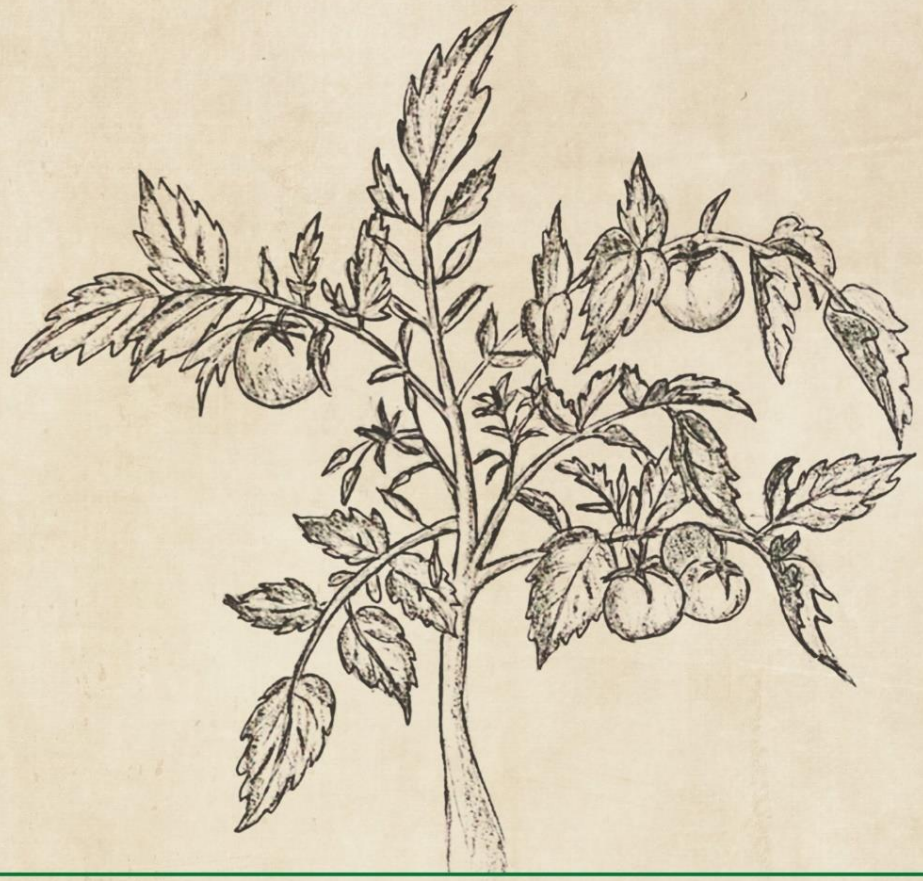
UAB
Universitat Autònoma de Barcelona

DYNAMICS OF VIRUS-HOST INTERACTIONS IN MIXED VIRAL INFECTIONS IN TOMATO PLANTS AND EFFECTS ON WHITEFLYMEDIATED DISSEMINATION

Irene Ontiveros Espinel

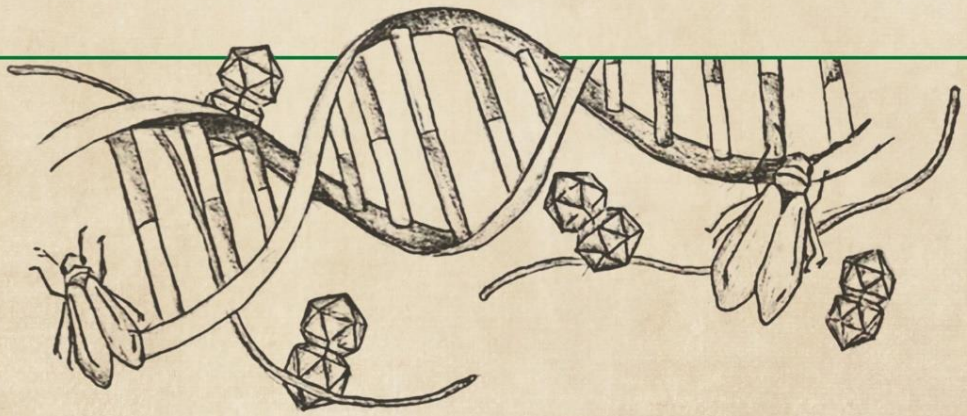
UAB
Universitat Autònoma de Barcelona



TESIS DOCTORAL

DYNAMICS OF VIRUS-HOST INTERACTIONS IN MIXED VIRAL INFECTIONS IN TOMATO PLANTS AND EFFECTS ON WHITEFLY-MEDIATED DISSEMINATION

Departamento de Biología Animal, Biología Vegetal y Ecología
Programa de Doctorado en Biología y Biotecnología Vegetal



Irene Ontiveros
Barcelona, 2023

UNIVERSIDAD AUTÓNOMA DE BARCELONA

Facultad de Biociencias

Departamento de Biología Animal, Biología Vegetal y Ecología

Programa de Doctorado en Biología y Biotecnología Vegetal

DYNAMICS OF VIRUS-HOST INTERACTIONS IN MIXED VIRAL INFECTIONS IN TOMATO PLANTS AND EFFECTS ON WHITEFLY-MEDIATED DISSEMINATION

TESIS DOCTORAL

Irene Ontiveros

Barcelona, 2023

UAB
Universitat Autònoma de Barcelona

CRAG
CENTRE FOR RESEARCH IN AGRICULTURAL GENOMICS

DYNAMICS OF VIRUS-HOST INTERACTIONS IN MIXED VIRAL INFECTIONS IN TOMATO PLANTS AND EFFECTS ON WHITEFLY-MEDIATED DISSEMINATION

Tesis doctoral presentada por Irene Ontiveros para acceder al grado de Doctora en el marco del programa de Doctorado en Biología y Biotecnología Vegetal de la Facultad de Biociencias de la Universidad Autónoma de Barcelona, bajo la co-dirección de los Dres. Juan José López-Moya y Juan Antonio Díaz Pendón.

Dr. Juan José López Moya
Director

Juan Antonio Díaz Pendón
Director

Barcelona, 2023

Este trabajo ha sido realizado a través de la beca FPI: BES-2017-080808, y se ha llevado a cabo principalmente en el laboratorio de Interacciones Planta-Patógeno del Instituto de Hortofruticultura Subtropical y Mediterránea “La Mayora” (IHSM-UMA-CSIC) en Málaga, con estancias en el laboratorio de Virología del Centro de Investigación en Agrigenómica CRAG (CSIC-IRTA-UAB-UB) de Barcelona, y bajo la supervisión conjunta en ambos casos de los Dres. Juan José López-Moya y. Juan Antonio Díaz Pendón. Parte del trabajo experimental se desarrolló durante una estancia breve en el laboratorio de Virología de la Universidad de Wageningen en Holanda.

AGRADECIMIENTOS

AGRADECIMIENTOS

Esta aventura comenzó siendo incierta y casi inesperada y ha terminado siendo una reafirmación de lo mucho que disfruto con la ciencia. Después de todo este tiempo solo queda cerrar esta etapa de la mejor manera posible, dando las gracias.

Agradecer en primer lugar a mis directores por guiarme, formarme y tratarme como una futura investigadora. Siempre he sentido vuestro apoyo.

Por supuesto, a todos mis compañeros de Virología, los que han pasado por ese laboratorio dejando huella y los que aún siguen compartiendo trabajo y risas dentro y fuera de él. Me llevo muchos amigos de ahí.

A toda la gente de La Mayora de servicio de invernaderos, en especial de la 3 y la 15, que me han ayudado tanto en que todo salga bien. Me lo habéis hecho todo más fácil, siempre disponibles.

A mis compañeros del laboratorio de virología del CRAG, por recibirme y ayudarme con una sonrisa. Y al grupo de ecología del control de plagas de La Mayora, por hacerme pasar muy buenos ratos y acogerme en vuestro “chalet”.

En general, agradecer a todos mis compañeros y amigos de La Mayora, de los servicios de invernaderos, mantenimiento de insectos, bioinformática, administración, etc., por hacerme un poco más fácil el día a día.

A mi pareja y a mis amigos de Madrid, siempre incondicionales a pesar de la distancia. Sois mi bote salvavidas.

Pero, sobre todo a mi familia. Siempre estáis y habéis estado ahí, apoyándome en esta y todas las aventuras, y las que están por llegar. Sin mis padres nunca habría sido posible llegar hasta aquí, gracias por vuestro esfuerzo y vuestro apoyo infinito.

Cada día retumba esta frase como un mantra en mi cabeza:

“No importa dónde estés sino dónde quieres llegar”

A mi familia y mis botes
salvavidas

GENERAL INDEX

General index

ABSTRACT

ABSTRACT

Viral diseases have an important impact on agriculture. Plant viruses parasitize susceptible host plants affecting their physiological functions, often leading to phenotypic changes, and also altering their interactions with other organisms, inducing direct or indirect effects that can compromise the productivity of the plant. Since the majority of these damaging plant diseases are caused by viruses transmitted by insect vectors, sensory alterations may mediate the host selection process by the vector. Consequently, affects the success of the pathogen dissemination. The frequent occurrence of viral infections with more than one virus in nature and agricultural environments increases the complexity of the interactions, and there is a need to complement the current knowledge about these relationships. The present thesis has considered the importance to understand the dynamic interactions occurring in a pathosystem involving mixed infections by *Tomato chlorosis virus* (ToCV) and *Tomato yellow leaf curl virus* (TYLCV) in tomato plants, and their common insect vector *Bemisia tabaci*. In the first chapter, the symptoms induced by both single- and mixed- infected tomato plants were recorded and compared for a better understanding of the dynamics of the phenotypic changes. We observed different symptomatic outcomes during mixed infection over time that ranged from mild symptoms at first stages to a later exacerbation of TYLCV symptoms. The synergistic interaction between both viruses at later stages of the infection suggested that the presence of TYLCV in both single- and mixed-infections played an important role in the *B. tabaci* preference behavior. Moreover, a series of choice assays were designed and performed under controlled conditions to determine the influence of visual and olfactory cues emitted by mixed-infected plants in whitefly preference responses. The results indicated that while olfactory cues have neutral effects, visual stimuli associated with a severe expression of TYLCV symptoms seemed to be more important in preference responses, enhancing the attractiveness to whiteflies. In the second chapter, we investigated changes in tomato transcriptome profile associated with single and mixed infections with ToCV and TYLCV. The time-course enrichment analysis showed significant dynamic changes in gene expression in tomato plants, affecting to processes related to photosynthesis,

hormone signal regulation, metabolism, and plant-pathogen interactions. In addition, the significantly enriched plant-pathogen interaction KEGG pathway was explored by focusing on the analysis of the *Hsp90-Sgt1* complex in ToCV-infected plants. We observed that ToCV accumulation in *Hsp90*- and *Sgt1*-silenced plants was approximately 4-fold greater than in control plants. Our findings confirmed that *Hsp90* and its co-chaperone *Sgt1* were necessary for an effective plant resistance response to ToCV infection. Finally, in the third chapter, we hypothesized about a possible cross-kingdom communication in the TYLCV-whitefly interaction through sRNAs present in virus infected tomato plants. Small RNAs from single and mixed infected tomato plants were mapped to the genome of *B. tabaci* to examine their potential to target whitefly genes. Several sRNAs that moderately accumulated in TYLCV-infected plants were found to potentially target genes in the insect. Among the selected matches, we identified three genes involved in neonicotinoid detoxification-related pathways. When analysed by RT-qPCR, the expression levels for the corresponding genes were approximately 3-fold lower in TYLCV-viruliferous whiteflies than in non-viruliferous ones, suggesting that the downregulation of the selected genes might be mediated by the TYLCV-derived sRNAs. Moreover, experiments with whiteflies feeding on an artificial diet were performed to functional validate the hypothetical role of the selected genes in survival when a toxic substance, such as nicotine, was included in the diet. The significantly lower survival rate observed suggested that the TYLCV-viruliferous condition of the whiteflies was critical for the detoxification of nicotine in our experimental setup. Overall, this study provided relevant information regarding the complexity of the interactions occurring during mixed viral infections, which could be important for a better understanding of viral pathogenesis and its ecological and epidemiological consequences.

RESUMEN

Las enfermedades virales causan un impacto importante en la agricultura. Los virus parasitan a plantas susceptibles afectando a sus funciones fisiológicas, conduciendo muchas veces a cambios fenotípicos, y también alterando sus interacciones con otros organismos, induciendo efectos directos o indirectos que pueden comprometer la productividad de la planta. Dado que la mayoría de estas enfermedades son causadas por virus transmitidos por insectos vectores, las alteraciones sensoriales pueden mediar en el proceso de selección del huésped por parte del vector y, en consecuencia, en el éxito de la diseminación del patógeno. La frecuente aparición de infecciones virales con más de un virus, en la naturaleza y en entornos agrícolas, aumenta la complejidad de las interacciones, y existe por tanto la necesidad de ampliar el conocimiento actual sobre estas relaciones. En la presente tesis se ha considerado la importancia de comprender las interacciones dinámicas que ocurren en un patosistema que involucra infecciones mixtas por el virus de la clorosis del tomate (ToCV) y el virus del enrollamiento de la hoja amarilla del tomate (TYLCV) en plantas de tomate, y su insecto vector común *Bemisia tabaci*. En el primer capítulo, se registraron y compararon los síntomas inducidos en plantas de tomate infectadas tanto con infecciones simples como mixtas, para una mejor comprensión de la dinámica de los cambios fenotípicos. Observamos diferentes resultados sintomáticos durante la infección mixta a lo largo del tiempo, que variaron desde síntomas leves en las primeras etapas hasta una exacerbación posterior de los síntomas de TYLCV. La interacción sinérgica entre ambos virus en etapas posteriores de la infección sugiere que la presencia de TYLCV tanto en infecciones únicas como mixtas juega un papel importante en el comportamiento de preferencia de *B. tabaci*. Además, se diseñaron y realizaron una serie de ensayos de elección en condiciones controladas para determinar la influencia de las señales visuales y olfativas emitidas por plantas infectadas mixtas en las respuestas de preferencia de la mosca blanca. Los resultados indicaron que, si bien las señales olfativas tienen efectos neutrales, los estímulos visuales asociados con una expresión grave de los síntomas de TYLCV parecían ser más importantes en las respuestas de preferencia al mejorar el atractivo para las moscas blancas. En el segundo capítulo, investigamos los cambios en el perfil

trasncriptómico del tomate asociados con infecciones únicas y mixtas con ToCV y TYLCV. El análisis de enriquecimiento a lo largo del tiempo mostró cambios dinámicos significativos en la expresión genética en plantas de tomate, que afectan a los procesos relacionados con la fotosíntesis, la regulación de señales hormonales, el metabolismo y las interacciones entre plantas y patógenos. Además, se exploró la vía KEGG de interacción planta-patógeno significativamente enriquecida, centrándose en el análisis del complejo *Hsp90-Sgt1* en plantas infectadas con ToCV. Observamos que la acumulación de ToCV en plantas silenciadas con *Hsp90* y *Sgt1* fue aproximadamente 4 veces mayor que en las plantas de control. Nuestros hallazgos confirmaron que *Hsp90* y su co-chaperona *Sgt1* son necesarios para una eficaz respuesta de resistencia de las plantas a la infección por ToCV. Finalmente, en el tercer capítulo, planteamos la hipótesis sobre una posible comunicación entre reinos en la interacción TYLCV-mosca blanca a través de sRNAs presentes en plantas de tomate infectadas con virus. Se mapearon sRNAs de plantas de tomate infectadas individuales y mixtas en el genoma de *B. tabaci* para examinar su potencial para mapear genes de mosca blanca. Se descubrió que varios sRNAs que se acumulaban moderadamente en plantas infectadas con TYLCV apuntaban potencialmente a genes del insecto. Entre las coincidencias seleccionadas, identificamos tres genes implicados en vías relacionadas con la desintoxicación de neonicotinoides. Cuando se analizaron mediante RT-qPCR, los niveles de expresión de los genes correspondientes fueron aproximadamente 3 veces más bajos en las moscas blancas virulíferas de TYLCV que en las no virulíferas, lo que sugiere que la regulación negativa de los genes seleccionados podría estar mediada por los sRNAs derivados de TYLCV. Además, se realizaron experimentos con moscas blancas que se alimentaban de una dieta artificial para validar funcionalmente el papel hipotético de los genes seleccionados en la supervivencia cuando se incluía en la dieta una sustancia tóxica, como la nicotina. La tasa de supervivencia significativamente menor observada sugirió que la condición virulífera de TYLCV de las moscas blancas era crítica para la desintoxicación de la nicotina en nuestra configuración experimental. En general, este estudio proporciona información relevante sobre la complejidad de las interacciones que ocurren durante las infecciones virales mixtas, lo que podría

ser importante para una mejor comprensión de la patogénesis viral y sus consecuencias ecológicas y epidemiológicas.

RESUM

Les malalties virals causen un impacte important a l'agricultura. Els virus parasiten plantes susceptibles afectant les seves funcions fisiològiques, produint moltes vegades canvis fenotípics, i també alterant les seves interaccions amb altres organismes, amb efectes directes o indirectes que poden comprometre la productivitat de la planta. Atès que la majoria d'aquestes malalties són causades per virus transmesos per insectes vectors, alteracions sensorials poden intervenir en el procés de selecció de l'hoste per part del vector i, en conseqüència, en l'èxit de la disseminació del patogen. L'alta freqüència d'infeccions virals amb més d'un virus, tant a la natura i en entorns agrícoles, augmenta la complexitat de les interaccions, i per tant hi ha la necessitat d'ampliar el coneixement actual sobre aquestes relacions. En aquesta tesi, s'ha considerat la importància de comprendre les interaccions dinàmiques que ocorren en un patosistema que involucra infeccions mixtes pel virus de la clorosi del tomàquet (ToCV) i el virus de l'enrotllament de la fulla groga del tomàquet (TYLCV) en plantes de tomàquet, i el seu insecte vector comú Bemisia tabaci. Al primer capítol, es van registrar i comparar els símptomes induïts en plantes de tomàquet infectades tant amb infeccions simples com mixtes, per entendre millor la dinàmica dels canvis fenotípics. Observem diferents símptomes durant la infecció mixta al llarg del temps, que van variar des de lleus a les primeres etapes fins a una exacerbació posterior dels símptomes de TYLCV. La interacció sinèrgica entre tots dos virus en etapes tardanes de la infecció suggereix que la presència de TYLCV tant en infeccions individuals com mixtes, juga un paper important en el comportament de preferència de B. tabaci. A més, es van dissenyar i realitzar una sèrie d'assajos d'elecció en condicions controlades per determinar la influència dels senyals visuals i olfactius emesos per plantes amb infeccions mixtes a les respostes de preferència de la mosca blanca. Els resultats van indicar que, si bé els senyals olfactius tenen efectes neutrals, els estímuls visuals associats amb una expressió greu dels símptomes de TYLCV semblaven ser més importants en les respostes de preferència en millorar l'atracció de les mosques blanques. Al segon capítol, investiguem els canvis en el perfil transcriptòmic del tomàquet associats amb infeccions simples i mixtes amb ToCV i TYLCV. L'anàlisi d'enriquiment al llarg del temps va mostrar canvis dinàmics

significatius a l'expressió gènica en plantes de tomàquet, que afecten els processos relacionats amb la fotosíntesi, la regulació de senyals hormonals, el metabolisme i les interaccions entre plantes i patògens. A més, es va explorar la via significativament enriquida d'interacció planta-patogen a la base de dades KEGG, centrant-se en l'anàlisi del complex Hsp90-Sgt1 en plantes infectades amb ToCV. Observem que l'acumulació de ToCV en plantes silenciades amb Hsp90 i Sgt1, va ser aproximadament 4 vegades més gran que en plantes control. Les nostres troballes van confirmar que Hsp90 i la seva co-xaperona Sgt1 són necessàries per a una eficaç resposta de resistència de les plantes a la infecció per ToCV. Finalment, al tercer capítol, plantegem la hipòtesi d'una possible comunicació entre regnes a la interacció TYLCV-mosca blanca a través de sRNAs presents en plantes de tomàquet infectades amb virus. Es van mapejar sRNAs de plantes de tomàquet infectades individuals i mixtes al genoma de B. tabaci per examinar el seu potencial d'afectar gens de mosca blanca. Es va descobrir que diversos sRNAs que s'acumulaven moderadament en plantes infectades amb TYLCV apuntaven potencialment a gens de l'insecte. Entre les coincidències seleccionades, identifiquem tres gens implicats en vies relacionades amb la desintoxicació de neonicotinoides. Quan es van analitzar mitjançant RT-qPCR, els nivells d'expressió dels gens corresponents van ser aproximadament 3 vegades més baixos a les mosques blanques virulíferes amb TYLCV que a les no virulíferes, cosa que suggereix que la regulació negativa dels gens seleccionats podria estar mediada per els sRNA derivats de TYLCV. A més, es van realitzar experiments amb mosques blanques alimentades en una dieta artificial per validar funcionalment el paper hipotètic dels gens seleccionats a la supervivència, quan s'incloïa en la dieta una substància tòxica com la nicotina. La significativament menor taxa de supervivència observada, va suggerir que la condició virulífera per a TYLCV era crítica per a la detoxificació de nicotina de les mosques blanques a la nostra configuració experimental. En general, aquest estudi proporciona informació rellevant sobre la complexitat de les interaccions que tenen lloc durant les infeccions virals mixtes, cosa que podria ser important per a una millor comprensió de la patogènesi viral i les seves conseqüències ecològiques i epidemiològiques.

ABBREVIATIONS

ABBREVIATIONS

Acronyms of virus species mentioned

CMV	*Cucumber mosaic virus*
PepMV	*Pepino mosaic virus*
PVX	*Potato virus X*
PVY	*Potato virus Y*
SPCSV	Sweet potato chlorotic stunt virus
SPFMV	*Sweet potato feathery mottle virus*
SPMMV	*Sweet potato mild mottle virus*
ToBRFV	*Tomato brown rugose fruit virus*
ToCV	*Tomato chlorosis virus*
ToTV	*Tomato torrado virus*
TRV	*Tobacco rattle virus*
TSWV	*Tomato spotted wilt virus*
TYLCV	*Tomato yellow leaf curl virus*
TYLCVD	Tomato yellow leaf curl virus disease
ZYMV	*Zucchini yellow mosaic virus*

List of abbreviations in alphabetical order

AAP	acquisition access period
AGO	Argonaute proteins
AhR	aryl hydrocarbon receptor
BtaEF1α	*B. tabaci* elongation factor 1-α gene
BtaJH-sRNA	fragment of *B. tabaci* juvenile hormone gene
Bta-P450-sRNA	fragment of *B. tabaci* P450 gene
BtaVit-sRNA	fragment of *B. tabaci* vitellogenin gene
cDNA	complementary DNA
CP	coat protein
CPm	minor coat protein
CS	complementary sense
Ct	cycle threshold
DCL	Dicer-like
DEGs	differentially expressed genes
EPPO	European Plant Protection Organization
ER	endoplasmatic reticulum
GFP	gene fluorescent protein
GO	Gene ontology
Hsp70	heat shock protein 70
Hsp90	heat shock protein 90
IAP	inoculation access period
ICTV	International Committee on Taxonomy of Viruses
IR	intergenic region
KEGG	Kyoto Encyclopedia of Genes and Genomes
MEAM1	Middle East-Asia Minor 1
MED	Mediterranean
NGS	next-generation sequencing
NW	New World
ORFs	open reading frames
P450	cytochrome P450
RCR	rolling circle replication
RdRp	RNA-dependent RNA polymerase

Rep	replication initiator protein
RISC	RNA-induced silencing complex
RNAi	RNA interference
RTM	restricted TEV movement
RT–qPCR	Real-time reverse transcription polymerase chain reaction
Sgt1	suppressor of the G2 allele of *skp1*
SlEF1α	*S. lycopersicum* elongation factor 1-α gene
sRNAs	small RNAs
TRV-0	empty vector of TRV
VIGS	virus induced gene silencing
VS	viral sense
YEP	yeast extract peptone

INTRODUCTION

INTRODUCTION

Viruses are considered among the major causal agents of plant diseases worldwide in both natural and agricultural environments. Despite the adoption of different control strategies, virus disease epidemics threaten cultivated plants grown causing important economic losses in agriculture affecting the yield and quality of crops (Hull, 2002; Jones, 2021). The most important diseases induced by viruses in agriculture, in terms of number and importance of diseases they provoke, rely on vectors such as insects for their transmission (Fereres, 2015). The polyphagous condition of many of these insect vectors might contribute to the frequent occurrence of mixed infections. The presence of multiple viruses in plants is very common in nature and it can have an important impact on agriculture as some of the most damaging plant diseases are caused by mixed infections (Karyeija et al., 2000; Moreno & López-Moya, 2020; Syller, 2012). However, interactions between viruses, vectors, and host plants are often unknown and poorly studied in mixed infections due to their complexity. In general, virus infections can differ in symptoms and other alterations that might be conducive to virus dissemination by insect vectors. Thus, the study of the interactions in a pathosystem during mixed infections may be crucial for a better understanding of viral pathogenesis and its ecological and epidemiological consequences.

In this context, the present work is focused on the study of single and mixed infections by two emerging viruses that often co-occur in susceptible tomato (*Solanum lycopersicum* L.) crops: *Tomato yellow leaf curl virus* (TYLCV, genus *Begomovirus*, family *Geminiviridae*) and *Tomato chlorosis virus* (ToCV, genus *Crinivirus*, family *Closteroviridae*), as well as their interactions with their common insect vector *Bemisia tabaci* (*Hemiptera: Aleyrodidae*). TYLCV is considered one of the most devastating and widespread viruses of cultivated tomatoes in tropical and subtropical regions resulting in critical yield losses (García-Cano et al., 2010; Enrique Moriones & Navas-Castillo, 2010; Yan et al., 2021). In the case of ToCV, although the yellow leaf disorder caused by this crinivirus is considered often to have less economic importance than other viral diseases, it has been found infecting a wide range of economically important crops such as tomato, pepper

and potato (Fortes et al., 2012; Fortes & Navas-Castillo, 2012). Effects of single and mixed infections involving TYLCV and ToCV in tomato plants have been studied in this thesis to determine how the virus-induced changes might affect *B. tabaci* host preferences. The development of symptoms, their severity, and the relative abundance of each virus were analysed through time in addition to the influence of virus-induced visual and olfactory changes in host plant traits on the whitefly vector's preference. General dynamic changes in accumulation patterns of small RNAs (sRNAs) and in the expression of tomato genes associated with these viral infections were also investigated, providing new insights to identify potential genes for designing future disease control strategies. Finally, the possibilities of cross-kingdom communication mediated by sRNAs between plants infected by viruses and the insect vector *B. tabaci* were explored. The results presented in this thesis intend to contribute to better understand the infections in tomato plants caused by TYLCV, by ToCV and by both of them as mixed infections, and to evaluate their complex interactions between viruses and with their host plants and whitefly vectors.

In the following sections, I will introduce key aspects of the studied pathosystems that might help to orientate, interpret and discuss the results obtained, focusing on the global impact of plant virus diseases in agriculture, on the infections by TYLCV and ToCV in tomato crops, the viral interactions in mixed infections, the effects of viral infections in *B. tabaci* behaviour, and the main molecular processes involved in the host response to viral infections, to conclude with an innovative approach to consider the relationship between elements of the pathosystem through a possible cross kingdom communication.

Impact of plant viruses in agriculture

In natural conditions, viruses as parasitic organisms form part of ecosystems, with a contribution to their ecological balance that often goes unnoticeable (Roossinck, 2013). This balanced situation has been drastically altered in agroecosystems, existing even historical evidences supporting an association between the origins of agriculture and the expansion of pathogenic plant viruses (Gibbs et al., 2008). Indeed, virology as a scientific discipline started with research done with a plant pathogen affecting a crop plant (van Kammen, 1999).

Currently, a century and a quarter later, plant viruses still represent a global and emerging agricultural problem, resulting in a negative economic impact worldwide, as they are responsible for significant damage to a wide range of crops including cereals, fruit crops, and vegetables. Agricultural globalization nowadays drives even more severity to the spreading of several viruses and their vectors that favour viral disease outbreaks with dramatic consequences for food production and ecosystem functioning. Often, this impact might be more significant in tropical and subtropical regions where constant surveillance and early identification of emerging plant viruses are neccesary for a better disease management (Hilaire et al., 2022; R. A. C. Jones & Naidu, 2019).

As a consequence of those factors, a great number of emerging viruses have been reported in different parts of the world with special predominance of the whitefly-transmitted geminiviruses. These viruses are collectively becoming one of the major threats for crop production, followed by aphid- and thrips-transmitted infections (Ertunc, 2020; Rojas & Gilbertson, 2008). Some examples might include relevant viruses such as begomoviruses responsible for cassava mosaic disease in cassava, or for tomato yellow leaf curl disease; potyviruses such as *Zucchini yellow mosaic virus* (ZYMV) or criniviruses as for example *Sweet potato chlorotic stunt virus* (SPCSV) responsible of sweet potato virus disease that result in synergistic interactions in mixed infections with an increment of the severity of symptoms(Coutts et al., 2011; R. A. C. Jones & Naidu, 2019; E. Moriones & Navas-Castillo, 2000; Jesús Navas-Castillo et al., 2011; Rojas et al., 2018; Untiveros et al., 2007).

Despite the fact that many known plant diseases can be already important limiting factor for a wide range of crops, the evolving situation force to keep a constant vigilance for the study and identification of new pathogens and diseases caused by them that could affect the different crops. As a clear example it can be mentioned the diseases of tomato (*Solanum lycopersicum*) crops, which are considered major issues during the decades as this species is considered one of the most economically important horticultural crops (FAO, 2021), with a high extension and production worldwide. Diseases induced by viral infections are considered of particular relevance with a considerable high number of described viruses that affect this crop. The intensification and production under greenhouse

conditions of tomatoes favour the survival of viruses and insect vectors by generating the appropriate conditions for a better spread. Thus, several emerging virus diseases have been described in greenhouse tomato production, including TYLCV (Juan Antonio Díaz-Pendón et al., 2010) and ToCV (William M Wintermantel & Wisler, 2006) both transmitted by whiteflies; *Pepino mosaic virus* (PepMV) (Hanssen & Thomma, 2010) efficiently transmitted by contact; or *Tomato spotted wilt virus* (TSWV) (Roselló et al., 1996) transmitted by thrips. Furthermore, other virus diseases have been identified in tomato crops in Spain such as those induced by *Tomato torrado virus* (ToTV) (Alfaro-Fernández et al., 2007; Amari et al., 2017; Verbeek et al., 2007), *Cucumber mosaic virus* (CMV) (Lavina et al., 1996), and more recently by *Tomato brown rugose fruit virus* (ToBRFV) (Alfaro-Fernández et al., 2021).

Tomato chlorosis virus (ToCV)

Tomato chlorosis virus (ToCV, genus *Crinivirus*, family *Closteroviridae*) is an emergent plant virus that causes economic losses in some important crops since it was first detected in greenhouse-grown tomato plants in Florida in 1996 (Wisler et al., 1998) showing a yellow leave disorder that was initially wrongly associated with physiological or nutritional disorders, indirectly proving the intrinsic difficulty to identify the causal agent of many yellowing or chlorotic diseases. In contrast with other criniviruses, ToCV can be transmitted by four different whiteflies in two genera. It is vectored in a semi-persistent manner by *Trialeurodes vaporariorum* (Westwood) and the MEAM1 (Middle East-Asia Minor 1) and MED (Mediterranean) complex of cryptic species of *Bemisia tabaci*, and less efficiently by *T. abutilonea* (Halderman) and *B. tabaci* NW (New World) (William M Wintermantel & Wisler, 2006; Wisler et al., 1998).

As the cultivation of tomato under greenhouses have been stablished worldwide, the European Plant Protection Organization (EPPO) currently recollects that ToCV is present in 10 countries of Africa, 7 of America, 11 of Asia, being China the most representative, and 12 of Europe including Spain and Portugal (Fig.1). Although not all of the reported outbreaks have been exclusively associated with greenhouse tomato productions, their severity is driven by the highly enhanced vector-mediated spread occurring under controlled conditions. In addition to its

impact on tomato, this virus has been reported to naturally infect a wide range of wild and cultivated plants, such as some other economically important crops including potato (*Solanum tuberosum*) (Fortes & Navas-Castillo, 2012), pepper (*Capsicum annuum*) (Lozano et al., 2004), tobacco (*Nicotiana tabacum*) (Fiallo-Olivé et al., 2014) and eggplant (*Solanum melongena*) (Zhou et al., 2015).

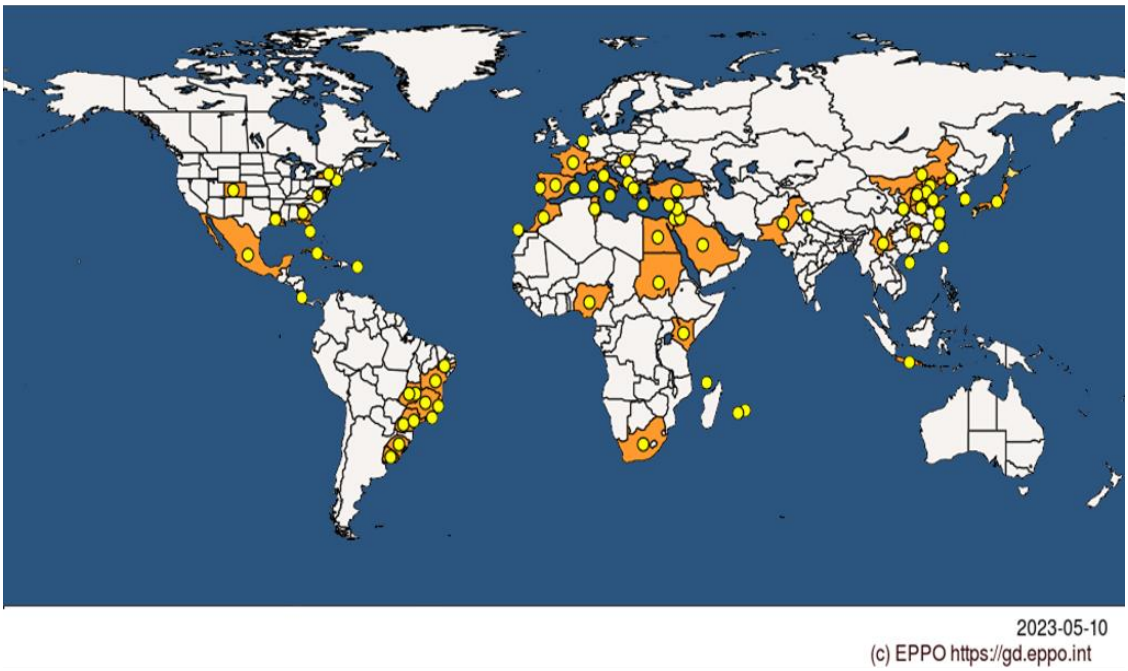


Fig 1. Geographical distribution of ToCV. Coloured areas and dots represent the regions where the virus is currently present. Source: EPPO (2023).

Overall, ToCV induced interveinal chlorosis or mild yellowing and stunting, sometimes including mild leaf curling and irregular elongation of leaves as for sweet pepper (Fortes et al., 2012; Lozano et al., 2004). In tomato, these symptoms usually begin a week after the infection displaying chlorotic areas on the lower older leaves, that progress forward to the upper part of the plant (Ontiveros et al., 2022). In addition, the curved appearance or rolling of older leaves and brown necrotic spots can be found in later stages of the infection. Infected tomato plants showed early senescence and less vigour with losses of fruit yields due to the delay on ripening (Fiallo-Olivé & Navas-Castillo, 2023).

As other criniviruses ToCV is a phloem-limited virus with a bipartite genome (RNA 1 and RNA 2) of positive single-stranded RNAs, individually encapsidated in elongated flexuous particles. RNA 1 comprises four open reading frames (ORFs), being from 5' to 3' the ORFs 1a and 1b that encode proteins associated to viral

replication (1a with protease, methyl transferase and helicase domains, and 1b being the RNA dependent RNA polymerase), followed by the ORF2 that encodes the efficient RNA silencing suppressor protein p22, and a small protein p6. By contrast, RNA 2 is organized into nine ORFs and encodes proteins putatively involved in other functions like virus encapsidation, whitefly transmission, cell-to-cell movement and also in silencing suppression activity, such as a small upstream protein p4, followed by Hsp70, p8, p59, p9, CP and CPm, p27 and p7 (Fig. 2) (Cañizares et al., 2008; Lozano et al., 2006, 2007; Wintermantel et al., 2005).

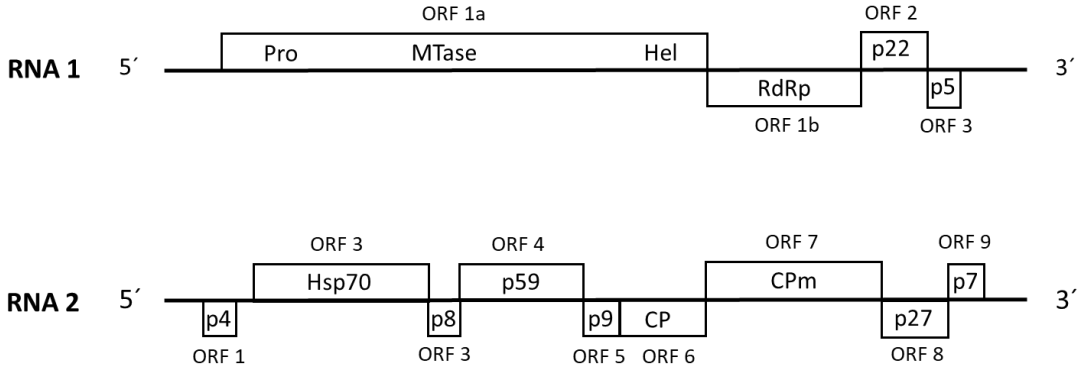


Fig. *2*. Scheme of the genomic organization of ToCV RNA1 and RNA2. Open reading frames (ORFs) and their proteins are represented by boxes.

Tomato yellow leaf curl virus (TYLCV)

Tomato yellow leaf curl virus (TYLCV, genus *Begomovirus*, family *Geminiviridae*) is considered one of the most devastating and widespread viruses of cultivated tomatoes in tropical and subtropical regions of the world, causing huge crop losses. It is responsible for the omato yellow leaf curl virus disease (TYLCVD), in addition to other related begomoviruses, which together are a limiting factor in tomato production since the first outbreaks of this disease were reported in the late 1920s in Israel (Cohen & Antignus, 1994; E. Moriones & Navas-Castillo, 2000). In general terms, begomoviruses are capable to rapidly adapt to several environments due to their high recombination and mutation rates, resulting in a complex taxonomy, which in the case of TYLCV results in identifying up to seven different strains of the virus with distinctive characteristics below the species demarcation criteria. As TYLCV is transmitted in a circulative, persistent manner by the MEAM1 and MED complex of cryptic species of *Bemisia tabaci*, the

widespread of this insect vector and the continuous trend for sharing agricultural resources contributed to the spread of this pathogen around the world (Fereres, 2015; Rojas et al., 2018).

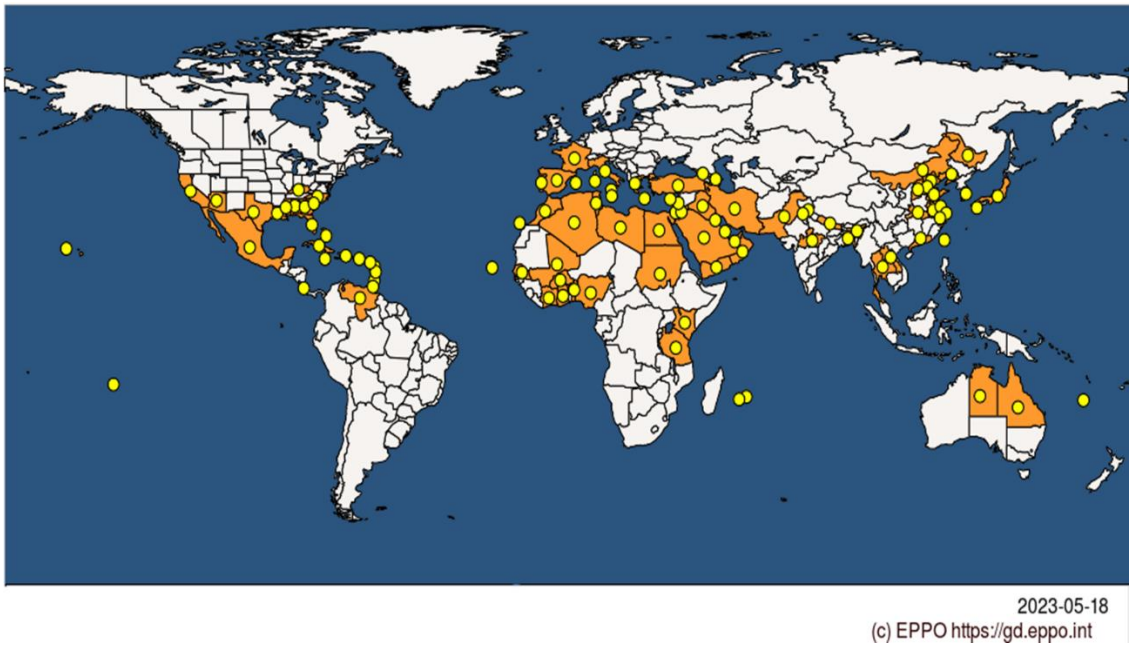


Fig. 3. Geographical distribution of TYLCV. Coloured areas and dots represent the regions where the virus is currently present. Source: EPPO (2023).

Nowadays, TYLCV is widespread across the Mediterranean basin, being present in 18 countries of Africa and 15 of Europe. It is also distributed across Australia and East Asia, being present in almost 22 countries in this last region, and it has been detected in the Caribbean and temperate regions of America (EPPO, 2023) (Fig. 3). Although this virus has been detected in a wide range of plant species including economically important crops such as common bean (*Phaseolus vulgaris*) (Jesús Navas-Castillo et al., 1999) and pepper (*C. annuum*) (Reina et al., 1999), TYLCD is mainly associated with tomato. Tomato plants affected by TYLCV exhibit characteristic symptoms of stunting, yellowing, and upward curling of leaves that appear a few weeks after the infection (E. Moriones & Navas-Castillo, 2000; Ontiveros et al., 2022). Moreover, the premature dropping of flowers and the reduction of marketable fruits can result in 100% yield lost when infections occur during early stages (Yan et al., 2021).

According to the criteria of the International Committee on Taxonomy of Viruses (ICTV), a complex of TYLCV virus species and their strains have been associated

with TYLCD. Except for two cases of related viruses that possess bipartite genomes, TYLCVs have a monopartite circular, single-stranded DNA (ssDNA) genome with six partially overlapping ORFs. An intergenic region (IR) of about 300 nt acts as a bidirectional promoter and separates the ORFs V1 and V2, which are on the virion-sense strand, and the complementary-sense strand ORFs C1 to C4 (Fig. 4). The encoded protein of ORF V1 are responsible for movement and encapsidation of the genome (capsid protein), and V2 was identified as a suppressor of gene silencing (Zrachya et al., 2007). The C1 protein is the Rep protein, involved in viral replication (Noris et al., 1996), and C2 protein is a host-range factor and considered as a suppressor of gene silencing in TYLCV-China (Dong et al., 2003; Wartig et al., 1997). As C1, C3 protein is associated with replication and C4 is involved in symptom expression and virus movement (Jupin et al., 1994; Wartig et al., 1997). The stem-loop structure of the IR contains a conserved region used for the replication initiator protein (Rep) to initiate the rolling circle replication (RCR) (Prasad et al., 2020). Recent reports showed that the TYLCV genome contain additional ORFs besides the canonical ones described to date such C5, C7and V3 that play an important role in viral pathogenicity and suppression of RNA silencing (Gong et al., 2021; H. Liu et al., 2023; S. Zhao et al., 2022).

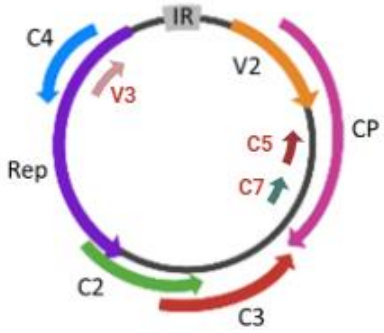


Fig. 4. Scheme of the genome organization of TYLCV. The ORFs coded by viral orientation (V2, CP) and complementary sense orientation (C4, Rep, C2, C3) strands are represented, in addition to additional ORFs C5, C7 and V3.

Bemisia tabaci (Gennadius)

When dealing with the impact of viral diseases in agriculture, a special emphasis must be given to emerging viruses, usually spread via insect vectors, particularly through transmission by whiteflies such as *B. tabaci*. This vector is a phloem-feeding insect that was first named the tobacco whitefly as it was described in 1889 as a tobacco pest in Greece (Gennadius, 1889). A century and a third later, it is nowadays considered one of the most damaging insect vectors in agriculture as it has been reported in more than 600 different plants (both crops and ornamental plants) and as a vector of a wide range of viruses (Antignus, 2010; Costa et al., 1973; D. R. Jones, 2003; Laurence Alfred Mound & Halsey, 1978). Therefore, the polyphagous nature and global distribution of *B. tabaci* result in high economic losses associated to direct and indirect damage caused by this insect acting as pest and vector.

B. tabaci is considered a cryptic complex of species comprising differences in genetics and biological characteristics such as attraction behaviour and host plant rates (De Barro et al., 2011; Perring, 2001; Xu et al., 2010). Although the first outbreaks of viruses transmitted by *B. tabaci* in the Mediterranean Basin were associated to the MEAM1 specie, later different *B. tabaci* populations were found coexisting in local areas of the south of the Iberian Peninsula (Guirao et al., 1997; Simón et al., 2007). The presence of the MED specie of *B. tabaci* have been reported in several Mediterranean countries, and more recently in farther regions such as China and North and Central America (Barbosa et al., 2015; Brown et al., 2005; Horowitz et al., 2003; L. P. Zhang et al., 2005). Consequently to the global severe infestations recorded, avoiding further dispersal and control of this whitefly has been one of the main topics for plant health during recent years. However, intensive use of insecticides, such as nicotinoids in horticultural production has resulted in an apparently paradoxical effect, with reduction of susceptibility *of B. tabaci* to these chemical products once they were developing resistances after repeated exposure with a negative impact on management strategies for control (Oliveira et al., 2001; Palumbo et al., 2001).

Mixed infections

Efficient transmission mechanisms mediated by insect vectors and the overlapping host ranges of many viruses are two factors that may contribute to the more frequent presence of multiple viruses in the same plant. Although these mixed infections are very common in both natural and agricultural environments, sometimes inducing important diseases with ecological and economic consequences (Moreno & López-Moya, 2020), there is a gap in the understanding of their complex interactions compared with often much better knowledge of the individual infections.

Multiple interactions occur during mixed infections with a direct impact on the host plant, and also a potential influence on the insect vector(s), including their behaviour. Viruses involved in mixed infections can be either genetically related or unrelated and they can interact in different ways, which might range from synergism to antagonism (DaPalma et al., 2010; Syller, 2012). A third possible situation occur when coexisting viruses do not influence on each other, referred as neutralism (Mascia & Gallitelli, 2016; Syller & Grupa, 2016). In most cases, antagonistic interactions occur when the presence of one virus is detrimental for at least one of the other present viruses, for instance with a decrease in the replication or transmission rates (Chávez-Calvillo et al., 2016; Syller & Grupa, 2016). By contrast, synergism increase the fitness of one or more of the viruses, which may result in an increment of virus titres and often with more severe symptoms in the host plant than those observed in single infections with the same viruses (García-Cano et al., 2006; Mascia & Gallitelli, 2016; Ontiveros et al., 2022; Syller, 2012). Consequently, synergistic interactions usually have a high economic impact when affect commercial crops. Contrary, antagonism in some cases can be even very useful to prevent infections by a closely related virus as part of cross-protection strategies, in which an attenuated strain serves to avoid or diminish the damage of other severe strain (Agüero et al., 2018; Moreno & López-Moya, 2020).

Significant diseases caused by mixed infections have been reported affecting diverse groups of plants during many years. The synergistic interaction between *Potato virus Y* (PVY) and *Potato virus X* (PVX) in tobacco plants (*Nicotiana*

tabacum) is a good example of an increment of the severity of symptoms when the two viruses coexist in the same plant (Bance, 1991; Rochow & Ross, 1955). Another example of important crops highly affected by multiple viral infections is sweet potato (Clark et al., 2012). Some mixed viral infection that dramatically affect the production of this crop have been reported, for example the synergism between isolates of SPCSV and *Sweet potato feathery mottle virus* (SPFMV), and between SPCSV and *Sweet potato mild mottle virus* (SPMMV).

In case of tomato, several mixed infections have been reported affecting this crop in different parts of the world, including our country and closer Mediterranean regions (García-Andrés et al., 2007; Gómez et al., 2009; Monci et al., 2002), for which their presence in co-infections might be associated with high infestations of *B. tabaci* (Navas-Castillo et al., 2000). More recently, mixed infections caused by TYLCV and ToCV in tomatoes have been studied in different regions where the distribution of these two viruses, and consequently populations of *B. tabaci* vectors, overlapped (Gul-Seker & Elibuyuk, 2019; Li et al., 2021; Martínez-Zubiaur et al., 2008; Zhao et al., 2014). Although mixed infections with these non-related viruses are expected to be common in crop areas where both are present, the epidemiological consequences that may rise are still unpredictable since a recent publication suggest a possible break of resistance to TYLCV conferred by the Ty-1 gene in mixed infected plants with ToCV (Fortes et al., 2023).

Effects of viral infections on their insect vector and host

The vector-borne transmission of viruses requires a close interaction between the insect vector and the plant since the vector has to be in contact with the infected plant to acquire the virus and subsequently transmit it to another host. Therefore, they depend on vector behaviour for a successful spread. However, this process might be altered by virus-host interactions during viral infections with important implications for transmission.

Virus infection often induces a wide range of changes in the plant physiology that depends on the infection (single or mixed) and, in the case of mixed infections, on the different interactions between coexisting viruses. Although vector-borne viruses differ in their transmission, such viral-induced changes may alter the sensory cues emitted by the infected plant that mediate vector behaviour, for

instance by modifying the frequency and nature of interactions between host and vector (Fereres & Moreno, 2009; Johnston & Martini, 2020; Mauck et al., 2016). Since vectors such as whiteflies use olfactory and visual cues to first select a host plant, previously to landing and probing the plant, there is a recent concern about how changes in these cues emitted by the plant after a viral infection may influence on insect vectors behaviour (Fang et al., 2013; Fereres et al., 2016; Shi et al., 2018). However, only a few studies have documented such effects in mixed viral infections (review in Moreno & López-Moya, 2020; Ontiveros et al., 2022) which usually result in different phenotypes that alter fitness and virus transmission by their vector in a distinctively manner compared to single infections (Gautam et al., 2020; Syller, 2014; Wintermantel et al., 2008).

The molecular details underlying virus interactions, mainly in mixed infections, are in most cases relatively unknown. In addition to the modulation of volatiles and visual cues, virus infections induce deep alterations of the host plant transcriptome and also of the abundance and profile of sRNAs (Alcaide et al., 2022; Matsumura & Kormelink, 2023). The economic importance of synergistic interactions and the development of next-generation sequencing (NGS) in recent years has allowed a better understanding of the mechanisms of interaction between plants, viruses and insects (Qiao et al., 2023; Vargas-Mejía et al., 2020).

Important biological functions such as the regulation of gene expression and the defense against viruses are modulated by sRNAs inducing the silencing of specific genes by RNA silencing pathways. During viral infections, dsRNA are synthesized by the RNA-dependent RNA polymerase (RdRp) as a necessary step in genome replication, and in some cases their presence as aberrant could trigger a molecular response to be inactivated by a sequence-specific degradation (Baulcome, 2004). sRNAs, include microRNAs (miRNAs) and small interfering RNAs (siRNAs) of 21, 22, and 24 nt that mediate RNA silencing pathways, being the two major groups of sRNAs described in plants. In a simplified scheme, these and other sRNAs are involved in several silencing processes that result in the inhibition of gene expression either at transcriptional or post-transcriptional levels (Brodersen & Voinnet, 2006; Diaz-Pendon et al., 2007; Ding, 2010). The produced dsRNAs during viral infection, either replication intermediate forms of RNA viruses, or structured parts of transcripts for both RNA

and DNA viruses, can be processed by Dicer-like (DCL) dsRNA-specific endoribonucleases into 20-25 nt virus-derived small RNAs (viral sRNAs). There are four major DCL elements in most plants, with DCL1 processes miRNAs, while the sRNAs of 21nt, 22nt, and 24nt are produced by DCL4, DCL2, and DCL3, respectively. Argonaute proteins (AGO) provide slicing activity to the RNA-induced silencing complex (RISC) and bind these siRNAs to guide them for degradation or translation arrest of the corresponding target mRNAs in a sequence-specific manner (Diaz-Pendon et al., 2007; Ding, 2010).

The role of sRNAs in post-transcriptional silencing of mRNAs has been well documented not only in plants but also in insects, even leading to novel approaches for insect pest management. The evidence of the presence of plant-derived sRNAs in some insect sRNAs databases (Zhang et al., 2012), and the studies that reveal a modulation of gene expression in insects after the ingestion of sRNAs from plants (H. Wang et al., 2017), support the idea that transferring sRNAs between different species can represent a new system to connect the regulation of functions between distantly related organisms. Therefore, a phenomenon denominated cross-kingdom RNAi could be a way of communication between extracellular interacting organisms that might facilitate gene silencing in unrelated organisms (Matsumura & Kormelink, 2023; Weiberg et al., 2015).

The cross-kingdom transfer and action of sRNAs might be favoured by the conserved RNA silencing mechanisms between several organisms, including plants and insects such as *Drosophila melanogaster* (Ding, 2010; Jensen et al., 1999). In recent years, this type of communication has been reported to influence in a wide range of host-pathogen interactions. Some of these examples include fungi-plant pathosystems (Hua et al., 2018; Weiberg et al., 2013) and plant-insect interactions (Zhang et al., 2019; Zhao et al., 2014). Therefore, although further studies in different pathosystems are necessary to better understand the implications of these cross-kingdom interactions, their knowledge and application through RNA interference (RNAi) technology can provide a powerful tool to explore new strategies aiming to identify, and eventually silence, important genes in vectors that might be useful for disease control in agriculture.

Despite being still poorly studied and understood, the possibility of cross-kingdom communication at the molecular level constitutes an attractive hypothesis that might help to explain aspects of the complex virus-host-vector interactions, governing for instance behavioural mechanisms of vectors with potentially important ecological consequences for the dissemination of diseases. In evolutionary terms, viruses could benefit from the capacity to indirectly alter the behaviour of vectors towards being conducive for transmission, using their capacity to infect the host plant as a channel to deliver the "message" to the insect feeding on the same plant, acting as a bridge organism for the communication between virus and vector. In this scenario, sRNAs can be ideal vehicles for this cross-kingdom dialogue, as proposed in an interesting recently published theoretical frame (Matsumura & Kormelink, 2023).

OBJECTIVES

OBJECTIVES

The overall objective of this thesis was to improve the understanding of the dynamics of virus-plant-vector interactions, at a molecular and phenotypic level, in a complex pathosystem of mixed infections with two non-taxonomical related viruses (TYLCV and ToCV) in tomato plants, and their common vector *Bemisia tabaci*. To achieve this general goal, the following specific objectives were contemplated:

1. Evaluation of the effects of virus-virus interactions in mixed infections involving TYLCV and ToCV in tomato plants to determine their influence on whitefly vector´s preference.

2. Identification and functional validation of transcriptomic responses of tomato plants to single and mixed infections.

3. Study of dynamic changes in accumulation patterns of small RNAs (sRNAs) during mixed infections in tomato plants.

4. Exploration of the potential of cross-kingdom sRNAs communication in plant-virus-vector interactions in a context of TYLCV viral infections in tomato plants.

MATERIALS AND METHODS

MATERIALS AND METHODS

Plant and insect materials, and growth conditions

The tomato (*Solanum lycopersicum*) line used to develop all experiments of this work was the virus-susceptible cv. “Moneymaker”. In addition, plants of the wild tomato relative species *Solanum habrochaites,* former *S. glabratum,* (accession PI 134418) were used for preliminary control tests in Y-tube olfactometer experiments. All seeds were provided by the Instituto de Hortofruticultura Subtropical y Mediterránea, La Mayora (IHSM), CSIC-UMA. Seeds were surface-sterilized by a 1 h incubation with diluted commercial bleach at 50% (final sodium hypochlorite concentration ~ 2,4 %) and then extensively rinsed with sterilized mili-Q water. Sterile seeds were sown in plastic pots of 20 cm diameter containing vermiculite and were maintained in an insect-free chamber under controlled conditions. Chamber light intensity was set at 250 μmol·$s^{-1}m^{-2}$ with a 16 h/d photoperiod (25°C day /20 °C night) and 70% relative humidity. After 7-10 days, plants were individually transferred to 12 cm diameter pots and placed inside a greenhouse under natural light and at controlled temperature (25 ± 2 °C). Both test plants and virus source plants were maintained under the greenhouse-controlled conditions mentioned.

The virus-free colonies of *Bemisia tabaci* MED species (former Q biotype), originated from individuals collected during field visits in Malaga, were reared on melon plants (*Cucumis melo* L. “ANC42”) from IHSM-UMA-CSIC seedbank collection. Plants sown in 20 cm plastic pots were placed in wooden cages covered with insect-proof nets and all colonies were maintained in an insect-proof glasshouse with temperature control (22 to 27 °C day and 17 to 20 °C night) and light supplementation when needed.

Virus sources

The viruses used in this study were the Israel strain of the geminivirus *Tomato yellow leaf curl virus* (TYLCV) (GenBank accession number AJ489258) and the isolate Pl-1-2 of the crinivirus *Tomato chlorosis virus* (ToCV). This isolate was initially detected in a naturally infected tomato plant during a field sampling in a

commercial tomato crop in Malaga in 1997 and it was maintained at IHSM by periodic transmissions with *B.tabaci*. Conversely, *Agrobacterium tumefaciens*-mediated puncture inoculation technique (agroinoculation) was used to obtain TYLCV infected source plants, using the infectious clone provided by the virology department of IHSM-CSIC “La Mayora”. In addition, *Tobacco rattle virus* (TRV) (Liu et al., 2002) was used as vector for virus induced gene silencing (VIGS).

Agrobacterium-mediated virus inoculation

Agrobacterium tumefaciens strain GV3101 was used to inoculate TYLCV, TRV and the TRV- constructs for VIGS experiments according to the procedure previously described by (Ratcliff et al., 2001). Bacterial cultures from the glycerol stock were grown on a yeast extract peptone (YEP) liquid medium at 28 °C at 200 rpm for 48h, containing 1 µl/ml of the antibiotic required (kanamycin (50µg/mL) and rifampicin (25µg/mL)). Once the culture reached an optical density between 0.8 and 1.0 measured at λ = 600 (OD_{600}), it was centrifuged at 3500 rpm for 20 min at 4 °C and then re-suspended in inoculation medium (10 mM of MgCl2, 10 mM of MES pH 5.6, 150 mM of acetosyringone) with an OD_{600} adjusted at 1.0. Tomato plants at the three-leaf growth stage were inoculated in the axillary buds of the first or second true leaf, using a needle syringe, and then maintained under the aforementioned greenhouse conditions. For VIGS constructions, tomato plants were agroinoculated by mixing an equal volume of *pTRV1* and *pTRV2*-insert. Similarly, positive control plants were agro-inoculated with *A. tumefaciens* cultures carrying *pTRV1* and empty *pTRV2* (*TRV-0*). Seven days after inoculation, plants were infected with ToCV by *B.tabaci*-mediated inoculation as described below.

Whitefly-mediated virus inoculation

Adults of *B. tabaci* MED species from colonies maintained at IHSM-CSIC La Mayora were used for whitefly-mediated virus inoculation. To obtain viruliferous whiteflies, virus-free individuals were mass given a 24h acquisition access period (AAP) on virus source plants 4 weeks after their infection with either TYLCV or ToCV (single infections) or with both viruses (mixed infections) within insect-proof cages. As previously described by García Cano et al., (2015), different inoculation procedures may follow virus acquisition: mass whitefly inoculation or individual

whitefly inoculation. Both methodologies can be performance for the establishment of single infections. In case of mass inoculation, viruliferous whiteflies were transferred to an insect-proof cage with healthy test plants at the three-leaf growth stage for a 48h inoculation access period (IAP). A total of about 100 viruliferous whiteflies per test plant were released. Conversely, 20 viruliferous and 20 non-viruliferous whiteflies per test plants (three-leaf growth stage) in clip-on cages were used for individual whitefly inoculations. To obtain co-infected plants, 40 viruliferous whiteflies containing 20 with ToCV and 20 with TYLCV were established. Clip-on cages were removed after 48h of IAP and the infested leaf was cut at 7 days to avoid eclosion of possible eggs laid by adults during IAP. Same procedure was used to obtain mock-inoculated (healthy) plants except that non-viruliferous whiteflies were used for inoculation of test tomato plants. All healthy, single- and mixed-infected plants were maintained until verified infection conditions in a greenhouse protected with an insect-proof net.

Artificial feeding

The liquid diet employed in this study was mainly composed of sterilized water with 15% sucrose. According to the experiments, the diet can be mixed with 45 µg/ml of the selected virus-derived sRNAs (vsRNAs) duplexes (commercially provided), and either a concentration of 0.01% nicotine or without nicotine, reaching a total volume of 100 µl. The diet was contained in between two stretched layers of Parafilm covering the end of 50 mL centrifuge tubes. Groups of 30 whiteflies were released inside each tube before adding the diet to the parafilm sachet. To favour feeding of the insects, the tubes were orientated toward a light source at approximately 20 cm distance.

To evaluate responses of non-viruliferous and TYLCV-viruliferous whiteflies to nicotine, survival of insects after feeding on artificial diet for 48h was recorded during different periods (i.e. 1h, 2h, 6h, 24h, 48h). ToCV-viruliferous whiteflies were used as control. Similarly, the hypothetical role of selected vsRNAs from TYLCV to induce cross-kingdom gene silencing in *B. tabaci* was tested following the same procedure, except that synthetic vsRNAs were added to the diet with the nicotine and that the insects tested were non-viruliferous whiteflies. All experiments were developed in an insect proof chamber under controlled

conditions (250 µmol·s^{-1}m^{-2} with a 16 h/d photoperiod (25°C day /20 °C night) and 70% relative humidity).

Evaluation of symptoms and virus detection

The severity of symptoms for control, single and mixed infected plants with TYLCV and ToCV were visually assessed at 2, 7, 14, 21 and 28 days post inoculation (dpi), using a 0 to 5 scale, where 0 represented asymptomatic plants and 5 was assigned to the most severe symptoms observed, as described by Ferrero et al. (2020).

All infected plants used in this study were analysed by tissue blot hybridization 3 weeks after inoculation to verify infectious condition. Petiole cross-sections of tomato samples were printed on positively charged nylon membranes (Roche Diagnostics) and hybridized with probes specific to TYLCV-Is and to ToCV previously described by (Fortes et al., 2012; Navas-Castillo et al., 1999), respectively. In addition, the TRV-specific probe described by Landeo-Ríos et al. (2017) was used to check the presence of TRV in tomato plants during the VIGS assays.

Virus quantification and evaluation of gene expression by RT- q PCR

Real-time reverse transcription polymerase chain reaction (RT–qPCR) was employed for several analyses during the present work: to estimate viral titres in control and infected plants with TYLCV, ToCV, or TRV, and to assess gene expression levels of target silenced genes in tomato plants and in *B. tabaci* insects.

For each infection condition, time point, and replica, the second most recently expanded leaf from the apex of six plants was pooled and used for the following analysis. In all cases, three biological replicates were processed per condition and time point. In the case of insect samples, to confirm the downregulation of *B. tabaci* target genes five replicates of 30 whiteflies were used. Total DNA of plant samples and RNA of plant and insect tissues were isolated by using DNeasy Plant Mini Kit (Quiagen). and Trizol reagent (Ambion), respectively, and then treated with RNase-Free DNase (Quiagen) and DNase-Free RNase (Roche),

according to the manufacturer's instructions. Its quality and concentration were subsequently estimated in a NanoDrop One spectrophotometer (Thermo Scientific, MA, USA). The complementary DNA (cDNA) of each sample was synthesize from 500 ng of total RNA using BioRAD iScript™ cDNA Synthesis Kit in a reaction volume of 20 µl. Each quantitative PCR amplification was done with a template of 1 µl of cDNA or 500 ng of total DNA in a PowerUp SYBR Green PCR kit with a final volume of 15 µl and run on a QuantStudio 5 thermocycler (ThermoFisher). The following cycling conditions were used: 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min.

In the case of plant samples, relative quantification of specific RNAs and TYLCV DNA were normalized to the elongation factor 1-α gene (SlEF1 α) and tomato 25S ribosomal RNA genes, respectively (Li et al., 2013; López-Ráez et al., 2010; Rodríguez-Negrete et al., 2014). Similarly, elongation factor 1-α gene of *B. tabaci* (BtaEF1 α) was selected as reference gene for insect samples. Data were analysed via the comparative cycle threshold (Ct) method, as described by Livak & Schmittgen (2001).

Gene-specific primers used to determine TYLCV, ToCV and TRV accumulation are listed in Supplementary Table S1. The relative expression of silenced genes in tomato by VIGS were estimated using the specific primers showed in Supplementary Table S2. In the case of *B.tabaci* targeted genes, their relative expression was estimated using primer pairs listed in Supplementary Table S3. All of them were designed by using Primer Blast tool available online (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer pairs for tomato PR-P6 and Pin-II amplification were reported by Sarmento et al. (2011) and Uppalapati et al. (2005), respectively. Each primer pair efficiency was evaluated by a standard curve with six points and three replicates to obtain efficient rates (*E*) (*E* = 10 (1/slope)-1, expressed as percentages).

Whitefly dual choice bioassays

Whitefly host preference behaviour was assessed under dual-choice conditions following the experimental design described in Ontiveros et al. (2022). Dual choice assays were performed by pairwise comparisons of tomato leaflets from the 10-leaf-growth stage of mock-inoculated plants and single and co-infected plants with ToCV and TYLCV at 21 dpi. For each condition, four leaflets were placed in the corners of a plastic cage (25 x 25 cm) equidistant to a flight release platform placed in the middle of the cage. As described by Rodríguez-López et al. (2011), leaflets were individually inserted in a petri dish (2 cm x 1 cm) filled with nutrient solution to maintain leaflet turgor during the experiments. The experimental design of the cages is illustrated in figure 5. After a short period on ice (no more than 30 seconds) to facilitate insect manipulation, a single adult of *B. tabaci* was place on the flight release platform and only the first choice was recorded. Each whitefly was tested only once and choosing periods longer than 15 min were excluded. A total of 60 whiteflies were individually released per pairwise comparison and both non-viruliferous and viruliferous (with ToCV, TYLCV or both) whiteflies were tested without sex distinction. Before the beginning of the experiments, all insects were individually collected with a controlled-vacuum hand trap for a starvation period of 1 h.

To confirm the influence of visual cues caused by TYLCV infections on *B. tabaci* host preferences, dual choice assays were performed using asymptomatic TYLCV-infected leaflets and leaflets of tomato plants infected with TYLCV at 21 dpi showing severe expression of symptoms. Similarly, to evaluate feeding behaviour in presence of healthy and TYLCV-infected tomato leaflets, a group of 30 adults were released per cage and the number of whiteflies landed on each healthy or TYLCV-infected leaflet was counted at 2, 8, 24 and 48 h after release. The experiment was replicated 12 times. Non-viruliferous and ToCV- and TYLCV-viruliferous whiteflies were tested in both dual-choice assays developed in an insect-proof chamber under controlled conditions (250 $\mu mol \cdot s^{-1} m^{-2}$ with a 16 h/d photoperiod (25°C day /20 °C night) and 70% relative humidity).

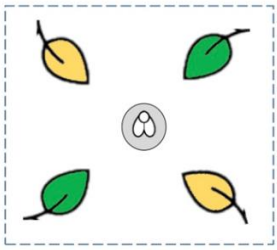


Fig. 5. Experimental design of dual-choice arena cages. A total of four leaflets per pairwise comparison are disposed surrounding the flight release platform. Leaflets belonged to the two treatments compared (represented by blue and yellow for TYLCV- and ToCV-infected, respectively) were placed alternately for each test.

Whitefly olfactometer choice bioassays

Whitefly responses to volatile cues emitted by healthy and TYLCV-infected tomato plants in the absence of visual stimuli were tested using a Y-tube olfactometer (Analytical Research Systems, Gainesville, FL). The olfactometer consisted of a glass Y-tube (28 cm long stem and 20 cm long arms) connected to two separate glass chambers (40 cm height and 20 cm inner diameter) through silicone tubes (Fig. 6). A single test plant was placed in each glass jar connected to an air delivery system (ARS, Gainesville, FL) to ensure a purified and humidified airflow. Each whitefly was briefly placed on ice (as described above) before being settle in the intersection of both arms of the Y-tube. Later, an input flow was pulled across the silicone tubes from the air delivery system to the jars and then to the Y-tube. Thus, the whitefly was able to make a choice based on the reception of the volatiles emitted by the plants by moving through one of the arms. A choice was considered when the insect crossed a limit line at 6 cm into either arm for more than 3 min. If the stablished choice was not completed after 15 min, the whitefly was excluded from the sampling and replaced. An airflow of 0.3 liters per minute generated with a vacuum pump connected to the olfactometer was chosen for the assays. To avoid visual stimuli during the experiments, both glass chambers were covered. As for dual-choice assays, a total of 60 adults each viruliferous and non-viruliferous were tested as replicates and previously starved for 1h before the beginning of the experiments. A preliminary control test was used to validate experimental parameters using *S. habrochaites* accession number PI 134418 compared with tomato cultivar Moneymaker plants. As described in previous studies *B. tabaci* is repelled by *S.*

habrochaites (Bleeker et al., 2009; Momotaz et al., 2010), thus a total of 35 whiteflies out of 40 preferred Moneymaker plants. New plants were used for each of the replications alternating the spatial orientation of the infected and mock-inoculated plants. Bioassays were conducted in a room chamber with light intensity at 250 µ/s/m^2 and maintained at 27° C and 70% relative humidity.

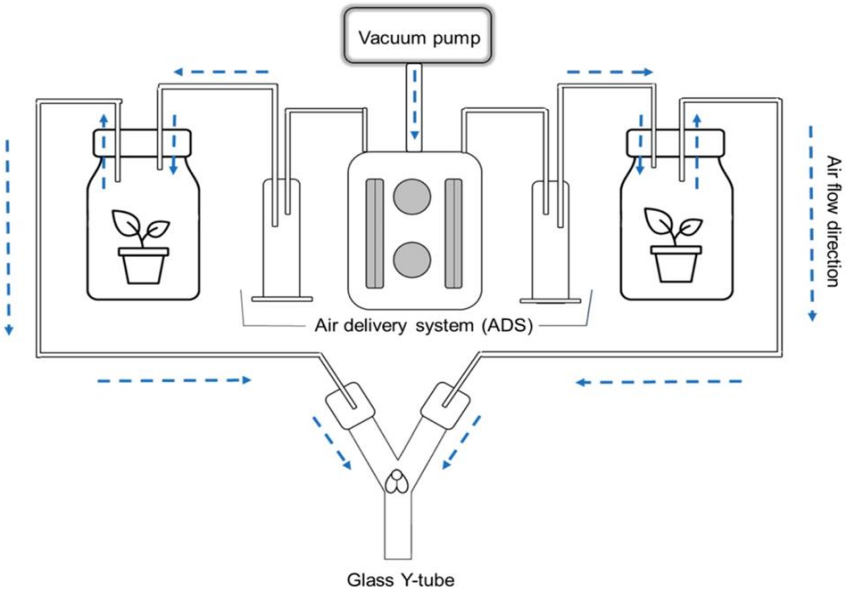


Fig. 6. Set-up of the Y-tube olfactometer used to test *B. tabaci* preferences to volatiles emitted by mock or TYLCV-infected plants. Blue arrows represent the airflow direction.

Measurement of leaf coloration and colored sticky traps assays

Optical methods based on image analysis were used to estimate differences in color between leaves of healthy (mock-inoculated), ToCV- and TYLCV-infected tomato leaves using a colorimeter (model CR-400; Konica Minolta, Inc., Tokio, Japan). Three random measures per leaf with an aperture of 1 cm diameter were recorded and the color attributes of the samples were evaluated with CIE Lab three-dimensional color space. In this color space, the *L** value indicates lightness which goes from black (*L* = 0) to white (*L* = 100). Chroma (*C**) is calculated from *a**- *b** plane, which depends of L* values, and represents colour saturation. The *a** value characterizes the color of the region of green (-*a**) to red (+*a**), and the *b** value indicates the color in the range from blue (-*b**) to yellow (+*b**). Numerical values of *a** and *b** were converted to hue angles (*H*) in the

Konica Minolta CR-400 Utility software. The *H* value is expressed in degrees: 0° (+*a* axis) represents red; 90° (+*b* axis) represents yellow; 180° (-*a* axis) is green and 270° (-*b* axis) represent blue colour (Barrantes et al., 2016; Sacks & Francis, 2001). The colorimeter was previously calibrated following the manufacturer's instructions and each *L** and *H* value was calculated by the formulas described by Sacks & Francis, (2001).

Coloured sticky cards were designed to conduct choice assays to evaluate the influence of visual stimuli emitted by TYLCV symptomatic leaves on *B. tabaci* in absence of olfactory cues. A total number of 24 cards (8x10 cm) coloured to mimic TYLCV symptomatic leaves at 21 dpi (yellowish colour) and asymptomatic leaves (greenish colour) were alternatively disposed on both sides of a cardboard surface (21x29.7 cm) and used for each replicate. Three glass boxes were disposed in a greenhouse under natural light and controlled temperature (25 ± 2°C) to test either non-viruliferous, ToCV- or TYLCV- infected whiteflies visual responses. Two sticky cardboards were suspended from the top of each glass box (65 x 77 x 56 cm) as illustrated in figure 7 and groups of 100 whiteflies were released per comparison. The cards were collected after 8 h to record the number of whiteflies trapped on each section. The assays were replicated three times for either viruliferous or non-viruliferous whiteflies.

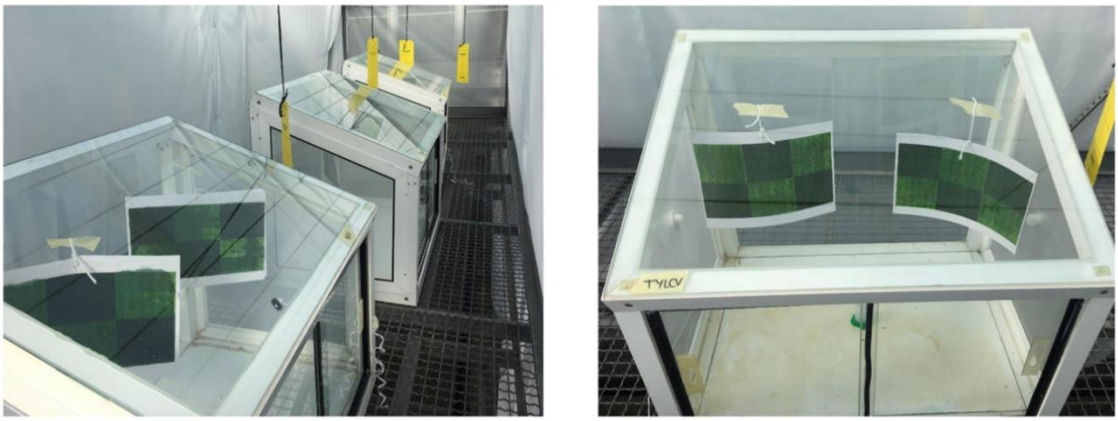


Fig. 7. Sticky traps experiment designed using colored cards mimicking healthy and TYLCV-infected leaves.

Libraries construction and sequencing

RNA-sequencing technology was used in this study not only to obtain a deep insight into the gene expression profile and the dynamic changes in accumulation patterns of sRNAs during co-infections with ToCV and TYLCV, but also to explore whether sRNAs derived from TYLCV could mediate cross-kingdom RNAi to suppress *B. tabaci* genes. Both RNA-seq and sRNA libraries were generated and sequenced at CNAG (Centro Nacional de Análisis Genómico, Barcelona, Spain) using Illumina Hi-Seq 2000 (Illumina, Inc) in pair-end mode with a read length of 76 bp for RNA-seq libraries and using 50 bp single reads for sRNA libraries as described by Piedra-Aguilera et al. (2019).

Plant leaves tissues sampled from mock-inoculated, single- and mixed-infected plants (same set of plants used for evaluation of symptoms and virus detection) at 2, 7, and 14 dpi were used to construct the RNA-seq libraries, meanwhile samples at 7 and 14 were collected for the sRNA libraries. In both cases, the second youngest leaf from the apex of six plants was harvested and leaf tissues for each infection status and time point were pooled and immediately frozen in liquid nitrogen and maintained at -80°C. Three and two biological replicates were processed per condition and time point for RNA-seq and sRNA libraries, respectively. Total RNA was isolated with Trizol reagent (Ambion) as previously described, and plants were analysed by tissue blot hybridization followed by RT-qPCR to confirm the presence of viral RNA in infected tomato plants at each time point.

RNA-seq data analysis and evaluation of gene expression differences

RNA-seq raw data files obtained after sequencing were processed using Trimmomatic to remove adapters and low quality reads. Trimmed reads were aligned to the current version of tomato reference genome (SL4.0) and annotation ITAG4.0 available at Sol Genomics Network website (SGN, http://solgenomics.net) (Fernandez-Pozo et al., 2015).

Detection of differentially expressed genes (DEGs) for each comparison and time point was performed using the R package DESeq2 (Love et al., 2014).

Experiment replicates were previously checked by Principal Components (PCA) to determine the correlation among the different biological replicates. Genes with an adjusted *p* value <0.05 (false discovery rate (FDR)) were considered differentially expressed and DEGs with 20% increase (log2 fold changes > 0.263) were considered up-regulated, while those with 20% decrease (log2 fold changes < -0.322) were considered down-regulated. Gene Ontology (GO) analysis was conducted to classify and extract information on biological functions and pathways in which up- and down-regulated DEGs identified in response to single and mixed infections were involved. In addition, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways analysis was performed to support the identification of potential metabolic pathways. Both enrichment analyses were conducted using the bioinformatics tool g: Profiler (Raudvere et al., 2019).

Reverse analysis of candidate genes by VIGS

Virus induced gene silencing (VIGS) vector derived from the tobacco rattle virus (TRV) was used to silence *Sgt1* (Solyc06g036420) and *Hsp90* (Solyc07g047790) genes and the three loci *RTM1* (Solyc09g083020), *RTM2* (Solyc11g071560), and *RTM3* (Solyc11g071700) according to the pTRV1 and pTRV2 vectors previously described in Liu et al. (2002). Gene-specific PCR primer pairs were designed with the corresponding restriction sites for cloning using Primer Blast tool available online (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Supplementary Table S2) and fragments were subjected to BLAST analysis against tomato genome to avoid off-target silencing. A 430 bp fragment of *Hsp90* gene was synthesize and amplify by RT-PCR using the primers LK35A/ LK36A and the resulting PCR product was cloned into the BamHI-SacI site within the multiple cloning site of the *pTRV2* vector. In addition, the set primers LK22A/LK23A, LK2/LK3 and LK8/LK9 were used to amplify a 235 bp fragment of *Sgt1* and fragments of 420 bp and 410 bp of *RTM1* and *RTM3*, respectively. Corresponding fragments were cloned into EcoRI-XhoI site of the *pTRV2* vector. Similarly, a 300 bp fragment of *RTM2* (LK4/LK5) was inserted into SmaI-cut *pTRV2*. Recombinant plasmids were transformed into *Escherichia coli* strain DH5α (Invitrogen) and corresponding clones (*pTRV2-Sgt1*, *pTRV2-Hsp90*, *pTRV2-RTM1*, *pTRV2-RTM2* and *pTRV2-RTM3*) were selected to be transformed into *Agrobacterium tumefaciens* strain

GV3101. Both *E.coli* and *A. tumefaciens* transformations were developed by electroporation (25 mF, 200 Ω, 1,200 V) in a Gene Pulser XCell (Bio-Rad) as described in Navas-Hermosilla et al. (2021). Clones were verified by restriction enzyme digestion and by sequencing.

Identification of sRNAs from TYLCV and *B. tabaci* target genes

Trimmed reads data of sRNA from TYLCV-infected leaves at 7 and 14 dpi were mapped against the reference genome of *B. tabaci* MED species. A list of genes was obtained following same procedure as for RNA-seq data and PCA analysis was performed to determine the correlation among the different biological replicates. Alignments of selected sRNAs were visualized using the IGV 2.3 software (Integrative Genomics Viewer) (Robinson et al., 2011) showing a piece of complete information as count details, depth of the reads displayed, and the nucleotides that differ from the reference sequence. The IGV tool allowed identifying the location of target genes in the genome and the specific sequences of the sRNAs. The same analysis was performed using resulted cleaned reads from mock-inoculated and ToCV-infected leaves as a control since no predicted targeting genes were found. The followed specific duplex sRNAs were commercially synthesized (Sigma-Aldrich) for artificial diet assays: sRNAs target of Bta001158 (Cytochrome P450), Bta001692 (Aryl hydrocarbon receptor), and Bta028144 (Neuroligin-1); sRNAs target of the endogenous *B. tabaci* genes Bta017585 (Vitellogenin) and Bta008678 (Juvenile hormone), and a predesigned sRNA for GFP used as controls. All sRNAs were ordered in a concentration of 100 µM (5 OD). The specific sequences of the sRNAs and lengths are described in Supplementary Table S4.

Statistical analysis

Statistical analysis in this thesis were performed in IBM SPSS version 26.0 (IBM, Armonk, NY) and R for Windows (R Foundation for Statistical Computing, Vienna, Austria).

Means of severity of symptoms and the relative quantification of the genes PR-P6 and Pin II, was analysed via one-way ANOVA (Supplementary Tables S5 and S6). However, to estimate statistical differences ($p<0.05$) of TYLCV and ToCV

accumulation between single- and double-infected plants data were analysed by the Student-t-test (Supplementary Table S7). For the dual choice assays, host settling preferences of viruliferous and non-viruliferous whiteflies were analysed by applying a χ^2 test to the data of each pairwise comparison. The χ^2 test was also used to evaluate host preference behaviour of *B. tabaci* in the olfactometer assays. Colorimeter data were submitted to the Shapiro-Wilk normality test and then compared via one-ways ANOVA to correlate colour components of mock-inoculated and single-infected leaves with ToCV and TYLCV (Supplementary Table S8). Numbers of whiteflies collected from sticky cards were analysed by a Wald test ($P < 0.05$) with a general linear model following a Poisson distribution.

For the VIGS experiments, accumulation of ToCV and TRV as well as the relative expression of silenced genes were analysed via one-way ANOVA (Supplementary Table S9). The relative expression of target genes of *B. tabaci* (P450, AhR and neuroligin) and the two endogenous control genes (vitellogenin and juvenile hormone) in either viruliferous and non-viruliferous individuals was analysed by the Student-t-test (Supplementary Table S10). By contrast, one-way ANOVA statistical analysis was used to estimate the relative expression of these putative target genes and the two endogenous control *B. tabaci* genes after feeding on artificial diet with sRNAs (Supplementary Table S11). Kaplan-Meier statistical analysis was used to estimate survival rates of *B. tabaci.*

CHAPTER 1

Co-infection of tomato plants with tomato yellow leaf curl virus and tomato chlorosis virus affects the interaction with host and whiteflies

RESULTS

Mixed infections of ToCV and TYLCV induce more severe symptoms in tomato plants than single infections at late time points.

Under our growing conditions, tomato plants infected by TYLCV and ToCV in single or coinfection started displaying symptoms at about 7 dpi (Fig. 8). ToCV-infected plants exhibited only slight symptoms of interveinal chlorosis and mild yellowing with no evidence of stunting, always with a rating ≤ 2. As expected, tomato plants infected with TYLCV exhibited significantly more severe symptoms than ToCV-infected plants (Table S5 and Fig. 9), with scores of about 4 at 28 dpi, and symptoms included leaf curling, yellowing, and plant stunting.

However, we observed that ToCV and TYLCV produce different symptomatic outcomes during mixed infection over time. Symptoms ranged from moderate (values 1 to 2) to severe (values 4 to 5), suggesting that antagonism and synergism occurred respectively at early (weeks 1 and 2) and late stages (from week 3 onward) of mixed infection with the two viruses. The development of symptoms resembled those of single-infected plants with ToCV during the first observations but were followed by a dramatic increase in severity (severe curling and extensive yellowing in upper leaves) that led to necrosis of the newly emerging leaves and sometimes even to the death of the plant in the final stages (Fig. 9), a situation that was not observed in any of the plants inoculated solely with one virus.

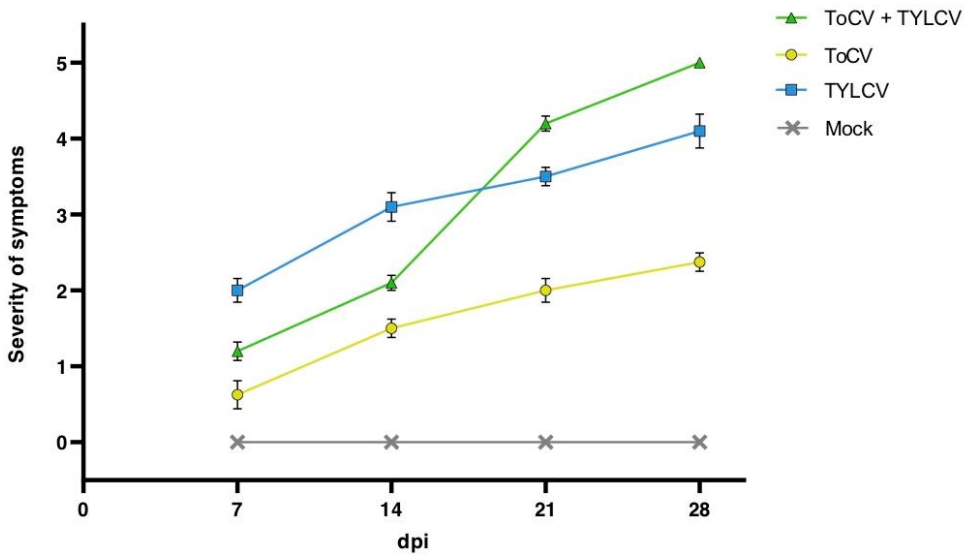


Fig. 8. Evolution of symptoms in single- and mixed-infected plants. Mean severity of symptoms (from 0 = absence to 5 = maximum severity) observed on different days post-inoculation (dpi) in tomato cv. Moneymaker plants singly infected with *Tomato yellow leaf curl virus* (TYLCV) or *Tomato chlorosis virus* (ToCV), co-infected with both viruses (ToCV+TYLCV), or mock-inoculated. Mean ± standard error values corresponding to six plants per treatment are indicated.

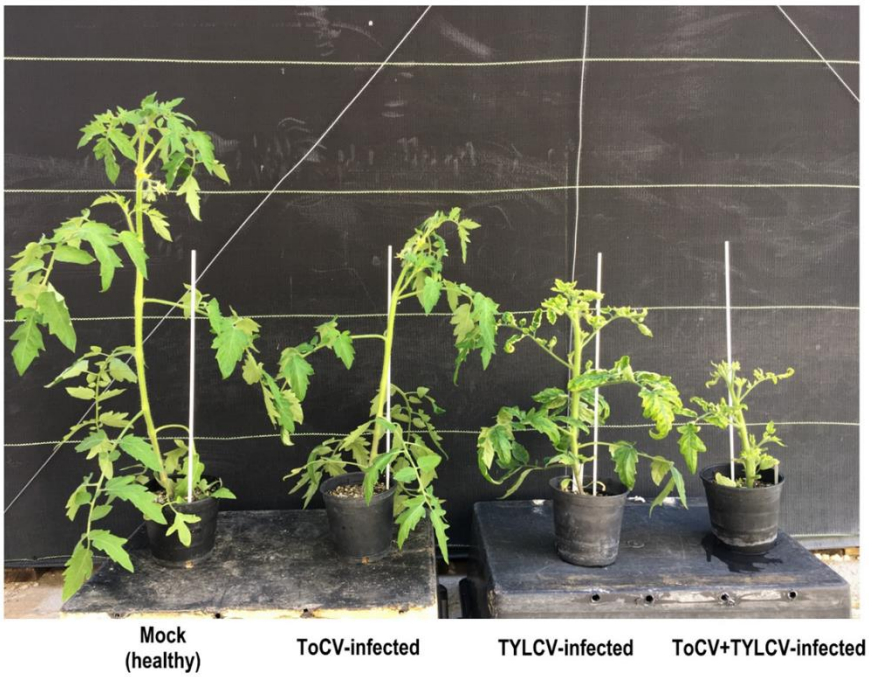


Fig. 9. Symptoms following inoculation of tomato plants infected with Tomato yellow leaf curl virus (TYLCV) and Tomato chlorosis virus (ToCV) in single and mixed infection at 21 days postinoculation: Mock-inoculated (Mock); single ToCV (ToCV), single TYLCV (TYLCV) and mixed co-inoculation with TYLCV and ToCV displaying synergism (TYLCV+ToCV).

Time-dependent antagonistic and synergistic interactions correlate with significant changes in ToCV accumulation.

To determine whether symptom severity could be correlated with viral accumulation in infected tissues, we estimated the relative abundance of TYLCV and ToCV in single- and mixed-infected tomato plants by qPCR and at 7, 14, and 21 dpi (Fig. 10A and B). Samples at 28 dpi were not considered because severe necrosis in many apical leaves of co-infected plants precluded sampling of enough material for representative analysis.

The results showed that the pattern of TYLCV DNA accumulation was similar between single infection and coinfection with ToCV (Table S7), where the total amount of viral DNA increased drastically from 7 to 14 dpi and slightly decreased at 21 dpi (Fig. 10A). The same trend was observed for ToCV RNA accumulation in single infection, reaching a peak at 14 dpi with a slight decrease at 21 dpi. However, a pattern of reduced ToCV RNA in coinfection with TYLCV was observed at 7 and 14 dpi compared with plants infected by ToCV alone, and the opposite trend was observed at 21 dpi: ToCV reached a higher accumulation in coinfection compared with single infection (Fig. 10B).

Taken together, these results suggest that changes in symptoms of mixed-infected plants between antagonism (14 dpi) and synergism (21 dpi) seemed to correlate with the dynamics of ToCV accumulation: The relative abundance of ToCV significantly increased in mixed-infected plants at 21 dpi (Fig. 10B) when the increment of the severity of symptoms suggests a synergistic interaction between both viruses (Fig. 8).

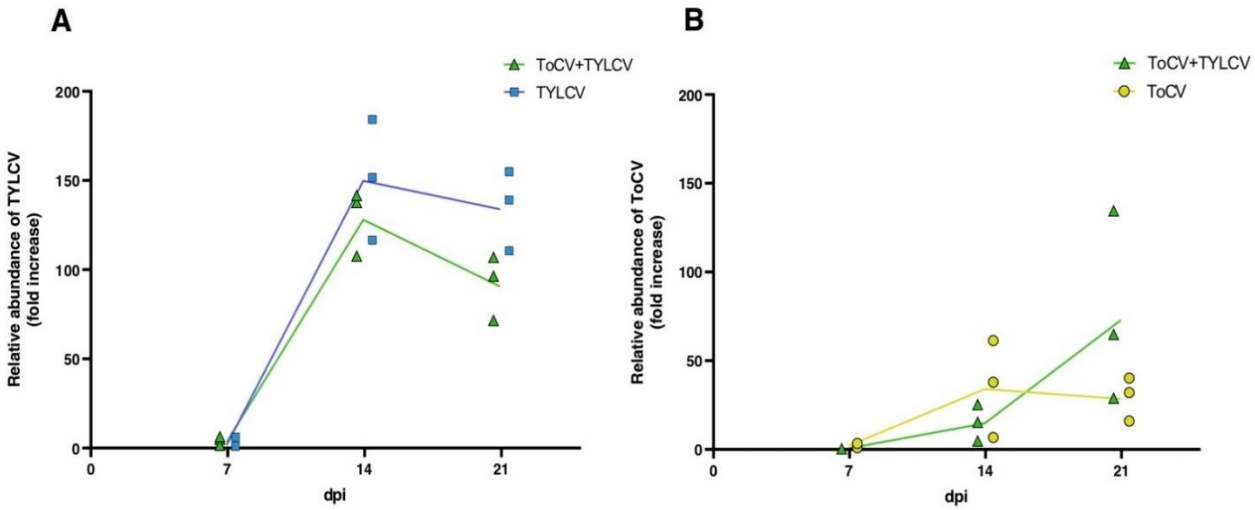


Fig. 10. Kinetics of viral loads in single- and mixed-infected plants. Relative quantification of *Tomato yellow leaf curl virus* (TYLCV) (A) and *Tomato chlorosis virus* (ToCV) (B), showing viral loads in single-infected (blue for TYLCV and yellow for ToCV) and mixed-infected (green) plants at 7, 14, and 21 days postinoculation (dpi). Each point represents the relative quantification of viral accumulation in the independent biological replicates with pooled samples of six infected plants per treatment. Mean values are indicated by the horizontal lines. The relative quantifications were calculated using the comparative cycle threshold method (Livak and Schmittgen ,2001). The co-infected samples at 7 dpi were chosen as calibrators for comparisons. Relative quantification of specific RNAs and TYLCV DNA were normalized to the expression of the elongation factor 1-α (SlEF1α) and the tomato 25S ribosomal RNA genes, respectively.

Dynamics of expression corresponding to defense-related genes in single and mixed infections.

To investigate the putative involvement of known pathways corresponding to plant immune responses, we monitored PR-P6 and Pin-II gene expressions. PR-P6 and Pin-II are respectively considered molecular markers for two major pathways: systemic acquired resistance related to responses mediated by salicylic acid (SA) and induced systemic resistance related to responses mediated by jasmonic acid (JA) (Ament et al., 2004; Wasternack et al., 2006). To verify whether the expression of these genes could be triggered by the different viral infections alone or combined, we analyzed their relative expression by reverse transcription qPCR in systemic leaves at 7, 14, and 21 dpi.

We observed no significant differences in PR-P6 expression pat- terns at 7 dpi between single-infected and coinfected plants. Furthermore, the PR-P6 RNA levels peaked at 14 dpi in infected plants with TYLCV alone, with significantly higher values than those detected in plants either coinfected or infected only with

ToCV (Table S6), except for one ToCV sample that showed a similar value to those shown by TYLCV. This variability might reflect local differences related to the sampling process and the irregular distribution of criniviruses. After these peaks in single infections, PR-P6 expression significantly decreased at 21 dpi for plants infected with TYLCV or with ToCV alone, but the values continued to increase in co-infected plants, resulting in higher levels compared with single infections (Fig. 11A). This delay in the pattern of PR-P6 expression for mixed-infected plants roughly followed symptom severity, suggesting that its expression might be correlated with the severity of symptoms.

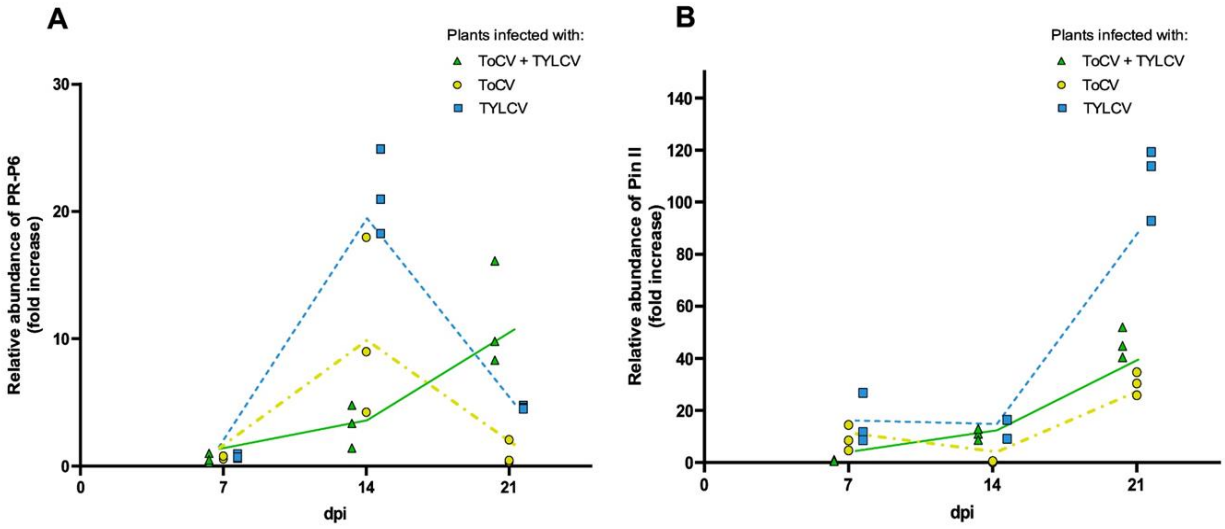


Fig. 11. Relative accumulation of the salicylic acid (SA) responsive PR-P6 gene (A) and the wound-induced proteinase inhibitor II (Pin-II) (B) in single- and mixed-infected tomato plants at 7, 14 and 21 dpi. Each point represents the relative quantification of the levels of transcript corresponding to independent biological replicates after pooling samples of six infected plants per treatment. Mean values are indicated by the horizontal lines. The relative quantifications were calculated using the comparative cycle threshold method (Livak & Schmittgen, 2001). The co-infected samples at 7 dpi were chosen as calibrators for comparisons. Relative quantification of specific RNAs was normalized to the expression of the elongation factor 1-α (SlEF1α) gene.

No significant differences in Pin-II RNA expression were observed at 7 dpi between singly infected and co-infected plants, whereas at 14 dpi a significant lower expression was observed in infected plants with ToCV than in TYLCV singly or co-infected tomato plants (Fig. 11B). The Pin-II RNA accumulation reached its maximum at 21 dpi for all treatments, with the highest induction levels detected in plants infected with TYLCV (Fig. 11B, Table S6).

The presence of TYLCV conditions the host plant preference responses of *B. tabaci*.

To investigate whether viral infections might interfere with vector attraction, we tested whiteflies' preferences by comparing infected and mock-inoculated plants at 21 dpi. This time point was chosen based on the differential expression of Pin-II and PR-P6 in the conditions analyzed, which suggested that the plants were clearly responding to the infections (Fig. 11). The experiments were repeated independently with naive whiteflies (non-viruliferous) and with whiteflies previously fed on virus-infected plants, therefore becoming viruliferous for either one of the individual viruses considered. Significantly, more non-viruliferous and ToCV-viruliferous whiteflies preferred to land on infected leaves than on control leaves both for TYLCV and for mixed infections. A similar pattern was found for TYLCV-viruliferous whiteflies with a preferential attraction to the leaves of plants with TYLCV, whereas plants harbouring mixed infections were not significantly more attractive than uninfected controls, although the number of whiteflies was consistently higher on infected plants. In contrast, whiteflies did not discriminate between leaves from plants infected with ToCV alone and mock leaves or TYLCV from mixed infection, regardless of the non-viruliferous or viruliferous condition of the insects (Fig. 12). Overall, enhanced whitefly attraction was observed toward TYLCV single- or mixed-infected plants com- pared with plants infected by ToCV, suggesting that the presence of TYLCV might drive host attractiveness, either alone or in mixed infection with ToCV and regardless of their viruliferous condition as either naive or ToCV-viruliferous. Interestingly, although the trends are similar, the TYLCV-viruliferous whiteflies discriminated significantly only between leaflets of mock and TYLCV-infected plants, and it seems that the preferences shared by non-viruliferous and ToCV-viruliferous whiteflies were less marked in this case, suggesting a possible effect of the virus presence in the behaviour of the vector.

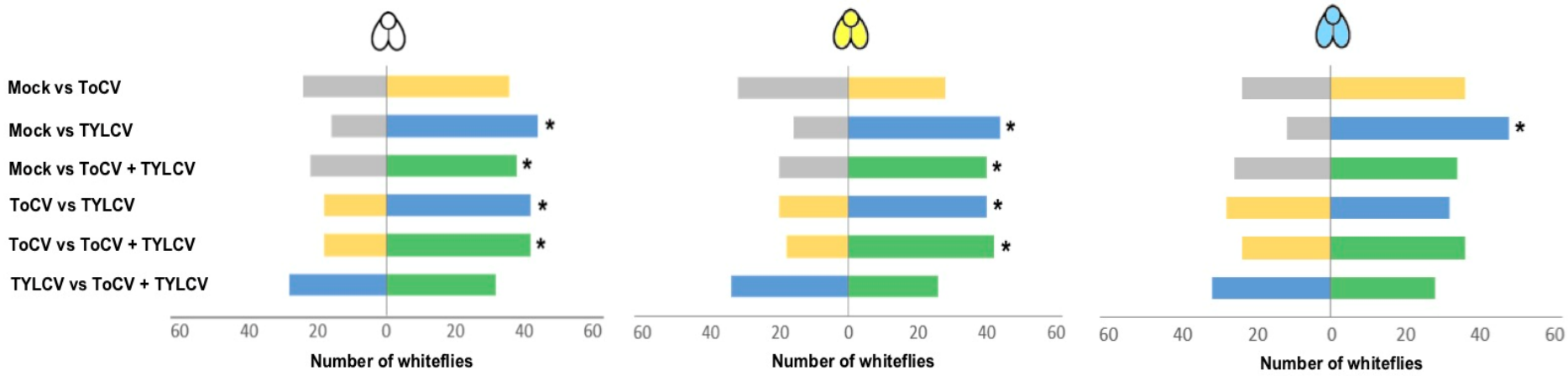


Fig. 12. Viruliferous and non-viruliferous *Bemisia tabaci* preference responses in a dual-choice bioassay. Number of whiteflies that landed on TYLCV-infected (blue), ToCV-infected (yellow), mixed-infected (green) or mock-inoculated (grey) leaflets were recorded in each pairwise comparisons. Whitefly icons represent the infectious status of the whiteflies tested: white for non-viruliferous and blue and yellow for TYLCV- and ToCV-viruliferous whiteflies, respectively. Asterisks indicate significant differences between pairs of treatments ($p<0.05$) based on chi square analysis.

Visual cues rather than olfactory stimuli are responsible for whiteflies' attraction toward TYLCV-infected tomato plants.

We performed a series of assays to determine whether the TYLCV infection induces attraction based on odour or visual cues among whitefly vectors in tomato plants. Because the infection with TYLCV was shown to be determinant in the arena studies reported previously, we concentrated our efforts on comparing mock and TYLCV-infected plants.

In the Y-tube olfactometer bioassays, volatiles emitted from either plants systemically infected with TYLCV or mock-inoculated plants flowed through both arms of the Y-tube. Individual non-viruliferous and viruliferous whiteflies were released from the base of Y-tube stem and allowed to make the choice to fly toward either arm of the Y-tube, to prevent visual detection of the plants. Whiteflies showed no preference for either mock or TYLCV-infected plants, suggesting that olfactory cues have neutral effects in the initial choice of *B. tabaci* (Fig. 13A). Furthermore, we tested the whiteflies' preference in dual assays for mock-inoculated versus TYLCV-infected leaflets at 4 dpi, when the symptoms were not yet developed. The results showed no significant differences in the preferences of either viruliferous or non-viruliferous whiteflies (Fig. 14) suggesting that visual cues are responsible for the attraction of whiteflies to TYLCV-infected plants.

We then investigated the response of whiteflies to colored sticky traps in absence of olfactory stimuli (Fig. 13B). Comparing TYLCV symptomatic plants with mock-inoculated or asymptomatic infected plants (before the onset of symptoms), we observed that TYLCV infection had a significant effect on plant coloration. There was no significant difference in the mean values of parameters describing the color between mock-inoculated and asymptomatic TYLCV-infected leaves, whereas TYLCV symptomatic plants revealed significant differences in a*, b*, L*, and H (Table S8). This difference reflects an increase in yellow coloration in TYLCV-infected plants. When traps displaying the different colors were used in choice experiments, significantly more whiteflies were caught on the card sectors mimicking TYLCV symptomatic leaves than on sectors representing the color of asymptomatic plants, and the differences were maintained for both viruliferous

and non-viruliferous whiteflies (Fig. 13B). Overall, these results suggest that visual signals associated with the color yellow seem to play an important role in whiteflies' preference responses.

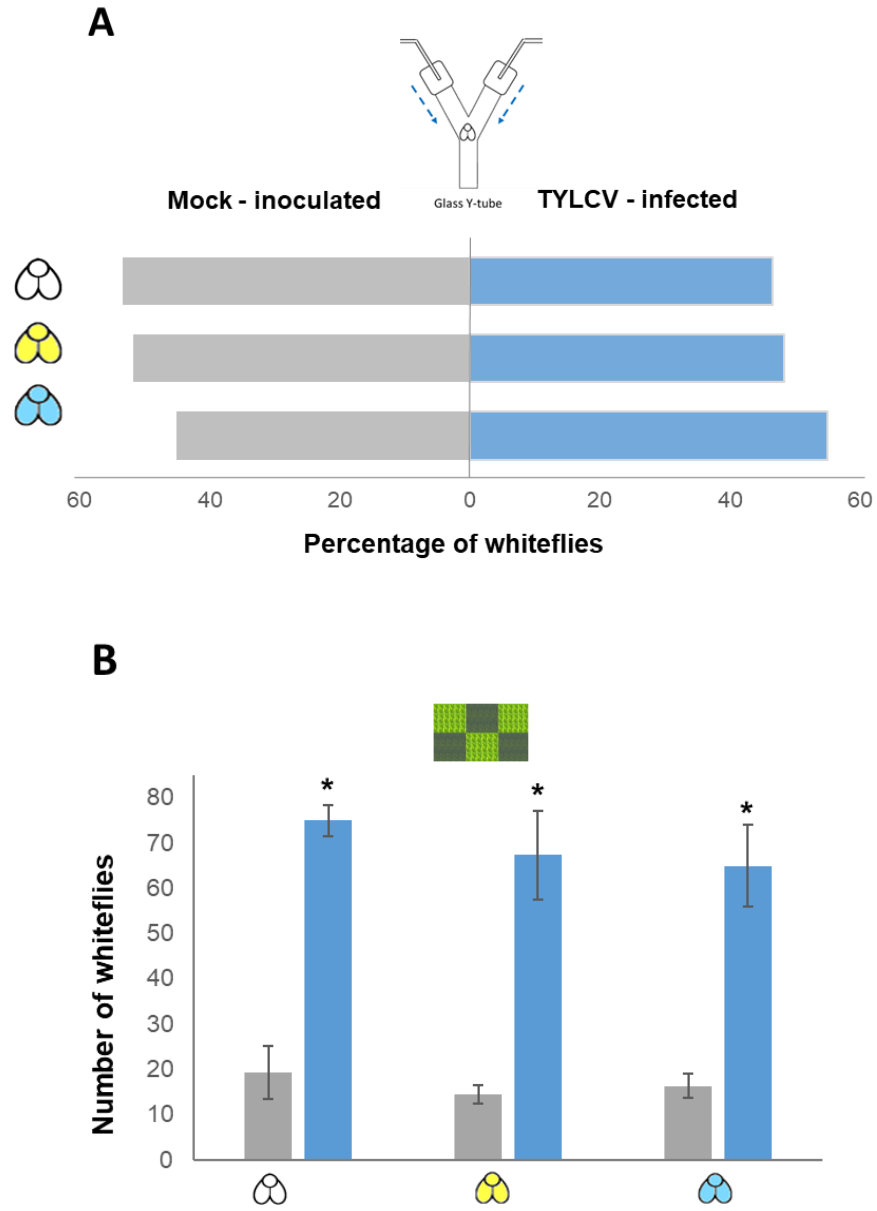


Fig. 13. Behavioral responses of *Bemisia tabaci* to olfactory and visual stimuli. A, Percentage of whiteflies responding to volatiles coming from mock- inoculated and Tomato yellow leaf curl virus (TYLCV)-infected plants connected to a Y-tube olfactometer with a continuous active air flow. Individual adult whiteflies were released in the principal arm, and their responses were recorded individually during a 15-min interval. A positive response was considered when whiteflies remained for 3 min across the border line of the lateral tubes. A total of 60 replicates (whiteflies) per test were used. B, Visual attractiveness of *B. tabaci* toward colored sticky cards. Bars represent the mean ± SE of whiteflies collected on cards colored to mimic TYLCV symptomatic leaves com- pared with mock-inoculated leaves. Whitefly icons represent the infectious condition of the whiteflies tested: white for non-viruliferous and blue and yellow for TYLCV- and Tomato chlorosis virus (ToCV)-viruliferous whiteflies, respectively.

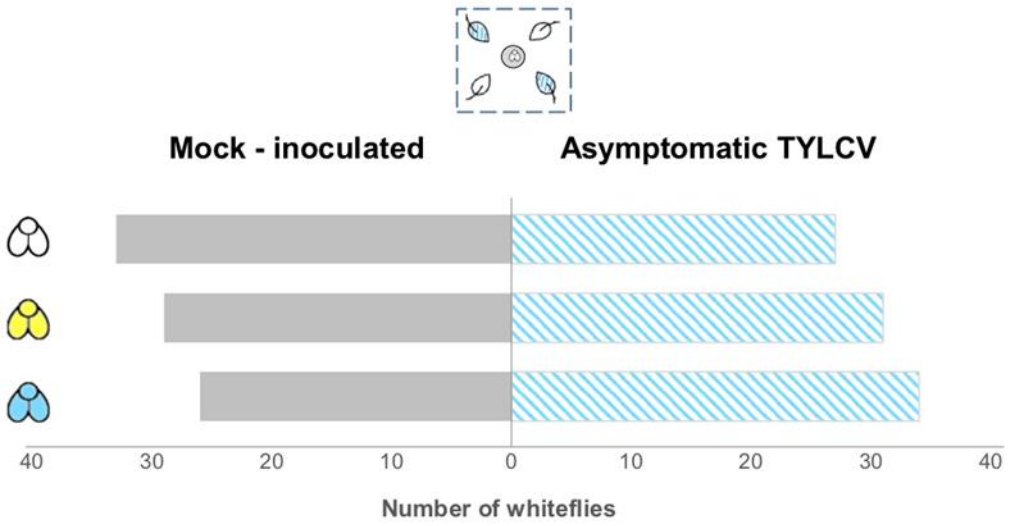


Fig. 14. Whitefly preference responses for asymptomatic TYLCV-infected versus mock-inoculated plants in a dual-choice bioassay. Whitefly icons represent the infectious status of the whiteflies tested: white for non-viruliferous and blue and yellow for TYLCV and ToCV-viruliferous whiteflies, respectively.

DISCUSSION

In this study, we found a synergistic interaction between TYLCV and ToCV, characterized by an increase in the severity of symptoms in the late stages of the observation, with a significant increase in ToCV RNA accumulation in plants co-infected with TYLCV. Previous reports studying mixed infections by TYLCV and ToCV (Jie Li et al., 2021; Seo et al., 2018) showed a reasonable level of coincidence with our results. However, these studies were performed in other experimental conditions, in particular with different tomato cultivars, virus isolates, and even inoculation procedures (grafting vs. whitefly). All these differences may cause discrepancies in outcomes, making difficult their direct comparison. In the first of these two previous studies, the authors focused on differential gene expression associated with the development of distinct symptoms induced by ToCV and TYLCV in tomato plants (‘Tenten’), testing only samples in upper leaves at 8 weeks after grafting infections and giving no information about earlier time points (Seo et al., 2018). The second article reported clear synergistic effects in a different cultivar of tomato (‘Zhong Za 9’) at late time points in the coinfection (Jie Li et al., 2021). This coincides with our data in a different cultivar of tomato (Moneymaker), but under our conditions we also noticed that the synergism was preceded by antagonism in the early stages (7

and 14 dpi) not reported in the different periods of observation during the experiments described in the aforementioned articles.

These differences could be attributed to additional divergences beyond the plant materials, such as the virus isolates used (local Qingdao field isolates were used for the studies in China, and we used viruses previously characterized in Spain) and also to the growing conditions, particularly the temperature, which was higher (27 ± 2◦C) in the greenhouse experiments conducted in China (Li et al. 2021) than in our growth chambers. Indeed, temperature has previously been shown to affect synergistic interactions (Aguilar et al., 2015) and is a key factor for virus–virus interactions (Alcaide et al., 2021). In the case of TYLCV + ToCV, the differences might affect only the dynamics to reach the final stages when the outcomes are mostly coincidental, although this hypothesis will require further experiments and appears to be irrelevant for the clear synergism at later time points.

Among the numerous examples of viral synergistic interactions in mixed infections, the crinivirus ToCV appeared to cause interesting interactions with unrelated viruses. Indeed, mixed infections of ToCV and Tomato spotted wilt virus (TSWV) of susceptible tomato plants exacerbated ToCV-associated symptoms with a remarkable increase in ToCV accumulation, whereas TSWV accumulation was not altered, but the resistance in tomato cultivars carrying the Sw-5 gene was compromised by preinfection with ToCV (García-Cano et al., 2006). Also, Wintermantel et al. (2008) reported that coinfection of ToCV with the crinivirus *Tomato infectious chlorosis virus* (TICV) altered the accumulation of each virus in a host-specific manner: Whereas in *Nicotiana benthamiana* TICV and ToCV titers increased and decreased, respectively, in *Physalis wrightii* the titers of both TICV and ToCV decreased in comparison with single infections (Wintermantel et al., 2008). However, a different scenario was found in the early stage of infection in which mixed infections repressed the symptoms induced by TYLCV and ToCV accumulation compared with the corresponding single-inoculated plants, showing an asymmetric synergistic effect between both viruses. A recent study demonstrated that interactions between synergistically interacting viruses can be asymmetric (Tatineni et al., 2019). Moreover, both antagonistic and synergistic interactions were observed between unrelated *Papaya ringspot virus* (PRSV) and

Papaya mosaic virus (PapMV), depending on their order of infection. Synergism occurred in plants inoculated first with PRSV and later with PapMV, but antagonism occurred if PapMV was first inoculated, a situation that could derive from the prior activation of the SA-associated defense response against PRSV (Chávez-Calvillo et al., 2016). Interestingly, in our case we found that the asymmetric synergistic effect between both viruses correlated with a higher accumulation of ToCV and expression of PR-P6, although it is difficult to ascertain whether the altered expression of PR-P6 is a direct cause or a consequence of the exacerbated disease symptoms or virus accumulation.

The molecular details of the mechanism underlying the interaction between these two viruses have not yet been clarified; how- ever, we might speculate that blockage of the RNA silencing antiviral response by viral suppressors of RNA silencing could be part of the mechanisms involved in synergism and antagonism in plants (Carrington et al., 2001). Similarly to other plant viruses, ToCV and TYLCV encode multiple viral suppressors of RNA silencing (Cañizares et al., 2008; Luna et al., 2012), which may act at different stages of infection (Díaz-Pendón & Ding, 2008), although these interactions may occur for more complex reasons than the action of a few viral proteins (Latham & Wilson, 2008). In a previous work involving mixed infection of TYLCV and ToCV, the differential gene expression associated with the development of distinct symptoms induced by ToCV and TYLCV in tomato plants was explored (Seo et al., 2018). Unfortunately, as mentioned earlier, the analyzed samples corresponded to late time points in the infection process, and consequently any possible asymmetric synergistic effect between both viruses was not observed.

Recent studies have uncovered novel strategies that insect-borne plant viruses might adopt to manipulate the host defenses, such as the emission of plant volatile organic compounds, or improve the plant's nutritional quality to improve the suitability of the vector and its attraction to the infected host plant, thereby affecting plant–insect interactions (Pan et al., 2021; Pieterse et al., 2012; Ponzio et al., 2013). For example, infection of tobacco plants with *Tomato yellow leaf curl China virus* and Arabidopsis with *Cucumber mosaic virus* could repress the JA-mediated defenses against the whitefly and aphid, respectively. Jasmonate is commonly considered in studies of the effects of virus infection on plant–insect

interactions, mainly as a result of its direct involvement in plant defense against insect herbivores and production of a volatile blend (Lewsey et al., 2010; Luan et al., 2013; Tong Zhang et al., 2012). Similarly, the manipulation of the SA-signaling pathway by viruses may affect host attractiveness to insect herbivores (Chisholm et al., 2018; Tomitaka et al., 2015). Our data showed that whiteflies did not discriminate between plants infected with TYLCV alone or mixed- infected plants, regardless of the non-viruliferous or viruliferous condition of the insects (Fig. 12), despite the fact that the mixed infection causes more severe symptoms than in a single virus infection with up-and-down regulation of expression of SA-related gene PR-P6 and JA-related gene Pin-II, respectively. It seems reasonable to postulate that in our pathosystem the vector attraction may not depend on the induction of the signaling pathway of the host hormone JA or SA. Additional studies are needed to draw more definitive conclusions on the impact of host hormones on whitefly preference.

In our dual choice experiment, a preferential whitefly attraction to the leaves of plants with TYLCV or mixed infections was observed, whereas plants with single ToCV infections were not significantly more attractive than uninfected controls (Fig. 14), regardless of whitefly viruliferous condition, suggesting that TYLCV drives host attractiveness in mixed infection with ToCV. For example, the fact that plants with mixed infections were more attractive than plants with ToCV infections or uninfected controls suggests that ToCV present in co-infected plants might benefit from enhanced whitefly transmission opportunities created by TYLCV-driven changes in host traits, thus providing more opportunities for ToCV to interact with insects, which might favour their spread.

We hypothesized that these whitefly preferences were probably mediated by plant-derived olfactory and visual cues, because white- flies use both to locate, select, and accept their host plants. Our data obtained in the Y-tube experiment showed that volatiles emitted by tomato plants infected with TYLCV had neutral effects in viruliferous and non-viruliferous whiteflies compared with mock-inoculated plants. However, our findings differed from those of Johnston & Martini (2020) who also worked on the TYLCV–tomato pathosystem, where *B. tabaci* attraction to olfactory cues from TYLCV symptomatic tomato plants was reported. They also differed from those who suggest that begomoviruses can manipulate

whitefly olfactory response by switching their preference to non-infected plants once the virus is acquired (Fang et al., 2013; Legarrea et al., 2015). However, this type of manipulation induced by begomovirus was not observed for ToCV, where non-viruliferous *B. tabaci* MEAM1 preferred volatiles from mock-inoculated tomato plants, whereas viruliferous whiteflies showed no preference (Fereres et al., 2016). Conversely, Shi et al. (2018) found that non-viruliferous MEAM1 were attracted to ToCV-infected plants, and viruliferous MED preferred to settle on non-infected plants rather than on ToCV-infected plants. This inconsistency indicates that virus effects on host–vector communication are complex and may include effects on the host phenotype and vector physiology.

Our subsequent data showed that the leaf color profiles were altered to a much greater extent by TYLCV than by ToCV or mock-inoculated plants, suggesting that attraction based on visual cues drives selection of *B. tabaci*. Moreover, the significant differences in H and b* values found in measurements of the external color of TYLCV-infected plants revealed a higher expression of a yellow color than either healthy, ToCV-infected, or asymptomatic TYLCV-infected leaflets, which may increase the attractiveness of *B. tabaci*. In fact, this visual influence is consistent with the work developed by Mound (1962) and Johnston & Martini (2020).

Johnston & Martini (2020), which documented that whiteflies are strongly attracted to the color yellow, suggesting that it may influence the host selection mechanism. Recently these results have been supported by their application in many management strategies to control whitefly populations as seen, for example, in the use of yellow sticky traps or the development of optical barriers to manipulate the sunlight spectrum (Antignus, 2010; Gerling & Horowitz, 1984; Moreau & Isman, 2011).

Our study constitutes a further effort to investigate the consequences of coinfection of ToCV and TYLCV on whitefly-related plant traits. TYLCV and ToCV are two emerging viruses with similar ecological niches; both are transmitted by the same vector *B. tabaci* and have overlapping host ranges. Therefore, mixed infections of TYLCV and ToCV are expected to be common in crops in areas where these two viruses are present, with important epidemiological

consequences that may be unpredictable. In fact, superinfection of TSWV-resistant plants carrying the Sw5 with ToCV resulted in a breakdown of the resistance to TSWV (García-Cano et al., 2006). Moreover, recent studies have reported the emergence of different aggressive TYLCV (TYLCV-IS76 and TYLCV-IL [IT:- Sic23:16]) outbreaks in resistant tomatoes carrying the TY-1 gene in Spain, Morocco, and Italy (Belabess et al., 2015; Panno et al., 2018; Torre et al., 2018). Because ToCV is widely distributed in these areas, mixed infections of resistant-breaking variants of TYLCV and ToCV could compromise the resistance conferred by TY-1 and should be seen as a warning for tomato breeders and growers in Mediterranean countries. Interestingly, Ty-1 resistance was shown to be compromised upon a mixed infection of TYLCV with CMV (Butterbach et al., 2014). The complexities of these kinds of mixed viral infections go beyond the interactions between the viruses involved and the host plant infected by them and also can influence the behaviour of the insect vectors. We believe these studies yield relevant information about the impact of mixed infections in the spread of both viruses, which could be important for achieving a better understanding of the epidemiological consequences of ToCV and TYLCV mixed infections.

CHAPTER 2

Analysis of gene expression in susceptible tomato plants after whitefly-mediated co-inoculation with tomato chlorosis virus and tomato yellow leaf curl virus

RESULTS

Identification of differentially expressed genes in response to single- and mixed-infections at different time points

To investigate changes in tomato transcriptome profile associated with single and mixed infections with ToCV and TYLCV, Illumina libraries were sequenced from total RNA of mock and infected plants at 2, 7 and 14 dpi. Reads were generated and mapped to the tomato reference genome (SL 4.0). Principal component (PCA) analysis revealed a clear clustering of transcript profiles in three groups corresponding to the stage of the infection. Interestingly, no discrimination between mock-inoculated plants at 14 dpi and either infected or mock-inoculated plants at 7 dpi was found in the analysis (Fig. 15). This finding suggests that viral infection at 7 dpi and the effects of *B. tabaci* feeding after 7 dpi did not altered the overall gene expression.

The differential expression analysis of genes between mock and infected plants revealed that the number of DEGs in response to single infections with ToCV was 2009, 561, and 5937 at 2, 7, and 14 dpi, respectively, and 1701, 157, and 4498 in case of TYLCV. In mixed infected plants, 2467, 419, and 4444 DEGs at 2, 7, and 14 dpi, respectively (Fig. 16A) were observed. In this time-course analysis, a gradual decrease in the number of DEGs detected in both single and mixed infections between 2 and 7 dpi were observed, compared to the control (mock-inoculated) followed by a significant increase at 14 dpi. Moreover, the lowest number of DEGs was consistently found at 7 dpi in all cases, which agrees with the findings of PCA analysis. These results suggest that while the effect of the

virus presence (or viruses in case of mixed infections) had only a slight influence on the gene expression of the plant at early stages, it became stronger at 14 dpi. The percentage of up-regulated and down-regulated DEGs were similar in single infections compared with the control, with the exceptions of the DEGs in response to TYLCV at 2 and 7 dpi where the pattern observed a higher percentage of up-regulated genes. In case of mixed infections, similar percentages of up- and down-regulated genes were observed at 7 dpi, while more than twice up-regulated genes were identified at 2 and 14 dpi (Fig. 16A).

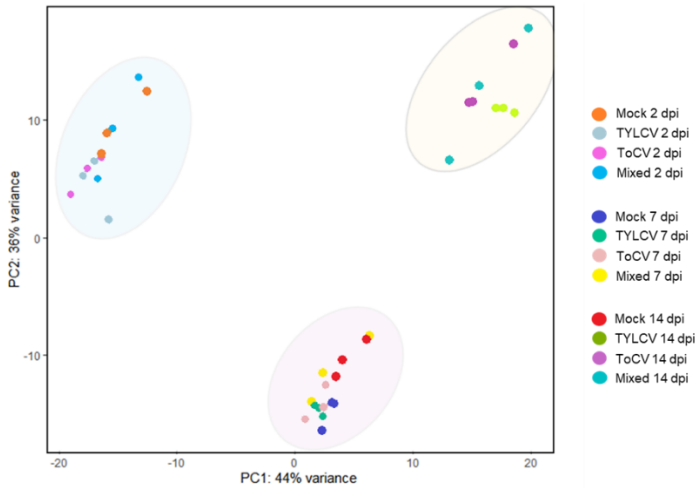


Fig. 15. Principal component analysis of the RNA-seq data.

In contrast with the mentioned results, the differential expression enrichment analysis between single- and mixed-infected plants showed quite different patterns. While the number of DEGs between ToCV- and mixed infected plants increased from early to later stages of the infection, identifying 206, 306, and 622 DEGs at 2, 7, and 14 dpi, respectively, opposite trends were observed when compared to plants with single infections of TYLCV. In this case, the higher number of DEGs (530) was observed at 2 dpi which decreases at later stages of the infection with 196 DEGs at 7 dpi and 323 at 14 dpi (Fig 16 B). Therefore, our results suggest that transcriptional changes in mixed infected plants change over time showing more similarities with single infections with ToCV at the early stages of the infection (2 dpi) and fewer differences in gene expression at 7 and 14 dpi when compared to TYLCV-infected plants.

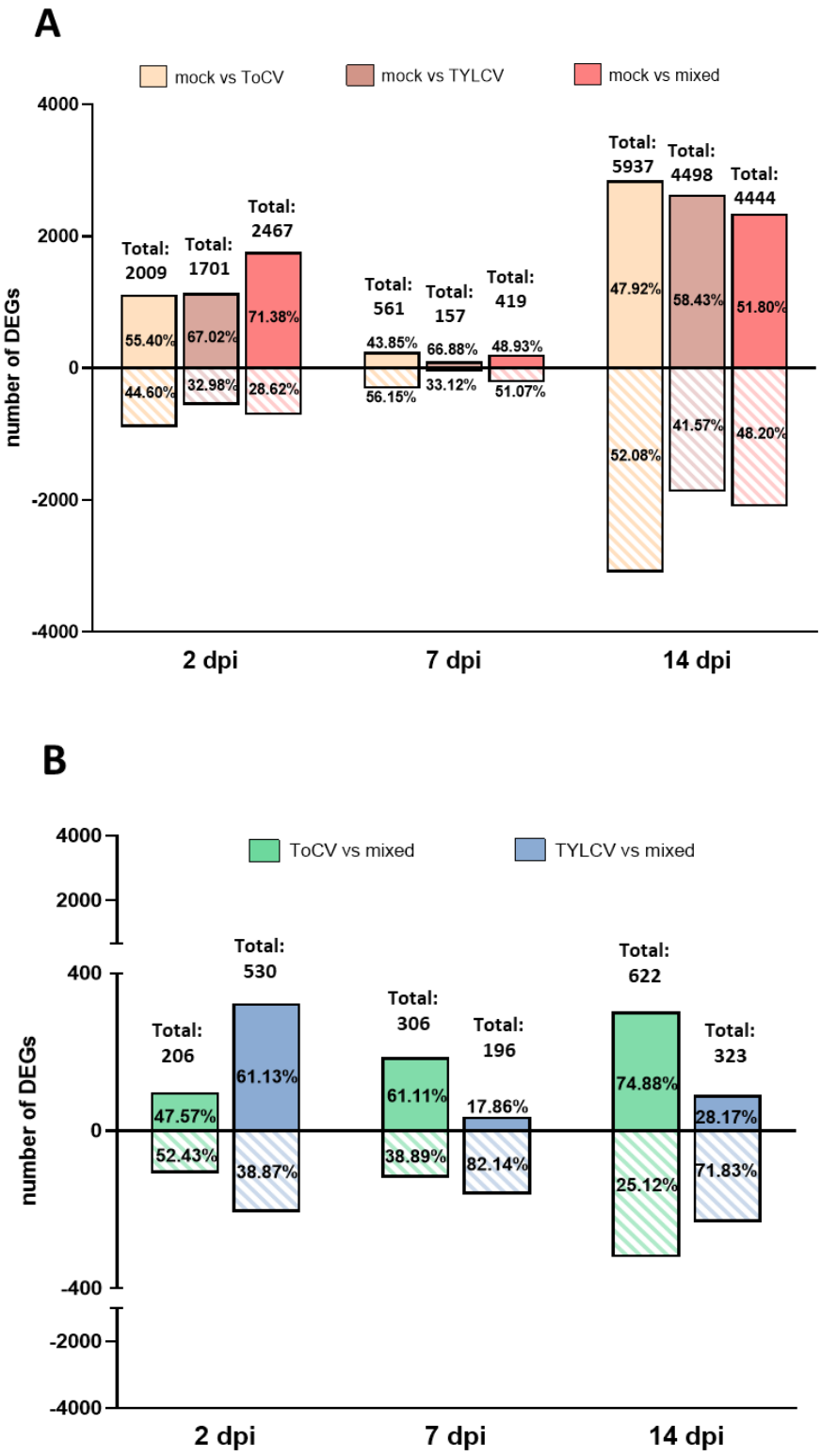


Fig. 16. Comparison of differentially expressed genes (DEGs) in response to single- and mixed-infections with ToCV and TYLCV at different days post inoculation (dpi). (A) Number of up- and down-regulated DEGs in response to single and mixed infections compared to uninfected plants. (B) Number of up- and down-regulated DEGs in response to single infections compared to mixed-infected plants.

Functional classification of DEGs by GO enrichment analysis

Gene ontology (GO) enrichment analysis was conducted to determine the functional roles of the DEGs at each time point. A total of 419, 396 and 528 GO terms were significantly enriched at 2 dpi in ToCV-, TYLCV-, and mixed-infected plants, respectively, with higher number of enriched GO terms for up-regulated genes in TYLCV- and mixed-infected plants and similar percentages of up- and down-regulated genes in ToCV-infected plants. As expected, the lowest number of GO terms was found at 7 dpi with only 120, 55, and 76 GO terms identified in ToCV-, TYLCV-, and mixed-infected plants, respectively, in clear contrast with those enriched at 14 dpi that reached 534 terms for ToCV-infected, 412 for TYLCV-infected and 490 terms for mixed-infected plants.

Several GO terms shared between single and mixed infections, and also among the different time points, were identified. The majority of the terms identified from up-regulated genes of mixed-infected plants at 2 dpi were related to chloroplast and photosynthesis processes, as well as to ligase and oxidoreductase activity, some of them also represented in single-infected plants (Fig. 17 A). Meanwhile, terms related to mitochondrial processes and response to reactive oxygen species were exclusively observed in mixed-infected plants. Regarding down-regulated genes at 2 dpi, common terms between single and mixed infections were observed related to cell communication, endoplasmic reticulum, lipid biosynthetic processes, and hormone-mediated signalling pathways as those involving jasmonic acid (JA) (Fig 17 B). Out of the 76 terms significantly enriched in mixed-infected plants at 7 dpi, the terms for up-regulated genes were mainly related to chloroplast and photosynthesis processes, while terms related to oxidoreductase and chitinase activity and heat shock protein binding were identified for down-regulated genes.

Among the GO terms identified at 14 dpi, the majority of them enriched for up-regulated genes in both single- and mixed-infected plants were related to defense response and hormone-mediated signalling pathways. Moreover, terms related to cell communication, transport and ubiquitin-protein activity were also commonly observed in infected plants, while terms related to vacuole, kinase activity and mitochondrial processes were identified only in mixed-infected plants

(Fig 17 A). In contrast, most of the enriched terms for down-regulated genes observed were common for single- and mixed-infected plants, some of the most relevant related to fatty acid, lipid, carotenoid and terpenoid biosynthetic processes, as well as to chloroplast and photosynthesis processes (Fig. 17 B).

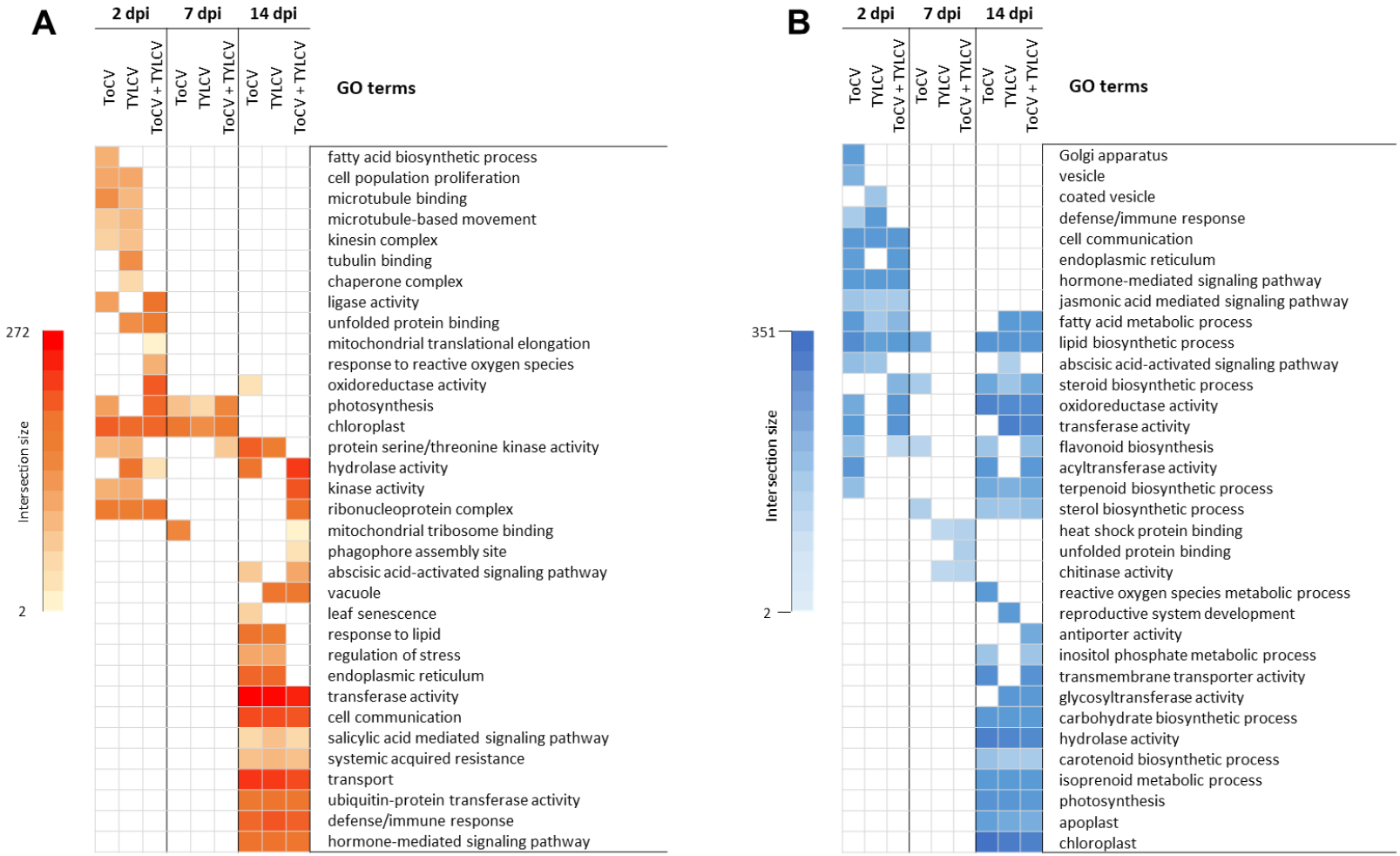


Fig. 17. Gene Ontology (GO) enrichment analysis of DE genes in response to single and mixed infection with ToCV and TYLCV at 2, 7 and 14 dpi. To analyze GO term enrichment, the DEGs were divided into up- (A) and down-regulated (B). Each cell is coloured based on the number of genes associated with each GO term.

Top KEGG pathways influenced by single and mixed infections

KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis was also conducted to assist the identification of potential pathways, using the same bioinformatics tool employed in the GO enrichment analysis.

In general terms, our analysis revealed a greater number of KEGG pathways for down-regulated genes compared to up-regulated genes in all cases except for mixed-infected plants where 28 KEGG pathways for up-regulated genes and 19 for down-regulated genes were identified at 2 dpi. Notably, several pathways were identified only in mixed-infected plants at this time point, such as riboflavin metabolism, carotenoid biosynthesis, pentose and phosphate pathway, sulfur metabolism and photosynthesis. Furthermore, some other pathways including ribosome, biosynthesis of amino acids, and biosynthesis of cofactors, purine and nucleotide metabolism were commonly identified in both single- and mixed-infected plants (Fig. 18 A). Among the top KEGG pathways from down-regulated genes at 2 dpi, most of them were common for single- and mixed-infected plants including MAPK signalling pathway, plant hormone signal transduction, cysteine, methionine and phenylalanine metabolism and steroid and flavonoid biosynthesis (Fig. 18 B).

The KEGG enrichment analysis for mixed-infected plants at 7 dpi revealed 3 and 9 pathways for up- and down-regulated genes, respectively. The most representative pathway category identified for up-regulated genes included photosynthesis (Fig. 18 A) while amino sugar metabolism, fatty acid elongation, cysteine and methionine metabolism and pathways related with processing proteins in endoplasmatic reticulum (ER) were identified for down-regulated genes (Fig. 18 B).

The number of pathways identified in mixed infections at 14 dpi was significantly higher for down-regulated genes compared with those for up-regulated genes, with 46 and 9 KEGG pathways respectively. Pathways related with hormone regulation, MAPK signalling and plant-pathogen interaction was enriched for up-regulated genes at 14 dpi in all infections (Fig. 18 A). Moreover, pathways related with photosynthesis, pyruvate, carotenoid and fatty acid biosynthesis, as well as

citrate cycle and carbon metabolism were associated with down-regulated genes in both single- and mixed-infected plants (Fig. 18 B).

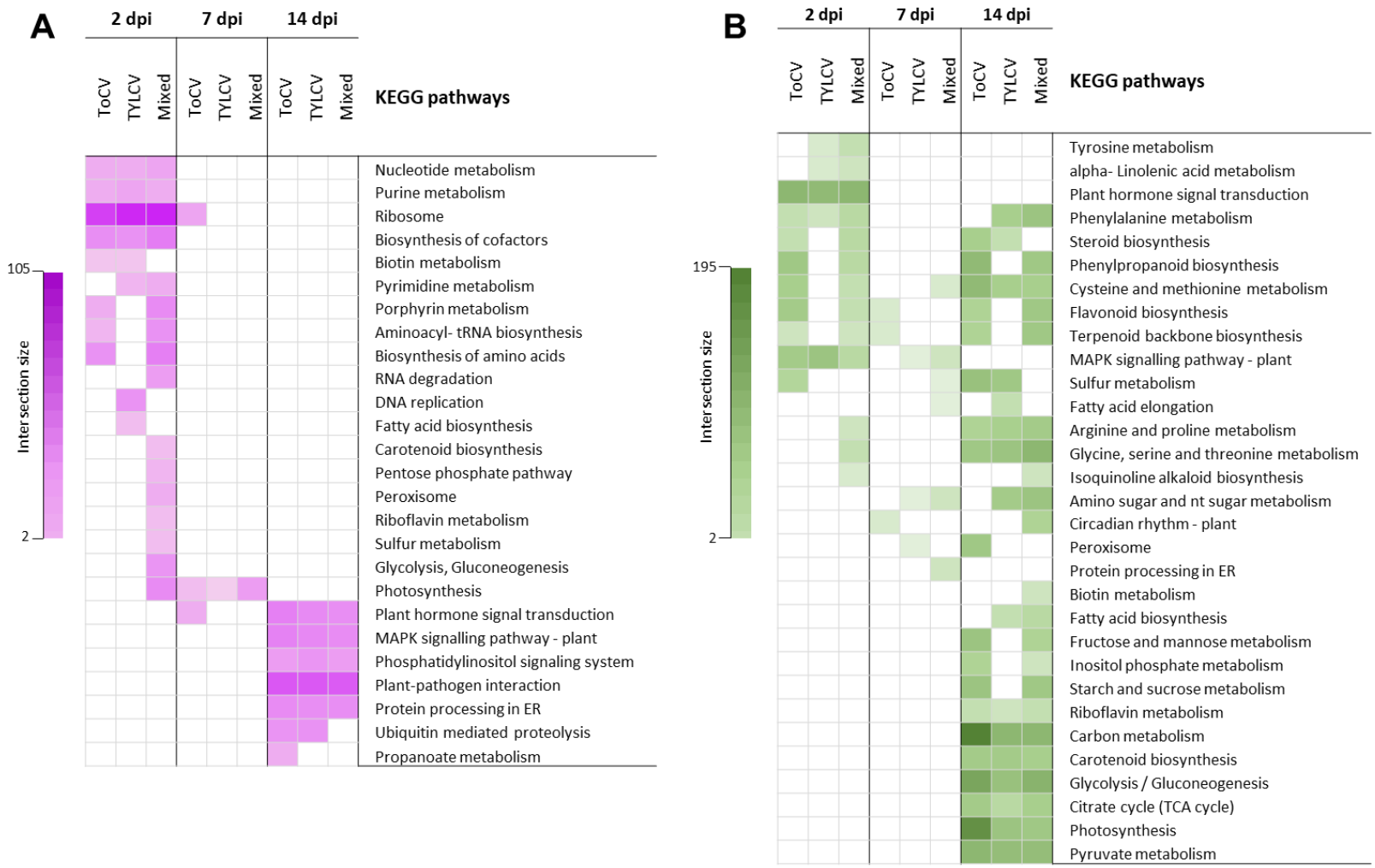


Fig. 18. Top Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched with up- (A) and down-regulated (B) DEGs in response to single and mixed infection with TYLCV and ToCV at different time points.

Silencing of genes related to defense response in single infections with ToCV

To further investigate the regulation of the plant immune system against pathogens, the significantly enriched plant-pathogen interaction KEGG pathway was explored. Among the up-regulated DEGs included in this pathway at 14 dpi, we decided to focus on the analysis of the mostly unknown expression profile of the *Hsp90-Sgt1* complex in ToCV-infected plants. Furthermore, the expression pattern of additional selected genes with annotated functions of resistance to pathogens was studied, which include the three RTM (restricted TEV movement) genes: *RTM1, RTM2*, and *RTM3*; implicated in long-distance movement of virus particles. Therefore, we investigated whether the silencing of the chaperone *Hsp90* and its co-chaperone *Sgt1*, both involved in plant immunity, and the RTM-resistant genes might affect viral RNA accumulation in tomato plants. The induced silencing of the selected target genes was achieved through VIGS with a TRV-based vector.

First, the gene expression of the selected genes was analysed, finding that the levels of mRNAs were reduced in the silenced plants compared to TRV- and ToCV-infected control plants 14 days after the inoculation of the virus (Fig 19). Although only mild phenotypes were observed for the *RTMs*- and *Hsp90*-silenced plants, silencing of *Sgt1* resulted in lethality at 21 dpi (Fig. 20), thus gene expression levels and viral RNA accumulation were assessed earlier, at 10 days after the inoculation of the virus vector. Then, results from the analysis of ToCV RNA accumulation showed that although the RNA-seq analysis revealed that RTM1 gene was up-regulated in ToCV-infected plants at 14 dpi, no differences in the relative abundance of ToCV between control and RTMs-silenced plants were found (Fig. 21 A). However, viral accumulation in *Hsp90*- and *Sgt1*-silenced plants was approximately 4-fold greater than in TRV-vector plants (Fig. 21 B), which suggests that *Hsp90* and *Sgt1* play a role in basal resistance against ToCV.

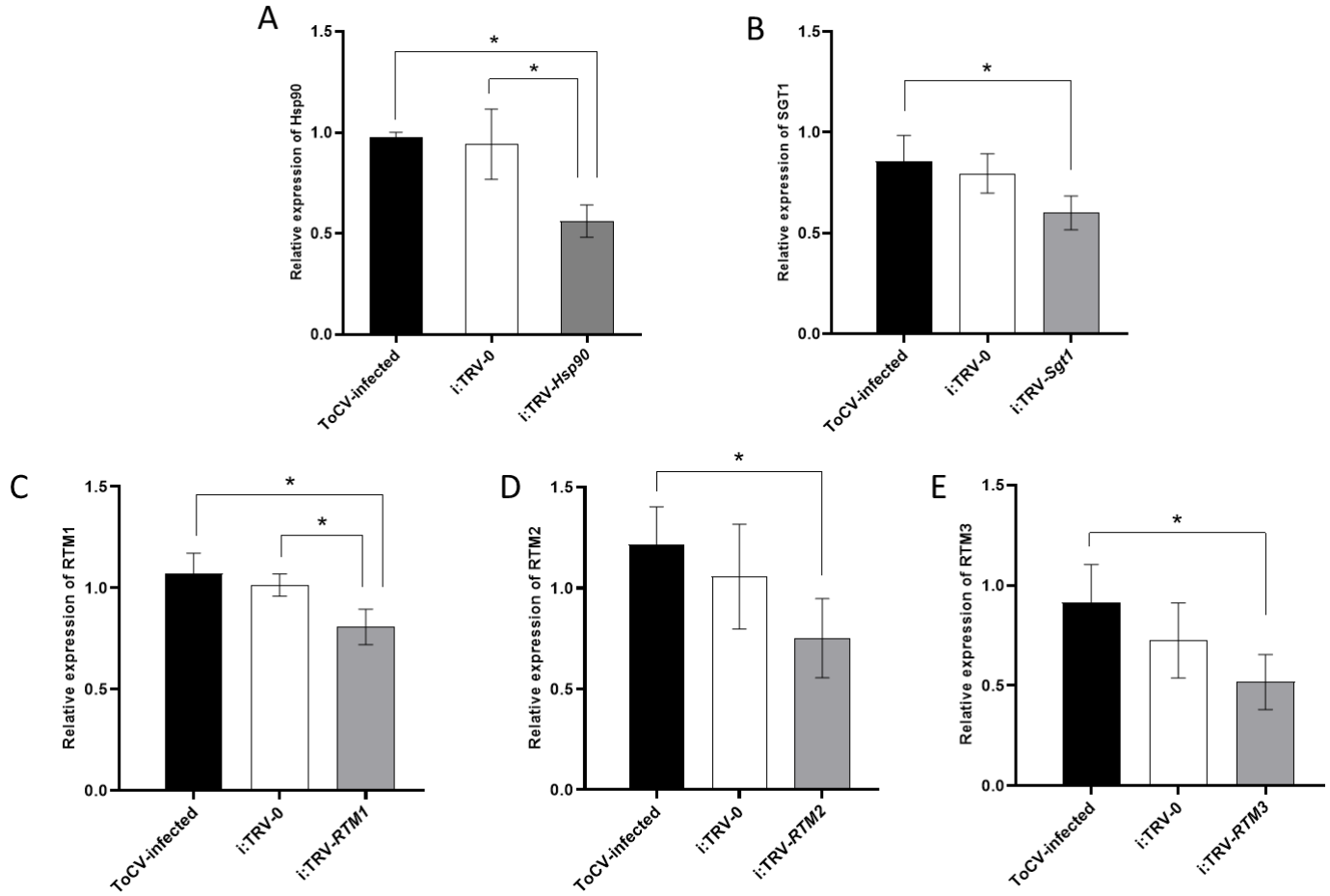


Fig. 19. Relative accumulation of the chaperone *Hsp90* (A) and its co-chaperone *Sgt1* (B) and the three RTM resistance genes selected (*RTM1* (C), *RTM2* (D) and *RTM3* (E)) in the selected controls (ToCV-infected and plants with TRV vector (i:TRV-0)) and silenced plants at 14 dpi. ToCV infected plants with empty TRV vector are named i:TRV-0; i:TRV-Hsp90 and :TRV-Sgt1 corresponds to ToCV infected plant with Hsp90 and Sgt1 constructions in TRV, respectively; i:TRV-RTM1, i:TRV-RTM2 and i:TRV-RTM3 correspond to ToCV infected plant with RTMs constructions in TRV.

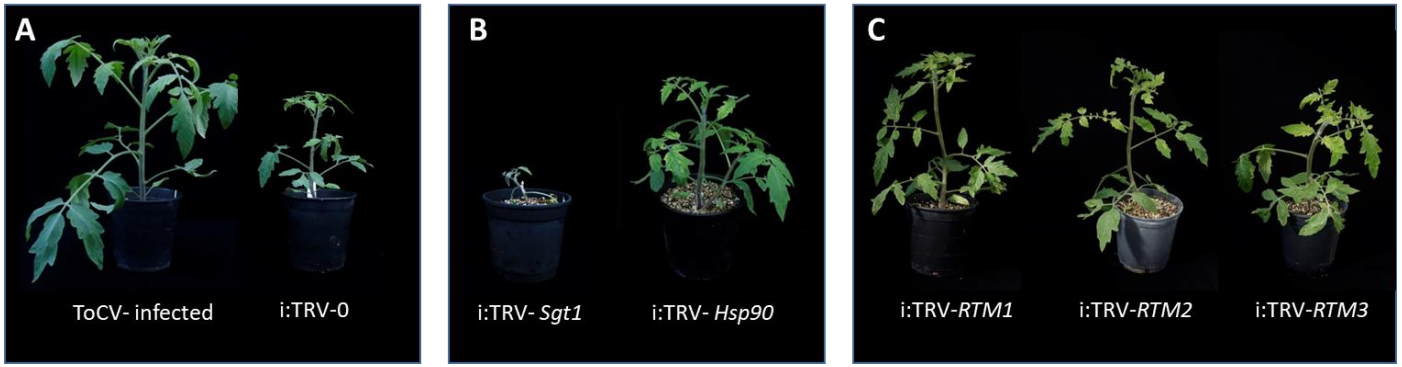


Fig. 20. Control (ToCV-infected and i:TRV-0) (A) and silenced plants at 14 dpi. Silencing of *Sgt1* (i:TRV-*Sgt1*), *Hsp90* (i:TRV-*Hsp90*) (B) and the RTMs genes (i:TRV-*RTM1*, i:TRV-*RTM2* and i:TRV-*RTM3*) (C) are represented.

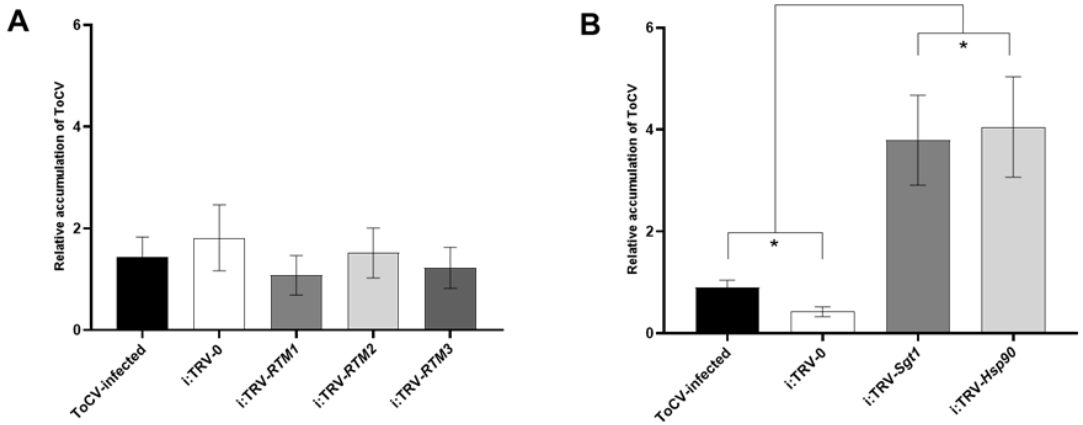


Fig. 21. Relative quantification of ToCV in both control and silenced plants at 14 dpi. (A) Efficiency of RTMs silencing, showing viral loads between control (ToCV-infected and plants with TRV vector (i:TRV-0)) and RTMs-silenced plants (i:TRV-*RTM1*, i:TRV-*RTM2* and i:TRV-*RTM3*). (B) Efficiency of *Sgt1* and *Hsp90* silencing, showing viral loads between control (ToCV-infected and i:TRV-0 plants) and *Sgt1*- (i:TRV-*Sgt1*) and *Hsp90*-silenced plants (i:TRV-*Hsp90*). ToCV infected plants with empty TRV vector are named i:TRV-0; i:TRV-Hsp90 and :TRV-Sgt1 corresponds to ToCV infected plant with Hsp90 and Sgt1 constructions in TRV, respectively; i:TRV-RTM1, i:TRV-RTM2 and i:TRV-RTM3 correspond to ToCV infected plant with RTMs constructions in TRV.

Accumulation patterns of 21-, 22-, and 24-nt sRNAs in mixed-infected tomato plants

To further explore molecular characterization of mixed infections with ToCV and TYLCV, general dynamic changes in accumulation patterns of sRNAs were analysed compared to single-infections. Same samples of single- and mixed-infected tomato plants at 14 dpi used to determine the transcriptome profile were employed to sRNAs sequencing. Data of the sRNAs profile of TYLCV-infected plants, used in this study as control, was previously described by Piedra-Aguilera et al. (2019). The total normalized number of sRNA reads of 20-25 nt that mapped in the TYLCV and ToCV genome was very similar in either single- or mixed-infected samples. Results showed 674.710 and 683.452 reads in the TYLCV genome of single and mixed-infected samples, respectively, and in the case of ToCV, 869.447 reads were obtained for single-infected samples and 220.129 reads for the mixed ones, 75-80% of them mapped to RNA2. No significant differences were observed between the two replicates of each viral infection.

Size distribution results showed that vsRNAs of 21- and 22-nt were the most abundant in both single and mixed infections. No significant differences were found in the size distribution pattern of TYLCV-sRNAs where 21-nt size represented more than 50% of the 20-25 nt vsRNAs followed by 22-nt size with approximately 30% (Fig. 22 A). Similar percentages were observed in the RNA1 pattern of ToCV except for mixed infections, where greater differences in the percentages between 21- and 22-nt vsRNAs were observed (Fig. 22 B). The RNA2 of ToCV showed a different size distribution pattern between single- and mixed-infected samples with a higher percentage of 22-nt over 21-nt vsRNAs in single infections (Fig. 22 B). In all cases, 20-nt and 24-nt were the third and fourth most representative size classes.

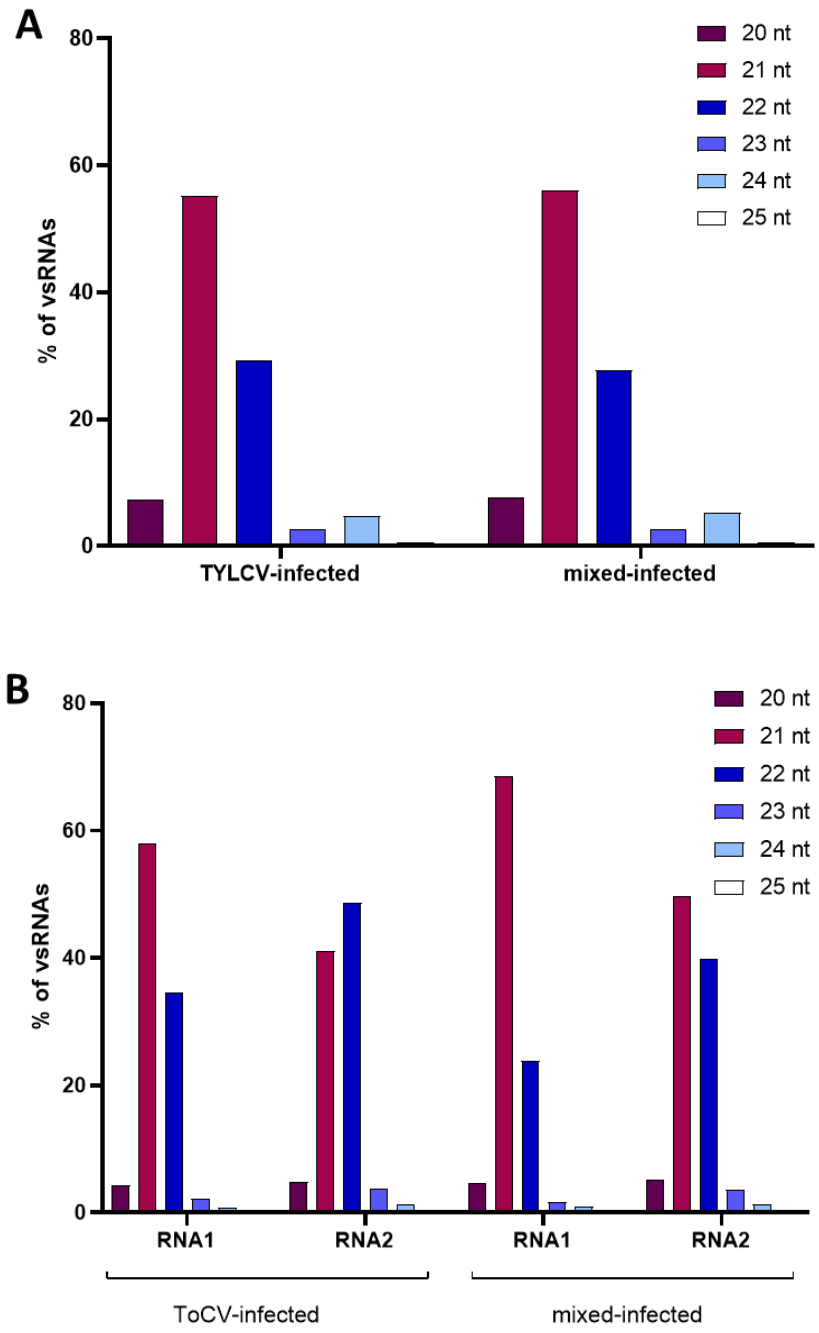


Fig. 22. Size distribution of vsRNAs present in single and mixed-infected plants with TYLCV and ToCV. Percentage of TYLCV (A) and ToCV (b) vsRNA size from 20 nt to 25 nt relative to the total sRNA reads in the indicated genome component, differenciating RNA1 and RNA2 in the case of ToCV. The mean of two independent biological replicates was used to represent bars corresponding to each size.

Distribution of vsRNAs along viral genome of TYLCV and ToCV

To have an overview of sRNA distribution along the virus genome, MISIS tool (Seguin et al., 2014) was used to display sRNA reads as an histogram which indicates not only the nucleotide positions but also the sense of the sRNAs (Fig. 23). Results revealed that the distribution pattern of 20-25 nt vsRNAs along the TYLCV genome and the RNA1 of ToCV was similar between single- and mixed-infected samples. In the case of TYLCV, although sRNAs mapped in both viral sense (VS) and complementary sense (CS) of the circular genome of TYLCV, the Rep and Cp encoding regions showed a higher percentage of sRNAs. The analysis of sRNA distribution along the ToCV genome revealed that most sRNAs mapped in RNA2 (80%) with a relatively higher accumulation in regions encoding Hsp70 and CPm. Moreover, the heterogeneous distribution of sense and antisense vsRNAs (Fig. 23) was observed in all cases. Our results showed consistency between the distribution patterns of the two biological replicates of each infection.

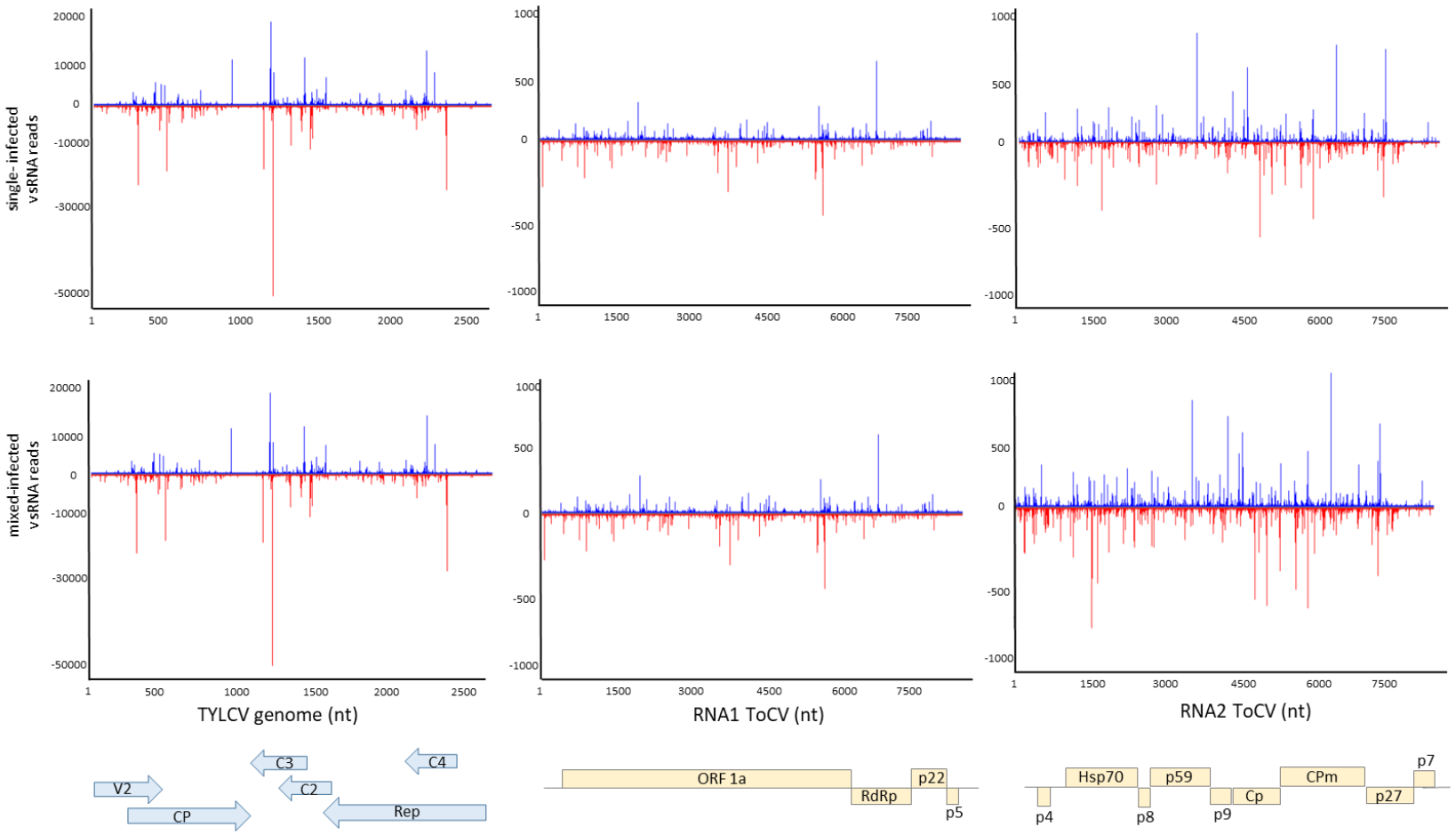


Fig. 23. Maps corresponding to vsRNAs of single (uppers panels) and mixed-infections (lower panels) with TYLCV and ToCV. Number of total vsRNA reads (20-25 nt) identified at each position of the genomes of TYLCV (left represented in a linearized format) or ToCV genome (bipartite, differentiating RNA1 and RNA2, as indicated below. Bars represented in blue correspond to sense reads and those represented in red the antisense reads. Plots represent one biological replicate.

DISCUSSION

Transcriptome sequencing technology had been relevant to explore and identify changes in the host plant associated with several pathways as defense response, regulation of hormones, and photosynthesis in different pathosystems, including mixed infections. Moreover, molecular mechanisms involved in tomato responses to TYLCV and ToCV have been previously studied in other cultivars of tomato and at specific time point of the infection (Seo et al., 2018; Zhong Wang et al., 2009; Yue et al., 2021). However, the time course analysis of transcriptional changes in host plant associated with viral infection has not been extensively explored so far. Although it is complicated to generally define the metabolic responses triggered by mixed viral infection due to the complexity of virus-host interactions, our work aimed to provide a comprehensive understanding of the changes in the tomato transcriptome throughout single and mixed infections with TYLCV and ToCV contributing to the study of molecular mechanisms underlying plant-virus interactions. Results obtained of the analysis of transcriptional responses of tomato plants at different time points after *Bemisia tabaci*-mediated infection revealed significant dynamic changes in the gene expression of plants over time in all types of infections assayed. A similar number of differentially expressed genes in either single or mixed infections with TYLCV and ToCV was observed when compared with the mock-inoculated plants (Fig. 16 A). While a large number of DEGs were identified at the early (2 dpi) and late stages (14 dpi) of the infection, a critical decrease was observed at 7 dpi. This reduction coincided with the results of the PCA analysis which revealed that mock-inoculated plants at 14 dpi and either infected or mock-inoculated plants at 7 dpi formed a unique cluster, indicating that viral infection at 7 dpi and the effects of *B. tabaci* feeding after 7 dpi does not alter the overall gene expression. By contrast, comparison of the DEGs between mixed and single infections with either ToCV or TYLCV revealed a different pattern during the infection process with a significant reduction of DEGs at 2 and 14 dpi. Transcriptome analysis at 2 dpi revealed higher number of DEGs identified in mixed infected plants when comparing to single infections with TYLCV than with ToCV, contrary to the results showed at 14 dpi (Fig 16 B). These results might correlate with the symptomatic outcomes observed during mixed infections over time, previously published

(Ontiveros et al., 2022) and described in chapter I, where the plants showed slight symptoms similar to single ToCV-infected plants during the first observations followed by an increase of severity that resembled more to TYLCV infection.

During the infection, plant viruses require host machinery to replicate and spread through the plant inducing several metabolic changes (Llave, 2016; Nelson & Citovsky, 2005; Zanardo et al., 2019). By contrast, plants are able to detect and respond to viral infections by the induction of the expression of genes involved in different defense responses reducing other cellular activities such as photosynthesis (Nomura et al., 2012). In this study, the significantly enriched GO terms and KEGG pathways identified in the different stages of the infection indicated that viral infection influence in several biological processes, most of them related to photosynthesis, hormone signal regulation, metabolism, and plant-pathogen interactions. The activation of plant defense responses induced by a viral infection trigger a complex networks of hormone signalling pathways, mainly the SA- and JA- mediated signalling pathways (Bari, R., & Jones, 2009; Pieterse & Van Loon, 1999). Results obtained in this work showed this up-regulation of DEGs related to plant hormone pathways in all infected plants at 14 dpi, however, some of these hormone-related pathways were down-regulated at 2 dpi. In agreement with previous studies reported that some insect-borne plant virus infections might influence insect-plant interactions, for example by repressing JA-mediated defences against vectors (Lewsey et al., 2010; Zhang et al., 2012), it seems that either single or mixed infections with ToCV or TYLCV influence on JA-mediated gene expression with possible consequences on virus transmission by the vector.

Moreover, other major processes were also up- or down-regulated during the infection. Several studies reported that genes related to photosynthesis and pigment metabolism were down-regulated after a viral infection (Hanssen et al., 2011; Lu et al., 2012; Nomura et al., 2012). Interestingly, in this study, a dynamic regulation of DEGs involved in photosynthesis processes, mainly in mixed infections, was observed being up-regulated at 2 and 7 dpi and significantly down-regulated at 14 dpi as shown in figure 18. These results at the beginning of the infection might be explained in relation to the study reported by Liu et al. (2014) which revealed the up-regulation of genes associated with chlorophyll

degradation, and thus photosynthesis, in response to *African cassava mosaic virus* infection. Moreover, Lu et al. (2012) described that processes related to photosynthesis, pigment metabolism and plant-pathogen interactions were associated with development of symptoms induced by a viral infection.

Given the complexity of virus-host interactions, defining the metabolic responses triggered by mixed viral infection in a general sense can be challenging. Therefore, we focused on the significantly enriched plant-pathogen interaction KEGG pathway to better study the role of the *Hsp90-Sgt1* complex and the *RTM1, RTM2*, and *RTM3* genes in ToCV infections, which might set the basis to further studies in mixed infections. The RTM resistance genes and *Hsp90-Sgt1* complex plays a critical role in regulating the plant immune system against pathogens, including plant viruses (Cosson et al., 2012; Mahajan et al., 1998; Shirasu, 2009). In this study, a significant increase in *RTM1*, *Hsp90* and *Sgt1* expression was observed at 14 dpi following ToCV infection, however, no differences in ToCV accumulation were found in *RTMs*-silenced plants. By contrast, silencing of *Hsp90* and *Sgt1* resulted in a higher level of ToCV accumulation (Fig. 21). Additionally, silencing of *Sgt1* through VIGS led to cell death, whereas *Hsp90*-silenced plants showed no noticeable differences when compared to control *TRV*-infected plants. These results are in line with those of Moshe et al. (2016), who demonstrated that silencing the tomato *Hsp90* and *Sgt1* genes resulted in increased TYLCV accumulation. However, while these authors reported that silencing of both genes led to cell death, we observed this phenotype only in *Sgt1* tomato plants (Fig. 20). Overall, these data strongly support the hypothesis that *Hsp90* and its co-chaperone *Sgt1* are necessary for an effective plant resistance response to ToCV infection.

The primary defense mechanisms in plants against viruses is antiviral RNA silencing, triggered by viral dsRNA processed into 21-25 nt sRNAs (Ding, 2023). The accumulation and distribution patterns of vsRNAs have been studied in several viral infections, including TYLCV (Piedra-Aguilera et al., 2019); however, the genomic distribution of vsRNAs in mixed infections is relatively unknown. In the mentioned study (Piedra-Aguilera et al., 2019) the size distribution of 20-25 nt vsRNAs and their organization through the genome of TYLCV in single-infected plants showed the same pattern as the results obtained in this work for

mixed infections (Fig. 22 and Fig. 23). Moreover, positions and percentages of vsRNAs in RNA1 of ToCV are remarkably similar between single and mixed infected plants. The conducted transcriptome analysis showed that single infections with ToCV induced higher expression of DCL2b (Solyc11g008540) compared with mixed infected plants, contrary to DCL4 (Solyc07g005030) which was highly up-regulated in mixed infected. It is well documented the role of DCL2 and DCL4 to process dsRNAs to generate 22- and 21-nt sRNAs in Arabidopsis, respectively (Ding, 2023; Wang et al., 2018). Moreover, Wang et al. (2018) reported that slDCL2b were one of the most abundant members of DCL2 over the four ones found in tomato. Interestingly, these results correlate with the opposite patterns of percentages of 21- and 22-nt vsRNAs found in RNA2 of ToCV for either single- and mixed-infected tomato plants.

Although further research would be needed for a better understanding of the implications of mechanisms involved in this complex pathosystem, this study provides new insights into the molecular responses that occur in tomato plants co-infected with ToCV and TYLCV that could help in identifying potential gene targets to develop new strategies for disease control.

CHAPTER 3

Evidence of cross-kingdom communication of small RNAS derived from TYLCV with *Bemisia tabaci*

RESULTS

Comparative bioinformatics analysis of sRNAs

Cross-kingdom RNAi has recently emerged as a novel mode of communication between organisms that are distantly related. This fascinating phenomenon has demonstrated its pivotal role in regulating the interaction between pathogens/pests and host immunity in plants. It raises an intriguing hypothesis that plant viruses might employ a similar communication strategy with their insect vectors, utilizing sRNAs produced in the infected plants where they feed. Therefore, it is reasonable to hypothesize that sRNAs derived from TYLCV and/or ToCV mediate cross-kingdom RNAi to suppress *Bemisia* genes and enhance its spread. To explore this scenario, first an in silico analysis of sRNAs derived from single- and mixed-infected tomato plants with ToCV and TYLCV at 7 and 14 dpi tomato plants infected (see chapter II) was performed to search potential targets in the *B. tabaci* (MED) genome. The result of this bioinformatic analysis identified in several *B. tabaci* genes that could potentially serve as targets for sRNAs originating from TYLCV-infected tomato plants. These sRNAs were directly derived from viral sequences. Interestingly, no matches between sRNAs from uninfected or ToCV-infected tomato samples and the *B. tabaci* genes were found. These findings strongly suggested compelling evidence of cross-kingdom communication through sRNA in the complex interplay between TYLCV and the whitefly vector.

The GO analysis revealed that at least six of the potentially targeted genes were involved in detoxification pathways and genes related to nicotinic acetylcholine

receptors (Table S13). From each of these two pathways, three genes were selected by choosing those with the highest count numbers and a size between 21 and 22 nt and the expression levels in both non-viruliferous and TYLCV-viruliferous whiteflies. Preliminary results obtained at Wageningen University (data not shown) led us to finally focused on three of these genes related to detoxification pathways identified: Bta001158, Bta001692 and Bta028144. These genes correspond to a cytochrome P450, an aryl hydrocarbon receptor (AhR) and a neuroligin gene, respectively (Fig. 24). Subsequent analysis using RT-qPCR revealed that the expression levels of the selected genes in TYLCV-viruliferous whiteflies were approximately 3-fold lower than in non-viruliferous ones (Fig 25 A), suggesting that the downregulation of the selected genes might be mediated by sRNAs derived from TYLCV.

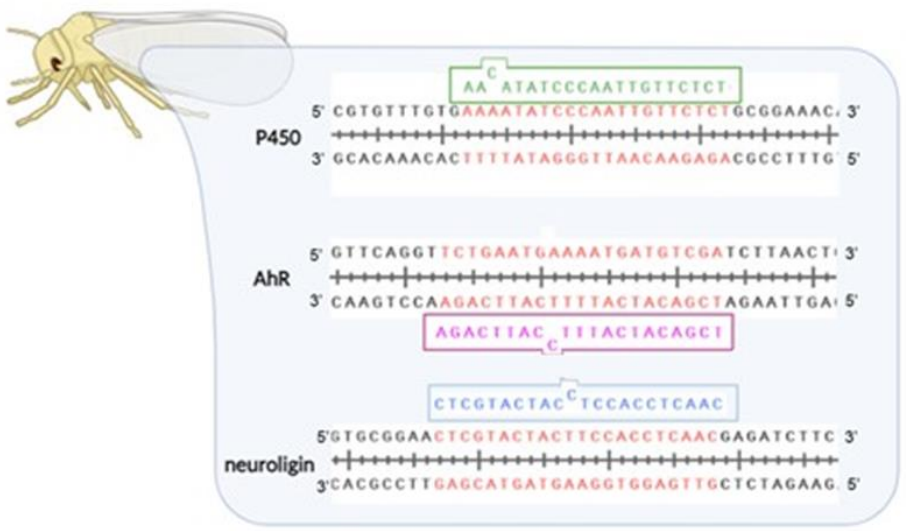


Fig. 24. Schematic representation of matches corresponding to TYLCV-derived sRNAs on selected *B. tabaci* genes identified as putative target genes involved in neonicotinoid detoxification-related pathways: P450, AhR and neuroligin. Sequences inside boxes represent the sRNAs found in TYLCV-infected plants.

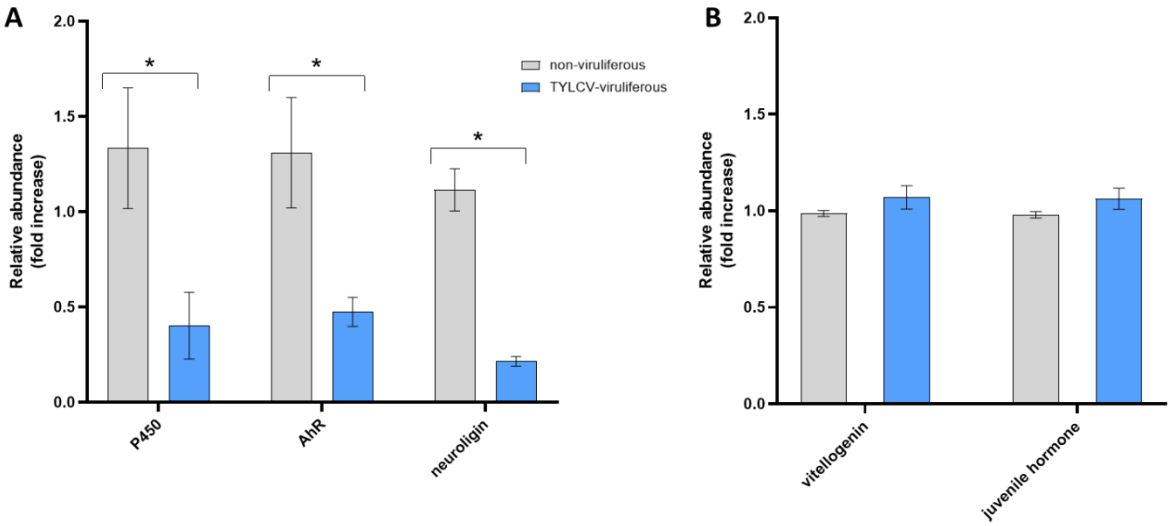


Fig. 25. Relative expression levels of *B. tabaci* target genes in non-viruliferous and TYLCV-virulíferous whiteflies. Bars represent mean ± standad error of the relative expression of cytochrome P450, AhR and neuroligin (A), in addition to the control genes corresponding to vitellogenin and juvenile hormone. The relative quantifications were calculated using the comparative cycle threshold method (Livak and Schmittgen ,2001). Relative quantification of specific RNAs was normalized to the expression of the elongation factor 1-α (SIEF1α) gene. Asterisks indicate significant differences ($p<0.05$) based on one-way ANOVA analysis.

Evaluation of viruliferous and non-viruliferous whiteflies' responses to nicotine artificial diet

As mentioned above, the three selected genes Bta001158, Bta001692, and Bta028144 were identified as genes related to the detoxification of nicotine and neonicotinoids, more specifically they corresponded to cytochrome P450, aryl hydrocarbon receptor (AhR) and neuroligin genes respectively. Thus, experiments with feeding of whiteflies on an artificial diet were performed to functionally validate the hypothetical role of the selected genes in survival when a toxic substance was included in the diet. Briefly, I studied the response of viruliferous and non-viruliferous whiteflies to the incorporation of nicotine (0.01%) in the feeding sucrose-containing solution. Groups of 30 whiteflies per tube were allowed to feed on an artificial diet containing 15% sucrose and 0.01% of nicotine for a 48 h time-course observation period, along with the corresponding controls (no nicotine) in parallel. Results showed that TYLCV-viruliferous whiteflies seemed to be more susceptible than non-viruliferous whiteflies to the presence of the toxic substance, as the survival rate was significantly lower in the groups fed with the nicotine-containing solution. The effect was observed since the first hour of exposure to nicotine, and after 24h of feeding on the artificial diet, results showed that more than 50% of the whiteflies (viruliferous and non-viruliferous) died reaching 100% of mortality at 48h (Figure 26 A). As an additional control, and considering that no predicted ToCV-vsRNAs targeting whitefly genes were found in our bioinformatics analysis, ToCV-viruliferous whiteflies were used as control in equivalent experiments, showing as expected no differences in survival rates between non-viruliferous and ToCV-viruliferous whiteflies (Figure 26 B). Thus, our results suggested that the TYLCV-viruliferous condition of the whiteflies had a detrimental effect on their ability to detoxify nicotine in our experimental setup with artificial diet.

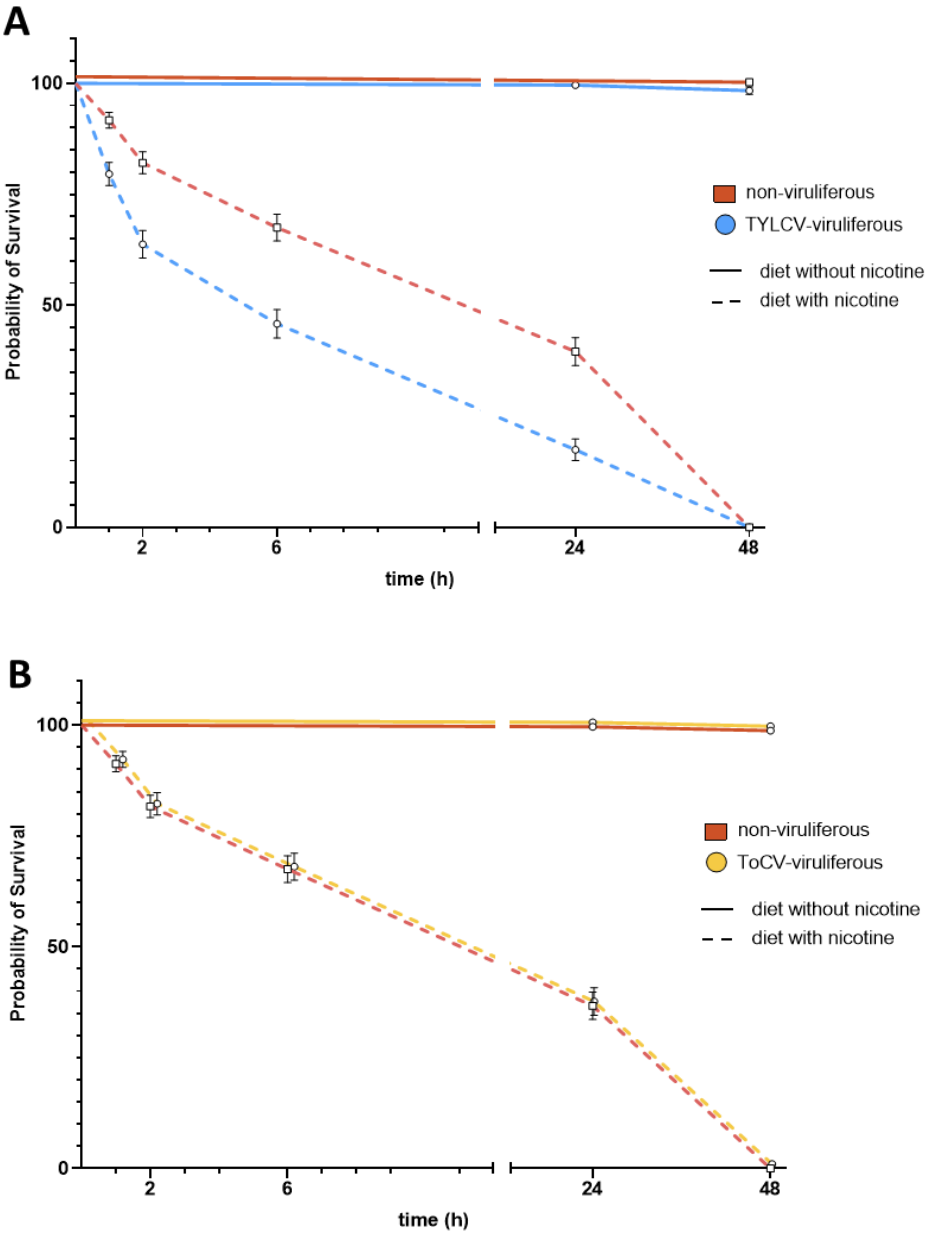


Fig. 26. Survivor dynamic of non-viruliferous whiteflies versus TYLCV-viruliferous (A) and ToCV-viruliferous (B) whiteflies in response to nicotine (0,001%). An artificial diet containing sucrose 15% was used as a control and the number of whiteflies (alive or death) were recorded after 1h, 2h, 6h, and 24h of exposure to nicotine.

Evaluation of TYLCV-viruliferous whiteflies' behaviour under 48h-dual-choice conditions

To investigate whether the acquisition of TYLCV may influence the host preference behaviour of its vector, a dual-choice experiment was performed under controlled conditions with healthy (control) and TYLCV-infected leaflets. The number of whiteflies settled on either control or infected leaflets was recorded at 2h, 24h, and 48h to assess the rate of migration of the insect vector. Groups of 30 TYLCV-viruliferous and 30 non-viruliferous whiteflies were tested using the same procedure as described for dual-choice assays described in Chapter I. As expected and previously confirmed in chapter I, TYLCV-infected leaflets were the first choice of the whiteflies showing double of individuals landed compared to the recorded on healthy leaflets. However, trends changed after 24h with more whiteflies settled on healthy than on infected leaflets showing more significant differences at 48 h (Fig. 27). A similar behaviour was observed in either non-viruliferous and TYLCV-viruliferous whiteflies. This result suggests that TYLCV interacts with *Bemisia* in a way that promotes virus acquisition (as suggested by feeding on TYLCV infected plants immediately after the first choice), and subsequently facilitates migration to other uninfected hosts (as the number of settled whiteflies at later times indicated, likely involving a transfer from infected (take off) to healthy plants (landing and settling)) in a manner conducive to transmission.

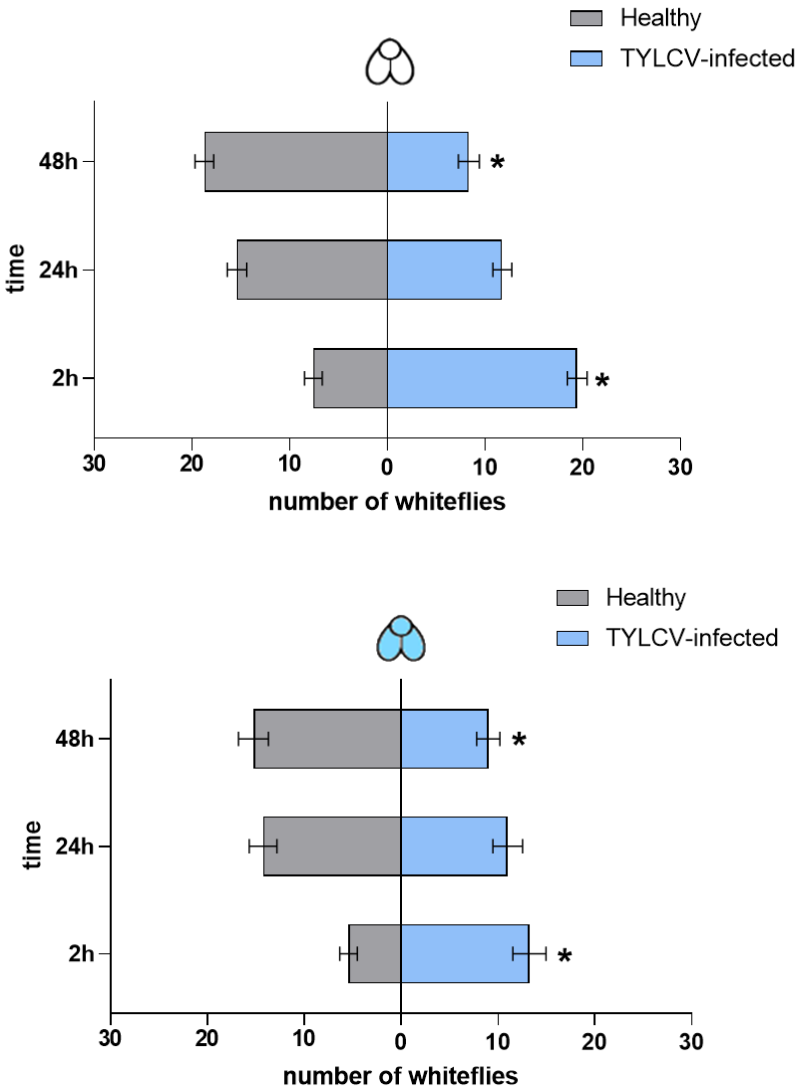


Fig. 27. Whitefly preference responses for mock-inoculated (healthy) versus TYLCV-infected versus plants in a dual-choice bioassay. Whitefly icons represent the infectious status of the whiteflies tested: white for non-viruliferous and blue for TYLCV whiteflies, respectively. Asterisks indicate significant differences between pairs of treatments ($p<0.05$) based on chi-square analysis.

Survival rates of *B. tabaci* after silencing target genes by artificial diet with vsRNAs

To validate the role of the identified TYLCV-derived sRNAs in *B. tabaci* nicotine detoxification, similar experiments as mentioned in the previous paragraph were performed by directly feeding specific duplex RNAs (Table S13) mixed with nicotine (0.01%) to whiteflies. Before performing the experiments, the potential vsRNAs were amplified from TYLCV-infected whiteflies and subsequently sequenced to verify nucleotide identity with *B. tabaci* target genes. Artificial diets with sucrose 15% with and without nicotine were used as controls, as well as diets supplemented with synthesized fragments of a 21-nt length of the following *B. tabaci* genes:Bta001158 (P450), Bta017585 (vitellogenin), and Bta008678 (juvenile hormone) , from now on named as BtaP450-sRNA, BtaVit-sRNA and BtaJH-sRNA, respectively.

Mortality rates of whiteflies and expression levels of P450 (Bta001158), aryl hydrocarbon receptor (AhR), and neuroligin (Bta028144), in addition to the previously mentioned control genes corresponding to vitellogenin and juvenile hormone, were analysed after 24h of feeding in an artificial diet containing sRNAs. Results showed that the relative expression of both targets (P450, AhR and neuroligin) and control (vitellogenin and juvenile hormone) genes significantly decreased compared with sucrose and GFP diet (Fig. 28), suggesting the silence of these genes by artificial diet with sRNAs. No differences in mortality rates were observed compared with the controls.

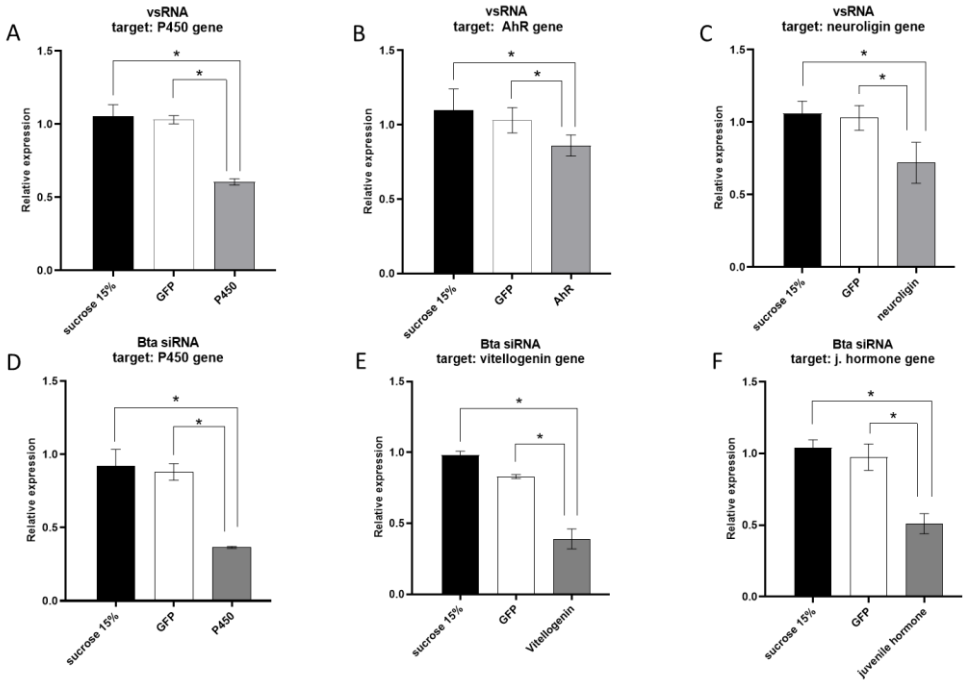


Fig. 28. Relative accumulation of the *B. tabaci* genes target of control and TYLCV-derived sRNAs. Expression levels relative to P450 (A), AhR (B) and neuroligin (C), after 24h of feeding on artificial diet supplemented with sRNA compared with sucrose diet and a diet containing GFP. In addition, the relative expression of control genes corresponding to P450 (D), vitellogenin (E) and juvenile hormone (F) were analysed after feeding on a diet supplemented with *B. tabaci*-derived sRNAs. The artificial diets in A and D contained sRNAs targetting the same P450 gene, with one mismatch in the vsRNA case (A) and no mismatches in the Bta siRNA (D).

Analysis of Kaplan-Meier survival curves showed significant differences in the survival of individuals feeding on control diets compared with diets with vs-RNAs. No differences in survival rates were observed in individuals fed on diets supplemented with Bta-sRNAs, except for Bta-P450-sRNA (Fig. 29 A). However, silencing of P450, AhR, and neuroligin with vs-sRNAs resulted in lower survival compared with controls with around 50% of dead individuals after 6h, which reached around 25% at 24h. Similar survival rates were observed in whiteflies fed with vs-sRNAs target of AhR and neuroligin, and with BtaP450-sRNA, by contrast to the whiteflies fed with vs-sRNAs target of P450 whose probability of survival drastically decreased after 6h of feeding (Fig. 29 B). After 48h of feeding, 100% of mortality was observed in all cases except with the no-nicotine diet used as control (Fig. 29). Overall, this study showed that oral ingestion of synthetized TYLCV-sRNAs targeting for silencing specific genes in *B. tabaci* increased r susceptibility to nicotine and also that cross-kingdom RNAi could function as a

ubiquitous mechanism with significant implications for the transmission and spread of TYLCV.

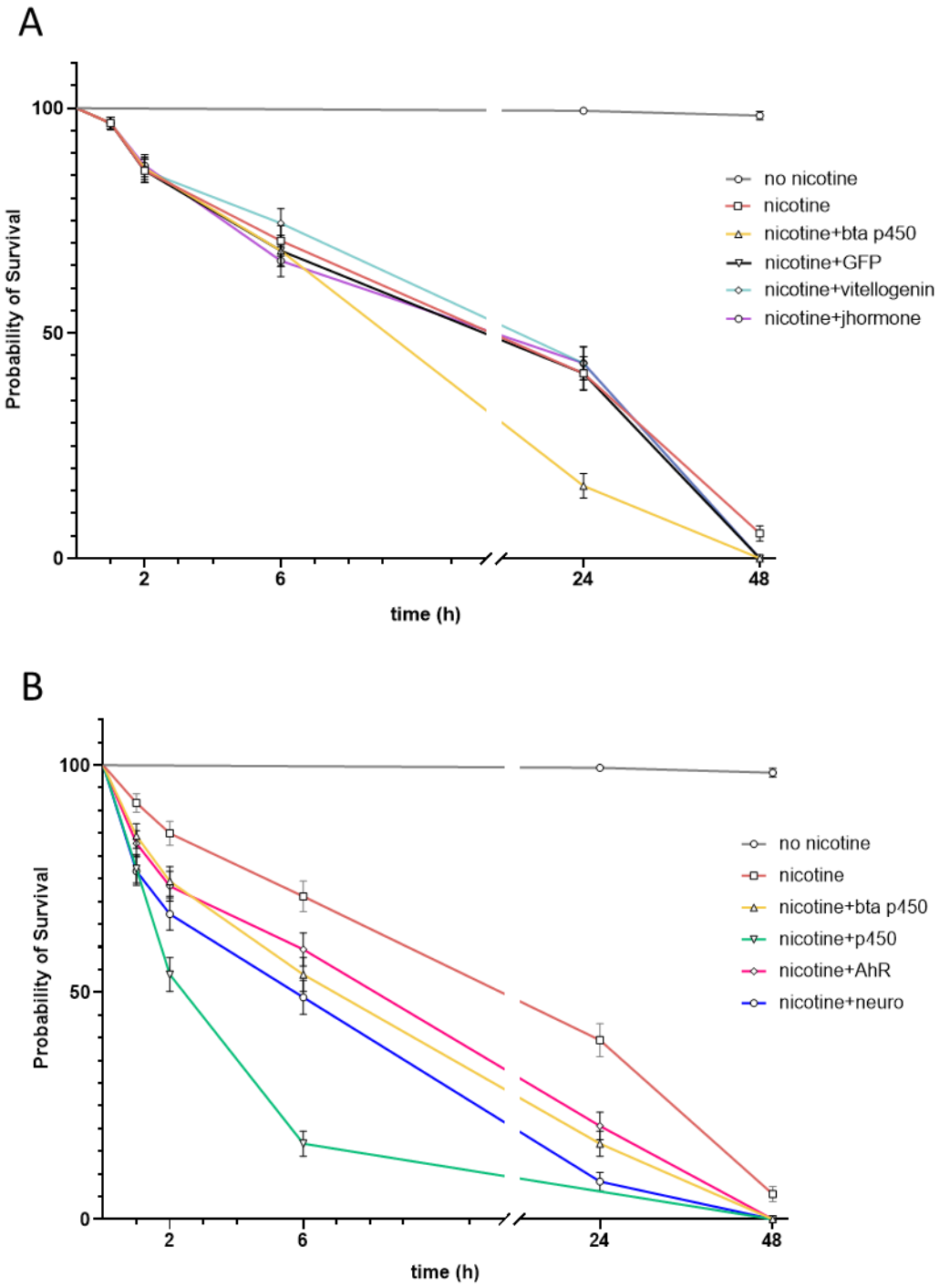


Fig. 29. Survival of *B. tabaci* to nicotine (0, 01%) when fed on sRNAs artificial diet. Lines represent Kaplan-Meier analysis survivor curves of whiteflies after feeding on either control (A) or TYLCV-derived sRNAs (B). The number of whiteflies (alive or death) were recorded after 1h, 2h, 6h, 24h and 48h of exposure to nicotine.

DISCUSSION

As previously discussed in chapter II, sRNAs play an important role in regulating gene expression in eukaryotic and bacteria. Recent studies evidenced that these sRNAs can be transmitted not only within an individual organism but also across interacting species, connecting different kingdoms. This cross-kingdom communication has been reported in several host-pathogen interactions, mainly between fungal and host plants. Fungal pathogens such as *Botrytis cinera* are able to transfer sRNAs to manipulate host genes to suppress host immunity and promoting the infection (Weiberg et al., 2013). More recently, the transport of sRNAs from host plant sRNAs into pathogen was also found in *Verticillum dahliae* (Tao Zhang et al., 2016) supporting the evidence of a bidirectional fungal-host sRNAs transmission. Similarly, other cross-kingdom communications have been recently studied, such as the transport of sRNAs from an insect fungal pathogen to its host, inducing gene silencing (Cui et al., 2019), or the detection of whitefly-specific sRNAs in tomato host plants (van Kleeff et al., 2016). However, despite these studies, no evidence of cross-kingdom sRNAs communication between virus-infected plants and their insect vector have been reported so far.

High-throughput sequencing used in this study allowed the identification of targets for TYLCV-derived sRNAs in *B. tabaci* genome. Interestingly, some of the targeted genes were involved in neonicotinoid detoxification-related pathways. The study of detoxification related genes in *B. tabaci* have been recently expanded over the las years, probably due the association of cytochrome P450 monooxygenase genes to the increased resistance of whiteflies to insecticides, including neonicotinoids (Jones et al., 2011; Karunker et al., 2008; Xie et al., 2018; Zhao et al., 2023). Thus, Neonicotinoids act on insect nicotinic acetylcholine receptors (AChR), confined to their nervous system, showing high toxicity (Matsuda et al., 2001). Significate differences found in the expression levels of tested target genes between non-viruliferous and TYLCV-viruliferous whiteflies (Fig. 25 A) suggest that their downregulation can be mediated indeed by TYLCV-sRNAs. Hence, the higher mortality observed in TYLCV-viruliferous whiteflies in response to nicotine (0.01%) supports the evidence of a possible cross-kingdom gene silencing in *B.tabaci* by virus-derived sRNAs.

The mentioned development of resistance of *B. tabaci* to several types of available insecticides led to the research on alternative strategies of control. RNA interference (RNAi) strategy, in which dsRNA is introduced to a target organism to induce specific gene silencing, has been proven to be a potential new approach for whitefly control (Grover et al., 2019; Shelby et al., 2020). The application of this RNAi technology by ingesting dsRNAs allowed the target of several specific genes of *B. tabaci* providing a better understanding of gene function for the control of this vector population (Upadhyay et al., 2011; C. Zhang et al., 2017). Previous studies have tested the silencing of genes related to insecticide resistance in *B. tabaci* by artificial diet with dsRNAs. For example, Li et al. (2015) showed a successful silencing of P450 CYP6CM1 (related to the resistance of *B. tabaci* to neonicotinoids) through the feeding of *B. tabaci* (MED and MEAM1) on an artificial diet with synthetized dsRNAs of this gene. However, they suggest a low efficiency of targeting the P450 CYP6CM1 gene in MED *B. tabaci* control, as they showed no significant differences in mortality rates in response to either imidacloprid or nicotine after feeding on an artificial diet. By contrast, our results presented in this chapter showed that silencing of P450, AhR, and neuroligin by artificial diet experiments resulted in higher mortality of whiteflies fed with vs-dsRNAs in response to nicotine. This could indicate that providing directly sRNAs, either being TYLCV-derived or artificial, was a more efficient strategy for effective gene silencing than the delivery of dsRNA to the insect, perhaps due to the longer period of time required to exert the effect.

As previously discussed, the bidirectional influence of sRNAs has been reported in several studies, mainly between plants and fungal pathogens. However, to the best of our knowledge there are no previous studies that explore the possible potential effects of sRNAs cross-kingdom communication in tripartite interactions between plants, viruses and insect vectors. A recent review has considered this possibility as a very attractive hypothesis, although highlighting the absence of experimental evidences (Matsumura & Kormelink, 2023). Since a viral infection may induce changes in the host plant that mediate insect vector behaviour, it is tempting to speculate about the role played by the increased susceptibility to perceive the toxicity caused by nicotine: the vectors might be stimulated to move away from the TYLCV-infected plants, despite the first higher attractiveness. This

hypothesis agrees with the observation that whiteflies, after a first choice towards probing on infected plants (and hence, being able to acquire the virus), they later tend to move to uninfected plants in order to clear out the increased susceptibility to nicotine, and where at the same time they can hypothetically succeed to inoculate the virus. In other words, the targeting of nicotine-detoxification genes could be leading to behavioural changes in the insect resulting in transmission-conducive responses. The similar behaviour observed on both viruliferous and non-viruliferous whiteflies may be due to the change in viruliferous condition after landing on TYLCV-infected plants. Overall, our results manifest that despite the initial attractiveness of *B. tabaci* for TYLCV-infected plants (Ontiveros et al., 2022) they preferred later to migrate onto healthy tomato plants, suggesting that trans-kingdom communication of sRNAs may have an impact on insect vector feeding behaviour with consequences on viral transmission. Are these effects specific for TYLCV-tomato-*B. tabaci,* or could they be more general and apply to other virus-host-vector relationships? It is early to say, but at least for the ToCV-tomato-*B. tabaci* pathosystem we didn't identify any relevant targeting of vector genes using sRNAs generated in infected plants. This difference might reflect simply the quite big taxonomical distance between the begomovirus TYLCV and the crinivirus ToCV, but also could be due to other reasons, like the divergence in transmission mode, circulative for TYLCV and semi persistent for ToCV: in the first case, the longer acquisition, retention and inoculation periods could provide evolutionary grounds for the alteration of vector behaviour, while they would be irrelevant for the fastest acquisition and inoculation periods of ToCV.

Although further research is necessary to explore several mechanistic aspects, results showed in this study might have a significant impact on our understanding plant-virus-vector interactions and could lead to new technological approached based on sRNA manipulation.

CONCLUSIONS

CONCLUSIONS

1- The co-infection of tomato plants with *Tomato yellow leaf curl virus* (TYLCV) and *Tomato chlorosis virus* (ToCV) displayed different symptomatic outcomes during mixed infections over time. Initially, co-infected plants exhibited milder symptoms compared to single infections. However, as the infection advanced, the symptoms became more severe, indicating a complex interplay between antagonistic and synergistic interactions at different infection stages. This shift in symptom severity correlated with a significant increase in ToCV accumulation during later infection stages.

2- Whiteflies exhibited a strong preference for symptomatic leaflets from plants infected either with TYLCV alone or in co-infection with ToCV, as opposed to mock-inoculated or ToCV-infected leaflets. Furthermore, our findings suggested that this preference was primarily driven by visual cues associated with the yellow coloration of TYLCV symptomatic leaves, rather than olfactory stimuli. This outcome also implies that ToCV, when present in mixed infections, could potentially benefit from enhanced whitefly transmission opportunities created by TYLCV.

3- The transcriptomic analysis unveiled distinct temporal patterns in gene expression in response to single and mixed infections with ToCV and TYLCV. While viral infection at 7 days post-inoculation (dpi) and the effects of whitefly feeding after 7 dpi had minimal impact on overall gene expression, significant changes were observed at 14 dpi. This suggests that the influence of the viruses on gene expression becomes more pronounced at later stages of infection.

4- Silencing of the chaperone *Hsp90* and its co-chaperone *Sgt1* genes in tomato plants resulted in increased susceptibility to ToCV infection, indicating that these genes are crucial for the plant's basal resistance against ToCV. In contrast, the silencing of RTM resistance genes did not exert a significant influence on ToCV RNA accumulation.

5- The total number of sRNA reads in the 20-25 nt size range that mapped to TYLCV and ToCV genomes exhibited striking similarities in both single and mixed infections. Specifically, 21- and 22-nt sRNAs were the most abundant in both types of infections, followed by 20-nt and 24-nt sRNAs. However, an intriguing exception was observed in single ToCV infections, where there was a notable increase in the percentage of 22-nt sRNAs compared to 21-nt sRNAs. This indicates that the size distribution of sRNAs in ToCV RNA2 was influenced by the presence of mixed infections, possibly reflecting variations in the host plant's response to mixed viral infections.

6- *In silico* analysis of sRNAs from single- and mixed-infected tomato plants with TYLCV and ToCV revealed potential targets in *Bemisia tabaci* (MED) that could be targeted by vsRNAs from TYLCV. Furthermore, several of these target genes were associated with detoxification pathways and they exhibited downregulation in TYLCV-viruliferous whiteflies. This downregulation correlated with an increased susceptibility of these whiteflies to nicotine toxicity, suggesting a possible role for vsRNA derived from TYLCV in suppressing these detoxification-related genes.

7- Silencing of the specific genes linked to detoxification pathways in whiteflies using TYLCV-derived siRNAs resulted in higher susceptibility to nicotine. This outcome offers compelling evidence of cross-kingdom communication between TYLCV and its whitefly vector through sRNAs, shedding light on the intricate interactions between plant viruses, their hosts, and insect vectors, with potential implications for virus transmission.

ANNEXES

ANNEXES

Annex I

This annex includes the published article corresponding to Chapter I.

Authors and Affiliations

- Irene Ontiveros[1 2]
- Juan José López-Moya[2 3]
- Juan Antonio Díaz-Pendón[1 †]

1. [1]Instituto de Hortofruticultura Subtropical y Mediterránea “La Mayora,” Universidad de Málaga Consejo Superior de Investigaciones Científicas, Estación Experimental “La Mayora,” E-29750 Algarrobo-Costa, Málaga, Spain
2. [2]Centre for Research in Agricultural Genomics, CSIC-IRTA-UAB-UB, Campus UAB Bellaterra, Barcelona, Spain
3. [3]Consejo Superior de Investigaciones Científicas, Barcelona, Spain

Phytopathology® • 2022 • 112:944-952 • https://doi.org/10.1094/PHYTO-08-21-0341-R

Virology

e-Xtra*

Coinfection of Tomato Plants with *Tomato yellow leaf curl virus* and *Tomato chlorosis virus* Affects the Interaction with Host and Whiteflies

Irene Ontiveros,[1,2] Juan José López-Moya,[2,3] and Juan Antonio Díaz-Pendón[1,†]

[1] Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora," Universidad de Málaga Consejo Superior de Investigaciones Científicas, Estación Experimental "La Mayora," E-29750 Algarrobo-Costa, Málaga, Spain
[2] Centre for Research in Agricultural Genomics, CSIC-IRTA-UAB-UB, Campus UAB Bellaterra, Barcelona, Spain
[3] Consejo Superior de Investigaciones Científicas, Barcelona, Spain
Accepted for publication 20 October 2021.

ABSTRACT

Susceptible plants infected by single or multiple viruses can differ in symptoms and other alterations influencing virus dissemination. Furthermore, behavior of viruliferous vectors may be altered in certain cases to favor acquisition and inoculation processes conductive to virus transmission. We explored single and mixed infections frequently occurring in tomato crops, caused by two viruses transmitted by the whitefly *Bemisia tabaci*: *Tomato yellow leaf curl virus* (TYLCV, *Begomovirus*, Geminiviridae) and *Tomato chlorosis virus* (ToCV, *Crinivirus*, Closteroviridae). Coinfection of both viruses in tomato plants showed more severe symptoms at late stages compared with single infections, although at earlier stages the interaction began with attenuation. This asymmetric synergism correlated with the dynamics of ToCV accumulation and expression of the salicylic acid responsive gene PR-P6. Visual and olfactory cues in whitefly preference were evaluated under controlled conditions in choice assays, testing viruliferous and nonviruliferous adult whiteflies. In experiments allowing both visual and olfactory cues, whiteflies preferred symptomatic leaflets from plants infected either with TYLCV alone or with TYLCV and ToCV, over those infected with ToCV alone or noninfected leaflets, suggesting that TYLCV drove host selection. Odor cues tested in Y-tube olfactometer assays showed neutral effects on whiteflies' preference, and bioassays comparing the attractiveness of colored sticky cards confirmed preference for sectors colored to mimic TYLCV symptomatic leaves compared with asymptomatic leaves. Our results show that the presence of coinfecting viruses affect the host and could alter the behavior of insect vectors.

Keywords: *Bemisia tabaci*, coinfection, host choice, olfaction, synergism, *Tomato chlorosis virus*, *Tomato yellow leaf curl virus*, virology, visual

Most plant viruses causing economically important diseases in agriculture rely on vectors such as insects for their transmission. The process of virus transmission is highly influenced by their interactions with both the host and the vector (Hogenhout et al. 2008; Ng and Falk 2006). Virus infection often induces changes in host plants that are conducive to vector transmission. Such changes in the plant physiology alter visual and olfactory cues from infected plants that mediate vector attractiveness, preference, and feeding behavior (Fereres and Moreno 2009; Johnston and Martini 2020; Mauck et al. 2016). In addition, some plant viruses can directly affect vector behavior and response to plant cues with ecological and epidemiological consequences (Blanc and Michalakis 2016; Eigenbrode et al. 2018; Moreno-Delafuente et al. 2013).

Although in recent years there have been an increasing number of studies on the effects of plant virus infection on host plants that appear to increase transmission by insect vectors, only a few studies have documented such effects in natural systems where mixed viral infections are very common (Moreno and López-Moya 2020). Interactions between plant viruses in mixed infections may range from antagonism to synergism (DaPalma et al. 2010; Moreno and López-Moya 2020; Syller 2012). In most cases, antagonistic interactions occur when the presence of one virus is detrimental for at least one of the other coinfecting viruses present, for instance with a decrease in the replication or transmission rate (Chávez-Calvillo et al. 2016; Syller and Grupa 2016). Conversely, synergistic interactions increase the fitness of one or more of the viruses, which may result in increases of virus titers, often with more severe symptoms in the host plant, although the impact on insect vector behavior is often unknown (García-Cano et al. 2006; Mascia and Gallitelli 2016; Syller 2012). A third situation is neutralism, when coexisting viruses do not influence each other (Mascia and Gallitelli 2016; Syller and Grupa 2016). The outcome of such interactions may have important epidemiological consequences that merit being explored experimentally.

This study focuses on single and mixed virus infection by two emerging viruses that often co-occur in susceptible tomato (*Solanum lycopersicum* L.) crops: *Tomato yellow leaf curl virus* (TYLCV, genus *Begomovirus*, family Geminiviridae) and *Tomato chlorosis virus* (ToCV, genus *Crinivirus*, family Closteroviridae), both transmitted by the whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae). TYLCV is one of the most devastating and widespread viruses of cultivated tomatoes in tropical and subtropical regions. Tomato plants affected by TYLCV exhibit characteristic symptoms of stunting, yellowing, and upward curling of leaves, and they suffer premature dropping of flowers and reduction of marketable fruits that can result in 100% yield loss when infections occur during early growth stages (Yan et al. 2021). In contrast, symptoms caused by ToCV are less severe and usually appear irregularly distributed in tomato-infected plants, including interveinal yellowing

†Corresponding author: J. A. Díaz-Pendón; diazpendon@eelm.csic.es

Funding: This study was financially supported by the project AGL2016-75529-R funded by MCIN/AEI/10.13039/501100011033 and by "ERDF A way of making Europe", and project PID2019-105692RB-100 funded by MCIN/AEI/10.13039/501100011033. I. Ontiveros was recipient of grant BES-2017-080808 funded by MCIN/AEI/10.13039/501100011033 and by "ESF Investing in your future". Research at the Centre for Research in Agricultural Genomics was also supported by grants SEV-2015-0533 and CEX2019-000902-S funded by MCIN/AEI/10.13039/501100011033, and by the CERCA Programme/Generalitat de Catalunya.

*The *e-Xtra* logo stands for "electronic extra" and indicates there are supplementary materials published online.

The author(s) declare no conflict of interest.

chlorotic areas, bronzing, and red patches. The yellow leaf disorder syndrome caused by this crinivirus is often similar to abiotic or nutritional disorders and seems to have less economic importance than other viral diseases. Emergence of ToCV and TYLCV has been associated with the global spread of the whitefly *B. tabaci* in tropical and warm regions worldwide (Fereres 2015; Fiallo-Olivé and Navas-Castillo 2019; Gilbertson et al. 2015; Rojas et al. 2018). TYLCV has a single-stranded circular DNA genome and is transmitted in a persistent manner, which means the virus moves through the insect vector from the midguts to the salivary glands via haemolymph, thus having a latency period within the vector before transmission, and can remain viruliferous for several days (Díaz-Pendón et al. 2010; Hogenhout et al. 2008). Meanwhile, ToCV has a bipartite genome of positive single-stranded RNA and is transmitted in a semipersistent manner, thus the virus is retained in the foregut of the whitefly for a few hours to days and can be transmitted readily after acquisition (Ng and Falk 2006; Orfanidou et al. 2016).

The interest for studying mixed infections caused by TYLCV and ToCV is demonstrated by a recent independent publication in which a detailed description of the synergistic interaction between the two pathogens is provided (Li et al. 2021). The authors found no differences in transmission efficiencies under near-saturating conditions for either of the two viruses when they compared acquisitions from single- and mixed-infected plants (Li et al. 2021).

The goal of the present study was to evaluate the effects of single and mixed infections involving TYLCV and ToCV in tomato plants to determine how the virus-induced changes might affect the whitefly vector's preference. To do so, we first analyzed the development of symptoms, their severity and relative abundance of each virus through time, mainly confirming previously published observations in the independent study mentioned previously (Li et al. 2021). Then, we examined under our conditions whether the individual or simultaneous presence of these viruses might affect host plant traits that influence visual and olfactory cues for the vector, and we tested them on both viruliferous and nonviruliferous whiteflies. With this knowledge we gain a better understanding of ToCV and TYLCV mixed infections in tomato plants and evaluate their impact on vector–virus–host plant relationships that could help us design measures for disease control.

MATERIALS AND METHODS

Tomato plants and whitefly colony. The insect vector and tomato lines used in this work were the *B. tabaci* and virus-susceptible tomato 'Moneymaker'. In addition, plants of the wild tomato *Solanum habrochaites* former *S. glabratum* (accession PI 134418) were used for the preliminary control tests in Y-tube olfactometer experiments. All seeds were provided by the Instituto de Hortofruticultura Subtropical y Mediterránea Consejo Superior de Investigaciones Científicas (IHSM-CSIC) germplasm collection. Plants were individually sown in plastic pots of 12 cm diameter containing vermiculite and were grown in an insect-free growth chamber with light intensity at 250 µmol/s/m^2 with a 16 h/day photoperiod (25°C day/20°C night) and 70% relative humidity. Virus-free *B. tabaci* Mediterranean (MED) individuals were obtained from a colony originating from individuals collected during field visits in Malaga (Spain) and reared on melon plants (*Cucumis melo* L. 'ANC42', IHSM seedbank collection) in wooden cages covered with insect-proof nets, in an insect-proof glasshouse with temperature control (22 to 27°C day and 17 to 20°C night) and light supplementation when needed.

Virus isolates and virus inoculation. The viruses used in this study were the ToCV isolate Pl-1-2 (García-Cano et al. 2010) maintained at IHSM-CSIC "La Mayora" in tomato cultivar Moneymaker by periodic transmission with *B. tabaci* and the Israel strain of TYLCV (GenBank accession number AJ489258). To obtain TYLCV-viruliferous whiteflies, nonviruliferous whiteflies were given a 24-h acquisition access period (AAP) on tomato plants 4 weeks after their agroinoculation with TYLCV, as previously described by Pereira-Carvalho et al. (2015). Similarly, viruliferous whiteflies with ToCV were obtained by 24 h AAP on ToCV-infected tomato plants maintained by whitefly-mediated inoculations. After the AAP, whiteflies were transferred to healthy plants at the three-leaf growth stage for a 48-h inoculation access period (IAP). The same procedure was used for obtaining healthy (mock-inoculated) tomato plants, except that nonviruliferous whiteflies were placed on noninfected plants. Transmission of each virus for the establishment of single infection was performed with 20 viruliferous and 20 nonviruliferous whiteflies per test plant in clip-on cages. Coinfections were established with 40 viruliferous whiteflies, 20 containing ToCV and 20 TYLCV. After the IAP, the clip cages were removed and the infested leaf was excised from the plant at 7 days to avoid eclosion of eggs putatively laid by adults during the IAP. The plants were maintained in an insect-free growth chamber. Virus-inoculated plants were checked by tissue blot hybridization 3 weeks after inoculation to verify infection condition as described by Navas-Castillo et al. (1999) and Fortes et al. (2012). Similarly, viruliferous whiteflies were also regularly checked by PCR to verify their viruliferous condition (Macedo et al. 2015).

Evaluation of symptoms and virus quantification. Symptom severities of infected and control plants were recorded at 7, 14, 21, and 28 days postinoculation (dpi) on a 0 to 5 scale, where 0 was assigned to asymptomatic plants and 5 was assigned to the most severe symptoms observed, as described by Ferrero et al. (2020). The second most recently expanded leaf from the apex was harvested at 7, 14, and 21 dpi. For each infection condition, time point, and replica, the leaf tissue from six infected plants was pooled and used in downstream analysis. Three biological replicates were processed per condition and time point. Total DNA and RNA were isolated with the DNeasy Plant Mini Kit (Qiagen) and Trizol reagent (Ambion) and then treated with RNase-Free DNase (Qiagen) and DNase-Free RNase (Roche), respectively. The complementary DNAs (cDNAs) were generated from 500 ng of total RNAs with a BioRAD iScript cDNA Synthesis Kit in a reaction volume of 20 µl. Quantitative PCR (qPCR) was done with a template of 1 µl of cDNA or 500 ng of total DNA in a Takara SYBR Green PCR kit on a CFX96 Real-time PCR detection system (Bio-Rad, U.S.A.). The following cycling conditions were used: 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative quantification of specific RNAs and TYLCV DNA were normalized to the elongation factor 1-α gene and tomato 25S ribosomal RNA genes, respectively (López-Ráez et al. 2010; Rodríguez-Negrete et al. 2014). Primers for tomato PR-P6 and Pin-II amplification were reported by Sarmento et al. (2011) and Uppalapati et al. (2005). Gene-specific primer pairs for virus quantification were designed with the Primer Blast tool, available online (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The set primers MA1178 (5′-ACCGGGCGCAGTT CATACAA-3′)/MA1179 (5′-CCGACAAGAAACAGCGCTCC-3′) and MA1461 (5′-CCTGGATTGCAGAGGAAGATAGT-3′)/MA1462 (5′-TGGTACAACGTCATTGATGACGT-3′) were used to amplify 173 nucleotides of the ToCV coat protein gene and 152 nucleotides of the replication enhancer of TYLCV, respectively. Each primer pair was evaluated by a standard curve with six points and three replicates to obtain efficiency rates (*E*) of 102.56% (for ToCV) and 106.05% (for TYCLV) ($E = 10(1/\text{slope}) - 1$, expressed as percentages) with $R^2 = 0.99$ correlation values for the curves. Relative quantifications were measured via the comparative cycle threshold method, as described by Livak and Schmittgen (2001), with coinfected samples at 7 dpi used as a calibrator.

Whitefly dual and olfactometer choice bioassays. Dual choice assays were performed by pairwise comparisons of tomato leaflets (10-leaf-growth stage) of mock-inoculated plants and 21-dpi single and those coinfected with ToCV and TYLCV. For each comparison, four leaflets were placed in a plastic cage (25 × 25 cm)

equidistant to a flight release platform in the middle of the cage. The procedure was similar to that described by Rodríguez-López et al. (2011), and the experimental design of the cages is illustrated in Supplementary Figure S1. Each whitefly was briefly placed on ice before being placed on the release platform, and only the first choice was recorded. Choosing periods longer than 15 min were excluded, and each whitefly was tested only once. A total of 60 adults of *B. tabaci* (replicates) were individually released per pairwise comparison. Both viruliferous and nonviruliferous *B. tabaci* were used for each test (without sex distinction), and they were individually collected with a controlled-vacuum hand trap 1 h before the beginning of the experiments.

A Y-tube olfactometer (Analytical Research Systems, Gainesville, FL) was used to test whether volatile cues are involved in whitefly choice responses in the absence of visual stimuli (Supplementary Fig. S2). The olfactometer consisted of a glass Y-tube (28-cm-long stem and 20-cm-long arms) connected to two separate glass chambers (40 cm height and 20 cm inner diameter) through silicone tubes. Each glass jar contained a single test plant and was connected to an air delivery system (ARS, Gainesville, FL) to ensure a purified and humidified airflow. After a short period on ice, a single whitefly was placed in the intersection of both arms of the Y-tube, and later an input flow was pulled across the silicone tubing from the glass chambers to the Y-tube. Thus, the whitefly was able to make a choice based on the reception of the volatiles emitted by the plants by moving through one of the arms. Crossing a limit line at 6 cm into either arm for more than 3 min was considered a choice. The insect was excluded from the sampling and replaced after 15 min if the established choice was not completed. An airflow of 0.3 liters per minute was chosen for the assays and generated with a vacuum pump connected to the olfactometer. A preliminary control test was used to validate experimental parameters, verifying repellences to *S. habrochaites* accession number PI 134418 (Bleeker et al. 2009, 2011; Momotaz et al. 2010), compared with tomato cultivar Moneymaker plants. A total of 35 whiteflies out of 40 preferred Moneymaker plants.

Whitefly adults were starved for 1 h before the experiments began, and a total of 60 adults each of nonviruliferous, ToCV-viruliferous and TYLCV-viruliferous whiteflies per comparison were individually used as replicates. Both glass chambers were covered to avoid whitefly detection of visual cues during the experiments. Dual choice and olfactometer tests for all comparisons were run in parallel, with new plants used for each of the replications and systematic alternation of the spatial orientations of infected and mock-inoculated plants. The bioassays were conducted in a room chamber with light intensity at 250 µmol/s/m^2, maintained at 27°C and 70% relative humidity.

Effects of plant infection on leaf coloration and evaluation of colored sticky traps. Optical methods based on image analysis were used to estimate differences in color between leaves of healthy (mock-inoculated), ToCV-infected, and TYLCV-infected tomato plants. Three random measures per leaf were recorded with a colorimeter (model CR-400; Konica Minolta, Inc., Tokyo, Japan) with a 1-cm-diameter aperture. The CIE Lab three-dimensional color space was used to evaluate color attributes of the samples. In this color space, the L^* value indicates lightness, varying from black ($L = 0$) to white ($L = 100$); the a^* value characterizes the color of the region from green ($-a^*$) to red ($+a^*$), and the b^* value indicates the color in the range from blue ($-b^*$) to yellow ($+b^*$). Numerical values of a^* and b^* were converted to hue angles (H) in the Konica Minolta CR-400 Utility software. The H value is expressed in degrees: 0° ($+a$ axis) represents red, 90° ($+b$ axis) represents yellow, 180° ($-a$ axis) represents green, and 270° ($-b$ axis) represents blue (Barrantes et al. 2016; Sacks and Francis 2001). The colorimeter was previously calibrated according to the manufacturer's instructions, and each L^* and H value was calculated by the formulas described by Sacks and Francis (2001).

Colored sticky cards were designed to conduct choice assays to evaluate the influence of visual stimuli on *B. tabaci* in absence of olfactory cues. A total of 24 cards (8 × 10 cm) colored to mimic TYLCV symptomatic leaves at 21 dpi and asymptomatic leaves were placed alternately on both sides of a cardboard surface (21 × 29.7 cm) and used for each replicate. Three glass boxes were placed inside a greenhouse under natural light and at a controlled temperature (25 ± 2°C) to test the visual responses of whiteflies, either nonviruliferous or viruliferous, for ToCV or TYLCV. Two sticky cardboards were suspended from the top of each glass box (65 × 77 × 56 cm), and groups of 100 whiteflies were released per comparison. The cards were collected after 8 h to record the number of whiteflies trapped on each sector. The assays were replicated three times for the different categories of viruliferous or nonviruliferous whiteflies.

Data analysis. Statistical analysis was performed in IBM SPSS version 26.0 (IBM, Armonk, NY) and R for Windows (R Foundation for Statistical Computing, Vienna, Austria). Means of severity of symptoms and the relative quantification of the responsive genes PR-P6 and Pin-II were analyzed via one-way ANOVA. An independent t test was performed to estimate statistical differences of TYLCV and ToCV accumulation between single- and double-infected plants. For the dual choice assays, we analyzed the host-settling preferences of viruliferous and nonviruliferous *B. tabaci* by applying a χ^2 test to the data of each pairwise comparison. The χ^2 test was also used to evaluate host preference behavior of whiteflies in the olfactometer assays. Colorimeter data were submitted to the Shapiro-Wilk normality test and then compared via one-way ANOVA to correlate color components of mock-inoculated and single-infected leaves with ToCV and TYLCV. Numbers of whiteflies collected from sticky cards were analyzed by a Wald test ($P < 0.05$) with a general linear model following a Poisson distribution.

RESULTS

Mixed infections of ToCV and TYLCV induce more severe symptoms in tomato plants than single infections at late time points. Under our growing conditions tomato plants infected by TYLCV and ToCV in single or coinfection started displaying symptoms at about 7 dpi (Fig. 1). ToCV-infected plants exhibited only slight symptoms of interveinal chlorosis and mild yellowing with no evidence of stunting, always with a rating ≤2. As expected, tomato plants infected with TYLCV exhibited significantly more severe symptoms than ToCV-infected plants (Supplementary Table S1 and Supplementary Fig. S3), with scores of about 4 at 28 dpi, and symptoms included leaf curling, yellowing, and plant stunting.

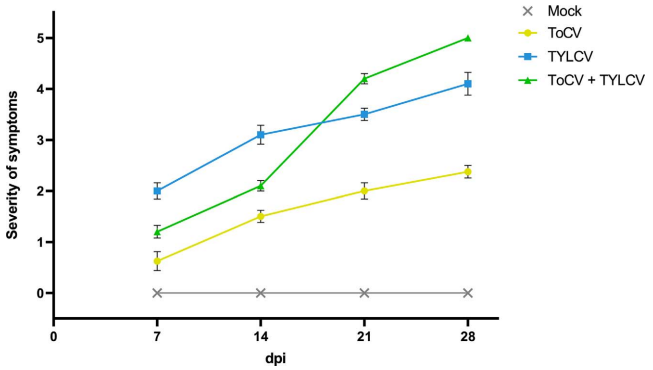


Fig. 1. Evolution of symptoms in single- and mixed-infected plants. Mean severity of symptoms (from 0 = absence to 5 = maximum severity) observed on different days postinoculation (dpi) in tomato cultivar Moneymaker plants singly infected with *Tomato yellow leaf curl virus* (TYLCV) or *Tomato chlorosis virus* (ToCV), coinfected with both viruses (ToCV + TYLCV), or mock-inoculated. Mean ± standard error values corresponding to six plants per treatment are indicated.

However, we observed that ToCV and TYLCV produce different symptomatic outcomes during mixed infection over time. Symptoms ranged from moderate (values 1 to 2) to severe (values 4 to 5), suggesting that antagonism and synergism occurred respectively at early (weeks 1 and 2) and late stages (from week 3 onward) of mixed infection with the two viruses. The development of symptoms resembled those of single-infected plants with ToCV during the first observations but were followed by a dramatic increase in severity (severe curling and extensive yellowing in upper leaves) that led to necrosis of the newly emerging leaves and sometimes even to the death of the plant in the final stages (Fig. 1), a situation that was not observed in any of the plants inoculated solely with one virus.

Time-dependent antagonistic and synergistic interactions correlate with significant changes in ToCV accumulation. To determine whether symptom severity could be correlated with viral accumulation in infected tissues, we estimated the relative abundance of TYLCV and ToCV in single- and mixed-infected tomato plants by qPCR and at 7, 14, and 21 dpi (Fig. 2A and B). Samples at 28 dpi were not considered because severe necrosis in many apical leaves of coinfected plants precluded sampling of enough material for representative analysis.

The results showed that the pattern of TYLCV DNA accumulation was similar between single infection and coinfection with ToCV (Supplementary Table S2), where the total amount of viral DNA increased drastically from 7 to 14 dpi and slightly decreased at 21 dpi (Fig. 2A). The same trend was observed for ToCV RNA accumulation in single infection, reaching a peak at 14 dpi with a slight decrease at 21 dpi. However, a pattern of reduced ToCV RNA in coinfection with TYLCV was observed at 7 and 14 dpi compared with plants infected by ToCV alone, and the opposite trend was observed at 21 dpi: ToCV reached a higher accumulation in coinfection compared with single infection (Fig. 2B).

Taken together, these results suggest that changes in symptoms of mixed-infected plants between antagonism (14 dpi) and synergism (21 dpi) seemed to correlate with the dynamics of ToCV accumulation: The relative abundance of ToCV significantly increased in mixed-infected plants at 21 dpi (Fig. 2B) when the increment of the severity of symptoms suggests a synergistic interaction between both viruses (Fig. 1).

Dynamics of expression corresponding to defense-related genes in single and mixed infections. To investigate the putative involvement of known pathways corresponding to plant immune responses, we monitored PR-P6 and Pin-II gene expressions. PR-P6 and Pin-II are respectively considered molecular markers for two major pathways: systemic acquired resistance related to responses mediated by salicylic acid (SA) and induced systemic resistance related to responses mediated by jasmonic acid (JA) (Ament et al. 2004; Wasternack et al. 2006). To verify whether the expression of these genes could be triggered by the different viral infections alone or combined, we analyzed their relative expression by reverse transcription qPCR in systemic leaves at 7, 14, and 21 dpi.

We observed no significant differences in PR-P6 expression patterns at 7 dpi between single-infected and coinfected plants. Furthermore, the PR-P6 RNA levels peaked at 14 dpi in infected plants with TYLCV alone, with significantly higher values than those detected in plants either coinfected or infected only with ToCV (Supplementary Table S3), except for one ToCV sample that showed a similar value to those shown by TYLCV. This variability might reflect local differences related to the sampling process and the irregular distribution of criniviruses. After these peaks in single infections, PR-P6 expression significantly decreased at 21 dpi for plants infected with TYLCV or with ToCV alone (Supplementary Table S3), but the values continued to increase in coinfected plants, resulting in higher levels compared with single infections (Fig. 3A). This delay in the pattern of PR-P6 expression for mixed-infected plants roughly followed symptom severity, suggesting that its expression might be correlated with the severity of symptoms.

No significant differences in Pin-II RNA expression were observed at 7 dpi between singly infected and coinfected plants, whereas at 14 dpi a significant lower expression was observed in infected plants with ToCV than in TYLCV singly or coinfected tomato plants (Fig. 3B, Supplementary Table S3). The Pin-II RNA accumulation reached its maximum at 21 dpi for all treatments, with the highest induction levels detected in plants infected with TYLCV (Fig. 3B, Supplementary Table S3).

The presence of TYLCV conditions the host plant preference responses of *B. tabaci*. To investigate whether viral infections might interfere with vector attraction, we tested whiteflies' preferences by comparing infected and mock-inoculated plants at 21 dpi. This time point was chosen based on the differential

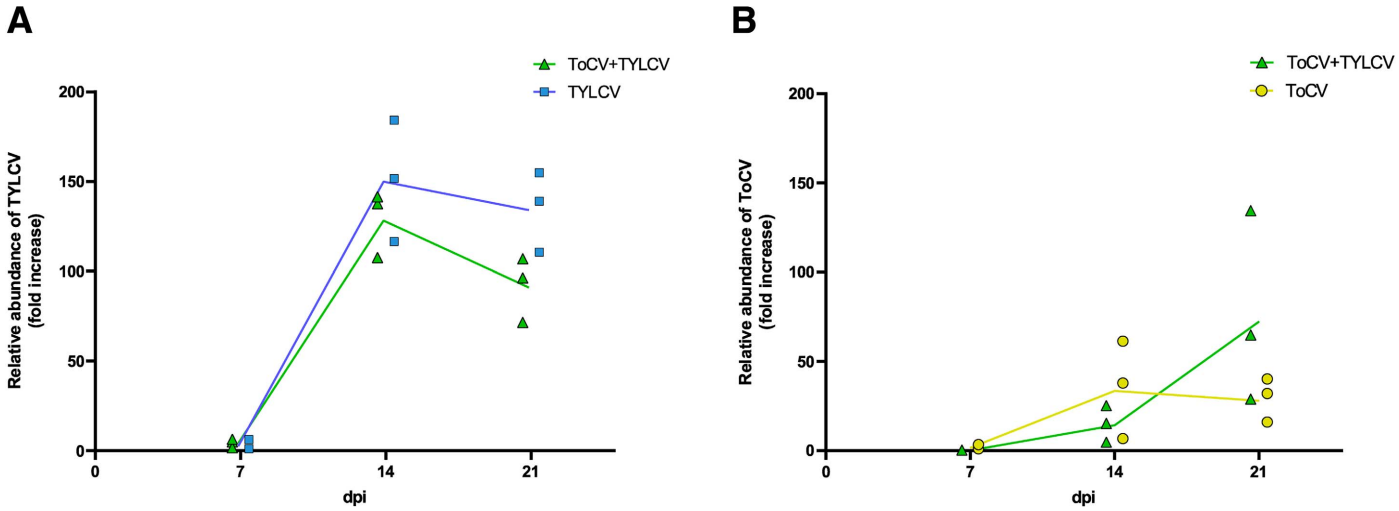


Fig. 2. Kinetics of viral loads in single- and mixed-infected plants. Relative quantification of **A,** *Tomato yellow leaf curl virus* (TYLCV) and **B,** *Tomato chlorosis virus* (ToCV), showing viral loads in single-infected (blue for TYLCV and yellow for ToCV) and mixed-infected (green) plants at 7, 14, and 21 days postinoculation (dpi). Each point represents the relative quantification of viral accumulation in the independent biological replicates with pooled samples of six infected plants per treatment. Mean values are indicated by the represented lines. The relative quantifications were calculated via the comparative cycle threshold method (Livak and Schmittgen 2001). The coinfected samples at 7 dpi were chosen as calibrators for comparisons. Relative quantification of specific RNAs and TYLCV DNA were normalized to the expression of the elongation factor 1-α and the tomato 25S ribosomal RNA genes, respectively.

expression of Pin-II and PR-P6 in the conditions analyzed, which suggested that the plants were clearly responding to the infections (Fig. 4). The experiments were repeated independently with naive whiteflies (nonviruliferous) and with whiteflies previously fed on virus-infected plants, therefore becoming viruliferous for either one of the individual viruses considered. Significantly, more nonviruliferous and ToCV-viruliferous whiteflies preferred to land on infected leaves than on control leaves both for TYLCV and for mixed infections. A similar pattern was found for TYLCV-viruliferous whiteflies with a preferential attraction to the leaves of plants with TYLCV, whereas plants harboring mixed infections were not significantly more attractive than uninfected controls, although the number of whiteflies was consistently higher on infected plants. In contrast, whiteflies did not discriminate between leaves from plants infected with ToCV alone and mock leaves or TYLCV from mixed infection, regardless of the nonviruliferous or viruliferous condition of the insects (Fig. 4). Overall, enhanced whitefly attraction was observed toward TYLCV single- or mixed-infected plants compared with plants infected by ToCV, suggesting that the presence of TYLCV might drive host attractiveness, either alone or in mixed infection with ToCV and regardless of their viruliferous condition as either naive or ToCV-viruliferous. Interestingly, although the trends are similar, the TYLCV-viruliferous whiteflies discriminated significantly only between leaflets of mock and TYLCV-infected plants, and it seems that the preferences shared by nonviruliferous and ToCV-viruliferous whiteflies were less marked in this case, suggesting a possible effect of the virus presence in the behavior of the vector.

Visual cues rather than olfactory stimuli are responsible for whiteflies' attraction toward TYLCV-infected tomato plants. We performed a series of assays to determine whether the TYLCV infection induces attraction based on odor or visual cues among whitefly vectors in tomato plants. Because the infection with TYLCV was shown to be determinant in the arena studies reported previously, we concentrated our efforts on comparing mock and TYLCV-infected plants.

In the Y-tube olfactometer bioassays, volatiles emitted from either plants systemically infected with TYLCV or mock-inoculated plants flowed through both arms of the Y-tube. Individual nonviruliferous and viruliferous whiteflies were released from the base of Y-tube stem and allowed to make the choice to fly toward either arm of the Y-tube, to prevent visual detection of the plants. Whiteflies showed no preference for either mock or TYLCV-infected plants, suggesting that olfactory cues have neutral effects in the

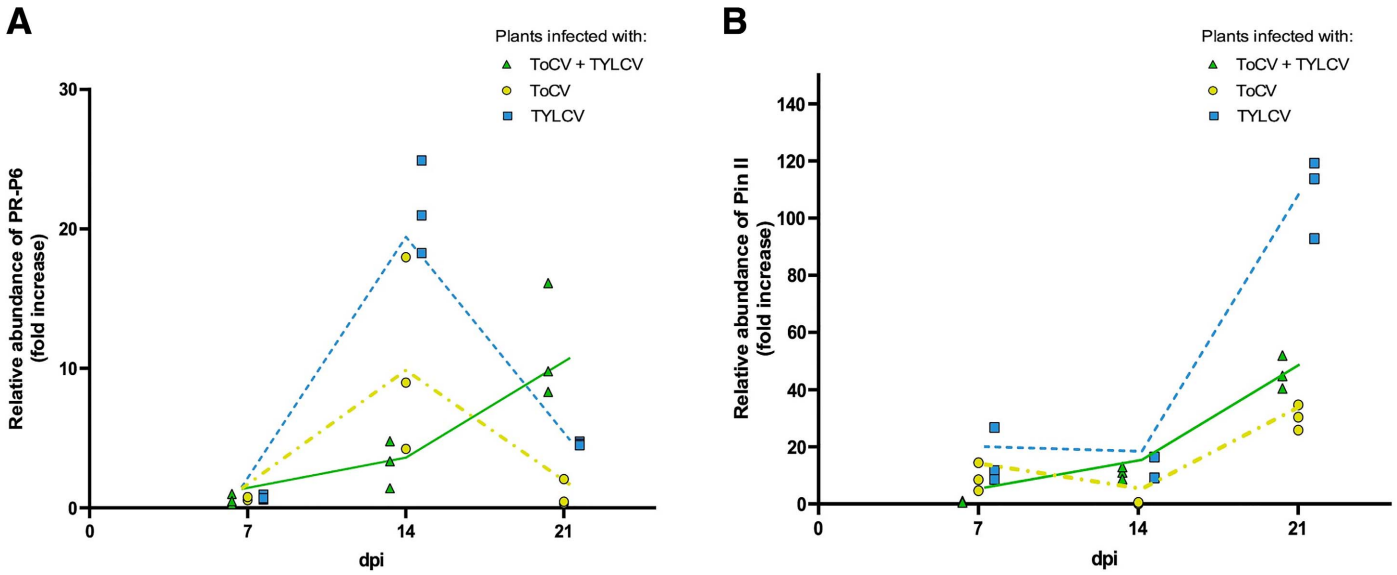


Fig. 3. Relative accumulation of **A,** the salicylic acid–responsive PR-P6 gene and **B,** the wound-induced proteinase inhibitor II (Pin-II) in single- and mixed-infected tomato plants at 7, 14, and 21 days postinoculation (dpi). Each point represents the relative quantification of the levels of transcript corresponding to independent biological replicates after samples of six infected plants per treatment were pooled. Mean values are indicated by the represented lines. The relative quantifications were calculated via the comparative cycle threshold method (Livak and Schmittgen 2001). The coinfected samples at 7 dpi were chosen as calibrators for comparisons. Relative quantification of specific RNAs was normalized to the expression of the elongation factor 1-α gene.

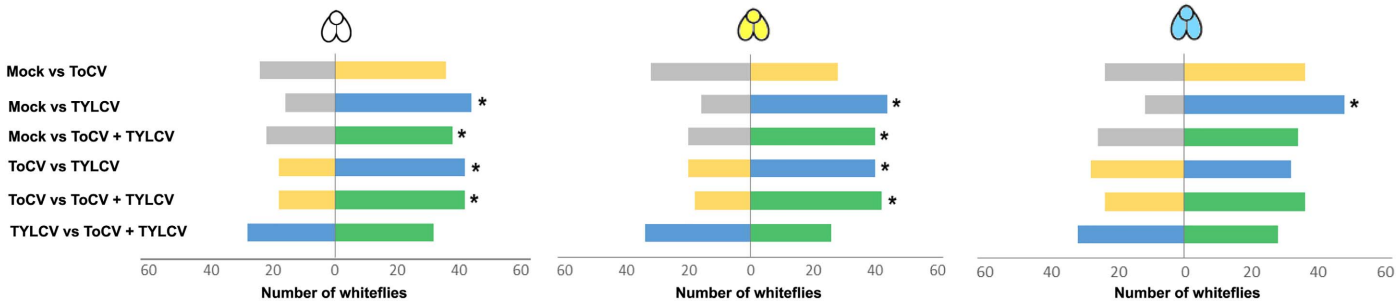


Fig. 4. Viruliferous and nonviruliferous *Bemisia tabaci* preference responses in a dual choice bioassay. Number of whiteflies that landed on *Tomato yellow leaf curl virus* (TYLCV)-infected (blue), *Tomato chlorosis virus* (ToCV)-infected (yellow), mixed-infected (green), or mock-inoculated (gray) leaflets were recorded in each pairwise comparison. Whitefly icons represent the infectious condition of the whiteflies tested: white for nonviruliferous and blue and yellow for TYLCV- and ToCV-viruliferous whiteflies, respectively. Asterisks indicate significant differences between pairs of treatments ($P < 0.05$) based on χ^2 analysis.

initial choice of *B. tabaci* (Fig. 5A). Furthermore, we tested the whiteflies' preference in dual assays for mock-inoculated versus TYLCV-infected leaflets at 4 dpi, when the symptoms were not yet developed. The results showed no significant differences in the preferences of either viruliferous or nonviruliferous whiteflies (Supplementary Fig. S4) suggesting that visual cues are responsible for the attraction of whiteflies to TYLCV-infected plants.

We then investigated the response of whiteflies to colored sticky traps in absence of olfactory stimuli (Supplementary Fig. S5). Comparing TYLCV symptomatic plants with mock-inoculated or asymptomatic infected plants (before the onset of symptoms), we observed that TYLCV infection had a significant effect on plant coloration. There was no significant difference in the mean values of parameters describing the color between mock-inoculated and asymptomatic TYLCV-infected leaves, whereas TYLCV symptomatic plants revealed significant differences in a^*, b^*, L^*, and H (Table 1). This difference reflects an increase in yellow coloration in TYLCV-infected plants. When traps displaying the different colors were used in choice experiments, significantly more whiteflies were caught on the card sectors mimicking TYLCV symptomatic leaves than on sectors representing the color of asymptomatic plants, and the differences were maintained for both viruliferous and nonviruliferous whiteflies (Fig. 5B). Overall, these results suggest that visual signals associated with the color yellow seem to play an important role in whiteflies' preference responses.

DISCUSSION

In this study, we found a synergistic interaction between TYLCV and ToCV, characterized by an increase in the severity of symptoms in the late stages of the observation, with a significant increase in ToCV RNA accumulation in plants coinfected with TYLCV. Previous reports studying mixed infections by TYLCV and ToCV (Li et al. 2021; Seo et al. 2018) showed a reasonable level of coincidence with our results. However, these studies were performed in other experimental conditions, in particular with different tomato cultivars, virus isolates, and even inoculation procedures (grafting vs. whitefly). All these differences may cause discrepancies in outcomes, making difficult their direct comparison. In the first of these two previous studies, the authors focused on differential gene expression associated with the development of distinct symptoms induced by ToCV and TYLCV in tomato plants ('Tenten'), testing only samples in upper leaves at 8 weeks after grafting infections and giving no information about earlier time points (Seo et al. 2018). The second article reported clear synergistic effects in a different cultivar of tomato ('Zhong Za 9') at late time points in the coinfection (Li et al. 2021). This coincides with our data in a different cultivar of tomato (Moneymaker), but under our conditions we also noticed that the synergism was preceded by antagonism in the early stages (7 and 14 dpi) not reported in the different periods of observation during the experiments described in the aforementioned articles.

These differences could be attributed to additional divergences beyond the plant materials, such as the virus isolates used (local Qingdao field isolates were used for the studies in China, and we used viruses previously characterized in Spain) and also to the growing conditions, particularly the temperature, which was higher (27 ± 2°C) in the greenhouse experiments conducted in China (Li et al. 2021) than in our growth chambers. Indeed, temperature has previously been shown to affect synergistic interactions (Aguilar et al. 2015) and is a key factor for virus–virus interactions (Alcaide et al. 2021). In the case of TYLCV + ToCV, the differences might affect only the dynamics to reach the final stages when the outcomes are mostly coincidental, although this hypothesis will require further experiments and appears to be irrelevant for the clear synergism at later time points.

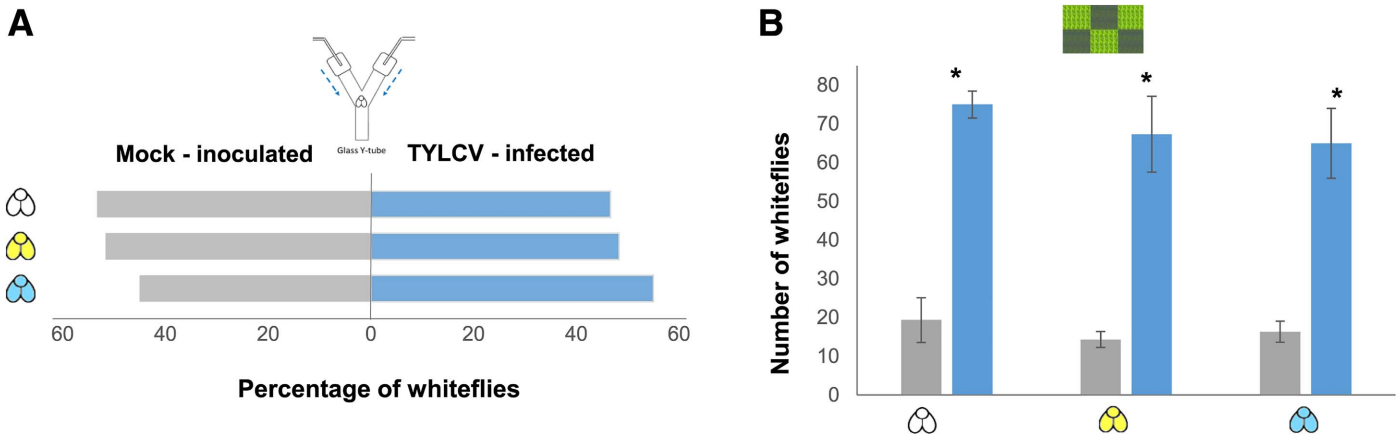


Fig. 5. Behavioral responses of *Bemisia tabaci* to olfactory and visual stimuli. **A,** Percentage of whiteflies responding to volatiles coming from mock-inoculated and *Tomato yellow leaf curl virus* (TYLCV)-infected plants connected to a Y-tube olfactometer with a continuous active air flow. Individual adult whiteflies were released in the principal arm, and their responses were recorded individually during a 15-min interval. A positive response was considered when whiteflies remained for 3 min across the border line of the lateral tubes. A total of 60 replicates (whiteflies) per test were used. **B,** Visual attractiveness of *B. tabaci* toward colored sticky cards. Bars represent the mean ± SE of whiteflies collected on cards colored to mimic TYLCV symptomatic leaves compared with mock-inoculated leaves. Whitefly icons represent the infectious condition of the whiteflies tested: white for nonviruliferous and blue and yellow for TYLCV- and *Tomato chlorosis virus* (ToCV)-viruliferous whiteflies, respectively.

TABLE 1. Mean and standard deviation values of color differences corresponding to mock-inoculated, asymptomatic, and symptomatic *Tomato yellow leaf curl virus* (TYLCV)-infected tomato leaves

Parameter	Mock	Asymptomatic TYLCV-infected	TYLCV symptomatic
L^*	35.83 ± 1.03 m[z]	38.49 ± 0.46 m	44.78 ± 1.08 n
a^*	−16.27 ± 0.84 m	−17.99 ± 0.39 m	−13.71 ± 0.72 n
b^*	23.92 ± 1.37 m	27.19 ± 0.61 m	32.805 ± 0.72 n
H	124.27 ± 0.48 m	123.5 ± 0.17 m	112.68 ± 0.29 n

[z] Significant differences are indicated by different letters according to one-way analysis of variance with $P < 0.05$.

Among the numerous examples of viral synergistic interactions in mixed infections, the crinivirus ToCV appeared to cause interesting interactions with unrelated viruses. Indeed, mixed infections of ToCV and *Tomato spotted wilt virus* (TSWV) of susceptible tomato plants exacerbated ToCV-associated symptoms with a remarkable increase in ToCV accumulation, whereas TSWV accumulation was not altered, but the resistance in tomato cultivars carrying the Sw-5 gene was compromised by preinfection with ToCV (García-Cano et al. 2006). Also, Wintermantel et al. (2008) reported that coinfection of ToCV with the crinivirus *Tomato infectious chlorosis virus* (TICV) altered the accumulation of each virus in a host-specific manner: Whereas in *Nicotiana benthamiana* TICV and ToCV titers increased and decreased, respectively, in *Physalis wrightii* the titers of both TICV and ToCV decreased in comparison with single infections (Wintermantel et al. 2008). However, a different scenario was found in the early stage of infection in which mixed infections repressed the symptoms induced by TYLCV and ToCV accumulation compared with the corresponding single-inoculated plants, showing an asymmetric synergistic effect between both viruses. A recent study demonstrated that interactions between synergistically interacting viruses can be asymmetric (Tatineni et al. 2019). Moreover, both antagonistic and synergistic interactions were observed between unrelated *Papaya ringspot virus* (PRSV) and *Papaya mosaic virus* (PapMV), depending on their order of infection. Synergism occurred in plants inoculated first with PRSV and later with PapMV, but antagonism occurred if PapMV was first inoculated, a situation that could derive from the prior activation of the SA-associated defense response against PRSV (Chávez-Calvillo et al. 2016). Interestingly, in our case we found that the asymmetric synergistic effect between both viruses correlated with a higher accumulation of ToCV and expression of PR-P6, although it is difficult to ascertain whether the altered expression of PR-P6 is a direct cause or a consequence of the exacerbated disease symptoms or virus accumulation.

The molecular details of the mechanism underlying the interaction between these two viruses have not yet been clarified; however, we might speculate that blockage of the RNA silencing antiviral response by viral suppressors of RNA silencing could be part of the mechanisms involved in synergism and antagonism in plants (Carrington et al. 2001). Similarly to other plant viruses, ToCV and TYLCV encode multiple viral suppressors of RNA silencing (Cañizares et al. 2008; Luna et al. 2012), which may act at different stages of infection (Díaz-Pendón and Ding 2008), although these interactions may occur for more complex reasons than the action of a few viral proteins (Latham and Wilson 2008). In a previous work involving mixed infection of TYLCV and ToCV, the differential gene expression associated with the development of distinct symptoms induced by ToCV and TYLCV in tomato plants was explored (Seo et al. 2018). Unfortunately, as mentioned earlier, the analyzed samples corresponded to late time points in the infection process, and consequently any possible asymmetric synergistic effect between both viruses was not observed.

Recent studies have uncovered novel strategies that insect-borne plant viruses might adopt to manipulate the host defenses, such as the emission of plant volatile organic compounds, or improve the plant's nutritional quality to improve the suitability of the vector and its attraction to the infected host plant, thereby affecting plant–insect interactions (Pan et al. 2021; Pieterse et al. 2012; Ponzio et al. 2013). For example, infection of tobacco plants with *Tomato yellow leaf curl China virus* and *Arabidopsis* with *Cucumber mosaic virus* could repress the JA-mediated defenses against the whitefly and aphid, respectively. Jasmonate is commonly considered in studies of the effects of virus infection on plant–insect interactions, mainly as a result of its direct involvement in plant defense against insect herbivores and production of a volatile blend (Lewsey et al. 2010; Luan et al. 2013; Zhang et al. 2012). Similarly, the manipulation of the SA-signaling pathway by viruses may affect host attractiveness to insect herbivores (Chisholm et al. 2018; Tomitaka et al. 2015). Our data showed that whiteflies did not discriminate between plants infected with TYLCV alone or mixed-infected plants, regardless of the nonviruliferous or viruliferous condition of the insects (Fig. 4), despite the fact that the mixed infection causes more severe symptoms than in a single virus infection with up-and-down regulation of expression of SA-related gene PR-P6 and JA-related gene Pin-II, respectively. It seems reasonable to postulate that in our pathosystem the vector attraction may not depend on the induction of the signaling pathway of the host hormone JA or SA. Additional studies are needed to draw more definitive conclusions on the impact of host hormones on whitefly preference.

In our dual choice experiment, a preferential whitefly attraction to the leaves of plants with TYLCV or mixed infections was observed, whereas plants with single ToCV infections were not significantly more attractive than uninfected controls (Fig. 4), regardless of whitefly viruliferous condition, suggesting that TYLCV drives host attractiveness in mixed infection with ToCV. For example, the fact that plants with mixed infections were more attractive than plants with ToCV infections or uninfected controls suggests that ToCV present in coinfected plants might benefit from enhanced whitefly transmission opportunities created by TYLCV-driven changes in host traits, thus providing more opportunities for ToCV to interact with insects, which might favor their spread.

We hypothesized that these whitefly preferences were probably mediated by plant-derived olfactory and visual cues, because whiteflies use both to locate, select, and accept their host plants. Our data obtained in the Y-tube experiment showed that volatiles emitted by tomato plants infected with TYLCV had neutral effects in viruliferous and nonviruliferous whiteflies compared with mock-inoculated plants. However, our findings differed from those of Johnston and Martini, (2020) who also worked on the TYLCV–tomato pathosystem, where *B. tabaci* attraction to olfactory cues from TYLCV symptomatic tomato plants was reported. They also differed from those who suggest that begomoviruses can manipulate whitefly olfactory response by switching their preference to noninfected plants once the virus is acquired (Fang et al. 2013; Legarrea et al. 2015). However, this type of manipulation induced by begomovirus was not observed for ToCV, where nonviruliferous *B. tabaci* MEAM1 preferred volatiles from mock-inoculated tomato plants, whereas viruliferous whiteflies showed no preference (Fereres et al. 2016). Conversely, Shi et al. (2018) found that nonviruliferous MEAM1 were attracted to ToCV-infected plants, and viruliferous MED preferred to settle on noninfected plants rather than on ToCV-infected plants. This inconsistency indicates that virus effects on host–vector communication are complex and may include effects on the host phenotype and vector physiology.

Our subsequent data showed that the leaf color profiles were altered to a much greater extent by TYLCV than by ToCV or mock-inoculated plants, suggesting that attraction based on visual cues drives selection of *B. tabaci*. Moreover, the significant differences in H and b^* values found in measurements of the external color of TYLCV-infected plants revealed a higher expression of a yellow color than either healthy, ToCV-infected, or asymptomatic TYLCV-infected leaflets, which may increase the attractiveness of *B. tabaci*. In fact, this visual influence is consistent with the work developed by Mound (1962) and Johnston and Martini (2020), which documented that whiteflies are strongly attracted to the color yellow, suggesting that it may influence the host selection mechanism. Recently these results have been supported by their application in many management strategies to control whitefly populations as seen, for example, in the use of yellow sticky traps or the development of optical barriers to manipulate the sunlight spectrum (Antignus 2010; Gerling and Horowitz 1984; Moreau and Isman 2011).

Our study constitutes a further effort to investigate the consequences of coinfection of ToCV and TYLCV on whitefly-related

plant traits. TYLCV and ToCV are two emerging viruses with similar ecological niches; both are transmitted by the same vector *B. tabaci* and have overlapping host ranges. Therefore, mixed infections of TYLCV and ToCV are expected to be common in crops in areas where these two viruses are present, with important epidemiological consequences that may be unpredictable. In fact, superinfection of TSWV-resistant plants carrying the Sw5 with ToCV resulted in a breakdown of the resistance to TSWV (García-Cano et al. 2006). Moreover, recent studies have reported the emergence of different aggressive TYLCV (TYLCV-IS76 and TYLCV-IL[IT:-Sic23:16]) outbreaks in resistant tomatoes carrying the TY-1 gene in Spain, Morocco, and Italy (Belabess et al. 2015; Panno et al. 2018; Torre et al. 2018). Because ToCV is widely distributed in these areas, mixed infections of resistant-breaking variants of TYLCV and ToCV could compromise the resistance conferred by TY-1 and should be seen as a warning for tomato breeders and growers in Mediterranean countries. Interestingly, Ty-1 resistance was shown to be compromised upon a mixed infection of TYLCV with CMV (Butterbach et al. 2014). The complexities of these kinds of mixed viral infections go beyond the interactions between the viruses involved and the host plant infected by them and also can influence the behavior of the insect vectors. We believe these studies yield relevant information about the impact of mixed infections in the spread of both viruses, which could be important for achieving a better understanding of the epidemiological consequences of ToCV and TYLCV mixed infections.

ACKNOWLEDGMENTS

We thank J. Navas Castillo and E. Moriones for providing the isolates of ToCV and TYLCV, respectively.

LITERATURE CITED

Aguilar, E., Allende, L., Del Toro, F. J., Chung, B.-N., Canto, T., and Tenllado, F. 2015. Effects of elevated CO_2 and temperature on pathogenicity determinants and virulence of *Potato virus X*/Potyvirus-associated synergism. Mol. Plant-Microbe Interact. 28:1364-1373.

Alcaide, C., Sardanyés, J., Elena, S. F., and Gómez, P. 2021. Increasing temperature alters the within-host competition of viral strains and influences virus genetic variability. Virus Evol. 7:veab017.

Ament, K., Kant, M. R., Sabelis, M. W., Haring, M. A., and Schuurink, R. C. 2004. Jasmonic acid is a key regulator of spider mite-induced volatile terpenoid and methyl salicylate emission in tomato. Plant Physiol. 135:2025-2037.

Antignus, Y. 2010. Optical manipulation for control of *Bemisia tabaci* and its vectored viruses in the greenhouse and open field. Pages 349-356 in: *Bemisia*: Bionomics and Management of a Global Pest. P. Stansly and S. Naranjo, eds. Springer, Dordrecht, The Netherlands.

Barrantes, W., López-Casado, G., García-Martínez, S., Alonso, A., Rubio, F., Ruiz, J. J., Fernández-Muñoz, R., Granell, A., and Monforte, A. J. 2016. Exploring new alleles involved in tomato fruit quality in an introgression line library of *Solanum pimpinellifolium*. Front. Plant Sci. 7:1172.

Belabess, Z., Dallot, S., El-Montaser, S., Granier, M., Majde, M., Tahiri, A., Blenzar, A., Urbino, C., and Peterschmitt, M. 2015. Monitoring the dynamics of emergence of a non-canonical recombinant of *Tomato yellow leaf curl virus* and displacement of its parental viruses in tomato. Virology 486:291-306.

Blanc, S., and Michalakis, Y. 2016. Manipulation of hosts and vectors by plant viruses and impact of the environment. Curr. Opin. Insect Sci. 16: 36-43.

Bleeker, P. M., Diergaarde, P. J., Ament, K., Guerra, J., Weidner, M., Schütz, S., de Both, M. T. J., Haring, M. A., and Schuurink, R. C. 2009. The role of specific tomato volatiles in tomato–whitefly interaction. Plant Physiol. 151:925-935.

Bleeker, P. M., Diergaarde, P. J., Ament, K., Schütz, S., Johne, B., Dijkink, J., Hiemstra, H., De Gelder, R., De Both, M. T. J., Sabelis, M. W., Haring, M. A., and Schuurink, R. C. 2011. Tomato-produced 7-epizingiberene and R-curcumene act as repellents to whiteflies. Phytochemistry 72:68-73.

Butterbach, P., Verlaan, M. G., Dullemans, A., Lohuis, D., Visser, R. G. F., Bai, Y., and Kormelink, R. 2014. Tomato yellow leaf curl virus resistance by Ty-1 involves increased cytosine methylation of viral genomes and is compromised by cucumber mosaic virus infection. Proc. Natl. Acad. Sci. USA 111:12942-12947.

Cañizares, M. C., Navas-Castillo, J., and Moriones, E. 2008. Multiple suppressors of RNA silencing encoded by both genomic RNAs of the crinivirus, *Tomato chlorosis virus*. Virology 379:168-174.

Carrington, J. C., Kasschau, K. D., and Johansen, L. K. 2001. Activation and suppression of RNA silencing by plant viruses. Virology 281:1-5.

Chávez-Calvillo, G., Contreras-Paredes, C. A., Mora-Macias, J., Noa-Carrazana, J. C., Serrano-Rubio, A. A., Dinkova, T. D., Carrillo-Tripp, M., and Silva-Rosales, L. 2016. Antagonism or synergism between papaya ringspot virus and papaya mosaic virus in *Carica papaya* is determined by their order of infection. Virology 489:179-191.

Chisholm, P. J., Sertsuvalkul, N., Casteel, C. L., and Crowder, D. W. 2018. Reciprocal plant-mediated interactions between a virus and a non-vector herbivore. Ecology 99:2139-2144.

DaPalma, T., Doonan, B. P., Trager, N. M., and Kasman, L. M. 2010. A systematic approach to virus–virus interactions. Virus Res. 149:1-9.

Díaz-Pendón, J. A., and Ding, S.-W. 2008. Direct and indirect roles of viral suppressors of RNA silencing in pathogenesis. Annu. Rev. Phytopathol. 46:303-326.

Díaz-Pendón, J. A., Cañizares, M. C., Moriones, E., Bejarano, E. R., Czosnek, H., and Navas-Castillo, J. 2010. Tomato yellow leaf curl viruses: Ménage à trois between the virus complex, the plant and the whitefly vector. Mol. Plant Pathol. 11:441-450.

Eigenbrode, S. D., Bosque-Pérez, N. A., and Davis, T. S. 2018. Insect-borne plant pathogens and their vectors: Ecology, evolution, and complex interactions. Annu. Rev. Entomol. 63:169-191.

Fang, Y., Jiao, X., Xie, W., Wang, S., Wu, Q., Shi, X., Chen, G., Su, Q., Yang, X., Pan, H., and Zhang, Y. 2013. *Tomato yellow leaf curl virus* alters the host preferences of its vector *Bemisia tabaci.* Sci. Rep. 3:2876.

Fereres, A. 2015. Insect vectors as drivers of plant virus emergence. Curr. Opin. Virol. 10:42-46.

Fereres, A., and Moreno, A. 2009. Behavioural aspects influencing plant virus transmission by homopteran insects. Virus Res. 141:158-168.

Fereres, A., Peñaflor, M. F. G. V., Favaro, C. F., Azevedo, K. E. X., Landi, C. H., Maluta, N. K. P., Bento, J. M. S., and Lopes, J. R. S. 2016. Tomato infection by whitefly-transmitted circulative and non-circulative viruses induce contrasting changes in plant volatiles and vector behaviour. Viruses 8:225.

Ferrero, V., Baeten, L., Blanco-Sánchez, L., Planelló, R., Díaz-Pendón, J. A., Rodríguez-Echeverría, S., Haegeman, A., and de la Peña, E. 2020. Complex patterns in tolerance and resistance to pests and diseases underpin the domestication of tomato. New Phytol. 226:254-266.

Fiallo-Olivé, E., and Navas-Castillo, J. 2019. Tomato chlorosis virus, an emergent plant virus still expanding its geographical and host ranges. Mol. Plant Pathol. 20:1307-1320.

Fortes, I. M., Moriones, E., and Navas-Castillo, J. 2012. Tomato chlorosis virus in pepper: Prevalence in commercial crops in southeastern Spain and symptomatology under experimental conditions. Plant Pathol. 61:994-1001.

García-Cano, E., Navas-Castillo, J., Moriones, E., and Fernández-Muñoz, R. 2010. Resistance to tomato chlorosis virus in wild tomato species that impair virus accumulation and disease symptom expression. Phytopathology 100:582-592.

García-Cano, E., Resende, R. O., Fernández-Muñoz, R., and Moriones, E. 2006. Synergistic interaction between *Tomato chlorosis virus* and *Tomato spotted wilt virus* results in breakdown of resistance in tomato. Phytopathology 96:1263-1269.

Gerling, D., and Horowitz, A. R. 1984. Yellow traps for evaluating the population levels and dispersal patterns of *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae). Ann. Entomol. Soc. Am. 77:753-759.

Gilbertson, R. L., Batuman, O., Webster, C. G., and Adkins, S. 2015. Role of the insect supervectors *Bemisia tabaci* and *Frankliniella occidentalis* in the emergence and global spread of plant viruses. Annu. Rev. Virol. 2:67-93.

Hogenhout, S. A., Ammar, E.-D., Whitfield, A. E., and Redinbaugh, M. G. 2008. Insect vector interactions with persistently transmitted viruses. Annu. Rev. Phytopathol. 46:327-359.

Johnston, N., and Martini, X. 2020. The influence of visual and olfactory cues in host selection for *Bemisia tabaci* biotype B in the presence or absence of *Tomato yellow leaf curl virus*. Insects 11:115.

Latham, J. R., and Wilson, A. K. 2008. Transcomplementation and synergism in plants: Implications for viral transgenes? Mol. Plant Pathol. 9:85-103.

Legarrea, S., Barman, A., Marchant, W., Diffie, S., and Srinivasan, R. 2015. Temporal effects of a *Begomovirus* infection and host plant resistance on the preference and development of an insect vector, *Bemisia tabaci*, and implications for epidemics. PLoS One 10: e0142114.

Lewsey, M. G., Murphy, A. M., MacLean, D., Dalchau, N., Westwood, J. H., Macaulay, K., Bennett, M. H., Moulin, M., Hanke, D. E., and Powell, G. 2010. Disruption of two defensive signaling pathways by a viral RNA silencing suppressor. Mol. Plant-Microbe Interact. 23:835-845.

Li, J., Wang, J., Ding, T., and Chu, D. 2021. Synergistic effects of a *Tomato chlorosis virus* and *Tomato yellow leaf curl virus* mixed infection on host tomato plants and the whitefly vector. Front. Plant Sci. 12:1032.

Livak, K. J., and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402-408.

López-Ráez, J. A., Verhage, A., Fernández, I., García, J. M., Azcón-Aguilar, C., Flors, V., and Pozo, M. J. 2010. Hormonal and transcriptional profiles highlight common and differential host responses to arbuscular mycorrhizal fungi and the regulation of the oxylipin pathway. J. Exp. Bot. 61: 2589-2601.

Luan, J., Yao, D., Zhang, T., Walling, L. L., Yang, M., Wang, Y., and Liu, S. 2013. Suppression of terpenoid synthesis in plants by a virus promotes its mutualism with vectors. Ecol. Lett. 16:390-398.

Luna, A. P., Morilla, G., Voinnet, O., and Bejarano, E. R. 2012. Functional analysis of gene-silencing suppressors from tomato yellow leaf curl disease viruses. Mol. Plant-Microbe Interact. 25:1294-1306.

Macedo, M. A., Michereff Filho, M., Navas-Castillo, J., and Inoue-Nagata, A. K. 2015. Host range and whitefly transmission efficiency of Tomato severe rugose virus and Tomato golden vein virus in tomato plants. Trop. Plant Pathol. 40:405-409.

Mascia, T., and Gallitelli, D. 2016. Synergies and antagonisms in virus interactions. Plant Sci. 252:176-192.

Mauck, K. E., De Moraes, C. M., and Mescher, M. C. 2016. Effects of pathogens on sensory-mediated interactions between plants and insect vectors. Curr. Opin. Plant Biol. 32:53-61.

Momotaz, A., Scott, J. W., and Schuster, D. J. 2010. Identification of quantitative trait loci conferring resistance to *Bemisia tabaci* in an f2 population of *Solanum lycopersicum* × *Solanum habrochaites* accession la1777. J. Am. Soc. Hortic. Sci. 135:134-142.

Moreau, T. L., and Isman, M. B. 2011. Trapping whiteflies? A comparison of greenhouse whitefly (*Trialeurodes vaporariorum*) responses to trap crops and yellow sticky traps. Pest Manag. Sci. 67:408-413.

Moreno, A. B., and López-Moya, J. J. 2020. When viruses play team sports: Mixed infections in plants. Phytopathology 110:29-48.

Moreno-Delafuente, A., Garzo, E., Moreno, A., and Fereres, A. 2013. A plant virus manipulates the behavior of its whitefly vector to enhance its transmission efficiency and spread. PLoS One 8:e61543.

Mound, L. A. 1962. Studies on the olfaction and colour sensitivity of *Bemisia tabaci* (Genn.) (Homoptera, Aleyrodidae). Entomol. Exp. Appl. 5:99-104.

Navas-Castillo, J., Sánchez-Campos, S., Díaz, J. A., Sáez-Alonso, E., and Moriones, E. 1999. Tomato yellow leaf curl virus-Is causes a novel disease of common bean and severe epidemics in tomato in Spain. Plant Dis. 83:29-32.

Ng, J. C. K., and Falk, B. W. 2006. Virus–vector interactions mediating nonpersistent and semipersistent transmission of plant viruses. Annu. Rev. Phytopathol. 44:183-212.

Orfanidou, C. G., Pappi, P. G., Efthimiou, K. E., Katis, N. I., and Maliogka, V. I. 2016. Transmission of Tomato chlorosis virus (ToCV) by *Bemisia tabaci* biotype Q and evaluation of four weed species as viral sources. Plant Dis. 100:2043-2049.

Pan, L., Miao, H., Wang, Q., Walling, L. L., and Liu, S. 2021. Virus-induced phytohormone dynamics and their effects on plant–insect interactions. New Phytol. 230:1305-1320.

Panno, S., Caruso, A. G., and Davino, S. 2018. The nucleotide sequence of a recombinant tomato yellow leaf curl virus strain frequently detected in Sicily isolated from tomato plants carrying the Ty-1 resistance gene. Arch. Virol. 163:795-797.

Pereira-Carvalho, R. C., Díaz-Pendón, J. A., Fonseca, M. E. N., Boiteux, L. S., Fernández-Muñoz, R., Moriones, E., and Resende, R. O. 2015. Recessive resistance derived from tomato cv. Tyking-limits drastically the spread of tomato yellow leaf curl virus. Viruses 7:2518-2533.

Pieterse, C. M. J., Van der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S. C. M. 2012. Hormonal modulation of plant immunity. Annu. Rev. Cell. Dev. Bio. 28:489-521.

Ponzio, C., Gols, R., Pieterse, C. M. J., and Dicke, M. 2013. Ecological and phytohormonal aspects of plant volatile emission in response to single and dual infestations with herbivores and phytopathogens. Funct. Ecol. 27: 587-598.

Rodríguez-López, M. J., Garzo, E., Bonani, J. P., Fereres, A., Fernández-Muñoz, R., and Moriones, E. 2011. Whitefly resistance traits derived from the wild tomato *Solanum pimpinellifolium* affect the preference and feeding behavior of *Bemisia tabaci* and reduce the spread of *Tomato yellow leaf curl virus*. Phytopathology 101:1191-1201.

Rodríguez-Negrete, E. A., Sánchez-Campos, S., Cañizares, M. C., Navas-Castillo, J., Moriones, E., Bejarano, E. R., and Grande-Pérez, A. 2014. A sensitive method for the quantification of virion-sense and complementary-sense DNA strands of circular single-stranded DNA viruses. Sci. Rep. 4:6438.

Rojas, M. R., Macedo, M. A., Maliano, M. R., Soto-Aguilar, M., Souza, J. O., Briddon, R. W., Kenyon, L., Rivera Bustamante, R. F., Zerbini, F. M., and Adkins, S. 2018. World management of geminiviruses. Annu. Rev. Phytopathol. 56:637-677.

Sacks, E. J., and Francis, D. M. 2001. Genetic and environmental variation for tomato flesh color in a population of modern breeding lines. J. Am. Soc. Hortic. Sci. 126:221-226.

Sarmento, R. A., Lemos, F., Bleeker, P. M., Schuurink, R. C., Pallini, A., Oliveira, M. G. A., Lima, E. R., Kant, M., Sabelis, M. W., and Janssen, A. 2011. A herbivore that manipulates plant defence. Ecol. Lett. 14:229-236.

Seo, J. K., Kim, M. K., Kwak, H. R., Choi, H. S., Nam, M., Choe, J., Choi, B., Han, S. J., Kang, J. H., & Jung, C. 2018. Molecular dissection of distinct symptoms induced by tomato chlorosis virus and tomato yellow leaf curl virus based on comparative transcriptome analysis. Virology 516:1-20.

Shi, X., Tang, X., Zhang, X., Zhang, D., Li, F., Yan, F., Zhang, Y., Zhou, X., and Liu, Y. 2018. Transmission efficiency, preference and behavior of *Bemisia tabaci* MEAM1 and MED under the influence of tomato chlorosis virus. Front. Plant Sci. 8:2271.

Syller, J. 2012. Facilitative and antagonistic interactions between plant viruses in mixed infections. Mol. Plant Pathol. 13:204-216.

Syller, J., and Grupa, A. 2016. Antagonistic within-host interactions between plant viruses: Molecular basis and impact on viral and host fitness. Mol. Plant Pathol. 17:769-782.

Tatineni, S., Alexander, J., Gupta, A. K., and French, R. 2019. Asymmetry in synergistic interaction between *Wheat streak mosaic virus* and *Triticum mosaic virus* in wheat. Mol. Plant-Microbe Interact. 32:336-350.

Tomitaka, Y., Abe, H., Sakurai, T., and Tsuda, S. 2015. Preference of the vector thrips *Frankliniella occidentalis* for plants infected with thrips-nontransmissible *Tomato spotted wilt virus*. J. Appl. Entomol. 139:250-259.

Torre, C., Donaire, L., Gómez-Aix, C., Juárez, M., Peterschmitt, M., Urbino, C., Hernando, Y., Agüero, J., and Aranda, M. A. 2018. Characterization of begomoviruses sampled during severe epidemics in tomato cultivars carrying the Ty-1 gene. Int. J. Mol. Sci. 19:2614.

Uppalapati, S. R., Ayoubi, P., Weng, H., Palmer, D. A., Mitchell, R. E., Jones, W., and Bender, C. L. 2005. The phytotoxin coronatine and methyl jasmonate impact multiple phytohormone pathways in tomato. Plant J. 42: 201-217.

Wasternack, C., Stenzel, I., Hause, B., Hause, G., Kutter, C., Maucher, H., Neumerkel, J., Feussner, I., and Miersch, O. 2006. The wound response in tomato–Role of jasmonic acid. J. Plant Physiol. 163:297-306.

Wintermantel, W. M., Cortez, A. A., Anchieta, A. G., Gulati-Sakhuja, A., and Hladky, L. L. 2008. Co-infection by two criniviruses alters accumulation of each virus in a host-specific manner and influences efficiency of virus transmission. Phytopathology 98:1340-1345.

Yan, Z., Wolters, A.-M. A., Navas-Castillo, J., and Bai, Y. 2021. The global dimension of tomato yellow leaf curl disease: Current status and breeding perspectives. Microorganisms 9:740.

Zhang, T., Luan, J., Qi, J., Huang, C., Li, M., Zhou, X., and Liu, S. 2012. Begomovirus–whitefly mutualism is achieved through repression of plant defences by a virus pathogenicity factor. Mol. Ecol. 21:1294-1304.

Annex II

This annex compiles supplementary tables corresponding to Chapters I, II and III of results.

Table S1. Gene-specific primers used to determine TYLCV, ToCV and TRV accumulation.

Target	Primer	Primer sequence (5´-3´)	Primer position	Efficiency rates (E)
ToCV-CP	MA1178	ACCGGGCGCAGTTCATACAA	1522-1541	102.56 % (Ontiveros et al, 2022)
	MA1179	CCGACAAGAAACAGCGCTCC	1697-1675	
TYLCV-Rep	MA1461	CCTGGATTGCAGAGGAAGATAGT	1705-1714	106.5% (Ontiveros et al, 2022)
	MA1462	TGGTACAACGTCATTGATGACGT	1856-1865	
TRV	LK61	CGGGCTAACAGTGCTCTTG	801-819	99.81%
	LK62	CTCCCTTGGTTCGTCGTAAC	934-915	

Table S2. Gene-specific primers used to amplify by RT-PCR and to determine expression levels of Sgt1, Hsp90 and RTMs genes.

	Target	Primer	Primer sequence (5´-3´)	Primer position	Efficiency rates (E)
RT-PCR					
	Sgt1	LK 22A	TAGTGAATTCATCCTGCATCTGAGTTACCG	416-435	
		LK23A	GCATCTCGAGGTTTCTTCACCTGGCACATC	650-631	
	Hsp90	LK35A	GATCGGATCCTTGAGCAGTTCTCCTTGTGT	2042-2061	
		LK36A	AGTCGAGCTCATTTCTGTCCACCAGCTTCA	2471-2452	
	RTM1	LK2	GGGGCAGTGGGAAACGATTA	**13-32**	
		LK3	ACTCTCGAGTCAACGGCAGAGTCACTCAC	392-385	
	RTM2	LK4	TGGATTCAAAGGGAGCTGCA	2-21	
		LK5	CGTCTCGAGTGCACTGATTTTCGTCTTGTCG	254-234	
	RTM3	LK8	CCACCAGAGGATCCTCAAACT	1-21	
		LK9	CCACTCGAGACCTCAGCATCAACAACACA	395-376	
q-PCR					
	Sgt1	LK53	CCAAGATGCTGACGAGGAC	1012-1030	101.74%
		LK54	CAGAGGATCGATTCTAGATCTCC	1178-1156	
	Hsp90	LK57	GGTGTTACTGAGCCTGAGC	422-441	104.73%
		LK58	GGCGAGTCATACCAATTCCTG	537-517	
	RTM1	LK12	TTGGCCAGACTACAACTTATGCT	5-27	107.79%
		LK13	ACTCTCAACGGCAGAGTCAC	108-89	
	RTM2	LK14	AAGCTAATGATCTCGCGGAAA	146-166	102.09%
		LK15	AGTTTACACGCATAACTCGGT	239-219	
	RTM3	LK18	ACAGCAGTTATTCGAAGGGCA	206-226	109.45%
		LK19	TATCGCCCTCAAGACGTTCA	375-356	

Table S3. Gene-specific primers used to determine expression levels of *B. tabaci* genes.

Target	Primer	Primer sequence (5´-3´)	Primer position	Efficiency rates (E)
Cytochrome P450	LK83	GGTCAGTGCGCAATCGTC	241-258	94.24%
	LK84	GCTCTTGGCGCTGATGTC	453-436	
AhCR	LK81	GGGTAATCCTGAGAAATGTTTCG	3-25	103.66%
	LK82	CTCAGCAGAGTGAAGCTGG	132-114	
Neuroligin	LK87	GGACCTGGAGTTCGGGTTC	804-822	104.36%
	LK88	CACCTCGAGGGTGGAGTC	978-961	
Vitellogenin	LK91	GTAGGTCTTGTAGGCGCAGG	753-772	94.65%
	LK92	CCAACCAATACGCCGATGAC	913-894	
Juvenile hormone	LK97	GCGACCGTCGGTACGTTATAG	932-912	107.75%
	LK98	GAATCGGCTCACCGTTATCACG	759-780	

Table S4. Specific sequences and lengths of the synthetized sRNAs used in artificial diet.

	Target gene	Gene description	Lenght (bp)	Strand	Sequence (5' - 3')
vsRNAs	Bta001158	Cytochrome P450	21	sense	AACAUAUCCCAAUUGUUCUCU [dT][dT]
			21	antisense	AGAGAACAAUUGGGAUAUGUU [dT][dT]
	Bta001692	Aryl hydrocarbon receptor	21	sense	UCUGAAUGGAAAUGAUGUCGA [dT][dT]
			21	antisense	UCGACAUCAUUUCCAUUCAGA [dT][dT]
	Bta028144	Neuroligin-1	22	sense	CUCGUACUACCUCCACCUCAAC [dT][dT]
			22	antisense	GUUGAGGUGGAGGUAGUACGAG [dT][dT]
sRNAs	Bta017585	Vitellogenin	21	sense	CCUACGCUUUCAUCGACUCUU [dT][dT]
			21	antisense	AAGAGUCGAUGAAAGCGUAGG [dT][dT]
	Bta008678	Juvenile hormone	21	sense	UAUAGCCGGUGACGUGGUCUU [dT][dT]
			21	antisense	AAGACCACGUCACCGGCUAUA [dT][dT]
	Bta001158	Cytochrome P450	21	sense	AAAAUAUCCCAAUUGUUCUCU [dT][dT]
			21	antisense	AGAGAACAAUUGGGAUAUUUU [dT][dT]
		GFP	21	sense	AAGCGUUCAACUAGCAGACCA [dT][dT]
			21	antisense	UGGUCUGCUAGUUGAACGCUU [dT][dT]

Table S5. Statistical analysis of variance of the evolution of symptoms in single- and mixed-infected plants at different days post-inoculation (dpi) which are summarized in Fig.1. Significant differences are indicated by different letters according to one-way ANOVA with $P < 0.05$.

	7 dpi			14 dpi			21 dpi			28 dpi		
Symptoms in plants infected with:	**Mean ± SE**	**ANOVA**		**Mean ± SE**	**ANOVA**		**Mean ± SE**	**ANOVA**		**Mean ± SE**	**ANOVA**	
		F	**P value**		**F**	**P value**		**F**	**P value**		**F**	**P value**
ToCV	0.625 ± 0.19 a			1.50 ± 0.12 a			2.00 ± 0.16 a			2.38 ± 0.12 a		
TYLCV	2.00 ± 0.16 b			3.10 ± 0.19 b			3.50 ± 0.12 b			4.10 ± 0.22 b		
ToCV + TYLCV	1.20 ± 0.12 c	26	< 0.001	2.10 ± 0.10 c	27.38	<0.001	4.20 ± 0.10 c	68.6	<0.001	5.00 ± 0.00 c	226.8	<0.001

Table S6. Statistical analysis of variance of the relative accumulation of the salicylic acid (SA) responsive PR-P6 gene and the wound-induced proteinase inhibitor II (Pin-II) at different days post-inoculation (dpi) which are summarized in Fig.3. Significant differences are indicated by different letters according to one-way ANOVA with $P < 0.05$.

	7 dpi			14 dpi			21 dpi		
Accumulation of PR-P6 in infected plants with:	**Mean ± SD**	**ANOVA**		**Mean ± SD**	**ANOVA**		**Mean ± SD**	**ANOVA**	
		F	**P value**		**F**	**P value**		**F**	**P value**
ToCV	0.63 ± 0.12 a			10.40 ± 6.98 b			0.95 ± 0.97 a		
TYLCV	0.75 ± 0.17 a			21.39 ± 3.34 a			4.62 ± 0.13 a		
ToCV + TYLCV	0.59 ± 0.37 a	0.08	0.922	7.02 ± 4.86 b	**12.05**	**0.008**	11.41 ± 4.14 b	**14.01**	**0.005**
	7 dpi			**14 dpi**			**21 dpi**		
Accumulation of Pin II in infected plants with:	**Mean ± SD**	**ANOVA**		**Mean ± SD**	**ANOVA**		**Mean ± SD**	**ANOVA**	
		F	**P value**		**F**	**P value**		**F**	**P value**
ToCV	9.19 ± 4.91 a			0.25 ± 0.26 b			30.28 ± 4.42 b		
TYLCV	15.66 ± 9.73 a			13.97 ± 4.25 a			108.61 ± 13.94 a		
ToCV + TYLCV	0.66 ± 0.30 a	4.28	0.07	10.85 ± 2.11 a	**20.68**	**0.002**	45.71 ± 5.84 b	**62.42**	**<0.001**

Table S7. Statistical analysis of the relative accumulation of TYLCV and ToCV in single- and mixed-infected plants at different days post-inoculation (dpi), which are summarized in Fig.2. Means of fold change ± SD and the values of the Student-t test with $P < 0.05$ are indicated.

TYLCV accumulation in plants infected with:	7 dpi			14 dpi			21 dpi		
	Mean fold change ± SD	**t**	**P value**	**Mean fold change ± SD**	**t**	**P value**	**Mean fold change ± SD**	**t**	**P value**
TYLCV	2.6 ± 3.11			150.8 ± 49.73			134.88 ± 22.41		
ToCV + TYLCV	4.28 ± 2.43	-0.74	0.503	128.94 ± 18.56	0.98	0.382	91.58 ± 28.72	2.60	0.060

ToCV accumulation in plants infected with:	7 dpi			14 dpi			21 dpi		
	Mean fold change ± SD	**t**	**P value**	**Mean fold change ± SD**	**t**	**P value**	**Mean fold change ± SD**	**t**	**P value**
ToCV	1.8 ± 1.41			35.34 ± 10.06			29.43 ± 12.26		
ToCV + TYLCV	0.29 ± 0.14	1.84	0.139	15.08 ± 8.26	1.20	0.296	76.06 ± 53.69	-1.47	0.216

Table S8. Mean and standard deviation values of color differences corresponding to mock-inoculated, asymptomatic, and symptomatic Tomato yellow leaf curl virus (TYLCV)-infected tomato leaves.

Parameter	Mock	Asymptomatic TYLCV-infected	TYLCV symptomatic
*L**	35.83 ± 1.03 m[z]	38.49 ± 0.46 m	44.78 ± 1.08 n
*a**	−16.27 ± 0.84 m	−17.99 ± 0.39 m	−13.71 ± 0.72 n
*b**	23.92 ± 1.37 m	27.19 ± 0.61 m	32.805 ± 0.72 n
H	124.27 ± 0.48 m	123.5 ± 0.17 m	112.68 ± 0.29 n

[z] Significant differences are indicated by different letters according to one-way analysis of variance with $P < 0.05$

Table S9. Statistical analysis of variance of the relative accumulation of ToCV and the gene expression of silenced genes in control (ToCV- and TRV-infected) and silenced tomato plants. Significant differences are indicated by different letters according to one-way ANOVA with P < 0.05.

	Mean fold change ± SD	ANOVA	
ToCV accumulation in plants:		**F**	**P value**
ToCV-infected	0.905 ± 0.140 a		
i:TRV-0	0.425 ± 0.0988 a		
i:TRV-Sgt1	4.06 ± 0.985 b		
i:TRV-Hsp90	3.85 ± 0.803 b	**35.27**	**<0.001**
ToCV accumulation in plants:		**F**	**P value**
ToCV-infected	1.38 ± 0.365 a		
i:TRV-0	1.62 ± 0.980 a		
i:TRV-RTM1	1.62 ± 1.20 a		
i:TRV-RTM2	1.47 ± 0.676 a		
i:TRV-RTM3	1.50 ± 0.924 a	**0.073**	**0.99**
Hsp90 expression in plants:		**F**	**P value**
ToCV-infected	0.975 ± 0.0238 a		
i:TRV-0	0.942 ± 0.175 a		
i:TRV-Hsp90	0.562 ± 0.0834 b	**16.6**	**<0.001**

Sgt1 expression in plants:		**F**	**P value**
ToCV-infected	0.86 ± 0.126 a		
i:TRV-0	0.798 ± 0.0974 a,b		
i:TRV-Sgt1	0.598 ± 0.0830 b	**6.98**	**<0.001**
RTM1 expression in plants:		**F**	**P value**
ToCV-infected	1.07 ± 0.105 a		
i:TRV-0	1.02 ± 0.0573 a		
i:TRV-RTM1	0.808 ± 0.0864 b	**12.98**	**<0.001**
RTM2 expression in plants:		**F**	**P value**
ToCV-infected	1.22 ± 0.189 a		
i:TRV-0	1.06 ± 0.259 a,b		
i:TRV-RTM2	0.754 ± 0.195 b	**5.873**	**0.0167**
RTM3 expression in plants:		**F**	**P value**
ToCV-infected	0.998 ± 0.207 a		
i:TRV-0	0.792 ± 0.207 a,b		
i:TRV-RTM3	0.562 ± 0.149 b	**5.255**	**0.0308**

Table S10. Statistical analysis of the relative expression of target genes and the two endogenous control *B. tabaci* genes in either viruliferous or non-viruliferous individuals Means of Cq ± SD and the values of the Student-t test with $P < 0.05$ are indicated.

P450 expression in *B. tabaci*:	**Mean fold change ± SD**	**t**	**P value**
non-viruliferous	1.34 ± 1.317		
TYLCV-viruliferous	0.404 ± 0.175	4.45	0.019

AhR expression in *B. tabaci*:	**Mean fold change ± SD**	**t**	**P value**
non-viruliferous	1.31 ± 0.29		
TYLCV-viruliferous	0.476 ± 0.076	4.82	0.031

Neuroligin expression in *B. tabaci*:	**Mean fold change ± SD**	**t**	**P value**
non-viruliferous	1.12 ± 0.11		
TYLCV-viruliferous	0.217 ± 0.025	13.74	0.003

Vitellogenin expression in *B. tabaci*:	**Mean fold change ± SD**	**t**	**P value**
non-viruliferous	0.955 ± 0.063		
TYLCV-viruliferous	1.10 ± 0.007	-3.31	0.181

JHormone expression in *B. tabaci*:	**Mean fold change ± SD**	**t**	**P value**
non-viruliferous	0.985 ± 0.021		
TYLCV-viruliferous	1.14 ± 0.063	-3.37	0.146

Table S11. Statistical analysis of variance of the relative expression of genes identified as putative target genes involved in neonicotinoid detoxification-related pathways and the two endogenous control *B. tabaci* genes after feeding on artificial diet with sRNAs. Means of Cq ± SD and significant differences are indicated by different letters according to one-way ANOVA with $P < 0.05$.

P450 expression in *B. tabaci* fed on:	**Mean fold change ± SD**	**ANOVA**	
		F	**P value**
sacarose	0.92 ± 0.113 a		
GFP	0.88 ± 0.566 a		
vsRNA P450	0.365 ± 0.007 b	35.82	0.008

AhR expression in *B. tabaci* fed on:	**Mean fold change ± SD**	**ANOVA**	
		F	**P value**
sacarose	1.06 ± 0.084 a		
GFP	1.03 ± 0.028 a		
vsRNA AhR	0.46 ± 0.042 b	69.98	0.003

Neuroligin expression in *B. tabaci* fed on:	**Mean fold change ± SD**	**ANOVA**	
		F	**P value**
sacarose	1.06 ± 0.085 a		
GFP	1.04 ± 0.078 a		
vsRNA neuroligin	0.67 ± 0.070 b	15.67	0.025

Vitellogenin expression in *B. tabaci* fed on:	**Mean fold change ± SD**	**ANOVA**	
		F	**P value**
sacarose	0.98 ± 0.028 a		
GFP	0.83 ± 0.014 a		
sRNA vitellogenin	0.39 ± 0.071 b	94.03	0.002

Juvenile hormone expression in *B. tabaci* fed on:	**Mean fold change ± SD**	**ANOVA**	
		F	**P value**
sacarose	1.04 ± 0.566 a		
GFP	0.975 ± 0.092 a		
sRNA j.hormone	0.51 ± 0.071 b	30.11	0.011

Table S12. Available genome resources for *B. tabaci* whitefly.

Source	Taxonomy	NCBI Accession	Reference	Online resources
National center for biotechnology infomation (NCBI)	*Bemisia tabaci* Reference genome isolate MEAM1 Annotation release ID: 100 & links to other resources (Total 10 genomes)	GCA_001854935.1	Chen, W., Hasegawa, D. K., Kaur, N., Kliot, A., Pinheiro, P. V., Luan, J., Stensmyr, M. C., Zheng, Y., Liu, W., Sun, H., Xu, Y., Luo, Y., Kruse, A., Yang, X., Kontsedalov, S., Lebedev, G., Fisher, T. W., Nelson, D. R., Hunter, W. B., Brown, J. K., … Fei, Z. (2016). The draft genome of whitefly *Bemisia tabaci* MEAM1, a global crop pest, provides novel insights into virus transmission, host adaptation, and insecticide resistance. BMC biology, 14(1), 110. https://doi.org/10.1186/s12915-016-0321-y	https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=7038
Whiteflygenomics / Cornell university	*Bemisia tabaci* MEAM1 and SSA-ECA	GCA_004919745.1	Chen, W., Wosula, E. N., Hasegawa, D. K., Casinga, C., Shirima, R. R., Fiaboe, K. K. M., Hanna, R., Fosto, A., Goergen, G., Tamò, M., Mahuku, G., Murithi, H. M., Tripathi, L., Mware, B., Kumar, L. P., Ntawuruhunga, P., Moyo, C., Yomeni, M., Boahen, S., Edet, M., … Fei, Z. (2019). Genome of the African cassava whitefly *Bemisia tabaci* and distribution and genetic diversity of cassava-colonizing whiteflies in Africa. Insect biochemistry and molecular biology, 110, 112–120. https://doi.org/10.1016/j.ibmb.2019.05.003	http://whiteflygenomics.org/cgi-bin/bta/index.cgi
Chinese Academy of Agricultural Science Institute of Vegetables and Flowers (CAAS)	*Bemisia tabaci* MED/Q	GCA_003994315.1	Xie, W., Chen, C., Yang, Z., Guo, L., Yang, X., Wang, D., Chen, M., Huang, J., Wen, Y., Zeng, Y., Liu, Y., Xia, J., Tian, L., Cui, H., Wu, Q., Wang, S., Xu, B., Li, X., Tan, X., Ghanim, M., … Zhang, Y. (2017). Genome sequencing of the sweetpotato whitefly *Bemisia tabaci* MED/Q. GigaScience, 6(5), 1–7. https://doi.org/10.1093/gigascience/gix018	http://gigadb.org/dataset/view/id/100286/token/etFfO6xzVU8Iv5Kk

e! EnsemblMetazoa / National Resource Institute (NRI)	*Bemisia tabaci* (Silverleaf whitefly, Asia II-5)	GCA_903994105.1	Campbell, L. I., Nwezeobi, J., van Brunschot, S. L., Kaweesi, T., Seal, S. E., Swamy, R. A. R., Namuddu, A., Maslen, G. L., Mugerwa, H., Armean, I. M., Haggerty, L., Martin, F. J., Malka, O., Santos-Garcia, D., Juravel, K., Morin, S., Stephens, M. E., Muhindira, P. V., Kersey, P. J., Maruthi, M. N., … Colvin, J. (2023). Comparative evolutionary analyses of eight whitefly *Bemisia tabaci* sensu lato genomes: cryptic species, agricultural pests and plant-virus vectors. BMC genomics, 24(1), 408. https://doi.org/10.1186/s12864-023-09474-3	https://metazoa.ensembl.org/index.html
	Bemisia tabaci (Silverleaf whitefly, SSA1-SG1 Ng)	GCA_903994125.1		
	Bemisia tabaci (Silverleaf whitefly, SSA1-SG1 Ug)	GCA_903994115.1		
	Bemisia tabaci (Silverleaf whitefly, SSA2 Ng)	GCA_902825415.1		
	Bemisia tabaci (Silverleaf whitefly, SSA3 Ng)	GCA_902825425.1		
	Bemisia tabaci (Silverleaf whitefly, Uganda-1)	GCA_903994095.1		
Pest Genome Iniciative (PGI) / Rothamsted Research Institute	*Bemisia tabaci* Q-type Almeria	GCA_918797505.1	Unpublished	https://www.pestgenomics.org/species/bemisia-tabaci

Table S13. List of sRNAs of TYLCV-infected plants identified that matches with *B. tabaci* genome.

Gene description	**Read lenght** (bp)	**vsRNA sequence**	**counts**	**match in *B. tabaci* genome ***
Nicotinic acetylcholine receptor	21	TGTTTCTCCATTTCTTTCTTCC	102	Bta019770
Protein kinase C epsilon type	21	GTAGCCATTAGGTGTCCAGGT	57	Bta010212
Neuroligin-1	22	CTCGTACTACCTCCACCTCAAC	989	Bta028144
Cytochrome P450 (Fragment)	21	AACATATCCCAATTGTTCTCT	643	Bta001158
Aryl hydrocarbon receptor	21	TCGACATCATTTCCATTCAGA	103	Bta001692
Arrestin	22	AGTAAATCAAGGTCCAACACAAG	107	Bta023674

Table S14. List of TYLCV-specific sRNAs target on the selected whitefly genes.

Gene description	Read lenght (bp)	vsRNA sequence	counts	match in *B. tabaci* genome *	match position in TYLCV genome
Cytochrome P450 (Fragment)	21	AACATATCCCAATTGTTCTCT	643	Bta001158	2560-2581
Aryl hydrocarbon receptor	21	TCGACATCATTTCCATTCAGA	103	Bta001692	1344-1365
Neuroligin-1	22	CTCGTACTACCTCCACCTCAAC	989	Bta028144	1504-1525

* Genome of *B. tabaci* MED available at http://whiteflygenomics.org

REFERENCES

REFERENCES

Agüero, J., Gómez-Aix, C., Sempere, R. N., García-Villalba, J., García-Núñez, J., Hernando, Y., & Aranda, M. A. (2018). Stable and broad spectrum cross-protection against Pepino mosaic virus attained by mixed infection. *Frontiers in Plant Science*, *9*, 1810.

Aguilar, E., Allende, L., Del Toro, F. J., Chung, B.-N., Canto, T., & Tenllado, F. (2015). Effects of elevated CO2 and temperature on pathogenicity determinants and virulence of Potato virus X/Potyvirus-associated synergism. *Molecular Plant-Microbe Interactions*, *28*(12), 1364–1373.

Alcaide, C., Donaire, L., & Aranda, M. A. (2022). Transcriptome analyses unveiled differential regulation of AGO and DCL genes by pepino mosaic virus strains. *Molecular Plant Pathology*, *23*(11), 1592–1607. https://doi.org/10.1111/mpp.13249

Alcaide, C., Sardanyés, J., Elena, S. F., & Gómez, P. (2021). Increasing temperature alters the within-host competition of viral strains and influences virus genetic variability. *Virus Evolution*, *7*(1), veab017.

Alfaro-Fernández, A, Córdoba-Sellés, C., Cebrián, M. C., Sánchez-Navarro, J. A., Espino, A., Martín, R., & Jordá, C. (2007). First report of tomato torrado virus in tomato in the Canary Islands, Spain. *Plant Disease*, *91*(8), 1060.

Alfaro-Fernández, Ana, Castillo, P., Sanahuja, E., Rodríguez-Salido, M. C., & Font, M. I. (2021). First report of Tomato brown rugose fruit virus in tomato in Spain. *Plant Disease*, *105*(2), 515.

Amari, K., Gonzalez-Ibeas, D., Gómez, P., Sempere, R. N., Sanchez-Pina, M. A., Pendon, J., & Moriones, E. (2017). Tomato torrado virus is transmitted by Bemisia tabaci and infects pepper and eggplant in addition to tomato. *Arch. Virol*, *9*.

Ament, K., Kant, M. R., Sabelis, M. W., Haring, M. A., & Schuurink, R. C. (2004). Jasmonic acid is a key regulator of spider mite-induced volatile terpenoid and methyl salicylate emission in tomato. *Plant Physiology*, *135*(4), 2025–2037. https://doi.org/10.1104/pp.104.048694

Antignus, Y. (2010). Optical manipulation for control of bemisia tabaci and its vectored viruses in the greenhouse and open field. In *Bemisia: Bionomics and Management of a Global Pest* (pp. 349–356). Springer Netherlands. https://doi.org/10.1007/978-90-481-2460-2_13

bance, V. B. (1991). Replication of potato virus X RNA is altered in coinfections with potato virus Y. *Virology*, *182*(2), 486–494.

Barbosa, L. da F., Yuki, V. A., Marubayashi, J. M., De Marchi, B. R., Perini, F. L., Pavan, M. A., de Barros, D. R., Ghanim, M., Moriones, E., & Navas-Castillo, J. (2015). First report of Bemisia tabaci Mediterranean (Q biotype) species in Brazil. *Pest Management Science*, *71*(4), 501–504.

Bari, R., & Jones, J. D. (2009). (2009). Role of plant hormones in plant defence responses. *Trends in Plant Science*, *69*(2), 473–488.

Barrantes, W., López-Casado, G., García-Martínez, S., Alonso, A., Rubio, F., Ruiz, J. J., Fernández-Muñoz, R., Granell, A., & Monforte, A. J. (2016). Exploring new alleles involved in tomato fruit quality in an introgression line library of Solanum pimpinellifolium. *Frontiers in Plant Science*, *7*, 1172.

Baulcome 2004_RNA silencing in plants. (n.d.).

Belabess, Z., Dallot, S., El-Montaser, S., Granier, M., Majde, M., Tahiri, A., Blenzar, A., Urbino, C., & Peterschmitt, M. (2015). Monitoring the dynamics of emergence of a non-canonical recombinant of Tomato yellow leaf curl virus and displacement of its parental viruses in tomato. *Virology*, *486*, 291–306.

Bleeker, P. M., Diergaarde, P. J., Ament, K., Guerra, J., Weidner, M., Schütz, S., de Both, M. T. J., Haring, M. A., & Schuurink, R. C. (2009). The role of specific tomato volatiles in tomato-whitefly interaction. *Plant Physiology*, *151*(2), 925–935. https://doi.org/10.1104/pp.109.142661

Brodersen, P., & Voinnet, O. (2006). The diversity of RNA silencing pathways in plants. *TRENDS in Genetics*, *22*(5), 268–280.

Brown, J. K., Dennehy, T. J., DeGain, B., Rogan, D., Harpold, G., Byrne, F., & Nichols, R. (2005). First report of the Q biotype of Bemisia tabaci (Gennadius) in the USA and resistance to insecticides in an Arizona population. *European Whitefly Studies Network Newsletter*.

Butterbach, P., Verlaan, M. G., Dullemans, A., Lohuis, D., Visser, R. G. F., Bai, Y., & Kormelink, R. (2014). Tomato yellow leaf curl virus resistance by Ty-1 involves increased cytosine methylation of viral genomes and is compromised by cucumber mosaic virus infection. *Proceedings of the National Academy of Sciences*, *111*(35), 12942–12947.

Cañizares, M. C., Navas-Castillo, J., & Moriones, E. (2008). Multiple suppressors of RNA silencing encoded by both genomic RNAs of the crinivirus, Tomato chlorosis virus. *Virology*, *379*(1), 168–174. https://doi.org/10.1016/j.virol.2008.06.020

Carrington, J. C., Kasschau, K. D., & Johansen, L. K. (2001). Activation and suppression of RNA silencing by plant viruses. *Virology*, *281*(1), 1–5.

Chávez-Calvillo, G., Contreras-Paredes, C. A., Mora-Macias, J., Noa-Carrazana, J. C., Serrano-Rubio, A. A., Dinkova, T. D., Carrillo-Tripp, M., & Silva-Rosales, L. (2016). Antagonism or synergism between papaya ringspot virus and papaya mosaic virus in Carica papaya is determined by their order of infection. *Virology*, *489*, 179–191. https://doi.org/10.1016/j.virol.2015.11.026

Chisholm, P. J., Sertsuvalkul, N., Casteel, C. L., & Crowder, D. W. (2018). *Reciprocal plant‐mediated interactions between a virus and a non‐vector herbivore*. Wiley Online Library.

Clark, C. A., Davis, J. A., Abad, J. A., Cuellar, W. J., Fuentes, S., Kreuze, J. F., Gibson, R. W., Mukasa, S. B., Tugume, A. K., & Tairo, F. D. (2012). Sweetpotato viruses: 15 years of progress on understanding and managing complex diseases. *Plant Disease*, *96*(2), 168–185.

Cohen, S., & Antignus, Y. (1994). Tomato yellow leaf curl virus, a whitefly-borne geminivirus of tomatoes. In *Advances in disease vector research* (pp. 259–288). Springer.

Cosson, P., Schurdi-Levraud, V., Le, Q. H., Sicard, O., Caballero, M., Roux, F., Le Gall, O., Candresse, T., & Revers, F. (2012). The RTM resistance to potyviruses in Arabidopsis thaliana: Natural variation of the RTM genes and evidence for the implication of additional genes. *PLoS ONE*, *7*(6). https://doi.org/10.1371/journal.pone.0039169

Costa, A. S., Costa, C. L., & Sauer, H. F. G. (1973). Outbreak of whitefly on crops in Parana and Sao Paulo. *Anais Da Sociedade Entomológica Do Brasil*, *2*(1), 20–30.

Coutts, B. A., Kehoe, M. A., & Jones, R. A. C. (2011). Minimising losses caused by Zucchini yellow mosaic virus in vegetable cucurbit crops in tropical, sub-tropical and Mediterranean environments through cultural methods and host resistance. *Virus Research*, *159*(2), 141–160.

Cui, C., Wang, Y., Liu, J., Zhao, J., Sun, P., & Wang, S. (2019). A fungal pathogen deploys a small silencing RNA that attenuates mosquito immunity and facilitates infection. *Nature Communications*, *10*(1), 4298.

DaPalma, T., Doonan, B. P., Trager, N. M., & Kasman, L. M. (2010). A systematic approach to virus-virus interactions. *Virus Research*, *149*(1), 1–9. https://doi.org/10.1016/j.virusres.2010.01.002

De Barro, P. J., Liu, S. S., Boykin, L. M., & Dinsdale, A. B. (2011). Bemisia tabaci: A statement of species status. *Annual Review of Entomology*, *56*, 1–19. https://doi.org/10.1146/annurev-ento-112408-085504

Diaz-Pendon, J. A., Li, F., Li, W.-X., & Ding, S.-W. (2007). Suppression of antiviral silencing by cucumber mosaic virus 2b protein in Arabidopsis is associated with drastically reduced accumulation of three classes of viral small interfering RNAs. *The Plant Cell*, *19*(6), 2053–2063.

Díaz-Pendón, Juan A, & Ding, S.-W. (2008). Direct and indirect roles of viral suppressors of RNA silencing in pathogenesis. *Annu. Rev. Phytopathol.*, *46*, 303–326.

Díaz-Pendón, Juan Antonio, Cañizares, M. C., Moriones, E., Bejarano, E. R., Czosnek, H., & Navas-Castillo, J. (2010). Tomato yellow leaf curl viruses: Ménage à trois between the virus complex, the plant and the whitefly vector. *Molecular Plant Pathology*, *11*(4), 441–450. https://doi.org/10.1111/j.1364-3703.2010.00618.x

Ding, S.-W. (2010). RNA-based antiviral immunity. *Nature Reviews Immunology*, *10*(9), 632–644.

Ding, S.-W. (2023). Transgene silencing, RNA interference, and the antiviral defense mechanism directed by small interfering RNAs. *Phytopathology®*, *113*(4), 616–625.

Dong, X., van Wezel, R., Stanley, J., & Hong, Y. (2003). Functional characterization of the nuclear localization signal for a suppressor of

posttranscriptional gene silencing. *Journal of Virology*, *77*(12), 7026–7033.

Ertunc, F. (2020). Emerging plant viruses. In *Emerging and reemerging viral pathogens* (pp. 1041–1062). Elsevier.

Fang, Y., Jiao, X., Xie, W., Wang, S., Wu, Q., Shi, X., Chen, G., Su, Q., Yang, X., Pan, H., & Zhang, Y. (2013). Tomato yellow leaf curl virus alters the host preferences of its vector bemisia tabaci. *Scientific Reports*, *3*, 1–5. https://doi.org/10.1038/srep02876

Fereres, A. (2015). Insect vectors as drivers of plant virus emergence. *Current Opinion in Virology*, *10*, 42–46.

Fereres, A., & Moreno, A. (2009). Behavioural aspects influencing plant virus transmission by homopteran insects. *Virus Research*, *141*(2), 158–168. https://doi.org/10.1016/j.virusres.2008.10.020

Fereres, A., Peñaflor, M. F. G. V., Favaro, C. F., Azevedo, K. E. X., Landi, C. H., Maluta, N. K. P., Bento, J. M. S., & Lopes, J. R. S. (2016). Tomato infection by whitefly-transmitted circulative and non-circulative viruses induce contrasting changes in plant volatiles and vector behaviour. *Viruses*, *8*(8), 2–5. https://doi.org/10.3390/v8080225

Fernandez-Pozo, N., Menda, N., Edwards, J. D., Saha, S., Tecle, I. Y., Strickler, S. R., Bombarely, A., Fisher-York, T., Pujar, A., & Foerster, H. (2015). The Sol Genomics Network (SGN)—from genotype to phenotype to breeding. *Nucleic Acids Research*, *43*(D1), D1036–D1041.

Ferrero, V., Baeten, L., Blanco-Sánchez, L., Planelló, R., Díaz-Pendón, J. A., Rodríguez-Echeverría, S., Haegeman, A., & de la Peña, E. (2020). Complex patterns in tolerance and resistance to pests and diseases underpin the domestication of tomato. *New Phytologist*, *226*(1), 254–266. https://doi.org/10.1111/nph.16353

Fiallo-Olivé, E., Espino, A. I., Botella-Guillén, M., Gómez-González, E., Reyes-Carlos, J. A., & Navas-Castillo, J. (2014). Tobacco: a new natural host of Tomato chlorosis virus in Spain. *Plant Disease*, *98*(8), 1162.

Fiallo-Olivé, E., & Navas-Castillo, J. (2023). Tomato chlorosis virus, a promiscuous virus with multiple host plants and whitefly vectors. *Annals of Applied Biology*, *182*(1), 29–36.

Fortes, I. M., Moriones, E., & Navas-Castillo, J. (2012). Tomato chlorosis virus in pepper: Prevalence in commercial crops in southeastern Spain and symptomatology under experimental conditions. *Plant Pathology*, *61*(5), 994–1001. https://doi.org/10.1111/j.1365-3059.2011.02584.x

Fortes, Isabel M, Fernández-Muñoz, R., & Moriones, E. (2023). The crinivirus tomato chlorosis virus compromises the control of tomato yellow leaf curl virus in tomato plants by the Ty-1 gene. *Phytopathology*, *ja*.

Fortes, Isabel M, & Navas-Castillo, J. (2012). Potato, an experimental and natural host of the crinivirus Tomato chlorosis virus. *European Journal of Plant Pathology*, *134*, 81–86.

García-Andrés, S., Accotto, G. P., Navas-Castillo, J., & Moriones, E. (2007).

Founder effect, plant host, and recombination shape the emergent population of begomoviruses that cause the tomato yellow leaf curl disease in the Mediterranean basin. *Virology*, *359*(2), 302–312.

García-Cano, E., Navas-castillo, J., Moriones, E., & Fernández-muñoz, R. (2010). Resistance to tomato chlorosis virus in wild tomato species that impair virus accumulation and disease symptom expression. *Phytopathology*, *100*(6), 582–592. https://doi.org/10.1094/PHYTO-100-6-0582

García-Cano, E., Resende, R. O., Fernández-Muñoz, R., & Moriones, E. (2006). Synergistic interaction between Tomato chlorosis virus and Tomato spotted wilt virus results in breakdown of resistance in tomato. *Phytopathology*, *96*(11), 1263–1269. https://doi.org/10.1094/PHYTO-96-1263

Gautam, S., Gadhave, K. R., Buck, J. W., Dutta, B., Coolong, T., Adkins, S., & Srinivasan, R. (2020). Virus-virus interactions in a plant host and in a hemipteran vector: Implications for vector fitness and virus epidemics. *Virus Research*, *286*(19), 198069. https://doi.org/10.1016/j.virusres.2020.198069

Gennadius, P. (1889). Disease of tobacco plantations in the Trikonia. *The Aleurodid of Tobacco. Ellenike Georgia*, *5*, 1–3.

Gerling, D., & Horowitz, A. R. (1984). Yellow traps for evaluating the population levels and dispersal patterns of Bemisia tabaci (Gennadius)(Homoptera: Aleyrodidae). *Annals of the Entomological Society of America*, *77*(6), 753–759.

Gibbs, A. J., Ohshima, K., Phillips, M. J., & Gibbs, M. J. (2008). The prehistory of potyviruses: their initial radiation was during the dawn of agriculture. *PloS One*, *3*(6), e2523.

Gómez, P., Sempere, R. N., Elena, S. F., & Aranda, M. A. (2009). Mixed infections of Pepino mosaic virus strains modulate the evolutionary dynamics of this emergent virus. *Journal of Virology*, *83*(23), 12378–12387.

Gong, P., Tan, H., Zhao, S., Li, H., Liu, H., Ma, Y., Zhang, X., Rong, J., Fu, X., & Lozano-Durán, R. (2021). Geminiviruses encode additional small proteins with specific subcellular localizations and virulence function. *Nature Communications*, *12*(1), 4278.

Grover, S., Jindal, V., Banta, G., Taning, C. N. T., Smagghe, G., & Christiaens, O. (2019). Potential of RNA interference in the study and management of the whitefly, Bemisia tabaci. *Archives of Insect Biochemistry and Physiology*, *100*(2), 1–17. https://doi.org/10.1002/arch.21522

Guirao, P., Beitia, F., & Cenis, J. L. (1997). Biotype determination of Spanish populations of Bemisia tabaci (Hemiptera: Aleyrodidae). *Bulletin of Entomological Research*, *87*(6), 587–593.

Gul-Seker, M., & Elibuyuk, I. O. (2019). Occurrence of Tomato yellow leaf curl virus and Tomato chlorosis virus mixed infections in protected tomato plants, Antalya, Turkey. *Phytoparasitica*, *47*(3), 441–449. https://doi.org/10.1007/s12600-019-00743-0

Hanssen, I. M., Peter van Esse, H., Ballester, A.-R., Hogewoning, S. W., Parra, N. O., Paeleman, A., Lievens, B., Bovy, A. G., & Thomma, B. P. H. J. (2011). Differential tomato transcriptomic responses induced by pepino mosaic virus isolates with differential aggressiveness. *Plant Physiology*, *156*(1), 301–318.

Hanssen, I. M., & Thomma, B. P. H. J. (2010). Pepino mosaic virus: a successful pathogen that rapidly evolved from emerging to endemic in tomato crops. *Molecular Plant Pathology*, *11*(2), 179–189.

Hilaire, J., Tindale, S., Jones, G., Pingarron-Cardenas, G., Bačnik, K., Ojo, M., & Frewer, L. J. (2022). Risk perception associated with an emerging agri-food risk in Europe: plant viruses in agriculture. *Agriculture & Food Security*, *11*(1), 21.

Horowitz, A. R., Denholm, I., Gorman, K., Cenis, J. L., Kontsedalov, S., & Ishaaya, I. (2003). Biotype Q of Bemisia tabaci identified in Israel. *Phytoparasitica*, *31*, 94–98.

Hua, C., Zhao, J.-H., & Guo, H.-S. (2018). Trans-kingdom RNA silencing in plant–fungal pathogen interactions. *Molecular Plant*, *11*(2), 235–244.

Hull, R. (2002). Mathews' Plant Virology,(4thedn) Academic Press, New York, human alimentary tract. *Nature*, *300*, 637–638.

Jensen, S., Gassama, M.-P., & Heidmann, T. (1999). Taming of transposable elements by homology-dependent gene silencing. *Nature Genetics*, *21*(2), 209–212.

Johnston, N., & Martini, X. (2020). The influence of visual and olfactory cues in host selection for bemisia tabaci biotype b in the presence or absence of tomato yellow leaf curl virus. *Insects*, *11*(2). https://doi.org/10.3390/insects11020115

Jones, C. M., Daniels, M., Andrews, M., Slater, R., Lind, R. J., Gorman, K., Williamson, M. S., & Denholm, I. (2011). Age-specific expression of a P450 monooxygenase (CYP6CM1) correlates with neonicotinoid resistance in Bemisia tabaci. *Pesticide Biochemistry and Physiology*, *101*(1), 53–58.

Jones, D. R. (2003). Plant viruses transmitted by whiteflies.pdf. *European Journal of Plant Pathology*, *109*(3), 195–219.

Jones, R. A. C. (2021). Global plant virus disease pandemics and epidemics. *Plants*, *10*(2), 1–41. https://doi.org/10.3390/plants10020233

Jones, R. A. C., & Naidu, R. A. (2019). Global dimensions of plant virus diseases: Current status and future perspectives. *Annual Review of Virology*, *6*, 387–409.

Jupin, I., De Kouchkovsky, F., Jouanneau, F., & Gronenborn, B. (1994). Movement of tomato yellow leaf curl geminivirus (TYLCV): involvement of the protein encoded by ORF C4. *Virology*, *204*(1), 82–90.

Karunker, I., Benting, J., Lueke, B., Ponge, T., Nauen, R., Roditakis, E., Vontas, J., Gorman, K., Denholm, I., & Morin, S. (2008). Over-expression of cytochrome P450 CYP6CM1 is associated with high resistance to

imidacloprid in the B and Q biotypes of Bemisia tabaci (Hemiptera: Aleyrodidae). *Insect Biochemistry and Molecular Biology*, *38*(6), 634–644.

Karyeija, R. F., Kreuze, J. F., Gibson, R. W., & Valkonen, J. P. T. (2000). Synergistic interactions of a potyvirus and a phloem-limited crinivirus in sweet potato plants. *Virology*, *269*(1), 26–36.

Landeo-Ríos, Y., Navas-Castillo, J., Moriones, E., & Cañizares, M. C. (2017). The heterologous expression of the p22 RNA silencing suppressor of the Crinivirus tomato chlorosis virus from tobacco rattle virus and potato virus X enhances disease severity but does not complement suppressor-defective mutant viruses. *Viruses*, *9*(12), 358.

Latham, J. R., & Wilson, A. K. (2008). Transcomplementation and synergism in plants: implications for viral transgenes? *Molecular Plant Pathology*, *9*(1), 85–103.

Lavina, A., Aramburu, J., & Moriones, E. (1996). Occurrence of tomato spotted wilt and cucumber mosaic viruses in field-grown tomato crops and associated weeds in northeastern Spain. *Plant Pathology*, *45*(5), 837–842.

Legarrea, S., Barman, A., Marchant, W., Diffie, S., & Srinivasan, R. (2015). Temporal effects of a Begomovirus infection and host plant resistance on the preference and development of an insect vector, Bemisia tabaci, and implications for epidemics. *PLoS ONE*, *10*(11), 1–19. https://doi.org/10.1371/journal.pone.0142114

Lewsey, M. G., Murphy, A. M., MacLean, D., Dalchau, N., Westwood, J. H., Macaulay, K., Bennett, M. H., Moulin, M., Hanke, D. E., & Powell, G. (2010). Disruption of two defensive signaling pathways by a viral RNA silencing suppressor. *Molecular Plant-Microbe Interactions*, *23*(7), 835–845.

Li, Jie, Wang, J., Ding, T., & Chu, D. (2021). Synergistic Effects of a Tomato chlorosis virus and Tomato yellow leaf curl virus Mixed Infection on Host Tomato Plants and the Whitefly Vector. *Frontiers in Plant Science*, *12*, 1032.

Li, Jingjing, Li, X., Bai, R., Shi, Y., Tang, Q., An, S., Song, Q., & Yan, F. (2015). RNA interference of the P450 CYP6CM1 gene has different efficacy in B and Q biotypes of Bemisia tabaci. *Pest Management Science*, *71*(8), 1175–1181. https://doi.org/10.1002/ps.3903

Li, R., Xie, W., Wang, S., Wu, Q., Yang, N., Yang, X., Pan, H., Zhou, X., Bai, L., & Xu, B. (2013). Reference gene selection for qRT-PCR analysis in the sweetpotato whitefly, Bemisia tabaci (Hemiptera: Aleyrodidae). *PloS One*, *8*(1), e53006.

Liu, H., Chang, Z., Zhao, S., Gong, P., Zhang, M., Lozano-Durán, R., Yan, H., Zhou, X., & Li, F. (2023). Functional identification of a novel C7 protein of tomato yellow leaf curl virus. *Virology*.

Liu, J., Yang, J., Bi, H., & Zhang, P. (2014). Why mosaic? Gene expression profiling of African cassava mosaic virus-infected cassava reveals the effect of chlorophyll degradation on symptom development. *Journal of*

Integrative Plant Biology, *56*(2), 122–132.

Liu, Y., Schiff, M., Marathe, R., & Dinesh-Kumar, S. P. (2002). Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus. *Plant Journal*, *30*(4), 415–429. https://doi.org/10.1046/j.1365-313X.2002.01297.x

Livak, K J, & Schmittgen, T. D. (2001). *Analysis of relative gene expression data using real time quantitative PCR and the 2-Ct method. Methods25: 402–408*.

Livak, Kenneth J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Llave, C. (2016). Dynamic cross-talk between host primary metabolism and viruses during infections in plants. *Current Opinion in Virology*, *19*, 50–55. https://doi.org/10.1016/j.coviro.2016.06.013

López-Ráez, J. A., Verhage, A., Fernández, I., García, J. M., Azcón-Aguilar, C., Flors, V., & Pozo, M. J. (2010). Hormonal and transcriptional profiles highlight common and differential host responses to arbuscular mycorrhizal fungi and the regulation of the oxylipin pathway. *Journal of Experimental Botany*, *61*(10), 2589–2601.

Love, M., Anders, S., & Huber, W. (2014). Differential analysis of count data–the DESeq2 package. *Genome Biol*, *15*(550), 10–1186.

Lozano, G., Moriones, E., & Navas-Castillo, J. (2004). First report of sweet pepper (Capsicum annuum) as a natural host plant for Tomato chlorosis virus. *Plant Disease*, *88*(2), 224.

Lozano, G., Moriones, E., & Navas-Castillo, J. (2006). Complete nucleotide sequence of the RNA2 of the crinivirus tomato chlorosis virus. *Archives of Virology*, *151*, 581–587.

Lozano, G., Moriones, E., & Navas-Castillo, J. (2007). Complete sequence of the RNA1 of a European isolate of tomato chlorosis virus. *Archives of Virology*, *152*(4), 839–842.

Lu, J., Du, Z. X., Kong, J., Chen, L. N., Qiu, Y. H., Li, G. F., Meng, X. H., & Zhu, S. F. (2012). Transcriptome Analysis of Nicotiana tabacum Infected by Cucumber mosaic virus during Systemic Symptom Development. *PLoS ONE*, *7*(8). https://doi.org/10.1371/journal.pone.0043447

Luan, J., Yao, D., Zhang, T., Walling, L. L., Yang, M., Wang, Y., & Liu, S. (2013). Suppression of terpenoid synthesis in plants by a virus promotes its mutualism with vectors. *Ecology Letters*, *16*(3), 390–398.

Luna, A. P., Morilla, G., Voinnet, O., & Bejarano, E. R. (2012). Functional analysis of gene-silencing suppressors from tomato yellow leaf curl disease viruses. *Molecular Plant-Microbe Interactions*, *25*(10), 1294–1306.

Mahajan, S. K., Chisholm, S. T., Whitham, S. A., & Carrington, J. C. (1998). Identification and characterization of a locus (RTM1) that restricts long-distance movement of tobacco etch virus in Arabidopsis thaliana. *Plant*

Journal, *14*(2), 177–186. https://doi.org/10.1046/j.1365-313X.1998.00105.x

Martínez-Zubiaur, Y., Fiallo-Olivé, E., Carrillo-Tripp, J., & Rivera-Bustamante, R. (2008). First report of Tomato chlorosis virus infecting tomato in single and mixed infections with Tomato yellow leaf curl virus in Cuba. *Plant Disease*, *92*(5), 836.

Mascia, T., & Gallitelli, D. (2016). Synergies and antagonisms in virus interactions. *Plant Science*, *252*(July 2016), 176–192. https://doi.org/10.1016/j.plantsci.2016.07.015

Matsuda, K., Buckingham, S. D., Kleier, D., Rauh, J. J., Grauso, M., & Sattelle, D. B. (2001). Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends in Pharmacological Sciences*, *22*(11), 573–580.

Matsumura, E. E., & Kormelink, R. (2023). Small Talk: On the Possible Role of Trans-Kingdom Small RNAs during Plant–Virus–Vector Tritrophic Communication. *Plants*, *12*(6), 1–18. https://doi.org/10.3390/plants12061411

Mauck, K. E., De Moraes, C. M., & Mescher, M. C. (2016). Effects of pathogens on sensory-mediated interactions between plants and insect vectors. *Current Opinion in Plant Biology*, *32*, 53–61. https://doi.org/10.1016/j.pbi.2016.06.012

Momotaz, A., Scott, J. W., & Schuster, D. J. (2010). Identification of quantitative trait loci conferring resistance to bemisia tabaci in an f2 population of solanum lycopersicum · solanum habrochaites accession la1777. *Journal of the American Society for Horticultural Science*, *135*(2), 134–142. https://doi.org/10.21273/jashs.135.2.134

Monci, F., Sánchez-Campos, S., Navas-Castillo, J., & Moriones, E. (2002). A natural recombinant between the geminiviruses Tomato yellow leaf curl Sardinia virus and Tomato yellow leaf curl virus exhibits a novel pathogenic phenotype and is becoming prevalent in Spanish populations. *Virology*, *303*(2), 317–326. https://doi.org/10.1006/viro.2002.1633

Moreau, T. L., & Isman, M. B. (2011). Trapping whiteflies? A comparison of greenhouse whitefly (Trialeurodes vaporariorum) responses to trap crops and yellow sticky traps. *Pest Management Science*, *67*(4), 408–413. https://doi.org/10.1002/ps.2078

Moreno, A. B., & López-Moya, J. J. (2020). When viruses play team sports: Mixed infections in plants. *Phytopathology*, *110*(1), 29–48. https://doi.org/10.1094/PHYTO-07-19-0250-FI

Moriones, E., & Navas-Castillo, J. (2000). Tomato yellow leaf curl virus, an emerging virus complex causing epidemics worldwide. *Virus Research*, *71*(1–2), 123–134. https://doi.org/10.1016/S0168-1702(00)00193-3

Moriones, Enrique, & Navas-Castillo, J. (2010). Tomato yellow leaf curl disease epidemics. *Bemisia: Bionomics and Management of a Global Pest*, 259–282.

Moshe, A., Gorovits, R., Liu, Y., & Czosnek, H. (2016). Tomato plant cell death induced by inhibition of HSP90 is alleviated by Tomato yellow leaf curl virus infection. *Molecular Plant Pathology*, *17*(2), 247–260. https://doi.org/10.1111/mpp.12275

Mound, Laurence A. (1962). Studies on the olfaction and colour sensitivity of Bemisia tabaci (Genn.)(Homoptera, Aleyrodidae). *Entomología Experimentalis et Applicata*, *5*(2), 99–104.

Mound, Laurence Alfred, & Halsey, S. H. (1978). *Whitefly of the world. A systematic catalogue of the Aleyrodidae (Homoptera) with host plant and natural enemy data.* John Wiley and Sons.

Navas-Castillo, J., Camero, R., Bueno, M., & Moriones, E. (2000). Severe yellowing outbreaks in tomato in Spain associated with infections of Tomato chlorosis virus. *Plant Disease*, *84*(8), 835–837. https://doi.org/10.1094/PDIS.2000.84.8.835

Navas-Castillo, Jesús, Fiallo-Olivé, E., & Sánchez-Campos, S. (2011). Emerging virus diseases transmitted by whiteflies. *Annual Review of Phytopathology*, *49*, 219–248. https://doi.org/10.1146/annurev-phyto-072910-095235

Navas-Castillo, Jesús, Sánchez-Campos, S., Díaz, J. A., Sáez-Alonso, E., & Moriones, E. (1999). Tomato yellow leaf curl virus-Is causes a novel disease of common bean and severe epidemics in tomato in Spain. *Plant Disease*, *83*(1), 29–32.

Navas-Hermosilla, E., Fiallo-Olivé, E., & Navas-Castillo, J. (2021). Infectious Clones of Tomato Chlorosis Virus: Toward Increasing Efficiency by Introducing the Hepatitis Delta Virus Ribozyme. *Frontiers in Microbiology*, *12*(July), 1–9. https://doi.org/10.3389/fmicb.2021.693457

Nelson, R. S., & Citovsky, V. (2005). Plant viruses. Invaders of cells and pirates of cellular pathways. *Plant Physiology*, *138*(4), 1809–1814.

Nomura, H., Komori, T., Uemura, S., Kanda, Y., Shimotani, K., Nakai, K., Furuichi, T., Takebayashi, K., Sugimoto, T., & Sano, S. (2012). Chloroplast-mediated activation of plant immune signalling in Arabidopsis. *Nature Communications*, *3*(1), 926.

Noris, E., Accotto, G. P., Tavazza, R., Brunetti, A., Crespi, S., & Tavazza, M. (1996). Resistance to tomato yellow leaf curl Geminivirus inNicotiana benthamianaPlants transformed with a truncated viral C1 gene. *Virology*, *224*(1), 130–138.

Oliveira, M. R. V, Henneberry, T. J., & Anderson, P. (2001). History, current status, and collaborative research projects for Bemisia tabaci $. In *Crop Protection* (Vol. 20).

Ontiveros, I., López-Moya, J. J., & Díaz-Pendón, J. A. (2022). Coinfection of Tomato Plants with Tomato yellow leaf curl virus and Tomato chlorosis virus Affects the Interaction with Host and Whiteflies. *Phytopathology*®, PHYTO-08.

Palumbo, J. C., Horowitz, A. R., & Prabhaker, N. (2001). Insecticidal control and resistance management for Bemisia tabaci. *Crop Protection*, *20*(9), 739–765.

Pan, L., Miao, H., Wang, Q., Walling, L. L., & Liu, S. (2021). Virus-induced phytohormone dynamics and their effects on plant–insect interactions. *New Phytologist*, *230*(4), 1305–1320.

Panno, S., Caruso, A. G., & Davino, S. (2018). The nucleotide sequence of a recombinant tomato yellow leaf curl virus strain frequently detected in Sicily isolated from tomato plants carrying the Ty-1 resistance gene. *Archives of Virology*, *163*(3), 795–797.

Perring, T. M. (2001). The Bemisia tabaci species complex. *Crop Protection*, *20*(9), 725–737.

Piedra-Aguilera, Á., Jiao, C., Luna, A. P., Villanueva, F., Dabad, M., Esteve-Codina, A., Díaz-Pendón, J. A., Fei, Z., Bejarano, E. R., & Castillo, A. G. (2019). Integrated single-base resolution maps of transcriptome, sRNAome and methylome of Tomato yellow leaf curl virus (TYLCV) in tomato. *Scientific Reports*, *9*(1), 1–16. https://doi.org/10.1038/s41598-019-39239-6

Pieterse, C. M. J., Van der Does, D., Zamioudis, C., Leon-Reyes, A., & Van Wees, S. C. M. (2012). Hormonal modulation of plant immunity. *Annual Review of Cell and Developmental Biology*, *28*, 489–521.

Pieterse, C. M. J., & Van Loon, L. C. (1999). Salicylic acid-independent plant defence pathways. *Trends in Plant Science*, *4*(2), 52–58.

Ponzio, C., Gols, R., Pieterse, C. M. J., & Dicke, M. (2013). Ecological and phytohormonal aspects of plant volatile emission in response to single and dual infestations with herbivores and phytopathogens. *Functional Ecology*, *27*(3), 587–598.

Prasad, A., Sharma, N., Hari-Gowthem, G., Muthamilarasan, M., & Prasad, M. (2020). Tomato Yellow Leaf Curl Virus: Impact, Challenges, and Management. *Trends in Plant Science*, *25*(9), 897–911. https://doi.org/10.1016/j.tplants.2020.03.015

Qiao, N., Liu, Y., Liu, J., Zhang, D., Chi, W., Li, J., Zhu, X., Liu, H., & Li, F. (2023). Antagonism of tomato spotted wilt virus against tomato yellow leaf curl virus in Nicotiana benthamiana detected by transcriptome analysis. *Genes & Genomics*, *45*(1), 23–37.

Ratcliff, F., Martin-Hernandez, A. M., & Baulcombe, D. C. (2001). Technical advance: tobacco rattle virus as a vector for analysis of gene function by silencing. *The Plant Journal*, *25*(2), 237–245.

Raudvere, U., Kolberg, L., Kuzmin, I., Arak, T., Adler, P., Peterson, H., & Vilo, J. (2019). g: Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). *Nucleic Acids Research*, *47*(W1), W191–W198.

Reina, J., Morilla, G., Bejarano, E. R., Rodríguez, M. D., & Janssen, D. (1999). First report of Capsicum annuum plants infected by tomato yellow leaf curl

virus. *Plant Disease*, *83*(12), 1176.

Robinson, J. T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E. S., Getz, G., & Mesirov, J. P. (2011). Integrative genomics viewer. *Nature Biotechnology*, *29*(1), 24–26.

Rochow, W. F., & Ross, A. F. (1955). Virus multiplication in plants doubly infected by potato viruses X and Y. *Virology*, *1*(1), 10–27.

Rodríguez-López, M. J., Garzo, E., Bonani, J. P., Fereres, A., Fernández-Muñoz, R., & Moriones, E. (2011). Whitefly resistance traits derived from the wild tomato Solanum pimpinellifolium affect the preference and feeding behavior of Bemisia tabaci and reduce the spread of Tomato yellow leaf curl virus. *Phytopathology*, *101*(10), 1191–1201. https://doi.org/10.1094/PHYTO-01-11-0028

Rodríguez-Negrete, E. A., Sánchez-Campos, S., Cañizares, M. C., Navas-Castillo, J., Moriones, E., Bejarano, E. R., & Grande-Pérez, A. (2014). A sensitive method for the quantification of virion-sense and complementary-sense DNA strands of circular single-stranded DNA viruses. *Scientific Reports*, *4*, 1–9. https://doi.org/10.1038/srep06438

Rojas, M. R., & Gilbertson, R. L. (2008). Emerging plant viruses: a diversity of mechanisms and opportunities. *Plant Virus Evolution*, 27–51.

Rojas, M. R., Macedo, M. A., Maliano, M. R., Soto-Aguilar, M., Souza, J. O., Briddon, R. W., Kenyon, L., Rivera Bustamante, R. F., Zerbini, F. M., & Adkins, S. (2018). World management of geminiviruses. *Annual Review of Phytopathology*, *56*, 637–677.

Roossinck, M. J. (2013). Plant virus ecology. *PLoS Pathogens*, *9*(5), e1003304.

Roselló, S., Díez, M. J., & Nuez, F. (1996). Viral diseases causing the greatest economic losses to the tomato crop. I. The Tomato spotted wilt virus—a review. *Scientia Horticulturae*, *67*(3–4), 117–150.

Sacks, E. J., & Francis, D. M. (2001). Genetic and environmental variation for tomato flesh color in a population of modern breeding lines. *Journal of the American Society for Horticultural Science*, *126*(2), 221–226. https://doi.org/10.21273/jashs.126.2.221

Sarmento, R. A., Lemos, F., Bleeker, P. M., Schuurink, R. C., Pallini, A., Oliveira, M. G. A., Lima, E. R., Kant, M., Sabelis, M. W., & Janssen, A. (2011). A herbivore that manipulates plant defence. *Ecology Letters*, *14*(3), 229–236. https://doi.org/10.1111/j.1461-0248.2010.01575.x

Seguin, J., Otten, P., Baerlocher, L., Farinelli, L., & Pooggin, M. M. (2014). MISIS: a bioinformatics tool to view and analyze maps of small RNAs derived from viruses and genomic loci generating multiple small RNAs. *Journal of Virological Methods*, *195*, 120–122.

Seo, J. K., Kim, M. K., Kwak, H. R., Choi, H. S., Nam, M., Choe, J., Choi, B., Han, S. J., Kang, J. H., & Jung, C. (2018). Molecular dissection of distinct symptoms induced by tomato chlorosis virus and tomato yellow leaf curl virus based on comparative transcriptome analysis. *Virology*,

516(December 2017), 1–20. https://doi.org/10.1016/j.virol.2018.01.001

Shelby, E. A., Moss, J. B., Andreason, S. A., Simmons, A. M., Moore, A. J., & Moore, P. J. (2020). Debugging: Strategies and considerations for efficient RNAi-mediated control of the whitefly Bemisia tabaci. *Insects*, *11*(11), 723.

Shi, X., Tang, X., Zhang, X., Zhang, D., Li, F., Yan, F., Zhang, Y., Zhou, X., & Liu, Y. (2018). Transmission efficiency, preference and behavior of Bemisia tabaci MEAM1 and MED under the influence of Tomato chlorosis virus. *Frontiers in Plant Science*, *8*, 2271.

Shirasu, K. (2009). The HSP90-SGT1 chaperone complex for NLR immune sensors. *Annual Review of Plant Biology*, *60*, 139–164. https://doi.org/10.1146/annurev.arplant.59.032607.092906

Simón, B., Cenis, J. L., & De La Rúa, P. (2007). Distribution patterns of the Q and B biotypes of Bemisia tabaci in the Mediterranean Basin based on microsatellite variation. *Entomologia Experimentalis et Applicata*, *124*(3), 327–336.

Syller, J. (2012). Facilitative and antagonistic interactions between plant viruses in mixed infections. *Molecular Plant Pathology*, *13*(2), 204–216. https://doi.org/10.1111/j.1364-3703.2011.00734.x

Syller, J. (2014). Biological and molecular events associated with simultaneous transmission of plant viruses by invertebrate and fungal vectors. *Molecular Plant Pathology*, *15*(4), 417–426.

Syller, J., & Grupa, A. (2016). Antagonistic within-host interactions between plant viruses: Molecular basis and impact on viral and host fitness. *Molecular Plant Pathology*, *17*(5), 769–782. https://doi.org/10.1111/mpp.12322

Tatineni, S., Alexander, J., Gupta, A. K., & French, R. (2019). Asymmetry in Synergistic Interaction between Wheat streak mosaic virus and Triticum mosaic virus in Wheat. *Molecular Plant-Microbe Interactions*, *32*(3), 336–350.

Tomitaka, Y., Abe, H., Sakurai, T., & Tsuda, S. (2015). Preference of the vector thrips F rankliniella occidentalis for plants infected with thrips-non-transmissible T omato spotted wilt virus. *Journal of Applied Entomology*, *139*(4), 250–259.

Torre, C., Donaire, L., Gómez-Aix, C., Juárez, M., Peterschmitt, M., Urbino, C., Hernando, Y., Agüero, J., & Aranda, M. A. (2018). Characterization of begomoviruses sampled during severe epidemics in tomato cultivars carrying the Ty-1 Gene. *International Journal of Molecular Sciences*, *19*(9), 2614.

Untiveros, M., Fuentes, S., & Salazar, L. F. (2007). Synergistic interaction of Sweet potato chlorotic stunt virus (Crinivirus) with carla-, cucumo-, ipomo-, and potyviruses infecting sweet potato. *Plant Disease*, *91*(6), 669–676.

Upadhyay, S. K., Chandrashekar, K., Thakur, N., Verma, P. C., Borgio, J. F., Singh, P. K., & Tuli, R. (2011). RNA interference for the control of whiteflies

(Bemisia tabaci) by oral route. *Journal of Biosciences*, *36*(1), 153–161. https://doi.org/10.1007/s12038-011-9009-1

Uppalapati, S. R., Ayoubi, P., Weng, H., Palmer, D. A., Mitchell, R. E., Jones, W., & Bender, C. L. (2005). The phytotoxin coronatine and methyl jasmonate impact multiple phytohormone pathways in tomato. *Plant Journal*, *42*(2), 201–217. https://doi.org/10.1111/j.1365-313X.2005.02366.x

van Kammen, A. (1999). Beijerinck's contribution to the virus concept—an introduction. In *100 years of virology: The birth and growth of a discipline* (pp. 1–8). Springer.

van Kleeff, P. J. M., Galland, M., Schuurink, R. C., & Bleeker, P. M. (2016). Small RNAs from Bemisia tabaci are transferred to solanum lycopersicum phloem during feeding. *Frontiers in Plant Science*, *7*(NOVEMBER2016), 1–12. https://doi.org/10.3389/fpls.2016.01759

Vargas-Mejía, P., Vega-Arreguín, J., Chávez-Calvillo, G., Ibarra-Laclette, E., & Silva-Rosales, L. (2020). Differential accumulation of innate-and adaptive-immune-response-derived transcripts during antagonism between Papaya ringspot virus and Papaya mosaic virus. *Viruses*, *12*(2), 230.

Verbeek, M., Dullemans, A. M., Van den Heuvel, J., Maris, P. C., & Van der Vlugt, R. A. A. (2007). Identification and characterisation of tomato torrado virus, a new plant picorna-like virus from tomato. *Archives of Virology*, *152*, 881–890.

Wang, H., Zhang, C., Dou, Y., Yu, B., Liu, Y., Heng-Moss, T. M., Lu, G., Wachholtz, M., Bradshaw, J. D., & Twigg, P. (2017). Insect and plant-derived miRNAs in greenbug (Schizaphis graminum) and yellow sugarcane aphid (Sipha flava) revealed by deep sequencing. *Gene*, *599*, 68–77.

Wang, Zhengming, Hardcastle, T. J., Pastor, A. C., Yip, W. H., Tang, S., & Baulcombe, D. C. (2018). A novel DCL2-dependent miRNA pathway in tomato affects susceptibility to RNA viruses. *Genes & Development*, *32*(17–18), 1155–1160.

Wang, Zhong, Gerstein, M., & Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics*, *10*(1), 57–63.

Wartig, L., Kheyr-Pour, A., Noris, E., De Kouchkovsky, F., Jouanneau, F., Gronenborn, B., & Jupin, I. (1997). Genetic analysis of the monopartite tomato yellow leaf curl geminivirus: roles of V1, V2, and C2 ORFs in viral pathogenesis. *Virology*, *228*(2), 132–140.

Wasternack, C., Stenzel, I., Hause, B., Hause, G., Kutter, C., Maucher, H., Neumerkel, J., Feussner, I., & Miersch, O. (2006). The wound response in tomato–role of jasmonic acid. *Journal of Plant Physiology*, *163*(3), 297–306.

Weiberg, A., Bellinger, M., & Jin, H. (2015). Conversations between kingdoms: small RNAs. *Current Opinion in Biotechnology*, *32*, 207–215.

Weiberg, A., Wang, M., Lin, F.-M., Zhao, H., Zhang, Z., Kaloshian, I., Huang, H.-D., & Jin, H. (2013). Fungal small RNAs suppress plant immunity by

hijacking host RNA interference pathways. *Science*, *342*(6154), 118–123.

Wintermantel, W M, Wisler, G. C., Anchieta, A. G., Liu, H.-Y., Karasev, A. V, & Tzanetakis, I. E. (2005). The complete nucleotide sequence and genome organization of Tomato chlorosis virus. *Archives of Virology*, *150*(11), 2287–2298.

Wintermantel, William M, Cortez, A. A., Anchieta, A. G., Gulati-Sakhuja, A., & Hladky, L. L. (2008). Co-infection by two criniviruses alters accumulation of each virus in a host-specific manner and influences efficiency of virus transmission. *Phytopathology*, *98*(12), 1340–1345.

Wintermantel, William M, & Wisler, G. C. (2006). Vector specificity, host range, and genetic diversity of Tomato chlorosis virus. *Plant Disease*, *90*(6), 814–819.

Wisler, G. C., Li, R. H., Liu, H.-Y., Lowry, D. S., & Duffus, J. E. (1998). Tomato chlorosis virus: a new whitefly-transmitted, phloem-limited, bipartite closterovirus of tomato. *Phytopathology*, *88*(5), 402–409.

Xie, W., Yang, X., Chen, C., Yang, Z., Guo, L., Wang, D., Huang, J., Zhang, H., Wen, Y., Zhao, J., Wu, Q., Wang, S., Coates, B. S., Zhou, X., & Zhang, Y. (2018). The invasive MED/Q Bemisia tabaci genome: A tale of gene loss and gene gain. *BMC Genomics*, *19*(1), 1–15. https://doi.org/10.1186/s12864-018-4448-9

Xu, J., De Barro, P. J., & Liu, S. S. (2010). Reproductive incompatibility among genetic groups of Bemisia tabaci supports the proposition that the whitefly is a cryptic species complex. *Bulletin of Entomological Research*, *100*(3), 359–366.

Yan, Z., Wolters, A.-M. A., Navas-Castillo, J., & Bai, Y. (2021). The global dimension of tomato yellow leaf curl disease: Current status and breeding perspectives. *Microorganisms*, *9*(4), 740.

Yue, H., Huang, L.-P., Ding-Yi-Hui Lu, Z.-H., Zhang, Z. Z., Zhang, D.-Y., Zheng, L.-M., Gao, Y., Tan, X.-Q., Zhou, X.-G., & Shi, X.-B. (2021). Integrated Analysis of microRNA and mRNA Transcriptome Reveals the Molecular Mechanism of Solanum lycopersicum Response to Bemisia tabaci and Tomato chlorosis virus. *Frontiers in Microbiology*, *12*.

Zanardo, L. G., de Souza, G. B., & Alves, M. S. (2019). Transcriptomics of plant–virus interactions: a review. *Theoretical and Experimental Plant Physiology*, *31*(1), 103–125. https://doi.org/10.1007/s40626-019-00143-z

Zhang, C., Yan, S.-Q., Shen, B.-B., Ali, S., Wang, X.-M., Jin, F.-L., Cuthbertson, A. G. S., & Qiu, B.-L. (2017). RNAi knock-down of the Bemisia tabaci Toll gene (BtToll) increases mortality after challenge with destruxin A. *Molecular Immunology*, *88*, 164–173.

Zhang, L., Jing, X., Chen, W., Wang, Y., Lin, J., Zheng, L., Dong, Y., Zhou, L., Li, F., & Yang, F. (2019). Host plant-derived miRNAs potentially modulate the development of a cosmopolitan insect pest, Plutella xylostella. *Biomolecules*, *9*(10), 602.

Zhang, L. P., Zhang, Y. J., Zhang, W. J., Wu, Q. J., Xu, B. Y., & Chu, D. (2005). Analysis of genetic diversity among different geographical populations and determination of biotypes of Bemisia tabaci in China. *Journal of Applied Entomology*, *129*(3), 121–128.

Zhang, Tao, Jin, Y., Zhao, J.-H., Gao, F., Zhou, B.-J., Fang, Y.-Y., & Guo, H.-S. (2016). Host-induced gene silencing of the target gene in fungal cells confers effective resistance to the cotton wilt disease pathogen Verticillium dahliae. *Molecular Plant*, *9*(6), 939–942.

Zhang, Tong, LUAN, J., QI, J., HUANG, C., Li, M., ZHOU, X., & LIU, S. (2012). Begomovirus–whitefly mutualism is achieved through repression of plant defences by a virus pathogenicity factor. *Molecular Ecology*, *21*(5), 1294–1304.

Zhang, Y., Wiggins, B. E., Lawrence, C., Petrick, J., Ivashuta, S., & Heck, G. (2012). Analysis of plant-derived miRNAs in animal small RNA datasets. *BMC Genomics*, *13*, 1–8.

Zhao, J., Sun, X., Dai, H., Zhang, X., Zhang, D., & Zhu, X. (2023). Changes in Gene Expression of Whiteflies, Bemisia tabaci MED Feeding on Tomato Plants Infected by One of the Criniviruses, Tomato Chlorosis Virus through Transcriptome Analysis. *International Journal of Genomics*, *2023*.

Zhao, L., Li, G., Liu, Y., Guo, J., Wei, J., & Zhu, X. (2014). Molecular identification on mixed infections of Tomato chlorosis virus and Tomato yellow leaf curl virus. *China Vegetables*, *12*, 15–20.

Zhao, S., Gong, P., Ren, Y., Liu, H., Li, H., Li, F., & Zhou, X. (2022). The novel C5 protein from tomato yellow leaf curl virus is a virulence factor and suppressor of gene silencing. *Stress Biology*, *2*(1), 19.

Zhou, Y., Yan, J. Y., Qiao, G. H., Liu, M., Zhang, W., & Li, X. H. (2015). First report of Tomato chlorosis virus infecting eggplant (Solanum melongena) in China. *Plant Disease*, *99*(11), 1657.

Zrachya, A., Glick, E., Levy, Y., Arazi, T., Citovsky, V., & Gafni, Y. (2007). Suppressor of RNA silencing encoded by Tomato yellow leaf curl virus-Israel. *Virology*, *358*(1), 159–165.