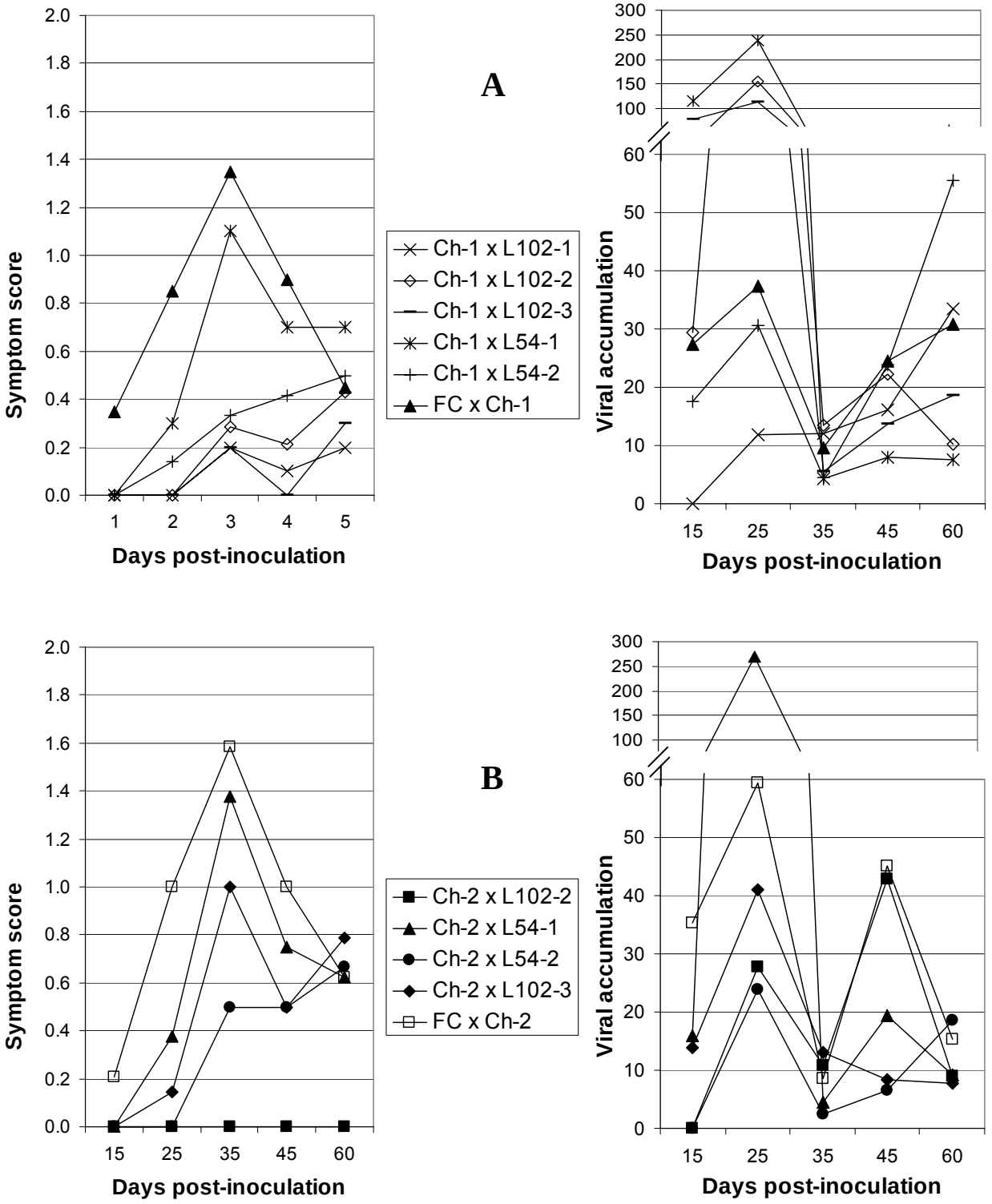

Fig.5. Average symptom score (left) and viral accumulation (right) in Ch-1 x UPV16991 line crosses (A) and Ch-2 x UPV16991 line crosses (B), after whitefly-mediated inoculation with *Tomato yellow leaf curl virus*. Heterozygotes for the *Ty-1* gene are included to facilitate comparisons. Symptom score was evaluated using a scale from 0 (symptomless) to 4 (severe symptoms). Viral accumulation is expressed in ng viral DNA/μg total DNA extracted.

3.4. Identification of a CAPS marker tightly linked to the Tomato yellow leaf curl disease resistance gene *Ty-1* in tomato

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FULL RESEARCH PAPER

Identification of a CAPS marker tightly linked to the Tomato yellow leaf curl disease resistance gene *Ty-1* in tomato

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Abstract During the process of breeding programmes, several resistance genes have been introgressed into tomato (*Solanum lycopersicum*) cultivars from different wild tomato relatives. A number of these resistance genes have been mapped to chromosome 6. Among them, *Ty-1* and *Mi*, which confer resistance to Tomato yellow leaf curl disease and to *Meloidogyne* spp., respectively, are in most cases incorporated in commercial hybrids. Several molecular markers tightly linked to *Mi* have been identified. This study was conducted in order to find an informative molecular marker linked to *Ty-1*. Six markers mapped in the same region as *Ty-1* were analysed in plant material carrying different combinations of *Ty-1* and *Mi* alleles. Three of the six markers revealed polymorphism among the assayed accessions. One allele of JB-1 marker showed association with *Ty-1*. Furthermore, the presence of *Mi* did not interfere with the results. The analysis of several accessions of wild tomato relatives with the three polymorphic markers allowed the establishment of the origin of the alleles found in cultivated plant material, showing that introgressions from *S. lycopersicum, S. pimpinellifolium* and *S. habrochaites* will not interfere with the results of this marker which tags *Ty-1*. Furthermore this analysis enabled the location of CT21, the RFLP marker from which JB-1 was designed.

Keywords Marker-assisted selection · *Mi* gene · *Solanum lycopersicum* · TYLCD

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Introduction

Tomato yellow leaf curl disease (TYLCD) causes important yield losses in tomato (*Solanum lycopersicum*) crops all over the world (Picó, Díez, & Nuez, 1996; Pilowsky & Cohen, 2000). This disease is caused by different viral species, all members of the genus *Begomovirus* (family Geminiviridae). Nine species from different geographical areas have been described and five more are considered tentative species (Fauquet & Stanley, 2005). Resistance to this disease has been identified in some wild tomato relatives such as *Solanum pimpinellifolium*, *Solanum habrochaites*, *Solanum peruvianum* and *Solanum chilense* (reviewed in Laterrot, 1992; Picó, Díez, & Nuez, 1996; Picó et al., 1999; Pilowsky & Cohen, 2000). The genetic basis of the resistance, which depends on the species, ranges from a single incompletely-dominant gene to a polygenic recessive pattern (Lapidot & Friedmann, 2000).

The resistance derived from accession LA1969 of *S. chilense*, has been thoroughly studied by Zamir et al. (1994); a major incompletely dominant gene, *Ty-1*, and two or more modifier genes control the resistance to TYLCD in this accession. The major gene, *Ty-1*, maps to chromosome 6, concretely to the region around markers TG297 and TG97. The resistance found in LA1969 has been introgressed into the cultivated species and several lines partially resistant to TYLCD have been developed (Laterrot, 1995; Michelson, Zamir, & Czosnek, 1994; Zamir et al., 1994). Most of the commercial cultivars resistant to TYLCD carry *Ty-1* gene. The availability of molecular markers tightly linked to this gene would allow the screening of putative resistant genotypes without inoculation with the pathogen, thereby shortening the length of breeding programmes. To date it has not been reported a concrete marker always linked to the presence of *Ty-1* gene. Several resistance genes have been mapped in tomato chromosome 6, apart from *Ty-1*, (reviewed in Zhang, Khan, Niño-Liu, & Foolad, 2002). The region around TG297 and TG97 markers holds some of these genes, i.e., *Mi*, *Cf-2* and *Cf-5* and *Ty-1*. This region has been studied in detail, initially with the purpose of cloning the *Mi* gene (Kaloshian et al., 1998; Liharska, Hontelez, van Kammen, Zabel, & Koornneef, 1997; Messeguer et al., 1991; van Daelen et al., 1993; van Wordragen et al., 1994).

Mi is a single dominant gene which confers resistance to the main species of the genus *Meloidogyne* (Gilbert, 1958). During the 1940s, this gene was transferred to cultivated tomato from *S. peruvianum* PI128657 (Bailey, 1941). Just one F_1 plant, derived from accession PI128657, was obtained (Smith, 1944). Breeding programmes were thus continued with this single plant. However, these programmes were developed from different backcrosses of the F_1 plant to *S. lycopersicum* at the Hawaiian Experimental Station (HES) and also at the University of California (Davis). The result was the release of the first resistant cultivars: VFN8 in California and Anahu and other HES lines in Hawaii. So, commercially available resistant plant materials are derived from one of these two original cultivars (Medina-Filho & Tanksley, 1983).

In *S. lycopersicum* plant material with introgressions for the region containing *Mi*, recombination is severely suppressed (Ho et al., 1992; Liharska, Koornneef, van Wordragen, van Kammen, & Zabel, 1996; Messeguer et al., 1991). As a consequence, tomato plants containing *Mi* retain a large introgression of *S. peruvianum*, although variable in length among cultivars (Messeguer et al., 1991). In order to facilitate breeding programmes, several molecular markers tightly linked to *Mi* have been identified, such as the acid phosphatase-1 (*Aps-1*) gene (Rick & Fobes, 1974) as well as RFLP (Ho et al., 1992; Klein-Lankhorst et al., 1991a; Messeguer et al., 1991) and RAPD (Klein-Lankhorst, Vermunt, Weide, Liharska, & Zabel, 1991b) markers. Even more specific PCR-based markers have also been developed (Kaloshian et al., 1998; Williamson, Ho, Wu, Miller, & Kaloshian, 1994), which are more suitable for routine analysis.

The purpose of the research reported here was to develop a molecular marker tightly linked to the resistance gene *Ty-1*. As has already been stated, the region containing *Ty-1* and *Mi* is genetically very short. It is very feasible that repression of recombination occurs also for introgressions from *S. chilense*, so it is likely that large amounts of *S. chilense* DNA are kept in resistant plant material derived from LA1969. Therefore, the molecular markers linked to *Mi* could be useful as markers for *Ty-1*, if alleles from *S. peruvianum* and *S. chilense* differed for these markers. On the other hand, if the alleles from these two species were the same for these markers, their use could lead to false positive results.

We have assayed different plant material for some of the markers that are tightly linked to *Mi* and, in addition, some other markers previously described for this region of chromosome 6. We describe a marker with one allele tightly associated with *Ty-1*.

Materials and methods

Plant material

Plant materials employed in this study along with their sources and main characteristics are listed in

Table 1 Plant material analysed

Species	Accession	*Mi*[a]	*Ty-1*[b]	Aps-1[c]	REX-1[d]	JB-1[e]	Source[f]
S. lycopersicum	UPV21183	*mi/mi*	*ty-1/ty-1*	1	1	1	1
	UPV21745	*mi/mi*	*ty-1/ty-1*	1	1	1	1
	FC	*mi/mi*	*ty-1/ty-1*	1	1	1	1
	Gévora	*Mi/Mi*	*ty-1/ty-1*	1	2	1	2
	H1124	*Mi/Mi*	*ty-1/ty-1*	2	2	2	2
	Fitó 1	*Mi/Mi*	*ty-1/ty-1*	1	2	1	3
	Fitó 2	*Mi/Mi*	*ty-1/ty-1*	2	2	2	3
	Fitó 3	*mi/mi*	*Ty-1/Ty-1*	2	1	3	3
	Fitó 4	*Mi/Mi*	*Ty-1/Ty-1*	2	2	3	3
	SC	*mi/mi*	*Ty-1/Ty-1*	2	2	3	4
	Boludo	*Mi/mi*	*Ty-1/ty-1*	1/2[g]	1/2	3	5
	Anastasia	*Mi/mi*	*Ty-1/ty-1*	1/2	2	3	5
	TY197	*mi/mi*	*ty-1/ty-1*	1	1-2[h]	1	6
	LA3473	*mi/mi*	*Ty-1/Ty-1*	2	3	3	7
	UPV21008[i]	*mi/mi*	*ty-1/ty-1*	1	1	1	1
S. peruvianum	PI128657	*Mi/Mi*	*ty-1/ty-1*	2	2-2/3	3	8
	UPV20196	*mi/mi*	*ty-1/ty-1*	2	2	3	1
	UPV20340	*mi/mi*	*ty-1/ty-1*	2	2	3	1
	UPV20342	*mi/mi*	*ty-1/ty-1*	2	3	3	1
	UPV20345	*mi/mi*	*ty-1/ty-1*	2	2	3	1
S. chilense	LA1969	*mi/mi*	*Ty-1/Ty-1*	2	2	3	7
	LA2884	*mi/mi*	*ty-1/ty-1*	2	2	3	7
	UPV20304	*mi/mi*	*ty-1/ty-1*	2	2	3	1
	UPV20306	*mi/mi*	*ty-1/ty-1*	2	2	3	1
	UPV20310	*mi/mi*	*ty-1/ty-1*	2	2	3	1
	UPV20320	*mi/mi*	*ty-1/ty-1*	2	2	3	1
	UPV20328	*mi/mi*	*ty-1/ty-1*	2	2	3	1
	UPV20329	*mi/mi*	*ty-1/ty-1*	2	2	3	1
	UPV20336	*mi/mi*	*ty-1/ty-1*	2	2	3	1
S. habrochaites	LA0386	*mi/mi*	*ty-1/ty-1*	3	2	D_1^j	7
	LA1777	*mi/mi*	*ty-1/ty-1*	3	2	D_2	7
	UPV16910a	*mi/mi*	*ty-1/ty-1*	2	2	D_3	1
	UPV17046 E	*mi/mi*	*ty-1/ty-1*	2	2	D_4	1
S. pimpinellifolium	LA1636	*mi/mi*	*ty-1/ty-1*	2	1	2	7
	LA1670	*mi/mi*	*ty-1/ty-1*	2	1	2	7
	LA2182	*mi/mi*	*ty-1/ty-1*	2	1	1	7
	LA2188	*mi/mi*	*ty-1/ty-1*	2	1	1	7
	LA2725	*mi/mi*	*ty-1/ty-1*	2	1	2	7
	PI 390728	*mi/mi*	*ty-1/ty-1*	2	1	1	9
	PI 127807	*mi/mi*	*ty-1/ty-1*	2	1	2	9

[a] Alleles for the *Mi* gene: *Mi* resistant allele, *mi* susceptible allele

[b] Alleles for the *Ty-1* gene: *Ty-1* resistant allele, *ty-1* susceptible allele

[c] Alleles for the Aps-1 marker (see results for description)

[d] Alleles for the REX-1 marker (see results for description)

[e] Allele for the JB-1 marker (see results for description)

[f] Source: 1: Genebank of the Institute for the Conservation and Improvement of Agrodiversity (COMAV), Valencia, Spain; 2: J. Gragera, Sevicio de Investigación y Desarrollo Tecnológico, (SIDT) Badajoz, Spain; 3: Semillas Fitó S.A., Barcelona, Spain; 4: Plant material with this genetic composition belongs to a seed company; 5: Seminis Vegetable Seeds, Murcia, Spain; 6: Dr. M. Pilowsky, Volcani Center, Rehovot, Israel; 7: Tomato Genetics Resource Center (TGRC), University of California, Davis; 8: United States Department of Agriculture (USDA); 9: Australian Plant Genetic Resource Information Service (AusPGRIS), corresponding genebank codes are AUSTRCF311996 (PI 390728) and AUSTRCF312128 (PI127807)

[g] Bars separate alleles present in heterozygous individuals

[h] Hyphens separate different patterns for different individuals of a concrete plant material

[i] This accession was formerly classified as *L. esculentum* var. *cerasiforme*

[j] D: Alleles different than the ones described for the rest of the species

Table 1. Between five and seven plants per accession were analysed. UPV21183 and UPV21745 are local tomato varieties and FC is a breeding line; none of them should have introgressions from any wild species. Gevora and H1124 are breeding lines homozygous for *Mi* and susceptible to TYLCD. Fitó 1, 2, 3 and 4 are breeding lines homozygous for *Ty-1* and/or *Mi*. SC is a breeding line homozygous for *Ty-1*. Boludo and Anastasia are commercial hybrids which are heterozygous for *Ty-1* and *Mi*. TY197 is a breeding line with resistance to TYLCD derived from *S. peruvianum* (Lapidot et al., 1997). LA3473 is a breeding line with resistance to TYLCD derived from LA1969, so carrying *Ty-1* (Michelson et al., 1994). UPV21008 is an accession of *S. lycopersicum* (formerly *Lycopersicon esculentum* var. *cerasiforme*). PI128657 and LA1969 are the sources of *Mi* and *Ty-1*, respectively. The remaining accessions of wild species are either resistant or susceptible to TYLCD and *Meloidogyne*, but in no case do they carry *Ty-1* or *Mi*.

DNA extraction

Plant DNA used for analysis was extracted from 75 mg of fresh tissue, following the procedure described by Doyle & Doyle (1990) with some modifications.

Markers

Markers employed, primer sequences and the basis of their design are listed in Table 2.

Amplification and restriction conditions

The PCR reaction was carried out in a total volume of 25 µl containing: 1× buffer recommended by suppliers, 2.5 mM $MgCl_2$, 0.5 µM of each primer, 0.4 mM dNTPs, 1 U of *Taq* polymerase and 40 ng of template DNA. The amplification was carried out in an Eppendorf Martercycler Thermal Cycler with the following conditions (except for marker JB-1): 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by an extension step of 10 min at 72°C. For JB-1, the optimum conditions for amplification were: 20 cycles of 94°C for 10 s, 55°C for 30 s and 72°C for 70 s, 10 cycles of 94°C for 10 s, 53°C for 30 s and 72°C for 70 s, followed by an extension step of 10 min at 72°C. Restrictions of 10 µl of the amplified products were performed overnight, in a total volume of 25 µl with 5 U of the corresponding enzyme, using buffers recommended by the suppliers at the recommended temperature. Digestion products were analysed by agarose gel electrophoresis (2% agarose w/v with TBE 1× buffer) and visualized by ethidium bromide staining. All reagents employed were supplied by Roche Diagnostics (Manheim, Germany).

Results

Screening for markers linked to *Ty-1*

PCR amplification of DNA from tomato accessions (*S. lycopersicum* plant material in Table 1) and subsequent digestions, when possible, were

Table 2 Markers of the region of gene *Ty-1* assayed

Marker	Primer sequence	Design basis	Restriction enzymes
Aps-1	ApsF: 5′-GGCAGGAGAATATGCCAAAA-3′ ApsR: 5′-CGTTCCATTCTCAACCCATT-3′	Designed based on a genomic clone (Williamson & Colwell, 1991)	*Taq*I
REX-1	REX-F1: 5′-TCGGAGCCTTGGTCTGAATT-3′ REX-R3: 5′-ATGCCAGAGATGATTCGTGA-3′	Williamson et al. (1994)	*Taq*I
JB-1	JB1F: 5′-AACCATTATCCGGTTCACTC-3′ JB1R: 5′-TTTCCATTCCTTGTTTCTCTG-3′	Designed based on RFLP CT21[a]	*Taq*I
CT216	CT216F 5′-ATTCTCCGGCGAGCCAAATC-3′ CT216R 5′-TTGTCTTCTTCTTCTAGTCGAC-3′	Designed based on RFLP CT216[a]	*Taq*I and *Hinf*I
CT119	CT119F: 5′-TCAGGTATCGAACCAAAACC-3′ CT119R: 5′-TAAAAGGTTCATCCTAATAC-3′	Dixon et al. (1995)	–
GP79	GP79F: 5′-TGTTCTCTAGTATCTCATCC-3′ GP79R: 5′-GGATTGTGATGTCGAGTTGC-3′	Dixon et al. (1995)	*Taq*I and *Tru*9I

[a] Information about these markers can be found at: http://www.sgn.cornell.edu

carried out using the primers and enzymes listed in Table 2. Clear amplification products were obtained for all the markers tested, except for CT119. However, for markers CT216 and GP79, polymorphism was not detected with the restriction enzymes employed.

For Aps-1 marker, two different alleles appeared in *S. lycopersicum* plant material (Fig. 1a): both showed a common band of approximately 700 pb and differed in a band slightly smaller than 300 pb (allele 1) or slightly larger than 300 pb (allele 2). Both alleles were codominant. All *S. lycopersicum* plant material without introgressions from wild species that were tested in this assay showed allele 1, so this must be the allele that corresponds to the cultivated species. TY197 also showed the*S. lycopersicum* allele for the Aps-1 marker. Plant materials that were homozygous for *Mi* exhibited either allele 1 or allele 2 for this marker. It has already been reported that DNA fragments introgressed from *S. peruvianum* kept by plant materials with this gene are variable in length, depending on their origin. Generally, lines derived from VFN8 maintain the *S. peruvianum* allele for locus *Aps-1*, while in those derived from HES lines, recombination occurred between *Mi* and *Aps-1* and they have the *S. lycopersicum* allele for *Aps-1* (Messeguer et al., 1991). Lines which were homozygous for *Ty-1* showed allele 2 for the *Aps-1* marker. The commercial hybrids, which were heterozygous for *Ty-1* and *Mi*, showed the heterozygous pattern for the *Aps-1* marker.

For the REX-1 marker (Fig. 1b), three different alleles appeared in *S. lycopersicum* plant material. Two of these alleles were previously described by Williamson et al. (1994): allele 1 consisted of a band of 750 pb and allele 2 of two bands of approximately 570 and 160 pb. These two alleles were co-dominant. We found one more allele, allele 3, which presented three bands of 350, 220 and 160 pb. All plant material without introgressions from any wild species showed allele 1 and all lines homozygous for *Mi*, allele 2, as previously described by Williamson et al. (1994). TY197 presented alternatively alleles 1 and 2, i.e., some plants kept *S. lycopersicum* allele while others kept *S. peruvianum* allele. Boludo, heterozygous for *Mi*, showed both allele 1 and 2. However, Anastasia, which is also heterozygous for *Mi*, was homozygous for allele 2. In lines which were homozygous for *Ty-1* but which did not carry *Mi*, alleles 1, 2 and 3 appeared alternatively.

Three different alleles appeared for JB-1 marker (Fig. 1c). All three had a common band of approximately 400 pb, which was the only band present for allele 1; allele 2 also consisted

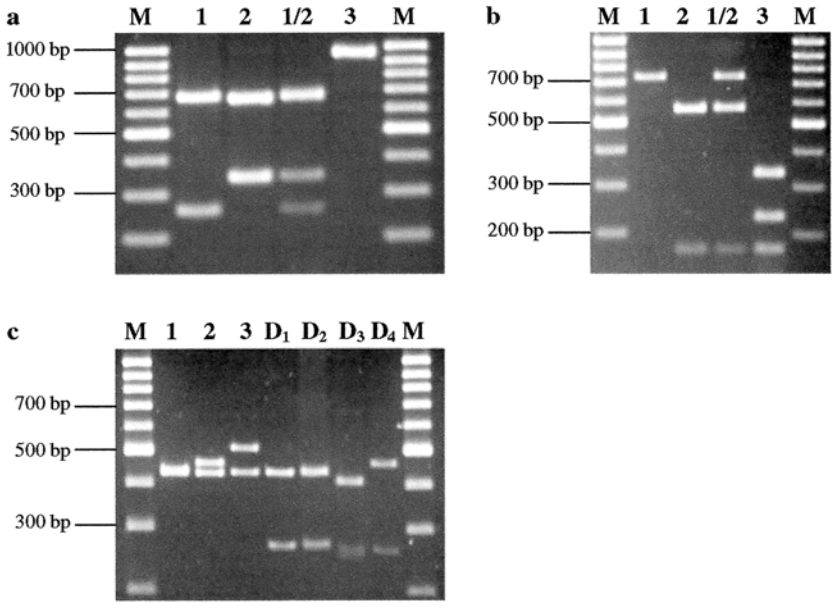

Fig. 1 Alleles for the markers Aps-1 (**a**), REX-1 (**b**) and JB-1 (**c**). Alleles are represented as coded in Table 1. M: DNA molecular weight marker (O'GeneRuler™ 100 bp DNA Ladder, Fermentas, Canada)

of a band slightly larger than 400 pb and allele 3 had a band of 500 pb. Allele 2 and allele 3 were co-dominant and dominant over allele 1. All *S. lycopersicum* lines without introgressions from wild species showed allele 1. This allele was also present in TY197 and in lines that, carrying *Mi*, showed allele 1 for the *Aps-1* marker. Lines that carried *Mi* and had the large introgression from *S. peruvianum* showed allele 2 for the JB-1 marker. All lines with *Ty-1* showed allele 3, independent of the presence of *Mi*.

Alleles for tomato and different wild tomato relatives

For the Aps-1 marker, *S. lycopersicum* accessions without introgressions showed allele 1. The rest of the species assayed showed allele 2, except for two *S. habrochaites* accessions, which showed a new allele (allele 3). This allele appeared as a result of the lack of a restriction site for *Taq*I in the amplification product (Fig. 1a).

Allele 1 for the REX-1 marker was present for all accessions of *S. lycopersicum* without introgressions and *S. pimpinellifolium*. Allele 2 was shown by all *S. chilense* and *S. habrochaites* accessions. Most *S. peruvianum* accessions had allele 2, as previously described (Williamson et al., 1994). However, we detected a new allele (allele 3) in some accessions of this species.

For the JB-1 marker, *S. lycopersicum* accessions without introgressions showed allele 1. *Solanum pimpinellifolium* accessions showed, alternatively, allele 1 or allele 2. All *S. chilense* accessions showed allele 3. This allele also appeared in all assayed *S. peruvianum* accessions. In *S. habrochaites*, several new alleles appeared in the different accessions (Fig. 1c), but all of them were distinguishable from the three alleles described for the rest of the species. The fact that allele 3 of the JB-1 marker is associated with *Ty-1* allows the use of this marker to tag the presence of this resistance allele. Moreover, the pattern obtained in different wild species shows that fragments in this region from these species will not interfere with the results for this marker.

Discussion

Molecular markers have many applications in plant breeding (Lörz & Wenzel, 2005; Nuez & Carillo, 2000). The availability of molecular markers linked to genes which confer desirable traits allows the shortening of breeding programmes. Several resistance genes identified in different wild tomato relatives have been introgressed into the cultivated species. Some of them map to chromosome 6 and are genetically very close. Identifying an allele of a marker associated specifically to one of these resistance genes can be complicated. Some breeding lines incorporate several genes from different wild species, and different wild species often share the same allele for a marker. This can lead to false positive results. *Ty-1* gene, which confers resistance to TYLCD, has been introgressed from *S. chilense* accession LA1969. We have identified a CAPS marker tightly linked to *Ty-1*.

Locus 1 of acid phosphatase (*Aps-1*) was the first isozyme employed as a marker for *Mi*. Knowledge of this locus at the sequence level (Williamson & Colwell, 1991) allowed us to develop a PCR-based marker. The results with this marker for plant material homozygous for *Mi* coincide with results previously reported (Messeguer et al., 1991); these plant materials showed, alternatively, allele 1 or 2, corresponding to alleles found in *S. lycopersicum* and *S. peruvianum*, respectively. For lines containing *Ty-1*, the alleles of this marker coincide with the alleles in plants carrying *Mi* along with the larger introgression of *S. peruvianum*. Analyses of alleles for the different wild tomato relatives support these results, given that *S. peruvianum* and *S. chilense* have the same allele for this marker. Furthermore, all species tested have this same allele, except *S. lycopersicum*, so introgressions from other species could lead to false positive results with this marker. Comparison at the sequence level of the amplification products obtained here from *S. chilense* and the sequence from *S. peruvianum* (GI:170369) showed that it would be possible to design a molecular marker that allowed the distinguishing of alleles for both species (data not shown). However, we also tested the Aps-1 marker on plants that did not present

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the *Mi* locus. In some cases, recombination occurred between Aps-1 and *Ty-1*, given that some lines that were selected based on the presence of allele 2 of Aps-1 were susceptible to TYLCD when inoculated (data not shown). So, Aps-1 is not very useful as a marker for *Ty-1* given that it is not tightly linked to this gene, and the presence of other genes, in the same region from different wild species can lead to false positive results.

REX-1 marker has previously been reported as being tightly linked to *Mi* (Williamson et al., 1994). All homozygous plant materials carrying *Mi* showed allele 2. Six plants of accession PI128657, the donor of *Mi*, were analysed, and all but one showed allele 2 for the REX-1 marker; the exception was a heterozygous plant for alleles 2 and 3. Kaloshian et al. (1998) also found considerable polymorphism within accessions of *S. peruvianum* for many RFLP and PCR markers. Given that all plants carrying *Mi* showed allele 2 at the REX-1 locus, this must be the allele inherited from PI128657 in the single F_1 plant from which all plants carrying *Mi* descend. None of the *S. lycopersicum* plant materials without *Mi* showed allele 2, except for SC, which carried *Ty-1* but not *Mi*. Furthermore, it has been reported that begomovirus-resistant lines derived from *S. habrochaites* that are susceptible to *M. incognita* give false positive results for the REX-1 marker (El Mehrach et al., 2005). These authors studied the REX-1 marker at the sequence level, detecting the same single nucleotide polymorphism (SNP) associated with a *Taq*I restriction site in plant material with introgressions from *S. peruvianum* and *S. habrochaites*. Our results support this finding, given that all *S. habrochaites* accessions tested in this experiment showed allele 2 for the REX-1 marker. Resistance to TYLCV derived from *S. habrochaites* has been mapped to chromosome 11 (Hanson et al., 2000). However, introgressions in chromosome 6 must have been retained in resistant plant material developed from this source. Lines which were homozygous for *Ty-1* and did not carry *Mi*, showed, alternatively, alleles 1, 2 or 3. Allele 1 (*S. lycopersicum* allele) appeared in some breeding lines coded as Fitó 3. Allele 2 was present in SC, introgressed from *S. chilense*. Allele 3 was shown by LA3473; El Mehrach et al. (2005) found 2 *Taq*I restriction sites in the amplification product of the REX-1 locus of this accession, which would result in the three bands observed in our results. LA3474 is a breeding line derived from an initial cross between *S. lycopersicum* cv. M82-1-8 and *S. chilense* LA1969 (Michelson et al., 1994). Given that allele 3 is not the *S. chilense* allele for this marker, the presence of this allele in accession LA3474 must be due to the background of cv. M82-1-8. TY197 showed alternatively alleles 1 and 2, so indicating that along the breeding programme developed to derive this line, some plants have retained the *S. lycopersicum* allele while others have inherited the *S. peruvianum* allele. Anastasia and Boludo, which were heterozygous for *Mi* and *Ty-1*, showed different patterns. Anastasia was homozygous for allele 2, whereas Boludo showed alleles 1 and 2 for this marker. These are commercial cultivars, so we do not know their genealogy. However, these results can be explained. Anastasia must have been developed from the cross of one line carrying *Mi* and another one carrying *Ty-1*. Each of the alleles present in Anastasia would derive from one of these lines. The allele from the line carrying *Mi* would be the *S. peruvianum* allele, while the allele from the line carrying *Ty-1* would be the *S. chilense* allele. On the contrary, Boludo must have been derived from a cross between a line carrying both *Mi* and *Ty-1*, and another line with the *S. lycopersicum* alleles for both genes. It is not therefore possible to determine the *S. peruvianum* or *S. chilense* origin of allele 2 for REX-1 in Boludo. In any case, marker REX-1 is not useful in marker-assisted selection for *Ty-1*. Allele 2, which is present in *S. chilense* LA1969, the source of this gene, is not frequently introgressed along with *Ty-1*. So REX-1 marker and *Ty-1* are not tightly linked.

One allele of JB-1 marker is always associated to *Ty-1*. All *S. lycopersicum* plant material carrying *Ty-1*, whether homozygous or heterozygous, showed allele 3 for this marker. This allele is also present in all accessions tested belonging to *S. chilense*, among them LA1969, the source of *Ty-1*. None of the *S. lycopersicum* plant materials without *Ty-1* showed this allele. Among them, plant material which did not carry *Mi*, showed

allele 1, which is the *S. lycopersicum* allele. In plant material carrying *Mi*, alleles 1 and 2 appeared alternatively; allele 1 was present in lines with the small introgression from *S. peruvianum* (Gevora and Fitó 1), whereas allele 2 was shown by lines that retained the larger introgression (H1124 and Fitó 2). However, all accessions of *S. peruvianum*, including PI128657, showed allele 3. Therefore the JB-1 region has not been introgressed in *S. lycopersicum* along with *Mi*, so the presence of this gene in cultivated plant material will not interfere with the results of this marker which tags *Ty-1*. Allele 3 is not present in the rest of the wild species analysed—*S. pimpinellifolium* and *S. habrochaites*—so introgressions from these species will not interfere, either.

Regarding the order of the markers and genes involved in this study, the results reported in this paper allow the location of CT21 (the RFLP marker from which JB-1 was designed) to be identified. The relative positions of *Mi*, REX-1 and Aps-1 have been previously reported (Fig. 2a). The first studies that were developed in order to locate *Mi* established that this locus resided near the centromere in chromosome 6 and was tightly linked to *Aps-1* (Gilbert, 1958; Rick & Fobes, 1974). Later, a higher resolution map around the *Mi* gene was established, in which *Mi* and *Aps-1* could be separated (Messeguer et al., 1991). Further studies based on radiation-induced deletion mapping supported the conclusion that *Mi* is separated from *Aps-1* by the centromere, mapping *Aps-1* on the long arm of chromosome 6 (Liharska et al., 1997). The location of *Mi* below REX-1 was established by Kaloshian et al. (1998). The relative position of CT21, though, was not clear: this RFLP marker has been mapped together with TG231 and *Aps-1* (Tanksley et al., 1992). The lines tested here that carried *Mi* retained introgressions from *S. peruvianum* that were variable in length, maintaining either the *S. peruvianum* allele only for the REX-1 marker or for both REX-1 and Aps-1 markers. In no case did these plant materials contain the *S. peruvianum* PI128657 allele for JB-1 marker (Fig. 2b).

Messeguer et al. (1991) classified *Meloidogyne*-resistant cultivars into three categories with respect to the amount of linked *peruvianum* DNA retained around the *Mi* gene. Two of these groups of cultivars did not contain the *S. peruvianum* allele at *Aps-1* nor any of the markers past this point. The group of cultivars with the largest introgression from PI128657 retained *S. peruvianum* alleles for markers between TG297 and TG231. We have analysed some plant material belonging to this category. These plant materials contained the *peruvianum* allele for *Aps-1*. However, in no case did they retain the *S. peruvianum* allele for the JB-1 marker. Therefore, we conclude that JB-1 is located beyond the TG231 marker. This position of CT21 is consistent with the results found in lines carrying *Ty-1*. Introgressions from *S. chilense* in these lines are

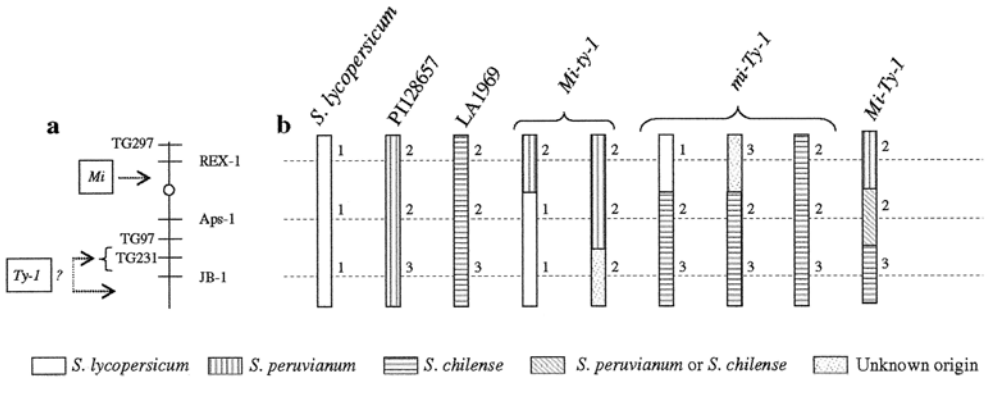

Fig. 2 (**a**) Diagram of *Mi/Ty-1* region of tomato chromosome 6, showing the relative order of markers and genes (framed). They are not on a genetic or physical scale. The centromere is indicated by a white circle. (**b**) Genetic composition of this region in the plant material used in this study. Introgressions from wild tomato relatives are represented by bars. Numbers on the right side of each bar indicate the allele as coded in Table 1

also variable in length. As stated above, introgressions always included the *S. chilense* allele for the JB-1 marker. In most of the plant material tested which carried *Ty-1*, the *S. chilense* allele for the *Aps-1* was also present. Moreover, even the allele for the REX-1 marker was introgressed from *S. chilense* along with *Ty-1* in some lines also carrying the *chilense* allele at *Aps-1*. The position of *Ty-1* with respect to JB-1 remains unclear. Zamir et al. (1994) located this locus below TG97, which is in accordance with the results obtained here.

The results reported here have allowed the identification of an allele of the JB-1 marker linked to *Ty-1*. This marker is more useful in marker-assisted selection than those previously used such as *Aps-1*, given that the presence of *Mi* or some other genes introgressed from wild tomato relatives will not interfere.

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4. CONCLUSIONES

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1. La resistencia a la enfermedad del rizado amarillo del tomate (Tomato yellow leaf curl disease, TYLCD) en líneas de mejora desarrolladas a partir de las entradas LA1932 y LA1938 de *Solanum chilense* es efectiva frente a las especies *Tomato yellow leaf curl Sardinia virus* (TYLCSV) y *Tomato yellow leaf curl virus* (TYLCV). Sin embargo, la resistencia a ambas especies parece tener un control genético distinto, ya que de 12 líneas evaluadas, sólo seis de ellas han mostrado resistencia a ambas especies, mientras que las seis restantes han sido resistentes a TYLCSV, pero susceptibles a TYLCV.

2. Las seis líneas de mejora resistentes a TYLCSV y TYLCV presentan un nivel elevado de resistencia parcial, consistente en la atenuación en la manifestación de síntomas y retraso en el momento de aparición de los mismos, además de en la reducción de la acumulación viral. Por otra parte, no se producen pérdidas significativas en el rendimiento como consecuencia de la infección por TYLCV. Las características agronómicas de estas líneas las hacen apropiadas como parentales para el desarrollo de híbridos con elevada resistencia a TYLCSV y TYLCV.

3. La resistencia parcial a TYLCV en la línea L102, derivada de la entrada UPV16991 de *S. pimpinellifolium*, está controlada por un gen con recesividad parcial y penetración incompleta. Estos resultados deben ser considerados a la hora del empleo de esta resistencia en programas de mejora. El control monogénico supone una ventaja para la introgresión de la resistencia en el fondo genético de la especie cultivada. Sin embargo, debido a la penetración incompleta y la recesividad parcial, se recomienda el uso de esta resistencia en homocigosis o en combinación con resistencia procedente de otras fuentes.

4. La expresión de esta resistencia depende considerablemente del fondo genético de *S. lycopersicum* en el que se introgresa. Los mayores niveles de resistencia se obtienen en cruces con las líneas de mayor vigor. En este sentido, en el proceso para el desarrollo de híbridos portadores de esta resistencia será necesario seleccionar parentales vigorosos y considerar, además de la heterosis del híbrido, la expresión de la resistencia.

5. Existen diferencias en el nivel de resistencia y en el control genético de la misma entre diferentes líneas de mejora derivadas de la entrada UPV16991 de *S. pimpinellifolium*. El

gen o los genes que controlan la resistencia en la línea L54 muestran penetración completa, a diferencia del gen presente en la línea L102. Resulta, por tanto, de interés continuar con los estudios encaminados a determinar el control genético de la resistencia de la línea L54.

6. El nivel de resistencia a TYLCV en materiales que combinan el gen *Ty-1* y el gen derivado de UPV16991, ambos en heterocigosis, es superior al mostrado por los heterocigotos para cada uno de estos genes. Incluso uno de los híbridos desarrollados presenta mayores niveles de resistencia que los homocigotos para cada uno de los genes. Por lo tanto, es posible conseguir materiales con niveles elevados de resistencia combinando la resistencia derivada de UPV16991 y el gen *Ty-1*. Esta es la aproximación más práctica para la utilización de la resistencia derivada de UPV16991 en el desarrollo de híbridos, ya que evita la necesidad de fijar los genes de resistencia en ambos parentales.

7. Se ha identificado un marcador molecular, JB-1, tipo CAPS (*Cleaved Amplified Polymorphic DNA*) ligado al gen de resistencia *Ty-1*. La presencia del gen *Mi*, frecuentemente introgresado a partir de *S. peruvianum* en los híbridos comerciales de tomate y muy próximo genéticamente al gen *Ty-1*, no interfiere con los resultados para este marcador. El análisis de varias entradas de especies silvestres relacionadas con el tomate ha permitido determinar que las introgresiones de otros genes en esta región procedentes de *S. lycopersicum* (antes *Lycopersicon esculentum* var. *cerasiforme*), *S. habrochaites* y *S. pimpinellifolium* tampoco interfieren con los resultados.

8. El análisis de los marcadores *Aps-1*, REX-1 y JB-1, los tres localizados en la región del gen *Ty-1*, en materiales con introgresiones procedentes de distintas especies silvestres relacionadas con el tomate ha permitido determinar la posición relativa del marcador CT21 a partir del cual se desarrolló el marcador JB-1.

5. OTRAS PUBLICACIONES

5. OTRAS PUBLICACIONES

Publicaciones relacionadas con la tesis presentadas a congresos

Breeding lines resistant to TYLCV derived from *Lycopersicon chilense*

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