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Implications of Mixed Infections of Two Strains
of a Plant Virus on its Epidemiology,
Evolution and Interaction with the Host

Implicaciones de las Infecciones Mixtas de dos Cepas
de un Virus de Plantas en su Epidemiología,
Evolución e Interacción con el Huésped

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“Implications of mixed infections of two strains of a plant virus on its epidemiology, evolution and interaction with the host”

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“La prueba final del conocimiento es tu capacidad de transmitirlo a otra persona”

Richard P. Feynman (1918 – 1988)

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Resumen

Resumen

Los virus de plantas causan enfermedades importantes en los cultivos de todo el mundo, siendo responsables de pérdidas económicas significativas al reducir el rendimiento y calidad de las cosechas (Jones, 2021; Jones and Naidu, 2019; Loebenstein, 2008; Patil, 2021). Las infecciones mixtas de virus de plantas son muy comunes en la naturaleza, conduciendo a una variedad de interacciones entre los virus implicados que pueden tener efectos de gran alcance en la ecología y evolución de la enfermedad (Alcaide *et al.*, 2020). De hecho, algunas de las enfermedades de plantas más destructivas están causadas por infecciones mixtas de dos virus en la misma planta (Goodman and Ross, 1974; Jones, 2021; Rentería-Canett *et al.*, 2011; Syller, 2012). Por tanto, el estudio de las infecciones mixtas es necesario para entender el impacto que las interacciones virus-virus y virus-huésped pueden tener en la epidemiología y dinámicas evolutivas de las poblaciones virales implicadas.

En este contexto, este trabajo se ha centrado en el virus del mosaico del pepino dulce (*pepino mosaic virus*; PepMV), el cual produce una enfermedad que afecta al cultivo del tomate y que causa importantes pérdidas económicas en Europa y América (Gómez *et al.*, 2012; Hanssen and Thomma, 2010). PepMV es un virus flexuoso filamentoso que pertenece al género *Potexvirus* (familia *Alphaflexiviridae*). Este virus fue descrito por primera vez en 1974 en Perú afectando a cultivos de pepino dulce (*Solanum muricatum*) (Jones *et al.*, 1980). En 1999, PepMV se encontró infectando a cultivos de tomate en Holanda (van der Vlugt *et al.*, 2000). Desde entonces se ha descrito infectando plantas de tomate en otros países. En la actualidad, de acuerdo con la Organización Europea de Protección de Plantas (*European Plant Protection Organization*; EPPO), este virus está presente en 33 países, encontrándose extendido en España, Italia, Marruecos y Perú (EPPO 2021). El genoma de PepMV es una cadena simple de ARN de polaridad positiva de aproximadamente 6.4 kb de longitud con cinco marcos abiertos de lectura (*open reading frames*; ORFs) (Aguilar *et al.*, 2002). El ORF1 codifica una ARN polimerasa dependiente de ARN (RdRp). Los ORFs 2, 3 y 4 constituyen el triple bloque de genes (*triple gene block*; TGB) que codifica las proteínas TGB1, TGB2 y

TGB3, implicadas en el movimiento célula a célula y a larga distancia del virus (Morozov and Solovyev, 2003). Por último, el ORF5 codifica la proteína de la cápsida del virus (*coat protein*; CP), implicada en el movimiento a larga distancia del virus y en la encapsidación del ARN viral (Agirrezabala *et al.*, 2015; Morozov and Solovyev, 2003). Además, tanto la TGB1 como la CP actúan como supresores del silenciamiento (Mathioudakis *et al.*, 2014) y todas las TGBs en su conjunto y la CP también se encargan de la formación de los complejos de replicación virales (Linnik *et al.*, 2013). En cuanto a la diversidad genética de PepMV, se han descrito cinco cepas hasta el momento: la Peruana original (LP), la Europea (EU), la Americana (US1), la Chilena (CH2) y la nueva Peruana (PES) (Hanssen and Thomma, 2010; Moreno-Perez *et al.*, 2014). En España, la cepa EU fue predominante hasta 2004 (Cotillon *et al.*, 2002; Pagán *et al.*, 2006), cuando la cepa CH2 emergió y desplazó rápidamente a la EU (Gómez *et al.*, 2009), lo cual ocurrió también en otros países europeos y en Norte América (Hanssen *et al.*, 2008; Ling *et al.*, 2013; Pospieszny and Borodynko, 2006). Durante los años posteriores, la situación epidemiológica de PepMV en los cultivos de tomate se ha mantenido estable en España, con la cepa CH2 siendo la predominante y la cepa EU circulando principalmente en infecciones mixtas (Gómez-Aix *et al.*, 2019). En infecciones mixtas de dos aislados de PepMV-EU y PepMV-CH2 se observó una relación de antagonismo asimétrico entre ambos, en la que la acumulación de PepMV-CH2 se redujo significativamente en la presencia de PepMV-EU en infecciones mixtas, mientras que la acumulación de PepMV-EU no varió entre infecciones simples y mixtas (Gómez *et al.*, 2009). Esta relación de antagonismo asimétrico en infecciones mixtas podría explicar el patrón epidemiológico encontrado en los cultivos de tomate, donde los aislados de PepMV-EU se mantienen en infecciones mixtas a pesar de su menor eficacia biológica *in planta*.

Con estos antecedentes, el objetivo principal de la tesis fue profundizar en la caracterización de las interacciones virus-virus y virus-huésped en infecciones mixtas de aislados de PepMV de dos cepas distintas. Estas interacciones pueden variar dependiendo del aislado del virus, el tipo de infección y la ecología del huésped. Por tanto, esta tesis ha abordado el estudio de la interacción entre PepMV-

EU y PepMV-CH2 desde diferentes perspectivas con distintos aislados, bajo distintas condiciones abióticas, a diferentes tiempos post-inoculación, después de someter a la población a varios pases y teniendo en cuenta la interacción con el huésped.

Más específicamente, los objetivos de esta tesis han sido:

1. Analizar la diversidad genética de la población de PepMV en Murcia a lo largo de distintas temporadas de producción de tomate y evaluar la eficacia biológica de seis aislados de PepMV-CH2 de diferentes localidades en infecciones simples y mixtas.

2. Estudiar el efecto de un factor abiótico, la temperatura, en la diversidad genética y la acumulación viral de dos aislados de dos cepas de PepMV.

3. Determinar los resultados de las súper infecciones de dos cepas de PepMV, así como la influencia de las infecciones mixtas en la transmisión y diversificación del virus.

4. Describir la respuesta transcriptómica de plantas de tomate a infecciones simples y mixtas de PepMV.

Los resultados del primer objetivo se describen en el capítulo uno de esta tesis. Primero estudié el patrón epidemiológico de PepMV en dos localidades del sureste español, observando que era similar al descrito con anterioridad, donde las infecciones por aislados de PepMV-CH2 eran mayoritarias y aquellas causadas por aislados de PepMV-EU se encontraban principalmente en infecciones mixtas. Sin embargo, se identificó un patrón epidemiológico diferente entre ambas localidades, Mazarrón y Águilas, a pesar de su cercanía geográfica. Mientras que en Águilas solo se identificaron aislados de la cepa PepMV-CH2, en Mazarrón se encontraron aislados pertenecientes a las dos cepas, CH2 y EU. Además, en un análisis filogenético de la población de PepMV-CH2 se encontró una clara diferenciación genética atendiendo a la localidad, observando a su vez una mayor diversidad genética en los aislados provenientes de Mazarrón, donde estaban coexistiendo aislados de ambas cepas. Determinamos entonces la eficacia biológica de seis

aislados de PepMV-CH2 que provenían de las dos localidades, tres de Mazarrón, de plantas con infecciones mixtas, y tres de Águilas, de plantas con infecciones simples. La eficacia biológica de estos aislados de PepMV-CH2 se determinó en presencia y ausencia de un aislado PepMV-EU, observando un coste en la eficacia biológica asociado a la presencia de PepMV-EU independientemente del origen de PepMV-CH2 y, por tanto, concluí que no había una adaptación genética de los aislados de PepMV-CH2 que procedían de infecciones mixtas en campo.

El segundo objetivo se ha abordado como se describe en el capítulo dos de esta tesis doctoral. Para ello se inocularon plantas de tomate con un aislado de PepMV-EU y otro de PepMV-CH2 en infecciones simples y mixtas bajo dos condiciones de temperatura (20ºC y 30ºC). En cuanto a la acumulación viral, se observó que en términos generales PepMV-CH2 se acumuló a mayores niveles que PepMV-EU, teniendo el tiempo y la temperatura un papel importante en la acumulación viral y en el antagonismo asimétrico. La acumulación viral tanto de PepMV-EU como de PepMV-CH2 fue más productiva a 20ºC que a 30ºC, pero además también se vio afectada por el tiempo, ya que a 60 días post-inoculación (dpi) se dejó de observar una interacción antagonista asimétrica entre ambos en infecciones mixtas. En cuanto a la ocurrencia de mutaciones, la mayoría de ellas se encontraron en frecuencias bajas (entre 0.01 y 0.1). En general, la población de PepMV-CH2 acumuló un mayor número de mutaciones que la población de PepMV-EU, no encontrándose diferencias entre infecciones simples y mixtas a 20ºC. Sin embargo, el número medio de mutaciones en las poblaciones virales se incrementó significativamente con la temperatura, siendo a su vez dependiente del aislado. De esta manera, se encontró que a 30ºC la población de PepMV-EU *in planta* tuvo un mayor número de mutaciones en infecciones simples comparado con infecciones mixtas, mientras que en el caso de PepMV-CH2, un mayor número de mutaciones se encontraron en infecciones mixtas con respecto a simples. Por tanto, pudimos concluir que las poblaciones de ambos aislados de PepMV difirieron en su carga mutacional de manera dependiente de tipo de infección y de temperatura.

El tercer objetivo se desarrolló como se describe en el capítulo tres de esta tesis. Primero se determinó que la edad de la planta tenía un efecto en la acumulación viral, aunque no afectó al antagonismo asimétrico en infecciones mixtas. En plantas pre-inoculadas con PepMV-EU se observó que al realizar una segunda inoculación con PepMV-CH2, este reducía su acumulación. Además, esta reducción era dependiente del tiempo transcurrido entre la primera y segunda inoculación. En esas mismas plantas, la acumulación de PepMV-EU no se vio afectada tras la inoculación de PepMV-CH2. Por otro lado, se observó que en plantas pre-infectadas con PepMV-CH2, la acumulación de PepMV-EU se vio estimulada comparada con la determinada en infecciones simples. A su vez, la acumulación de PepMV-CH2 en esas plantas no se vio reprimida tras la inoculación de PepMV-EU excepto cuando se realizó tras un lapso corto de tiempo (3 días). Así, los resultados en este objetivo indicaron una interacción sinergística que era favorable para PepMV-EU cuando PepMV-CH2 se inoculaba primero, y una relación antagonística contra PepMV-CH2 en infecciones mixtas con PepMV-EU. Otro de los sub-objetivos en este apartado era determinar el efecto de la infección mixta en la transmisión mecánica del virus. Para ello se usaron distintas fuentes de inóculo que provenían de plantas infectadas con un solo virus, con ambos virus a la vez (infecciones simultáneas) o con ambos virus en infecciones desfasadas (súper infecciones). Así se consiguió una batería de extractos con un amplio rango de cargas virales que se usaron como inóculo. Al analizar el número de plantas infectadas por cada uno de los extractos, obtuve una fuerte correlación positiva entre acumulación y transmisión de virus, no encontrando un sesgo diferencial entre infecciones simples y mixtas. Estos resultados sugirieron por tanto que las infecciones mixtas pueden tener un impacto importante en la transmisión del virus a través de la modulación de la acumulación viral que producen. El último sub-objetivo de esta parte de mi tesis fue el estudio del efecto de las infecciones mixtas en la diversificación genética de PepMV. En este caso formulé la hipótesis de que la reducción de la acumulación de PepMV-CH2 en las infecciones mixtas podría dar lugar a deriva genética asociada a cuellos de botella durante la transmisión (Acosta-Leal *et al.*, 2011; Kutnjak *et al.*, 2017). Se secuenciaron amplicones de una región de la RdRp de PepMV de tres linajes de

plantas infectadas con PepMV-EU, PepMV-CH2 o ambos a lo largo de ocho pases y se compararon las secuencias con los clones infecciosos usados para iniciar cada linaje. No se encontró correlación entre el número de pase y el número de mutaciones ni tampoco diferenciación entre los linajes. Sí que se encontraron diferencias significativas en el número medio de variantes entre PepMV-EU y PepMV-CH2, con una mayor diversidad nucleotídica para PepMV-CH2. Sin embargo, la nube de variantes *in planta* se mantuvo constante a lo largo de los pases y las infecciones mixtas no se pudieron asociar con evidentes cuellos de botella genéticos.

El cuarto y último objetivo de esta tesis se desarrolló como se describe en el anexo uno. Se llevó a cabo un análisis transcriptómico de plantas de tomate control e infectadas con PepMV-EU, PepMV-CH2 o ambos, a distintos tiempos post-inoculación. En general, las infecciones simples de PepMV-CH2 y las infecciones mixtas tuvieron efectos más profundos en el transcriptoma del huésped que las infecciones simples de PepMV-EU, lo cual también concuerda con los títulos virales de cada uno *in planta*. Cuando comparamos las infecciones simples, observamos que cada cepa modulaba el transcriptoma de manera diferencial, aunque ambas coincidían en una mayor desregulación de genes a tiempos tempranos (7 dpi). Al comparar las infecciones simples con respecto a las mixtas, encontramos que a 7 dpi las infecciones mixtas causaban alteraciones transcriptómicas que parecían ser la suma de aquellas causadas por las infecciones simples por separado. Sin embargo, a 14 y 21 dpi, las infecciones mixtas indujeron cambios en el transcriptoma del huésped que eran diferentes a la suma de las alteraciones causadas en cada infección simple. A continuación, centré mi interés en la búsqueda de genes del huésped que pudieran estar implicados en la relación de antagonismo asimétrico entre PepMV-EU y PepMV-CH2. Para ello identifiqué los genes regulados al alza por PepMV-EU (en infecciones simples o mixtas) pero no por PepMV-CH2. Dentro de esa lista de genes estaban algunos genes implicados en silenciamiento (*AGO1a*, *AGO2a*, *DCL2b* y *DCL2d*) y, concretamente, me centré en *AGO2* por estar diferencialmente regulado de manera significativa entre plantas infectadas por PepMV-EU y PepMV-CH2. El patrón de expresión de *AGO2* se validó de forma cuantitativa en plantas de

tomate y de *Nicotiana benthamiana*. El mismo tipo de experimento se realizó en plantas mutantes *ago2* de *N. benthamiana*, que fueron híper-susceptibles a la infección por PepMV, pero no mostraron un cambio de tendencia en la interacción antagonista entre PepMV-EU y PepMV-CH2.

Por tanto, las conclusiones que se desprenden de esta tesis doctoral son:

1. El patrón epidemiológico de PepMV en el cultivo de tomate del sureste español se ha mantenido a lo largo de los años, con aislados de PepMV-CH2 siendo predominantes y aislados de PepMV-EU persistiendo en menor proporción y principalmente en infecciones mixtas.

2. Dos áreas productoras de tomate geográficamente próximas mostraron un patrón de infección por PepMV diferente; se encontraron infecciones simples en Águilas mientras que en Mazarrón se observaron infecciones simples y mixtas.

3. La variabilidad genética de la población de PepMV-CH2 fue mayor en Mazarrón, donde las infecciones mixtas persistieron.

4. Los aislados PepMV-CH2 en plantas de tomate mostraron un coste en su eficacia biológica asociado a la presencia de un aislado PepMV-EU. Este coste en la eficacia biológica fue independiente del origen del aislado PepMV-CH2 (infecciones simples o mixtas).

5. En términos generales, la acumulación de PepMV-CH2 *in planta* fue mayor que la de PepMV-EU, aunque el tiempo post-infección y la temperatura tuvieron un papel relevante en la acumulación de PepMV, siendo esta más productiva a 20ºC que a 30ºC.

6. El antagonismo asimétrico entre PepMV-EU y PepMV-CH2 fue dependiente del tiempo, siendo particularmente evidente a tiempos tempranos post-infección.

7. La población de PepMV-CH2 *in planta* mostró un mayor número variantes que la población de PepMV-EU. Sin embargo, no se encontraron diferencias en el número de mutaciones entre infecciones simples y mixtas a 20ºC ni para PepMV-EU ni para PepMV-CH2.

8. El número medio de mutaciones se incrementó de manera significativa con la temperatura. La población de PepMV-EU *in planta* acumuló un mayor número de mutaciones en infecciones simples, mientras que la población de PepMV-CH2 acumuló un mayor número de mutaciones en infecciones mixtas. Por tanto, la carga mutacional fue dependiente de la temperatura y de la cepa viral.

9. La edad de la planta tuvo un efecto en la acumulación de PepMV-CH2, observando más acumulación viral cuanto mayor era la planta.

10. En plantas pre-inoculadas con PepMV-EU y retadas con PepMV-CH2, la acumulación de este último se reprimió en comparación con las infecciones simples, de forma similar a lo que se observó en infecciones mixtas simultáneas. Sin embargo, la acumulación de PepMV-EU no cambió significativamente despúes de la inoculación con el virus reto.

11. En plantas pre-inoculadas con PepMV-CH2 y retadas con PepMV-EU, la acumulación de este último se estimuló en comparación con las infecciones simples. En este caso, la acumulación de PepMV-CH2 no se reprime, al menos a tiempos tardíos después de la infección con el virus reto.

12. Se encontró una fuerte correlación positiva entre la acumulación de virus y su transmisión. Por tanto, las infecciones mixtas pueden tener un impacto importante en la transmisión del virus debido al efecto que pueden causar en la acumulación viral.

13. Durante varios pases seriados en plantas de tomate, el número de variantes *in planta* fue mayor para PepMV-CH2 que para PepMV-EU, aunque éste permaneció bastante constante con los pases, sin que las infecciones mixtas estuvieran asociadas con evidentes cuellos de botella genéticos.

14. Cada cepa viral moduló el transcriptoma del huésped de manera diferencial, aunque para ambas cepas se encontraron alteraciones transcriptómicas más profundas a tiempos de infección tempranos.

15. Las infecciones mixtas indujeron cambios en el transcriptoma del huésped que fueron diferentes a la suma de las alteraciones causadas por cada infección simple a 14 y 21 dpi, pero no a tiempos tempranos (7 dpi).

16. *AGO1a*, *AGO2a*, *DCL2b* y *DCL2d* fueron los principales determinantes de la diferenciación entre la infección de PepMV-EU y PepMV-CH2. Sin embargo, solo se encontraron diferencias estadísticamente significativas en los FPKMs escalados de *AGO2a* a 7 dpi.

17. *AGO2a* tiene un papel claro en la infección de PepMV, pero no parece ser el responsable directo o el único responsable del antagonismo entre PepMV-EU y PepMV-CH2, al menos en *N. benthamiana*.

Abstract

Abstract

Pepino mosaic virus (PepMV) is a single stranded (ss) positive sense (+) RNA potexvirus that affects tomato crops worldwide. In this thesis, I have studied virus-virus and virus-host interactions in mixed strain PepMV infections. These interactions can vary depending on virus isolate, type of infection and host ecology. So far, an *in planta* antagonistic interaction has been described between an isolate of the Chilean (CH2) strain and an isolate of the European (EU) strain, in which PepMV-CH2 accumulation was repressed during mixed infections. This antagonistic interaction was found to occur with different CH2 isolates, regardless of their origin. Subsequently, the effect that an abiotic factor, such as temperature, could have on the interaction between PepMV-EU and PepMV-CH2 was studied. Temperature had an effect on viral accumulation, with PepMV infection being more productive at the lower temperature (20ºC *versus* 30ºC). In turn, infection time also played a role in the interaction between PepMV-EU and PepMV-CH2, since at long post-inoculation times the antagonistic interaction between both isolates was no longer observed. Regarding the mutational load, the average number of variants in viral populations increased significantly with temperature, being in turn isolate-dependent.

The role of super-infections in the asymmetric antagonism shown by PepMV-EU and PepMV-CH2 was also studied. PepMV-EU had a protective effect against PepMV-CH2 super-infection, however, PepMV-EU accumulation was increased in plants pre-inoculated with PepMV-CH2. Regarding the effect of the type of infection (single or mixed) on viral transmission, a strong positive correlation was observed between viral accumulation and transmission, regardless of the type of infection in the inoculum source. The genetic diversity of PepMV-EU and PepMV-CH2 in single and mixed infections was also analysed in a serial passaging assay. For PepMV-EU, no significant differences were found in the average number of nucleotide substitutions between single and mixed infections. However, a higher number of substitutions was found in single PepMV-CH2 infections compared to

mixed infections. Comparing PepMV-EU and PepMV-CH2, a higher nucleotide diversity was found for PepMV-CH2 population *in planta*.

Finally, in an attempt to understand the mechanism(s) underlying the virus-virus interaction between PepMV-EU and PepMV-CH2, a transcriptomic analysis of tomato plants infected with PepMV-EU, PepMV-CH2 or both, was carried out. First, transcriptomes of single infected plants were compared, showing that each viral strain differentially modulated the host transcriptome and that the most pronounced alterations occurred at early times post-infection. It was also shown that the host transcriptome response to mixed infections differed from single infections at late post-inoculation times. A search for host genes that could potentially be involved in the antagonism between PepMV-EU and PepMV-CH2 was performed. It was found that *AGO1a*, *AGO2a*, *DCL2b* and *DCL2d* genes, which are involved in the antiviral silencing pathway, were upregulated during PepMV-EU infection, but not during PepMV-CH2 infection, at early post-infection times. The pattern of *AGO2a* expression was validated in tomato and *Nicotiana benthamiana* plants; however, *N. benthamiana ago2* mutant plants, while hyper-susceptible to PepMV, did not show a change in the trend of the antagonistic interaction between the isolates of the two PepMV strains.

List of abbreviations

List of abbreviations

Virus abbreviations (in alphabetical order)

AltMV	alternanthera mosaic virus
BYDV	barley yellow dwarf virus
CCMV	cowpea chlorotic mottle virus
CMV	cucumber mosaic virus
CPsV	citrus psorosis virus
CVYV	cucumber vein yellowing virus
CYDV	cereal yellow dwarf virus
CymMV	cymbidium mosaic virus
CYSDV	cucurbit yellow stunting disorder virus
GRAV	groundnut rosette assistor virus
GRV	groundnut rosette virus
MCMV	maize chlorotic mottle virus
MDMV	maize dwarf mosaic virus
NMV	narcissus mosaic virus
PAMV	potato aucuba mosaic virus
PepMV	pepino mosaic virus
PLRV	potato leaf roll virus
PVA	potato virus A
PVX	potato virus X
PVY	potato virus Y
RSV	rice strip virus
RTBV	rice tungro bacilliform virus
RTSV	rice tungro spherical virus
SCMV	sugarcane mosaic virus
SMV	soybean mosaic virus
SPCSV	sweet potato chlorotic stunt virus
SPFMV	sweet potato feathery mottle virus
SPMMV	sweet potato mild mottle virus
SVX	scallion virus X
TAV	tomato aspermy virus

TCV	turnip crinkle virus
TEV	tobacco etch virus
TICV	tomato infectious chlorosis virus
TMGMV	tobacco mild green mottle virus
TMV	tobacco mosaic virus
ToBRFV	tomato brown rugose fruit virus
ToCV	tomato chlorosis virus
ToMV	tomato mosaic virus
TSWV	tomato spotted wilt virus
ToTV	tomato torrado virus
TuMV	turnip mosaic virus
TYLCV	tomato yellow leaf curl virus
WMV	watermelon mosaic virus
WSMV	wheat streak mosaic virus

Other abbreviations (in alphabetical order)

3’ UTR	3’ untranslated region
5’ UTR	5’ untranslated region
AG	Águilas
AGO	Argonaute
BIC	Bayesian information criterion
cDNA	complementary DNA
CP	capsid protein
DCL	Dicer-like
DEG	differentially expressed gene
dsRNA	double stranded RNA
EPPO	European Plant Protection Organization
FPKM	fragments per kilo base per million mapped reads
GLM	general linear model
GO	Gene Ontology
gRNA	genomic RNA
INDEL	inserted or deleted nucleotide
LRT	likelihood-ratio test

miRNA	microRNA
mRNA	messenger RNA
MLND	maize lethal necrosis disease
MOI	multiplicity of infection
MZ	Mazarrón
NGS	next generation sequencing
ORF	open reading frame
PCA	principal component analysis
PCR	polymerase chain reaction
RDR	Host RNA dependent RNA polymerase
RdRp	Viral RNA dependent RNA polymerase
RISC	RNA-induced silencing complexes
RT	reverse transcription
RTD	rice tungro disease
RT-qPCR	quantitative reverse transcription PCR
sgRNA	subgenomic RNA
SIE	superinfection exclusion
SNP	single nucleotide polymorphism
SPVD	sweet potato virus disease
siRNA	small interfering RNA
sRNA	small RNA
TGB	triple gene block
tRNA	total RNA
VL	viral load
vRNA	viral RNA
vsiRNA	virus-derived small interfering RNA
VSR	viral suppressor of RNA silencing
YDD	yellow dwarf disease

1. Introduction

1. Introduction

Plant viruses are responsible for important crop diseases worldwide, causing significant economic losses by reducing yield and quality of crops (Jones, 2021; Jones and Naidu, 2019; Loebenstein, 2008; Patil, 2021). Mixed infections of plant viruses are very common in nature, which usually lead to a range of ecological interactions between viruses that may consequently have far-reaching implications on the disease ecology and evolution (Alcaide *et al.*, 2020). In fact, some of the most destructive plant diseases are caused by mixed infections of two viruses (Goodman and Ross, 1974; Jones, 2021; Rentería-Canett *et al.*, 2011; Syller, 2012). Then the study of mixed infections is necessary to shed light on the epidemiology and evolution of the viruses involved and to determine the impact that virus-virus and virus-host interactions can have on them. However, although there is a growing body of literature dealing with mixed infections of viruses belonging to different species, mixed infections of viruses from different strains of the same species are often overlooked.

In this context, this work has focused on pepino mosaic virus (PepMV), which induces a disease affecting tomato crops causing important economic losses in Europe and America (Gómez *et al.*, 2012; Hanssen and Thomma, 2010). PepMV is a filamentous flexuous virus belonging to the genus *Potexvirus*, in the family *Alphaflexiviridae*. The population structure and nucleotide diversity of PepMV in different tomato producing areas in Spain have been studied for this thesis, in addition to experimentally examine the nucleotide diversity of two isolates belonging to different strains of PepMV in single and mixed infections under two different environmental conditions. The relationship between these PepMV isolates in co-infections and in over infections have been also investigated, characterizing their ability to induce heterologous cross-protection and their transmission and nucleotide diversity after passaging. Finally, the virus-virus interaction in mixed infections has been evaluated with respect to its host. The results presented in this thesis contribute to understand the maintenance of mixed infections of PepMV

strains in tomato crops and the epidemiological patterns of the PepMV disease observed in the southeast of Spain in the last years.

In the following sections I will introduce some aspects needed for the understanding, interpretation and discussion of the results, focusing on the PepMV biology and strain diversity, the viral interactions in mixed infections, the effect of environmental conditions in these interactions and the main host process and factors controlling host susceptibility to viruses.

1.1. Viruses infecting tomato crops in Spain

Tomato is one of the main horticultural crops in extension and production worldwide, constituting a major component on our agricultural economy. Spain is the eighth largest tomato producer in the world, with 55,470 ha of cultivated area and 4,312,900 Tm of production (FAOSTAT, 2020). The three main tomato producing areas in Spain are Extremadura (1,782,549 Tm), Andalucía (1,738,684 Tm) and Murcia (217,187 Tm), with also a significant production in Navarra, Comunidad Valenciana and Canarias (MAPA 2020).

Tomato plants can be affected by several pests and diseases that affect negatively the yield and/or the quality of the product, causing important economic losses. Among tomato diseases, those that are caused by viruses are of particular relevance because of the epidemic impact in crops that are often complex to control. This difficulty is mainly due to the combination of multiple factors that interfere with the lack of an early detection and effectiveness of the countermeasures. In particular, the impact of tomato viral diseases varies depending on the virus or viruses involved, environmental conditions, tomato cultivar and cultural practices, among other factors (Hanssen *et al.*, 2010). In Spain, viruses causing major losses in tomato crops are: tomato yellow leaf curl virus (TYLCV), one of the begomoviruses causing the tomato yellow leaf curl disease that is whitefly transmitted (Díaz-Pendón *et al.*, 2010); the potexvirus PepMV, transmitted efficiently by contact (Gómez-Aix *et al.*, 2019; Hanssen *et al.*, 2009); the crinivirus

tomato chlorosis virus (ToCV), also whitefly transmitted (Fortes *et al.*, 2012) and the tospovirus tomato spotted wilt virus (TSWV), transmitted by thrips (Roselló *et al.*, 1996). In addition, other virus-induced diseases have been described in tomato crops in Spain, such as those caused by: cucumber mosaic virus (CMV), potato virus Y (PVY), tomato mosaic virus (ToMV) and tomato torrado virus (ToTV) (Velasco *et al.*, 2020; Verbeek *et al.*, 2007). Recently, it has been identified the presence of tomato brown rugose fruit virus (ToBRFV) in Spain (Alfaro-Fernández *et al.*, 2020), declared as present, with few occurrences (EPPO, 2021).

1.2. Pepino mosaic virus (PepMV)

PepMV was first described in Peru affecting pepino dulce (*Solanum muricatum*) crops in 1974 (Jones *et al.*, 1980). In 1999, PepMV was found infecting tomato crops in The Netherlands (van der Vlugt *et al.*, 2000). One year later, it was described infecting tomato plants in Spain (Jordá *et al.*, 2001) and in other European countries; UK, Germany, and France (EPPO, 2000). According to the European Plant Protection Organization (EPPO), PepMV is present in 33 countries, being widespread in Spain, Italy, Morocco and Peru (EPPO, 2021) (Figure 1).

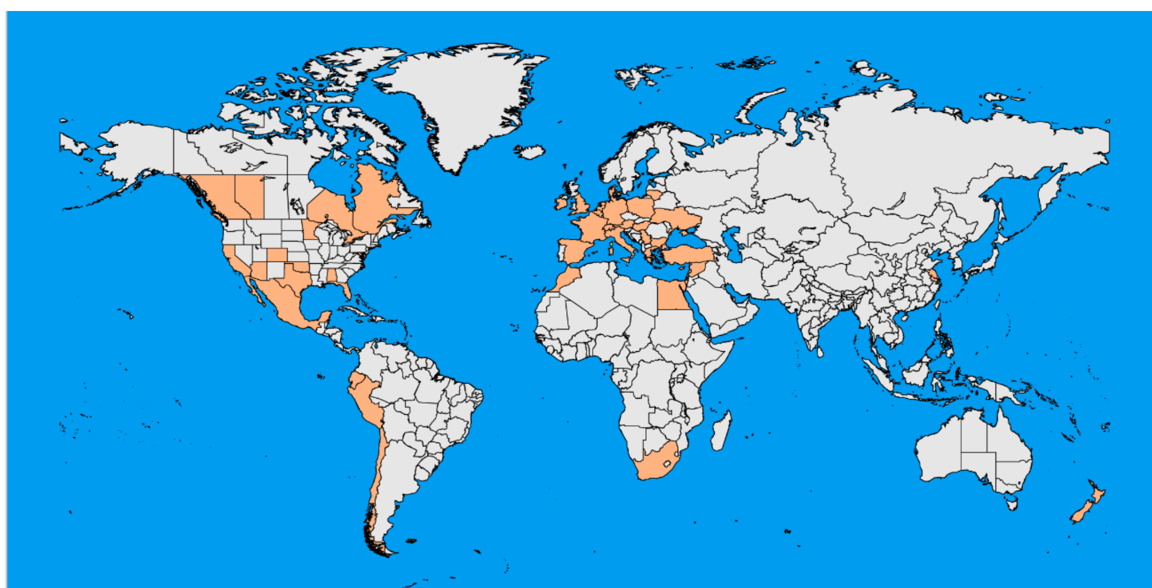

Figure 1. PepMV distribution worldwide. Countries/states where PepMV is currently present are filled in orange. Source: EPPO (2021).

The host range of PepMV mainly comprises *Solanaceae* species: pepino dulce, tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), eggplant (*Solanum melongena*); species from the genera *Capsicum*, *Datura*, *Nicotiana* and *Physalis* (Blystad *et al.*, 2015; Gómez *et al.*, 2009; Hanssen and Thomma, 2010; Jones *et al.*, 1980) besides some weed species (Córdoba *et al.*, 2004). PepMV symptoms either in tomato plants or fruits can vary depending on the environmental conditions and the viral isolate (Blystad *et al.*, 2015; Hanssen and Thomma, 2010; Sempere *et al.*, 2016). In leaves, PepMV infection is associated to bubbling, chlorosis and even yellowing and necrosis. While fruit marbling and discoloration have been observed in fruits, decreasing the commercial value of fruits from infected plants (Hanssen and Thomma, 2010; Spence *et al.*, 2006). Regarding the transmission mode, PepMV can be efficiently mechanically transmitted (Jones *et al.*, 1980), which is epidemiologically relevant since during tomato cultivation it will be easy to transmit the virus by contact through hands, contaminated tools, clothing or plant-to-plant contact (Hanssen and Thomma, 2010). Additionally, it has been reported that this virus can be transmitted by seeds with an efficiency ranging between 0.026 and 2 % (Córdoba-Sellés *et al.*, 2007; Hanssen and Thomma, 2010). Its transmission has been also associated to the presence of the fungus *Olpidium virulentus* (Alfaro-Fernández *et al.*, 2010), bumblebees (Shipp *et al.*, 2008) and whiteflies (Noël *et al.*, 2014) in a non-specific manner. Finally, PepMV transmission has also been reported through contaminated water (Mehle *et al.*, 2014).

1.2.1. Genomic organization of PepMV

The PepMV genome is a single stranded RNA of positive polarity of approximately 6.4 kb in length with a cap in the 5’ end, a polyadenylated tail in the 3’ end and five open reading frames (ORFs) flanked by two untranslated regions (5’UTR and 3’UTR) (Figure 2) (Aguilar *et al.*, 2002). The ORF1 is the only ORF that is translated directly from the genomic RNA (gRNA), whilst the ORFs 2, 3, 4 and 5 are translated from 3 subgenomic RNAs (sgRNAs) (Figure 2). The PepMV mechanism of expression is similar to that of other potexviruses; the ORF1 encodes a RNA dependent RNA polymerase (RdRp), which synthetizes the strain of negative

polarity of the genomic RNA, gRNA(-), using as template the gRNA, and also the three sgRNAs co-terminal with the viral 3'UTR. The ORFs 2, 3 and 4 encode the proteins of the triple gene block (TGB1, TGB2 and TGB3) that are translated from the sgRNAs 1 (TGB1) and 2 (TGB2 and TGB3). Finally, the ORF 5 encodes the capsid protein (CP) that is translated from the sgRNA 3 (Figure 2) (Verchot-Lubicz *et al.*, 2007).

RdRp protein has three functional domains: a methyltransferase domain, which attaches the 5'Cap to the viral RNAs; a helicase domain that splits RNA duplexes during replication; and an RNA polymerase domain that synthetizes the new RNA strains (Meng and Lee, 2017). The TGB proteins are involved in the building of the viral replication complexes and in the viral cell to cell and long distance movement (Linnik *et al.*, 2013; Morozov and Solovyev, 2003; Park *et al.*, 2014) and are not necessary for viral replication (Beck *et al.*, 1991; Morozov and Solovyev, 2003; Tilsner *et al.*, 2012; Verchot *et al.*, 1998) or encapsidation (Tilsner *et al.*, 2012). The TGB1 of potexviruses acts as silencing suppressor (Bayne *et al.*, 2005; Mathioudakis *et al.*, 2014) and is also involved in the disassembly of the virions (Rodionova *et al.*, 2003). TGB2 and TGB3 modify endomembranes of the endoplasmic reticulum and are able to recruit TGB1 to plasmodesmata (Tilsner *et al.*, 2013). Finally, the potexvirus CP has a structural role, being the subunit that oligomerizes to form the viral capsid (Agirrezabala *et al.*, 2015). The CP is involved in viral cell to cell and long distance movements and in the suppression of RNA silencing (Mathioudakis *et al.*, 2014; Morozov and Solovyev, 2003; Sempere *et al.*, 2011). In addition, the CP acts coordinately with TGBs to form the viral replication complexes (Linnik *et al.*, 2013).

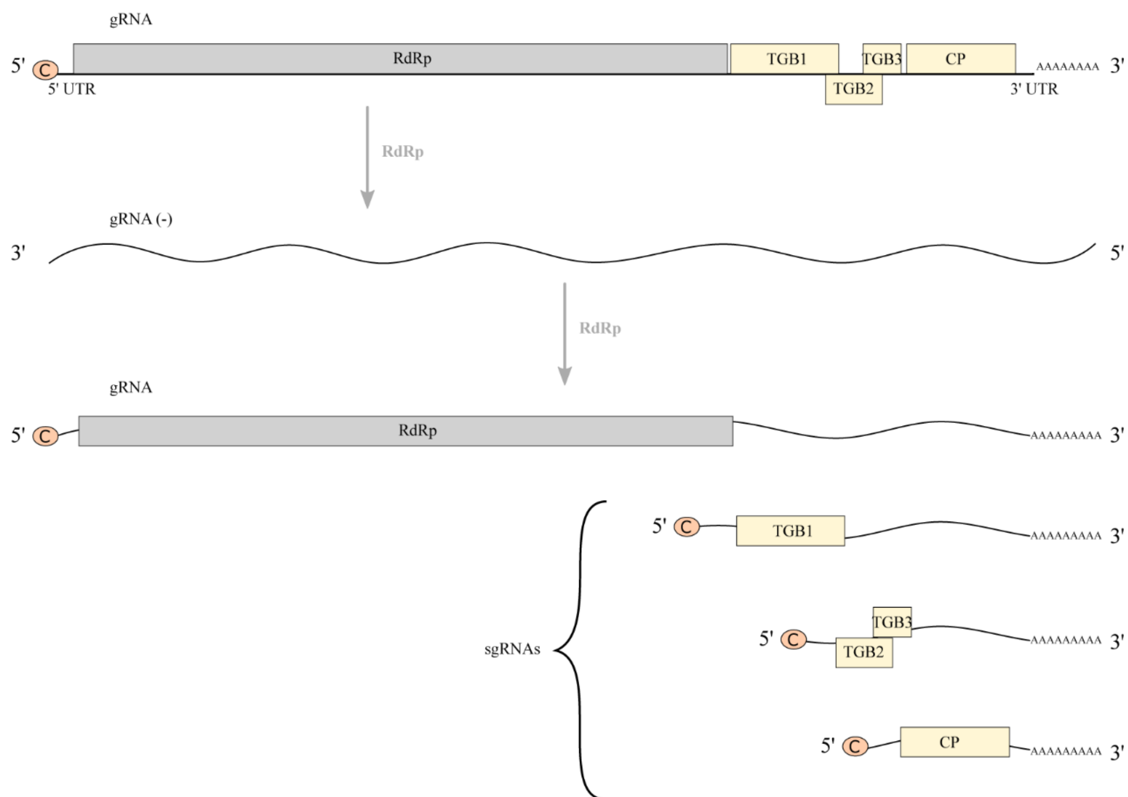

Figure 2. Genomic organization of PepMV and replication strategy. The genomic RNA (gRNA), the negative strand of the genomic RNA (gRNA (-)) and the three subgenomic RNAs (sgRNAs) of PepMV are represented. In the genomic RNA are shown the 5' cap (C), the 3' poly A tail, the 5' and 3' untranslated regions (5' UTR and 3' UTR) and the five open reading frames (ORFs), which encode: the RNA dependent RNA polymerase (RdRp), the triple gene block (TGB1, TGB2 and TGB3) and the capsid (CP) proteins. TGB1 is translated from the first sgRNA, TGB2 and TGB3 from the second sgRNA and the CP from the third sgRNA (shown in descending order).

1.2.2. PepMV genetic diversity

Phylogenetic analyses for each PepMV protein showed that the replicase was closely related with narcissus mosaic virus (NMV) and scallion virus X (SVX) replicases; the TGB1 with that of potato aucuba mosaic virus (PAMV); the TGB2 with that of cymbidium mosaic virus (CymMV); the TGB3 with that of potato virus X (PVX) and the CP with that of NMV, with a 75.3 %, 57.8 %, 66.3 %, 67.2 % and 79.3 % of homology, respectively (Cotillon *et al.*, 2002). Analyses comparing sequences from PepMV isolates that were originally collected from pepino or

tomato plants showed that the tomato isolates were genetically very homogeneous, sharing over 99 % of nucleotide identity, whilst only about a 94-97 % of nucleotide identity was found between pepino and tomato isolates (Mumford and Metcalfe, 2001; Verhoeven *et al.*, 2003; Van Der Vlugt *et al.*, 2002). Then, PepMV tomato isolates were considered a different strain (European strain) from the pepino isolates (original Peruvian strain) (Mumford and Metcalfe, 2001; Verhoeven *et al.*, 2003) (Table 1). In 2005, two isolates infecting tomato crops in US were sequenced, PepMV-US1 and US2, which showed 82 and 79 % of nucleotide identity with the European isolates respectively and were assumed to be representatives of two new strains (Maroon-Lango *et al.*, 2005). Later, a new isolate from Chile was described, PepMV-CH2, sharing 79 % of nucleotide identity with the original Peruvian and the European isolates and 78 and 90 % of identity with the US1 and US2 strains, respectively (Ling, 2007) (Table 1). Then, phylogenetic analysis suggested that US2 seemed to be a recombinant of US1 and CH2 (Hanssen and Thomma, 2010; Ling, 2007). In 2014, during a field survey in wild tomato plant species in Peru, PepMV-PES was identified as a new strain, with 79-82 % of nucleotide identity with the EU, LP and CH2 strains and 87 % with the US1 strain (Table 1) (Moreno-Perez *et al.*, 2014). Thus, five PepMV strains have been described so far: the original Peruvian (LP); the European (EU); the American (US1); the Chilean (CH2) and the new Peruvian (PES) (Figure 3) (Hanssen and Thomma, 2010; Moreno-Perez *et al.*, 2014).

Table 1. Nucleotide identities between the genomic sequences of isolates of the different PepMV strains.

PepMV strain	**Genbank accession**	LP	CH2	US1	US2	PES
EU	AJ438767	95	79	82	79	82
LP	AJ606361	-	79	82	79	82
CH2	DQ000985		-	78	90	79
US1	AY509926			-	86	87
US2	AY509927				-	81
PES	HG313805					-

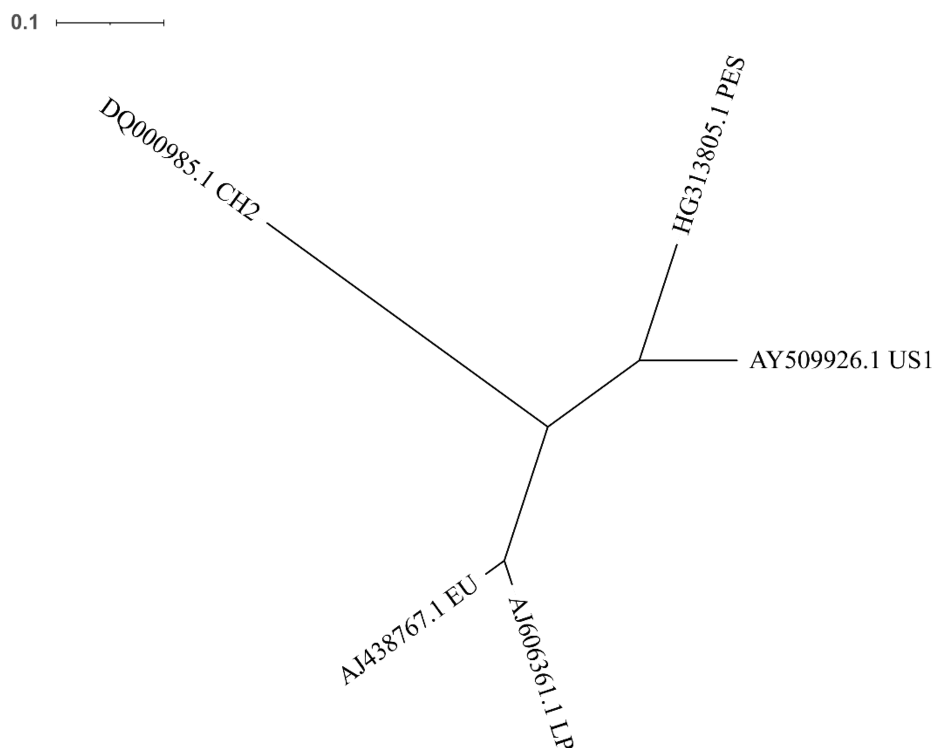

Figure 3. Phylogenetic tree of a representative isolate (accession number shown) of each PepMV strain described, LP (original Peruvian), EU (European), US1 (American), CH2 (Chilean), and PES (new Peruvian). The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model using MEGA7. The bootstrap of each node is 100 (not shown) and branch lengths represent the number of substitutions per site. Three differentiated clades are shown, one including both EU and LP isolates, which are very close each other; another clade with US1 and PES isolates, and the third one with the CH2 isolate, which is the most distant isolate to the others.

1.2.3. PepMV epidemics

From the first identification of PepMV infecting tomato crops in The Netherlands in 1999, it spread worldwide rapidly, affecting mainly the greenhouse tomato production (Blystad *et al.*, 2015; Hanssen and Thomma, 2010). In Spain, the EU strain was predominant until 2004 (Aguilar *et al.*, 2002; Cotillon *et al.*, 2002; Pagán *et al.*, 2006), when the CH2 strain emerged and rapidly displaced the EU strain (Gómez *et al.*, 2009) as in other European countries (Gómez *et al.*, 2009; Hanssen *et al.*, 2008; Pospieszny and Borodynko, 2006; Tiberini *et al.*, 2011). In the

case of North America, strain displacement took place around 2010 (Ling *et al.*, 2008; Ling *et al.*, 2013). However, the EU strain appeared to remain in the PepMV populations of tomato crops in Europe, but mainly detected in mixed infections with the CH2 strain (Gómez *et al.*, 2009; Hanssen *et al.*, 2008; Pagán *et al.*, 2006). In 2008, the US1 strain was also reported in Spain and for first time outside North America (Alfaro-Fernández *et al.*, 2008). During the following years, the epidemiological situation of PepMV in tomato crops appeared to be steady, with the CH2 strain being the dominant one (Davino *et al.*, 2017; Gómez-Aix *et al.*, 2019; Stankovic *et al.*, 2020), the EU strain still circulating mainly in co-infections, and with only a few occurrences of the US1 strain in the Canary Islands (Gómez-Aix *et al.*, 2019). This epidemiological pattern of PepMV infections in tomato crops has been explained by the fact that CH2 isolates appear to have a better *in planta* fitness than EU isolates in single infections; in contrast, the viral load of isolates of the CH2 strain is significantly reduced in the presence of an EU isolate in mixed infections, while the EU isolate has similar RNA accumulation in single and mixed infections (Gómez *et al.*, 2009). Additionally, the assessment of the effect of PepMV infection on plant fresh weight revealed that either the viral (EU or CH2) isolate or the type of infection (single or mixed infection) can induce a different response compared to the mock inoculated plants (Gómez *et al.*, 2009), and may also be influenced by the tomato cultivar (Agüero *et al.*, 2018).

1.3. Mixed infections

Mixed infections are hereby defined as those infections caused by more than one virus. This type of infection is very common in nature, including cultivated and wild plants (Alcaide *et al.*, 2020; Dutt *et al.*, 2021; Syller, 2012; Tollenaere *et al.*, 2016). Many of the studies of plant-virus interactions are carried out focusing on single infections, leaving a gap in the understanding of the complexity that mixed infections can bring into the ecology and evolutionary dynamics of the viral diseases. During a mixed infection, multiple pathogens coexist, sharing the same host and in some cases competing for the limited resources for their transmission,

development, survival and reproduction (Dutt *et al.*, 2021). Then, multiple infections can affect the virus-host interactions, being important to characterize the relationship between the viruses involved in an infection and the effect that this relationship may have in the host, being the plant a spatially structured space where viruses can be co-inhabiting the same cells or distributed in different areas of the host (Alcaide *et al.*, 2020). The distribution of the viruses in their hosts will condition the type of interactions that they can maintain, which can affect the produced symptomatology, virus transmission, viral loads, resistance breakdown in resistant cultivars, host range of the involved viruses and their virulence (Alcaide *et al.*, 2020; Moreno and López-Moya, 2020; Syller, 2012; Tollenaere *et al.*, 2016). In this sense, host plant species and even cultivar have been described to affect the disease outcome of mixed infections (González-Jara *et al.*, 2004; Méndez-Lozano *et al.*, 2003; Moreno and López-Moya, 2020; Tatineni *et al.*, 2010; Wintermantel *et al.*, 2008). This indicates that epidemiological patterns can be modulated by the presence of other overlapping cultivated or wild plant species in the surrounding crops, which can act as reservoirs and inoculum sources for the crop infections (Córdoba *et al.*, 2004). In addition, agronomic practices can be determinant for the control or dispersion of viral infections (Lefeuvre *et al.*, 2019; Rubio *et al.*, 2020). Therefore, compelling evidence shows that several factors may explain observed epidemiological patterns of plant viruses; it is needed to analyse their combinatorial effects, and their role in the evolutionary dynamics of viral populations. Mixed infections are complex systems with unpredictable consequences that may have far-reaching epidemiological outcomes.

1.3.1. Some examples of mixed viral infections resulting in important crop diseases

Plant virus epidemics and pandemics have been described over the years affecting different crops (Jones, 2021; Moreno and López-Moya, 2020); some significant examples are caused by mixed infections and are described below.

Maize lethal necrosis disease (MLND) became a pandemic disease affecting maize crops in Africa, Asia, South America and Europe. This disease causes necrosis in leaves and shoots, resulting in plant death. It is caused by a mixed infection between maize chlorotic mottle virus (MCMV) and another virus belonging to the family *Potyviridae* that can be either sugarcane mosaic virus (SCMV), maize dwarf mosaic virus (MDMV) or wheat streak mosaic virus (WSMV) (Jones, 2021; Redinbaugh and Stewart, 2018).

Yellow dwarf disease (YDD) is an epidemic disease affecting wheat crops in Europe, Africa, Asia, Oceania and America. In wheat plants, symptoms of this disease are associated with yellowing-leaves, reduced root and plant growth, and decreased seed quality. It can also affect other crops such as barley and oat. YDD is caused by barley yellow dwarf virus (BYDV) and cereal yellow dwarf virus (CYDV) in single or mixed infections (Jones, 2021). In this case, mixed infections allow the transencapsidation of these viruses, increasing their transmission through different vectors (Creamer and Falk, 1990; Moreno and López-Moya, 2020).

Rice tungro disease (RTD) is the most devastating disease caused by viruses affecting rice crops in different regions of Asia. Symptoms of this disease include leaves stripping and yellowing, plant stunting and severe impact on grain formation, leading to yields reduced over 70 % (Azzam and Chancellor, 2002; Bunawan *et al.*, 2014). RTD is caused by a mixed infection between rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV) (Bunawan *et al.*, 2014).

Sweet potato virus disease (SPVD) affects sweet potato crops in Africa, Asia and America (Jones, 2021). SPVD is caused by a mixed infection between sweet potato chlorotic stunt virus (SPCSV) and a member of the family *Potyviridae*, which is usually sweet potato feathery mottle virus (SPFMV) or sweet potato mild mottle virus (SPMMV), and associates with symptoms like plant stunting, severe malformations and mosaics in leaves, which contrasts with the symptoms induced by these viruses separately, much milder (Tairo *et al.*, 2005).

Those described above are just a few examples of the main diseases caused by mixed viral infections, although many others have been reported affecting important

crops such as cassava, tomato or grapevine (Gómez *et al.*, 2010; Martelli, 2014; Moreno and López-Moya, 2020; Pita *et al.*, 2001; Zinga *et al.*, 2013). All these examples highlight the importance of mixed infections and the impact they have in the outcomes of the viral disease and therefore the importance of studying the mixed infection as a whole, taking into account the possible virus-virus and virus-host interactions that can occur.

1.3.2. Types of *in planta* virus-virus interactions

Within mixed infections, it would be worth to distinguish between coinfections and sequential infections in the same plant. A coinfection is produced when two or more viruses infect a host at the same time, that is, simultaneously. Sequential infections occur when there is a lag of time in the viruses arrival; one virus infects a host, and thereafter, the same host is infected by another virus (Hull, 2014; Syller, 2012; Syller and Grupa, 2016). Regardless the virus arrival, when multiple viruses are infecting the same plant, the potential virus-virus interactions can be classified in direct or host-mediated interactions (Alcaide *et al.*, 2020; DaPalma *et al.*, 2010; Tollenaere *et al.*, 2016). Direct interactions are those in which nucleic acids or proteins of the viruses involved in the infection physically interact among each other; therefore, in that case it is necessary that both viruses infect the same cells (DaPalma *et al.*, 2010). Host-mediated or indirect interactions result in alterations in the host environment produced by one virus that influence the other virus involved in the infection (DaPalma *et al.*, 2010; Tollenaere *et al.*, 2016).

These viral interactions have been also classified into synergistic (facilitation) and antagonistic (interference or competition), according to the effect on host symptomatology and/or viral accumulation. Synergistic interactions lead to an increased multiplication and/or pathogenicity of at least one of the viruses involved. Antagonistic interactions harm at least one of the viruses involved in the infection and/or decrease the symptoms produced in the host compared with single infections (Syller, 2012; Syller and Grupa, 2016). Within these interactions there are different combinations in accordance with the viral fitness of each virus. In this sense, it

would be possible to classify interactions in neutral, neutral antagonistic, neutral synergistic, inverse, double synergistic and double antagonistic (Figure 4) (Alcaide *et al.*, 2020). Related viruses have usually been reported to interact antagonistically, although there are some exceptions (Rentería-Canett *et al.*, 2011; Wintermantel *et al.*, 2008). There are two terms used in antagonistic interactions depending on the moment in which the double infection occurs in the same host. In particular, it will depend on whether the infection is a coinfection or a superinfection. Superinfections can produce superinfection exclusion (SIE), which is the exclusion of a virus in a pre-infected host by another related virus. From an applied point of view, this SIE is associated to the phenomenon of cross-protection, where a plant is pre-infected by a mild strain of a virus, which prevents a secondary infection by a severe strain (Hull, 2014). In the case of coinfections, mutual exclusion can occur, separating spatially both viruses in the host (Syller, 2012).

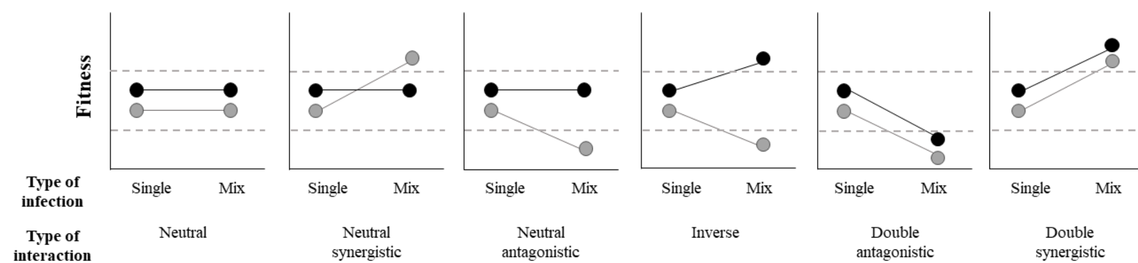

Figure 4. Types of virus-virus interactions depending on the fitness of the viruses involved. Virus-virus interactions during mixed infections can lead to changes in the viral fitness of each of the viruses with respect to single infections. Interactions can be classified in: neutral, neutral synergistic, neutral antagonistic, inverse, double antagonistic and double synergistic. Figure modified from Alcaide et al., 2020.

1.3.3. Mixed infections and *in planta* virus load and virus transmission

Mixed infections can affect different viral traits such as viral load and transmission through a range of processes (Moreno and López-Moya, 2020; Syller, 2012) that, in turn, could influence the viral fitness of each virus, reducing or increasing the population size, compared to single infections. These effects may constrain or enhance the evolvability and adaptation of the viral populations,

shaping their genetic structure and evolutionary dynamics. Regarding the effects of mixed infections on viral load, the type of interaction between the viruses involved in the infection will determine the disease outcome (Alcaide *et al.*, 2020). Virus accumulation is one of the most affected traits during mixed infections (Alcaide *et al.*, 2020; Mascia and Gallitelli, 2016; Moreno and López-Moya, 2020; Syller, 2012). Thus, the viral load of at least one of the viruses involved in a mixed infection can vary compared to a single infection, as it has been described in the previous section (1.3.2). At the same time, viral load can affect the efficiency of viral transmission, *e.g.* the coinfection of ToCV and tomato infectious chlorosis virus (TICV) causes changes in the viral accumulation of both viruses, which directly affect the efficiency of their transmission (Wintermantel *et al.*, 2008). Another example is the combination of cucurbit yellow stunting disorder virus (CYSDV) and watermelon mosaic virus (WMV), where WMV showed a lower accumulation and CYSDV a higher accumulation in mixed infections, with an unaltered transmission efficiency for WMV, and a higher CYSDV transmission at late times post-infection, suggesting that viral accumulation and transmission efficiency may or may not be connected depending on the virus (Domingo-Calap *et al.*, 2020).

There is evidence that some viruses benefit from the presence of others to acquire new transmission ways (Rochow, 1972). For example, mixed infections can lead the vector-borne transmission of a virus species that was unable to be transmitted in single infections (Rochow, 1972), suggesting that viral transmission in mixed infections can be selective (a vector transmits selectively only a virus) or non-selective (a vector transmits simultaneously two viruses from doubly infected plants). Additionally, there are viruses that may depend on others (helper virus) for their transmission (Mascia and Gallitelli, 2016; Moreno and López-Moya, 2020). For example, groundnut rosette virus (GRV) depends on its satellite and on groundnut rosette assistor virus (GRAV) for its transmission (Murant, 1990). Also, this is the case of the MAV strain of BYDV (BYDV-MAV), which can be trans-encapsidated by the CP of the RPV strain of CYDV (CYDV-RPV), allowing the transmission of BYDV-MAV by the vector of CYDV-RPV, *Rhopalosiphon padi* (Creamer and Falk, 1990).

1.4. Genetic, ecological and demographical processes defining viral population diversity and evolution

Viral variants can be produced during viral genome replication and they are genetically different from their ancestors. This capacity of variation is the basis of the biological success of viruses (Andino and Domingo, 2015; Pagán and García-Arenal, 2018). RNA viruses have the potential to stablish very diverse populations due to their small genomes, large population sizes and high replication and mutation rates (Pagán and García-Arenal, 2018; Sanjuán and Domingo-Calap, 2016). Most of the produced variants are deleterious, which can only be compensated with very high offspring numbers. Then, the combination of both factors gives rise to the genetic diversity that viruses need to adapt to new environments (Holmes, 2009). Although the variation potential of viruses is very high, viral populations might be genetically stable (García-Arenal *et al.*, 2003) and the frequency of each variant might change over time and under different environments, modifying the genetic structure of viral populations. In the following sections, the mechanisms that generate genetic variability and the processes that determine the genetic structure of viral populations will be introduced, as well as the potential implications of mixed infections on plant disease ecology and evolution.

1.4.1. Mechanisms generating genetic diversity

There are three main mechanisms that can generate genetic variability in virus populations; mutation, recombination and reassortment (Roossinck, 1997) (Figure 5). Mutation is the process by which nucleotides are either inserted, deleted (INDELs) or modified during the replication of a nucleic acid sequence, leading to variants in the population. Mutations can be neutral, deleterious or beneficial depending on the ecological context, ultimately having or not an effect on viral fitness. A mutation-selection balance is maintained among variants of a population, since most of these mutations will have a deleterious effect on the individual, although they are necessary for obtaining the genetic variability that allow viruses to evolve and adapt to environmental changes (Domingo *et al.*, 2012; García-Arenal

et al., 2003). RNA viruses exhibit high mutation rates (Drake *et al.*, 1998; Sanjuán and Domingo-Calap, 2016), which is in part due to the short generation times and the error-prone RNA-dependent RNA polymerases (RdRp) used by viruses (Sanjuán and Domingo-Calap, 2021). However, mutation rate of some DNA viruses is similar to those of RNA viruses, as it depends on other factors apart from the polymerase fidelity, like the replication speed and the viral genomic architecture (Duffy *et al.*, 2008). Mutation rates of RNA viruses that use RdRps are in the range of 0.03-2 mutations per genome per replication cycle (m/g/r) (Drake *et al.*, 1998; Drake and Holland, 1999; Duffy *et al.*, 2008; Tromas and Elena, 2010). These mutation rates are high enough to consider mutation as the principal mechanism involved in the evolution of most RNA viruses (Holmes, 2009). For plant RNA viruses, mutation rates appear to be at the lower limit of the range for RNA viruses (Tromas and Elena, 2010); for example, in the case of tobacco etch virus (TEV), the mutation rate has been estimated to be around 0.28 m/g/r (Sanjuán *et al.*, 2009; Tromas and Elena, 2010), similar to that of tobacco mosaic virus (TMV), 0.1-0.13 m/g/r (Malpica *et al.*, 2002). However, these estimations are specific to plant-virus combinations, and while mixed infections have been identified as ecological drivers affecting the viral fitness populations, it remains unknown the potential effects of mixed infections on viral mutation rates.

Recombination is the process by which nucleotide sequence fragments are exchanged between the strands of different genetic variants during the replication process, generating a mixed RNA molecule (García-Arenal *et al.*, 2001; Sanjuán and Domingo-Calap, 2021). Recombination rates are highly variable depending on the type of viral nucleic acid (Holmes, 2009) and in all cases it needs at least two molecules co-infecting the same cell, which means that the multiplicity of infection (MOI; *i.e.* the number of viral molecules infecting a cell) should be more than 1 (González-Jara *et al.*, 2009; Syller and Grupa, 2016; Zwart *et al.*, 2013). Thus, mixed infections are an essential requirement to recombination events, along with the ability of both viruses or variants to co-infect the same cell. Recombination can be produced between related viruses and even between variants of the same virus,

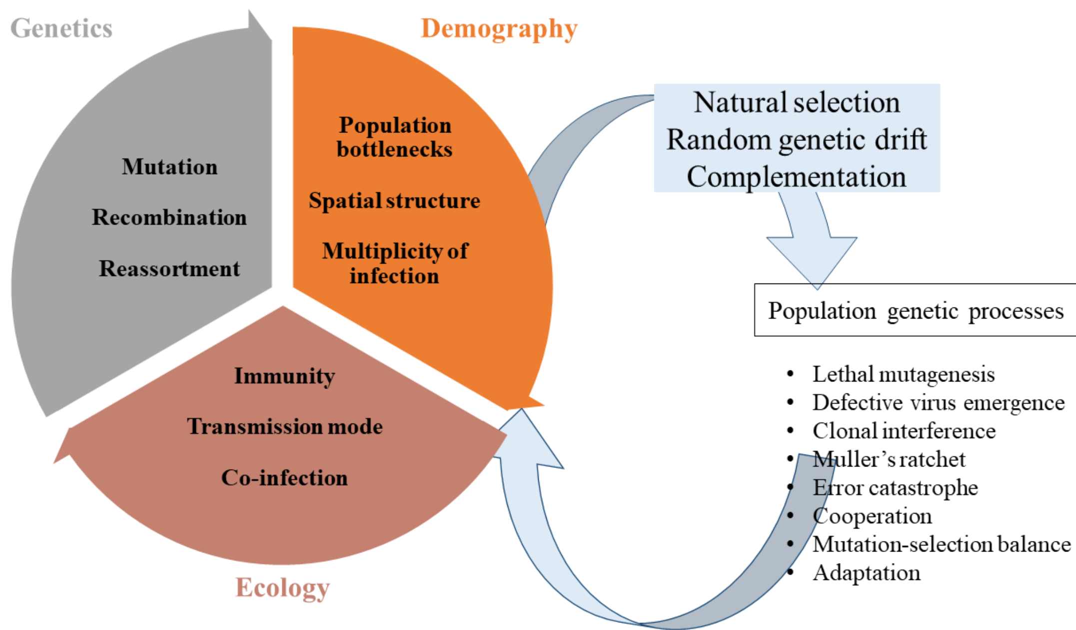

Figure 5. Genetic, ecological and demographical processes determining viral population diversity. Mutation, recombination and reassortment are the sources of genetic variation. The impact of natural selection, genetic drift and complementation on these new genetic variants are determined by demographical and ecological processes, which at the same time can lead to a series of population genetic processes that feedback on viral genetics, ecology and demography. Figure modified from Sanjuán and Domingo-Calap, 2021.

but it is not as frequent as expected because it has to deal with the handicap of SIE among related viruses. In addition, recombination can also occur between unrelated viruses, producing major genetic changes (Rubio *et al.*, 2020; Sanjuán and Domingo-Calap, 2021). There are certain plant virus families where recombination is important as source of genetic diversity, such as *Bromoviridae*, *Luteoviridae*, *Potyviridae*, *Closteroviridae*, *Virgaviridae*, *Secoviridae* and *Geminiviridae* (Codoñer and Elena, 2008; Ferriol *et al.*, 2014; Lefeuvre and Moriones, 2015; MacFarlane, 1997; Moonan *et al.*, 2000; Revers *et al.*, 1996; Rubio *et al.*, 2001). Recombination rates for plant viruses have been estimated in the range of 10^{-5} to 10^{-8} (Froissart *et al.*, 2005; Tromas *et al.*, 2014), indicating that for some viruses, recombination can be a source of genetic diversity as important as mutation (Pagán and García-Arenal, 2018).

Reassortment or pseudorecombination is the segment exchange in viruses with segmented or multipartite genomes. Similar to recombination events, in the case of reassortment, several viruses have to co-infect the same cell (Holmes, 2009). However, this process is a specific mechanism that is carried out in certain type of genomes, including *Begomovirus*, *Bromovirus*, *Fabavirus* and *Cucumovirus* genera (Codoñer and Elena, 2008; Ferriol *et al.*, 2014; Lefeuvre and Moriones, 2015).

1.4.2. Processes determining the genetic structure of viral populations

Beyond the mechanisms that generate genetic diversity in viral populations, there are demographic and ecological processes shaping the genetic structure of those populations (Figure 5). In the absence of migration, the main forces determining the frequency of each genetic type in the population are: natural selection, genetic drift and to a lesser extent complementation (García-Arenal *et al.*, 2001). Note that the strength of these forces can be modulated by diverse factors (Figure 5).

Natural selection is the process by which the variants in a population are selected based on their fitness; in a given environment the variant with a highest fitness will increase its frequency in the population (positive selection), and those variants with lower fitness will decrease (purifying or negative selection) (García-Arenal *et al.*, 2001; Pagán and García-Arenal, 2018). Therefore, natural selection will purge the disadvantageous variants and will keep beneficious variants, maintaining those with greater fitness in the population. For that, populations have to be large enough to ensure that each variant will have offspring in the next generation. Negative selection will favour the maintenance of certain conserved regions in the genome that might have a role in the viral biological cycle. For example, some amino acids of the capsid protein have been described as essential for the assembly and stabilization of viral capsid (Agirrezabala *et al.*, 2015; Altschuh *et al.*, 1987) or for the cell to cell movement (Navarro *et al.*, 2020). Although positive selection is less frequently observed, it has been mainly associated to resistance breakdown (García-Arenal *et al.*, 2001). For instance, a TMV strain which

overcomes resistance factors, increases its frequency when resistant cultivars are grown and decreases in susceptible cultivars, where other TMV strains could be fitter for that environment (Pelham *et al.*, 1970). In the case of co-infections, if one variant has a higher fitness than the other, both can co-exist if there are beneficious interactions between them (Leeks *et al.*, 2018).

Genetic drift is the process by which the allelic frequencies of a population change randomly. It occurs when the effective population size, the number of individuals that have progeny, is not large enough. This process has been associated to bottlenecks during viral transmission or colonization, either due to the shortage of new available hosts or the bottlenecks associated with transmission between hosts (Gutiérrez *et al.*, 2012; Pagán and García-Arenal, 2018; Sacristán *et al.*, 2003). In addition, the genetic drift can be produced by the founder effect, where there are few individuals that found or establish a new population (García-Arenal *et al.*, 2001; Pagán and García-Arenal, 2018). In this scenario, when the population is constrained by a threshold, the fitter types may not be the ones transmitted, resulting in viral populations that are increasingly dominated by less fit types (Muller's ratchet) (Muller, 1964). This can lead to the mutation meltdown and disappearance of the population (García-Arenal *et al.*, 2001; Lynch *et al.*, 1993). This Muller´s ratchet effect has been reported for TEV in *Chenopodium quinoa* (de la Iglesia and Elena, 2007), and also in mixed infections of TMV and tobacco mild green mottle virus (TMGMV), where the TMV population size was drastically reduced, avoiding the elimination of deleterious mutations and leading to mutational meltdown of the population (Fraile *et al.*, 1997). Several examples of population bottlenecks have been associated with viral cell-to-cell movement, between-host transmission, host range expansion or colonization of new geographical areas (Ali *et al.*, 2006; Fraile *et al.*, 1997; Kutnjak *et al.*, 2017; Li and Roossinck, 2004; Sacristán *et al.*, 2003). Among them, it has been shown that TMV and CMV pass through bottlenecks during plant colonization, reducing the number of the founder individuals colonizing a new leaf (Li and Roossinck, 2004; Sacristán *et al.*, 2003). However, not all the viruses experience bottlenecks during within host colonization (Monsion *et al.*, 2008), and not all transmission modes generate the same bottlenecks; mechanical

transmission for example produces less restrictive bottlenecks than aphid or vertical transmission (da Silva *et al.*, 2020). In that sense, changes in viral transmission influenced by mixed infections could impact indirectly on the viral diversity in accordance with the transmission mode. For example, within-host viral diversity can be higher in vertically transmitted viruses through tubers in comparison with those transmitted by aphids (da Silva *et al.*, 2020). In the case of two plant viruses mechanically transmitted, bottlenecks produced during between-host transmission suggest that viral populations can remain stable, regardless of the host species. For example, Ali and Roossinck (2017) showed that after ten passages of CMV and cowpea chlorotic mottle virus (CCMV) in single and mixed infections, the nucleotide variation of the RNA 3 was in general higher in single than in mixed infections without important changes during passages (Ali and Roossinck, 2017). In the case of two isolates of PepMV, Sp13 and PS5, similar nucleotide diversities were found in single and mixed infections after eight passages in tomato plants, also showing stability along passages (Agüero *et al.*, 2018).

Complementation allows defective types to be maintained in the population thanks to other competent types which complement them. Therefore, complementation can efficiently counteract natural selection favouring the maintenance of lethal mutants (García-Arenal *et al.*, 2001; Sanjuán, 2021). This process requires mixed infections, whether from related (even variants of the same virus) or unrelated viruses, and has been described in some plant viruses such as TMV and tomato aspermy virus (TAV) (Fraile *et al.*, 2008; Moreno *et al.*, 1997). It has been also described for soybean mosaic virus (SMV), where mixed infections of two strains resulted in complementation, allowing the systemic spread of an avirulent strain (Mansky *et al.*, 1995).

While natural selection and genetic drift decrease variability within a population and increase variability between populations (Pagán and García-Arenal, 2018), complementation allows the existence of defective types and therefore the maintenance of a higher genetic diversity in the population due to the accumulation of mutations, which can ultimately lead to error catastrophe (Sanjuán, 2021).

1.4.3. Temperature as an ecological factor affecting virus infections

Plants respond to abiotic stresses, such as salinity, floods, drought, radiation and extreme temperatures, with different strategies that affect plant development and growth (Zhang *et al.*, 2021). The combination of abiotic and biotic stresses in a host can be expected to occur at similar timescales, having a reciprocal effect (or eco-evolutionary feedbacks) between them. This highlights the importance of these combinations in a realistic and complex scenario, where it is necessary to understand how abiotic and biotic factors can combine to affect the eco-evolutionary dynamics of the viral populations. To date, it is practically unknown how abiotic factors could affect the viral evolution. Recently, it has been shown that viruses can evolve in situations of abiotic stress, such as drought, shifting from parasitism to mutualism, and thus, promoting host survival under that stress condition (González *et al.*, 2021). Another important abiotic factor is temperature, which can affect plant physiology (Went, 1953), and thus influence (positively or negatively) the viral infection progress in the plant. For example, temperature can affect the infection rate, *i.e.* the number of plants in which the infection was stablished after inoculation, whereas at high temperatures the infection rate of cucumber vein yellowing virus (CVYV) and potato leaf roll virus (PLRV) increased (Chung *et al.*, 2016; Ruiz *et al.*, 2006), on the contrary in the case of potato virus A (PVA) and PVY-O the infection rate was reduced (Chung *et al.*, 2016). Temperature can also affect the speed of the viral systemic infection (Choi *et al.*, 2017). There is also evidence that temperature affects viral accumulation, *e.g.* PepMV and citrus psorosis virus (CPsV) had higher accumulation at high temperatures (Sempere *et al.*, 2016; Velázquez *et al.*, 2010), and others like alternanthera mosaic virus (AltMV) at low temperatures (Lim *et al.*, 2010). Notably, high temperatures can reduce host symptoms expression (Chellappan *et al.*, 2005; Valkonen, 1997), which in some cases is related with the viral load (Velázquez *et al.*, 2010). And lastly, there is also an association of high temperatures with resistance breaking (Erickson *et al.*, 1999; Király *et al.*, 2008; Moury *et al.*, 1998; Siddiqui *et al.*, 2008; Soler *et al.*, 1998). Although the mechanisms behind these effects are unknown, it seems that there is a relationship with the effectiveness of RNA silencing, which has been described to be less

effective at low temperatures (Chellappan *et al.*, 2005; Ma *et al.*, 2016; Siddiqui *et al.*, 2008; Szittya *et al.*, 2003; Velázquez *et al.*, 2010).

1.5. RNA silencing as the main host mechanism controlling *in planta* virus fitness

RNA silencing is assumed to be the first layer of plant defence against viruses and other invading nucleic acids, and suppression of RNA silencing has been shown to be at the basis of synergistic interactions among viruses in numerous cases (see below). RNA silencing refers to a number of inhibition pathways mediated by small RNAs (sRNAs) which are sequence-specific and that can act at transcriptional (avoiding directly the transcription of a gene by chromatin modifications) or at post-transcriptional (producing RNA cleavage or translational repression) levels (Martínez de Alba *et al.*, 2013; Pumplin and Voinnet, 2013). In plants, two major groups of sRNAs have been described, microRNAs (miRNAs) and small interfering RNAs (siRNAs), which are involved in three silencing pathways: cytoplasmic siRNA-directed RNA degradation pathway, which controls exogenous nucleic acids and therefore participates in the defence against viruses; endogenous messenger RNAs (mRNAs) silencing pathway directed by miRNAs, which serves as a self-regulatory mechanism, controlling the mRNA levels of the endogenous host genes; siRNA-directed DNA methylation and transcription suppression pathway, which is involved in histone modifications and is responsible for controlling transposons (Baulcombe, 2004; Liu *et al.*, 2017; Martínez de Alba *et al.*, 2013).

1.5.1. RNA silencing in plants mediated by sRNAs

The siRNA-directed RNA silencing mechanism (also called RNA interference, RNAi), has been described in a great number of eukaryotic organisms as a defence mechanism against invasive nucleic acids, including DNA and RNA viruses, satellite RNAs and viroids (Diaz-Pendon *et al.*, 2007; Ding and Voinnet, 2007; Minoia *et al.*, 2014; Pumplin and Voinnet, 2013; Shimura *et al.*, 2011). During viral

infections, double stranded RNAs (dsRNAs) are produced from different viral origins and are processed by RNAseIII Dicer-like (DCL) endonucleases into 21 to 24 nt virus-derived small interfering RNAs (vsiRNAs) (Ding, 2010). Then, vsiRNAs can be incorporated into effector complexes called RNA-induced silencing complexes (RISC), where one strand of a vsiRNA can be loaded into an Argonaute (AGO) protein (Ding and Voinnet, 2007; Pantaleo *et al.*, 2007), being used as a guide by the AGO protein, which cleaves target ssRNAs (Tolia and Joshua-Tor, 2007) (Figure 6). In addition, silencing can move from cell to cell through plasmodesmata or long distance through the phloem thanks to the host RNA-dependent RNA polymerases (RDRs), which amplify the silencing signal producing "secondary" vsiRNAs using a ssRNA molecule as a template (Pumplin and Voinnet, 2013; Voinnet, 2005). In general, miRNAs are mainly involved in plant development and growth, protein degradation and stress response (Liu *et al.*, 2017; Voinnet, 2009) and although the siRNA-directed RNA degradation pathway is the main mechanism of viral defence, the miRNA pathway can also have an important role (Liu *et al.*, 2017). miRNAs are processed by DCL proteins into RNAs of 20 - 24 nt and are incorporated in RISC complexes similar to siRNAs (Liu *et al.*, 2017; Voinnet, 2009). In plants, the role of miRNAs in antiviral response can be direct, targeting viral RNA directly, or indirect, triggering the biogenesis of siRNAs involved in the antiviral defence (Liu *et al.*, 2017).

1.5.2. Viruses as inducers and targets of silencing

In the same way that plants have developed the RNA silencing mechanism as antiviral response, viruses have evolved means to avoid it using viral suppressors of RNA silencing (VSRs) that condition the host susceptibility favouring replication, viral movement and symptoms development by interfering with the host silencing mechanism (Burgyán and Havelda, 2011; Díaz-Pendón and Ding, 2008; Garcia-Ruiz, 2018; Kontra *et al.*, 2016). VSRs have been found in almost all plant virus genera, are coded by the virus and most of the times they are multifunctional, having an additional role for the virus. The mechanism behind each VSR varies depending on the virus; the most frequent strategy is ds siRNAs sequestration by the VSR but

there are others, such as the inhibition of the viral RNA recognition by the DCL proteins or the prevention of the RISC assembly by the sequestration of one of its essential components by the VSR (Burgyán and Havelda, 2011). For example, the p19 of tombusviruses is able to reduce RNA silencing through its binding to dsRNAs and ds siRNAs, preventing the virus-induced systemic silencing (Silhavy *et al.*, 2002) (Figure 6). Other example is the P0 protein of poleroviruses, which is able to target AGO1 and mediates its degradation, avoiding the formation of the RISC complex (Baumberger *et al.*, 2007; Bortolamiol *et al.*, 2007) or the HCPro protein of potyviruses, which apart from recruiting AGO1 is able to use it in pro-viral functions (Pollari *et al.*, 2020). Therefore, the mechanisms of silencing suppression are based on the degradation of the main components of gene silencing such as DCL, AGO and RDR proteins, and also in the binding and sequestration of siRNAs and miRNAs (Garcia-Ruiz *et al.*, 2015; Del Toro *et al.*, 2017).

However, at the same time, plants have evolved to prevent the inhibitory effects of viral suppressors on gene silencing (Ghoshal and Sanfaçon, 2015). For that, plants encode factors that affect directly VSRs and have developed mechanisms that regulate the expression of components of the silencing pathway. For example, this is the case of the calmodulin-like protein (rgs-CaM) from *Nicotiana tabacum*, which is able to degrade by autophagy the CMV silencing suppressor 2b (Jeon *et al.*, 2017). Similarly, the *A. thaliana* NBR1 receptor is able to degrade, also by autophagy, the potyviral VSR Hc-Pro, reducing the accumulation of viruses like turnip mosaic virus (TuMV) and WMV (Hafrén *et al.*, 2018). Therefore, the antiviral response of the plant and the mechanisms used by the viruses to bypass the antiviral defence of the host have coevolved to allow a balance between the virus and the host. Mixed infections can alter this balance depending on the type of interactions between the viruses involved in the infection. In the case of synergistic interactions, the VSR of one virus can promote the infection of the second virus, taking advantage the latter of the ability of the former to inhibit or reduce the silencing mechanism of the plant (Lee *et al.*, 2021; Pruss *et al.*, 1997; Siddiqui *et al.*, 2011; Xu *et al.*, 2020; Yang and Ravelonandro, 2002). In the case of antagonistic interactions, typical of related viruses, it is necessary to differentiate between super-infections and co-

infections (see section 1.3.2). In the case of super-infections, the vsiRNAs produced by the first virus can activate the silencing mechanism of the host, which is prepared when the second virus infects it, being rapidly recognised by the RNA silencing machinery (Figure 6), promoting SIE and even in some cases cross-protection (Laufer *et al.*, 2018; Ratcliff *et al.*, 1999; Syller and Grupa, 2016). In the case of co-infections, the same mechanism can be triggered, resulting in the spatial separation of both viruses (Dietrich and Maiss, 2003; Laufer *et al.*, 2018; Syller and Grupa, 2016).

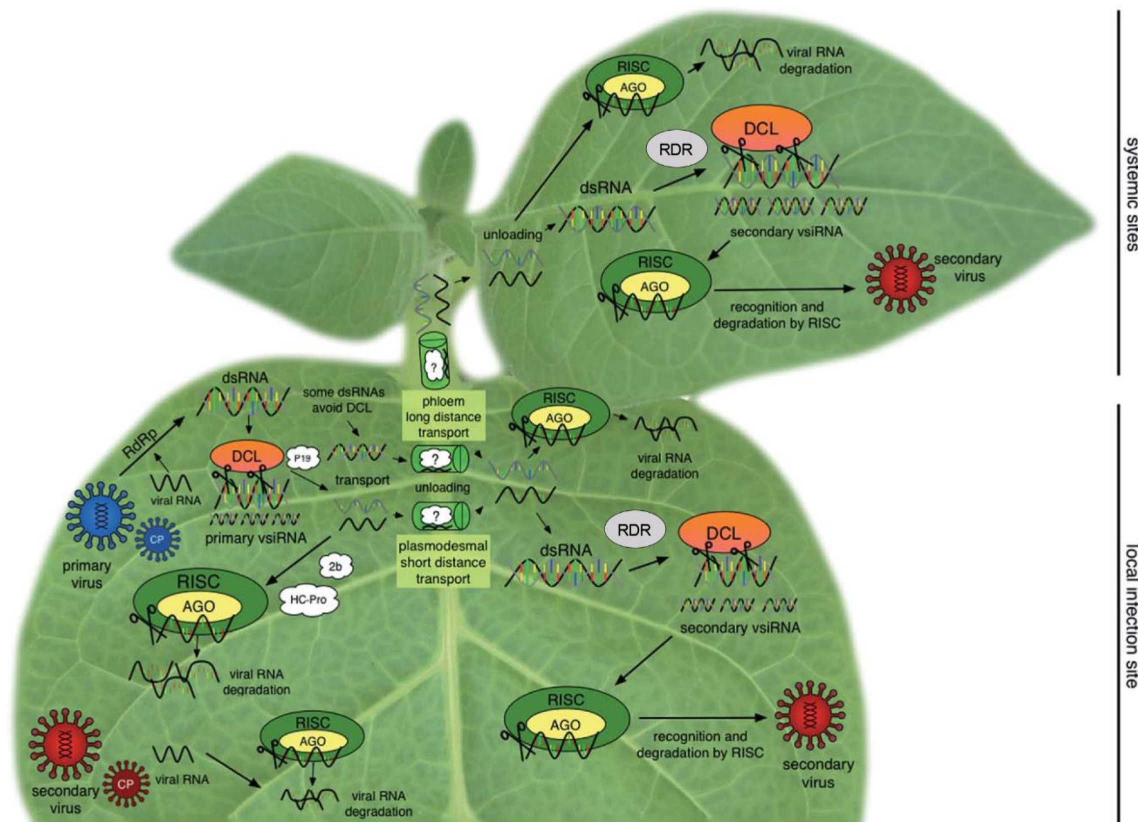

Figure 6. A simplified model of the siRNA silencing mechanism in plants. During viral replication, most viruses form dsRNAs, which are processed by DCL in vsiRNAs. These vsiRNAs are loaded into an AGO protein, which is part of the RISC complex, and target ssRNAs are cleaved. The silencing signal can move short and long distance through transport pathways. In the case of a mixed antagonistic infection, a primary virus (blue) triggers the RNA silencing pathway, which can interfere with a subsequent infection by the secondary virus (red). Figure modified from Syller and Grupa, 2016

2. Objectives

2. Objectives

The overall objective of this thesis was to study the virus-virus and virus-host interactions of PepMV isolates of two strains in mixed infections. The immediate background of this thesis included the observation that natural populations of PepMV in Spain were mainly composed of isolates belonging to the EU strain until the year 2005, when the CH2 strain began to prevail without completely displacing the EU strain, with EU isolates being mainly found in mixed infections. The background also included the description of a higher *in planta* fitness of a PepMV-CH2 isolate compared to that of a PepMV-EU isolate in single infections, but repression of the PepMV-CH2 isolate accumulation in mixed infections with the PepMV-EU isolate, defining an asymmetric antagonism. Whether the phenomenon could be generalised to other CH2 isolates and to a variety of ecological conditions was unknown, as the outcomes of virus infections can vary depending on the virus isolate, type of infection, virus-host interactions and host ecology. Thus, this thesis has addressed the PepMV-EU and PepMV-CH2 interaction from different perspectives (i) with different isolates, (ii) under different abiotic conditions, (iii) at different post-inoculation times, (iv) after passages, and (v) taking into account the interaction with the host.

More specifically, the objectives of this thesis have been:

1. To analyse the genetic diversity of PepMV populations in Murcia over tomato production seasons and to evaluate the fitness of six PepMV-CH2 isolates from different locations in single and mixed infections.

2. To study of the effect of an abiotic factor, temperature, on the genetic diversity and viral accumulation of two isolates of two strains of PepMV.

3. To identify the outcomes of over-infections of two PepMV strains, as well as the influence of mixed infections on virus transmission and diversification.

4. To describe the transcriptomic responses of tomato plants to PepMV in single and mixed infections.

3. Results

3.1. Chapter I. Long-term cocirculation of two strains of pepino mosaic virus in tomato crops and its effect on population genetic variability

3.1. Chapter I. Results

Long-term cocirculation of two strains of pepino mosaic virus in tomato crops and its effect on population genetic variability

C. Alcaide, M. P. Rabadán, M. Juárez, and P. Gómez

Phytopathology, 12 September 2019 | doi: 10.1094/PHYTO-07-19-0247-FI

Mixed viral infections are common in plants, and the evolutionary dynamics of viral populations may differ depending on whether the infection is caused by single or multiple viral strains. However, comparative studies of single and mixed infections using viral populations in comparable agricultural and geographical locations are lacking. Here, we monitored the occurrence of pepino mosaic virus (PepMV) in tomato crops in two major tomato-producing areas in Murcia (southeastern Spain), supporting evidence showing that PepMV disease-affected plants had single infections of the Chilean 2 (CH2) strain in one area and the other area exhibited long-term (13 years) coexistence of the CH2 and European (EU) strains. We hypothesized that circulating strains of PepMV might be modulating the differentiation between them and shaping the evolutionary dynamics of PepMV populations. Our phylogenetic analysis of 106 CH2 isolates randomly selected from both areas showed a remarkable divergence between the CH2 isolates, with increased nucleotide variability in the geographical area where both strains cocirculate. Furthermore, the potential virus-virus interaction was studied further by constructing six full-length infectious CH2 clones from both areas, and assessing their viral fitness in the presence and absence of an EU type isolate. All CH2 clones showed decreased fitness in mixed infections and although complete genome sequencing indicated a nucleotide divergence of those CH2 clones by area, the magnitude of the fitness response was irrespective of the CH2 origin. Overall, these results suggest that although agroecological cropping practices may be particularly important for explaining the evolutionary dynamics of PepMV in tomato crops, the cocirculation of both strains may have implications on the genetic variability of PepMV populations.

3.2. Chapter II. Increasing temperature alters the within-host competition of viral strains and influences virus genetic variability

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3.2. Chapter II: Results.

Increasing temperature alters the within-host competition of viral strains and influences virus genetic variability

Cristina Alcaide, Josep Sardanyés, Santiago F. Elena, and Pedro Gómez

Virus Evolution, 23 February 2021 | doi: 10.1093/ve/veab017

Environmental conditions can affect viral accumulation, virulence and adaptation, which have implications in the disease outcomes and efficiency of control measures. Concurrently, mixed viral infections are relevant in plants, being their epidemiology shaped by within-host virus–virus interactions. However, the extent in which the combined effect of variations in abiotic components of the plant ecological niche and the prevalence of mixed infections affect the evolutionary dynamics of viral populations is not well understood. Here, we explore the interplay between ecological and evolutionary factors during viral infections and show that isolates of two strains of *Pepino mosaic potexvirus* coexisted in tomato plants in a temperature-dependent continuum between neutral and antagonistic interactions. After a long-term infection, the mutational analysis of the evolved viral genomes revealed strain-specific single-nucleotide polymorphisms that were modulated by the interaction between the type of infection and temperature. These results suggest that the temperature is an ecological driver of virus-virus interactions, with an effect on the genetic diversity of individual viruses that are co-infecting an individual host. This research provides insights into the effect that changes in host growth temperatures might have on the evolutionary dynamics of viral populations in mixed infections.

3.3. Chapter III. Determinants of persistent patterns of pepino mosaic virus mixed infections

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3.3. Chapter III. Results

Determinants of persistent patterns of pepino mosaic virus mixed infections

Cristina Alcaide and Miguel A. Aranda

Frontiers in Microbiology, 06 July 2021 | doi: 10.3389/fmicb.2021.694492

Pepino mosaic virus (PepMV) has become a pandemic virus in tomato crops, causing important economic losses worldwide. In Spain, isolates of the EU and CH2 strains co-circulate, with PepMV-EU predominantly found in mixed infections. Simultaneous in planta mixed infections result in an asymmetric antagonism against PepMV-CH2, but the outcome of over-infections has never been tested. PepMV-EU and PepMVCH2 time-lagged inoculations were performed, and viral accumulation was measured 10 days after challenge inoculation. PepMV-EU had a protective effect over PepMVCH2; in contrast, the accumulation of PepMV-EU increased in plants pre-inoculated with PepMV-CH2 as compared to single infections. We also studied the effect of the type of infection on viral transmission. Independently of the nature of the infection (single or mixed), we observed a strong positive correlation between virus accumulation in the source plant and transmission, excluding mixed infection effects different than modulating viral accumulation. Finally, in order to determine the genetic variability of PepMV strains in single and mixed infections, a 430 nucleotide region was RT-PCR amplified from samples from a serial passages experiment and deep-sequenced. No significant differences were found in the number of nucleotide substitutions between single and mixed infections for PepMV-EU; in contrast, significant differences were found for PepMV-CH2, which was more variable in single than in mixed infections. Comparing PepMV-EU with PepMV-CH2, a higher nucleotide diversity was found for PepMV-CH2. Collectively, our data strongly suggest that PepMV mixed infections can impact the virus epidemiology by modulating *in planta* virus strain accumulation and diversification.

4. Conclusions

4. Conclusions

The following conclusions can be drawn from results obtained in this work:

1. The strain distribution of PepMV in tomato in Southeast Spain remained stable over the years, with PepMV-CH2 isolates being predominant and PepMV-EU isolates persisting in lower proportion and mainly in mixed infections.

2. Two tomato-producing areas that are geographically close to each other showed a different pattern of PepMV infections; single PepMV-CH2 infections were found in Águilas and mixed PepMV-EU and PepMV-CH2 infections were mostly observed in Mazarrón.

3. The Mazarrón PepMV-CH2 population, where mixed infections persist, had a larger intrapopulation nucleotide diversity than that in Águilas.

4. PepMV-CH2 isolates showed a fitness cost associated with the presence of a PepMV-EU isolate in tomato plants. This fitness cost was irrespective of the origin of the CH2 isolate (either from single or mixed infection).

5. PepMV-CH2 viral load was higher than that of PepMV-EU *in planta*, although time post-infection and temperature played an important role in PepMV accumulation, being PepMV infection more productive at 20ºC than at 30ºC.

6. The asymmetric antagonism between PepMV-EU and PepMV-CH2 is time-dependent, being particularly evident at early times post-infection.

7. PepMV-CH2 within-plant population displayed a higher number of variants than PepMV-EU population. However, no differences in the number of mutations were found between single and mixed infections at 20ºC neither for PepMV-EU nor for PepMV-CH2.

8. The mean number of mutations increased significantly with temperature. PepMV-EU population *in planta* had a higher number of mutations in single infections, whilst PepMV-CH2 population had a higher number of mutations in

mixed infections. Therefore, the mutational load was temperature- and strain-dependent.

9. The age of the plant had an effect on PepMV-CH2 accumulation, showing more viral accumulation the older the plant was.

10. In plants pre-inoculated with PepMV-EU and challenged with PepMV-CH2, the accumulation of the latter appeared to be repressed as compared to single infections, similarly to simultaneous mixed infections. However, PepMV-EU accumulation did not change significantly after challenge inoculation.

11. In plants pre-inoculated with PepMV-CH2 and challenged with PepMV-EU, the accumulation of the latter appeared to be stimulated as compared to single infections. In this case, PepMV-CH2 accumulation was not repressed, at least at later times after the challenge.

12. There was a strong positive correlation between virus accumulation and transmission. Therefore, mixed infections can have an important impact on virus transmission through their effects on virus accumulation in the plant source of inoculum.

13. During serial passaging in tomato plants, the number of *in planta* variants was larger for PepMV-CH2 than for PepMV-EU, although it remained rather constant with passaging, with mixed infections not associating with evident genetic bottlenecks.

14. Each viral strain modulated the host transcriptome differentially, with deeper transcriptomic alterations occurring at early infection times.

15. Mixed infections induced host transcriptome changes that are different than the sum of alterations caused by each single infections at 14 and 21 dpi, but not at early times (7 dpi).

16. *AGO1a*, *AGO2a*, *DCL2b* and *DCL2d* appeared to be principal determinants of the differentiation between PepMV-EU and PepMV-CH2 infections. However,

statistically significant differences were found only for *AGO2a* scaled FPKMs at 7 dpi.

17. *AGO2a* has a clear role in PepMV infection, but it does not seem to be a direct or only responsible for the PepMV-EU *vs* -CH2 antagonism, at least in *N. benthamiana*.

5. Annexes

5.1. Annex I

This annex compiles results of the current line of research. The data presented below is necessary to complete the scientific unit of the doctoral thesis since it helps to establish conclusions and discuss the results described in chapters I, II and III.

Infections by viruses can produce alterations in the host transcriptome (Gómez-Aix *et al.*, 2016; Hanssen *et al.*, 2011; Seo *et al.*, 2018), which in turn can depend on factors such as the time post-infection, type of infection or host ecology. So far, transcriptomic perturbations associated with PepMV infections have been studied using a tomato GeneChip microarray (Hanssen *et al.*, 2011). Different transcriptomic responses have been described for two PepMV-CH2 isolates producing different symptomatology in the host. In general terms, PepMV infection repressed genes related to photosynthesis in addition to deregulate the RNA silencing pathway (Hanssen *et al.*, 2011). RNA silencing is central for antiviral response (Baulcombe, 2004) and some of the host proteins involved in it are: RNAseIII Dicer-like (DCL), Argonaute (AGO) or RNA-dependent RNA polymerase (RDR) proteins (Ding, 2010; Ding and Voinnet, 2007; Pumplin and Voinnet, 2013). There are several members of each of these protein families with demonstrated antiviral functions, such as AGO1, AGO2, AGO4, AGO5, AGO7, AGO10, DCL2, DCL4, RDR1 and RDR6 (Carbonell and Carrington, 2015; Katsarou *et al.*, 2019; Liu *et al.*, 2009; Wassenegger and Krczal, 2006; Willmann *et al.*, 2011).

In spite that PepMV mixed strain infections have been studied from different perspectives, the effects that they may have on the host transcriptome are unknown. The results presented below describe the transcriptome response of tomato plants to PepMV single and mixed infections, with a particular focus on genes encoding the RNA silencing pathway components.

We first studied the differentially expressed genes (DEGs) between mock and PepMV-infected plants, finding that PepMV-CH2 and mixed infections produced stronger alterations on the host transcriptome than PepMV-EU single infection.

After that, we determined that single infections of PepMV-EU and PepMV-CH2 differentially modulate the tomato transcriptome in a strain-dependent manner, showing in both cases stronger transcriptomic alterations during early infection times. Comparing single and mixed PepMV infections, we found that both caused similar transcriptomic perturbations at early infection times, but clearly differed at later times. Then we look for host genes that could be involved in the antagonism between PepMV-EU and PepMV-CH2 and we found the *AGO2a* gene, among others. Then we decided to analyse other host genes implicated in the RNA silencing pathway, such as DCL, RDR and other AGO genes. We performed a heatmap and a clustering analysis of the scaled fragments per kilo base per million mapped reads (FPKM) at 7 days post-inoculation (dpi) (Figure S1). Three clear groups were observed depending on the sampling time, although at 7 dpi, the PepMV-EU and mixed infections grouped together and not with the PepMV-CH2 infection, being apparently *AGO1a*, *AGO2a*, *DCL2b and DCL2d*, the principal determinants of this differentiation (Figure S1).

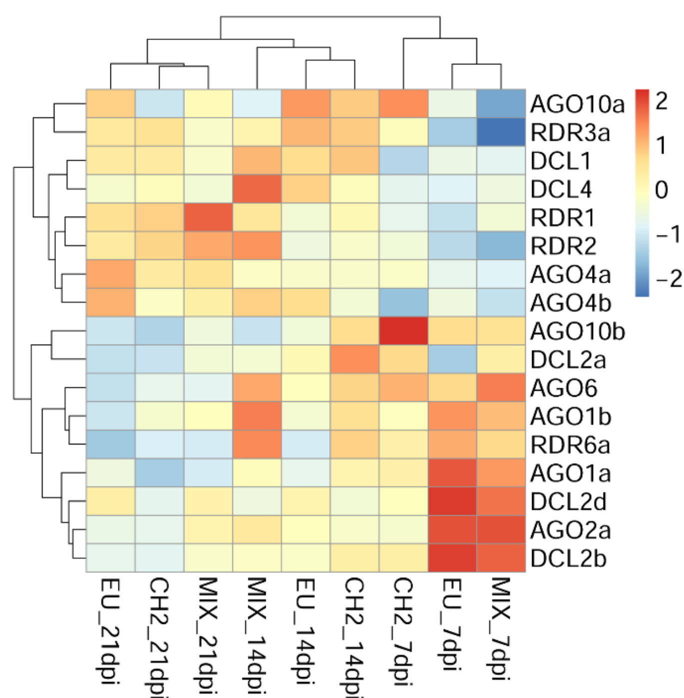

Figure S1. Heatmap of the genes involved in the RNA silencing pathway that are expressed in tomato plants. Scaled fragments per kilo base per million mapped reads (FPKM) values were used as input and are represented by colours. Red means higher expression and blue lower.

We then determined the differences in the scaled FPKMs of these four genes at 7 dpi and we found only statistically significant differences for *AGO2a*, showing PepMV-EU and mixed infections high expression levels compared to PepMV-CH2 single infection. To validate these expression pattern in tomato plants, a relative RT-qPCR was carried out, confirming the RNA-seq data. The same verification was performed in *N. benthamiana* plants, showing that *AGO2a* expression pattern was not exactly the same as in tomato plants although we also found significant differences at 7 dpi between PepMV-EU infected (single or mixed) and PepMV-CH2 infected plants. Then we took advantage of *ago2* knocked out *N. benthamiana* plants to study the role of this gene in PepMV infection. Higher accumulation of PepMV was found in *ago2* plants compared with wild type plants (Figure S2), showing that *ago2* plants were hyper-susceptible to PepMV. However, we did not find any changes in the antagonistic interaction between PepMV-EU and PepMV-CH2 (Figure S2), concluding that although AGO2a plays a role in PepMV infection, it is not the direct or only determinant of PepMV-EU and PepMV-CH2 antagonism, at least in *N. benthamiana*.

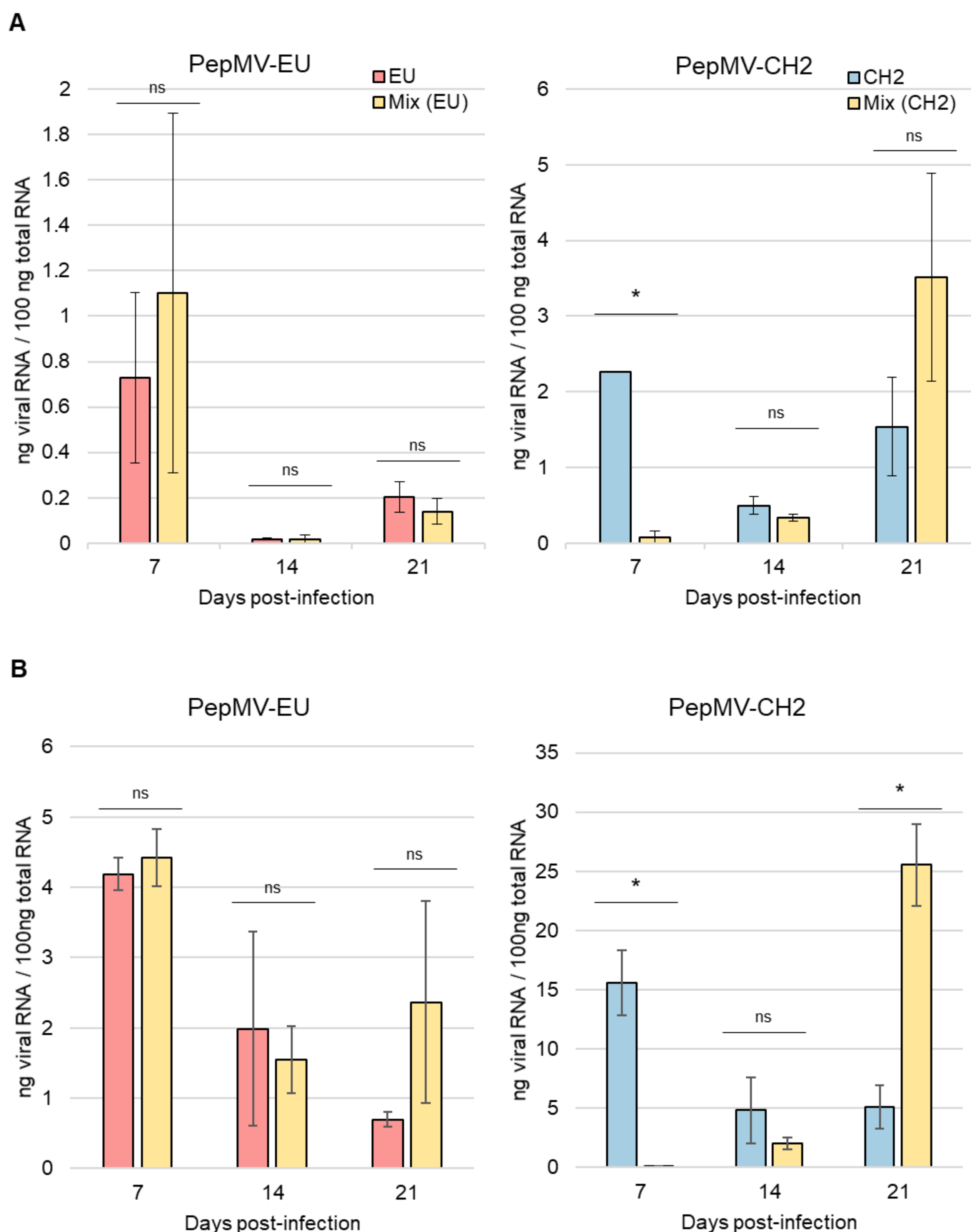

Figure S2. PepMV accumulation in *Nicotiana benthamiana* plants. Accumulation of PepMV-EU in single (red) and mixed (yellow) infections (left) or PepMV-CH2 in single (blue) and mixed (yellow) infections (right) at different times post-inoculation in **(A)** wild type and **(B)** *ago2 N. benthamiana* plants. Asterisks show significance level (* = p < 0.05; ns = no significant).

5.2. Annex II

The annex II includes the supplementary figures and tables corresponding to Chapters I, II and III of results.

- Supplementary Table 1 belonging to Chapter I of results.

Area			Year			
			2007	2008	2014	2015
Mazarrón	Nº of PepMV infected samples / total analysed samples		29/37	29/30	137/140	27/35
	Single infection	PepMV-EU	0	1	3	0
		PepMV-CH2	19	14	54	22
	Mixed infection (EU + CH2)		10	14	80	5
Águilas	Nº of PepMV infected samples / total analysed samples		9/28	23/52	80/120	0/25
	Single infection	PepMV-EU	0	0	0	0
		PepMV-CH2	9	23	80	0
	Mixed infection (EU + CH2)		0	0	0	0

Table S1. Number of tomato leaf samples examined in this study to determine the occurrence of PepMV. Samples are grouped by areas in different years and the type of infections (single or mixed).

- Supplementary Table 1 belonging to Chapter II of results.

PepMV strain	Temperature	Infection type	Replicate	SNP genome position	Frequency	Amino acid change
CH2	20ºC	Single	R1	2742	0.535548	R>K
CH2	20ºC	Single	R1	1349	0.211603	A>T
CH2	20ºC	Single	R1	566	0.192214	L>I
CH2	20ºC	Single	R1	1350	0.143142	A>V
CH2	20ºC	Single	R1	1404	0.114742	V>A
CH2	20ºC	Single	R1	1312	0.049679	D>E
CH2	20ºC	Single	R1	1419	0.037254	V>A
CH2	20ºC	Single	R1	560	0.036282	T>A
CH2	20ºC	Single	R1	3400	0.029944	Syn
CH2	20ºC	Single	R1	1412	0.028931	G>S
CH2	20ºC	Single	R1	545	0.023525	E>K
CH2	20ºC	Single	R1	3379	0.023281	Syn
CH2	20ºC	Single	R1	1413	0.018658	G>D
CH2	20ºC	Single	R1	4851	0.017452	V>F

CH2	20ºC	Single	R1	4736	0.017166	Syn
CH2	20ºC	Single	R1	1111	0.017019	Syn
CH2	20ºC	Single	R1	1326	0.015762	A>D
CH2	20ºC	Single	R1	1340	0.015483	I>V
CH2	20ºC	Single	R1	286	0.013928	Syn
CH2	20ºC	Single	R1	643	0.011348	Syn
CH2	20ºC	Single	R1	1403	0.011009	V>I
CH2	20ºC	Single	R2	1403	0.57941	V>I
CH2	20ºC	Single	R2	2742	0.528565	R>K
CH2	20ºC	Single	R2	1412	0.109702	G>S
CH2	20ºC	Single	R2	4003	0.106812	Syn
CH2	20ºC	Single	R2	679	0.101215	Syn
CH2	20ºC	Single	R2	1349	0.051402	A>T
CH2	20ºC	Single	R2	2914	0.041405	Syn
CH2	20ºC	Single	R2	4988	0.04093	Syn
CH2	20ºC	Single	R2	1393	0.040149	L>F
CH2	20ºC	Single	R2	1325	0.029358	A>S
CH2	20ºC	Single	R2	545	0.024903	E>K
CH2	20ºC	Single	R2	1404	0.019632	V>A
CH2	20ºC	Single	R2	798	0.016926	L>H
CH2	20ºC	Single	R2	4835	0.016264	Syn
CH2	20ºC	Single	R2	328	0.014719	Syn
CH2	20ºC	Single	R2	560	0.013662	T>A
CH2	20ºC	Single	R2	928	0.013655	Syn
CH2	20ºC	Single	R2	1326	0.01339	A>D
CH2	20ºC	Single	R2	1312	0.012082	D>E
CH2	20ºC	Single	R2	826	0.01201	Syn
CH2	20ºC	Single	R2	4694	0.011733	Syn
CH2	20ºC	Single	R2	341	0.011627	Syn
CH2	20ºC	Single	R2	2452	0.011333	Syn
CH2	20ºC	Single	R2	1338	0.010884	E>G
CH2	20ºC	Single	R3	1349	0.514458	A>T
CH2	20ºC	Single	R3	2742	0.28165	R>K
CH2	20ºC	Single	R3	1394	0.103091	T>A
CH2	20ºC	Single	R3	4343	0.08991	L>F
CH2	20ºC	Single	R3	1350	0.076624	A>V
CH2	20ºC	Single	R3	1403	0.066934	V>I
CH2	20ºC	Single	R3	2936	0.061578	D>N
CH2	20ºC	Single	R3	3163	0.045935	Syn
CH2	20ºC	Single	R3	1326	0.042617	A>D
CH2	20ºC	Single	R3	1425	0.034915	L>S
CH2	20ºC	Single	R3	5756	0.031199	I>V
CH2	20ºC	Single	R3	545	0.029313	E>K
CH2	20ºC	Single	R3	740	0.027239	E>K
CH2	20ºC	Single	R3	2482	0.022344	Syn
CH2	20ºC	Single	R3	1370	0.021961	T>A

CH2	20ºC	Single	R3	1396	0.01661	Syn
CH2	20ºC	Single	R3	4186	0.013915	Syn
CH2	20ºC	Single	R3	4894	0.012573	A>G
CH2	20ºC	Single	R3	1312	0.011821	D>E
CH2	20ºC	Single	R3	1312	0.011757	D>E
CH2	20ºC	Mixed	R1	2742	0.488147	R>K
CH2	20ºC	Mixed	R1	1349	0.342911	A>T
CH2	20ºC	Mixed	R1	1412	0.197082	G>S
CH2	20ºC	Mixed	R1	3943	0.170935	Syn
CH2	20ºC	Mixed	R1	1424	0.104451	L>V
CH2	20ºC	Mixed	R1	4419	0.068472	NC
CH2	20ºC	Mixed	R1	1425	0.066775	L>S
CH2	20ºC	Mixed	R1	2908	0.058186	Syn
CH2	20ºC	Mixed	R1	1350	0.042538	A>V
CH2	20ºC	Mixed	R1	1338	0.039318	E>G
CH2	20ºC	Mixed	R1	5791	0.028811	Syn
CH2	20ºC	Mixed	R1	1403	0.026765	V>I
CH2	20ºC	Mixed	R1	4992	0.019626	L>I
CH2	20ºC	Mixed	R1	5166	0.016067	Syn
CH2	20ºC	Mixed	R1	1933	0.015145	Syn
CH2	20ºC	Mixed	R1	1882	0.013097	Syn
CH2	20ºC	Mixed	R1	1404	0.013021	V>A
CH2	20ºC	Mixed	R1	749	0.012541	D>N
CH2	20ºC	Mixed	R1	2425	0.011047	Syn
CH2	20ºC	Mixed	R1	751	0.011006	D>E
CH2	20ºC	Mixed	R1	97	0.01028	Syn
CH2	20ºC	Mixed	R1	2431	0.010004	Syn
CH2	20ºC	Mixed	R2	2742	0.663107	R>K
CH2	20ºC	Mixed	R2	560	0.212599	T>A
CH2	20ºC	Mixed	R2	1412	0.208156	G>S
CH2	20ºC	Mixed	R2	1413	0.200663	G>D
CH2	20ºC	Mixed	R2	4087	0.193453	Syn
CH2	20ºC	Mixed	R2	1312	0.1872	D>E
CH2	20ºC	Mixed	R2	1349	0.066257	A>T
CH2	20ºC	Mixed	R2	4132	0.062946	Syn
CH2	20ºC	Mixed	R2	1621	0.050653	Syn
CH2	20ºC	Mixed	R2	1561	0.027433	Syn
CH2	20ºC	Mixed	R2	4643	0.020456	Syn
CH2	20ºC	Mixed	R2	1791	0.016513	R>K
CH2	20ºC	Mixed	R2	1403	0.014989	V>I
CH2	20ºC	Mixed	R2	292	0.013261	Syn
CH2	20ºC	Mixed	R2	4419	0.012963	NC
CH2	20ºC	Mixed	R2	2500	0.011851	Syn
CH2	20ºC	Mixed	R2	773	0.011364	V>I
CH2	20ºC	Mixed	R3	2742	0.48174	R>K
CH2	20ºC	Mixed	R3	1419	0.250231	V>A

CH2	20ºC	Mixed	R3	1424	0.223915	L>V
CH2	20ºC	Mixed	R3	2404	0.218551	Syn
CH2	20ºC	Mixed	R3	412	0.200654	Syn
CH2	20ºC	Mixed	R3	1371	0.184048	T>K
CH2	20ºC	Mixed	R3	2821	0.055287	Syn
CH2	20ºC	Mixed	R3	575	0.049257	R>C
CH2	20ºC	Mixed	R3	1349	0.025808	A>T
CH2	20ºC	Mixed	R3	5009	0.02525	Syn
CH2	20ºC	Mixed	R3	1338	0.022049	E>G
CH2	20ºC	Mixed	R3	1350	0.016655	A>E
CH2	20ºC	Mixed	R3	2890	0.0157	Syn
CH2	20ºC	Mixed	R3	1963	0.015162	Syn
CH2	20ºC	Mixed	R3	1365	0.014831	A>V
CH2	20ºC	Mixed	R3	2074	0.014639	Syn
CH2	20ºC	Mixed	R3	1312	0.011645	D>E
CH2	20ºC	Mixed	R3	1426	0.011558	L>F
CH2	20ºC	Mixed	R3	3571	0.011516	Syn
CH2	20ºC	Mixed	R3	545	0.011348	E>K
CH2	20ºC	Mixed	R3	4496	0.010216	Syn
CH2	20ºC	Mixed	R3	6100	0.010184	Syn
CH2	30ºC	Single	R1	2742	0.99233	R>K
CH2	30ºC	Single	R1	1312	0.838541	D>E
CH2	30ºC	Single	R1	5756	0.232488	I>V
CH2	30ºC	Single	R1	1325	0.226016	A>S
CH2	30ºC	Single	R1	1312	0.160364	D>E
CH2	30ºC	Single	R1	773	0.118393	V>I
CH2	30ºC	Single	R1	2356	0.055639	Syn
CH2	30ºC	Single	R1	1121	0.054317	A>S
CH2	30ºC	Single	R1	4016	0.035438	Syn
CH2	30ºC	Single	R1	2512	0.029586	Syn
CH2	30ºC	Single	R1	1483	0.028437	L>F
CH2	30ºC	Single	R1	1120	0.027494	Syn
CH2	30ºC	Single	R1	1591	0.025696	Syn
CH2	30ºC	Single	R1	2860	0.019233	Syn
CH2	30ºC	Single	R1	3685	0.017108	Syn
CH2	30ºC	Single	R1	2257	0.016295	Syn
CH2	30ºC	Single	R1	4240	0.01293	Syn
CH2	30ºC	Single	R1	3982	0.012324	Syn
CH2	30ºC	Single	R1	2627	0.011299	Syn
CH2	30ºC	Single	R2	2742	0.732341	R>K
CH2	30ºC	Single	R2	1312	0.555608	D>E
CH2	30ºC	Single	R2	1312	0.443501	D>E
CH2	30ºC	Single	R2	773	0.341787	V>I
CH2	30ºC	Single	R2	2936	0.27032	D>N
CH2	30ºC	Single	R2	4966	0.262857	T>I
CH2	30ºC	Single	R2	4057	0.159751	Syn

CH2	30ºC	Single	R2	2206	0.159345	Syn
CH2	30ºC	Single	R2	1759	0.126046	Syn
CH2	30ºC	Single	R2	941	0.100774	Q>K
CH2	30ºC	Single	R2	3646	0.096043	Syn
CH2	30ºC	Single	R2	5012	0.091475	Syn
CH2	30ºC	Single	R2	1121	0.072349	A>S
CH2	30ºC	Single	R2	3022	0.069281	Syn
CH2	30ºC	Single	R2	4387	0.067437	Syn
CH2	30ºC	Single	R2	1834	0.066049	Syn
CH2	30ºC	Single	R2	5034	0.065298	G>S
CH2	30ºC	Single	R2	1099	0.052798	Syn
CH2	30ºC	Single	R2	6070	0.038242	Syn
CH2	30ºC	Single	R2	5650	0.018475	Syn
CH2	30ºC	Single	R2	5620	0.017818	NC
CH2	30ºC	Single	R2	5134	0.017525	A>T
CH2	30ºC	Single	R2	1867	0.016235	Syn
CH2	30ºC	Single	R2	1609	0.014538	Syn
CH2	30ºC	Single	R2	2386	0.012649	Syn
CH2	30ºC	Single	R2	2811	0.012367	K>R
CH2	30ºC	Single	R2	1121	0.012365	A>T
CH2	30ºC	Single	R2	880	0.011987	Syn
CH2	30ºC	Single	R2	5159	0.011666	I>T
CH2	30ºC	Single	R2	4138	0.011309	Syn
CH2	30ºC	Single	R2	2290	0.010589	Syn
CH2	30ºC	Single	R3	2742	0.963591	R>K
CH2	30ºC	Single	R3	1312	0.554882	D>E
CH2	30ºC	Single	R3	1312	0.444534	D>E
CH2	30ºC	Single	R3	1487	0.201813	S>P
CH2	30ºC	Single	R3	2754	0.189475	L>S
CH2	30ºC	Single	R3	2107	0.173126	Syn
CH2	30ºC	Single	R3	1633	0.17225	Syn
CH2	30ºC	Single	R3	4416	0.170452	NC
CH2	30ºC	Single	R3	773	0.164403	V>I
CH2	30ºC	Single	R3	5824	0.160386	Syn
CH2	30ºC	Single	R3	1483	0.144965	L>F
CH2	30ºC	Single	R3	5710	0.143869	Syn
CH2	30ºC	Single	R3	454	0.134274	Syn
CH2	30ºC	Single	R3	1483	0.111017	L>F
CH2	30ºC	Single	R3	1576	0.07788	Syn
CH2	30ºC	Single	R3	5538	0.058625	A>E
CH2	30ºC	Single	R3	3620	0.043401	Syn
CH2	30ºC	Single	R3	941	0.034691	Q>K
CH2	30ºC	Single	R3	1487	0.032322	S>T
CH2	30ºC	Single	R3	381	0.012493	R>K
CH2	30ºC	Single	R3	2470	0.011886	Syn
CH2	30ºC	Single	R3	2968	0.01019	Syn

CH2	30ºC	Mixed	R1	1312	0.723023	D>E
CH2	30ºC	Mixed	R1	2742	0.66898	R>K
CH2	30ºC	Mixed	R1	1312	0.276615	D>E
CH2	30ºC	Mixed	R1	4946	0.248172	Syn
CH2	30ºC	Mixed	R1	2696	0.242478	Syn
CH2	30ºC	Mixed	R1	1371	0.22489	T>K
CH2	30ºC	Mixed	R1	4638	0.207083	S>T
CH2	30ºC	Mixed	R1	773	0.192976	V>I
CH2	30ºC	Mixed	R1	545	0.15867	E>K
CH2	30ºC	Mixed	R1	4985	0.131516	Syn
CH2	30ºC	Mixed	R1	154	0.09166	Syn
CH2	30ºC	Mixed	R1	4054	0.088815	Syn
CH2	30ºC	Mixed	R1	229	0.046916	Syn
CH2	30ºC	Mixed	R1	2512	0.046539	Syn
CH2	30ºC	Mixed	R1	5175	0.046174	Syn
CH2	30ºC	Mixed	R1	1121	0.042719	A>S
CH2	30ºC	Mixed	R1	2644	0.035154	Syn
CH2	30ºC	Mixed	R1	3334	0.031217	Syn
CH2	30ºC	Mixed	R1	2167	0.028662	Syn
CH2	30ºC	Mixed	R1	1325	0.028394	A>P
CH2	30ºC	Mixed	R1	2206	0.026564	Syn
CH2	30ºC	Mixed	R1	4409	0.026244	NC
CH2	30ºC	Mixed	R1	4471	0.024688	F>Y
CH2	30ºC	Mixed	R1	5884	0.023167	Syn
CH2	30ºC	Mixed	R1	4477	0.016709	H>P
CH2	30ºC	Mixed	R1	3661	0.014519	Syn
CH2	30ºC	Mixed	R1	4411	0.014469	NC
CH2	30ºC	Mixed	R1	4189	0.014245	Syn
CH2	30ºC	Mixed	R1	5057	0.013062	Syn
CH2	30ºC	Mixed	R1	5466	0.012888	Syn(2)/Q>L(3)
CH2	30ºC	Mixed	R1	2563	0.012197	Syn
CH2	30ºC	Mixed	R1	1079	0.011283	Syn
CH2	30ºC	Mixed	R1	1481	0.011124	Syn
CH2	30ºC	Mixed	R1	5280	0.010575	Syn
CH2	30ºC	Mixed	R1	2936	0.010168	D>N
CH2	30ºC	Mixed	R2	1312	0.886015	D>E
CH2	30ºC	Mixed	R2	2742	0.532242	R>K
CH2	30ºC	Mixed	R2	5329	0.463066	K>Q
CH2	30ºC	Mixed	R2	1312	0.113325	D>E
CH2	30ºC	Mixed	R2	773	0.112474	V>I
CH2	30ºC	Mixed	R2	3559	0.103911	Syn
CH2	30ºC	Mixed	R2	2936	0.099402	D>N
CH2	30ºC	Mixed	R2	1009	0.074298	Syn
CH2	30ºC	Mixed	R2	1487	0.066805	S>P
CH2	30ºC	Mixed	R2	529	0.056401	Syn
CH2	30ºC	Mixed	R2	6100	0.034816	Syn

CH2	30ºC	Mixed	R2	403	0.027707	Syn
CH2	30ºC	Mixed	R2	4036	0.026002	Syn
CH2	30ºC	Mixed	R2	2812	0.025046	Syn
CH2	30ºC	Mixed	R2	2552	0.023293	Syn
CH2	30ºC	Mixed	R2	6007	0.023011	Syn
CH2	30ºC	Mixed	R2	381	0.021777	R>K
CH2	30ºC	Mixed	R2	5356	0.021064	I>F(2)/Syn(3)
CH2	30ºC	Mixed	R2	1814	0.020225	L>F
CH2	30ºC	Mixed	R2	2470	0.017421	Syn
CH2	30ºC	Mixed	R2	418	0.017104	Syn
CH2	30ºC	Mixed	R2	2341	0.01689	Syn
CH2	30ºC	Mixed	R2	3307	0.015458	Syn
CH2	30ºC	Mixed	R2	3904	0.014077	Syn
CH2	30ºC	Mixed	R2	376	0.013004	Syn
CH2	30ºC	Mixed	R2	2695	0.012665	Syn
CH2	30ºC	Mixed	R2	3964	0.012439	Syn
CH2	30ºC	Mixed	R2	5965	0.01128	Syn
CH2	30ºC	Mixed	R2	4357	0.011011	Syn
CH2	30ºC	Mixed	R2	1265	0.010661	Syn
CH2	30ºC	Mixed	R3	2742	0.811013	R>K
CH2	30ºC	Mixed	R3	1312	0.716879	D>E
CH2	30ºC	Mixed	R3	1312	0.282575	D>E
CH2	30ºC	Mixed	R3	3172	0.132084	Syn
CH2	30ºC	Mixed	R3	3517	0.086118	Syn
CH2	30ºC	Mixed	R3	4643	0.079916	Syn
CH2	30ºC	Mixed	R3	3421	0.069456	Syn
CH2	30ºC	Mixed	R3	1483	0.068272	L>F
CH2	30ºC	Mixed	R3	4416	0.064298	NC
CH2	30ºC	Mixed	R3	3226	0.062723	Syn
CH2	30ºC	Mixed	R3	490	0.062338	Syn
CH2	30ºC	Mixed	R3	4411	0.058797	NC
CH2	30ºC	Mixed	R3	3565	0.051629	Syn
CH2	30ºC	Mixed	R3	5935	0.048121	Syn
CH2	30ºC	Mixed	R3	1778	0.044558	Syn
CH2	30ºC	Mixed	R3	4559	0.040753	Syn
CH2	30ºC	Mixed	R3	3964	0.040342	Syn
CH2	30ºC	Mixed	R3	4493	0.040261	Syn
CH2	30ºC	Mixed	R3	1927	0.039051	Syn
CH2	30ºC	Mixed	R3	4925	0.036892	Syn
CH2	30ºC	Mixed	R3	4409	0.036506	NC
CH2	30ºC	Mixed	R3	3317	0.03646	Syn
CH2	30ºC	Mixed	R3	5154	0.033788	Syn
CH2	30ºC	Mixed	R3	4033	0.032291	Syn
CH2	30ºC	Mixed	R3	1850	0.031748	A>T
CH2	30ºC	Mixed	R3	2320	0.028022	Syn
CH2	30ºC	Mixed	R3	4418	0.027327	NC

CH2	30ºC	Mixed	R3	2620	0.024618	Syn
CH2	30ºC	Mixed	R3	4410	0.024432	NC
CH2	30ºC	Mixed	R3	6208	0.023563	Syn
CH2	30ºC	Mixed	R3	191	0.021938	Syn
CH2	30ºC	Mixed	R3	4408	0.021103	NC
CH2	30ºC	Mixed	R3	773	0.018124	V>I
CH2	30ºC	Mixed	R3	1580	0.017674	E>K
CH2	30ºC	Mixed	R3	1506	0.015004	R>Q
CH2	30ºC	Mixed	R3	1237	0.014453	Syn
CH2	30ºC	Mixed	R3	4966	0.014185	T>I
CH2	30ºC	Mixed	R3	994	0.01413	Syn
CH2	30ºC	Mixed	R3	381	0.012978	R>K
CH2	30ºC	Mixed	R3	865	0.012373	Syn
CH2	30ºC	Mixed	R3	5295	0.012186	Syn
CH2	30ºC	Mixed	R3	589	0.011827	Syn
CH2	30ºC	Mixed	R3	4649	0.011637	Syn
CH2	30ºC	Mixed	R3	2754	0.011384	L>S
CH2	30ºC	Mixed	R3	2644	0.010864	Syn
CH2	30ºC	Mixed	R3	1114	0.010833	Syn
CH2	30ºC	Mixed	R3	4934	0.010297	E>D
EU	20ºC	Single	R1	1797	0.073149	E>K
EU	20ºC	Single	R1	4226	0.049148	Syn
EU	20ºC	Single	R1	2648	0.030996	Syn
EU	20ºC	Single	R1	2897	0.027023	Syn
EU	20ºC	Single	R1	4519	0.024078	I>V
EU	20ºC	Single	R1	5914	0.022859	Syn
EU	20ºC	Single	R1	4295	0.021007	Syn
EU	20ºC	Single	R1	38	0.020227	NC
EU	20ºC	Single	R1	3944	0.018115	Syn
EU	20ºC	Single	R1	6145	0.015996	Syn
EU	20ºC	Single	R1	1043	0.015406	Syn
EU	20ºC	Single	R1	3485	0.014913	Syn
EU	20ºC	Single	R1	4969	0.013921	T>A
EU	20ºC	Single	R1	1742	0.012268	Syn
EU	20ºC	Single	R1	1532	0.01113	N>K
EU	20ºC	Single	R1	3135	0.011091	Syn
EU	20ºC	Single	R1	3938	0.010594	Syn
EU	20ºC	Single	R2	2636	0.203647	Syn
EU	20ºC	Single	R2	212	0.118703	Syn
EU	20ºC	Single	R2	3299	0.092898	Syn
EU	20ºC	Single	R2	1837	0.02398	V>G
EU	20ºC	Single	R2	4550	0.017364	R>K
EU	20ºC	Single	R2	2531	0.017117	Syn
EU	20ºC	Single	R2	4933	0.016053	E>K
EU	20ºC	Single	R2	5180	0.015719	Syn
EU	20ºC	Single	R2	5019	0.015521	Syn

EU	20ºC	Single	R2	1589	0.013681	I>M
EU	20ºC	Single	R2	2817	0.011868	V>I
EU	20ºC	Single	R2	2507	0.011557	Syn
EU	20ºC	Single	R3	308	0.073321	Syn
EU	20ºC	Single	R3	2812	0.017754	P>Q
EU	20ºC	Single	R3	1620	0.016324	D>N
EU	20ºC	Single	R3	713	0.015242	Syn
EU	20ºC	Single	R3	1406	0.014109	Syn
EU	20ºC	Single	R3	5281	0.013189	Syn
EU	20ºC	Single	R3	5037	0.012041	Syn
EU	20ºC	Single	R3	5411	0.011485	R>C(2)/Syn(3)
EU	20ºC	Single	R3	1532	0.010595	N>K
EU	20ºC	Mixed	R1	81	0.24777	NC
EU	20ºC	Mixed	R1	4653	0.167333	Syn
EU	20ºC	Mixed	R1	1824	0.100537	D>N
EU	20ºC	Mixed	R1	2004	0.046381	S>G
EU	20ºC	Mixed	R1	1112	0.039044	Syn
EU	20ºC	Mixed	R1	2969	0.025765	Syn
EU	20ºC	Mixed	R1	4226	0.025736	Syn
EU	20ºC	Mixed	R1	4499	0.019673	G>V
EU	20ºC	Mixed	R1	5403	0.017761	S>I(2)/A>S(3)
EU	20ºC	Mixed	R1	1368	0.015015	T>A
EU	20ºC	Mixed	R1	1532	0.013849	N>K
EU	20ºC	Mixed	R1	302	0.012838	Syn
EU	20ºC	Mixed	R1	4499	0.011633	G>E
EU	20ºC	Mixed	R1	4250	0.011529	Syn
EU	20ºC	Mixed	R1	681	0.011165	E>K
EU	20ºC	Mixed	R2	5077	0.14008	Syn
EU	20ºC	Mixed	R2	1620	0.043765	D>N
EU	20ºC	Mixed	R2	5998	0.028662	Syn
EU	20ºC	Mixed	R2	4370	0.019477	Syn
EU	20ºC	Mixed	R2	4714	0.015111	Syn
EU	20ºC	Mixed	R2	4499	0.014984	G>V
EU	20ºC	Mixed	R2	3581	0.012407	Syn
EU	20ºC	Mixed	R3	1695	0.251946	A>T
EU	20ºC	Mixed	R3	4989	0.235214	Syn
EU	20ºC	Mixed	R3	1526	0.072138	Syn
EU	20ºC	Mixed	R3	1802	0.044656	Syn
EU	20ºC	Mixed	R3	1298	0.042541	Syn
EU	20ºC	Mixed	R3	3545	0.036352	Syn
EU	20ºC	Mixed	R3	5203	0.031681	F>L
EU	20ºC	Mixed	R3	4845	0.028365	Syn
EU	20ºC	Mixed	R3	1532	0.028123	N>K
EU	20ºC	Mixed	R3	779	0.019937	Syn
EU	20ºC	Mixed	R3	5308	0.018181	Syn
EU	20ºC	Mixed	R3	6100	0.016365	Syn

EU	20ºC	Mixed	R3	4395	0.010617	A>P
EU	30ºC	Single	R1	2786	0.230744	Syn
EU	30ºC	Single	R1	1455	0.221925	I>L
EU	30ºC	Single	R1	4752	0.113595	Syn
EU	30ºC	Single	R1	1418	0.106893	Syn
EU	30ºC	Single	R1	3972	0.068751	H>Y
EU	30ºC	Single	R1	1106	0.068529	Syn
EU	30ºC	Single	R1	2984	0.059274	Syn
EU	30ºC	Single	R1	1991	0.053341	Syn
EU	30ºC	Single	R1	4068	0.051209	K>Q
EU	30ºC	Single	R1	2695	0.031971	N>S
EU	30ºC	Single	R1	3830	0.02608	Syn
EU	30ºC	Single	R1	4211	0.021733	Syn
EU	30ºC	Single	R1	68	0.021343	NC
EU	30ºC	Single	R1	1025	0.020469	Syn
EU	30ºC	Single	R1	5224	0.016586	Syn
EU	30ºC	Single	R1	5079	0.014007	Syn
EU	30ºC	Single	R1	5896	0.013853	Syn
EU	30ºC	Single	R1	3956	0.013102	Syn
EU	30ºC	Single	R1	1837	0.012024	V>G
EU	30ºC	Single	R1	179	0.012009	Syn
EU	30ºC	Single	R1	1492	0.011883	Q>L
EU	30ºC	Single	R1	1475	0.011801	E>D
EU	30ºC	Single	R2	5356	0.19772	Syn(2)/S>F(3)
EU	30ºC	Single	R2	3972	0.190263	H>Y
EU	30ºC	Single	R2	1455	0.174081	I>L
EU	30ºC	Single	R2	5998	0.071382	Syn
EU	30ºC	Single	R2	1455	0.068251	I>L
EU	30ºC	Single	R2	4456	0.066289	Syn
EU	30ºC	Single	R2	4068	0.057419	K>Q
EU	30ºC	Single	R2	6306	0.040549	A>V
EU	30ºC	Single	R2	5236	0.036218	Syn
EU	30ºC	Single	R2	6172	0.029895	Syn
EU	30ºC	Single	R2	5857	0.029421	Syn
EU	30ºC	Single	R2	1494	0.027229	V>L
EU	30ºC	Single	R2	4611	0.022307	Syn
EU	30ºC	Single	R2	4628	0.021049	S>L
EU	30ºC	Single	R2	4499	0.018263	G>V
EU	30ºC	Single	R2	5791	0.018089	Syn
EU	30ºC	Single	R2	4499	0.016965	G>E
EU	30ºC	Single	R2	5761	0.016249	Syn
EU	30ºC	Single	R2	5615	0.013299	NC
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EU	30ºC	Single	R2	4987	0.011494	Syn
EU	30ºC	Single	R2	5730	0.011443	T>R
EU	30ºC	Single	R2	623	0.011257	Syn
EU	30ºC	Single	R2	5947	0.01107	Syn
EU	30ºC	Single	R2	2486	0.010387	Syn
EU	30ºC	Single	R2	6226	0.010341	Syn
EU	30ºC	Single	R2	5893	0.010337	Syn
EU	30ºC	Single	R2	4798	0.010262	F>L
EU	30ºC	Single	R2	6189	0.010151	E>A
EU	30ºC	Single	R2	1589	0.010137	I>M
EU	30ºC	Single	R2	1532	0.010055	N>K
EU	30ºC	Single	R2	5025	0.010042	Syn
EU	30ºC	Single	R3	1475	0.126648	E>D
EU	30ºC	Single	R3	3972	0.074042	H>Y
EU	30ºC	Single	R3	5632	0.040931	NC
EU	30ºC	Single	R3	962	0.032311	Syn
EU	30ºC	Single	R3	6265	0.031784	Syn
EU	30ºC	Single	R3	864	0.030816	Syn
EU	30ºC	Single	R3	1106	0.026031	Syn
EU	30ºC	Single	R3	4965	0.025629	Syn
EU	30ºC	Single	R3	179	0.024194	Syn
EU	30ºC	Single	R3	5960	0.023758	Syn
EU	30ºC	Single	R3	1455	0.022342	I>L
EU	30ºC	Single	R3	4465	0.021448	Syn
EU	30ºC	Single	R3	4725	0.019677	Syn
EU	30ºC	Single	R3	1443	0.018662	R>G
EU	30ºC	Single	R3	1970	0.01858	Syn
EU	30ºC	Single	R3	2345	0.017151	Syn
EU	30ºC	Single	R3	2699	0.017107	Syn
EU	30ºC	Single	R3	5482	0.016945	Syn(2)/I>T(3)
EU	30ºC	Single	R3	671	0.016227	Syn
EU	30ºC	Single	R3	6189	0.016097	E>A
EU	30ºC	Single	R3	2420	0.014198	Syn
EU	30ºC	Single	R3	1532	0.014176	N>K
EU	30ºC	Single	R3	5974	0.013528	Syn
EU	30ºC	Single	R3	5745	0.011162	S>N
EU	30ºC	Single	R3	5660	0.010206	T>P
EU	30ºC	Mixed	R1	5731	0.099662	Syn
EU	30ºC	Mixed	R1	4069	0.099495	K>M
EU	30ºC	Mixed	R1	3972	0.048719	H>Y
EU	30ºC	Mixed	R1	2695	0.034289	N>S
EU	30ºC	Mixed	R1	4686	0.031732	Syn
EU	30ºC	Mixed	R1	1607	0.027778	Syn
EU	30ºC	Mixed	R1	4102	0.022862	K>R
EU	30ºC	Mixed	R1	5170	0.021017	Syn
EU	30ºC	Mixed	R1	2672	0.020517	Syn

EU	30ºC	Mixed	R1	5519	0.019923	Syn
EU	30ºC	Mixed	R1	299	0.019367	Syn
EU	30ºC	Mixed	R1	5257	0.016104	Syn
EU	30ºC	Mixed	R1	5189	0.015974	A>S
EU	30ºC	Mixed	R1	1592	0.014691	Syn
EU	30ºC	Mixed	R1	1532	0.012652	N>K
EU	30ºC	Mixed	R1	5621	0.012615	NC
EU	30ºC	Mixed	R1	3359	0.010923	Syn
EU	30ºC	Mixed	R1	4881	0.010361	E>D
EU	30ºC	Mixed	R2	1475	0.109237	E>D
EU	30ºC	Mixed	R2	4222	0.077166	H>R
EU	30ºC	Mixed	R2	67	0.070517	NC
EU	30ºC	Mixed	R2	2695	0.038984	N>S
EU	30ºC	Mixed	R2	4274	0.038454	Syn
EU	30ºC	Mixed	R2	2170	0.035942	F>Y
EU	30ºC	Mixed	R2	4068	0.03496	K>Q
EU	30ºC	Mixed	R2	5954	0.033201	S>P
EU	30ºC	Mixed	R2	4499	0.022833	G>V
EU	30ºC	Mixed	R2	6154	0.018475	Syn
EU	30ºC	Mixed	R2	1106	0.014516	Syn
EU	30ºC	Mixed	R2	1526	0.014308	Syn
EU	30ºC	Mixed	R2	1797	0.013891	E>K
EU	30ºC	Mixed	R2	1640	0.013677	Syn
EU	30ºC	Mixed	R2	2411	0.013664	Syn
EU	30ºC	Mixed	R2	2891	0.013562	Syn
EU	30ºC	Mixed	R2	3972	0.012736	H>Y
EU	30ºC	Mixed	R2	4340	0.012564	Syn
EU	30ºC	Mixed	R2	5329	0.012214	Syn
EU	30ºC	Mixed	R2	4238	0.010908	Syn
EU	30ºC	Mixed	R2	2193	0.010892	V>I
EU	30ºC	Mixed	R2	5728	0.010714	Syn
EU	30ºC	Mixed	R3	2927	0.035954	Syn
EU	30ºC	Mixed	R3	5998	0.025657	Syn
EU	30ºC	Mixed	R3	5740	0.021267	Syn
EU	30ºC	Mixed	R3	2828	0.018464	Syn
EU	30ºC	Mixed	R3	5941	0.016283	Syn
EU	30ºC	Mixed	R3	3428	0.014013	Syn
EU	30ºC	Mixed	R3	4241	0.01226	Syn
EU	30ºC	Mixed	R3	5854	0.012155	Syn

- Supplementary figures and tables belonging to Chapter III of results.

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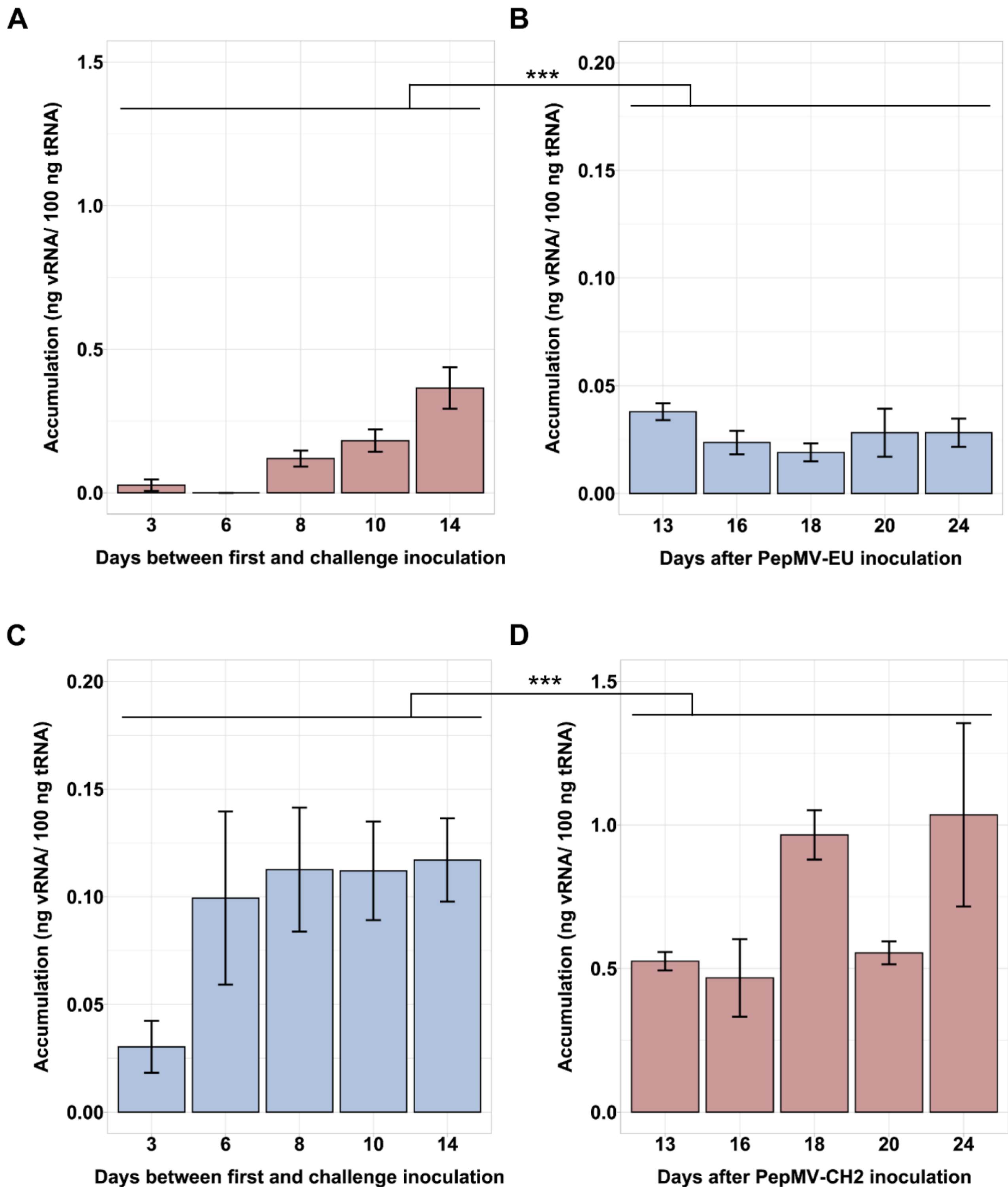
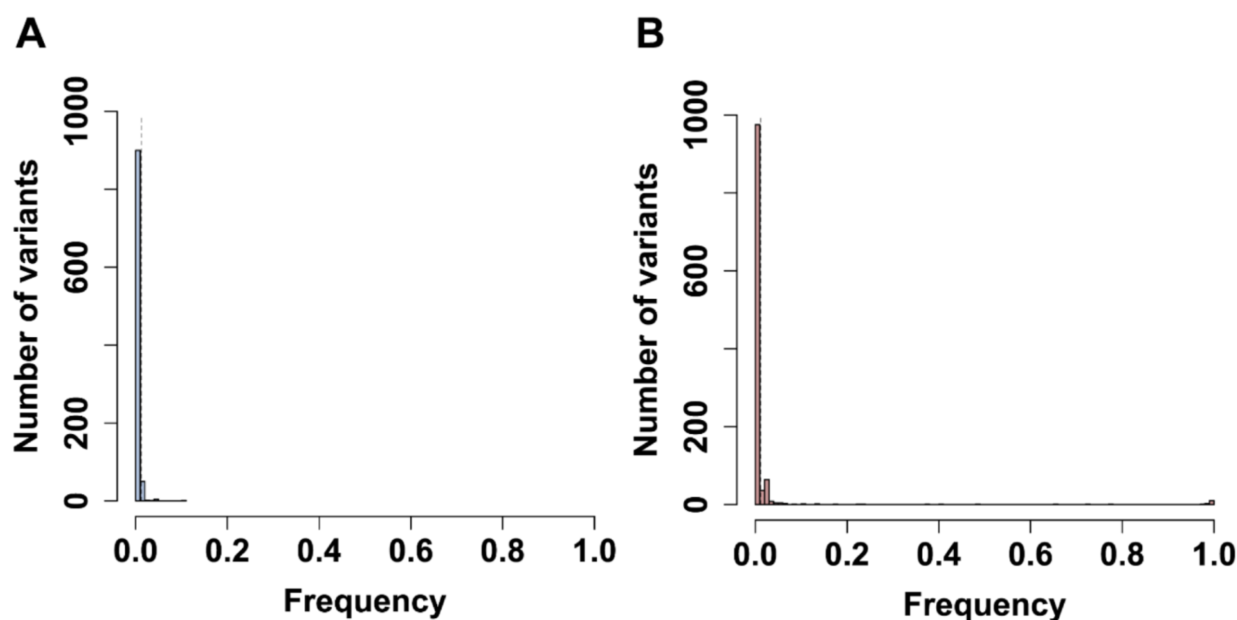
Supplementary Material

Supplementary Figures



Supplementary Material

Supplementary Figure 1. Effect of PepMV-CH2 **(A, D)** or PepMV-EU **(B, C)** over-infections on virus accumulation. PepMV-CH2 **(A)** and PepMV-EU **(D)** accumulation was measured in plants pre-inoculated with PepMV-EU and challenged with PepMV-CH2. Also PepMV-CH2 **(B)** and PepMV-EU **(C)** accumulation was measured in plants pre-inoculated with PepMV-CH2 and challenged with PepMV-EU. Over-inoculations were carried out at different times after pre-inoculation (3, 6, 8, 10 and 14 days). Viral accumulation was determined for 3 plants for each time point by absolute RT-qPCR and shown as ng viral RNA / 100 ng total RNA; each bar represents the mean and its standard deviation. Asterisks shows significance level (*** = $p < 0.001$).



Supplementary Figure 2. Number of variants and their frequencies found in PepMV-EU **(A)** and PepMV-CH2 **(B)** populations. SNPs with a frequency lower than 0.01 were excluded from the analysis (left to dashed line).

Suplementary Table 1

Source plant	Accumulation[1]	Nº of infected plants	Strain	Infection type
Single EU	1,12E-01	14	EU	Single
Single EU dilution 1:5	2,23E-02	11	EU	Single
Single EU dilution 1:25	4,46E-03	9	EU	Single
Simultaneous EU + CH2	1,35E-01	14	EU	Mixed
Simultaneous EU + CH2 dilution 1:5	2,70E-02	11	EU	Mixed
Simultaneous EU + CH2 dilution 1:25	5,41E-03	7	EU	Mixed
Overinfection CH2	4,70E-02	15	EU	Mixed
Overinfection CH2 dilution 1:5	9,39E-03	14	EU	Mixed
Overinfection CH2 dilution 1:25	1,88E-03	6	EU	Mixed

Overinfection EU	6,55E-01	18	EU	Mixed
Overinfection EU dilution 1:5	1,31E-01	17	EU	Mixed
Overinfection EU dilution 1:25	2,62E-02	16	EU	Mixed
Single CH2	1,22E+00	16	CH2	Single
Single CH2 dilution 1:5	2,45E-01	13	CH2	Single
Single CH2 dilution 1:25	4,90E-02	12	CH2	Single
Simultaneous EU + CH2	7,86E-02	17	CH2	Mixed
Simultaneous EU + CH2 dilution 1:5	1,57E-02	17	CH2	Mixed
Simultaneous EU + CH2 dilution 1:25	3,14E-03	10	CH2	Mixed
Overinfection CH2	2,75E-05	0	CH2	Mixed
Overinfection CH2 dilution 1:5	5,50E-06	0	CH2	Mixed
Overinfection CH2 dilution 1:25	1,10E-06	0	CH2	Mixed
Overinfection EU	1,86E+00	18	CH2	Mixed
Overinfection EU dilution 1:5	3,72E-01	17	CH2	Mixed
Overinfection EU dilution 1:25	7,43E-02	15	CH2	Mixed

[1] Viral accumulation (ng viral RNA / 100 ng total RNA) in the different inoculum sources is shown, including that for two single infections, one simultaneous mixed-infection, two over-infections and their dilutions.

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