Caracterización de aislados del virus del bronceado del tomate (TSWV) que superan las resistencias de los genes *Sw-5* en tomate y *Tsw* en pimiento. Identificación de una fuente de tolerancia en pimiento

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El Doctor D. Carmelo López del Rincón, Profesor Titular de Genética del Departamento de Biotecnología de la Universidad Politécnica de Valencia adscrito al Instituto de conservación y Mejora de la Agrodiversidad Valenciana (COMAV-UPV) y el Doctor D. Luis Rubio Miguélez, Investigador científico del Instituto Valenciano de Investigaciones Agrarias (IVIA).

CERTIFICAN:

Que la presente memoria titulada "Caracterización de aislados del virus del bronceado del tomate (TSWV) que superan las resistencias de los genes *Sw-5* en tomate y *Tsw* en pimiento. Identificación de una fuente de tolerancia en pimiento", realizada por Dª Diana Elvira Debreczeni bajo su dirección, constituye su Memoria de Tesis para optar al grado de Doctor.

Y para que así conste a los efectos oportunos, firman el presente certificado en Valencia, a 27 de Febrero de 2015.

Carmelo López del Rincón   Luis Rubio Miguélez

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Resumen

El virus del bronceado del tomate (*Tomato spotted wilt virus*, TSWV) es uno de los virus más extendidos y de mayor importancia económica del mundo. Infecta a un gran número de especies vegetales, siendo los cultivos de tomate y pimiento los más afectados. Este virus se transmite de una planta a otra por medio de varias especies de trips en una manera propagativa y circulativa, siendo *Flankliniella occidentalis* el vector más eficaz. El cultivo de variedades resistente de tomate y pimiento (en los que se han introducido por mejora genética los genes *Sw-5* y *Tsw*, respectivamente) permitió el control de la enfermedad, ya que estos genes inducen una respuesta hipersensible impidiendo la infección sistémica del virus (resistencia total). Sin embargo, con frecuencia aparecen aislados del virus que son capaces de superar estas resistencias. Para obtener un control de la enfermedad más eficaz y duradero es necesario: A) la caracterización genética y biológica de los aislados del TSWV que superan y no superan las resistencias; B) el estudio de los factores evolutivos y epidemiológicos implicados en la aparición y establecimiento de los aislados que superan las resistencias; C) el desarrollo de herramientas que permitan la detección y cuantificación de estos aislados virales; y D) la evaluación de nuevas fuentes de resistencia (total o parcial, que dificulten la infección y multiplicación viral) o tolerancia (total o parcial, que dificulten la aparición de síntomas y daños aunque no tengan un efecto en la infección viral).

En este trabajo se han caracterizado biológicamente y molecularmente diferentes aislados del TSWV procedentes de cultivos de tomate y pimiento de España. Se determinó la secuencia nucleotídica del genoma completo de tres aislados españoles correspondientes a tres biotipos: biotipo N (incapaz de infectar variedades resistentes de tomate o pimiento); biotipo T (supera la resistencia *Sw-5* de tomate); y P (supera la resistencia *Tsw* de pimiento). Los resultados mostraron que no había una correlación entre la variación genética y la capacidad de superar la resistencia.

Estas secuencias nucleotídicas y otras obtenidas de la base de datos Genbank se utilizaron para desarrollar una técnica basada en la RT-PCR cuantitativa (RT-qPCR) que permite detectar el virus con un alto grado de sensibilidad y cuantificar en un amplio rango dinámico (10^3-10^{10} moléculas de RNA viral por cada ng de RNA total). Se diseñaron los iniciadores y una sonda TaqMan MGB a partir de segmentos de secuencia conservados para que fueran válidos para todos los aislados del TSWV. El hecho de no encontrar correlación entre biotipo y genotipo impide que se pueda desarrollar un método molecular para diferenciar los aislados que superan y no superan las resistencias. En su lugar se desarrollaron dos sondas Taqman que permitían cuantificar diferencialmente variantes genéticas del virus en infecciones mixtas.

La RT-qPCR se utilizó para evaluar la acumulación de varios aislados del TSWV en tomate sin *Sw-5*, pimiento sin *Tsw* y *Datura stramonium* (que actúa como reservorio del virus), así como, en su principal vector *F. occidentalis* que se usó para evaluar la eficiencia de la transmisión. Se observó que la superación de la resistencia no suponía un coste en la eficacia biológica (fitness) tanto en la multiplicación del virus en estas plantas como en la transmisión por trips. Por tanto, los aislados que superan las

resistencias tienen la misma capacidad de dispersión en campo que los aislados convencionales.

Por último, se utilizó la RT-qPCR y la información obtenida para evaluar la capacidad de resistencia y tolerancia de una accesión de *Capsicum baccatum* al TSWV. Además de considerar la variabilidad biológica y genética del virus, se desarrolló un nuevo enfoque basado en el análisis de datos longitudinales (tomados de los mismos individuos a lo largo del tiempo). El nivel de resistencia se estimó a partir de dos variables: A) la eficacia absoluta, calculada a partir de la variación en el tiempo del título viral, cuantificado por RT-qPCR; y B) la infectividad, como la mediana del tiempo que tarda el virus en ser detectado en una planta por ELISA. El nivel de tolerancia se estimó como la mediana del tiempo que tarda una planta en mostrar síntomas graves. Se realizó un ensayo en el que se inocularon cantidades equimolares de dos aislados del TSWV (uno que supera y otro que no supera la resisencia del gen *Tsw*) en lotes de 30 plantas de la nueva accesión de *C. baccatum*, una variedad de pimiento susceptible y una variedad con el gen de resistencia *Tsw*. Los resultados mostraron que esta nueva accesión es parcialmente resistente (menor infectividad y acumulación viral que la variedad susceptible) y totalmente tolerante (ninguna planta manifestó síntomas graves) tanto para aislados del TSWV convencionales como para aquellos capaces de infectar e inducir síntomas severos en variedades con el gen *Tsw*. Por tanto, esta nueva variedad de *C. baccatum* es un buen candidato para usarlo en programas de mejora genética de pimiento (*C. annuum*).

Resum

El virus del bronzejat de la tomaca (*Tomato spotted wilt virus*, TSWV) és un dels virus més estesos i de major importància econòmica del món. Infecta a un gran nombre d'espècies vegetals i els cultius de tomaca i pebrot són alguns dels més afectats. Aquest virus es transmet d'una planta a una altra, per mitjà de diverses espècies de trips de manera propagativa i circulativa, i *Flankliniella occidentalis* és el vector més eficaç. El cultiu de varietats resistents de tomaca i pebrot (en els quals s'han introduït per millora genètica els gens *Sw-5* i *Tsw*, respectivament) va permetre el control de la malaltia, ja que aquests gens indueixen una resposta hipersensible i impedeixen la infecció sistèmica del virus (resistència total). No obstant açò, amb freqüència apareixen aïllats del virus que són capaços de superar aquestes resistències. Per a obtenir un control de la malaltia més eficaç i durador és necessari: A) la caracterització genètica i biològica dels aïllats del TSWV que superen i no superen les resistències; B) l'estudi dels factors evolutius i epidemiològics implicats en l'aparició i establiment dels aïllats que superen les resistències; C) el desenvolupament d'eines que permeten la detecció i quantificació d'aquests aïllats virals; i D) l'avaluació de noves fonts de resistència (total o parcial, que dificulten la infecció i multiplicació viral) o tolerància (total o parcial, que dificulten l'aparició de símptomes i danys encara que no tinguen un efecte en la infecció viral).

En aquest treball s'han caracteritzat biològicament i molecularment diferents aïllats del TSWV procedents de cultius de tomaca i pebrot d'Espanya. Es va determinar la seqüència nucleotídica del genoma complet de tres aïllats espanyols corresponents a tres biotips: biotip N (incapaç d'infectar varietats resistents de tomaca o pebrot); biotip T (supera la resistència *Sw-5* de tomaca); i P (supera la resistència *Tsw* de pebrot). Els resultats van mostrar que no hi havia una correlació entre la variació genètica i la capacitat de superar la resistència.

Aquestes seqüències nucleotídiques i altres obtingudes de la base de dades *Genbank* es van utilitzar per a desenvolupar una tècnica basada en la RT-PCR quantitativa (RT-qPCR) que permet detectar el virus amb un alt grau de sensibilitat i quantificar en un ampli rang dinàmic (10^3-10^{10} molècules de RNA viral per cada ng de RNA total). Es van dissenyar els iniciadors i una sonda TaqMan MGB a partir de segments de seqüència conservats perquè foren vàlids per a tots els aïllats del TSWV. El fet de no trobar correlació entre biotip i genotip impedeix que es puga desenvolupar un mètode molecular per a diferenciar els aïllats que superen i no superen les resistències. En el seu lloc es van desenvolupar dos sondes TaqMan que permetien quantificar diferencialment variants genètiques del virus en infeccions mixtes.

La RT-qPCR es va utilitzar per a avaluar l'acumulació de diversos aïllats del TSWV en tomaca sense *Sw-5*, pebrot sense Tsw i *Datura stramonium* (que actua com a reservori del virus) així com, en el seu principal vector *F. occidentalis* que es va usar per a avaluar l'eficiència de la transmissió. Es va observar que la superació de la resistència no suposava un cost en l'eficàcia biològica (*fitness*) tant en la multiplicació del virus en aquestes plantes com en la transmissió per trips. Per tant, els aïllats que

superen les resistències tenen la mateixa capacitat de dispersió en camp que els aïllats convencionals.

Finalment, es va utilitzar la RT-qPCR i la informació obtinguda per a avaluar la capacitat de resistència i tolerància d'una accessió de *Capsicum baccatum* al TSWV. A més de considerar la variabilitat biològica i genètica del virus, es va desenvolupar un nou enfocament basat en l'anàlisi de dades longitudinals (presos dels mateixos individus al llarg del temps). El nivell de resistència es va estimar a partir de dues variables: A) l'eficàcia absoluta, calculada a partir de la variació en el temps del títol viral, quantificat per RT-qPCR; i B) la infectivitat, com la mitjana del temps que es tarda a detectar el virus en una planta per mitjèr d'ELISA. El nivell de tolerància es va estimar com la mitjana del temps que tarda una planta a mostrar símptomes greus. Es va realitzar un assaig en el qual s'inocularen quantitats equimolars de dos aïllats del TSWV (un que supera i un altre que no supera la resistència del gen Tsw) en lots de 30 plantes de la nova accessió de *C. baccatum,* una varietat de pebrot susceptible i una varietat amb el gen de resistència *Tsw*. Els resultats van mostrar que aquesta nova accessió és parcialment resistent (menor infectivitat i acumulació viral que la varietat susceptible) i totalment tolerant (cap planta va manifestar símptomes greus) tant per a aïllats del TSWV convencionals com per a aquells capaços d'infectar i induir símptomes severs en varietats amb el gen *Tsw*. Per tant, aquesta nova varietat de *C. baccatum* és un bon candidat per a ser usat en programes de millora genètica de pebrot (*C. annuum*).

Abstract

Tomato spotted wilt virus (TSWV) is one of the most widespread and economically important viruses worldwide. It infects a large number of plant species, being the tomato and pepper the most affected. TSWV is transmitted from one plant to another by various species of thrips in a circulative and propagative manner, being *Flankliniella occidentalis* the main vector. The best strategy for disease control in tomato and pepper has been breeding resistant cultivars, but only tomato with the gene *Sw-5* and pepper with gene *Tsw* have been effective against a wide spectrum of TSWV isolates, but resistance-breaking isolates often arise. To obtain a more effective and durable control is necessary: A) the genetic and biological characterization of conventional and resistance-breaking isolates of TSWV; B) the understanding the evolutionary and epidemiological factors involved in the emergence and dispersion of resistance-breaking isolates; C) the development of tools for viral detection and quantification; and D) evaluation of new sources of resistance (total or partial, estimated as the difficulty to viral infection or/and accumulation) or tolerance (total or partial, estimated as the difficulty to symptoms development and damage without affecting viral infection).

In this work, TSWV isolates from Spanish tomato and pepper crops have been biologically and molecularly characterized. The complete genome of three TSWV isolates with different biotypes were sequenced: N (unable to infect *Sw-5* resistant tomato and *Tsw* resistant pepper), T (tomato *Sw-5* resistance-breaking), and P (pepper *Tsw* resistance-breaking). No correlation between genetic variation and the ability of overcoming resistance was found.

These nucleotide sequences and others retrieved from the GenBank database were used to develop quantitative RT-PCR (RT-qPCR), with high sensitivity and dynamic range (10^3-10^{10} viral RNA molecules per ng of total RNA). Primers and one TaqMan MGB were designed from conserved sequence stretches with the aim of detecting and quantifying any TSWV isolate. It was not possible to develop a molecular method to differentiate between conventional and resistance-breaking isolates since there is no correlation between genotype and biotype. Instead, two TaqMan MGB probes were designed to quantify the two main genotypes (according to the M segment) in mixed infections.

RT-qPCR was used to evaluate TSWV accumulation in non-resistant tomato, non-resistant pepper and *Datura stramonium* (an important reservoir), as well as in the main vector *F. occidentalis*, which was considered to evaluate transmission efficiency. The results showed that resistance breakdown was not associated to a fitness cost (tradeoff) in the infectivity of susceptible hosts or transmissibility by thrips and that the resistance-breaking isolates have the same potential for dispersion in field as the conventional isolates.

Finally, the information obtained from the previous studies and the RT-qPCR technique were used to evaluate the resistance and tolerance to TSWV of a new accession of *Capsicum baccatum*. In addition to considering the genetic and biological

variation of TSWV, a new approach was used based on analysis of longitudinal data (those measured in the same individuals over time). The resistance level was estimated with two variables: A) absolute fitness, calculated from the variation in time of the viral titer, quantified by RT-qPCR; and B) infectition survival, the median time in which the virus was detected in a plant by ELISA. The tolerance level was estimated as symptom survival, the median time in which a plant developed severe symptoms. This analysis showed that this new accession is partially resistant and totally tolerant to TSWV conventional and resistance-breaking isolates and therefore is a good candidate for a pepper (*C. annuum*) breeding program.

INTRODUCCIÓN

1. Importancia económica de los cultivos de tomate y pimiento

La producción de tomate (*Solanum lycopersicum*) y pimiento (*Capsicum annuum*) ha experimentado un aumento constante a nivel mundial durante las últimas décadas debido a que son consumidos durante todo el año. Al aumento de la producción ha contribuido la fuerte inversión a nivel tecnológico y la obtención de variedades con mayores rendimientos por hectárea. El tomate es la hortaliza con mayor volumen de producción a nivel mundial, seguido por sandía, cebolla, col, pepino, berenjena, zanahoria, melón y pimiento que ocupa el noveno lugar en cuanto a producción (Tabla 1) (FAOSTAT, 2015).

Tabla 1. Producción mundial de hortalizas en toneladas.

Hortaliza	Producción mundial (t)
Tomate	161.793.834
Sandía	105.372.341
Cebolla	82.851.732
Col	70.104.972
Pepino	65.134.078
Berenjena	48.424.295
Zanahoria	36.917.246
Melón	31.925.787
Pimiento	31.171.567
Lechuga	24.946.142

Según la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAOSTAT, 2015), el cultivo de tomate durante el año 2012 alcanzó una producción a nivel mundial de casi 162 millones de toneladas, producidas en 4,8 millones de hectáreas. El principal país productor fue China, que produjo 50 millones de toneladas, lo que representó el 30% de la producción mundial seguido de India, Estados Unidos y Turquía. España ocupó el octavo lugar con una producción de 4 millones de toneladas y el segundo lugar dentro de la Unión Europea tras Italia, tanto por la superficie cultivada (48.800 hectáreas) como por la producción obtenida (Tabla 2).

Tabla 2. Producción de tomate por países durante el año 2012 (FAOSTAT, 2015)

País	Producción (t)
China	50.000.000
India	17.500.000
Estados Unidos	13.207.000
Turquía	11.350.000
Egipto	8.625.220
Irán	6.000.000
Italia	5.131.980
España	4.007.000
Brasil	3.873.990
Méjico	3.433.570

También según la FAO, el cultivo de pimiento durante el año 2012 alcanzó una producción a nivel mundial de 31 millones de toneladas, producidas en 1,9 millones de

hectáreas. El principal país productor fue también China con 16 millones de toneladas, lo que representa más del 50% de la producción mundial seguido de Méjico y Turquía. España ocupó la sexta posición a nivel mundial en cuanto a producción, y la primera a nivel europeo, con más de 1 millón de toneladas producidas en 18.100 hectáreas (Tabla 3).

Tabla 3. Producción de pimiento por países durante el año 2012 (FAOSTAT, 2015)

País	Producción (t)
China	16.000.000
Méjico	2.379.740
Turquía	2.072.130
Indonesia	1.656.620
Estados Unidos	1.064.800
España	1.023.700
Egipto	650.054
Nigeria	500.000
Argelia	426.566
Etiopía	402.109

En España la mayor parte del cultivo de tomate se realiza en regadío y al aire libre y, aproximadamente, un tercio de la superficie total se cultiva en invernadero, lo que posibilita unos elevados rendimientos. Sin embargo, en el caso del pimiento el cultivo en condiciones protegidas representa entorno al 50% de la superficie total, concentrándose su producción, principalmente, en las provincias de Almería y Murcia.

2. Factores limitantes de los cultivos de tomate y pimiento en España

Aunque en la actualidad se dispone de un conjunto de variedades de tomate y pimiento mejoradas, con elevados rendimientos y adaptadas a las exigencias de cada mercado, existen diferentes problemas de naturaleza biótica o abiótica que limitan el rendimiento económico. La constante necesidad de aumentar la producción de estos cultivos ha obligado a utilizar suelos agrícolas, aguas y climas que pueden favorecer situaciones de estrés relacionados con la salinidad, sequía o temperaturas extremas entre otras. Sin embargo, el desarrollo de algunas prácticas culturales ha minimizado las incidencias de los factores abióticos sobre estos cultivos. Se dispone de infraestructuras que permiten cultivar en zonas en las que el clima no es el idóneo del cultivo y de productos fitosanitarios que permiten solventar cualquier problema causado por la deficiencia de nutrientes. Así mismo, se han puesto a punto técnicas de polinización mediante el uso de determinados insectos que permiten o mejoran el cuajado de los frutos en condiciones adversas y aumentan la producción. Además, se han desarrollado variedades con menores necesidades hídricas y nutritivas, con mayor resistencia a temperaturas extremas, a la salinidad o a iones tóxicos. Todos estos avances han permitido reducir en gran medida los factores abióticos a los que se ven sometidos los cultivos de tomate y pimiento, pero no sucede lo mismo en el caso de los factores bióticos.

En cuanto a las plagas, las más importantes en ambos cultivos son las causadas por pulgones, moscas blancas, trips, araña roja, minadores de hojas y orugas de lepidópteros, que merman los rendimientos de los cultivos por tener efectos negativos en su desarrollo. Además, tanto pulgones como trips y moscas blancas son importantes vectores transmisores de virus. Cabe destacar la transmisión del virus del bronceado del tomate (*Tomato spotted wilt virus*, TSWV) por trips y la del virus del rizado amarillo del tomate (*Tomato yellow leaf curl virus*, TYLCV) por mosca blanca, que causan importantes epidemias y limitan el cultivo tanto de tomate como de pimiento.

En el caso de los parásitos del suelo, los daños más importantes los producen distintos nematodos del género *Meloidogyne*. Atacan a la planta produciendo nódulos en las raíces que provocan la obstrucción de los vasos y dificultan la absorción de nutrientes lo que provoca un menor desarrollo de la planta y el marchitamiento a temperaturas elevadas. El control requiere de la desinfección del suelo a partir de la solarización y biofumigación.

Con respecto a las enfermedades infecciosas, estos cultivos pueden verse afectados por algunos hongos como botritis (*Botrytis cinérea* P.), oídio (*Leveillula taurica* L.), podredumbre blanca (*Sclerotinia sclerotiorum* B.), fusarium (*Fusarium oxysporum* S.), mildiu (*Phytophtora capsici* L.) y verticilium (*Verticilium dahliae* K.). Adquieren especial importancia los daños causados por *Botrytis cinérea* en el cultivo bajo invernadero y *Phytophtora capsici*, el agente causal de la enfermedad fúngica más extendida del pimiento (Gil Ortega et al. 1990). En el caso de las enfermedades de origen bacteriano, las más importantes son las causadas por especies pertenecientes a los géneros *Clavibacter, Pseudomonas, Xanthomonas* y *Erwinia*, aunque ninguna de ellas se caracteriza por causar daños de importancia en los cultivos de tomate o pimiento de nuestro país. Estos dos tipos de patologías, fúngicas y bacterianas, son de relativo fácil control mediante la aplicación de productos fitosanitarios.

Sin embargo, si nos referimos a las enfermedades víricas podemos destacar la gran complejidad que presenta su control en campo, debido a la facilidad de transmisión y a la ausencia de tratamientos curativos. Por este motivo y por las grandes pérdidas económicas que provocan, son consideradas uno de los problemas de naturaleza biótica de mayor relevancia Las enfermedades de etiología viral provocan grandes pérdidas económicas en ambos cultivos en casi todas las campañas. En ocasiones la incidencia de las enfermedades es tan fuerte que obliga a los agricultores al cambio de las especies cultivadas e incluso cuando la intensidad del ataque es muy grave, al abandono del cultivo. De entre las enfermedades víricas que afectan a ambos cultivos aquellas que producen o han producido un mayor impacto económico son las causadas por el TSWV, el virus del mosaico del pepino (*Cucumber mosaic virus*, CMV), el virus Y de la patata (*Potato virus Y*, PVY). Otros virus que provocan pérdidas importantes son el virus del mosaico del pepino dulce (*Pepino mosaic virus*, PepMV) y el virus del mosaico del tomate (*Tomato mosaic virus, ToMV).*

El cultivo intensivo en invernadero durante casi todo el año, el movimiento de material vegetal entre países y las condiciones climáticas en los invernaderos,

favorables para el establecimiento de los vectores y el desarrollo de nuevas enfermedades, parecen ser las causas directas de la aparición e incremento de enfermedades víricas y de sus vectores transmisores. TSWV, virus del que se ocupa esta tesis, provoca graves epidemias en los cultivos de tomate y pimiento en España, sobretodo en toda la franja del litoral mediterráneo.

3. El virus del bronceado del tomate (TSWV): historia e importancia económica

La enfermedad del bronceado fue observada por primera vez en plantas de tomate en Australia en 1906 (Sakimura 1962), aunque no se realizó una descripción detallada de esta nueva enfermedad hasta 1915 (Brittlebank 1919). A partir de entonces la enfermedad se describió en Estados Unidos y Canadá (Illingworth 1931). Sin embargo, no fue hasta 1930 cuando se descubrió que el agente que provoca dicha enfermedad es un virus, al que se denominó *Tomato spotted wilt virus* (TSWV) (Samuel et al. 1930), aunque ha recibido numerosos nombres como Carcova virus, Vira cabeça virus, Pineapple yellow spot virus, Peanut bud necrosis virus, Kat river disease virus, Komnek virus, Pineapple side rot virus, Lycopersicon virus 3, Lycopersicon virus 7, Markhorka tip chlorosis virus y Tip blight virus, (Rosello et al. 1994). En Europa, el TSWV fue observado por primera vez en 1931 en el Reino Unido y a partir de 1987 se propagó por diferentes países europeos como Holanda, Francia, Italia y España, siendo la Costa Mediterránea la más afectada (Roselló et al. 1996). Actualmente, el TSWV está presente en zonas de clima templado, tropical y subtropical y se puede considerar que su distribución es mundial, si exceptuamos la parte más al norte del hemisferio (Figura 1).
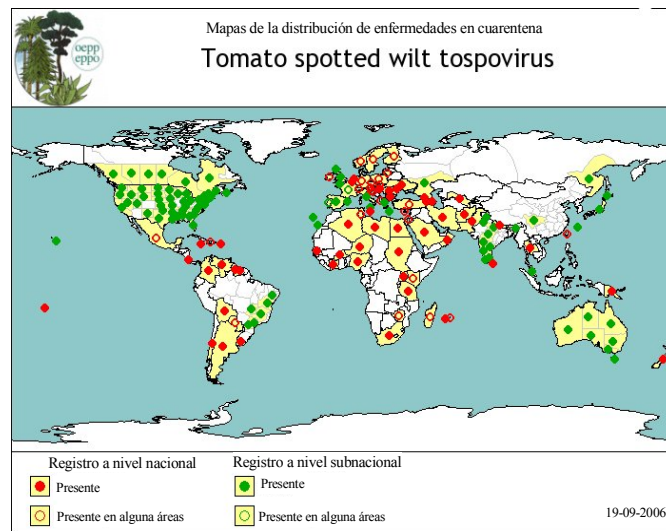
Figura 1. Distribución del TSWV
(http://www.eppo.org/QUARANTINE/virus/Tomato_spotted_wilt_virus/TSWV00_map.htm)

Las pérdidas económicas ocasionadas por TSWV en la agricultura son enormes, debido a su distribución geográfica, a su amplia gama de plantas huéspedes y a los graves daños que infligen en las plantas infectadas. El TSWV es una de las diez virosis

más devastadoras, ya que es capaz de infectar más de 1.090 especies de plantas, pertenecientes a 85 familias botánicas (15 familias de plantas monocotiledóneas, 69 familias de plantas dicotiledóneas y una familia de pteridofitas) (Parrella et al. 2003; Pappu et al. 2009). El TSWV es un patógeno de cuarentena que se añadió a la lista A2 de la EPPO (Organización Europea para la Protección de las Plantas) en 1997 (http://www.eppo.int/QUARANTINE/virus/Tomato_spotted_wilt_virus/TSWV00_ds.pdf).

4. Gama de huéspedes y síntomas

Los cultivos más frecuentemente y severamente afectados por el TSWV son tomate, pimiento y lechuga, pero supone también una amenaza importante para otros cultivos como patata, tabaco, cacahuete, alcachofa, haba, papaya, piña, berenjena, col, pepino, guisante, espinaca, apio, coliflor, etc. (Turina et al. 2012) y para varias especies de plantas ornamentales como dalia, begonia, violeta africana, gladiolo, crisantemo, caléndula, etc.

Figura 2. Sintomatología del TSWV en distintas especies hortícolas y ornamentales
Pimiento: http://www.omafra.gov.on.ca/english/crops/facts/14-001.htm
Tomate: http://www.infojardin.com/foro/showpost.php?p=2543867&postcount=10;
        http://enfermedadesdeltomate.blogspot.com.es/2011/11/enfermedades-del-tomate.html
Cacahuete: http://www.apsnet.org/edcenter/K-12/NewsViews/Article%20Images/Forms/DispForm.aspx?ID=140
Tabaco: http://www.crosscreekseed.com/wp-content/uploads/2012/11/tomato-spotted-wilt-virus3.jpg
Crisantemo: http://www.separationsnow.com/details/ezine/sepspec20385ezine/Biosafety-of-transgenic-plants.html?
Gloxinea: http://vegetablemdonline.ppath.cornell.edu/factsheets/Virus_SpottedWilt.htm
Lechuga: http://ucanr.edu/blogs/SalinasValleyAgriculture/index.cfm?tagname=thrips

Los síntomas varían dependiendo de la especie infectada, pero también del genotipo, del estado de desarrollo de la planta al ser infectada, del aislado viral y las condiciones ambientales (luminosidad, humedad y sobretodo temperatura) (Adkins 2000). La infección se establece como sistémica en la mayoría de cultivos, siendo los

daños más notorios cuando la planta está en los primeros estadios de desarrollo, provocando a menudo, un enanismo acusado y quemaduras en la planta que a veces provocan su muerte (Figura 2). Un síntoma característico de la infección por TSWV es la presencia de lesiones necróticas en las hojas de color del bronce (o marrón púrpura) del que deriva el nombre de "bronceado" en España y "peste negra" en Argentina. En las hojas es habitual encontrar anillos necróticos y fuertes líneas sinuosas con formas geométricas de color más claro o amarillo sobre el fondo verde de la hoja. Los frutos suelen presentar manchas y anillos concéntricos de color verde, amarillo o tonos de color más claro sobre el color del fruto maduro (Rodríguez et. al. 2007).

En tomate y pimiento es frecuente observar anillos cloróticos que posteriormente necrosan y fuertes líneas sinuosas que forman como un arabesco de dibujos geométricos de color más claro o amarillo en contraste con el verde de la hoja. Ocasionalmente aparece amarilleamiento del brote y necrosis apical con aborto de las flores en desarrollo. Los frutos muestran moteado redondo de color verde, amarillo o una tonalidad más clara respecto al rojo del fruto maduro, presentado forma de anillos concéntricos con abullonado; hecho que impide su comercialización debido a un descenso de la calidad (Aramburu y Aos 2001).

5. Transmisión del TSWV

Tomato spotted wilt virus se trasmite de forma natural por insectos del orden *Thysanoptera* (suborden Terebrantia; familia *Thripidae*), comúnmente conocidos como trips, pero no por semilla. Existen más de 5.000 especies de trips pero sólo se han encontrado nueve especies como vectores de este virus: *Frankliniella bispinosa, F. fusca, F. intonsa, F. occidentalis, F. schultzei, F. cephalica Scirtothrips dorsalis, Thrips setosus,* y *T. tabaci*, siendo la especie *F. occidentalis* (Figura 3A) la más eficaz por su polifagia, su amplia distribución geográfica y su alta eficiencia en la transmisión con respecto a las otras especies (Pappu et al. 2009).
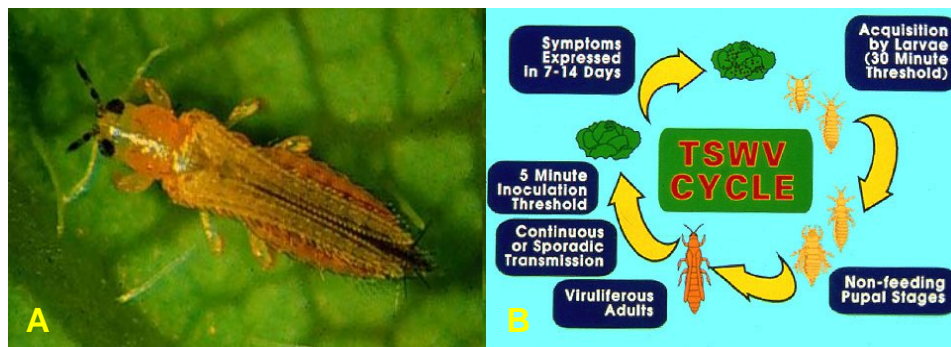
Figura 3. A. El vector F. occidentalis en el estado adulto; B. Esquema del ciclo de transmisión del TSWV por trips. http://www.apsnet.org/online/feature/tospovirus)

De hecho, la rápida expansión que experimentó *F. occidentalis* por todo el mundo hace unas décadas fue una de las principales causas de la reemergencia de TSWV como una de las principales virosis en el mundo.

La transmisión natural del virus mediante el insecto vector es de tipo persistente, circulativa y propagativa (Peters et al. 1995).Para que un trips adulto se convierta en transmisor, la larva (primera fase o al inicio de la segunda fase larval) tiene que haber adquirido el virus previamente, mediante la alimentación en un tejido vegetal infectado. El virus se multiplica en las células intestinales, pasa a las glándulas salivares y de allí puede ser trasmitido a la planta con relativa eficacia durante el resto de la vida del insecto (Figura 3B).

La proximidad entre las células intestinales y las glándulas salivares en esta fase inmadura facilita el movimiento del virus, mientras que en el insecto adulto, la formación de barreras entre el intestino y las glándulas salivares, así como la mayor distancia entre ambos impiden que el virus llegue a las glándulas salivares (Adkins 2000, Whitfield et al. 2005), lo cual explica que un trips sólo pueda ser virulífero si ha adquirido el virus en estado larvario y no como adulto. El factor determinante en la eficiencia de trasmisión se relaciona, precisamente, con la acumulación del virus desde el estadio larvario hasta la etapa adulta. Los virus pueden ser adquiridos con periodos de alimentación en plantas infectadas de tan solo 5 minutos, pero cuanto más larga sea esta fase, más eficiente será posteriormente la transmisión. Los adultos son capaces de transmitirlo en un intervalo de inoculación de tan sólo 5 o 10 minutos, este fenómeno dota al virus de un gran potencial de dispersión, debido a que con pocos trips virulíferos se pueden llegar a infectar un gran número de plantas. En algunas especies, el periodo de transmisión puede extenderse a lo largo de toda la vida del insecto (30-40 días), pero la tasa de transmisión es máxima entre 22 y 30 días. El virus no se transmite a la descendencia (Inoue et al. 2004).

6. Taxonomía

Tomato spotted wilt virus es el miembro tipo del género *Tospovirus* de la familia *Bunyaviridae*. La familia *Bunyaviridae* incluye 97 especies repartidas en 5 géneros: *Hantavirus* (24 especies), *Nairovirus* (7 especies), *Orthobunyavirus* (48 especies), *Phlebovirus* (9 especies) y *Tospovirus* (9 especies). La mayoría de los miembros de la familia son transmitidos por artrópodos de un modo persistente y se replican en el vector. TSWV es la especie tipo del género *Tospovirus,* siendo el único género que afecta a vegetales, ya que los virus de los otros géneros de la familia afectan a mamíferos incluyendo humanos (Briese et al. 2013, Granval y Gracia 1999, King et al. 2011, Rodríguez et al. 2007).

7. Viriones y genoma viral

7.1. Estructura de las partículas virales

Las partículas virales o viriones del TSWV son casi esféricas, con un diámetro que oscila entre 80 y 120 nm (Best y Palk 1964, Sin 2004). Están rodeadas por una envuelta lipídica procedente del huésped que presenta en la superficie las glicoproteínas virales G_N y G_C (Figura 4) (de Haan et al. 1989, de Haan et al. 1991, King et al. 2011). En el interior hay tres segmentos de RNA denominados L, M y S. Cada RNA presenta secuencias parcialmente complementarias entre los extremos 5' y 3' lo que les permite adoptar conformaciones pseudocirculares o de tipo *panhandle* (Parrella et al. 2003). Estas regiones terminales están muy conservadas y constituyen importantes señales para la transcripción y replicación del virus. Además, cada segmento de RNA está encapsidado por múltiples copias de la proteína de la nucleocápsida (N) y asociado a un número bajo de moléculas de la proteína L (la RNA polimerasa dependiente de RNA o RdRp) para formar estructuras ribonucleoproteícas denominadas nucleocápsidas.
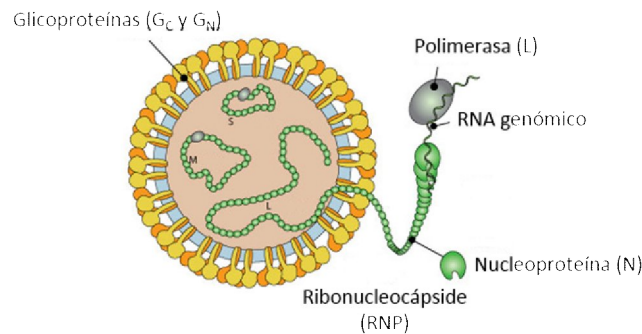
Figura 4. Estructura de una partícula del TSWV, donde se observan los tres fragmentos de RNA viral L, M y S. G_N y G_C, glicoproteínas; N, proteína de la cápside y L, RNA polimerasa dependiente de RNA. Fuente: http://viralzone.expasy.org/all_by_species/253.html.

7.2. Estructura y funciones del genoma

El genoma del TSWV consta de tres segmentos de RNA de cadena simple y de polaridad negativa o ambisentido, denominados L (de la palabra inglesa Large), M (Medium) y S (Small) (Figura 5). En total, el genoma posee cinco marcos de lectura abierta (ORFs, *Open Reading Frames*) con capacidad para codificar seis proteínas.

El RNA del segmento L, con un tamaño de 8,9 kb, es de polaridad negativa y monocistrónico y en la hebra complementaria codifica la RdRp de 331 kDa (de Haan et al. 1991, Granval y Gracia 1999, Kormelink et al. 1992). Los RNAs de los segmentos M y S son de polaridad ambisentido y cada uno de ellos contiene dos marcos de lectura abierta separados por una región intergénica rica en adeninas y uracilos (A + U) y de tamaño variable. El RNA del segmento M tiene un tamaño de 4,9 kb y en el extremo 5´ de la cadena viral codifica la proteína no estructural NSm de 33,6 kDa, implicada en el

movimiento viral célula a célula (Kormelink et al. 1992, Sin 2004), mientras que la hebra complementaria codifica la poliproteína de 127,4 kDa precursora de las glicoproteínas G_N (58 kDa) y G_C (78 kDa). Estas proteínas están implicadas en la maduración y en el ensamblaje de los viriones tanto en las plantas como en los trips y en la transmisión por trips (Adkins 2000).
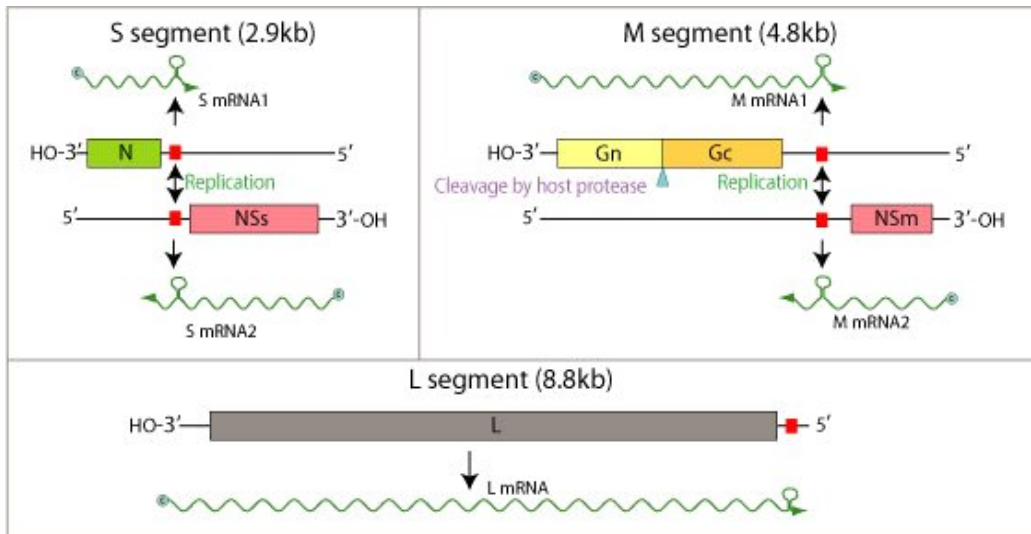
Figura 5. Organización genómica y estrategia de expresión del TSWV. vRNA representa la forma predominante del RNA en el virión. vcRNA representa la hebra complementaria. Los rectángulos de colores sobre el RNA indican los marcos de lectura abierta expresados a partir del vRNA o del vcRNA.

Por último, el RNA del segmento S tiene un tamaño de 2,9 kb y en el extremo 5´ de la cadena viral codifica la proteína no estructural NSs de 52,4 kDa, responsable de la inducción de síntomas y superación del mecanismo de defensa de la planta basado en el silenciamiento del RNA (Takeda et al. 2002), mientras que la hebra complementaria codifica la proteína N de la nucleocápsida de 28,8 kDa, que encapsida el RNA viral y, posiblemente, facilita el movimiento del virus a larga distancia.(de Haan et al. 1990, Kormelink et al. 1991). Los cinco genes virales se expresan a través de RNAs mensajeros que presentan una estructura tipo capezura (cap) en el extremo 5'.

8. Emergencia y evolución del virus

Los virus de plantas causan cada año importantes pérdidas económicas en los cultivos agrícolas de todo el mundo. El mayor impacto lo provocan las enfermedades emergentes, definidas como aquéllas cuya incidencia aumenta debido a cambios duraderos en su epidemiología tras invadir una población huésped, y su impacto en la población huésped suele ser particularmente grave. Al igual que en las enfermedades de humanos y animales, las enfermedades de las plantas más destructivas son las emergentes (Elena et al. 2014, García-Arenal 2013, Woolhouse 2002).

La estructura y los cambios genéticos de las poblaciones virales y su evolución está determinada por la interacción de las cinco principales fuerzas evolutivas: la mutación, el intercambio genético (recombinación y reordenamiento de segmentos genómicos), la selección natural, la deriva genética y la migración (Moya et al. 2004). La mutación y el intercambio genético entre aislados divergentes son generadores de diversidad genética mientras la selección natural, deriva genética y flujo genético limitan y modulan la diversidad genética de las poblaciones virales. El conocimiento de los factores implicados en la evolución de los virus es crucial para entender los procesos implicados en la biología molecular y la epidemiología (incluido la emergencia de nuevas virosis), así como para desarrollar estrategias de control que sean más eficaces y duraderas (García-Arenal et al. 2001, Moya et al. 2004).

Los virus con genomas de RNA tienen un gran potencial para la variación genética y para la evolución y adaptación a las nuevas condiciones y ambientes debido a sus altas tasas de mutación (10^5 veces más altas que las de sus huéspedes), debido a la ausencia de actividad correctora de la RNA polimerasa, y debido a la capacidad de replicarse rápidamente y generar poblaciones muy grandes (García-Arenal et al. 2001). El intercambio genético es un proceso importante en los ribovirus, especialmente en los de cadena positiva (Nagy 2008) y se postula que además de aumentar la diversidad genética tiene un papel importante en la eliminación de variantes genéticas deletéreas.

Las poblaciones del TSWV presentan una variabilidad genética moderada dentro de los virus de plantas (García-Arenal et al. 2001, López et al. 2011, Tsompana et al. 2005). A pesar de ello, el TSWV ha mostrado tener una gran capacidad de adaptación y ser capaz de superar las resistencias genéticas que se han usado para su control (López et al. 2011, Margaria et al. 2007, Tentchev et al. 2011). Así mismo, se han descrito eventos de recombinación y reordenamiento en aislados naturales del TSWV (Lian et al. 2013, Margaria et al. 2014, Tentchev et al. 2011).

9. Diagnóstico y cuantificación del TSWV

Para estudiar la epidemiología del virus, así como para desarrollar y aplicar estrategias de control de la enfermedad es necesario disponer de métodos de detección rápidos, sensibles y específicos. Tradicionalmente se han utilizado técnicas de diagnóstico costosas y poco específicas. Una de ellas es la transmisión mecánica del virus a plantas testigo, siendo *Petunia hybrida Vilm* una de las más utilizadas pos sus característicos síntomas, consistentes en pequeñas lesiones locales marrones o negras, que se observan en tan sólo dos días después de la inoculación (Roselló et al. 1996). También ha sido muy común la transmisión del virus mediante trips, pero el difícil manejo de estos insectos la convierte en una alternativa poco práctica y restringe su uso como método de diagnóstico (Rodríguez 2007). Por otra parte, la morfología propia del virus y las inclusiones citoplasmáticas asociadas a la infección permiten la identificación mediante microscopía electrónica, aunque solamente a nivel de género. Además se trata de una técnica muy laboriosa y costosa que requiere de personal especializado lo que limita su aplicabilidad (Rodríguez 2007).

La purificación de las proteínas N y G_N (Verkleij y Peters 1983) permitió la producción de anticuerpos policlonales que dieron lugar a la utilización de métodos serológicos. En 1989, Sherwood y colaboradores desarrollaron el primer anticuerpo monoclonal para detectar virus de plantas con envoltura lipídica. En este momento, el uso de antisueros de buena calidad y técnicas inmunoenzimáticas con mayor sensibilidad permitieron la diferenciación y caracterización de las distintas especies del género *Tospovirus* (Granval and Gracia 1999). La técnica serológica DAS-ELISA (*Double antibody sándwich Enzyme-Linked Immuno Sorbent Assay*) es la más utilizada actualmente para la detección del TSWV (Cho et al. 1988, Huguenot et al. 1990, Wu et al. 2009). Se basa en la detección de la proteína de la cápsida del virus, inmovilizada sobre una fase sólida mediante anticuerpos conjugados con una enzima, la cual hidroliza un sustrato dando lugar a un producto coloreado cuya acumulación puede ser medida espectrofotométricamente. Los anticuerpos pueden ser policlonales (Cho et al. 1988, Ghotbi et al. 2005) o monoclonales (Huguenot et al. 1990, Wu et al. 2009). La técnica de ELISA presenta como ventaja que es un método fiable, específico, económico, permite procesar y analizar simultáneamente un gran número de muestras y se puede llevar a cabo una cuantificación relativa del título viral.

Para la detección del TSWV por hibridación molecular se han desarrollado distintas técnicas (Huguenot et al. 1990, Ronco et al. 1989, Saldarelli et al. 1996). Se basan en la capacidad de apareamiento específico entre dos cadenas sencillas de DNA o RNA, de manera que se puede sintetizar una sonda correspondiente a una zona del genoma del virus que esté marcada radiactivamente, o conjugada con un fluoróforo o con enzimas que den reacciones colorimétricas o quimioluminiscentes, cuya intensidad es proporcional a la cantidad de sonda. Al igual que con la técnica ELISA, se pueden analizar simultáneamente muchas muestras y realizar una cuantificación relativa, aunque es más versátil ya que se pueden obtener diferentes grados de especificidad modificando las condiciones o sintetizando las sondas a partir de las diferentes zonas genómicas.

Para la detección de TSWV, también, se han desarrollado varias modalidades de RT-PCR (*Reverse Transcriptase-Polymerase Chain Reaction*), tanto cualitativas como cuantitativas (Mason et al. 2003, Mumford et al. 1994, Mumford et al. 1996, Weekes et al. 1996). Esta técnica se basa en producir primero DNA a partir de RNA con una transcriptasa reversa y luego amplificar un fragmento de DNA usando una DNA polimerasa mediante ciclos cortos de desnaturalización y renaturalización, de manera que se producen un gran número de copias de dicho fragmento de DNA que se pueden detectar mediante electroforesis, fluoróforos o técnicas colorimétricas. Este método también es muy sensible y versátil, pudiéndose llevar a cabo un gran control de la especificidad a partir del diseño de iniciadores. Sin embargo, su gran sensibilidad se convierte a veces en un inconveniente, por la facilidad de detectar falsos positivos debido a contaminaciones de restos de antiguos productos de RT-PCR.

La RT-PCR en tiempo real o cuantitativa (RT-qPCR) es un método altamente sensible que permite una cuantificación absoluta y relativa del RNA viral. La técnica detecta el producto amplificado en cada ciclo, por lo que evita la manipulación de la

muestra una vez finalizada la PCR y se reduce significativamente el riesgo de contaminación. La posibilidad de obtener una estimación del número de copias de RNA diana presente en una muestra, hace de la RT-PCR a tiempo real una herramienta indispensable y poderosa para estudiar diferentes aspectos de la biología del virus. Los productos de RT-PCR a tiempo real más ampliamente utilizados se basan en dos químicas diferentes: en una de ellas, el DNA sintetizado es detectado directamente con el fluoróforo SYBR®Green I que se une con gran afinidad al surco menor del DNA bicatenario y produce resultados muy precisos en la cuantificación del producto, mientras que en la otra (sondas TaqMan), el DNA hibrida con una sonda marcada con un fluoróforo donante (*reporter*) en el extremo 5', que emite fluorescencia al ser excitado, y una aceptor (*quencher*) en el extremo 3', que absorbe la fluorescencia liberada por el donante. Para que esto ocurra, las moléculas donante y aceptora deben estar espacialmente próximas y el espectro de emisión de la primera se ha de solapar con el espectro de absorción de la segunda. Mientas la sonda está intacta, la fluorescencia emitida por el donante es absorbida por el aceptor. Durante la amplificación de DNA, la Taq DNA polimerasa se desplaza a lo largo de la cadena en su acción de síntesis y su actividad 5' exonucleasa, hidroliza el extremo 5' de la sonda, separando el fluorocromo donante del aceptor, por lo que la fluorescencia del primero no se absorbe y puede ser medida (Bustin 2004). Un tipo de sonda TaqMan es la MGB (*Minor Groove Binder*) de menor tamaño (13-20 nt) lo que permite un mayor control de la especificidad (Kutyavin et al. 2000). La cantidad de diana puede ser determinada mediante una cuantificación absoluta o relativa. En la primera, el número de moléculas de ácido nucleico diana presentes en una muestra se determina utilizando una curva estándar construida a partir de la amplificación previa de concentraciones conocidas de la diana. En cambio, en la segunda, la cantidad relativa de ácidos nucleicos diana en diferentes muestras se compara con un control interno, aunque no se proporciona ningún dato sobre el número real de copias diana presentes en cada muestra.

Se han desarollado varias tecnicas de RT-qPCR con las que se pueden detectar y cuantificar (de manera relativa) el segmento genómico S de TSWV en plantas y trips (Boonham et al. 2002, Dietzgen et al. 2005, Roberts et al. 2000) pero el diseño de iniciadores y sondas se basaba solamente en unas pocas secuencias y la RT-qPCR no funciona para todos los aislados de TSWV.

10. Métodos de control de la enfermedad

El control del TSWV presenta dificultades por el amplio rango de huéspedes tanto para el virus como los vectores y por la gran eficacia de los vectores en la transmisión. Como no se ha encontrado ninguna estrategia totalmente efectiva, el control debe basarse en una combinación de medidas culturales y métodos preventivos que, aunque por sí solo no son capaces de controlar la enfermedad, pueden disminuir su incidencia, para lo cual es importante conocer los factores implicados en la epidemiología del virus. Estos métodos se basan en impedir o reducir la propagación del virus, mediante la eliminación de material vegetal infectado tanto de los cultivos como de las malas

hierbas cercanas que pudieran actuar de reservorios, la inclusión de distancias de seguridad en los cultivos, la evitación de solapamiento de cultivos susceptibles o la utilización de cultivos no susceptibles como barrera (Coutts et al. 2005).

El control del transporte del material vegetal, mediante servicios de cuarentena y certificación y el control de los vectores de transmisión del virus aplicando métodos biológicos, químicos y físicos son también estrategias integradas que favorecen el control del TSWV. En cuanto a los medios físicos destacan el uso de acolchados reflectantes de radiación ultravioleta, la utilización de mallas finas en puertas y ventanas de los invernaderos y la utilización de placas pegajosas, las cuales permiten detectar precozmente la presencia de trips en los invernaderos y adoptar otras medidas de control rápidamente. Los tratamientos químicos, que implican la utilización de insecticidas se han mostrado por sí solos poco eficaces, debido a que las poblaciones del vector son poco sensibles a estos productos y a su hábito de vivir dentro de las flores (Rodríguez et al. 2007). Además, se ha descrito la aparición en los trips de resistencias a insecticidas. A pesar de ello, la utilización combinada de insecticidas con las medidas culturales descritas anteriormente han permitido alterar las poblaciones de trips y disminuir la incidencia del virus (Fanigliulo et al. 2007). El control biológico de los trips a través de la utilización de insectos depredadores como los antocóridos del género *Orius* (Figura 6) y diferentes especies de míridos (*Dicyphus tamaninii* y *Macrolophus caliginosus*) es una alternativa cada vez más extendida en toda Europa y, principalmente, en los cultivos hortícolas del área Mediterránea (Bosco et al. 2008).

Figura 6. Depredadores de trips: antocóridos del género Orius
(http://www4.gipuzkoa.net/Corporac/Agricultura/Manzanos/eus/05_c.asp?id=52)

En cualquier caso, ninguno de estos métodos resulta por sí solo totalmente eficiente, por lo que la mejor estrategia a largo plazo para controlar la enfermedad consiste en el desarrollo de variedades con resistencias al TSWV obtenidas mediante mejora genética o mediante ingeniería genética.

El método más efectivo de control ha sido el desarrollo de variedades con resistencias mediante mejora genética o la obtención de resistencia derivada del patógeno mediante ingeniería genética (Roselló et al. 1996). En las últimas décadas se

ha hecho un gran esfuerzo por todo el mundo para la obtención de variedades resistentes de varios cultivos. Se han encontrado varias fuentes de resistencia en tomate y pimiento, pero generalmente solamente eran efectivos con un limitado número de aislados del TSWV. La primera de ellas se detectó en *Lycopersicum pimpinellifolium* (Samuel et al. 1930), y estaba controlada por cinco genes (1a, 1b, 2, 3 y 4) que individualmente conferían resistencia, aunque rápidamente algunos aislados fueron capaces de superarla. En tomate, la resistencia más eficaz se encontró en *Solanum peruvianum*, proporcionada por el gen dominante *Sw-5*, que se caracteriza por desencadenar una respuesta de hipersensibilidad en la hoja que provoca la necrosis del tejido e impide la progresión del virus (Aramburu et al. 2000). El coeficiente de penetración de este gen se ha estimado en un 98,7% lo que determina que un pequeño porcentaje de plantas, inferior al 2%, pueda llegar a infectarse. La resistencia se muestra eficaz en toda la planta salvo en el fruto, donde la alimentación directa de trips infectivos induce la formación de lesiones locales que no son capaces de detener la progresión del virus. (Aramburu et al. 2007). Además, esta resistencia se ha visto superada por diversos aislados virales descritos, sucesivamente, en Hawai, Australia, Sudáfrica, España e Italia (Ciuffo et al. 2005, Margaria et al. 2004, Margaria et al. 2014, Melzer et al. 2012, Sharman y Persley 2006, Sivparsad y Gubba 2008). Los determinantes genéticos responsables de la superación de dicha resistencia se han asociado a un cambio aminoacídico en la proteína de movimiento NSm (Hallwass et al. 2014, López et al. 2011, Peiró et al. 2014). Por otra parte, también es superada por TSWV como consecuencia de la interacción de tipo sinérgico en infecciones mixtas con el virus de la clorosis del tomate (*Tomato chlorosis virus*, ToCV) (García-Cano et al. 2006). En pimiento se ha introducido la resistencia natural encontrada en *Capsicum chinense*, proporcionada por el gen dominante *Tsw*, que desencadena, igual que el gen *Sw-5*, una reacción de hipersensibilidad que impide la progresión del virus. Sin embargo, la eficacia de este gen puede ser afectada por factores como la cantidad de inóculo a la que están sometidas las plantas, el estadio fenológico de éstas, el aumento de la temperatura (Aramburu et al. 2007) y, sobretodo, la aparición de nuevos aislados virales capaces de superar dicha resistencia (Margaria et al. 2004, Roggero et al. 2002, Sharman y Persley 2006, Thomas-Carroll y Jones 2003), lo que constituye nuevos casos de reemergencia del virus. Los determinantes genéticos responsables de la superación de esta resistencia se localizan en el segmento S del genoma viral, concretamente en la proteína no estructural NSs (de Ronde et al. 2013, Tentchev et al. 2011). Desafortunadamente, hasta la fecha no se han podido identificar fuentes de resistencia efectiva en otros cultivos (Pappu et al. 2009).

La utilización de plantas transgénicas para controlar la enfermedad causada por el TSWV es otra alternativa que está muy desarrollada aunque actualmente no tenga una utilidad práctica. La expresión constitutiva del gen de la proteína de la cápsida (N) viral del TSWV ha permitido incorporar ciertos niveles de resistencia (Rodríguez et al. 2007). La utilización de construcciones sentido-antisentido para la producción de transcritos de RNA bicatenario también ha permitido obtener plantas resistentes a diversos virus incluido el TSWV (Bucher et al. 2006). No obstante, las reticencias populares y los impedimentos legales para la utilización de productos transgénicos, en

especial los dedicados al consumo humano, no han favorecido su implantación comercial y no parece previsible que lo hagan a corto plazo, por tanto, la utilización de resistencias naturales parece la solución más práctica para combatir la enfermedad.

JUSTIFICACIÓN Y OBJETIVOS

Los virus causan cada año considerables pérdidas económicas en cultivos agrícolas en todo el mundo. El mayor impacto de las enfermedades virales ocurre en los procesos de emergencia, en los que se produce un incremento exponencial de la incidencia del virus. La emergencia de virosis es cada vez más frecuente debido a varios factores: a) el gran tráfico internacional de material vegetal que lleva virus o vectores de virus en nuevas áreas, b) el cambio climático que influye en el área de distribución de plantas e insectos vectores, c) la agricultura intensiva con alta densidad de población de plantas con reducida diversidad genética que las hace altamente susceptibles a nuevas enfermedades, y d) la gran capacidad de los virus para una rápida evolución y adaptación. Actualmente, el control de las enfermedades virales se basa en medidas profilácticas encaminadas a impedir o dificultar la dispersión de los virus y en el cultivo de variedades resistentes obtenidas por mejora genética o ingeniería genética. Para poder aplicar estas medidas de control de la enfermedad es necesario por una parte estudiar los factores implicados en la epidemiología, emergencia y evolución de los virus y por otra parte desarollar técnicas moleculares para la detección específica y cuantificación del virus.

El virus del bronceado del tomate (*Tomato spotted wilt virus*, TSWV) es uno de los virus más dañinos en la agricultura. La protección más eficaz se ha conseguido utilizando variedades con los genes de resistencia *Sw-5* en tomate y *Tsw* en pimiento. Sin embargo, en varios países, incluido España, han aparecido aislados capaces de superar dichas resistencia, lo que constituye un caso de reemergencia del virus.

En esta tesis doctoral se ha abordado la determinación de la secuencia nucleotídica del genoma de aislados del TSWV incapaces o capaces de superar las resistencias proporcionadas por los genes *Sw-5* de tomate y *Tsw* de pimiento. Con el análisis *in silico* de estas secuencias y las que se encuentran en la base de datos GenBank se pretende: A) la evaluación de la variabilidad genética y biológica, que se tengan en cuenta en programas de mejora genética, y buscar un marcador molecular de la superación de estas resistencias; y B) el desarrollo de un método de detección y cuantificación del virus basado en RT-PCR cuantitativa en tiempo real. Así mismo, la cuantificación del virus posibilita: A) la evaluación y comparación de la eficacia biológica (fitness) de distintos aislados virales en la multiplicación en planta y en su transmisión por trips, lo que proporciona información sobre la capacidad de dispersión de los aislados que superan las resistencias; y B) la evaluación de la capacidad de resistencia y tolerancia de nuevas accesiones o variedades de tomate y pimiento contra los aislados del TSWV convencionales y los que superan las resistencias de los genes *Sw-5* y *Tsw*.

Los objetivos concretos de esta tesis doctoral son:

1. Determinación de la secuencia nucleotídica del genoma completo de tres aislados del TSWV: uno convencional, y dos que superan resistencias, *Sw-5* de tomate y *Tsw* de pimiento.

2. Desarrollo de un método de detección, discriminación y cuantificación de aislados del TSWV basado en RT-PCR cuantitativa en tiempo real. Aplicación a la evaluación de la multiplicación del virus en planta.

3. Evaluación de la transmission de aislados del TSWV, que superan y no superan las resistencias *Sw-5* de tomate y *Tsw* de pimiento, por su principal vector, el trips *Frankliniella occidentalis*.

4. Evaluación de la resistencia y tolerancia de una nueva accession de *Capsicum baccatum* contra aislados de TSWV que superan y no superan la resistencia *Tsw* de pimiento.

CAPÍTULO 1

Determinación de la secuencia nucleotídica del genoma completo de tres biotipos de TSWV: convencional, capaz de superar la resistencia *Sw-5* de tomate y capaz de superar la resisencia *Tsw* de pimiento.

Abstract

Tomato spotted wilt virus (TSWV) occurs worldwide and causes production losses in many important horticultural crops such as tomato and pepper. Breeding resistant cultivars has been the most successful method so far for TSWV disease control, but only two resistance genes have been found to confer resistance against a wide spectrum of TSWV isolates: *Sw-5* in tomato and *Tsw* in pepper. However, TSWV resistance-breaking isolates have emerged in different countries a few years after using resistant cultivars. In this paper we report the first complete nucleotide sequences of three Spanish TSWV isolates with different biotypes according to their abilities to overcoming resistance: LL-N.05 (wild type, WT), Pujol1TL3 (*Sw-5* resistance breaking, SBR) and PVR (*Tsw* resistance-breaking, TBR). The genome of these TSWV isolates consisted of three genomic segments: L (8913-8914 nt), M (4752-4825 nt) and S (2924-2961 nt). Variations in nucleotide sequences and genomic RNA lengths among the different virus biotypes are reported here. Phylogenetic analysis of the five TSWV open reading frames showed evidence of reassortment between genomic segments of LL-N.05 and Pujol1TL3, which was supported by analysis with different recombination-detecting algorithms.

Tomato spotted wilt virus (TSWV) is the type member of the genus *Tospovirus* which contains the only plant-infecting members of the family *Bunyaviridae* (Plyusnin et al. 2012). TSWV has a wide hosts range including more than 1000 species among weed species, ornamental and horticultural crops such as pepper (*Capsicum annuum*), potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*), peanut (*Arachis hypogaea*), lettuce (*Lactuca sativa*) and bean (*Phaseolus vulgaris*) (Hanssen et al. 2010, Pappu et al. 2009, Turina et al. 2012). The virus is naturally transmitted by several thrips species (*Thysanoptera: Thripidae)* in a persistent and propagative manner with *Frankliniella occidentalis* (Pergande) being its main vector (Debreczeni et al. 2014, Whitfield et al. 2005).

TSWV virions are quasi-spherical particles composed of an outer membrane envelope derived from the host with two embedded viral-coded glycoproteins (G_N and G_C). Virions contain several copies of the RNA dependent RNA polymerase (RdRp) and nucleoproteins which encapsidate three negative-sense or ambisense genomic RNA segments: segment L (~9 kb) encodes the RdRp; segment M (~5 kb) encodes the cell-to-cell movement protein (NSm) and a precursor of the surface glycoproteins (G_N/G_C) involved in TSWV transmission by thrips; and segment S (~3 kb) encodes a silencing suppressor (NSs) and the nucleocapsid protein (N) (Plyusnin et al. 2012).

TSWV is one of the most harmful plant viral pathogens, ranking second in the list of the most important plant viruses worldwide (Scholthof et al. 2011). Eradication or control of TSWV has proven to be difficult, while breeding for resistance is the most effective strategy for disease control. So far, only two genes, *Sw-5* introgressed in tomato (*Solanum lycopersicum*) and *Tsw* in pepper (*Capsicum annuum*), have conferred resistance against a wide spectrum of TSWV isolates (Pappu et al. 2009). However, resistance-breaking TSWV isolates have been reported in several countries after a few years of using these resistant cultivars harboring these genes (López et al. 2011, Margaria et al. 2007). Based on the ability of the virus to overcome the resistance conferred by these genes, TSWV isolates are classified in three biotypes: wild type (WT), which cannot infect tomato and pepper with the resistance genes *Sw-5* and *Tsw* respectively; *Sw-5* resistance-breaking (SBR), which can infect *Sw-5* resistant tomato but not *Tsw* resistant pepper; and *Tsw* resistance-breaking (TBR), which can infect *Tsw* resistant pepper but not *Sw-5* resistant tomato. Currently, no natural TSWV isolate infecting both *Sw-5* resistant tomato and *Tsw* resistant pepper has been reported.

Inoculation in resistant tomato or pepper of reassortants generated between WT and resistance-breaking isolates showed that the genetic determinants for overcoming tomato *Sw-5* resistance and pepper *Tsw* resistance are located in the M and S segments, respectively (Hoffmann et al. 2001, Jahn et al. 2000). Comparison of nucleotide and amino acid sequences of the M segment from WT and SRB isolates revealed that *Sw-5* resistance breakdown is related to substitutions C118Y or T120N in the TSWV NSm protein (López et al. 2011), which was demonstrated by transient expression of NSm in *Sw-5* resistant plants by using a heterologous viral system (Peiró et al. 2014). Transient expression confirmed that NSs is the avirulence protein triggering resistance in pepper

cultivars carrying the gene *Tsw* (de Ronde et al. 2014a, de Ronde et al. 2013) but the amino acid substations responsible for *Tsw* resistance breakdown remain unidentified.

Presently, no complete sequences of SBR and TBR isolates are available, although partial sequences have been elucidated (López et al. 2011, Margaria et al. 2007, Tentchev et al. 2011). The nucleotide sequences of the complete genomes has been determined for 17 WT isolates (Table 1): one from Brazil (de Haan et al. 1991, de Haan et al. 1990, Kormelink et al. 1992), two from China (Hu et al. 2011), 14 from South Korea (Lee et al. 2011, Lian et al. 2013) and two from Italy (Margaria et al. 2014). Here, we report the complete sequences of three isolates from Spain, corresponding to WT, SBR or TBR biotypes (Table 1).

Table 1. TSWV isolates whose genomes have been completely sequenced

Isolate	Origin	Biotype[a]	Host	GenBank accession number		
				L segment	M segment	S segment
LL-N.05	Spain	WT	Tomato	KP008128	FM163373	KP008129
Pujol1TL3	Spain	SBR	Tomato	KP008130	HM015520	KP008131
PVR	Spain	TBR	Pepper	KP008132	KP008133	KP008134
p105	Italy	WT	Pepper	KJ575620	KJ575621	DQ376178
p202/3wt	Italy	WT	Pepper	KJ575619	HQ830188	HQ830187
BR01	Brazil	WT	Tomato	NC_002052	NC_002050	NC_002051
YN	China	WT	Tomato	JF960237	JF960236	JF960235
CG-1	China	WT	Lettuce	JN664254	JN664253	JN664252
SK2004[b]	South Korea	WT	NP[b]	AB190813	AB190818	AB190819
NJ-JN	South Korea	WT	Tomato	HM581934	HM581935	HM581936
CY-CN1	South Korea	WT	Pepper1	HM581937	HM581938	HM581939
CY-CN2	South Korea	WT	Pepper2	HM581940	HM581941	HM581942
TSWV-4	South Korea	WT	Pepper	KC261947	KC261948	KC261949
TSWV-5	South Korea	WT	Stellaria aquatica	KC261950	KC261951	KC261952
TSWV-6	South Korea	WT	Stellaria media	KC261953	KC261954	KC261955
TSWV-7	South Korea	WT	Pepper	KC261956	KC261957	KC261958
TSWV-8	South Korea	WT	Lactuca indica	KC261959	KC261960	KC261961
TSWV-10	South Korea	WT	Stellaria_aquatica	KC261962	KC261963	KC261964
TSWV-12	South Korea	WT	Lettuce	KC261965	KC261966	KC261967
TSWV-16	South Korea	WT	Tomato	KC261968	KC261969	KC261970
TSWV-17	South Korea	WT	Stellaria media	KC261971	KC261972	KC261973
TSWV-18	South Korea	WT	Chrysanthemum	KC261974	KC261975	KC261976

[a]Biotypes: WT: wild type. SBR: tomato Sw-5 resistance-breaking. TBR: pepper Tsw resistance-breaking.
[b]The name and host of this isolate is not published (NP). The isolate was named in this work as SK2004.

Samples from tomato with and without *Sw-5* gene and from pepper with *Tsw* gene were collected in Canary Islands, North-Eastern and South-Eastern peninsular Spain. To obtain pure WT or resistance-breaking TSWV isolates free of other viruses, the samples were tested for TSWV and other viruses by ELISA and those infected by only TSWV were biologically cloned by mechanical inoculation in *Nicotiana glutinosa* to produce local lesions which were used to inoculate *Datura stramonium* (Debreczeni et al. 2014). To determine the biotype (WT, SBR or TBR), each TSWV isolate was introduced in tomato cultivars: 'Verdi' (Fitó), with *Sw-5*, and 'Marmande' (Fitó) without *Sw-5*; and pepper cultivars 'Spiro' and 'Divino' (Seminis), both with *Tsw*; and

'C804' (Fitó), without *Tsw*. For sequencing the complete genome, one isolate of each biotype was selected: LL-N.05, Pujol1TL3 and PVR corresponding to biotypes WT, SBR and TBR, respectively (Table 1). Total RNAs were purified from *D. stramonium* plants infected by these TSWV isolates by a protocol involving phenol/chloroform/isoamyl-alcohol extraction followed by isopropanol precipitation (Debreczeni et al. 2011). RT-PCR was performed with conserved primers (Supplementary Table S1) to amplify the complete S segment (primers S1 / S7), two overlapping fragments comprising the complete M segment (primers M1 / M7 and M6 / M12), and two overlapping fragments comprising the complete L segment (primers 1L_f / 3L_r and 2L_f / 1L_r). For RT-PCR, total RNA (~5 μg) was denatured by heating at 65ºC and chilling quickly on ice. First-strand cDNA synthesis was performed in a 20 μl reaction mixture containing the denatured RNA, 5 x first strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$), 1 mM DTT, 200 μM each of dNTPs, 0.4 μM of each primer, 40 U of RNAse OUT inhibitor and 100 U of SuperScript^{TM} II Reverse Transcriptase (Invitrogen) and incubating at 42ºC for 50 min. The reaction was inactivated by heating at 70°C for 15 min. Removal of RNA complementary to the cDNA was performed by adding 2 U of *Escherichia coli* RNase H (Invitrogen) and incubating at 37°C for 20 min. PCR was performed in a 50 μl reaction containing 5 x iProof HF buffer (BIORAD), 0.2 mM $MgCl_2$, 3% DMSO, 200 μM of each dNTP, 0.2 μM of each specific primer, and 1 U of iProof High-Fidelity DNA polymerase (BIORAD). The PCR conditions were: 1 cycle at 98 ºC for 30 s, followed by 5 cycles at 98 ºC for 10 s, 60 ºC for 20 s and 72 ºC for 30 s/kb, next, 30 cycles at 98 ºC for 10 s, 65 ºC for 20 s and 72 ºC for 30 s/kb, and a final extension step of 72 ºC for 10 min. Nucleotide sequences were determined with primers encompassing overlapping regions of about 1 kb covering the whole genome (Supplementary Table S1) by using a ABI 3130XL Genetic Analyzer (Life Technologies, USA).

The 5'- and 3'- terminal sequences were determined using the 5'/3' RACE Kit 2nd Generation (Roche). To avoid errors associated with RT-PCR and sequencing, each genomic region was amplified and sequenced at least twice in both directions.

The pool of sequences was assembled with Vector NTI (Lu and Moriyama 2004). The complete nucleotide sequences of segments L and S of TSWV isolates LL-N.05, Pujol1TL3 and PVR and segment M of PVR were deposited in the GenBank database under accession numbers KP008128-KP008134 and nucleotide sequences of segment M of isolates LL-N.05 and Pujol1TL3 were retrieved from GenBank under accession numbers FM163373 and HM015520, respectively (Table 1).

The three TSWV isolates LL-N.05, Pujol1TL3 and PVR have the typical genome organization of TSWV. The sizes of L, M and S segments were 8913-8914 nt, 4752-4825 nt and 2924-2961 nt, respectively. The length of the open reading frames (ORFs) and 5'- and 3'- untranslated regions (UTRs) were identical or almost identical for the three isolates: RdRP (8640 nt), NSm (909 nt), G_N/G_C (3408), NSs (1404 nt), and N (777 nt), L segment 5'-UTR (33 nt) and 3'-UTR (240-241 nt), M segment 5'-UTR (100 nt) and 3'-UTR (84 nt), and S segment 5'-UTR (88 nt) and 3'-UTR (151 nt).

However, the intergenic regions (IR) had different sizes for each isolate: M segment IR had 251, 324 or 280 nt and S segment IR 504, 541 and 531 nt, for LL-N.05, Pujol1TL3 or PVR, respectively. This shows that differences in RNA size were mostly caused by insertions and/or deletions of nucleotide sequences within the IR.

The complete sequences of isolates LL-N.05, Pujol1TL3 and PVR were aligned and compared with all complete sequences available for TSWV retrieved from GenBank (Table 1) by using the algorithm CLUSTALW implemented in the program MEGA V6.0 (Tamura et al. 2013). Nucleotide and amino acid sequence identities for different genomic regions between TSWV isolates were calculated from the p-distance (proportion of distinct nucleotides between two sequences) with MEGA V6.0 as $(1 - p\text{-}distance) \times 100$. Nucleotide identities between isolates LL-N.05, Pujol1TL3 and PVR and the others varied depending of the genomic region and isolates ranging from 85.2 to 100% for the non-coding regions and from 93.1 to 99.3% for the ORFs.

Phylogenetic trees of the nucleotide sequences of the five TSWV ORFs were inferred by the maximum likelihood (ML) method with the nucleotide substitution model best fit for each ORF and 1000 bootstrap replicates to estimate the statistical significance of each node, using the program MEGA V6.0. The phylogenetic relationships among the Spanish TSWV isolates and with the other TSWV isolates were very similar for the two ORFs within each segment (NSm and G_N/G_C of M segment and NSs and N of S segment) whereas were different for genomic segments L, M and S (Fig. 1), suggesting reassortment but no recombination. Thus, for ORF RdRp (L segment) isolate LL-N.05 was very close to isolate Pujol1TL3 and separated from isolate PVR, whereas for ORFs NSm and G_N/G_C (M segment) the three isolates were in different clades and for ORFs NSs and N (S segment) LL-N.05 was close to PVR and separated from Pujol1TL3. Interestingly, for the five ORFs the Spanish isolate Pujol1TL3 clustered with the Italian isolate p202/3wt and the Korean isolates TSWV-12 and TSWV-17 indicating a common origin and suggesting that reassortment occurred in the ancestor of these isolates before the virus migrated between these countries. The other Italian isolate p105 was also close to the Spanish isolates (PVR for RdRp and LL-N.05 for the other ORFs). Comparison of all isolates showed no correlation between geographic location and genetic relationships (Fig. 1), e.g. for G_N/G_C the Spanish isolate PVR and the Korean TSWV-4 (nucleotide identity 98.8 %) were in the same clade separated from the Spanish isolate Pujol1TL3 and the Korean TSWV-12 (nucleotide identity 98.9 %) which were included in another clade (nucleotide identities between isolates from different clades were lower than 94.0 %). This suggests several events of long distance migration (García-Arenal et al. 2001, Rubio et al. 2013). Potential genetic exchange was analyzed with the algorithms 3SeqBootScan, Chimaera, GENECON, Maxchi, RDP, SiScan, implemented in the RDP4 package (Martin et al. 2010).

RdRp

NSm

NSs

G_N/G_C

N
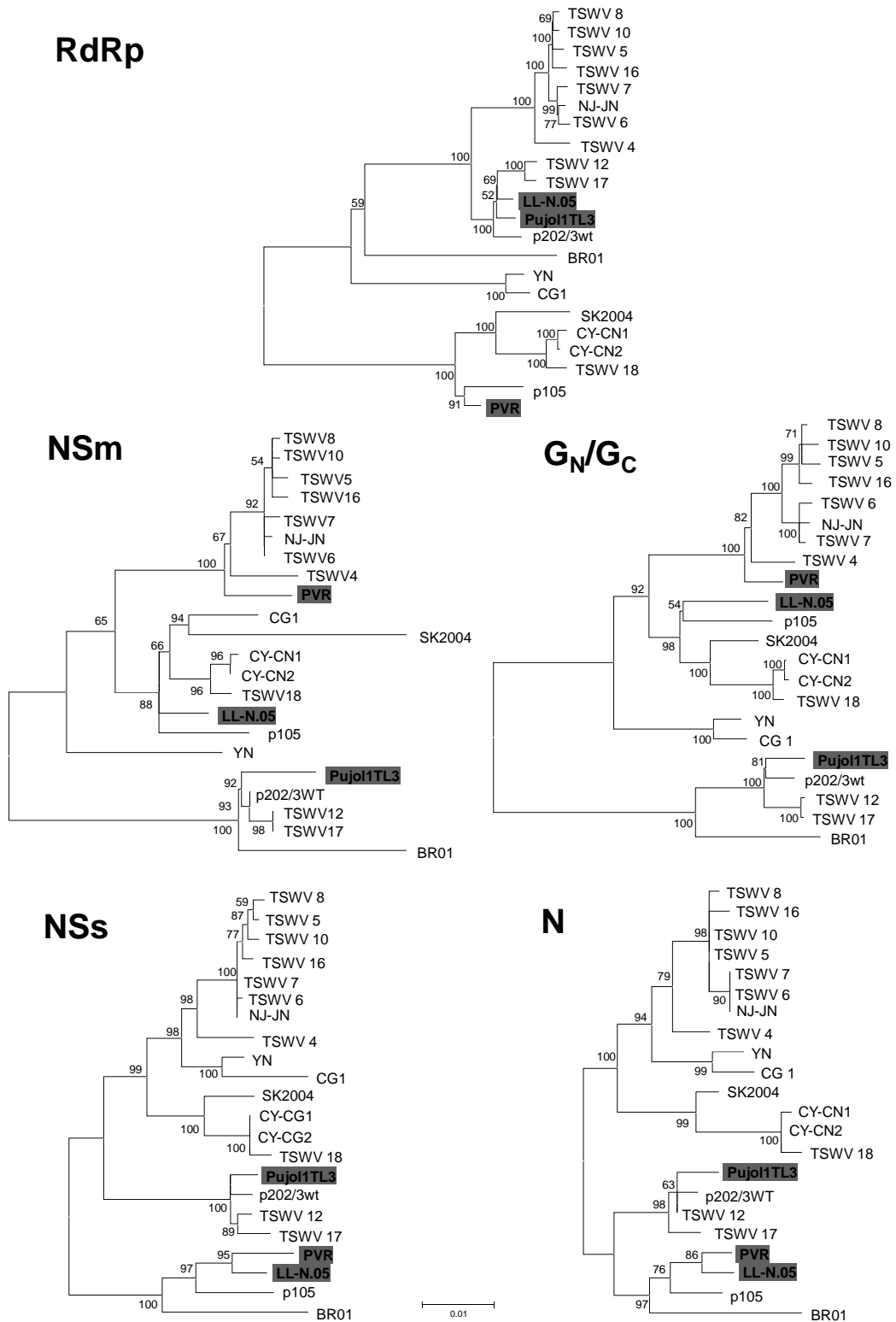
Figure 1. Unrooted maximum likelihood phylogenetic tree of the five ORFs (RdRp, NSm, G$_N$/G$_C$, NSs and N) of Tomato spotted wilt virus (TSWV) isolates whose complete genome has been sequenced (Table 1). Bootstrap values higher or equal to 50% are indicated in the nodes. TSWV isolates sequenced here are shadowed.

Analysis of concatenated sequences of the three genomic segments L, M and S for each isolate revealed, with at least five algorithms, that isolates LL-N.05 and Pujol1TL3 could result from a genetic exchange (likely a reassortment) with the segment L from an ancestor of the Korean isolate TSWV-12 and the segments M and S from ancestors of the Brazilian isolate BR01 or the Italian isolate p105. A likely reassorment involving the L segment has been also observed for the Italian isolates (Margaria et al. 2014); reassortment but no recombination were also found between Asiatic and European isolates (Tentchev et al. 2011); and several recombination and reassorment events were detected for the Korean isolates (Lian et al. 2013). Genetic exchange could represent an adaptive advantage (Qiu and Moyer 1999) or result from differences of fitness between genomic segments for different TSWV isolates (Qiu et al. 1998).

The genetic distances among TSWV isolates were uncorrelated to biotypes (resistance-breakdown ability) or plant hosts (Fig. 1 and Table 1). Thus, for ORF NSm (where the mutations responsible for the change of biotype WT to SBR are produced), there are WT isolates with lower nucleotide identity between them (e.g. p202/3wt and CG1, 94.8% identity) than the SBR isolate Pujol1TL3 with other WT isolates (e.g. p202/3wt, 98.9% identity). Likewise, for the ORF NSs (where the mutations responsible for change of biotype WT to TBR occurs) there are WT isolates with lower nucleotide identities (e.g. p105 and TSWV-10, 92.3% identity) than those between the TBR isolate PVR and some WT isolates (e.g. p105, 97.5% identity). This is in agreement with other studies which analyzed the nucleotide sequences of ORFs NSm or NSs from many TSWV isolates (López et al. 2011, Margaria et al. 2007, Tentchev et al. 2011). The probable cause is that resistance-breakdown (adaptation to resistant hosts) seems to have occurred several times independently and involve a few nucleotide changes (positively selected) as for other plant viruses (de Ronde et al. 2014b). Positive selection was identified in the position 118 of NSm (López et al. 2011), responsible of *Sw-5* resistance breakdown, and several sites of NSs although they could not be related to *Tsw* resistance breakdown (Margaria et al. 2007, Tentchev et al. 2011). TSWV adaptation to resistant hosts does not appear to affect its accumulation in thrips and its transmissibility (Debreczeni et al. 2014). With respect to plant hosts, some TSWV isolates were more closely related to isolates from other plant species than isolates from the same plant host. E.g. isolate TSWV-4 from pepper was closely related to isolate TSWV-10 from *Stellaria aquatica*, and isolate TSWV-16 from tomato, but distantly related to isolate CY-CN1 from pepper (Fig. 1).

The complete genome sequences of TSWV isolates with different ability to overcome resistance in tomato and pepper will be useful for further studies to understand emergence and evolution of TSWV adaptation to new hosts genotypes, which can be applied in breeding programs to develop durable and efficient resistance cultivars against TSWV.

Supplementary Table S1. Conserved primers among TSWV isolates used for RT-PCR and sequencing

Segment	Primers[a]	Polarity	Sequence 5'-3'	Position[b]
L	1L_F	+	Agagcaatcaggtaacaacg	1 - 20
	1b	-	Gtttggttatttatgctact	1564-1583
	2a	+	Atgcaaacactcaaagaatcaaat	911-934
	R1000	-	Tttctttctttgtatttgct	1000-1019
	Bf	+	Attattaacaagtttcggga	1501-1520
	2b	-	Cattacgaaatagatatctgccac	2825-2848
	Br	-	Atgctagtatttgctgtggt	2302-2321
	3a	+	Caggtacatgactaaagaaa	2268-2287
	Pol3	+	Taagcacaaatgccaagcc	2888-2906
	Cf	+	Gagaaaaatagattggtgga	3472-3491
	Pol4	-	Aaggtcatagagctcctctg	3692-3711
	2Ld	+	Gagcactttctacgttatctttggacaca	4025-4053
	Cr	-	Gtttgttgagcttttcttag	4285-4304
	3L_r	-	Tctaatactatcattcacttcaccaggaagca	4889-4920
	6a	+	Gtgataagagatttttttgaacaa	5053-5075
	Df	+	Gatagacgaggatgctgttt	5415-5434
	5b	-	Cttttgaccttaacactctatct	5634-5656
	7a	+	Gaacttagagaaaatactggacac	6369-6392
	Dr	-	Cagcagtgtccagtattttc	6378-6397
	Ef	+	Gaagaacaagatgaatcagg	6706-6725
	6b	-	Catagttccagagacataatttcc	6724-6747
	8a	+	Ggatttaatgacacagtagaa	7402-7422
	Er	-	Ctgaaaaaagaggactatac	7467-7486
	7b	-	Gatcatgatccaatcttattctcc	7778-7801
	1L_R	-	Agagcaatcaggtacaactaaaacatataa	8868-8897
M	M1	+	Agagcaatcagtgcatcagaaatatacctattatac	1-36
	M2	+	Gtagatacaaaccatcatatctcaaactgg	365-394
	M3	-	Tctttatcagctctgggtgaatcac	771-795
	M4	+	Caaggtgagacaaatccataggtggcc	1335-1361
	M5	-	Tgatgagtatgctcatgaagaacaac	1638-1663
	M6	+	Caggatcattcaagtttgcaatatttccag	2268-2297
	M7	-	Cttattggggatgtgaagaagcttgg	2566-2591
	M8	+	Gatgttaaccctaaaagagcttcctg	3029-3053
	M9	-	Gtctcaaatgcccatgtctatggctc	3348-3373
	M10	+	Gttataggataattatcttgtgtc	4130-4153
	M11	-	Ccagaggtttatgatgattctgctgag	4579-4065
	M12	-	Agagcaatcagtgcaaacaaaaaccttaatcc	4790-4821
S	S1	+	Agagcaattgtgtcataattttattc	1-26
	S2	-	Gaacctgtgcaaaagatgtgtgag	1113-1135
	S3	+	Tcctggaagatagactttgccag	1195-1217
	S4	-	Ccaaatttggccaaaattgtccctttc	1697-1723
	S5	+	Atttaacacactaagcaagcacaagc	1898-1923
	S6	-	Cattacagtgaaactcttaacaagttc	2038-2064
	S7	-	Agagcaattgtgtcaattttat	2895-2916
	S8	+	Gatcgagatgtgctataatcaagc	601-624

[a]Shadowed primers were used for RT-PCR and the rest for sequencing.
[b]Positions are referenced to TSWV isolate BR01 (Table 1).

CAPÍTULO 2

Desarrollo de un método de detección, discriminación y cuantificación de aislados de TSWV basado en RT-PCR en tiempo real. Aplicación a la evaluación de la multiplicación del virus en planta.

Abstract

A quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR) procedure using a general primer set and three TaqMan®MGB probes was developed for general and genotype-specific detection and quantification of the genomic M segment of *Tomato spotted wilt virus* (TSWV). Standard curves using RNA transcripts homologous to the three probes allowed reproducible quantitative assays with a wide dynamic range (10^3-10^{10} TSWV M segment RNA copies/ng of total RNA) and high sensitivity. This protocol was assayed with a battery of TSWV isolates, covering the range of the present known genetic variation, in single and/or mix infections in three plant hosts, as well as in the thrips vector *Frankliniella occidentalis*. This quantitative detection assay will be a valuable tool for molecular biology and epidemiology studies, diagnosis and disease control.

1. Introduction

Tomato spotted wilt virus (TSWV), the type member of the genus *Tospovirus* of the family *Bunyaviridae*, is one of the most economically significant plant viruses causing damage to diverse ornamental and vegetable crops worldwide (Adkins 2000). TSWV has a wide host range including more than 1000 plant species (Hanssen et al. 2010). It is transmitted in a persistent manner by several thrips species (*Thysanoptera*: *Thripidae*), with *Frankliniella occidentalis* (Pergande) being its main vector. The virus can only be acquired effectively by first instar larvae, and it can only be inoculated by second instar larvae and adult thrips, after a latent period during which TSWV reaches the salivary glands where it replicates (Wijkamp et al. 1993b). TSWV virions are quasi-spherical, measure 80-110 nm in diameter and are composed of an outer membrane envelope derived from the host, with two virus-coded glycoproteins (G_N and G_C) which are embedded and projected from the surface. Inside there are several copies of the RNA dependent RNA polymerase (RdRp) and nucleoproteins which encapsidate the genome. This consists of three single-stranded negative-sense or ambisense RNA segments: the L segment (~8.9 kb) encodes in negative sense a putative RNA-dependent RNA polymerase; the M segment (~4.8 kb) encodes in positive sense the plant cell-to-cell movement protein NSm, and in the negative sense the precursor of surface glycoproteins, G_N/G_C, involved in TSWV transmission by thrips; and the S segment (~2.9 kb) encodes, in the positive sense the silencing suppressor NSs, and in negative sense, the nucleoprotein N (Pappu et al. 2009).

Eradication or control of TSWV is a very difficult task due to its wide host range, its effective spread by thrips, and its great ability to evolve and adapt to new situations (Adkins 2000, Pappu et al. 2009). Breeding for resistance has been proven to be the most effective method of control. Although, only the resistance conferred by two genes, *Sw-5* in tomato (*Solanum lycopersicum*) and *Tsw* in pepper (*Capsicum annuum*), has been found to be efficient against a wide spectrum of TSWV isolates (Pappu et al. 2009). However, resistance-breaking TSWV isolates have been reported in several countries after a few years of using these resistant cultivars (López et al. 2011, Margaria et al. 2007).

Specific and sensitive detection as well as accurate estimation of the virus titer in plant hosts and thrips vectors are necessary to study different aspects of virus biology and epidemiology, as well as to develop and evaluate strategies of disease control. Quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR) has been widely used for diagnosis and quantitation of diverse human, animal and plant viruses (Mackay et al. 2002) because of its high sensitivity, specificity and reproducibility without the need for post-PCR sample processing. RT-qPCR has been developed to detect and quantify the genomic S segment of TSWV in plants (Mortimer-Jones et al. 2009, Roberts et al. 2000) and in thrips (Boonham et al. 2002), although it has only been assayed with genetically similar isolates without considering the worldwide genetic diversity of TSWV. Nucleotide analysis of a good number of worldwide TSWV isolates (López et al. 2011, Tsompana et al. 2005) showed a relatively high genetic variability with two main genotypes (groups of isolates based on

genetic similarity) named genotype A and genotype B. It would be valuable to develop a RT-qPCR valid for all TSWV isolates and/or able to discriminate these genotypes.

In this work, a RT-qPCR method for universal detection and quantitation of TSWV RNA of the genomic M segment was developed using primers and a TaqMan®MGB probe based on conserved sequences of the TSWV M segment. Also, genotype-specific TaqMan®MGB probes were designed to detect and monitor the accumulation of the two TSWV genotypes in single and mixed infections. These protocols were validated in different plant hosts and in thrips with different TSWV isolates in single and mix infections.

2. Materials and Methods

2.1. Virus isolates and biological assays

Twenty-six TSWV isolates, collected from tomato or pepper plants from Spain and the Brazilian isolate BR01 (kindly provided by Dr. Peters, Wageningen Agricultural University) were inoculated in plants of datura (*Datura stramonium*), tomato (cv. Robin, Royal Sluis, El Ejido, Almería, Spain) and/or pepper (cv. Dulce Italiano, Batlle, Molins de Rei, Barcelona, Spain) or were directly analyzed (Table 1). Plants were maintained in a chamber at a controlled temperature of 25 ºC with a 14:10 h photoperiod.

Table 1. Detection and RNA quantitation of several TSWV isolates infecting datura, tomato, pepper and the thrips vector, by RT-qPCR with TaqMan®MGB probes P_U, P_A and P_B

Host/vector[a]	Isolates[b]	Probe P_U[c] $C_t \pm SD$[d]	Copies[e]	Probe P_A[c] $C_t \pm SD$[d]	Copies[e]	Probe P_B[c] $C_t \pm SD$[d]	Copies[e]
Datura	GRAU (A)	16.93 ± 0.01	6.07×10^7	18.25 ± 0.02	2.06×10^7	-	0
	Mon1NL2 (A)	21.94 ± 0.06	1.88×10^6	23.39 ± 0.20	5.85×10^5		0
	ALPA (A)	17.74 ± 0.75	3.69×10^7	19.05 ± 0.04	1.18×10^7	-	0
	BR01 (B)	31.94 ± 0.33	1.85×10^3	-	0	32.57 ± 0.26	1.01×10^3
	Da1NL2 (B)	29.33 ± 0.25	1.13×10^4	-	0	31.50 ± 0.18	8.25×10^5
	Oller1TL3 (B)	19.70 ± 0.03	3.07×10^6	-	0	22.98 ± 0.01	5.71×10^7
Tomato	Mon1NL2 (A)	24.31 ± 0.18	6.55×10^6	23.33 ± 0.43	1.55×10^5	-	0
	ALPA (A)	23.08 ± 0.29	1.56×10^7	19.60 ± 0.50	2.56×10^6	-	0
	Oller1TL3 (B)	25.29 ± 0.08	3.32×10^6	-	0	25.74 ± 0.06	8.05×10^5
Pepper	Mon1NL2 (A)	23.83 ± 0.14	9.12×10^6	23.82 ± 0.06	1.07×10^5	-	0
	ALPA (A)	17.85 ± 0.04	4.60×10^8	17.71 ± 0.02	1.92×10^8	-	0
	Oller1TL3 (B)	22.83 ± 0.18	1.83×10^7		0	24.50 ± 0.03	1.90×10^6
Thrips	Mon1NL2 (A)	32.28 ± 0.01	3.59×10^5	26.28 ± 0.07	2.21×10^5	-	0
	ALPA (A)	23.57 ± 0.68	1.60×10^8	19.22 ± 0.71	3.12×10^7		0
	Oller1TL3 (B)	29.66 ± 0.03	2.20×10^6	-	0	25.54 ± 0.09	9.28×10^4

[a]Plant hosts: datura (Datura stramonium), tomato (Solanum lycopersicum) and pepper (Capsicum annuum), and the thrips vector Frankliniella occidentalis.
[b]TSWV isolate genotypes are between parentheses.
[c] TaqMan®MGB Probe P_U is general for TSWV and TaqMan®MGB probes P_A and P_B are specific for genotypes A and B, respectively.
[d]Average threshold cycle (C_t) and standard deviation (SD) obtained from two different assays using two replicates. Dashes indicate no virus detection.
[e]Number of TSWV M segment RNA copies / ng of total RNA from an infected plant or thrips.

Nucleotide sequences of the M segment were known for the tomato isolates GRAU (GenBank accession FM163370) and Mon1NL2 (HM015514) and the pepper isolate ALPA (HQ537114) which correspond to genotype A and the tomato isolates BR01 (GenBank accession S58512), Da1NL2 (HM015512) and Oller1TL3 (HM15519) of genotype B. These isolates were obtained from local lesions in *Nicotiana glutinosa* plants after three successive passages and then inoculated in datura plants to multiply the biological clones. The purpose was to obtain a highly homogeneous within-isolate population, which was confirmed by single strand conformation polymorphism analysis (data not shown).

Virus mechanical inoculation was performed by grinding TSWV-infected plant material in chilled inoculation buffer (0.01 M sodium phosphate buffer, pH 7.0) in a mortar, and rubbing this sap extract with carborundum onto plants with their first true leaves. For each experiment, virus accumulation was analyzed by RT-qPCR 15 days after inoculation, when infection was systemic.

Transmission assays were performed from TSWV-infected datura plants, leaving ten adult thrips (*F. occidentalis)* to reproduce and the first instar larva progeny was allowed to acquire the virus for 24 h. The thrips were then placed onto healthy plants to complete the development into adults. Finally, inoculation of TSWV by adults was tested using the Petunia (*Petunia x hybrida*) leaf disk assay (Wijkamp and Peters 1993a). This consisted of leaving individual adult thrips on petunia leaf disks during 24 h; following this the leaf discs were floated on water for 2 days at 25ºC. Thrips were scored as transmitters if the leaf discs developed local lesions.

To equalize TSWV inocula from different TSWV isolates, several datura plants were inoculated per each isolate and several leaf discs per each inoculated plant were analyzed by RT-qPCR leaving the plants alive. Plants with similar virus concentration were chosen for mechanical inoculation and transmission by thrips.

2.2. Design of primers and TaqMan®MGB probes

Nucleotide sequences of the complete M segment of 24 TSWV isolates from Brazil, the USA (California, Florida, North Carolina and Hawaii), Korea, the Netherlands and Spain were retrieved from GenBank (see accession numbers in Fig. 1) and aligned using the CLUSTAL W algorithm implemented in the program MEGA 4.0 (Kumar et al. 2008). The design of primers and TaqMan®MGB probes (Applied Biosystems, Austin, TX, USA) was aided by the program Primer Express® (Applied Biosystems). For universal detection and quantitation of TSWV (valid for all TSWV isolates), conserved sequence stretches located in the M segment of TSWV were chosen to design primers 1M_F and 1M_R (at positions 564-584 and 687-700, respectively, which encompassed a region of 137 nt) and a TaqMan®MGB probe, named P_U, at position 440-457 (Fig. 1).

GenBank Accessions	Forward primer 1M_F	Probe P_A	Probe P_U	Reverse primer 1M_R

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GenBank        Forward primer 1M_F                                                  Probe P_A                    Probe P_U                                          Reverse primer 1M_R
Accessions
               564                584                                              416                437   440                457                                 687                700
             FM163370 CCAACATGCCATCTGAAAAGCAAGTCATTCTGAAGGGTCAAGGGACAATAACTGATCCTATATGTTTTGTCTTTTATCTGAACTGGTCTATTCCAAAAATAAACAACACTCCAGAAAACTGCTGTCAGCTGCATTTG
             FM163371 ...........................................................................................................................................................
             FM015514 ..................................A........................................................................................................................
Genotype A   HQ537114 ...................................................................................................................T.......................................
             FM163373 ...........................................G.............................................................G.....G..T.....................................
             FM163372 ...........................................G.............................................................G.....G..T.....................................
             AB190818 ...........................................G........G..........................................A.........G.....G..T..T..................................
             AB010996 ...........................................G....T.......................................................G.....G..T.....................................
             AY744481 ...........................................G.............................................................G.....G..T.....................................
             AY744482 ...........................................G....................................T......................................G..T.....................................
             AY744483 ...........................................G....................................T......................G.....G..T.....................................
             AY744484 ...........................................G....................................T......................G.....G..T.....................................
             AY744485 ...........................................G........G....................................................G.....G..T.....................................
             AY744487 ...........................................G..............T.............................................G.....G..T.....................................
             AY744488 ...............G...........................G..............T.............................................G.....G..T.....................................
             AY744489 .......................................C..G..............T.............................................G.....G..T.....................................
             AY744490 ...........................................G..............T.............................................G.....G..T.....................................
             AY744491 ...........................................G..............T.............................................G.....G..T.....................................
             AY744492 ..................................A........................................................................................................................
             AY744493 ...........................................G...............................................................................................................
             AY870389 .........G.................................G..............T.............................................G.....G..T.....................................
             AY870390 ...........................................G..............T...........................A.................G.....G..T.....................................
             AF208498 ...........................................G...............................................................G.....G..T.....................................

             HM015519 ..............................C....................C..C.....T.......C...................G..G........C...........T..........
Genotype B   HM015512 ........T...................................................C..C.....T..................................G..G..G.......C...........T..........
             AF208497 ..............................C....................C..C.....T......................G..G.......C...........T..........
             AY744486 ..............................C....................C..C.....T......................G..G.......C...........T..........
             S58512   ...............G...............C.........G..........C..C.....T..................G..G..G.......C...........T..........
                                                                 TCCTATCTGCTTTGTTTT
                                                                 419              436
                                                                     Probe P_B.
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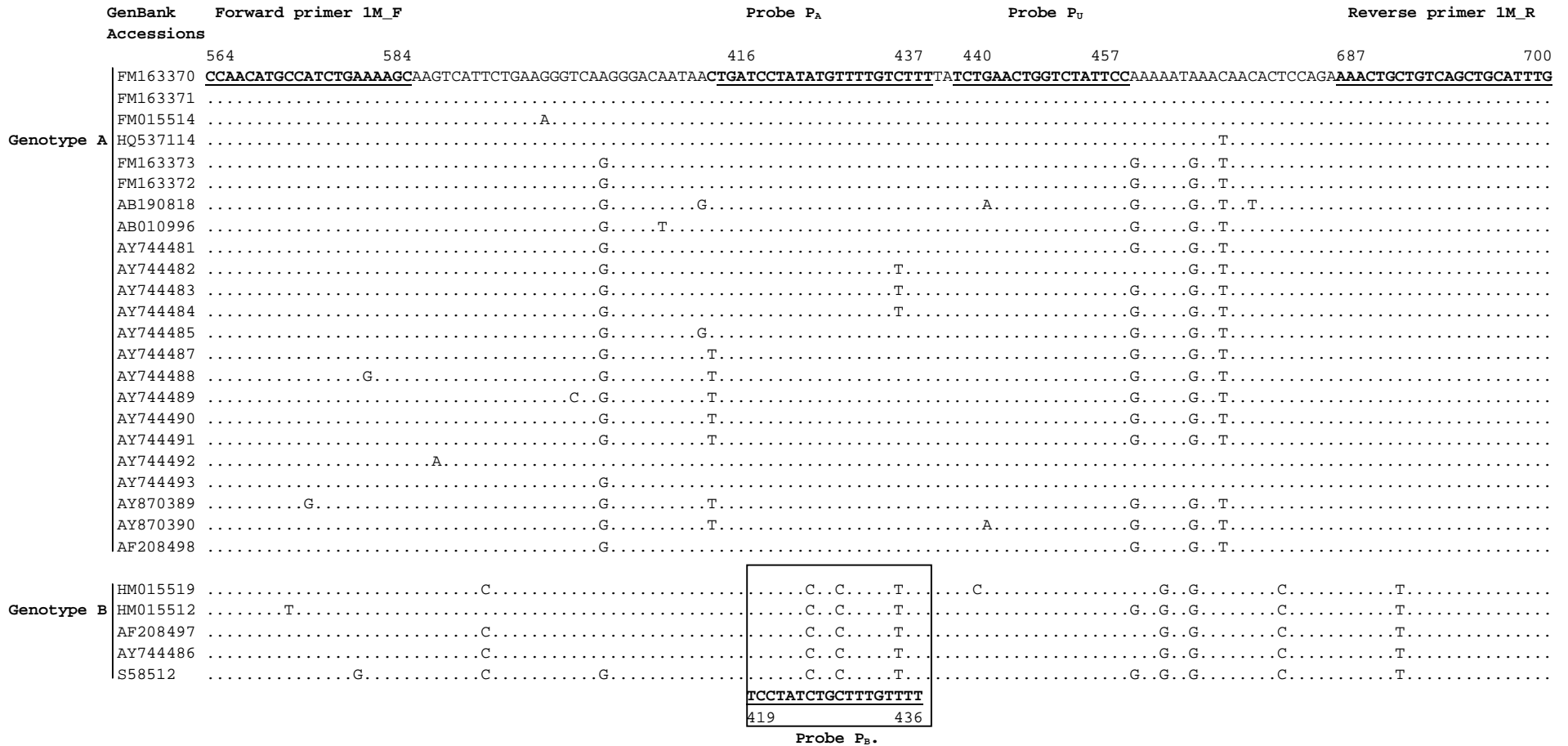
Fig. 1. Alignment of the M segment of different TSWV isolates for the purpose of designing primers and probes for RT-qPCR (only the sequence sites encompassed by the primers are shown). Nucleotide sequences of primers 1M_F and 1M_R, and TaqMan®MGB probes P_U, P_A and P_B are underlined in bold letters. Numbers indicate nucleotide positions in the M segment of isolate GRAU (GenBank accession FM163370), dots indicate nucleotide identity and boxes enclose the sequence regions considered for probe design.

To discriminate and separately quantify the two TSWV genotypes (López et al. 2011, Tsompana et al. 2005), two TaqMan®MGB probes were designed: named P_A (position 416-437) which is specific and conserved for TSWV isolates of genotype A (tagged with the fluorescent dye FAM); and P_B (position 419-436), which is specific and conserved for genotype B (tagged with the fluorescent dye VIC).

2.3. RNA extraction, RT-PCR and RT-qPCR

Total RNAs from 0.1 g of fresh leaf tissue from TSWV-infected and non-infected datura, tomato and pepper plants and from individual adult thrips able or unable to transmit TSWV were extracted using a standard protocol with phenol/chloroform/isoamilic alcohol (Ruiz-Ruiz et al. 2009a). Total RNA extracts were eluted in 20 µl of RNase-free water and treated with RNase-free DNase (Turbo DNA-free, Ambion, Applied Biosystems, Austin, TX, USA). RNA concentrations were measured in duplicate with the UV-Vis spectrophotometer Nanodrop 1000 (Thermo Scientific, Waltham, MA, USA) and adjusted to approximately 10 ng/µl to normalize the different extractions. Aliquots were stored at –80ºC until use.

To obtain a template for *in vitro* transcription, conventional one-step RT-PCR was set up as follows: total RNAs (3 µl, ~1 µg) were denatured by heating for 5 min at 95ºC and chilling on ice. The denatured RNAs were reverse-transcribed and amplified in a 20 µl reaction mixture containing PCR buffer, 1.5 mM $MgCl_2$, 1 mM of each of the four dNTPs, 0.2 µM of primers T7-1M_F (like primer 1M_F but including the T7 promoter sequence) and 1M_R, 3 U of RNase Out (Invitrogen, Carlsbad, CA, USA), 15 U of Superscript II Reverse Transcriptase (Invitrogen) and 0.75 U of Taq DNA polymerase (Invitrogen). The thermal cycling conditions were: 42ºC for 55 min, 94ºC for 2 min, followed by 40 cycles of 94ºC for 20 s, 55ºC for 20 s and 72ºC for 25 s.

RT-qPCR was performed in a LightCycler®480 (Roche Molecular Diagnostics, Basel, Switzerland) using 25 µl of a reaction mix that contained 12.5 µl LightCycler® 480 Probes Master (Roche Molecular Diagnostics), 4.38 µl of RNase-free water, 15 U of RT Multiscribe Reverse Transcriptase (Applied Biosystems), 2 U RNase Inhibitor (Applied Biosystems), 5 µM of each forward and reverse primer, 0.25 µM TaqMan®MGB probe, and 5 µl of total RNA (~50 ng). Cycling conditions consisted of reverse transcription at 48ºC for 30 min, incubation at 95ºC for 10 min, and 40 cycles of 95ºC for 15 s and 60ºC for 1 min. In each run the LightCycler® 480 software plotted the fluorescence intensity against the number of cycles and provided the threshold cycle (C_t) value. Each sample was analyzed in duplicate in two independent RT-qPCR assays and the mean Ct and the standard deviation (SD) for each sample were calculated. The variation coefficient within and between assays was calculated as percentage of the SD in comparison with the mean C_t value. Control samples included total RNAs from plants which were not inoculated with TSWV and thrips fed on non-infected plants, water instead of RNA extracts, and RNAs from TSWV-infected plants and TSWV-infected thrips but using no reverse transcriptase. RT-PCR and RT-qPCR products were

analyzed by electrophoresis in 3% agarose gels and stained with GelRed (Biotium, Hayward, CA, USA).

2.4. Preparation of RNA transcripts and standard curves

To determine the number of RNA copies of the TSWV M segment in the total RNA extracts, RNA transcripts of the selected region of this genomic segment were synthesized *in vitro*, and serial dilutions were used in real time RT-PCR assays to generate external standard curves. RNA transcripts were generated from RT-PCR products using the Megascript T7 Kit (Ambion) following the manufacturer's instructions. Transcripts were purified with the RNaid w/Spin Kit (Qbiogene, Quebec, Canada) and treated twice with RNase-free DNase (Turbo-DNA-free, Ambion).

The transcript RNA concentration and purity were evaluated by the spectrophotometer Nanodrop 1000 and the integrity by electrophoresis in 3% agarose gels. Conversion of microgram of single stranded RNA (ssRNA) to picomoles was performed considering the average molecular weight of a ribonucleotide (340 pg) and the number of bases of the transcript (N_b). The following formula was applied: pmol of ssRNA = µg of ssRNA x (10^6 pg/1µg) x (1 pmol/340 pg) x (1/ N_b). Avogrado's constant (6.023 x 10^{23} molecules/mol) was used to estimate the number of ssRNA copies. To generate external standard curves, 10-fold serial dilutions containing 10^{10}-10^1 RNA copies were prepared in total RNA extracts (10 ng/µl) from healthy datura plants and analyzed by RT-qPCR with the three designed probes: P_U (universal for all TSWV isolates) and P_A (specific for genotype A) were used with isolate GRAU (genotype A) transcripts and P_B (specific for genotype B) was used with isolate Oller1TL3 (genotype B) transcripts. RT-qPCR with and without reverse transcriptase were run in parallel to ensure the absence of DNA templates in transcript preparations. Standard curves for each TaqMan®MGB were constructed plotting C_t values per two replicates per standard dilution versus the logarithm of the RNA copy number. The amplification efficiency was calculated from the slope of the corresponding curve using the formula $10^{(-1/\text{slope of the standard curve})}$.

3. Results

3.1. Evaluation and comparison of TaqMan®MGB probes for detection, discrimination and accumulation of TSWV isolates in different plant hosts and the thrips vector

To estimate the number of TSWV M segment RNA copies, three standard curves were prepared with probes P_U, P_A and P_B using 10-fold serial dilutions of the cognate RNA transcripts, containing 10^{10}-10^1 copies. The three standard curves covered a wide dynamic range of eight log units of concentration and showed a strong linear relationship with a correlation coefficient greater than 0.990, as well as high amplification efficiency (> 99.5%). The variation coefficient was very low (<1.0%). This procedure enabled detection of as little as 10^2 TSWV M segment RNA copies,

however, quantitation of such a low number of copies was not reliable as it fell off the linear range of the standard curves. Therefore, the standard curves obtained enabled reliable quantitation of TSWV in RNA extracts containing between 10^3 and 10^{10} TSWV M segment RNA copies.

The performance of three designed TaqMan®MGB probes was evaluated by RT-qPCR using as template total RNA extracted from datura, tomato and pepper plants and the thrips vector infected with several TSWV isolates whose genomic M segment nucleotide sequences are known (Table 1). Virus RNA accumulation determined with probes P_A and P_B was similar to the accumulation estimated with the probe P_U for each TSWV isolate, showing some random variations. Thus, probe P_A reacted specifically with isolates Mon1NL2, GRAU and ALPA, having genotype A; probe P_B reacted only with isolates BR01, Da1NL2 and Oller1TL3, having genotype B; and probe P_U reacted with all these isolates (Table 1). RT-qPCR using total RNAs extracted from plants not inoculated with TSWV or adult thrips raised on healthy plants was negative for the three TaqMan®MGB probes.

Comparison of TSWV M segment RNA accumulation in the three plant hosts and thrips (Table 1) showed that ALPA accumulated in the three plant hosts and thrips (mean number of M RNA copies estimated with probe P_U = 1.68x10^8) more than isolates Mon1NL2 and Oller1TL3 (mean number of M segment RNA copies = 4.47x10^6 and 6.72x10^6, respectively). Accumulation of the three TSWV isolates was higher in pepper (mean number of M RNA copies = 1.62x10^8) than in tomato and datura (mean number of M RNA copies = 1.40x10^7 and 8.49x10^6, respectively), whereas accumulation in thrips varied for each isolate (3.59x10^5, 2.20x10^6 and 1.60x10^8 for Mon1NL2, Oller1TL3 and ALPA, respectively). The non-infected plants and the non-transmitter thrips gave negative results.

3.2. Discrimination of accumulation of TSWV genotypes in mix infections

The performance of probes P_A and P_B in detecting and quantifying genotypes A and B in mix infections was evaluated by two assays. In the first, total RNA extracts obtained from a datura plant infected by isolate Mon1NL2 (genotype A) and from another datura plant infected with Oller1TL3 (genotype B) were quantified by RT-qPCR with TaqMan®MGB probe P_U, mixed in different proportions (10:10, 10:1 and 1:10) and analyzed by RT-qPCR with TaqMan®MGB probes P_U, P_A and P_B (Table 2). The estimated TSWV M segment RNA quantity was similar for probes P_A and P_B, but a little higher for probe P_B, showing some small random variations. In the second assay, plants of datura, tomato and pepper were singly or doubly inoculated with equivalent quantities of these two isolates and analyzed by RT-qPCR with the three TaqMan®MGB probes (Table 2). For each plant host, only one plant resulted infected with both isolates whereas the other two doubly-inoculated plants were infected by only one isolate (Oller1TL3 for datura and Mon1NL2 for tomato and pepper).

Table 2. Discriminative detection and quantitation of M segment RNA of two TSWV isolates by RT-qPCR with TaqMan®MGB probes P_U, P_A and P_B

Host/template[a]	Isolate	Probe P_U[b]		Probe P_A[b]		Probe P_B[b]	
		$C_t\pm SD$[c]	Copies[d]	$C_t\pm SD$[c]	Copies[d]	$C_t\pm SD$[c]	Copies[d]
RNA mix							
10:10	Mon1NL2-Oller1TL3	19.49±0.01	1.01×10^8	20.03±0.03	6.80×10^7	20.51±0.10	1.45×10^7
1:10	Mon1NL2-Oller1TL3	20.76±0.17	4.20×10^7	21.88±0.04	1.92×10^7	21.34±0.14	8.13×10^8
10:1	Mon1NL2-Oller1TL3	18.69±0.05	1.76×10^8	19.39±0.06	1.08×10^8	22.97±0.01	5.71×10^7
Datura							
Plant 1	Mon1NL2	18.63±0.10	1.82×10^8	19.25±0.03	1.24×10^8	-	0
Plant 2	Oller1TL3	21.08±0.68	3.54×10^7	-	0	22.20±0.11	4.45×10^8
Plant 3	Mon1NL2-Oller1TL3	25.61±0.01	1.18×10^7	26.54±0.62	6.55×10^6	26.67±0.03	9.90×10^6
Plant 4	Mon1NL2-Oller1TL3	24.34±0.45	2.92×10^7	-	0	24.73±0.02	5.77×10^7
Plant 5	Mon1NL2-Oller1TL3	22.38±0.14	1.11×10^8	-	0	22,25±1.81	$2,96\times10^8$
Tomato							
Plant 1	Mon1NL2	23.35±0.01	1.27×10^7	22.58±0.01	1.27×10^6	-	0
Plant 2	Oller1TL3	25.23±0.01	3.46×10^6	-	0	25.74±0.06	8.05×10^5
Plant 3	Mon1NL2-Oller1TL3	22.59±0.57	2.25×10^7	22.53±0.04	1.32×10^6	-	0
Plant 4	Mon1NL2-Oller1TL3	26.05±0.01	1.95×10^6	25.88±0.14	1.29×10^5	-	0
Plant 5	Mon1NL2-Oller1TL3	24.16±0.11	7.26×10^6	24.65±0.22	3.05×10^5	25.09±1.17	1.49×10^6
Pepper							
Plant 1	Mon1NL2	23.99±0.11	8.17×10^6	24.55±0.03	3.23×10^5	-	0
Plant 2	Oller1TL3	22.83±0.18	1.83×10^7	-	0	24.50±0.03	1.90×10^6
Plant 3	Mon1NL2-Oller1TL3	26.01±0.01	2.01×10^6	25.72±0.64	1.02×10^5	-	0
Plant 4	Mon1NL2-Oller1TL3	27.96±0.10	5.22×10^5	27.30±0.18	4.82×10^4	27.27±0.04	2.80×10^5
Plant 5	Mon1NL2-Oller1TL3	24.63±0.48	5.37×10^6	23.82±0.06	5.35×10^5	-	0

[a]Two types of template were used: I) Total RNA extracts of isolate Mon1NL2 (genotype A) and isolate Oller1TL3 (genotype B) which were quantified by RT-qPCR with TaqMan®MGB probe P_U and mixed in different proportions (10:10, 1:10, 10:1); and II) RNA extracts from datura, tomato and pepper plants inoculated or co-inoculated with an equivalent quantity of isolates Mon1NL2 and Oller1TL3.

[b] TaqMan®MGB P_U is general for TSWV and TaqMan®MGB probes P_A and P_B are specific for genotypes A and B, respectively. Genotypes are based on sequence similarity.

[c]Average threshold cycle (C_t) and standard deviation (SD) obtained from two different assays using two replicates. Dashes indicate no virus detection.

[d]Number of TSWV M segment RNA copies / ng of total RNA of an infected plant.

The accumulation of each isolate showed small variations in different plants but these did not correlate to single and double infections. For example, in the tomato plants, the number of M segment RNA copies of Mon1NL2 estimated with probe P_A

was 1.29×10^5 to 1.32×10^6 in single infections and 3.05×10^5 in double infections (plant 5, Table 2). Those of Oller1TL3 estimated with probe P_B were 8.05×10^5 and 1.49×10^6 in single and double infections (plant 5, Table 2), respectively.

3.3. Evaluation of TSWV isolates from field

In addition to the six mentioned isolates (BR01, Mon1NL2, Da1NL2, GRAU, Oller1TL3 and ALPA), thirteen tomato and eight pepper isolates collected in the field were directly analyzed by RT-qPCR with the universal probe P_U and the genotype-specific probes P_A and P_B (Table 3).

Table 3. Detection and RNA quantitation of TSWV isolates directly collected from tomato and pepper fields by RT-qPCR with TaqMan®MGB probes P_U, P_A and P_B

Host	Isolate[a]	Probe P_U[b]		Probe P_A[b]		Probe P_B[b]	
		$C_t \pm SD$[c]	Copies[d]	$C_t \pm SD$[c]	Copies[d]	$C_t \pm SD$[c].	Copies[d]
Tomato	LloR05	20.77±0.13	7.89×10^6	21.12±0.06	6.73×10^6	-	0
Tomato	GG1	24.18±0.08	8.49×10^4	24.58±0.14	6.12×10^4	-	0
Tomato	Ayter1	20.49±1.82	5.34×10^6	21.57±1.54	5.68×10^5	-	0
Tomato	Ayter4	19.09±1.39	1.23×10^7	18.66±0.01	3.28×10^6	-	0
Tomato	G7L	23.39±0.22	5.05×10^5	23.25±0.66	1.44×10^5	-	0
Tomato	Font1	18.22±0.45	5.17×10^7	18.00±0.05	5.83×10^7	-	0
Tomato	Font3	29.58±0.35	1.85×10^4	30.43±0.15	1.06×10^4	-	0
Tomato	Font6	19.88±0.13	1.49×10^6	19.99±0.14	1.48×10^7	-	0
Tomato	Font7	30.73±0.04	8.26×10^3	-	0	32.41±1.44	3.37×10^3
Tomato	Font8	20.66±0.45	3.41×10^6	-	0	27.93±1.59	8.79×10^5
Tomato	MonP	24.01±0.49	2.00×10^5	25.19±0.12	3.56×10^4	-	0
Tomato	Mon	25.84±0.27	9.28×10^4	26.24±0.02	1.72×10^4	-	0
Tomato	MonC	18.35±1.15	1.92×10^7	16.66±0.56	1.36×10^7	-	0
Pepper	PLL3	18.72±0.34	1.30×10^7	18.78±0.33	3.07×10^6	-	0
Pepper	PM1	19.95±0.33	1.53×10^6	20.53±0.056	1.01×10^6	-	0
Pepper	PM4	27.23±0.10	9.70×10^4	-	0	29.18±0.13	2.52×10^4
Pepper	PVR1	19.27±0.04	2.42×10^6	19.13±0.042	2.68×10^6	-	0
Pepper	PVR2	28.86±0.00	3.16×10^4	28.83±0.00	3.22×10^4	-	0
Pepper	PVS1	22.33±0.24	2.93×10^6	22.88± 0.15	1.98×10^6	-	0
Pepper	SUS4	19.87±0.26	5.80×10^6	19.32± 0.04	2.08×10^6	-	0
Pepper	SUS8	16.80±0.84	4.98×10^7	17.49± 0.97	8.25×10^6	-	0

[a]TSWV isolates collected and directly analyzed from tomato (Solanum lycopersicum) and pepper (Capsicum annuum) plants from the field.
[b] TaqMan®MGB probe P_U is general for TSWV and TaqMan®MGB probes P_A and P_B are specific for genotypes A and B, respectively. Genotypes are based on sequence similarity.
[c]Average threshold cycle (C_t) and standard deviation (SD), obtained from two different assays using two replicates. Dashes indicate no virus detection.
[d]Number of TSWV M segment RNA copies / ng of total RNA of a infected plant.

TSWV M segment RNA accumulation determined with TaqMan®MGB probes P_A and P_B was approximate to that estimated with TaqMan®MGB probe P_U, confirming the previous results. The TSWV M segment RNA accumulation of different isolates

showed a great variation, ranging from 8.3×10^3 to 5.2×10^7 RNA copies when determined by RT-qPCR with probe P_U. The genotypes of these twenty-one TSWV Spanish isolates were determined by RT-qPCR with probes P_A and P_B. Eighteen had genotype A (eleven from tomato and seven from pepper) and three had genotype B (two from tomato and one from pepper). None of these isolates had both genotypes, suggesting the absence of mix infections (Table 3).

4. Discussion

Analysis of all available nucleotide sequences of TSWV M segment revealed two genotypes (groups of isolates), with nucleotide identities greater than 98% between isolates of the same genotype and about 93% between isolates of different genotypes (López et al. 2011, Tsompana et al. 2005). RT-qPCR primers and TaqMan®MGB probes were designed taking into account the worldwide genetic diversity of TSWV with the aim to obtain a method valid for all TSWV isolates and able to differentiate the two TSWV genotypes. The TaqMan®MGB were designed to contain a MGB (minor grove binding) group because its small size makes finding conserved sequence stretches more probable for all TSWV isolates and for each genotype (Kutyavin et al. 2000). Although, the use of RT-qPCR for plant viruses is rapidly increasing (Mackay et al. 2002), the design of primers and/or probes that takes into account the genetic variability has scarcely been carried out, e.g. *Citrus tristeza virus* (Ruiz-Ruiz et al. 2007, Ruiz-Ruiz et al. 2009b). However the low variability found in some viruses, e.g. *Citrus leaf bloth virus* (Vives et al. 2002) makes it unnecessary to take this into consideration (Ruiz-Ruiz et al. 2009a).

These primers and probes were assayed with TSWV isolates, covering the presently known genetic variation range, in different plant hosts and the thrips vectors. The C_t values obtained from experimental and field isolates were variable but all were within the dynamic range of the standard curve and enable reliable quantitation of TSWV M segment RNA. The ability of this RT-qPCR procedure to detect a wide spectrum of TSWV isolates with a high sensitivity and reproducibility makes it an appropriate tool for supporting control measures to limit TSWV disease spread based on quarantine, eradication and certification programs, which need to assure the complete virus-free status of propagative plant materials (Adkins 2000).

The detection and quantitation of different genomic segments (Roberts et al. 2000) and different genotypes can be used to study: a) the possible differential accumulation of genomic segments with respect to different aspect of virus biology (e.g. in plant hosts versus thrips vectors, resistance-breakdown, etc); b) reassortment of genomic segments (Qiu and Moyer 1999); and c) interactions between virus strains in mixed infections and fitness evaluation (Carrasco et al. 2007) which could provide information on the molecular biology, evolution and epidemiology of TSWV. This would be especially interesting to study those aspects related to the generation and dispersion of TSWV resistance-breaking isolates. Our results from RT-qPCR evaluation of plants co-inoculated with two TSWV isolates of different genotypes and TSWV

isolates collected from the field, as well as competition experiments (Aramburu et al. 2010) suggested that coexistence of two genotypes in the same plant is infrequent which contrasts with other plant viruses (Rubio et al. 2001, Sanchez-Campos et al. 1999). The discrimination of genotypes could be used for evaluation of control procedures such as cross protection assays (Sambade et al. 2002) and breeding resistant cultivars since they may behave differently according to the TSWV isolate.

CAPÍTULO 3

Evaluación de la transmission de aislados de TSWV, que superan y no superan las resistencias *Sw-5* de tomate y *Tsw* de pimiento, por su principal vector, el trips *Frankliniella occidentalis*.

Abstract

Tomato spotted wilt virus (TSWV) causes serious diseases of many economically important crops. Disease control has been achieved by breeding tomato and pepper cultivars with the resistance genes *Sw-5* and *Tsw*, respectively. However, TSWV isolates overcoming these genetic resistances have appeared in several countries. To evaluate the risk of spread of the resistance-breaking isolates, we tested their ability of transmission by the main vector of TSWV, the thrips *Frankliniella occidentalis*. We compared the transmission rate by thrips of six TSWV isolates of different biotype (able or unable to overcome this resistance inpepper and tomato), and with divergent genotypes (A and B). Our results indicate that transmission rate was related to the amount of virus acumulated in thrips but not to virus accumulation in the source plants on which thrips acquired the virus. No correlation was found between transmission efficiency by thrips and the genotype or between transmission efficiency and the ability of overcoming both resistance. This result suggests that resistance-breaking isolates have the same potential to be transmitted as the isolates unable to infect resistant tomato and pepper cultivars.

1. Introduction

Tomato spotted wilt virus (TSWV), the member type of the genus *Tospovirus*, is one of the most destructive plant viruses causing serious economic losses in many agricultural crops worldwide (Adkins 2000). TSWV has a wide host range including more than 1000 species (Hanssen et al. 2010) and is transmitted by several species of thrips (Thysanoptera: Thripidae), among which *Frankliniella occidentalis* (Pergande) is the main vector. TSWV can be transmitted by adults and to a lesser extent by second instar larvae, after a latent period during which the virus circulates in the vector and replicates in the vector's midgut, visceral muscle cells and salivary glands (Kritzman et al. 2002, Nagata et al. 1999, Ullman et al. 1992, Whitfield et al. 2005, Wijkamp and Peters 1993a, Wijkamp et al. 1993b). The triangular interactions between virus, plant host and thrips can be very complex affecting not only the transmission efficiency of TSWV, but can also have detrimental or beneficial effects in the reproduction and survival of the thrips (Belliure et al. 2005, Belliure et al. 2008, Inoue and Sakurai 2006).

TSWV genome consists of one negative-sense (L) and two ambisense (M and S) RNA segments: segment L encodes a putative RNA-dependent RNA polymerase; segment M encodes the cell-to-cell movement protein NSm, and the precursor of surface glycoproteins G_N/G_C, which are thought to be involved in TSWV transmission by thrips; and segment S encodes a silencing suppressor NSs and the nucleocapsid N (Plyusnin et al. 2012).

The wide host range of TSWV and efficient spread by thrips, their hidden way of life and the incoming numbers after insecticide sprays make the application of prophylactic measures difficult. The most efficient way to control the spread of TSWV is the use of resistant cultivars. So far, only two resistance genes (*Sw-5* in tomato and *Tsw* in pepper) have been effective in the control of TSWV (Adkins 2000, Pappu et al. 2009). However, *Sw-5* resistance-breaking (SRB) isolates have been reported in Australia, South Africa, Spain and Italy, and *Tsw* resistance-breaking (TRB) isolates in Australia, Brazil, USA, Italy and Spain (Aramburu and Marti 2003, López et al. 2011, Margaria et al. 2004, Tentchev et al. 2011).

Understanding the factors involved in the transmission of TSWV is crucial to assess the risk of spread of the resistance-breaking isolates, and to develop integrated management strategies for disease control based on combining breeding resistant cultivars, prophylactic measures and agronomical practices. In this study, we analysed the efficiency of transmission of TSWV by its main vector *F. occidentalis,* taking into account several factors, such as the accumulation of virus in source plants and in the thrips vector, the developmental stage of thrips (second instar larvae and adult), TSWV genetic variability and the ability to overcome resistance.

2. Materials and Methods

2.1. Virus isolates

Field samples from tomato and pepper plants apparently infected by TSWV were collected from commercial crops in Spain. These samples were tested for the presence of other viruses frequently infecting tomato and pepper in addition to TSWV and discarded when unwanted infections were found. Sixty-one samples infected with only TSWV were selected (41 from tomato and 20 from pepper). One group of samples was collected from pepper and tomato before resistant cultivars of tomato and pepper were introduced in commercial crops in Spain. A second group of samples was collected from resistant tomato plants in the two regions near Barcelona, where the first breaking of resistant in tomato was detected (Aramburu and Marti 2003). A third group of samples was collected from resistant pepper plants from Malaga, Murcia, Tenerife and Barcelona, after detection of resistance breaking in pepper in Almeria (Margaria et al. 2004). TSWV isolates were obtained from these samples, free of any other virus, after three serial passages of a single local lesion in *Nicotiana glutinosa* plants 3 or 4 days after inoculation (Aramburu et al. 2010). They were subsequently subcultured in *Datura stramonium* to produce systemic infection. The purpose of these serial passages was to get pure resistance breaking and non-breaking TSWV isolates from these samples.

To evaluate the ability to overcome tomato *Sw-5* and pepper *Tsw* resistance, these isolates were mechanically inoculated to tomato cultivars 'Verdi' (Fitó), heterozygous for *Sw-5* and 'Marmande' (Fitó) without *Sw-5*, to pepper cultivars 'Spiro' and 'Divino' (Seminis), both heterozygous for *Tsw* and 'C804' (Fitó) without *Tsw*. TSWV isolates were classified into three biotypes: wild-type (WT) isolates, which were unable to infect both tomato and pepper resistant cultivars, tomato SRB isolate and pepper TRB isolates. No isolate were found to be able to break resistance in resistant tomato and in pepper cultivars. In all case, different TSWV isolate obtained from the same sample were of the same biotype, indicating that any field sample was composed of a mixture of WT and SRB or TRB variants (Aramburu et al. 2010).

Nucleotide sequence analysis of TSWV M segment showed that these isolates can be classified into two main groups or genotypes, A and B, with nucleotide identities greater than 98% between isolates of the same genotype and about 93% between isolates of both genotypes (Debreczeni et al. 2011, López et al. 2011). Analysis of 21 TSWV samples directly collected in the field and analysed by real-time reverse-transcription polymerase chain reaction (RT-PCR) with two TaqMan probes, which detect separately genotype A and B with high sensibility (1000 TSWV RNA molecules), showed that these TSWV samples were composed of one genotype indicating no mixed infection (Debreczeni et al. 2011). The 61 TSWV isolates collected from tomato had biotypes WT (14 samples) with both genotypes A (7 samples) and B (7 samples) and SRB (27 samples) with both genotypes A (17 samples) and B (10 samples), whereas all samples from pepper with biotype WT (1 sample) and TRB (19 samples) were only associated with genotype A. Based on this classification, five Spanish TSWV isolates, (a) ALPA (biotype TRB, genotype A, GenBank accession

HQ537114), (b) GRAU (biotype SRB, genotype A, GenBank accession FM163370), (c) Mon1NL2 (biotype WT, genotype A, GenBank accession HM015514), (d) Oller1TL3 (biotype SRB, genotype B, GenBank accession HM15519), and (e) Da1NL2 (biotype WT, genotype B, GenBank accession HM015512), were selected and compared with the Brazilian isolate BR01 (biotype WT, genotype B, GenBank accession S58512), which has been widely used in many studies and can be considered as the reference TSWV isolate (De Avila et al. 1990).

These six TSWV isolates were inoculated after grinding of TSWV-infected plant material in a mortar in chilled inoculation buffer (0.01 M phosphate buffer, pH 7.0) by rubbing this sap extract with celite onto *D. stramonium* plants with four or five true leaves. The plants were maintained in a growth chamber with controlled conditions (25 ºC temperature; 60% relative humidity; 16:8 h light:dark).

2.2. Thrips and transmission assays

A stock culture of the thrips *F. occidentalis* was reared in transparent plastic jars closed with a lid where a hole was cut. This hole was covered with a piece of filter paper to avoid water condensation and to prevent thrips from escaping. A sponge was placed on the bottom of the jar to provide places for thrips to pupate. Thrips fed and oviposited on bean pods (*Phaseolus vulgaris* L.) coated with a water solution of 5% sugar and 0.1% amino acids (Isabion, Syngenta) that was let to dry before using the bean pods (Espinosa et al. 2002). The plastic jars with the thrips cultures were maintained at 24ºC.

Transmission assays were performed as follows: groups of eight female adult thrips (*F. occidentalis)* were allowed during 24 h to lay eggs on TSWV-infected *D. stramonium* plant, to obtain cohorts of thrips larvae that had already fed on infected plants during the first hours after emerging from the eggs. Such first instar larvae are known to become effective transmitters of TSWV (Ullman et al. 1992, van De Wetering et al. 1996). The thrips larvae were kept on the infected plants for 5 days. The ability to transmit TSWV by second instar larvae was tested using the Petunia leaf disk assay (Wijkamp and Peters 1993a). Each larva was placed on a leaf disk of Petunia (*Petunia x hybrida* cv. Multifora) in a 1.5-ml eppendorf tube during 48 h. Subsequently, those larva that did not become prepupae were placed on new Petunia leaf disk. Adults were also placed on Petunia leaf disks two times for an acquisition access period (AAP) of 48 h. After removing the larvae or adults, the leaf disks were placed individually on water in a Petri dish for 2 days at 27ºC. Larvae and adults were scored as transmitters when the leaf disks developed local lesions (annular red rings or red spots) which are produced as a hypersensitive response triggered by the virus multiplication. Transmission rate was calculated as number of transmitter thrips divided by total number of tested thrips. In total, 253 larvae and 179 adults were tested.

Two preliminary transmission experiments were performed with three TSWV isolates by using two source plants and about 40 adult thrips per isolate. No significant

differences of the transmission rate were found using the isolates in both experiments: (a) GRAU (23.8%); Mon1NL2 (21.4%), and (b) GRAU (22.5%) ; ALPA (41.4%). The next experiment, presented in detail in this article, was designed to compare the transmission of the five new isolates and the the BR01 isolate, and to relate the virus titre in source plants and thrips, and TSWV genotype and biotype with the rate of transmission. Two plants were inoculated to produce a source for each isolate: ALPA, Mon1NL3, Oller1TL3 and Da1NL2, and one plant for isolates GRAU and BR01.

2.3. Estimation of TSWV titer in source plants and adult thrips by real-time quantitative RT-PCR

The number of the molecules of TSWV RNA segment M was calculated by real-time RT-PCR as an estimate of TSWV titre. Total RNA from 0.1 g of fresh leaf tissue from TSWV-infected and non-infected *D. stramonium* plants and from individual adults thrips able or unable to transmit TSWV was extracted using a standard protocol with phenol:chloroform:isoamyl alcohol 25:24:1 (v:v:v) (Debreczeni et al. 2011). Real-time quantitative RT-PCR (RT-qPCR) was performed in a LightCycler$^®$480 (Roche Molecular Diagnostics, Indianapolis, IN, USA) using 25 μl of a reaction mix that contained 12.5 μl LightCycler$^®$480 Probe Master Mix (ROCHE), 4.38 μl of RNase-free water, 15 U RT Multiscribe Reverse Transcriptase (Life Technologies, Pockville, MD, USA), 2 U of RNase Inhibitor (Applied Biosystems, Foster City, CA, USA), 5 μM of primers 1M_F and 1M_R (Debreczeni et al. 2011), 0.25 μM TaqMan$^®$MGB probe and 5 μl of total RNA (~10 ng/μl). Cycling conditions consisted of reverse transcription at 48ºC for 30 min, incubation at 95ºC for 10 min and 45 cycles of 95ºC for 15 s and 60ºC for 1 min (Debreczeni et al. 2011).

2.4. Statistical analysis

Transmission rate by thrips was compared between the different isolates, and between the different biotypes and genotypes with a general linear mixed (GLM) model assuming binomial distribution of the variable transmission. Spearman's correlation test was performed to test the relationship between: (a) virus titer in source plants and transmission rate of TSWV by second instar larvae and adult thrips, (b) virus titer in source plants and viral accumulation in adult thrips and (c) viral accumulation in adult thrips and transmission rate by adult thrips. Transmission rate was compared between thrips larvae and adult thrips using Mann-Whitney U-test. Accumulation of the different TSWV in adult thrips was compared by using Kruskal-Wallis test. Developmental time was compared with one-way analisis of variance (ANOVA) and proportion of males:females was compared with χ^2 test.

3. Results

3.1. Performance of thrips on TSWV-infected plants

A total number of 464 larvae was obtained using 80 adult female thrips, and 179 of them reached adulthood. Thus, in our experimental conditions, the average reproductive rate was 5.8 (number of larvae born from each female adult) and the mean juvenile survival rate (proportion of larvae becoming adults with respect to the number of larvae emerged) was 38.6%. No significant differences were found between the rate of reproduction of thrips carrying different TSWV isolates (data not shown, Kruskal-Wallis test, p = 0.19).

Developmental time from the moment that the larvae emerged until their adulthood (on average 12.78 ± 0.15 days) was generally not different between larvae developing on plants infected with the different isolates. Only larvae developing on plants with Da1NL2 showed a longer developmental time (14.2 ± 0.48 days) than larvae from plants with ALPA (12.36 ± 0.19 days; one-way ANOVA, P=0.00077), Mon1NL2 (12.34 ± 0.26; one-way ANOVA, P=0.0065) and GRAU (12.07 ± 0.33; one-way ANOVA, P=0.0167). Overall, these results suggest that performance of thrips was not significantly different in plants infected with the different isolates tested.

Male thrips are mentioned as more efficient transmitters of TSWV than females (Rotenberg et al. 2009, van de Wetering et al. 1999). In our experiment, the proportion of males obtained (on average 34%) was lower than that of females (on average 66%) for all the isolates tested, and there were no significant differences in this proportion between the different isolates (proportion of males: ALPA: 28%; Da1NL2: 38%; Mon1NL2: 33%; Oller1TL3: 42%; GRAU: 45%; BR01: 27%; χ^2 tests, P> 0.39 in all comparisons).

3.2. Comparison of TSWV transmission by second instar larvae and adults

To evaluate the transmissibility of TSWV by the different developmental life stages of thrips, the ability to transmit the virus to Petunia leaf disks was tested for each individual second instar larvae. Once the larvae reached adulthood, their ability to transmit TSWV was also tested in the same way consecutively twice per each adult thrips. Adult thrips transmitting in the first AAP maintained the ability to transmit in the second AAP. The percentage of adult thrips transmitting TSWV was 43.5%, significantly higher (GLM, P=0.003) than the 8.1% transmitting thrips larvae observed. From all the non-transmitter second instar larvae, 40.7% were able to transmit TSWV when they became adults, whereas most (75.3%) of the transmitter second instar larvae did not lose their ability to transmit TSWV as adults during their pupal stage.

3.3. Effect of TSWV accumulation in source plants and in thrips on its transmissibility

Virus titer in the source plants (estimated by real-time qRT-PCR) varied from 2.00×10^8 to 1.17×10^{10} TSWV RNA molecules per microgram of total RNAs, which corresponded to C_t (threshold cycle) values within the same range found in another study (Roberts et al. 2000). Virus titer in the source plants were not significantly correlated either to virus titer in adult thrips (Spearman's test, P=0.111) or to transmission rate by second instar larvae (Spearman's test, P=0.310) or by adults (Spearman's test, P=0.276, Fig. 1).
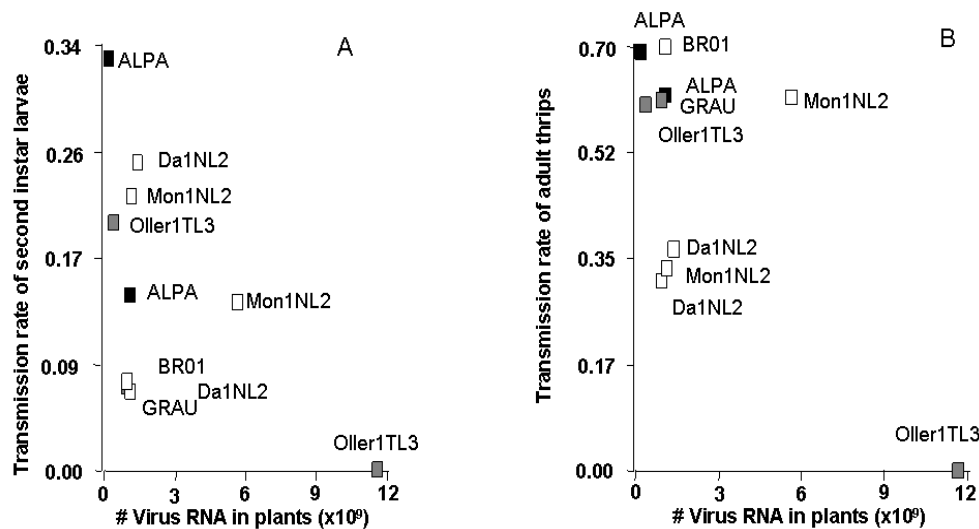
Figure 1. Correlation between accumulation of Tomato spotted wilt virus (TSWV) in source plants and transmission rate by thrips A) second instar larvae, B) adults. White squares: wild-type (WT) TSWV biotypes, black squares: Tsw resistance-breaking (TRB) biotype and grey squares: Sw-5 resistance-breaking (SRB) biotypes. Transmission rate was calculated as number of transmitter thrips divided by total number of tested thrips.

Virus titer in adult thrips varied from 1.14×10^3 to 3.6×10^7 TSWV RNA molecules per microgram of total RNA, which corresponded to C_t calues within a range found in previous estimations of TSWV in thrips (Boonham et al. 2002). Virus titres were significantly higher in transmitter adult thrips than in non-transmitter adults (Mann-Whithney U-test, P=0.00001, Fig. 2). Accordingly, virus titer in thrips were positively correlated to rate of transmission of TSWV (Spearman's test, correlation coefficient Rho=0.724; P=0.00001; Fig. 3).

Figure 2
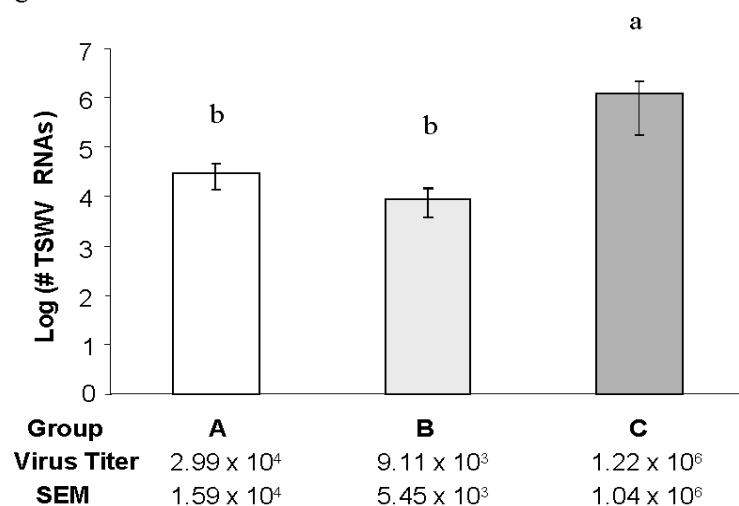
Figure 2. Average accumulation (plus standard error of the mean) of Tomato spotted wilt virus (TSWV) in thrips adults (as number of TSWV RNA molecules per microgram of total RNAs). A) Non-transmitter thrips excluding those without virus accumulation, B) non-transmitter thrips, including those without virus accumulation, and C) transmitter thrips. Bars accompanied by different letters in the graph were significantly different (Mann-Whitney U test).

3.4. Comparison between transmission of different TSWV isolates

The differences in viral accumulation of the different TSWV isolates observed in adult thrips were not statistically significant (Kruskal-Wallis test, P=0.717, Fig. 3). Also, no significant differences were found in the transmission rate between the different TSWV isolates either transmitted by thrips larvae (GLM, P=0.676) or by adult thrips (GLM, P=0.489, Fig. 3).

The same trend was found when data were grouped according to genotype or biotype. Thus, transmission rate was not significantly different between the two TSWV genotypes, either transmitted by thrips larvae (GLM, P=0.712) or by adult thrips (GLM, P=0.295). Similarly, transmission rate did not differed significantly between the three biotypes (WT, SRB and TRB) either when transmitted by thrips larvae (GLM, P=0.511) or by adult thrips (GLM, P=0.610).
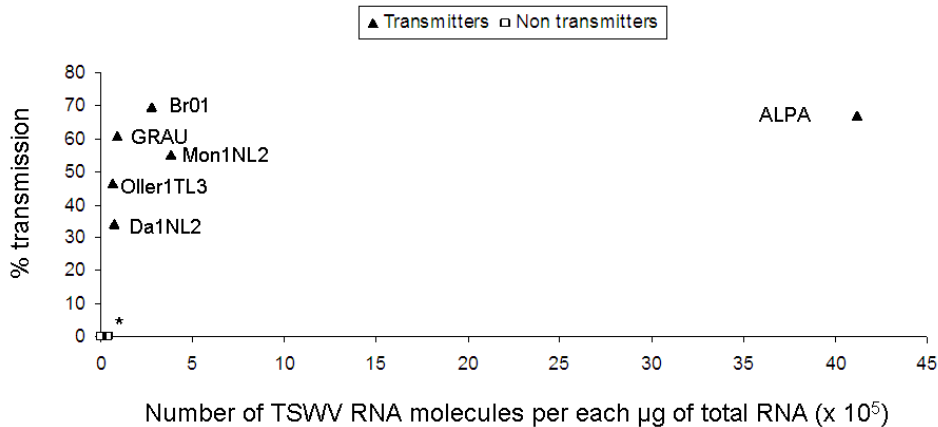
Figure 3. Transmission rate of Tomato spotted wilt virus (TSWV) by adult thrips (number of transmitter thrips divided by total number of tested thrips × 100) and virus accumulation in adult thrips (as number of TSWV RNA molecules per microgram of total RNA × 105). Black triangles: transmitter thrips and white squares: non-transmitter thrips. Each value represents the average transmission rate and average virus accumulation of transmitters and non-transmitter thrips from each TSWV isolate tested. *Non-transmitter thrips showed very low accumulation of TSWV (next to zero) for the six TSWV isolates, and isolate names are not included in the graph.

4. Discussion

TSWV displays a high level of biological diversity and a great ability of evolution and adaptation with respect to other plant viruses (Qiu and Moyer 1999, Tsompana et al. 2005). In the few years that resistant tomato or pepper cultivars have been used, TSWV isolates overcome such resistances have emerged in many areas. Evidence has been obtained that tomato resistance-breaking isolates have been generated several times by only one of two possible specific non-synonymous nucleotide substitutions in the gene encoding the movement protein NSm (López et al. 2011). However, mutation leading to the breakdown of resistance in pepper are still unknown (Margaria et al. 2007, Tentchev et al. 2011).

The generation of mutations associated to resistance breakdown is the first step of the process of emergence of isolates able to overcome resistance. This mutations would become a problem only if they are able to disperse and predominate in the viral population. Often, the plant defense system involved in resistance targets amino acid motifs that are functionally important for the virus life cycle and cause a strong negative selection against amino acid change (Moffett 2009). This negative selection makes it difficult that the virus can escape from the plant defense system and the production of resistance-breaking isolates. For this reason, the mutations associated to resistance breakdown could have fitness costs in non-resistant hosts and be associated to a decrease of the dispersion ability of TSWV (Dieckmann et al. 2002). To understand the progression of the resistance-breaking isolates, it is essential to evaluate the efficiency of transmission of the resistance-breaking isolates by thrips with respect to WT isolates and study the factors determining their transmissibility.

Our results indicated that TSWV accumulation in plants had no effect in the transmission efficiency of TSWV by thrips. A recent study described that *F. occidentalis* needed a threshold amount of TSWV particles in source plants to become viruliferous (Okazaki et al. 2011). This observation suggests that the virus titers of the source plants in our experiment were above the concentration threshold needed to allow transmission by thrips. This suggestion also means that possible fitness loss of TSWV in the host (eg. Replication and cell-to-cell movement) associated to the acquisition of the ability to infect resistant cultivars would have a small impact in the transmission of these isolates by thrips at least in conditions similar to those in our experiment.

Viral accumulation in *F. occidentalis* was positively correlated to the transmission rate of TSWV by adults. This confirms what had been observed in another study on TSWV transmissibility by *F. occidentalis* (Rotenberg et al. 2009). Therefore, viral accumulation in thrips seems to be one of the main factors affecting transmission rate by thrips, although other factors can have a role, for example, the differences in feeding behaviour between males and females (Nagata et al. 2002, Rotenberg et al. 2009, Stafford et al. 2011).

Adult thrips transmitted TSWV at a higher rate than larvae in our study. This is in agreement with a lower accumulation of TSWV in thrips larvae, when compared with thrips adults reported by Inoue et. al. 2004 and with previous studies describing lower transmission efficiency of larvae when compared with adult trips (Moritz et al. 2004). These results, together with the lower mobility of larvae in the field with respect to adults, mean that TSWV dispersion must be performed predominantly by adults. The transmission rate of TSWV by adult thrips obtained in our study ranged from 34% to 69%, which coincides with transmission rates described in previous works (Belliure et al. 2008, van de Wetering et al. 1999).

We used the Petunia leaf disk test to evaluate transmission rate of thrips. This method is the most frecuently used in studies on ability of transmission of TSWV and we chose it to standardise the transmission tests of all isolates independently from their host plants. However, we cannot discard the possibility of obtaining different transmission rates by using plants such as pepper and tomato. Nevertheless, in a previous study, WT and SRB isolates showed a similar transmission rate to tomato plants (Aramburu et al. 2010).

Finally, comparison of reproductive rate and developmental time of thrips, virus accumulation in thrips and transmission rates between different TSWV isolates suggested that genotype and biotype (WT and tomato and pepper resistance breaking) has the same potential to be transmitted by thrips. In Spain, SRB isolates have been confined to a small region of the Barcelona province (northeastern Spain) where they were detected for the first time in 2001, whereas TRB isolates have had a greater dispersion in Spain (Mediterranean coast of Spain and Canary Islands). These observations suggest that the differences in the spread of biotypes of TSWV observed in Spain cannot be explained on the basis to transmission efficiency by thrips.

CAPÍTULO 4

Evaluación de la resistencia y tolerancia de una nueva accession de *Capsicum baccatum* contra aislados de TSWV que superan y no superan la resistencia *Tsw* de pimiento.

Abstract

Tomato spotted wilt virus (TSWV) causes economically important losses in many crops, worldwide. In pepper (*Capsicum annuum*), the best method for disease control has been breeding resistant cultivars by introgression of gene *Tsw* from *C. chinense*. However, this resistance has two drawbacks: I) it is not efficient if plants are infected at early growth stages and under prolonged high temperatures, and II) it is rapidly overcome by TSWV evolution. In this work, we selected and evaluated a new accession from *C. baccattum*, named PIM26-1, by using a novel approach consisting in measuring how three parameters related to virus infection changed over time, in comparison to a susceptible pepper variety (Negral) and a resistant (with *Tsw*) accession (PI-159236): 1) The level of resistance to virus accumulation was estimated as an opposite to absolute fitness, $W=e^r$, being r the viral multiplication rate calculated by quantitative RT-PCR; 2) the level of resistance to virus infection was estimated as the Kaplan-Meier survival time for no infection by using DAS-ELISA to identify TSWV-infected plants; 3) the level of tolerance was estimated as the Kaplan-Meier survival time for no appearance of severe symptoms. Our results showed that the levels of both resistance parameters against TSWV wild type (WT) and *Tsw* resistance breaking (TBR) isolates were higher in PIM26-1 than in the susceptible pepper variety Negral and similar to the resistant variety PI-159236 against the TBR isolate. However, PIM26-1 showed a very high tolerance (none of the plants developed severe symptoms) to the WT and TBR isolates in contrast to Negral for WT and TBR or PI-159236 for TBR (most TSWV-inoculated plants developed severe symptoms). All this indicate that the new accession PIM26-1 is a good candidate for breeding programs to avoid damages caused by TSWV TBR isolates in pepper.

1. Introduction

Tomato spotted wilt virus (TSWV), the type member of the genus *Tospovirus* of the family *Bunyaviridae*, is one of the most widespread and economically important plant virus affecting many crops such as tomato, pepper, potato, tobacco, peanut, lettuce, bean and ornamental species (Pappu et al. 2009, Turina et al. 2012). TSWV has a wide host range including more than 1000 species and is transmitted in a persistent manner by several thrips species (*Thysanoptera*: *Thripidae*), with *Frankliniella occidentalis* (Pergande) being its main vector (Debreczeni et al. 2014, Whitfield et al. 2005).

TSWV virions are quasi-spherical composed of an outer membrane envelope derived from the host, with two embedded viral-coded glycoproteins (G_N and G_C). Inside there are several copies of the RNA dependent RNA polymerase (RdRp) and nucleoproteins which encapsidate the genome consisting of three negative-sense or ambisense RNA segments: Segment L (~9 kb) encodes a putative RNA-dependent RNA polymerase; segment M (~5 kb) encodes the cell-to-cell movement protein, NSm, and the precursor of surface glycoproteins, G_N/G_C, involved in TSWV transmission by thrips; and segment S (~3 kb) encodes a silencing suppressor, NSs, and the nucleocapsid, N (Plyusnin et al. 2012).

In pepper (*Capsicum annuum*), symptoms caused by TSWV infection vary depending on host genotype and include: stunting of the whole plant, chlorosis and necrosis of the new growth, apical downward leaf curling, mosaic or necrotic lesions on leaves, stems and fruits. The disease can cause the death of the plant or drastically reduce the proportion of marketable fruits (Boiteux 1995, Moury and Verdin 2012, Soler et al. 1998).

Introgression of genes conferring resistance or tolerance against viruses in commercial cultivars from wild relatives by plant breeding is considered the most efficient and simplest strategy for viral disease control, despite of being a long and costly process (Lecoq et al. 2004). Resistance is considered a host characteristic hindering virus infection and/or multiplication, whereas tolerance is considered a host characteristic allowing systemic viral infection while developing milder symptoms than those of more sensitive hosts. We consider total resistance as the absence of systemic infection and total tolerance as the absence of systemic severe symptoms.

In spite of great efforts and investments in pepper breeding programs, in over seven decades only the gene *Tsw*, identified in several *Capsicum chinense* accessions and mapped to the chromosome 10, was found to confer total resistance against a wide spectrum of TSWV isolates (Jahn et al. 2000). Plants carrying the gene *Tsw* inoculated with TSWV show a hypersensitive response (HR) consisting of a rapid plant cell death in and around the virus entry points to halt cell-to-cell viral movement and avoid systemic infection (Soler et al. 1999). This is manifested as discrete necrotic lesions followed by abscission of the inoculated leaves (Boiteux 1995).

However, *Tsw* fails to confer resistance in plants inoculated at early stages of development and subjected to prolonged high temperatures (>30ºC) (Moury et al. 1998, Soler et al. 1998, Soler et al. 1999). Another problem is due to the high evolutionary and adaptative capacity of TSWV (López et al. 2011, Tentchev et al. 2011, Tsompana et al. 2005) that allowed the emergence of resistance breaking isolates in many areas where resistant cultivars have been grown (Boiteux and Nagata 1993, Hobbs et al. 1994, Margaria et al. 2004, Roggero et al. 2002, Thomas-Carroll and Jones 2003).

The incomplete effectiveness of the gene *Tsw* in pepper, and the great ability of the virus to generate new virulent isolates have imposed the need to seek and evaluate new sources of resistance or tolerance to TSWV. Although, most breeding programs are aimed to find and implement total resistance, considering degrees of resistance (reduction of virus infectivity and/or multiplication) and/or tolerance (reduction of symptom severity) may be useful to rescue valuable phenotypes. This requires developing new analytical tools to asses the level of resistance and tolerance.

In this work, a new accession of *C. baccatum*, PIM26-1, was evaluated for resistance and tolerance to TSWV by measuring how different parameters related to the viral infection changed over time. This accession did not suppose an improvement in terms of resistance with respect to the accessions or cultivars with the gene *Tsw*. However, PIM26-1 showed a total tolerance not only to TSWV wild type (WT) but also to *Tsw*-resistance-breaking (TBR) isolates, which induce strong symptoms and damage in cultivars carrying the gene *Tsw*.

2. Material and methods

2.1. Plants and viruses

Three pepper accessions were selected from the germplasm collection from Institute for Conservation and Improvement of Valencian Agrodiversity (COMAV) in Valencia, Spain: PIM26-1 from *C. baccatum* (the new accession), PI-159236 from *C. chinense* (containing gene *Tsw*), which was used as a resistant control, and Negral from *C. annuum*, which was used as a susceptible and sensitive control.

Four TSWV isolates were recovered from a collection of biologically characterized TSWV isolates obtained from pepper fields in Eastern Spain (Debreczeni et al. 2014), which corresponded to two biotypes: Da1NL2, of biotype WT, and Pilar 1, Alm1 and PC916, of biotype TRB.

Mechanical inoculation of TSWV was performed by grinding 2 g of TSWV-infected tomato leaf tissue in 20 ml of 0.1 M sodium phosphate buffer (pH 7), containing 0.2% of sodium diethyldithiocarbamate trihydrate (DIECA) and 0.2% of carborundum (600 mesh). This preparation was rubbed with cotton-bud sticks to pepper plants with the sixth leaf fully expanded. Some plants were inoculated only with phosphate buffer and carborundum (mock-inoculation) or not inoculated to be used as negative controls and identify possible pathological effects caused by their cultivation in the growth room.

Plants were maintained in a growth room with controlled environmental conditions of 25°C/18°C day/night temperature, 60%/95% day/night relative humidity, and 60-85 μmol s^{-1} m^{-2} of irradiance from Sylvania Grolux fluorescent tubes, and a 14 h-10 h light/dark photoperiod.

2.2. Evaluation of parameters related to viral infection

Viral titer in plants was estimated by reverse transcription and quantitative polymerase chain reaction (RT-qPCR) of total RNAs with primers 1M_F (5'-CCAACATGCCATCTGAAAAGC-3') and 1M_R (5'-CAAATGCAGCTGACAGCAGTTT -3') and the TaqMan$^®$MGB probe P$_U$ (5'-6FAM-TCTGAACTGGTCTATTCC-3'). Total RNAs from 0.1 g of fresh leaf tissue from TSWV-infected and non-infected plants were purified by using a phenol-chloroform protocol eluted in 20 µl of RNase-free water, treated with RNase-free DNase(Turbo DNA-free, Ambion, Applied Biosystems, Austin, TX, USA), measured in duplicate with the UV-Vis spectrophotometer Nanodrop 1000 (Thermo Scientific, Waltham, MA, USA) and adjusted to 10 ng/µl to normalize the different extractions (Debreczeni et al. 2011).

RT-qPCR was performed in a LightCycler®480 (Roche Molecular Diagnostics, Indianapolis, IN, USA) using 25 µL of a reaction mix containing 12.5 µL LightCycler$^®$480 Probe Master Mix (ROCHE), 4.38 µL of RNase-free water, 15 U RT Multiscribe ReverseTranscriptase (Life Technologies, Rockville, MD, USA), 2 U of RNase inhibitor (Applied Biosystems, Foster City, CA, USA), 5 µM of each primer, 0.25 µM TaqMan®MGB probe and 5 µL of total RNA. Cycling conditions consisted of reverse transcription at 48ºC for 30 min, incubation at 95ºC for 10 min and 45 cycles of 95ºC for 15 s and 60ºC for 1 min. For absolute quantification (number of viral RNA molecules per ng of total RNA) a standard curve with serial dilutions of TSWV transcripts was used (Debreczeni et al. 2011).

TSWV-infected plants were identified by double-antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) with polyclonal antibodies (Loewe Biochemica GmbH, Sauerlach, Germany) by following the standard protocol (Clark and Adams 1977) with some modifications (Soler et al. 1999). Absorbance after serological reactions was measured at 405 nm with a microplate reader (model 550, Biorad, Hercules, California, USA). A sample was considered positive (infected) when the absorbance was higher than the mean absorbance of the blank controls (obtained from mock- or non-infected plants) plus three times the standard deviation.

Symptoms were visually evaluated and plants were classified into: asymptomatic, mild and severe based on the degree of stunting, and leaf yellowing and distortion with respect to mock-inoculated or non-infected plants.

2.3. Biological assays

In preliminary assays, the three pepper accessions were inoculated with the four TSWV isolates (10 plants per accession and isolate). At 15 days post inoculation (dpi), infectivity (proportion of infected plants) was determined by das-elisa and symptoms (proportion of plants with severe symptoms) were evaluated by visual observation.

In the final assay, leaf extracts of TSWV isolates Da1NL2 (WT) and Alm1 (TRB) were quantified by RT-qPCR, equalized to a concentration of 3×106 copies of viral RNA copies per ng of total RNA and mechanically inoculated to the three pepper accessions: Negral (susceptible), PI-159236 (with the resistance gene *Tsw*) and PIM26-1 (new accession) by using 200 µl of inoculum per plant. In total 180 plants were inoculated (30 plants per accession and isolate), 6 plants were mock-inoculated (2 plants per accession) and 6 plants were non-inoculated (2 plants per accession). At 7, 14, 21 and 28 days post inoculation (dpi), every plant was evaluated for symptoms and samples from the youngest leaves (not inoculated) were collected for each plant (768 samples) and analyzed by DAS-ELISA to detect TSWV infection (Table 1). Another part of the samples were used for estimation of virus titer by RT-qPCR from pools of five plants per accession and isolate to obtain six biological replicates (300 pooled samples). Per each sample two RT-qPCR replicates were performed.

2.4. Statistical analysis

Resistance and tolerance were evaluated as the host response to virus infection over time (in four periods: 7, 14, 21 and 28 dpi), depending upon two factors: viral biotype (WT and TBR) and plant genotype (Negral, PI-159236 and PIM26-1).

Resistance was estimated from two variables. The first variable was viral accumulation. Since the host exerts a pressure against TSWV accumulation, absolute fitness (*W*) as an inverse measure of the resistance level of each pepper genotype was used. In evolutionary biology, *W* measures the total number of surviving offspring of an individual or genotype in a given environment (Moya et al. 2004, Orr 2009). In the present study, we were not interested in comparing different viral genotypes in an environment but the performance of each viral genotype in different environments (pepper accessions) (Peña et al. 2014). *W* is calculated as $W = e^r$, where *r*, the Malthusian growth rate, is a normalized measure of the rate of virus accumulation, which was estimated as the slope of the lineal regression of the log-transformed values of the viral titer measured by RT-qPCR (log[number of viral RNA molecules +1]) over time (7, 14, 21 and 28 dpi). Data was obtained from six groups of five pooled plants (six replicates) per each viral biotype, plant genotype and time. *W* was analyzed by using a Generalized Linear Model (Molenberghs and Verbeke 2005), assuming that *W* follows a Gamma distribution and applying a log-link function (Hillung et al. 2013). Differences among treatment means were evaluated using mean standard errors, and a Bonferroni correction (Bonferroni 1936) was applied to protect against type I error.

The second variable to measure resistance was the variation over time (7, 14, 21 and 28 dpi) of the survival to viral infection (proportion of non-infected plants determined by DAS-ELISA) whose distribution was estimated with Kaplan-Meier survival curves (Kaplan and Meier 1958). Log-rank test (Peto and Peto 1972) with the Bonferroni correction (Bonferroni 1936) was used to compare survival distributions. The median survival time *Imd* (in which half of the inoculated plants were not infected) and the mean survival time *Im* (in which a single plant is expected to remain no infected) were used as measures of the resistance level to viral infection. Data were collected from 30 plants (replicates) per each viral biotype, plant genotype and time.

Tolerance was estimated as the opposite to symptom development over time. Kaplan-Meier survival analysis was used to evaluate the development of severe symptoms. The survival median time *Smd* (in which half of the plants did not present severe symptoms) and the mean survival time *Sm* (in which a single plant is expected to remain without severe symptoms) were used as a measure of the tolerance level. Data were collected from 30 plants (replicates) per each viral biotype, plant genotype and time.

All analyses were performed with *R Statistical* Software (http://www.r-project.org/) by using the packages: survival, stats and multcomp.

3. Results

In a preliminary assay, four TSWV isolates: PC-916 (biotype TBR), Pilar1 (TBR), Da1NL2 (WT) and Alm1 (TBR), whose titers were unknown, were inoculated in the pepper accessions: PIM26-1 (new accession), Negral (susceptible control) and PI-159236 (containing the resistance gene *Tsw*). At 15 dpi, all plants of Negral inoculated with the four isolates resulted infected, with most of them displaying severe symptoms (60, 80, 100 and 100% for isolates PC-916, Pilar1, Da1NL2 and Alm1, respectively). None of PI-159236 plants become infected with Da1NL2 as expected whereas most of them were infected with the TBR isolates (90, 100 and 100% for PC-916, Pilar1 and Alm1, respectively), with a variable number of plants showing severe symptoms (10, 90 and 90% for PC-916, Pilar1 and Alm1, respectively). The number of infected PIM26-1 plants was variable (10, 90, 20 and 80% for PC-916, Pilar1, Da1NL2 and Alm1, respectively) but none of these plants showed severe symptoms.

For a precise evaluation of resistance and tolerance, an assay was performed by inoculating equimolar quantities of TSWV isolates Da1NL2 (WT) and Alm1 (TBR) in three pepper accessions (PIM26-1, PI-159236 and Negral), and measuring overtime the viral titer by RT-qPCR, infectivity (proportion of infected plants) by ELISA and symptoms (proportion of plants with mild and severe symptoms).

1) Resistance measured as opposition to virus multiplication. TSWV multiplication was estimated for the WT (Da1NL2) and TBR (Alm1) isolates (Table 1 and Fig. 1). Except for the WT isolate, which did not infect the cultivar PI-159236 (with the resistance gene *Tsw*), both TSWV isolates showed an accumulation pattern

consisting of an exponential increase of viral titer reaching a maximum peak (mean titer= 2.8×10^6 viral molecules) at 21 dpi, followed by a decrease (mean titer 2.8×10^4 viral molecules) at 28 dpi.

Table 1. Analysis of three variables over time to evaluate resistance and tolerance of three pepper genotypes to two TSWV biotypes

		Negral[a]			PI-159236[a]			PIM26-1[a]		
		WT[b]	TBR[b]	C[b]	WT[b]	TBR[b]	C[b]	WT[b]	TBR[b]	C[b]
RT-qPCR[c]	N[d]	6	6	2	6	6	2	6	6	2
	7 dpi[e]	7.5 ± 4.5^3	8.9 ± 2.9^4	0	0	0	0	0	0	0
	14 dpi[e]	3.9 ± 0.9^4	8.2 ± 0.9^4	0	0	1.8 ± 0.6^4	0	1.8 ± 1.8^2	2.3 ± 1.3^4	0
	21 dpi[e]	1.7 ± 0.5^6	4.4 ± 0.7^6	0	0	5.8 ± 4.2^6	0	5.8 ± 2.9^5	1.7 ± 0.6^6	0
	28 dpi[e]	3.4 ± 1.6^4	9.7 ± 2.0^4	0	0	4.5 ± 1.8^3	0	5.2 ± 5.2^2	6.3 ± 5.7^3	0
ELISA[c]	N[d]	30	30	4	30	30	4	30	30	4
	7 dpi[e]	8	21	0	0	6	0	1	2	0
	14 dpi[e]	22	30	0	0	21	0	3	14	0
	21 dpi[e]	29	30	0	0	24	0	4	17	0
	28 dpi[e]	30	30	0	0	29	0	7	22	0
Symptoms[c]	N[d]	30	30	4	30	30	4	30	30	4
	7 dpi[e]	15(0)	23(1)	0	0	3(0)	0	0	0	0
	14 dpi[e]	21(2)	27(4)	0	0	15(4)	0	1(0)	4(0)	0
	21 dpi[e]	30(25)	30(30)	0	0	22(14)	0	3(0)	13(0)	0
	28 dpi[e]	30(30)	30(30)	0	0	30(25)	0	5(0)	21(0)	0

[a]Pepper genotypes: Negral (considered as susceptible), PI-159236 (with the resistance gene Tsw) and PIM26-1 (new accession).

[b]Inocula: TSWV isolates of biotype wild type (WT) and Tsw-resistance-breaking (TBR) and mock- or non-inoculated controls (C).

[c]Analysis. RT-qPCR to evaluate viral accumulation (mean viral titer for 6 replicates, corresponding to 6 groups of 5 plants or two groups of two plants for controls and standard error), ELISA to evaluate the number of TSWV-infected plants, and Symptoms, number of plants with symptoms (number of plants with severe symptoms is between parentheses) evaluated by visual inspection. Viral accumulation is presented simplified, ex. is $7.5\times10^3 \pm 4.5\times10^3$.

[d]N= number of replicates. RT-qPCR: 6 groups of 5 plants for WT and TBR and 2 groups of 2 plants for C, ELISA and symptoms: 30 individual plants for WT and TBR and 4 for C (2 mock- and 2 non- inoculated).

[e]Time of taking measurements: 7, 14, 21 and 28 days post-inoculation (dpi). Mean number of TSWV RNA molecules (for 6 replicates), number of TSWV-infected plants and number of plants with symptoms or severe symptoms (between parentheses) are indicated for each time.

Virus accumulation in the susceptible cultivar Negral occurred faster than in the other two pepper genotypes (PIM26-1 and PI-159236). Thus, viral titer in Negral was 7.5×10^3 and 8.9×10^4 for isolates WT and TRB, respectively; at 7 dpi whereas no accumulation was detected for the other two pepper accession at that time (Fig. 1). TSWV reached a peak at 21 dpi in the three pepper accessions: Negral (1.7×10^6 and 4.4×10^4 for WT and TRB isolates, respectively), PI-159236 (5.8×10^6 for isolate TRB) and PIM26-1 (5.8×10^5 and 1.7×10^6 for WT and TRB isolates, respectively).

A) WT isolate

B) TRB isolate

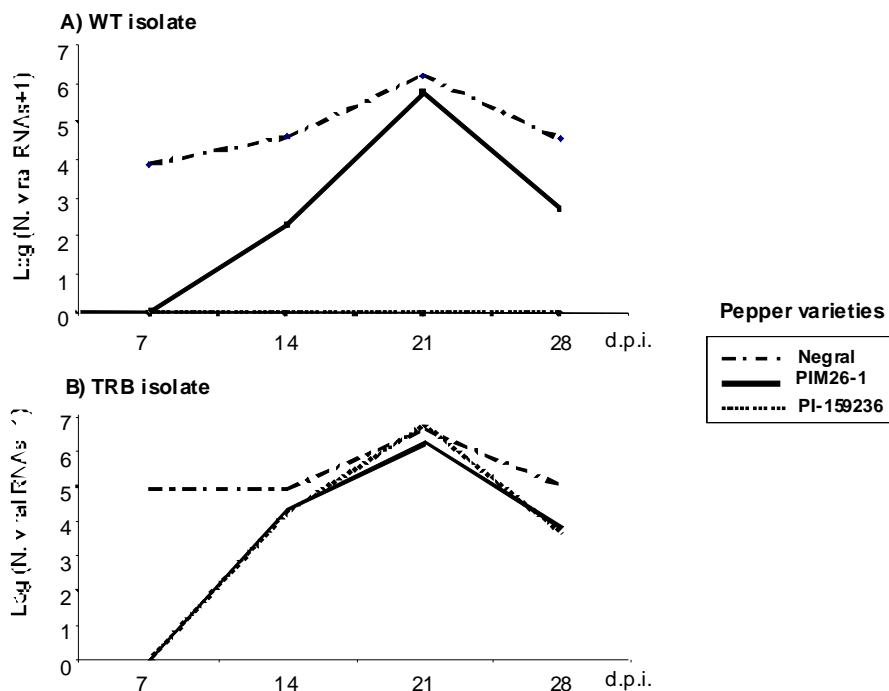**Pepper varieties**
- Negral
- PIM26-1
- PI-159236

Figure 1. Time-course accumulation, measured as (Log of number of viral RNA copies per ng of total RNA + 1), of two TSWV isolates: Da1NL2 (Biotype wild type, WT) and Alm1 (biotype Tsw resistance-breaking, TRB) in three pepper accessions: Negral (susceptible), PIM26-1 and PI-159236 (with the resistance gene Tsw). Mean values of six replicates per isolate and pepper accession are shown.

Absolute fitness was used as an opposed measure of the resistance level. Statistical analysis showed different resistance levels against isolate WT for the three pepper varieties (Table 2). As expected, the lowest *W* value was for PI-159236 containing the gen *Tsw* that confers total resistance to TSWV WT isolates (none of the plants were infected and therefore the accumulation remained zero over time).

Table 2. Absolute fitness (W) for evaluation of resistance levels to TSWV

TSWV biotype[a]	Pepper variety[b]	W (mean)[c]	GLM test[d]
WT	Negral	1.239±0.041	A
	PI-159236	1.000±0.000[e]	B
	PIM26-1	1.086±0.063	C
TBR	Negral	1.290 0.019	A
	PI-159236	1.204±0.047	B
	PIM26-1	1.157±0.056	B

[a]Three pepper varieties: Negral (susceptible), PI-159236 (with resistance gene Tsw) and PIM26-1 (new accession).
[b]Two TSWV isolates: wild type (WT) and Tsw resistance breaking (TBR).
[c]Mean and standard error of absolute fitness (W= e^r, being r the Malthusian growth rate) for six replicates (6 groups of 5 plants).
[d]For each virus biotype, different letters indicate significant differences according to a Gamma generalized linear model (overall p-value < 0.05 by using Bonferroni correction).
[e]Null accumulation as the virus never infected the host, indicating total resistance.

PIM26-1 had a lower *W* value (and therefore a higher resistance level) than the susceptible control Negral. With respect to the TRB isolate, both PI-159236 and PIM26-1 showed similar levels of resistance which were significantly higher than that of Negral.

2) Resistance measured as survival to virus infection. Fig. 2 shows Kaplan-Meier survival curves (proportion of plants non-infected by TSWV). Survival (probability of no infection) of the WT isolate in PI-159236 remained 100% since no plant became infected. This was expected since PI-159236 contains the gene *Tsw* conferring resistance against WT isolates. In the other two pepper genotypes survival decreased over time but being faster in the susceptible Negral (less than 0.5% of the plants remained non-infected at 21 dpi) than in the new accession, PIM26-1 (about 80% were not-infected at 21 dpi).

For the TBR isolate the three pepper genotypes became infected (Fig. 2). The most susceptible was Negral (all plants infected at 14 dpi) and survival decreased faster in PI-159236 (30% survival at 21 dpi) than in PIM26-1 (50% survival at 21 dpi).The median (*Imd*) and mean (*Im*) survival time for each TSWV biotype and pepper accession were used measures of the resistance level to viral infection (Table 3).

As expected, both *Imd and Im* of the WT isolate in PI-159236 could not be calculated (higher than the time used in this assay) since this pepper genotype presents total resistance and none of the plants became infected. The new accession, PIM26-1 showed much higher level of resistance (*Im*= 26.1) than that of Negral (*Im*= 14.2). Regarding the TRB isolate, the survival times for PI-159236 and PIM26-1 were not significantly different with *Imd* and *Im* values much higher than those for Negral.
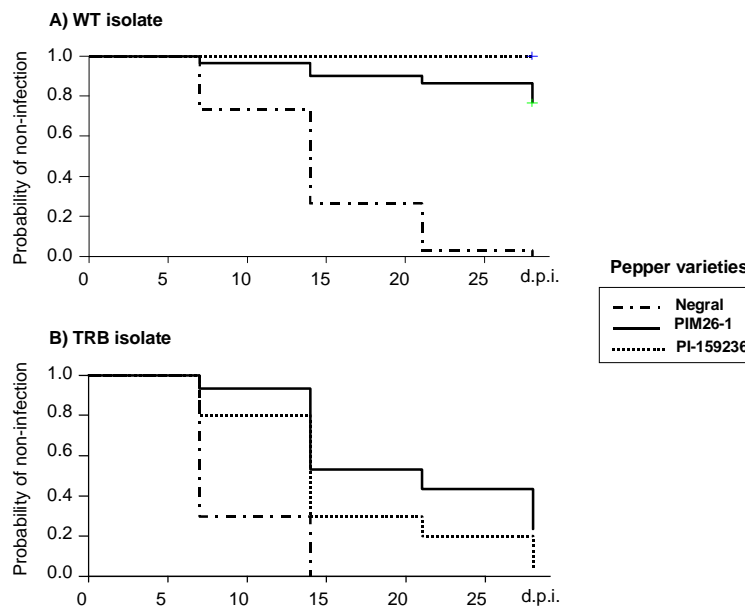
Figure 2. Kaplan-Meier survival curves showing the probability of no infection over time for two TSWV isolates: Da1NL2 (Biotype wild type, WT) and Alm1 (biotype Tsw resistance-breaking, TRB) and three pepper accessions: Negral (susceptible), PIM26-1 and PI-159236 (with the resistance gene Tsw). Thirty replicates were used per isolate and pepper accession.

Table 3. Survival time to viral infection for evaluation of resistance levels to TSWV

TSWV biotype[a]	Pepper variety[b]	lmd (median)[c]	lm (mean)[d]	Log-rank test[e]	Number of infected plants at 28 dpi
WT	Negral	14	14.2±1.0	A	30
	PI-159236	N[f]	N[f]	B	0
	PIM26-1	N[f]	26.1±0.9	C	7
TBR	Negral	7	9.1±0.6	A	30
	PI-159236	14	16.1±1.3	B	29
	PIM26-1	21	20.3±1.3	B	22

[a]Two TSWV biotypes: wild type (WT) and Tsw resistance breaking (TBR)

[b]Three pepper varieties: Negral (susceptible), PI-159236 (with resistance gene Tsw) and PIM26-1 (new accession)

[c]lmi, median survival time (that in which 50% of the plants remain non-infected) estimated according to a Kaplan-Meier survival analysis for 30 replicates (plants)

[d]lm, mean and standard error of survival time (that in which a single plant is expected to remain no infected) estimated according to a Kaplan-Meier survival analysis for 30 replicates (plants).

[e]For each virus biotype, different letters indicate significant differences according to a log-rank test (overall p-value < 0.05 by using Bonferroni correction).

[f]N means it cannot calculated indicating total resistance(no plant became infected)or almost total resistance.

3) Tolerance. The probability of plants showing no severe symptoms was evaluated for each time. As expected, all PI-159236 plants inoculated with the WT isolate remained asymptomatic since they were not infected due to their total resistance. Some Negral plants inoculated with the WT isolate showed severe symptoms at 7 dpi and the probability of showing severe symptoms increased over time (about 80% at 21 dpi and 100% at 28 dpi).
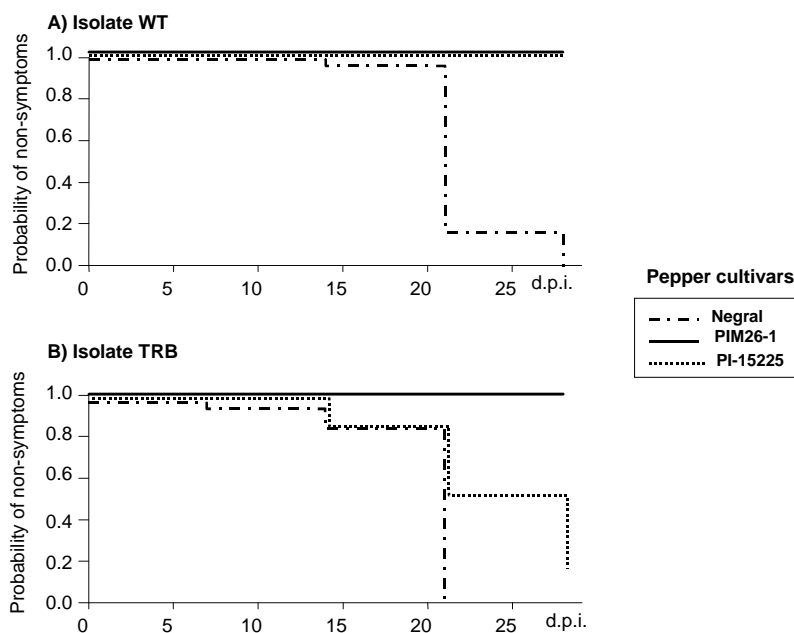

Figure 3. Kaplan-Meier survival curves showing the probability of no presence of severe symptoms over time for two TSWV isolates: Da1NL2 (Biotype wild type, WT) and Alm1 (biotype Tsw resistance-breaking, TRB) and three pepper accessions: Negral (susceptible), PIM26-1 and PI-159236 (with the resistance gene Tsw). Thirty replicates were used per isolate and pepper accession

In contrast, the new accession PIM26-1 inoculated with the WT isolate underwent a very slow increase of the number of plants with mild symptoms reaching less than 20% at 28 dpi (Table 1) and none of the plants developed severe symptoms (Fig. 3).

For the TRB isolate, Negral showed a similar response to the WT isolate with more than 80% of plants with severe symptoms after 21 dpi. PI-159236 showed a higher level of tolerance than Negral, with a lower number of plants with symptoms (about 30% mild and about 50% severe at 21 dpi) but almost all plants of both (Negral and PI-159236) had severe symptoms at 28 dpi. In contrast, PIM26-1 never developed severe symptoms, although 70% of these plants had mild symptoms at 28 dpi (Table 1 and Fig. 3).

Regarding the WT isolate, Negral showed a very low level of tolerance whereas PI-159236 and PIM26-1 showed total tolerance since none of the plants developed severe symptoms (Table 4). In this case, PIM26-1 showed a true tolerance since the virus infected and multiplied in the host, in contrast to PI-159236 which was never infected, therefore in this case is not tolerance but resistance. Regarding the TBR isolate, PI-15923 showed a little higher, yet statistically significant, tolerance than Negral whereas PIM26-1 had total tolerance (Table 4).

Table 4. Survival time to severe symptoms for evaluation of tolerance levels to TSWV

TSWV biotype[a]	Pepper variety[b]	Smd (median)[c]	Sm (mean)[d]	Log-rank test test[e]	Number of plants with severe symptoms at 28 dpi
WT	Negral	21	21.9±0.5	A	30
	PI-159236	N[f]	N[f]	B	0
	PIM26-1	N[f]	N[f]	B	0
TBR	Negral	21	19.8±0.6	A	30
	PI-159236	28	23.8±0.9	B	25
	PIM26-1	N[f]	N[f]	C	0

[a]Two TSWV biotypes: wild type (WT) and Tsw resistance breaking (TBR)

[b]Three pepper varieties: Negral (susceptible), PI-159236 (with resistance gene Tsw) and PIM26-1 (new accession)

[c]Smd, median survival time (that in which 50% of the plants remain did not developed severe symptoms) estimated according to a Kaplan-Meier survival analysis for 30 replicates (plants).

[d]Sm, mean and standard error of survival time (that in which a single plant is expected to remain without severe symptoms) estimated according to a Kaplan-Meier survival analysis for 30 replicates (plants).

[e]For each virus biotype, different letters indicate significant differences according to a log-rank test (overall p-value < 0.05 by using Bonferroni correction).

[f]N means it cannot calculated indicating total tolerance (no plant develop severe symptoms)or almost total tolerance.

4. Discussion

Introgression of gene *Tsw* into pepper cultivars by plant breeding has been the best method to control TSWV disease in pepper. This gene confers a total resistance against TSWV infection, although it is not efficient in some conditions (Moury et al. 1998, Soler et al. 1998, Soler et al. 1999) and not durable since TSWV can overcome this resistance after a few years of exposition (Tentchev et al. 2011).

Most breeders are aimed towards total resistance (none of the plants becomes infected). In the few cases that the resistance and tolerance levels were estimated (Galipienso et al. 2013, Rubio et al. 2003), they were usually analyzed by taking measures in a single time. These can be considered as snapshots of the host response and only provides incomplete and inaccurate information given the dynamic nature of biological processes. Evaluation of the viral infection and symptoms over time is important since the damage severity is highly correlated with the plant growth stage when the virus and/or symptoms become evident. In this work, the variation over time of the resistance and tolerance levels were evaluated and integrated. Resistance was evaluated by absolute fitness, *W*, from viral accumulation measured by RT-qPCR and the Kaplan-Meier survival time to viral infection, measured by DAS-ELISA. RT-qPCR is very sensitive and gives a very accurate estimate of viral titer (Debreczeni et al. 2011, Mackay et al. 2002) whereas DAS-ELISA is much less sensitive and, in spite that is considered semi-quantitative, provides a more limited information (in this case ELISA detects the virus if the titer has surpassed a certain threshold). In addition, data from RT-qPCR can be log-transformed to follow a normal distribution which translates into a higher statistical power than the count data from DAS-ELISA. However, RT-qPCR is much more expensive and laborious than DAS-ELISA and limits the number of replicates compared to DAS-ELISA. In the present work, six replicates (groups of five plants) were used for RT-qPCR vs 30 replicates (individual plants) for DAS-ELISA, reaching the same conclusion with both techniques. There was a high correlation between symptoms and infectivity. TSWV was not detected by ELISA only in two Negral plants inoculated with the WT isolate and analyzed at 21 dpi and one Negral plant inoculated with the TBR isolate and analyzed 7 dpi which showed mild symptoms. However these plants resulted infected when they were analyzed seven days latter and it did not affect the results since only severe symptoms were considered for tolerance evaluation. The most plausible cause is that the virus was present and induced mild symptoms but the titer did not reach a threshold for ELISA detection.

The TBR isolate accumulated more in the susceptible Negral than in the resistant PI-159236 suggesting that the acquisition of overcoming resistance conferred by gene *Tsw* did not suppose a fitness cost for TSWV (at least for these host genotypes) as reported for other plant viruses (Desbiez et al. 2003, Jenner et al. 2002), although there are also several cases with no fitness cost associated to resistance breakdown (Chain et al. 2007, Sorho et al. 2005).

Our results showed that the new accession, PIM26-1, has a certain resistance level for viral infection and accumulation against both TSWV biotypes: WT and TBR, much higher than the susceptible Negral, and similar to PI-159236 (with the resistance gene *Tsw*) against the TBR isolate. Therefore the new accession does not represent an improvement with respect to resistance because PI-159236 showed total resistance against WT isolates. However, the tolerance of PIM26-1 for both TSWV isolates was very high or total (none of the plants developed severe symptoms during the assay) in contrast to Negral for both isolates and PI-15236 for the TBR isolate, which ended with most plants developing severe symptoms.

The high tolerance of PIM26-1 was observed for different isolates in previous assays and in field (data not shown) suggesting that can be valid for a wide spectrum of TSWV isolates. The advantage of tolerance versus resistance is its durability. Resistance exerts a selective pressure favoring mutations increasing fitness in resistant cultivars (higher infectivity and/or multiplication) so that the virus would evolve to overcome the resistance under appropriate conditions (Garcia-Arenal and McDonald 2003). The *Tsw*-based resistance is not durable since TSWV resistance-breaking isolates have been detected in many areas after releasing resistant cultivars. Recently, the TSWV avirulence determinant of the *Tsw*-based resistance has been identified in the gene NSs (de Ronde et al. 2013). Nucleotide analyses suggest that mutations in several sites of this gene could trigger resistance breakdown (Margaria et al. 2007, Tentchev et al. 2011) in opposite to the breakdown of the resistance conferred by gene *Sw-5* in tomato which only one mutation in one of two loci are allowed in TSWV NSm gene (López et al. 2011, Peiró et al. 2014). Also, the loss of efficiency at higher temperatures or at early stages of growth could exert a partial selective pressure reducing virus fitness but allowing multiplication which may favor the emergence of resistance-breaking mutants such as it has been observed for RNA interference-mediated resistance (Lafforgue et al. 2011). In contrast, true tolerance (without decreasing virus infection and multiplication) might be more durable as the plant defenses would have a low negative effect on virus fitness and harming the host would decrease the probability of virus transmission to new plants. According to the avirulence hypothesis, parasites should evolve towards avirulence and the parasite fitness would be related to the host fitness. When virulence is related to virus multiplication, the tradeoff hypothesis suggests that virulence will evolve to a level at which virulence and transmission would balance to maximize the spread of the virus (Alizon et al. 2009). However, tolerant pepper plants carry the virus that can be transmitted to other crops.

In conclusion, this new accession PIM26-1, obtained from *C. baccatum*, can be used to avoid the damages by TSWV infection including those isolates able to infect pepper cultivars with the gene *Tsw*, widely used for disease control. It would be of great interest to obtain cultivars combining this tolerance with the *Tsw* resistance. This requires further research to identify the source of tolerance and the feasibility to incorporate it in commercial pepper cultivars.

DISCUSIÓN GENERAL

Uno de los métodos de control de enfermedades más utilizado es la mejora genética, basada en la incorporación en variedades cultivadas de genes que confieren resistencia a patógenos procedentes de especies silvestres. Dado que éste es un proceso largo y costoso, una de las principales cuestiones es la durabilidad de la resistencia, ya que en muchos casos los patógenos son capaces de evolucionar y adaptarse, infectando las variedades resistentes introducidas en campo dando lugar frecuentemente a fenómenos de emergencia donde la incidencia del patógeno escala drásticamente produciendo un efecto devastador en la agricultura. Por ello el estudio de los procesos evolutivos implicados en la superación de las resistencias y en la emergencia de enfermedades es un área de investigación relevante, tanto para desarrollar de resistencias más eficaces y duraderas, como para entender la epidemiología del patógeno y establecer estrategias de control de la enfermedad basadas en medidas profilácticas.

En el caso de los virus, se ha observado que aproximadamente dos tercios de las resistencias obtenidas son estables (García-Arenal y McDonald 2003). Esto no ocurre con TSWV, para el cual ha sido difícil conseguir fuentes de resistencia naturales que sean eficaces contra un espectro amplio de aislados virales. Así, a pesar del gran esfuerzo que han realizado instituciones públicas y privadas, solamente se han encontrado dos genes que aportan una resistencia eficaz: Sw-5 incorporado en tomate y Tsw en pimiento. En España el cultivo de variedades con estos genes supuso la solución a una problemática que suponía grandes pérdidas económicas. Sin embargo, a los pocos años aparecieron aislados del TSWV que eran capaces de superar la resistencia conferida por Sw-5 en tomate o por Tsw en pimiento. La gran capacidad evolutiva del TSWV para superar estas resistencias se ha constatado por evolución experimental, mediante pases seriados de aislados convencionales del TSWV en variedades resistentes (Latham y Jones 1998, Thomas-Carroll y Jones 2003). También se ha descrito que la infección mixta del TSWV con el virus de la clorosis del tomate (Tomato clorosis virus, ToCV) da lugar a una interacción sinergica que permite a aislados convencionales del TSWV infectar tomate con el gen Sw-5 (García-Cano et al. 2006).

La resistencia conferida por los genes *Sw-5* y *Tsw* se basa en una respuesta hipersensible que conlleva la necrosis de las células que rodean el sitio de entrada del virus que impiden la infección sistémica de la planta. Para poder entender como se ha generado la superación de la resistencia, el primer paso es la determinación del factor de avirulencia, es decir los componentes del virus que son reconocidas por el sistema defensivo de la planta para desencadenar la respuesta defensiva (Flor 1971). Desafortunadamente, hasta la fecha no se ha podido desarrollar un clon infectivo del TSWV que permitiera hacer estudios de genética reversa por lo que se ha recurrido a otras alternativas. Para determinar en qué segmentos genómicos del TSWV se encuentran los factores de avirulencia, se llevaron a cabo experimentos de reordenamiento (pseudorecombinación) coinoculando dos aislados, uno que supera y otro que no supera la resistencia, en una planta que produjera lesiones necróticas y cada lesión se inoculó en una planta que se infecta sistémicamente. De esta manera, se consiguió una colección de clones biológicos con todas las combinaciones posibles de

los segmentos genómicos de ambos aislados. Se constató que solamente los clones que tenían el segmento M procedente de un aislado que supera la resistencia *Sw-5* era capaz de infectar tomate con el gen *Sw-5* independientemente de la procedencia de los otros dos segmentos genómicos. Así mismo, se comprobó que solamente los clones que poseían el segmento S de un aislado que superaba la resistencia *Tsw* podían infectar pimiento con este gen de resistencia.

La comparación de las secuencias nucleotídicas del segmento M de distintos aislados del TSWV que superan y no superan la resistencia conferida por el gen *Sw-5* reveló que la superación de la resistencia podría producirse por una substitución aminoacídica en la posicion 218 o en la posición 220 (C118Y ó T120N) del gen Nsm, que codifica la proteína del movimiento viral (López et al. 2011). La demostración de que estas posiciones aminoacídicas son el determinante de la superación de la resistencia se llevó a cabo mediante un sistema genómico basado en el virus del mosaico de la alfalfa (*Alfalfa mosaic virus*, AMV) en el cual parte de la proteína de movimiento del AMV (aquella que no interacciona con la proteína de la cápsida) se sustituía por la misma parte de la proteína de movimiento del TSWV y se incoculaban plantas con y sin el gen *Sw-5* (Peiró et al. 2014). En el caso de pimiento, la comparación de las secuencias nucleotídicas no reveló ningún patrón que pudiera relacionarse con la superación de la resistencia, lo que sugiere que ésta podría ocurrir por substituciones nucleotídicas o aminoacídicas en multiples posiciones. Mediante experimentos de expresión transitoria de los dos genes del segmento S del TSWV se demostró que el determinante de avirulencia es la proteína NSs que actúa también como supresor de silenciamiento génico (de Ronde et al. 2013). El hecho de que la superación de las resistencias genéticas se produzca por unos pocos cambios aminoacídicos parece ser un rasgo común en los virus (Karasawa et al. 1999, Matsumoto et al. 2009, Meshi et al. 1989, Padgett y Beachy 1993). En otro tipo de resistencias (como aquellas basadas en el silenciamiento genético) se han encontrado que han actuado otros mecanismos evolutivos. Así, la superación de la resistencia por TSWV en plantas transgénicas, que expresan el gen N del TSWV, parece ocurrir mediante reordenamiento genómico (Qiu y Moyer 1999).

El siguiente paso consiste en estudiar cómo se producen los cambios genéticos que determinan la superación de la resistencia, en qué contexto y qué fuerzas evolutivas actúan para intentar evaluar la facilidad con la que se producen estos cambios, lo que afectará a la durabilidad de la resistencia. Los virus con genoma de RNA tienen tasas de reproducción y mutación muy elevadas lo que les permiten generar poblaciones que contienen un gran número de mutaciones dentro de una planta (Domingo et al. 1996). La superación de la resistencia conferida por el gen *Sw-5* se produce por un único cambio aminoacídico en dos posibles posiciones. En ambos casos el cambio aminoacídico solamente requiere un único cambio nucleotídico lo cual indica que estás mutaciones deben generarse continuamente. La inferencia de las relaciones filogenéticas del TSWV mostró que la superación de la resistencia conferida por *Sw-5* ha ocurrido al menos tres veces de manera independiente (López et al. 2011).

Ahora bien, cuando se generan las mutaciones responsables de la superación de la resistencia, éstas se encuentran en una proporción muy baja en la población viral dentro de la planta, por lo que debe haber algún proceso que haga aumentar su frecuencia en la población viral. La comparación de substituciones nucleotídicas no sinónimas (producen cambios aminoacídicos) y sinónimas (no afectan a la proteína) mostró que la substitución C118Y que ocurría solamente en los aislados del TSWV que superaban la resistencia del gen *Sw-5* estaba bajo selección positiva lo que corroboraba que se trata de una adaptación del virus para poder infectar plantas con dicha resistencia. Por otra parte, se observó que a excepción de los aislados del TSWV que superan la resistencia, las posiciones 118 y 120 de la proteína Nsm estaban muy conservadas. De hecho la posición 120 presentaba el mismo aminoácido para todos los virus del género *Tospovirus*. Esto sugiere que estás posiciones deben estar sometidas a una fuerte presión negativa en plantas sin la resistencia proporcionada por el gen *Sw-5* y que deben ser importantes para la función de la proteína Nsm. Usando el sistema genético heterólogo basado en el AMV que llevaba la proteína Nsm fusionada a una proteina fluorescente (*green flourescent protein*, GFP) se constató que las variantes de la proteína con la mutación 118Y o 120N producían halos de infección reducidos lo que significa una menor eficacia en el movimiento entre las células de la plantas (Peiró et al. 2014). Esta menor eficacia (fitness) en el movimiento intercelular indica que hay una presión de selección negativa y, por tanto, que estas mutaciones son eliminadas de la población viral, lo cual explicaría el porqué no se han encontrado en la naturaleza y sólo aparecen asociadas con resistencia aportada por el gen *Sw-5*.

La adaptación de un virus a un huésped resistente puede suponer un coste de manera que éste pierda eficacia (*tradeoff*) en huéspedes susceptibles (Agudelo-Romero et al. 2008) tal como se ha descrito para algunos virus (Desbiez et al. 2003, Jenner et al. 2002), aunque también hay caso en los que esto no ocurre (Chain et al. 2007, Sorho et al. 2005). En la naturaleza las plantas como hospedadoras y los virus como parásitos han estado coevolucionando en una especie de carrera armamentística. Las plantas han desarrollado genes que codifican proteínas que reconocen motivos específicos del genoma de los virus y desencadenan los mecanismos de defensa, mientras que los virus mutan en esos motivos para escapar de la detección de las plantas y poderlas infectar. La evolución ha favorecido las plantas con genes que reconozcan motivos conservados y esenciales para el ciclo del virus, de manera que la mutación de estos motivos suponga un detrimento de la eficacia del virus, lo que hace que la resistencia sea más duradera (Janzac et al. 2009). Esto explicaría en parte el porqué la mayoría de las resistencias contra virus de plantas son estables (García-Arenal y McDonald 2003). En el caso de los aislados que superan la resistencia en pimiento con el gen *Tsw* se desconocen los cambios nucleotídicos necesarios para la superación de la resistencia. Se han identificado varios aminoácidos de la proteína NSs bajo selección positiva (Margaria et al. 2007, Tentchev et al. 2011), pero no se ha podido correlacionar con la capacidad de superar esta resistencia. Existen evidencias de que la adaptación del virus a la resistencia *Tsw* de pimiento también podría estar acompañada de una pérdida de eficacia en la función de la proteína NSs. Se ha observado que aislados del TSWV que superan

la resistencia han perdido la actividad de supresión de silenciamiento (de Ronde et al. 2014a).

Finalmente, para que pueda ser considerado un caso de emergencia, y por tanto constituir un problema en la agricultura, los aislados virales que superan las resistencias deben tener capacidad de dispersarse en el campo. En esta tesis doctoral se realizaron ensayos biológicos para evaluar la eficacia (*fitness*) del TSWV. Se observó que algunos aislados que superan las resistencias *Sw-5* de tomate o *Tsw* de pimiento no presentaban diferencias estadísticamente significativas en su acumulación en *Datura stramonium* (uno de los principales reservorios de este virus) con respecto a aislados convencionales (datos no mostrados). También se constató que un aislado, capaz de infectar pimiento con la resistencia conferida por el gen *Tsw* no presentaba pérdidas en su capacidad de acumulación en dos variedades de pimiento que no tienen dicho gen (de Ronde et al. 2014a). Tampoco se encontraron diferencias estadísticamente significativas entre estos aislados del TSWV con respecto a la acumulación viral en trips y en su tasa de transmisión (Debreczeni et al. 2014).

Otro hecho interesante es que la capacidad de superar la resistencia del gen *Sw-5* se mantuvo estable durante sucesivos eventos de transmisión y multiplicación viral en variedades de tomate susceptibles (Aramburu et al. 2010). Todo esto sugiere que la superación de la resistencia no va asociada a un coste biológico (*tradeoff*) lo que es contradictorio con el análisis de las secuencias nucleotídicas en el que se detectó selección negativa en las posiciones aminoácidicas 118 y 120 de la proteína Nsm, responsables de la superación de la resistencia *Sw-5* en tomate. Una explicación podría ser que tras la fijación de las mutaciones 118Y y 120N, se hayan producido otras mutaciones compensatorias que bien restauren la función de movimiento de la proteína Nsm o que aumenten la replicación del virus. Mediante mutagénesis dirigida de la proteína Nsm se observó que otra mutación (130I) aumentaba el transporte intercelular en la variante de esta proteína que tenía la mutación 120N (con su movilidad reducida) y que solamente se daba una infección sistémica en plantas con el gen *Sw-5* cuando ambas mutaciones estaban presentes (Peiró et al. 2014). Todo lo anterior sugiere: (i) que los aislados que superan la resistencia tienen la misma capacidad de dispersarse, (ii) que el cultivo de variedades resistentes de tomate y pimiento favorecería un incremento en la incidencia de estos aislados virales, y (iii) que esta incidencia se mantendría aunque se dejaran de cultivar variedades resistentes. En España los aislados que superan la resistencia *Sw-5* de tomate han estado restringidos a dos comarcas de Barcelona, mientras que los aislados que superan la resistencia *Tsw* de pimiento se han dispersado por Andalucía, Murcia, Comunidad Valenciana, Cataluña y las Islas Canarias. Estos últimos aislados se han encontrado también en muchos más países que los primeros. Se podría especular dos posibles causas para la mayor distribución de los aislados que superan la resiencia de *Tsw*: A) el gen de avirulencia que codifica para la proteína NSs está sometida a una menor presión negativa o tiene una mayor robustez genética (Elena et al. 2006); y B) el tipo de resistencia, que pierde efectividad a altas temperatura, de manera que el cambio de temperaturas que sufren los cultivos podrían producir una expresión reducida del gen *Tsw* que disminuyese la presión negativa ejercida sobre el

virus y favoreciera la superación de la resistencia (Lafforgue et al. 2011). No obstante, hay que tener en cuenta que la epidemiología de un virus es muy compleja y puede depender de un gran número de factores, como las distintas especies vegetales silvestres y cultivos presentes en un agrosistema, la composición y dinámica de la poblaciones de trips (especies, genotipos), la introducción de nuevos aislados virales (tasa de migración) o interacciones con otros virus.

Otro de los puntos importantes que hay que considerar al tratar de obtener variedades con una resistencia eficaz y duradera es la utilización de un buen método de evaluación que tenga en cuenta las distintas variables implicadas. En esta tesis doctoral se desarrolló un método de RT-PCR cuantitativa (RT-qPCR) que se aplicó tanto para evaluar la resistencia de una nueva accesión de pimiento, como para estimar la eficacia del virus en su multiplicación en planta y en trips, que son variables importantes en la epidemiología del virus. Para que la técnica tuviera validez con todos los aislados del TSWV se tuvo en cuenta la variabilidad genética del virus y se diseñaron iniciadores y una sonda Taqman MGB (Debreczeni et al. 2011) a partir de secuencias conservadas. Se intentó diseñar sondas TaqMan que permitieran discriminar aislados que superan y no superan las resistencias. Sin embargo el análisis de las secuencias nucleotídicas obtenidas en esta tesis (Debreczeni et al. 2015) y de otros trabajos (López et al. 2011, Margaria et al. 2014, Tentchev et al. 2011, Tsompana et al. 2005) mostró que no había ninguna correlación entre las distancias genéticas y la superación de la resistencia, ya que ésta se puede producir por tan solo un único cambio aminoacidico y se ha constatado que ha ocurrido varias veces de manera independiente (López et al. 2011). No obstante se diseñaron dos sondas TaqMan que permitían cuantificar de manera independiente dos aislados pertenecientes a dos grupos genéticos distintos con respecto al segmento genómico M (Debreczeni et al. 2011). Por último, en esta tesis doctoral se desarrolló un nuevo enfoque para la evaluación de la resistencia y tolerancia. En la mayoría de los casos durante el proceso de mejora genética no se tiene en cuenta la variabilidad genética del patógeno. En este trabajo si se tuvo en cuenta. El análisis de las secuencias nucleotídicas de aislados del TSWV de todo el mundo (Debreczeni et al. 2015, López et al. 2011, Tsompana et al. 2005) mostró una variación genética baja como ocurre en un buen número de virus de plantas (García-Arenal et al. 2001) y como antes se mencionó no se encontró correlación entre la variabilidad genética y la capacidad de superar la resistencia. Por ello, para evaluar una nueva accesión de pimiento (*Capsicum baccatum*) se utilizaron solamente dos aislados virales: uno convencional y otro capaz de infectar variedades de pimiento con el gen *Tsw*.

Generalmente, los mejoradores genéticos buscan fuentes de resistencia monogénicas dominantes como las que se han utilizado en tomate y pimiento contra el TSWV (de Ronde et al. 2014b). La evaluación de estas resistencias se suele realizar mediante la observación de los síntomas y la detección del virus, normalmente por la técnica de ELISA. No obstante sería interesante, sobre todo para el TSWV en el que resulta difícil encontrar resistencias eficaces, la utilización de resistencias parciales, que reduzcan la multiplicación del virus, o tolerancias, en las cuales se reduzcan los daños causados por el virus aunque no afecten a la infectividad y multiplicación viral. Por ello,

en esta tesis se utilizó la RT-qPCR para comparar la multiplicación del virus en una nueva accesión de pimiento con respecto a una variedad susceptible y una variedad con el gen de resistencia *Tsw* (Soler & Debreczeni et al. 2015). También se consideró que sería interesante tener en cuenta el carácter dinámico del proceso y evaluar como varía con el tiempo la infectividad (porcentaje de plantas infectadas detectadas por ELISA), el título viral (acumulación viral estimada por RT-qPCR) y la sintomatología (porcentaje de plantas con síntomas severos). La técnica ELISA y la RT-qPCR se utilizaron para medir el nivel de resistencia. La técnica ELISA indica solamente si el título viral sobrepasa un umbral en cada planta, mientras que la RT-qPCR indica la cantidad precisa del virus, pero es mucho más cara, requiere más trabajo y por tanto es menos asequible para la evaluación rutinarias del gran número de plantas que se requieren en los programas de mejora genética. En esta tesis doctoral se observó que para esta accesión de pimiento se obtenía la misma información con ambas técnicas, pero habría que probarlo con otros virus y cultivos para ver si es suficiente la utilización de una técnica de detección y no es necesario una cuantificación más precisa.

La mayor originalidad de este trabajo es la utilización de una medida (un valor único), que refleje el carácter dinámico de los procesos biológicos, para comparar el comportamiento de la nueva variedad con las otras variedades. Así, la resistencia se evaluó como inverso de la eficacia absoluta (W) dependiente de la tasa de multiplicación del virus (Peña et al. 2014) o como el tiempo de supervivencia del virus a la infección utilizando las curvas de supervivencia de Kaplan-Meier (Kaplan y Meier 1958). La tolerancia se estimó como el tiempo de supervivencia del virus a síntomas intensos, también mediante las curvas de supervivencia de Kaplan-Meier.

Los resultados obtenidos fueron interesantes y pueden ser de gran utilidad para el control de la enfermead en los lugares con cultivos de pimiento donde se encuentren aislados del TSWV que superen la resistencia conferida por el gen *Tsw*. La nueva variedad de pimiento presentaba una resistencia parcial y una tolerancia total tanto con aislados del TSWV convencionales como con los aislados que superaban la resistencia conferida por el gen *Tsw*. La tolerancia podría ser más duradera ya que al no depender de la multiplicación del virus no habría una presión de selección para que el virus evolucione y aumente su virulencia. Por otra parte, las plantas tolerantes pueden actuar como reservorios del virus y como fuente de infección para otros cultivos. Sería interesante la obtención de variedades de pimiento que combinasen esta tolerancia y la resistencia del gen *Tsw*.

CONCLUSIONES GENERALES

En este trabajo se ha desarrollado una herramienta que permiten cuantificar el virus del bronceado del tomate (*Tomato spotted wilt virus*, TSWV) y se ha utilizado por una parte para estudiar algunos de los factores implicados en la emergencia de aislados que superan la resistencia conferida por los genes *Sw-5* en tomate y *Tsw* en pimiento; y por otra parte para evaluar la resistencia y tolerancia de una nueva accesión de pimiento a estos aislados del TSWV, lo que podría constituir un método de control de la enfermedad más duradero.

Las conclusiones de cada capítulo de esta tesis son:

1. La determinación y comparación de secuencias nucleotídicas del genoma de varios aislados del TSWV mostró que no había ninguna correlación entre la variación genética y la capacidad de superar la resistencia.

2. El desarrollo de un método basada en la RT-PCR cuantitativa (diseñado con las secuencias nucleotídicas) permite una detección muy sensible y una cuantificación precisa de manera universal para todos los aislados del TSWV y de manera diferencial para dos grupos genéticos del TSWV.

3. La superación de la resistencia no conllevó una pérdida de la eficacia en la acumulación viral en plantas sin resistencia, ni en la transmisión por trips. Esto sugiere que los aislados que superan la resistencia de los genes *Sw-5* en tomate y *Tsw* en pimiento tienen la misma capacidad de dispersión que los aislados convencionales.

4. Se desarrolló un nuevo método para evaluar los niveles de resistencia y tolerancia basados en el análisis a lo largo del tiempo del título viral (medido por RT-PCR cuantitativa), la infectividad (medido por ELISA) y la aparición de síntomas graves (por inspección visual). Se encontró que una accesión de *Capsicum Baccatum* presentaba una resistencia parcial y una tolerancia total tanto a los aislados convencionales del TSWV como a los aislados que superan la resistencia del gen *Tsw* de pimiento. Por tanto es un buen candidato para su implementación en un programa de mejora de pimiento.

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