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PATHOGENICITY AND HOST RANGE DETERMINANTS IN PLUM POX VIRUS INFECTION: A ROLE FOR P1 PROTEIN

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“Investigar es ver lo que otros también ven y pensar lo que nadie había pensado.”

Hans Adolf Krebs a Federico Mayor Zaragoza

A mis padres y mi hermana,

A Germán,

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http://martingallego.blogspot.com/2007/03/flor-de-melocotonero.html
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ABBREVIATIONS

Measure units: International units

ATPase	Adenosin triphosphatase
BrET	Ethidium Bromide
cDNA (ADNc)	copy DNA
CI	Cilindrical inclusions protein
CP	Capsid protein
cv	Cultivar
DASI-ELISA	Double antibody sandwich indirect
DLR	DNA loading Ratio
DNA	Deoxiribonucleic acid
DNase	Deoxiribonuclease
d.p.i.	Days post-inoculation
dNTPs	Equimolar mixture of the four deoxiribonuclotides
EDTA	Ethilene-diamin tetraacetic acid
ELISA	Enzyme-linked inmunosorbent assay
eIF	eucariotic traduction initiation factor
Fig.	Figure
GFP	Green fluorescent protein
HCPro	Helper component protein
HR	Hypersensitive response
IC	Immunocapture
IgG	Immunoglobuline G
kDa	Kilo Daltons
miRNA	Micro RNA
MLQ	Microcarrier loading quantity
MP	Movement protein
mRNA	Messenger RNA
NAT	Non-aphid transmissible
NCR	Non-coding region
NI	Nuclear inclusion
NOS	Nopaline sintase terminator signal
nt	Nucleotide
NTR	Non translated region
NTBD	NTP-binding domain
NTP	Nucleotide triphosphate
NTPase	Nucleotide triphosphatase
PABP	Poly-(A)-binding protein
O-GlcNAcylation	O-glicosil N-acylation
ORF	Open reading frame
PCR	Polimerase chain reaction
Poly-(A) tail	Polyadenosine tail
PR	Pathogenesis related protein
PTGS	Post-transcriptional gene silencing
RNA	Ribonucleic acid
RdRp	RNA dependent RNA polymerase
rgs-CaM	Calmodulin-related protein
RT	Reverse transcription
SDS-PAGE	Polyacrylamide gel electrophoresis in the presence of Sodium dodecil sulfate
SEL	Size exclusion limit
sRNA	Small RNA
siRNA	Small interferent RNA
ssRNA	Single stranded RNA
TBE	Tris-boric acid 90mM, Na2-EDTA 2mM pH8 Buffer
UTR	Untraslated region
UV	Ultraviolet
VIGS	Virus-induced gene silencing
wt	Wild type
3D	Tridimensional

VIRUSES CITED

CaMV	Cauliflower mosaic virus
CRLV	Carrot red leaf virus
CCMV	Cowpea chlorotic mottle virus
CMV	Cucumber mosaic virus
CymRSV	Cymbidium ringspot virus
CIRV	Carnation Italina ringspot virus
LMV	Lettuce mosaic virus
PPV	Plum pox virus
PSbMV	Pea seedborne mosaic virus
PVA	Potato virus A
PVBV	Pepper vein banding virus
PVX	Potato virus X
PVY	Potato virus Y
TEV	Tobacco etch virus
TuMV	Turnip mosaic virus
TVMV	Tobacco vein mottling virus
WSMV	Wheat streak mosaic virus

SPANISH SUMARY

Las enfermedades de las plantas están causadas por una gran diversidad de agentes patógenos que producen efectos negativos en las mismas y, en algunas ocasiones, los daños sufridos pueden ir acompañados de importantes pérdidas económicas. Se entiende por enfermedad la disfunción de un proceso causada por una acción continuada, con efectos deletéreos para el sistema viviente como consecuencia de la manifestación de síntomas (definición según el Comité de Terminología de la Sociedad Americana de Fitopatología) (Horsfall, 1977). Los patógenos causantes de la disfunción pueden tener naturalezas muy diferentes. Desde los hongos, considerados como el grupo de patógenos más importante desde el siglo XVIII, hasta los fitoplasmas, protozoos, o viroides (identificados en la segunda mitad del siglo XX), pasando por bacterias y virus (identificados a finales del siglo XIX) (García-Arenal and García, 1996).

En cuanto a los virus, se conocen más de setecientos capaces de infectar plantas, que se clasifican en diferentes grupos taxonómicos en función de la forma de expresión de su genoma, de la estructura de la partícula viral y de diversas características biológicas, como la forma de transmisión, la formación de cuerpos de inclusión o el espectro de huéspedes.

El éxito del establecimiento de una infección vírica en una planta depende de factores de la planta y del patógeno, así como de las condiciones ambientales. En los últimos años se ha hecho un gran esfuerzo para comprender las bases moleculares de la patogénesis viral. Se conocen muchos de los cambios morfológicos y bioquímicos que se producen en las plantas en respuesta a los virus, además de la mayor parte de los aspectos básicos de la biología molecular de los virus que representan a los principales grupos taxonómicos.

La sharka es probablemente la enfermedad más importante de frutales de frutos con hueso en Europa. El agente causal es el virus conocido internacionalmente como *Plum pox virus* (PPV) y ha sido el objeto de estudio de esta tesis. La enfermedad va acompañada de elevadas pérdidas económicas debidas tanto a la disminución de la cosecha como a la imposible comercialización de muchos de los frutos de los árboles infectados (Németh, 1994). De ahí, la importancia de desarrollar procedimientos de prevención y tratamiento de la enfermedad, para lo cual resulta fundamental la

caracterización de los factores virales y de la planta implicados en el establecimiento de la infección y en el desarrollo de los síntomas que muestran las plantas infectadas.

El conocimiento de la biología molecular de PPV ha progresado notablemente en los últimos años. La obtención de secuencias completas del genoma de distintos aislados virales (Laín *et al.*, 1989a; Maiss *et al.*, 1989; Teycheney *et al.*, 1989; Palkovics *et al.*, 1993; Sáenz *et al.*, 2000; Fanigliulo *et al.*, 2003; James and Varga, 2005; Myrta *et al.*, 2006) ha permitido clasificarlos en seis subgrupos o cepas, M, D, C, EA, W y Rec (Palkovics *et al.*, 1993; Nemchinov *et al.*, 1996; Glasa *et al.*, 2004; James *et al.*, 2005; Myrta *et al.*, 2006). Además, numerosos estudios han permitido asignar funciones relacionadas con replicación, movimiento del virus, inducción de síntomas o interferencia con los mecanismos de defensa de la planta a las diferentes proteínas del virus (Salvador *et al.*, 2006). Por otro lado, la aplicación de nuevas técnicas en el estudio de las interacciones proteína-proteína entre las distintas proteínas virales y con proteínas de la planta ha comenzado a dar los primeros resultados que ayudarán a definir el escenario en el que se produce el enfrentamiento entre el virus y su huésped para dar lugar, en algunos casos, a la infección, sintomática o no.

PPV pertenece al género *Potyvirus*, cuyo virus tipo es el virus Y de la patata (PVY), que junto con los géneros*, Rymovirus, Macluravirus, Ipomovirus, Bymovirus* y *Tritimovirus*, forman la familia *Potyviridae* que incluye un gran número de virus de plantas, muchos de ellos con gran relevancia económica (López-Moya and García, 1999). Todos los miembros de la familia comparten características como la morfología de varilla helicoidal flexible de la cápsida y la inducción de inclusiones en forma de rueda de molino en el citoplasma de las células infectadas. El genoma de PPV consiste en una molécula de ARN monocatenario de polaridad positiva de 9786 nucleótidos con la proteína viral VPg unida covalentemente a su extremo 5´ y una cola poli-(A) en su extremo 3´, que codifica una única poliproteína que es procesada proteolíticamente por tres proteasas virales (P1, HCPro y NIaPro) para dar lugar, al menos, a diez proteínas maduras (Salvador *et al.*, 2006). El espectro de huéspedes naturales de PPV está integrado por árboles frutales del género *Prunus*, aunque PPV también infecta un amplio número de especies herbáceas, entre las que se encuentran *Chenopodium phoetidum, Pisum sativum*, *Arabidopsis thaliana* y diversas especies del género *Nicotiana*.

Que un virus sea capaz de infectar una planta parece ser un caso excepcional, ya que normalmente la planta carece de algún factor necesario para el establecimiento de la

infección o existen eficaces barreras estructurales y mecanismos de defensa de la planta, lo que da lugar a que se establezca una interacción incompatible. Para que un virus complete su ciclo vital debe de llevar a cabo tres procesos principales: (i) replicación del genoma dentro de la célula, (ii) movimiento a las células vecinas a través de los plasmodesmos, y (iii) propagación por toda la planta a través del tejido vascular. Fallos en interacciones necesarias para cualquiera de estos pasos puede dar lugar a resistencia parcial o total a la infección y por tanto limitar el espectro de huéspedes del virus. Además, la capacidad de un virus para infectar una determinada planta depende de su habilidad para evadir los mecanismos de defensa de la planta, tanto los innatos, como los inducidos por factores del virus. Obviamente, el éxito o fracaso de estos mecanismos de defensa, y por tanto la susceptibilidad o resistencia de una determinada planta a un determinado virus va a depender también de interacciones específicas entre factores inductores del virus, factores defensivos de la planta y factores del virus capaces de interferir con esos mecanismos de defensa.

Nuestros objetivos se han dirigido a la identificación en el genoma de PPV de las regiones implicadas en la selección del espectro de huéspedes y en la patogenicidad en huéspedes herbáceos y leñosos.

Factores que definen las diferencias de patogenicidad en huéspedes herbáceos y leñosos de variantes virales obtenidas a partir del aislado PPV-PS, de la cepa M de PPV

Como consecuencia del alto rendimiento y de las elevadas tasas de mutación de la replicación del ARN viral, las poblaciones virales no tienen un único genotipo, sino que están constituidas por un conjunto dinámico de secuencias relacionadas que proporcionan grandes reservorios de variabilidad genética y fenotípica.

En trabajos previos de este laboratorio se habían aislado diferentes subaislados a partir del aislado original de raza M PPV-PS, que a pesar de su similitud de secuencia genómica (~99.9%) presentaban diferente infectividad en melocotonero GF305 y síntomas muy distintos en *Nicotiana clevelandii* y *Nicotiana occidentalis* (Sáenz *et al.*, 2000). La capacidad de los subaislados para producir síntomas severos en *N. clevelandii* y *N. occidentalis* se relacionó con un cambio puntual en el aminoácido 109 de la proteína HCPro. Sin embargo, el mismo aminoácido presente en los subaislados severos estaba también presente en los subaislados 1.3.1 y 2.1.1, que producían síntomas débiles en estos huéspedes, lo que indicaba que debían existir cambios adicionales responsables de la atenuación de los síntomas en las regiones no secuenciadas de los aislados débiles

(Sáenz *et al.*, 2000). Por otra parte, los subaislados severos eran infectivos en plántulas de melocotonero GF305, mientras que los subaislados débiles 1.3.1 y 2.1.1 no eran capaces de infectarlas (Sáenz *et al.*, 2000), lo que indica que los subaislados atenuados carecen de algunos elementos genéticos necesarios para la replicación y/o el movimiento viral en este huésped que sí están presentes en los aislados severos.

Uno de los objetivos de este trabajo ha sido la secuenciación completa del genoma del subaislado 1.3.1 para investigar la existencia de diferencias que expliquen el fenotipo suave en especies herbáceas y la ausencia de infectividad en melocotonero GF305. Se han encontrado siete cambios de nucleótido con respecto al clon ADN(c) de PPV-PS (PS-MCl). Además del cambio que causa la sustitución K109E en HCPro previamente descrita (Sáenz *et al.*, 2000) hay dos cambios que dan lugar a sustituciones de aminoácido en la proteína P1 y cuatro cambios que no afectan a la secuencia proteica, en la región 5´ no codificante y en las secuencias que codifican las proteínas CI, NIaVPg y NIb. El hecho de que la proteína P1 del subaislado 2.1.1, que causa también síntomas suaves en especies herbáceas y es incapaz de infectar melocotonero GF305, sea idéntica a la del subaislado 1.3.1, mientras que la de los subaislados 4.1.4 y 10_7, causantes de síntomas severos en *N. clevelandii* y *N. occidentalis* e infectivos en melocotonero GF305, tiene la misma secuencia de PS-MCl, sugiere que los dos cambios de aminoácido encontrados en P1 tienen una relevancia biológica significativa.

Para comprobar esta hipótesis, los cambios de aminoácido de la proteína P1 se introdujeron en el clon pICPPV-PSes (cPSes) construido para este trabajo. cPSes deriva de pGPPV-PSes, que contiene la secuencia HCPro de los subaislados severos (E109S232), y que, por tanto, causa síntomas fuertes en *N. clevelandii* y *N. occidentalis* e infecta eficientemente a melocotonero GF305 (Sáenz *et al.*, 2000). Los mutantes resultantes cPSes W29R (W29R), cPSes V139E (V139E) y cPSes W29RV139E (W29RV139E) se inocularon en plantas de *N. clevelandii, N. occidentalis* y melocotonero GF305. Mientras que el clon W29R producía síntomas severos en *N. clevelandii* parecidos a los producidos por cPSes, V139E se comportaba de manera similar al subaislado natural 1.3.1 dando lugar a una infección débil, y el clon W29RV139E producía síntomas intermedios. Los niveles de acumulación de cPSes y W29R eran similares y significativamente mayores que los de PPV-PS 1.3.1 y V139E. En hojas de *N. occidentalis* inoculadas con W29R se observaban lesiones necróticas similares a las inducidas por cPSes, pero no se observaban en hojas inoculadas con el subaislado 1.3.1 o el mutante V139E. La ausencia de lesiones necróticas se

correlacionaba con niveles de acumulación viral más bajos. El cambio W29R parece tener un efecto compensatorio sobre el cambio V139E, ya que el doble mutante W29RV139E inducía lesiones necróticas y se acumulaba a niveles similares a los de cPSes en las hojas inoculadas de *N. occidentalis.* Estos resultados demuestran que E en la posición 139 de la proteína P1 es un factor atenuante en la infección de PPV-PS en *N. clevelandii* y *N. occidentalis*, aunque el aminoácido presente en la posición 29 de P1 y otros factores fuera de P1, podrían modular el efecto del aminoácido 139 en la patogénesis de PPV en estas plantas.

Contrariamente a lo que ocurre en plantas herbáceas, cPSes y el mutante V139E mostraron una alta infectividad, 100% y 82% respectivamente, en melocotoneros GF305, y ambos virus producían síntomas severos muy similares y se acumulaban a niveles también parecidos en las plantas infectadas. La infectividad en melocotonero del mutante W29R (58%), así como su acumulación y sintomatología, eran significativamente menores que los de cPSes y V139E. Resulta interesante que aunque la mutación V139E por sí sola no afectaba de modo significativo a la infección de *Prunus*, sí era capaz de aumentar el efecto de la mutación W29R, de manera que el doble mutante W29RV139E se comportaba como el subaislado 1.3.1 y era completamente incapaz de infectar melocotoneros GF305 en nuestras condiciones experimentales. Así pues, aunque nuestros resultados demuestran que el aminoácido 29 de la P1 es especialmente relevante para la infección de *Nicotiana* y el aminoácido 139 lo es para *Prunus*, ambos aminoácidos parecen jugar papeles concertados en ambos tipos de huéspedes.

La segregación de diferentes variantes a partir del aislado PPV-PS original indica que está formado por una población viral compleja y la baja divergencia (aproximadamente 99,9%) entre los subaislados podría estar de acuerdo con una estructura genética de cuasiespecie. Sin embargo los cambios de aminoácido encontrados entre los subaislados no parecen seguir una distribución aleatoria, y la propagación en *N. clevelandii* de las variantes virales segregadas no da lugar a la recuperación de la complejidad del aislado PPV-PS original, lo que estaría en contra de que deriven originariamente de una única cuasisespecie. Por otra parte, la distancia genética entre los subaislados es mayor a medida que aumentan las diferencias en sintomatología, y la divergencia entre aislados con la misma sintomatología es considerablemente menor, lo que sugiere que el aislado PPV-PS original estaría compuesto por "nubes" de mutantes de distintas cuasiespecies.

Factores que definen las diferencias de patogenicidad en huéspedes herbáceos y leñosos de los aislados PPV-R y PPV-D, de a la cepa D de PPV

Se han identificado determinantes virales relacionados con la replicación viral, el movimiento célula a célula y a larga distancia, la producción de síntomas y la transmisión del virus en varias especies del género *Potyvirus* (Revers *et al.*, 1999). Sin embargo es muy poco lo que se conoce sobre factores relevantes para la capacidad de los potyvirus de infectar local y sistémicamente huéspedes leñosos. Estudios previos con virus quiméricos construidos a partir de los aislados virales PPV-R y PPV-PS han permitido identificar determinantes de patogenicidad de PPV tanto en especies herbáceas (Sáenz *et al.*, 2000) como leñosas (Dallot *et al.*, 2001), sin embargo la alta divergencia entre los genomas de ambos aislados, pertenecientes a distintas cepas, limitó la precisión en la localización de estos determinantes de patogenicidad.

En este trabajo se ha llevado a cabo la secuenciación completa del genoma y la construcción de un clon ADN(c) infectivo del aislado PPV-D perteneciente a la cepa D, que, al contrario que el clon del aislado de la misma cepa PPV-R (Riechmann *et al.*, 1990), es capaz de iniciar eficientemente la infección de árboles del género *Prunus* como el melocotonero *Prunus persicae cv* GF305, pero es incapaz de infectar el huésped herbáceo *N. clevelandii*. A continuación se construyeron virus recombinantes intercambiando fragmentos de los genomas de PPV-D y PPV-R con objeto de, aprovechando sus diferencias en el espectro de huéspedes y la gran similitud de sus genomas, identificar factores virales importantes para la adaptación al huésped. Para complementar este planteamiento experimental se estudió la adaptación evolutiva de uno de los virus híbridos, PPV-5´BD GFP (BD GFP), a *N. clevelandii* mediante la realización de pases seriados en este huésped. El análisis de las mutaciones fijadas en variantes adaptadas ha proporcionado información adicional acerca de las regiones virales relevantes para una infección eficiente de la planta herbácea.

Aunque el estudio de las diferentes quimeras entre PPV-D y PPV-R establece que los determinantes de patogenicidad se encuentran muy repartidos a lo largo del genoma de PPV, se ha comprobado que la región 3´-terminal de PPV-R, que incluye la región 3´ no codificante, los cistrones de las proteínas CP, NIb y parte del de la NIa, es suficiente para proporcionar al virus la capacidad de infectar *N. clevelandii.* Además, la concentración de cambios de aminoácido en la proteína CP y el efecto negativo de la deleción NAT en la patogenicidad de PPV en melocotonero sugieren que determinantes relevantes para la especificidad de huésped se localizan en la región N-terminal de la

proteína CP. Por otro lado se ha demostrado que importantes determinantes de patogenicidad se localizan en el tercio 5´-terminal del genoma del virus, en la región que codifica las proteínas P1, HCPro, P3 y casi la totalidad de 6K1. Hay que destacar que P1 y P3 (que podría ser funcional como parte del producto sin procesar P3-6K1), junto con la región N-terminal de la proteína CP, son las proteínas más variables entre los diferentes potyvirus, por lo que se había sugerido que podrían estar implicadas en la interacción virus-huésped. La fijación de mutaciones en residuos concretos de las proteínas P1, P3 y 6K1 durante la adaptación del virus quimérico derivado de PPV-D a plantas de *N. clevelandii* apoya la posibilidad de que estas proteínas estén de algún modo implicadas en la adaptación al huésped. Concretamente, la mutación que afecta al aminoácido 876 de la proteína P3 concuerda con una heterogeneidad existente entre los aislados naturales PPV-R y PPV-D, y se ha fijado en poblaciones virales provenientes de dos series independientes de pases de la quimera BD GFP en *N. clevelandii*.

Efecto del intercambio de la proteína P1 entre dos potyvirus en la patogenicidad y el espectro de huéspedes

Los potyvirus PPV y *Tobacco vein mottling virus* (TVMV) difieren en su espectro de huéspedes y en los síntomas que producen en sus huéspedes comunes. Como ya se ha mencionado, P1 es la proteína mas variable de los potyvirus, junto con la región N-terminal de la CP. La proteína P1 de TVMV tiene 34 aminoácidos menos que la de PPV y ambas proteínas comparten un 24,1% de identidad. Con objeto de profundizar en la relevancia de P1 en la patogenicidad viral y en la especificidad del espectro de huéspedes, se construyeron virus híbridos en los que la secuencia codificante de la proteína P1 de PPV se reemplazó, completamente o en parte, por la de la proteína P1 de TVMV. El progreso de la infección y la acumulación viral de los híbridos en diferentes huéspedes herbáceos eran muy similares a los de PPV silvestre, lo que demuestra que la proteína P1 de TVMV y la P1 híbrida PPV/TVMV son funcionalmente equivalentes a la proteína P1 de PPV en estas plantas a pesar de la gran divergencia entre sus secuencias. Esto indica que determinantes estructurales y/o de secuencia implicados en la organización intramolecular de P1 y/o en sus interacciones con otros factores virales o de la planta están conservados en PPV y TVMV y permiten contactos heterólogos funcionales. Por el contrario, ninguno de los híbridos fue capaz de infectar el huésped leñoso melocotonero GF305, un huésped específico de PPV, lo que indica que la proteína P1 de TVMV no es funcionalmente competente en este

huésped y pone de manifiesto la relevancia de la proteína P1 de potyvirus en la adaptación al huésped.

En resumen, en este trabajo de tesis hemos demostrado que aunque los determinantes de patogenicidad de PPV específicos de huésped se encuentran ampliamente distribuidos en el genoma, hay regiones concretas como el tercio 5´-terminal del genoma y la región N-terminal de la CP que contienen determinantes importantes para la infección tanto de especies herbáceas como leñosas. También hemos observado que, aunque los resultados obtenidos con las quimeras entre los aislados PPV-D y PPV-R revelan que la adaptación a un huésped va acompañada de la perdida de "fitness" en el otro, la existencia de aislados de PPV infectivos en huéspedes herbáceos y leñosos demuestra que los requerimientos para la infección de ambos huéspedes no son mutuamente excluyentes. Además hemos comprobado que cambios en los determinantes de patogenicidad del virus producen efectos en la sintomatología y la infectividad que varían en los distintos huéspedes, lo que sugiere que los estudios de patogenicidad realizados con huéspedes herbáceos podrían no ser extrapolables a la infección del huésped natural de PPV.

Finalmente se demuestra el papel de la proteína P1 tanto en la patogenicidad del virus como en la definición del espectro de huéspedes ya que además de encontrarse en la región 5´-terminal de PPV, se ha detectado un cambio no conservativo en un aminoácido que muestra heterogeneidad entre PPV-R y Dc tras la adaptación de una quimera de PPV con poco "fitness" en *N. clevelandii.* Por otro lado*,* mutaciones de la proteína P1 afectan tanto a la patogenicidad como a la infectividad de PPV y su sustitución por la P1 de TVMV no afecta a la infectividad en huéspedes herbáceos, aunque elimina la infectividad en melocotonero GF305. Estos datos sugieren fuertemente que el mecanismo de acción de la proteína P1 requiere de una interacción con factores de la planta. La identificación y caracterización de estas posibles interacciones ayudarán a esclarecer su mecanismo de acción durante la infección viral.

I. INTRODUCTION

I.1 General considerations

Plant pathology is the science of diagnosing and managing plant diseases. The "Disease Triangle" is a central concept of plant pathology for infectious diseases. It is based on the principle that disease is the result of an interaction between a host, a pathogen, and the environment conditions. Organisms that cause infectious diseases include fungi, oomycetes, bacteria, viruses, viroids, virus-like organisms, phytoplasmas, protozoa, nematodes and parasitic plants. Not included are insects, mites, vertebrate or other pests that affect plant health (García-Arenal *et al.*, 1996).

Plant viruses are responsible for a great number of plant diseases. The dissemination of plant virus diseases either by their natural vectors or through the propagation of infected material causes every year major economic losses throughout the world. We know more than seven hundred viruses with the ability to infect plants. They are classified in taxonomic groups depending on the nature and expression strategy of their genome, virion structure and on diverse biological characteristics, such as transmission, formation of inclusion bodies or host range (Hull, 2002).

Understanding the molecular biology of plant viruses, and the molecular basis of viral pathogenesis is fundamental for the control of virus propagation and the elaboration of antiviral strategies. In recent years, much of the morphologic and biochemical changes produced in the plant in response to viral infection have been described, and most aspects of the basic molecular biology of the representative viruses from the principal taxonomic groups have been unraveled. However, in spite of the great advances in our understanding of the structure, function and replication of the viral genomes, there is still little molecular knowledge on how virus and plant factors interact and how viruses induce disease symptoms in the host plant.

The genus *Potyvirus*, which together with the genera *Rymovirus*, *Macluravirus*, *Ipomovirus*, *Bymovirus* and *Tritimovirus* form the family *Potyviridae*, is the largest group of plant viruses (Ward and Shukla, 1991; López-Moya *et al.*, 1999). Potyviruses are among the most damaging plant viruses and they are broadly distributed throughout the world. Some of them have restricted natural and experimental host ranges; others may infect a considerable number of plant species distributed in many families including monocots and dicots. There are potyviruses that are able to infect the most economically important crops, including grain, legumes, forage, vegetables, fruits and ornamentals. Progress in knowledge of the molecular biology of the potyvirus group has been achieved by numerous independent studies with different viruses of the genus.

New advances in understanding virus-host interaction, pathogenicity and host range definition in any virus of this group will help to define the scenario in which the potyviruses and their hosts interact upon infection.

I.2 *Plum pox virus* and sharka disease*

Plum pox virus (PPV) is the member of the genus *Potyvirus* that causes sharka, a devastating disease of trees from several species of the genus *Prunus*. Owing to the great economic relevance of sharka, many of the studies on this virus have focused on practical objectives addressing the diagnosis and control of the disease (López-Moya *et al.*, 2000a). However, information has also been gained on basic aspects of PPV biology, which together with data obtained for related potyviruses (Riechmann *et al.*, 1992; Revers *et al.*, 1999; Urcuqui-Inchima *et al.*, 2001), have allowed a rather thorough molecular characterization of this virus. Sharka was first detected at 1917-1918 and described as a viral disease in 1932 (Atanasoff, 1932). Since then, the virus has progressively spread to a large part of the European continent, around the Mediterranean basin and to the near and Middle East (Roy and Smith, 1994). It has been found in South and North America (Chile, USA, Canada, and Argentina) (Roy *et al.*, 1994); (Levy *et al.*, 2000); (Thompson *et al.*, 2001; Dal Zotto *et al.*, 2006) and in Asia (Kazakhstan, China and Pakistan) (Spiegel *et al.*, 2004; Navrátil *et al.*, 2005; Kollerová *et al.*, 2006). PPV is transmitted in the field by aphids in a non-persistent manner, but exchanges of infected propagative plant material has probably been the main pathway of spread of sharka over long distances (Cambra *et al.*, 2006). In addition to stone fruit trees of the *Prunus* genus (Llácer and Cambra, 2006), several herbaceous species and woody species that do not belong to the genus *Prunus* have been identified as natural and/or experimental hosts for PPV (Németh, 1986; Virscek Marn *et al.*, 2004; Llácer, 2006; Polák, 2006), although the relevance of these species in the epidemiology of sharka is largely unknown (James and Thompson, 2006; Damsteegt *et al.*, 2007).

I.3 Genome organization, expression and replication of PPV*

PPV virions are long, flexuous and rod-shaped, of ~660-750 nm in length and ~12.5-20 nm in width, formed by a single coat protein (CP) of about 36 kDa arranged helicoidally around one molecule of ssRNA of positive polarity (Brunt *et al.*, 1996; Büchen-Osmond, 2004). The genomic RNA of PPV has a protein (VPg) linked to its 5´-

end (Riechmann *et al.*, 1989) and a long poly-(A) tail heterogeneous in size at its 3´ end (Laín *et al.*, 1988).

In the past the classification of PPV isolates was based on serological and biological properties, particularly on the symptoms caused in experimental herbaceous hosts (Sutic *et al.*, 1971; Kerlan and Dunez, 1979). More recently, the availability of a number of full-length genome sequences (Laín *et al.*, 1989a; Maiss *et al.*, 1989; Teycheney *et al.*, 1989; Palkovics *et al.*, 1993; Sáenz *et al.*, 2000; Fanigliulo *et al.*, 2003; James and Varga, 2005; Myrta *et al.*, 2006) and many partial ones has enabled a more reliable classification to be established. Six subgroups or strains of PPV have been described, M, D, C, EA, W and Rec (Palkovics *et al.*, 1993; Nemchinov *et al.*, 1996; Glasa *et al.*, 2004; James and Varga, 2005; Myrta *et al.*, 2006). The phylogenetic relationships between these strains have not been well established, although PPV-Rec could derive from several recombination events involving PPV-M and D strains, or ancestors of them (Cervera *et al.*, 1993; Glasa *et al.*, 2004). Evidence for RNA recombination inside the PPV genomic RNA (Guo *et al.*, 1998) or between the PPV genomic RNA and transgenes or virus vector-expressed sequences (Varrelmann *et al.*, 2000b) have been obtained in experimental systems.

The PPV genome contains a long open reading frame (ORF) that is translated from its second AUG codon, into a large polyprotein of 355.5 kDa (Riechmann *et al.*, 1991), probably by a cap-independent leaky scanning mechanism (Simón-Buela *et al.*, 1997a). However, data on other potyviruses point to a possible translation initiation by internal recognition of specific viral sequences (Niepel and Gallie, 1999; Zeenko and Gallie, 2005). The PPV polyprotein is co- and post-translationally cleaved by three virus-encoded proteinases to produce at least ten mature protein products (Fig. 1). The P1 proteinase and the helper component-proteinase (HCPro) cleave at their respective C termini autocatalytically (García *et al.*, 1993; Ravelonandro *et al.*, 1993). In agreement with previous results on other potyviruses (Verchot *et al.*, 1992), *in vitro* P1 processing takes place in a wheat germ system but not in a reticulocyte lysate one (García *et al.*, 1993), suggesting that a plant co-factor is required for P1 activity.

The C-terminal proteinase domain of the nuclear inclusion protein a (NIaPro) catalyses the processing of the rest of the PPV polyprotein (García *et al.*, 1989b). Target sites of the NIaPro proteinase are defined by sequences of seven amino acids, from –6 to +1 around the scissile bond (García *et al.*, 1989a; Martín *et al.*, 1990). They differ in cleavage efficiency and in susceptibility to *in cis* and *in trans* processing (García *et al.*,

1990), and studies carried out with the NIaPro proteinase of *Tobacco etch virus* (TEV) indicated that these specific features are also mainly defined by the –6 to +1 heptapeptide (Dougherty *et al*., 1989a; Dougherty and Parks, 1989b). However, features modulating the susceptibility to NIaPro cleavage have also been found outside the –6 to +1 heptapeptide sequence (García *et al*., 1989a; García *et al*., 1992). Thus, proteolytic maturation of the potyviral polyprotein appears to be a highly regulated process playing an important role in the control of infection, which could be a suitable target to develop new antiviral strategies against potyvirus in general, and PPV in particular. It has been reported that expression of the cysteine proteinase inhibitor orycystatin in *Nicotiana tabacum* interferes with TEV infection (Gutierrez-Campos *et al*., 1999). Cystatins have been shown to inhibit, although with low efficiency, the activity of PPV proteinases *in vitro*, but their effects *in vivo*, or that of other proteinase inhibitors have not been tested yet (García *et al*., 1993; Wen *et al*., 2004).

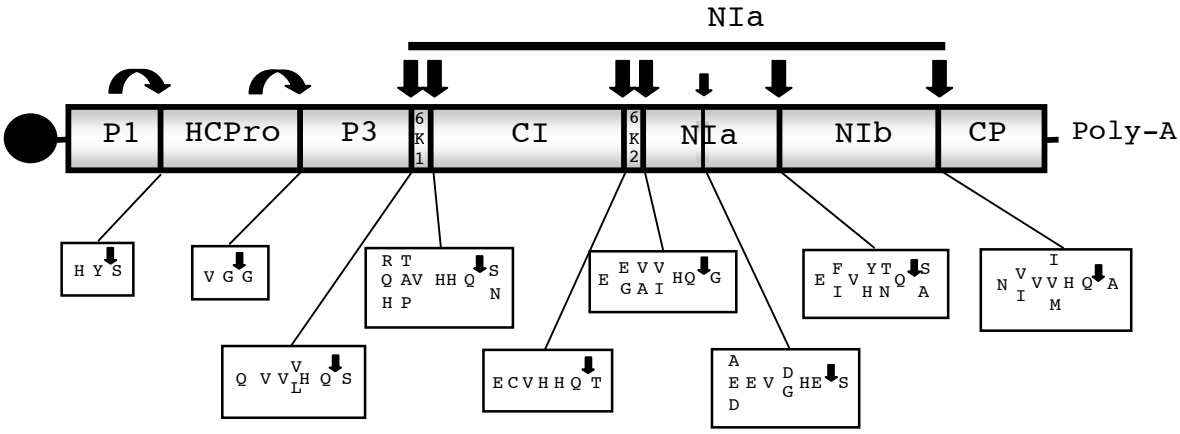


Figure 1. Genomic map of *Plum pox virus*. The open reading frame is represented by a rectangular box and the terminal VPg protein by a black circle; vertical lines and arrows indicate cleavage sites and the proteinase responsible for each cleavage. The mature protein products are indicated at their respective positions in the polyprotein. Amino acid sequences at the proteinase cleavage sites found in different PPV isolates (full-length sequences) are boxed below.

The 5' untranslated region (UTR) of PPV consists of 146 nucleotides. It starts with a tract of four A residues, but, apparently an intact 5' end is not essential for PPV replication, since PPV RNAs with mutations altering the number of 5' terminal A residues were able to infect *N. clevelandii* plants and the genomic 5' end is repaired by an unknown template-independent mechanism (Simón-Buela *et al*., 2000). Residues of the PPV 5' UTR essential for virus replication are confined to the first 35 residues,

whereas the deletion of long sequences located between nucleotides 39 and 145 did not alter the rate of infection or viral accumulation (Simón-Buela *et al.*, 1997b). However, these deletions affected viral fitness and pathogenesis. The 3' UTR of PPV consists of 220 nucleotides. No data are available on its relevance for PPV infection, but a determinant of disease symptom severity has been shown to be located in the 3' UTR of another potyvirus, *Tobacco vein mottling virus* (TVMV) (Rodríguez-Cerezo *et al.*, 1991).

A general feature of replication of plus-strand RNA viruses is that it takes place in association with intracellular membranes. In agreement with this, a crude membrane fraction from PPV-infected leaves has been shown to be able to synthesize viral RNA from endogenous template (Martín and García, 1991). Fractionation of this membrane extract by centrifugation in glycerol gradients indicated that PPV-specific RNA synthesis occurred in fractions enriched in endoplasmic reticulum and tonoplast vesicles (Martín *et al.*, 1995). A striking feature of potyvirus infections is that, although RNA replication takes place in cytoplasmic membranous structures, the RNA replicase NIb accumulates, together with the VPg-proteinase protein NIa, in the nucleus of infected cells (Carrington *et al.*, 1993; Li and Carrington, 1995). PPV NIa and NIb have been shown to form crystalline inclusions mainly in the nucleus, but also in the cytoplasm (Martín *et al.*, 1992). The functional meaning of the nuclear transport of the NI proteins is unknown. Although it may represent a way to deal with the excess of replication proteins expected from the expression strategy of potyviral genomes through polyprotein processing, a role of nuclear targeting in regulating the replication timing cannot be ruled out (Restrepo *et al.*, 1990).

I.4 Functions of PPV gene products*

The potyviral polyprotein gives rises to ten final proteins (Fig. 1). However, it is important to remark that the polyprotein processing as an expression strategy gives rise not only to the final mature proteins but also to a number of partially processed polyproteins, which can be used by the virus to optimize its infection capacity. For simplicity, the information has been divided here according to the final proteins, but data on partially processed products are also discussed in the appropriate sections. The reader is also referred to previous reviews on potyvirus in general or PPV in particular (Riechmann *et al.*, 1992; Revers *et al.*, 1999; López-Moya *et al.*, 2000a; Urcuqui-Inchima *et al.*, 2001).

P1

P1 occupies the first place in the potyviral polyprotein and is the most variable (in sequence and length) protein among the different potyviruses. However, the C-terminal region of this protein is relatively conserved and contains a serine proteinase domain extensively divergent from cellular proteinases (Verchot *et al.*, 1991). The catalytic triad and cleavage site preferences of P1 protein have been recently reviewed (Adams *et al.*, 2005a). P1 protein is a highly basic protein and RNA-binding activity has been reported for P1 protein from several potyviruses (Brantley and Hunt, 1993; Soumounou and Laliberté, 1994; Merits *et al.*, 1998), which could suggest a role in replication, translation and/or translocation of viral RNA. Deletion of the TEV P1 coding sequence and complementation on P1-expressing transgenic plants have shown that P1 functions *in trans* as an accessory factor for genome amplification (Verchot and Carrington, 1995b). P1 protein has been shown to contribute to the synergistic interaction between *Potato virus X* (PVX) and TEV (Pruss *et al.*, 1997), probably by enhancing the silencing suppression activity of HCPro (Rajamäki *et al.*, 2005). Work with PPV has demonstrated that this suppression-enhancing activity only takes place when P1 and HCPro are expressed in the same polyprotein (Valli *et al.*, 2006), contrasting with the *trans* activity on genome amplification reported for TEV.

On the other hand, the high sequence divergence of P1 and the detection of recombination events in its coding sequence that could be related with host adaptation, might suggest a role for this protein in pathogenicity and host range specificity (Valli *et al.*, 2007).

HCPro

The potyviral HCPro is a multifunctional protein (Maia *et al.*, 1996). It was initially identified as a factor required for plant-to-plant transmission by aphid vectors (Govier *et al.*, 1977). HCPro has also shown to be involved in genome amplification (Kasschau *et al.*, 1997), short- (Rojas *et al.*, 1997) and long- (Cronin *et al.*, 1995) distance movement, seed transmission (Johansen *et al.*, 1996), symptom induction (Gal-On and Raccah, 2000; Redondo *et al.*, 2001; Sáenz *et al.*, 2001) and in synergistic interactions with other viruses (Shi *et al.*, 1997; Yang and Ravelonandro, 2002; González-Jara *et al.*, 2004).

HCPro is a cysteine proteinase that cleaves at its C-end (Carrington *et al.*, 1989; García *et al.*, 1993; Ravelonandro *et al.*, 1993). In the case of TEV, the proteinase

activity itself, and not the mere separation of HCPro from the rest of the polyprotein, is essential for genome amplification (Kasschau and Carrington, 1995).

The potyviral HCPro was the first protein shown to interfere with the plant defense mechanism mediated by RNA silencing (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998). This activity has been confirmed for PPV HCPro (Tenllado *et al.*, 2003; Varrelmann *et al.*, 2007). HCPro has also been shown to interfere with miRNA-mediated regulation (Mallory *et al.*, 2002; Kasschau *et al.*, 2003). Moreover, HCPro is able to interact with RNA (Urcuqui-Inchima *et al.*, 2000), and this capacity could play a significant role in its silencing suppression activity. Although there is experimental evidence supporting the hypothesis that suppression of RNA silencing may be responsible for some other HCPro functions (Kasschau and Carrington, 2001; Sáenz *et al.*, 2002; González-Jara *et al.*, 2005), the dependence of HCPro functions on RNA silencing suppression activity might be less prominent than it was initially believed (Mlotshwa *et al.*, 2005).

HCPro is known to self-interact (Guo *et al.*, 1999) and recently, reconstructed 3D structures of the TEV HCPro have shown the oligomeric nature of this protein purified from infected plants (Plisson *et al.*, 2003; Ruiz-Ferrer *et al.*, 2005). Moreover, different interactions of HCPro with other virus and host proteins have also been described. Interactions of HCPro with CP (Peng *et al.*, 1998), virion particles (Manoussopoulos *et al.*, 2000; Torrance *et al.*, 2006) and aphid stylets (Blanc *et al.*, 1998) are probably involved in aphid transmission, although other functions of HCPro/CP interactions cannot be ruled out (Roudet-Tavert *et al.*, 2002). Interaction of HCPro with rgs-CaM is probably relevant for silencing suppression (Anandalakshmi *et al.*, 2000). The role of HCPro interaction with CI protein (Merits *et al.*, 1999; Guo *et al.*, 2001) and with two potato proteins, a RING finger protein, HIP1, and HIP2, is less obvious (Guo *et al.*, 2003). Moreover, the HCPro of *Lettuce mosaic virus* (LMV) has been shown to bind to the 20S proteosome and to inhibit its endonuclease activity, suggesting the existence of a novel type of defense and counter-defense interplay between potyviruses and their hosts (Ballut *et al.*, 2005).

P3

P3 protein is barely conserved among different potyviruses and there is not much information about the functions of this protein. It has been proposed to be involved in virus replication (Klein *et al.*, 1994). Moreover, it has been shown that TVMV P3 interact with the cytoplasmic cylindrical inclusions (Rodríguez-Cerezo *et al.*, 1993), but

there are also data showing interaction *in planta* of P3 with TEV nuclear inclusions (Langenberg and Zhang, 1997). Binding of P3 to P1, NIa and NIb of different potyviruses in heterologous systems has also been reported (Merits *et al.*, 1999; Guo *et al.*, 2001).

With respect to its function, the P3 protein, or a partially processed precursor of it (P3-6K1), has been reported to be a virulence determinant for genes involved in resistance to *Pea seedborne mosaic virus* (PSbMV) (Johansen *et al.*, 2001) and *Turnip mosaic virus* (TuMV) (Jenner *et al.*, 2002 ; Jenner *et al.*, 2003). Moreover, pathogenicity determinants for PPV infection in herbaceous (Sáenz *et al.*, 2000) and woody (Dallot *et al.*, 2001) hosts have also been localized in the P3-6K1 region.

6K1 peptide

6K1 is better conserved among potyviruses than the preceding P3 protein. It contains a hydrophobic region that could mediate membrane integration (Riechmann *et al.*, 1992). Cleavage between PPV P3 and 6K1 *in vitro* is only partial (García *et al.*, 1992) and appears not to be essential for virus viability, although mutations affecting the efficiency of processing at this site disturbed the time course and severity of the symptom induction process (Riechmann *et al.*, 1995). In the light of these results, a possible regulatory effect on the function of the partially processed P3-6K1 protein has been proposed for processing at the P3/6K1 junction (Riechmann *et al.*, 1995). However, mature 6K1 has been detected in PPV infected cells, suggesting that this peptide could play a functional role by itself (Waltermann and Maiss, 2006).

CI

A unique and typical feature of the potyviral infection is the accumulation of pinwheel-shaped cytoplasmic inclusion bodies, which are formed by the cylindrical inclusion protein (CI). CI is a member of the large group of proteins with an NTP-binding domain (NTBD). These proteins are widespread in nature, and most RNA viruses have, at least, one (Gorbalenya and Koonin, 1989; Laín *et al.*, 1989b; Kadaré and Haenni, 1997). PPV CI has NTPase and RNA helicase activities (Laín *et al.*, 1990, Laín *et al.*, 1991; Fernández *et al.*, 1995), which have been shown to be required for RNA replication (Fernández *et al.*, 1997). Self-interaction of PPV CI can take place in the absence of other viral proteins, and the N-terminal 177 amino acids appear to be responsible for this interaction (López *et al.*, 2001). Some data suggest that although PPV CI oligomerization is not relevant for its NTPase activity, it is necessary for an efficient RNA helicase activity (Gómez de Cedrón, 2004).

Genetic analysis (Carrington *et al.*, 1998) and electron microscopy visualization of CP- and CI-containing inclusion bodies positioned over the plasmodesmatal apertures (Rodríguez-Cerezo *et al.*, 1997; Roberts *et al.*, 1998) have revealed the involvement of CI in cell-to-cell movement of potyviruses. It is not known whether CI requires RNA helicase activity for its movement function, but it has additional requirements since point mutations at the N-terminal region of the PPV CI protein with no apparent effect on ATPase and RNA helicase activities *in vitro*, nor on virus replication in protoplasts, drastically impaired cell-to-cell spread of the virus (Gómez de Cedrón *et al.*, 2006). Moreover, these mutations caused a notable reduction in the strength of the CI self-interaction in a yeast Two-Hybrid system, suggesting that CI-CI interactions required for RNA replication and virus movement could be somewhat different (Gómez de Cedrón *et al.*, 2006).

In addition to its replication and movement functions, the CI protein of TuMV is the determinant of avirulence for a *Brassica napus* resistance gene (Jenner *et al.*, 2000; Jenner *et al.*, 2002) .

Three host factors interacting with the potyviral CI protein have been reported. The protein $P58^{IPK}$ from tobacco interacts with the helicase domain of the TEV CI protein (Bilgin *et al.*, 2003). The PPV CI protein has been found to interact with the photosystem I PSI-K protein, the product of the gene *psaK* of *N. benthamiana* (Jiménez *et al.*, 2006). Down regulation of the *psaK* gene led to higher PPV accumulation, suggesting a role for the CI-PSI-K interaction in PPV infection. The interaction of PPV CI with an unknown protein of *N. benthamiana* with a HIT type zinc finger domain, has been shown to have a positive effect on PPV infection (Jiménez, 2004).

6K2

6K2 resembles 6K1 in having a central hydrophobic domain. The TEV 6K2 protein has been shown to associate with endoplasmic reticulum membranes as an integral protein via this domain (Schaad *et al.*, 1997a). It has been proposed that 6K2 is involved in targeting the RNA replication complexes to membranous sites of replication (Schaad *et al.*, 1997a).

Moreover, it has been shown that the 6K2 protein of *Potato virus A* (PVA) affects viral long-distance movement and symptom induction independently and in a host-specific manner (Spetz and Valkonen, 2004).

NIa-VPg

The NIa protein was first identified as one of the components of the crystalline inclusions that accumulates in the nucleus of the cells infected with some potyviruses, including PPV (van Oosten and van Bakel, 1970; Knuhtsen *et al.*, 1974; Martín *et al.*, 1992), and as the proteinase responsible for the processing of the central and C-terminal regions of the potyviral polyprotein (Carrington and Dougherty, 1987). Later on, it has been shown that NIa is further processed to yield the VPg protein and a 27-kDa protein that has a proteolytic activity similar to NIa-associated activity (Dougherty and Parks, 1991).

NIa self-interaction and NIa-NIb interactions have been described for several potyviruses, although there are some discrepancies about the domains involved in the interactions (Fellers *et al.*, 1998; Daròs *et al.*, 1999; Guo *et al.*, 2001).

NIa has efficient and well defined nuclear localization signals, which appear to be relevant for virus viability, suggesting that NIa might have a, still unknown, role in the nucleus of the infected cells (see below) (Schaad *et al.*, 1996).

VPg is covalently linked to the viral RNA by a Tyr residue through a phosphodiester bond (Murphy *et al.*, 1991) and is exposed at one end of the virion (Puustinen *et al.*, 2002). Recent data have shown that VPg can be uridylylated by the RNA replicase NIb, suggesting that it can be used as a primer for initiation of RNA synthesis by a similar mechanism as proposed for picornavirus (Puustinen and Mäkinen, 2004; Anindya *et al.*, 2005).

RNA replication is not the only process in which VPg has a function. This protein is known to be a host-specific determinant for long-distance movement (Schaad *et al.*, 1997b; Rajamäki and Valkonen, 1999) and the virulence determinant for recessive resistance genes that encode translation initiation factors (Robaglia and Caranta, 2006). There is some experimental evidence suggesting that physical interaction between VPg and these translation initiation factors plays a role in potyvirus infection (Léonard *et al.*, 2000; Schaad *et al.*, 2000; Léonard *et al.*, 2004; Michon *et al.*, 2006). The infection step in which this interaction is involved is not known, but the fact that VPg replaces the cap structure typical of eukaryotic mRNAs, and its interaction with the poly-(A) binding protein (PABP), may suggest a possible involvement in translation.

Another cellular factor, a cysteine-rich protein of unknown function, has been shown to interact with VPg proteins of a diverse range of potyviruses (Dunoyer *et al.*,

2004). This interaction appears to affect symptom induction by potentiating virus movement, rather than virus replication.

There is experimental evidence of VPg phosphorylation by host kinases (Ivanov *et al.*, 2001), which can affect virion-bound VPg (Puustinen *et al.*, 2002). The relevance of this post-translational modification for the different functions of VPg is still unknown.

NIaPro

As we explained above, seven of the nine cleavage sites of the potyviral polyprotein are processed by NIaPro (Fig. 1). It has a catalytic cysteine residue but shares structural motifs with cellular serine proteinases (Bazan and Fletterick, 1988) and is related to the 3C proteinase of picornaviruses (Adams *et al.*, 2005a). The 3D structure of the TEV NIaPro proteinase has been solved at high resolution as a complex with either a substrate or a product peptide (Phan *et al.*, 2002), allowing the determinants of substrate specificity of the different potyviral NIaPro proteins to be elucidated (Adams *et al.*, 2005a; Tozser *et al.*, 2005).

NIaPro has also been shown to bind RNA non-specifically, suggesting its involvement in virus replication (Daròs and Carrington, 1997). Recently, a novel double-stranded DNA degradation activity has been reported for the NIaPro proteinases of TEV and *Pepper vein banding virus* (PVBV) (Anindya and Savithri, 2004). The Asp residue of the proteinase catalytic triad has been shown to be a crucial residue for DNase activity. The biological relevance of this activity is unknown, but it could be related to a possible function of NI proteins targeted to the nucleus (Anindya *et al.*, 2004).

NIb

The nuclear inclusion protein NIb is the viral RNA-dependent RNA polymerase responsible for genome replication of potyviruses (Hong and Hunt, 1996). In addition to its interaction with NIa, interactions of NIb with P1 and P3 have been detected in heterologous systems (Merits *et al.*, 1999). The functional relevance of these interactions in the formation of viral replication complexes is unknown. NIb is also responsible for VPg uridylylation (see above). The role of the interaction of NIb with PABP (Wang *et al.*, 2000) is also unknown, but it could be related to recognition of the 3'-terminal end of the genomic RNA to start synthesis of the minus strand RNA or to facilitate VPg uridylylation, which was previously described to require the presence of a poly-(A) template in poliovirus (Paul *et al.*, 1998).

CP

The primary function of the CPs of plant viruses is encapsidation of the viral genome. Potyviral CP is a three-domain protein with N- and C-terminal regions exposed on the virion surface (Allison *et al.*, 1985; Shukla *et al.*, 1988; Baratova *et al.*, 2001) and a conserved core domain that was shown to be essential for virus assembly (Dolja *et al.*, 1994; Varrelmann and Maiss, 2000a), plasmodesmatal gating (Rojas *et al.*, 1997), and cell-to-cell movement (Dolja *et al.*, 1995). The N-terminal region of CP is extremely variable among different potyviruses and is involved in viral long distance movement (Dolja *et al.*, 1994; Dolja *et al.*, 1995; Andersen and Johansen, 1998). It has been shown that an appropriate N-terminal net charge rather than a specific amino acid sequence is required for efficient potyvirus movement (López-Moya and Pirone, 1998; Arazi *et al.*, 2001; Kimalov *et al.*, 2004). Moreover, a conserved DAG motif in the N-terminal region of CP participates in the interaction of this protein with HCPro (Blanc *et al.*, 1997), and is essential for aphid transmission of potyviruses (Atreya *et al.*, 1990), although CP sequence requirements might vary for different potyviral HCPro proteins (López-Moya *et al.*, 1995).

The CPs of PVA (Ivanov *et al.*, 2001; Ivanov *et al.*, 2003) and PPV (Fernández-Fernández *et al.*, 2002) have been shown to be phosphorylated. This post-translational modification down-regulates the RNA binding capacity of CP, which suggests that it could be regulating the amount of genomic RNA allocated to translation, replication, propagation and encapsidation (Ivanov *et al.*, 2001). Moreover, PPV CP is also modified by *O*-GlcNAcylation, a modification that can be reciprocal of phosphorylation (Fernández-Fernández *et al.*, 2002; Chen *et al.*, 2005), suggesting that it could be the balance between phosphorylation and *O*-GlcNAcylation of CP which controls the fate of the genomic RNA.

Very recently, it has been reported that PVA CP displays NTPase activity (Rakitina *et al.*, 2005), but the functional relevance of this enzymatic activity is unknown.

* These sections are adapted from (Salvador *et al.*, 2006)

I.5 General aspects of plant virus host range and pathogenicity

Systemic infection of plants by viruses is an active process involving the interplay of specific viral and host factors (Dawson and Hilf, 1992; Carrington *et al.*, 1996; Seron and Haenni, 1996). It is the exception rather than the rule that viruses can successfully

infect plants. The existence in the plant of structural barriers that the virus is unable to surmount, the absence of factors required for virus life cycle or the activation of effective plant defense mechanisms will lead to the establishment of an incompatible interaction. A defective interaction at any of the infection steps can result in either partial or complete resistance to infection and hence limit the host range of the virus. In addition, the ability of a virus to establish infection within a host plant also depends on its ability to evade recognition and targeting by the host defense mechanisms. Two major active defense mechanisms are known to act against viral infections in plants, the one mediated by plant encoded resistance (R) genes confers protection against various pathogens including viruses, and RNA silencing, which is an ensemble of important cellular pathways involved in the control of gene expression and in defense against foreign nucleic acids. For the activation of the defense based in R genes, complementary pairs of dominant genes, one in the host (R gene) and the other in the pathogen (avirulence, or avr, gene), are required. Usually, R gene-mediated resistance is associated with induction of a a particular form of programmed cell death named hypersensitive response (HR) (Dangl and Jones, 2001). However, there are also cases in which R genes confer resistance without induction of HR (Hajimorad and Hill, 2001), and HR reactions that are not able to prevent virus propagation (Chandra-Shekara *et al.*, 2006). Loss or alteration of either the R or the avr gene leads to a compatible interaction and disease. An RNA silencing response in plants can be exogenously triggered by viruses and transgenes (Voinnet, 2005), and once triggered, RNA with homology to the inducer is specifically degraded not only locally but also systemically thanks to the existence of a mobile silencing signal (Palauqui *et al.*, 1997). Because RNA silencing acts as an antiviral mechanism in plants it is not surprising that many plant viruses encode suppressors of RNA silencing. The first virus-encoded silencing suppressor described was the potyviral HCPro protein (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau *et al.*, 1998), and since this initial demonstration, many other plant viral suppressors of silencing have been identified (Roth *et al.*, 2004; Qu and Morris, 2005). Hence viruses can be targets, inducers and suppressors of RNA silencing.

Plant viruses differ in the number of host species they infect. Some such as TuMV and *Cucumber mosaic virus* (CMV) infect a large number of species of different families, whereas others, such as *Carrot red leaf virus* (CRLV) and *Cowpea chlorotic mottle virus* (CCMV), have a more restricted host range and infect only a few plant species. As mentioned above, although the natural host range of PPV consists in stone

fruit trees of the *Prunus* genus it has a rather broad experimental host range including herbaceous species such as species of the *Nicotiana* genus, *Chenopodium foetidum*, *Pisum sativum or Arabidopsis thaliana*. Usually all isolates of a single viral species infect the same range of host species, but some individual isolates may be mostly confined to different members of that set of hosts (Gibbs and Cooper, 1995). The host range and virulence of a virus are usually among its most malleable characters. The factors that influence virus host range and pathogenicity have been studied using virus mutants as well as recombinant viruses constructed from closely related viruses (Rao and Davis, 1999). In many cases these factors concern one or more proteins involved in virus replication and/or transport, including coat protein, movement protein and/or other proteins conferring a function necessary for virus spread.

Induction of symptoms in the host plant can be the result of any step of the viral life cycle. An example of disease symptoms associated to a defensive response of the plant is the HR reaction mentioned above; it usually causes necrotic lesions surrounding the primary infection site, although when the resistance is not effective, systemic necrosis and, even plant death can also take place. Recently, the effects of viral silencing supressors on the accumulation of host miRNAs have been also associated with symptom induction (Kasschau *et al.*, 2003). The obligate intracellular nature of viruses and the high accumulation levels reached by viruses provide numerous opportunities for viral proteins and nucleic acids to interact and influence the activity of host factors. Thus, in addition to plant defensive responses, not only sequestration of plant factors by the virus replication machinery, but also collateral interactions that do not necessarily provide any advantage to the virus, can lead ultimately to the production of symptoms in the plant. Thus, virus effects range from non-specific changes in host gene expression to specific responses initiated by precise interactions between virus and host factors. There is still much information lacking regarding the influence of each viral protein and nucleic acid in triggering host responses and the signaling networks that are involved in them. In addition, for most viral proteins the identities of the host factors that are targets for interaction and participate in viral pathogenesis remain unknown. The final result of these interactions is usually a decrease of the rate of photosynthesis, an increase of the respiratory rate, disturbances of plant growth regulators, and, as a consequence of all these alterations, defects in plant growth and development.

I.6 PPV host range and pathogenicity

As mentioned above, PPV isolates are classified into six subgroups or strains. Most PPV isolates belong to the subgroups M and D. PPV M and D strains differ in their ability to infect peach. M isolates appear to cause, in general, faster epidemics and more severe symptoms in peach flowers, leaves and fruits than D isolates (Candresse and Cambra, 2006; Llácer *et al.*, 2006). However, peach-adapted PPV D isolates have also been detected, suggesting that some biological properties of PPV isolates are more dependent on isolate-specific traits than on their taxonomic status (Dallot *et al.*, 1998; Levy *et al.*, 2000). PPV Rec isolates have been mainly detected in plums. Although they can be experimentally transmitted to peach, they appear to resemble PPV D isolates in being less adapted than PPV M isolates to transmission to peach hosts (Glasa *et al.*, 2004). Since PPV Rec and PPV M isolates share the 3'-terminal region of the genome, sequences upstream of the NIb recombination site appear to be especially relevant for peach adaptation. Cherries were considered non-hosts of PPV for a long time. However, a number of PPV isolates infecting sour and sweet cherry trees have now been identified in several European countries and Turkey (Llácer *et al.*, 2006). All these isolates form a distinct monophyletic group, which has been defined as PPV C strain (Candresse and Cambra, 2006). Although PPV C isolates appear to be specifically adapted to cherry, they are also able to infect other *Prunus* species (Bodin *et al.*, 2003). PPV El Amar and PPV W317 are atypical PPV isolates that were isolated from apricot in Egypt (Wetzel *et al.*, 1991) and plum in Canada (James *et al.*, 2003), respectively. Their genome sequences largely diverge from each other and from isolates of other PPV strains, suggesting that they constitute independent evolutionary lineages. They have, therefore, been proposed as prototypes of PPV strains EA and W (Candresse and Cambra, 2006). Very little information is available about the biological and epidemiological properties of these PPV strains.

Extended generation times and hard length and space requirements of phenotypic assays have hampered molecular characterization of the interaction of PPV with its natural woody hosts. The ability of PPV to infect a number of herbaceous species makes experimental analysis more affordable. A recent study of PPV infection in a collection of *A. thaliana* accessions has revealed that multiple host factors are involved in the control of PPV infection (Decroocq *et al.*, 2006). Resistance of most Arabidopsis ecotypes preventing infection by a PPV C-type isolate, but not by other PPV isolates, appeared to be controlled by an R gene-mediated pathway. Restriction of long-distance

movement of PPV-EA and PPV-PS, an M-type isolate, involved the RTM genes, which were previously identified to cooperate also in the interference with TEV systemic movement (Whitham *et al.*, 2000). Another dominant resistance gene prevents systemic spread of the M-type PPV-PS isolate in *Arabidopsis* Cvi-1, and the ability of the D-type PPV-R isolate to break the resistance conferred by this gene probably depends on the sequence coding for the N-terminal region of CP (Decroocq *et al.*, 2006). Recessive resistance genes affecting long-distance spread of PPV-D type isolates in *Arabidopsis* ecotypes Cvi-1 and L*er* have also been identified. They probably code for host factors involved in virus movement (Decroocq *et al.*, 2006 and Sicard et al., submitted).

Other host factors required for PPV infection, even at a local level, are the translation initiation factors eIF(iso)4E (Decroocq *et al.*, 2006) and eIF(iso)4G1 (Nicaise *et al.*, 2007), in agreement with previous reports linking translation initiation factors with virus infection in various plant species (Robaglia *et al.*, 2006). Interestingly, an *eIF(iso)4E* ortholog cosegregates with a major quantitative trait locus of resistance to PPV in peach and apricot, suggesting that translation initiation factors also play an important role in the PPV infection of its natural woody hosts (Decroocq *et al.*, 2005).

Although some PPV isolates can infect both *Prunus* and herbaceous hosts, some others have lost the ability to infect their natural woody hosts after prolonged propagation in herbaceous plants, but the molecular basis of this deadaptation is unknown (Dallot *et al.*, 2001). There is also little information on genetic determinants of PPV pathogenesis in different hosts. Making use of chimeric viruses and mutants obtained by site-directed mutagenesis, it has been shown the importance of the P3-6K1 region for PPV pathogenicity both in herbaceous (Riechmann *et al.*, 1995; Sáenz *et al.*, 2000) and woody (Dallot *et al.*, 2001) hosts. P1 protein has also been proposed to play a relevant role in host adaptation (Valli *et al.*, 2006). As mentioned above, HCPro is another protein known to be involved in potyviral pathogenicity, likely as a consequence of its RNA silencing suppression activity (Kasschau *et al.*, 2003). In agreement with this, amino acid changes in HCPro have been shown to contribute to symptom enhancement of PPV in *N. clevelandii* (Sáenz *et al.*, 2001), and HCPro apprears to be a relevant factor for the restriction of PPV systemic spread in *N. tabacum* (Sáenz *et al.*, 2002; Alamillo *et al.*, 2006). Moreover, synergistic enhancement of PVX symptoms by PPV HCPro has also been described (Yang *et al.*, 2002; González-Jara *et al.*, 2005).

Very little is known about the biochemical basis of physiological disturbances associated with PPV infection. However, different analyses suggest that an oxidative stress in the apoplastic space produced by imbalance in the antioxidant system of infected leaves of susceptible peach and apricot cultivars may contribute to the deleterious effects caused by PPV infection (Díaz-Vivancos *et al.*, 2006).

I. 7 Objectives

The importance of the study of plant-pathogen interactions is widely recognized. The objectives of this thesis were focused in the identification of regions in the PPV virus genome involved in viral pathogenicity in herbaceous and woody hosts. For this purpose we followed different approaches based on the different phenotype, in herbaceous and *Prunus* hosts, of the infection caused by i) subisolates separated from the same isolate (PPV-PS), ii) PPV isolates belonging to the same strain (PPV-D and PPV-R), and iii) different potyviruses (PPV and TVMV).

Three types of analyses have been performed:

1. Identification of PPV factors responsible for the phenotypic differences found between different virus variants segregated from the PPV-PS isolate, which derived from a naturally infected peach tree.
2. Identification of PPV factors responsible for the different biological behavior of strain D isolates PPV-D (*Prunus*-adapted) and PPV-R (*N. clevelandii*-adapted) through the construction of chimeric viruses using cDNA clones of the parental viruses. We also forced the adaptation of one of the chimeras to *N. clevelandii* to further investigate PPV factors involved in host specificity.
3. Analysis of the effect of the replacement of PPV P1 protein cistron by the corresponding region of TVMV to investigate the involvement of this protein in host compatibility and pathogenicity.

II. MATERIALS AND METHODS

II.1 Plants

The infectivity assays and the determination of symptomatology were carried out in herbaceous species such as *Nicotiana benthamiana*, *Nicotiana clevelandii, Nicotiana occidentalis, Nicotiana tabacum* and *Chenopodiun phoetidum* and in the woody host *Prunus persicae* cv. GF305.

Plants were grown in a greenhouse maintained at 16 h of light with supplementary illumination and 19–23 ºC or in a climate-controlled chamber at 14 h of ligtht and 22 ºC or 16ºC.

II.2 Virus and bacterial strains

pGPPV (Riechmann *et al.*, 1990) and pIC PPV NK GFP (Fernández-Fernández *et al.*, 2001), which derive from the PPV-R isolate and pGPPVPSes (Sáenz *et al.*, 2001) which derives from the PPV-PS isolate, have been previously described. PPV-D derives from the PPV-Dideron isolate (Kerlan *et al.*, 1979). It was obtained from Dr. J.B. Quiot (ENSA-INRA, Bourdeaux) and maintained in GF305 peach trees.

Escherichia coli DH5α was used for cloning of the plasmids.

II.3 Plant inoculation

II.3.1 Hand inoculation

Leaves from previously infected plants were grinded with 5 mM sodium phosphate buffer pH 7·5 in an ice-cold pestle (1 g in 2 ml). The extract was centrifuged to eliminate tissue fragments in a tabletop centrifuge. Three plant leaves dusted with carborundum were inoculated with 15 μl of this extract.

II.3.2 Biolistic inoculation

The Helios Gene Gun System (Bio-Rad, Hercules, CA) was used for biolistic inoculation. Microcarrier cartridges were prepared with 1·0 μm gold particles coated with the different plasmids at DLR ratio of 2 μg DNA/mg gold and MLQ of 0·5 mg gold/shooting, according to the manufacturer's instructions. Helium pressure of 7·5 bar for herbaceous and of 10 bar for *P. persicae* cv. GF305 were used. Each cartridge was shot twice onto two leaves of each plant and two cartridges were shot for each plant.

In the coinoculation experiments, DNA from each plasmid pair was mixed up in 1 to 3 or 1 to 1 proportion to obtain 2 µg/µl total concentration and the DNA mixture was used for cartridge preparation and biolistic inoculation.

II.3.3 Serial passages

Young leaves of *N. clevelandii* plants were biolistically inoculated with pICPPV-5'BD GFP and collected by 30 d.p.i. Successive passages to *N. clevelandii* plants were carried out every 30 days by hand inoculation as described above. At each propagation step, leaves used as inoculum were checked for GFP expression.

II.3.4 Inoculation with viral transcripts

The recombinant plasmids were linearized with Pvu II (pGPPVR/PS1334esN and pGPPVR/PS1334esNP1Tm) or PstI and PvuII (pGPPV, pGPPVTmP1 and pGPPVTcP1). The digested DNAs were used as template for Capped transcription with the T7 mMessage mMachine (Ambion) or CAPScribe (Boehringer) kits following the manufacturers instructions. The yield and integrity of the transcripts were analyzed by agarose gel electrophoresis. Three primary leaves per plant were dusted with Carborundum and inoculated mechanically with 1.5 µl of the transcription reaction mixture diluted 1:1 with 5 mM sodium phosphate buffer, pH 7·5.

II.4 Nucleic acid preparation

II.4.1 Plasmid DNA preparation

Purification of plasmid DNA from *E. coli* was performed by the alkaline lysis method (Sambrook *et al.*, 1989).

II.5 Nucleic acid manipulation

II.5.1 DNA cloning

The DNA digestions with restriction enzymes were carried out following the manufacturers instructions. *E. coli* DNA polymerase Klenow fragment (New England Biolabs, NEB) was used for protruding 3´ ends digestion. DNA ligation was carried out with T4 DNA ligase (Fermentas).

II.5.2 DNA amplification by PCR

DNA amplification was carried out by means of the polymerase chain reaction (PCR) in a termocicler PTC-100™ (MJ Research, INC). The reaction volume was 25 μl and the enzyme Expand High Fidelity (Roche) was used following the manufacturer recommended protocol.

For the introduction of point mutations in a DNA fragment, an oligonucleotide with the appropriate mutation was used in the PCR reaction.

The Minielute PCR purification Kit (Qiagen) was used for PCR fragment purification.

II.5.3 DNA gel electrophoresis and DNA extraction

DNA fragments were separated by electrophoresis in 0·8% to 2% agarose gels, depending on the expected size, with 0·1 mg/ml of BrEt 0·5 x TBE buffer was used as a running buffer. Pictures of the gels were taken in a "GelDoc 2000" image capturer (BioRad) with a UV transiluminator.

The separated DNA fragments were extracted from the gel and purified with the "QiaexII" system from Qiagen.

II.6 Analysis of nucleic acids in infected plants

II.6.1 Immunocapture-RT-PCR (IC-RT-PCR)

Leaf extracts from infected plants homogenized in 5 mM sodium phosphate buffer, pH 7·5 (2 ml per g tissue) were incubated overnight at 4ºC and 2 h at 37ºC in tubes previously coated with anti-PPV IgGs, and then after two washing steps with PBS-Tween buffer (16 mM sodium phosphate buffer, 0·1 M NaCl, 0·5 g/L Tween 20, pH 7·2), RT-PCR was performed using the Titan kit (Roche Molecular Biochemical's).

GeneScan software was used for sequence analysis of the IC-PCR DNA fragments obtained using an automatic sequencing machine Abi Prism 3700.

II.7 Analysis of viral proteins in infected plants

II.7.1 Western-blot analysis

Proteins were extracted from leaf tissue in disruption buffer (Laemmli, 1970). Approximately 2 μg of total proteins were separated on a 12·5% SDS-PAGE (Laemmli, 1970) and electroblotted onto BioTrace®NT Pure Nitrocellulose Blotting Membrane (Pall

Corporation). Membranes were used for standard immuno-detection as previously described (García *et al.*, 1992) with anti-CP rabbit antiserum and anti-rabbit peroxidase conjugate (Jackson ImmunoReseach Laboratories). The peroxidase reaction was developed with the ECL kit (Amersham Pharmacia Biotech).

II.7.2 ELISA analysis

Leaves were collected and grinded with 5 mM sodium phosphate buffer pH 7·5 in an ice-cold pestle (2 ml per g tissue). The plant extract was diluted in 50 mM sodium carbonate buffer pH 9·6 and applied to 96 well plates (Maxisorp, Nunc). The assay was performed using the REALISA kit (Durviz) following the manufacturer's instructions. Color reaction was developed using *p*-nitrophenil phosphate as a substrate and the optical densities of samples were determined at 405 nm. A standard curve was obtained by including in the assay known amounts of purified PPV-R virions diluted in extract of healthy plants.

II.8 Plasmids

II.8.1 Construction of pICPPV-PSes and derived clones

As a first step to obtain plasmid pICPPV-Pses, two PPV cDNA fragments were amplified from pGPPVPSes (Sáenz *et al.*, 2001) by PCR using as primers 5´ CAGAAACTCGGAATGC3´ (primer 270, nt 2260-2275) and 5´TCCTG**C**AGATAACTTTTTTCAACCAG3´ (nt 2926-2901 with a G to C change indicated in bold, creating a PstI site, underlined) for one of the fragments and 5´ATCT**G**CAGGAATTGGAGCAAGC3´ (nt 2917-2938 with the C to G change in bold, and the introduced PstI site underlined) and 5´CGAACCAACGCCACTG3´ (primer 237, nt 4945-4930) for the second one. These two fragments were used as templates for a new PCR amplification with primers 270 and 237, and the product was cloned in pGEM-T to generate pGEM-PSes. A DNA fragment containing intron I from the ST-LS-1 gene of potato (Vancanneyt *et al.*, 1990) was PCR amplified from pGUS-intron plasmid as previously described by (López-Moya and García, 2000b). This PCR-amplified fragment was digested with PstI and NsiI (compatible with PstI), and cloned into the engineered PstI site of the PPV sequence of pGEM-PSes giving rise to pGEM-PSes-STLS1. In the next step, a cDNA fragment of to the 5' terminal region of the PPV-PS genome was amplified by PCR from pGPPVPSEes with primers 5´AAAATATAAAAACTCAACAC3´ (primer 29, nt 1-24) and 5´TGAACCACTATTGAACAG3´ (primer 317, nt 2609-2592), and cloned in the StuI site of p35SeNOSB (López-Moya and García, 2000b), between the CaMV 35S promoter and the

NOS terminator sequences, rendering p35S5´PSNOSB. Then, p35SNBSNOSB was obtained by inserting NdeI-BglII (PPV nt 309-2312) and BglII-SalI (PPV nt 2312-7633) fragments from pGPPVPSes into SalI/NdeI-digested p35S5´PSNOSB. Finally, a BglII-AspI fragment from pGEM-PSes-STLS1 (PPV nt 2312-4709) was ligated to BglI-BglII (inside the vector to PPV nt 2312) and AspI-BglI (PPV nt 4709 to inside the vector) fragments from p35SNBSNOSB to obtain the complete pICPPV-PSes clone.

A T231C mutation, causing the amino acid change W29R was introduced in pICPPV-PSes by mutagenic PCR. PPV DNA fragments 1-238 and 238-2608 were amplified by PCR from pICPPV-PSes with primers 29 and 5´GCAAAGGCC**G**GGACCCG3´ (nt 222-238) and primers 5´GGGTCC**C**GGCCTTTGCG3´ (nt 221-237) and 317 (the mutated positions are indicated in bold). The PCR products were used as templates for PCR amplification with primers 29 and 317 to obtain the PPV-PSes fragment 1-2608 containing the T231C mutation, which was cloned into StuI-digested p35SNOSB plasmid giving rise to p35S5´W29RNOSB. pICPPV-Pses W29R was finally obtained by ligating a NaeI–NdeI fragment (inside the vector to PPV nt 309) of p35S5´W29RNOSB with NdeI-BglII (PPV nt 309-2312) and BglII-NaeI (PPV nt 2312 to inside the vector) fragments from pICPPV-PSes.

As a first step to introduce the T562A mutation, which causes the amino acid change V139E, in pICPPV-PSes, a cDNA fragment spanning nt 1-2608 was amplified by IC-RT-PCR from extracts of *N. clevelandii* plants infected with the PPV-PS subisolate 1.3.1. The PCR product was cloned into StuI-digested p35SNOSB to obtain p35S5´1.3.1NOSB carrying nucleotides changes C231 and A562. Finally, pICPPV-PSes V139E was obtained by ligating a NdeI-BglII fragment (PPV nt 309-2312) with NaeI-NdeI (from inside the vector to PPV nt 309) and BglII-NaeI (PPV nt 2312 to inside the vector) fragments from pICPPV-PSes.

To construct the double mutant pICPPV-PSes W29RV139E, the PPV cDNA NdeI-BglII (nt 309-2312) and BglII-SalI (nt 2312-7633) fragments from pICPPV-PSes V139E were ligated with the vector-containing SalI-NdeI fragment from pICPPV-PSes W29R.

The accuracy of all the constructions was verified by restriction digestion analysis and DNA sequencing of all regions derived from PCR amplification.

II.8.2 Construction of an infectious PPV-D cDNA clone and PPV-D/R chimeras

As a first step for the construction of a full-length cDNA clone of the PPV-D isolate a PPV cDNA fragment was amplified by IC-RT-PCR from leaf extracts of PPV-D-infected GF305 peach using as primers 5´ATCT**G**CAGGAATTGGAGCAAGC3´ (nt 2917-2938 with a C to G change indicated in bold, creating a PstI site, underlined) and

5´GGGACAGTTGGTGCAAC3´(nt 3739-3723). This fragment was digested with PstI and SalI (nt 2922-3628) and cloned into pUC19 digested with the same enzymes giving rise to pUC-DSalPst. Then the PstI-PflMI fragment (PPV nt 2922-3156) from pICPPV-NK GFP including the intron I from the ST-LS-1 gene of potato (Vancanneyt *et al.*, 1990) was cloned into pUC-DSalPst digested with the same enzymes giving rise to pUC-DSalPstin.

To clone the 5´end sequence of PPV-D under the control of the CaMV 35S promoter a cDNA fragment was amplified by IC-RT-PCR from leaf extracts of PPV-D-infected GF305 peach using as primers 5´AAAATATAAAAACTCAACAC3´ (nt 1-20) and 5´TCCTG**C**AGATAACTTTTTTCAACC3´ (nt 2926-2903 with a G to C change indicated in bold, creating a PstI site, underlined). This fragment was digested with PstI and cloned into p35SeNOSB (López-Moya and García, 2000b) digested with Eco147I and PstI giving rise to p35S5´DNOS.

Next, cDNA fragments covering the rest of the PPV-D genome were amplified by IC-RT-PCR from leaf extracts of PPV-D-infected GF305 peach using primers designed on basis to the PPV-R sequence. Restriction fragments spanning the nucleotides 3628-6931, 6931-9021, and 3628-9786 were obtained by digestion of the IC-RT-PCR products with SalI-BamHI, BamHI-SacI, and SalI, respectively, and cloned into pUC19 or pUC18 giving rise to pUCSalBam, pUC-DBamSac, and pUC-DSalend. The chimeric full-length clone pICPPV-5´SD GFP was obtained by ligating a PvuII-PstI fragment from p35S5´DNOS (from inside the vector to PPV nt 2922) and a PstI-SalI fragment from pUC-DSalPstin (PPV nt 2922-3628) to a SalI-PvuII fragment from pICPPV-NK GFP (PPV nt 3628 to inside the vector). pICPPV-SBD GFP and pICPPV-5'BD GFP were the result of substituting a SalI-BamHI fragment from pUC-DSalBam (PPV nt 3628-6931) for the corresponding fragments of pICPPV-NK GFP and pICPPV-5´SD GFP, respectively. Replacement of the BamHI-SacI fragment of pICPPV-NK GFP (PPV nt 6931-9021) by the corresponding fragment from pUC-DBamSac yielded pICPPV-BSD, and a triple ligation of a SacI-NdeI fragment from pUC-DSacend (PPV nt 9021-9694) and NdeI-BglI (PPV nt 9694 to inside the vector) and BglI-SacI (from inside the vector to PPV nt 9021) fragments from pICPPV-BSD gave rise to pICPPV-BND. Finally, the complete PPV-D clone pICPPV-Dc was obtained by a triple ligation of a XbaI-SalI fragment from pICPPV-5´SD GFP (from inside the vector to PPV nt 3628), a SalI-BamHI fragment from pUCSalBam (PPV nt 3628-6931), and a BamHI-XbaI fragment from pICPPV-BND (PPV nt 6931 to inside the vector).

pICPPV-5´BsD GFP was obtained by ligating a SalI-BglI fragment (PPV nt 3629 to inside the vector) from pICPPV-NK GFP, a BglI-BstBI fragment (from inside the vector to

PPV nt 2038) from pICPPV-5´SD GFP and a BstBI-SalI fragment (PPV nt 2038-3629) from pICPPV. pICPPV-BsSD was constructed by a similar triple ligation, but in this case the BglI-BstBI fragment was from pICPPV and the BstBI-SalI and the SalI-BglI fragments were from pICPPV-5´SD GFP.

The genomic sequence of PPV-D isolate was determined by direct sequencing of cDNA fragments amplified by IC-RT-PCR from leaf extracts of infected peach GF305 plants. Heterogeneities respect to the published sequences of PPV-R and PPV Dideron were confirmed by sequencing of a second IC-RT-PCR product. We found a few punctual sequence differences between different IC-RT-PCR products, which could be due to sequence heterogeneities in the virus population or to PCR mistakes. In these cases, the sequence of pICPPV-Dc corresponded to the majority IC-RT-PCR sequence, except for A413 and G662, which implied silent changes with respect to the predominant T413 and G662. The regions spanning nt 2924-3156 and 9694-9786 cloned in pICPPV-Dc derived from the PPV-R clone pICPPV-NK GFP. No sequence heterogeneities were found between PPV-R and PPV-D in these regions, although we cannot rule out the existence of differences in the last 7 nt, which were not unambiguously determined for PPV-D.

II.8.3 Construction of PPV/TVMV chimeras.

Due to the lack of common restriction enzymes sites in the P1 cistrons of PPV and TVMV, the gene splicing by overlap extension method (Horton *et al.*, 1989) was used for the construction of pGPPVTmP1 and pGPPVTcP1 (Fig. 1). For pGPPVTmP1 construction, TVMV cDNA from pXbS7 (Domier *et al.*, 1989) was used as template for PCR amplification with primers 5´CTA**GCCATGGCAACCATTCACTCAG**3´ (primer A), which contains a NcoI site (underlined) to facilitate cloning and the nucleotides that correspond to P1 coding sequences from TVMV P1 (nt 205-226), and 5´*TTGCC****TTGGC*****TATATTAGCCAC**3´(primer B), in which the first 10 nt (in italics) and the last 17 nt (in bold) correspond to P1 coding sequences from PPV (nt 699 to 690) and TVMV (nt 602 to 586), respectively. A PCR fragment partially complementary to the TVMV fragment amplified with primers A and B was amplified with primers 5´**CTAATATA*****GCCAA***GGCAAATGG3´ (primer B´), in which the first 13 nt (in bold) and the last 14 nt (in italics) correspond to P1 coding sequences from TVMV (nt 590 to 602) and PPV (nt 690-703), respectively, and 5´*CGCATTAGTTCAC*3´ (primer D, PPV nt 1378-1390), using as template pGG5S6N, a partial PPV cDNA clone (PPV nt 1-3628), in which the first AUG of the large ORF was mutated and the surroundings of the second AUG were

engineered to display a NcoI restriction site (Simón-Buela *et al.*, 1997a). The resulting PCR fragments were used as templates for a second PCR round with primers A and D. The product of this PCR was digested with NcoI and BsaBI and cloned in pGG5S6N, yielding pGG5S6NtmP1Δ54. New data available from John Shaw (University of Kentucky, personal communication, genebank acession number NC_001768.1), suggested that the actual initiation codon of TVMV polyprotein was in position 154-156. A new TVMV fragment was amplified by PCR from pXb57 with primers 5´CTAG**CCATGGCAGCAACAATGATC**3´ (primer C, TVMV nt 154-171 in bold) and B. The PCR product was digested with NcoI and MunI and substituted for the corresponding sequence of pGG5S6NTmP1Δ54 to obtain pGG5S6NTmP1. Finally, pGPPVTmP1 was obtained by introducing the BglI-DraIII fragment of pGG5S6NtmP1containing the chimeric P1 coding sequence into pGPPV.

For pGPPVTcP1 cloning, first PCR round to obtain partially overlapping TVMV and PPV fragments were performed on pXbS7 with primers A and 5´*GCCTGGGTC***TGAGAAGTGAGTCG3´** (primer E, TVMV nt 978-965, corresponding to the 3'end of P1 sequence and the first 3 nt of HCPro cistron, in bold, and PPV nt 1082-1074, in italics), and on pGG5S6N with primers 5´**CACTTCTCA***GACCCAGGCAAAC*3´ (primer E´, TVMV nt 970-978, in bold, and PPV nt 1074-1086, in italics) and D. The products of these PCRs were used for the recombinant PCR with primers A and D, and the resulting fragment was cloned in pGG5S6N after NcoI/BsaBI digestion, yielding pGG5S6NTcP1Δ54. In order to obtain a recombinant clone with the correct TVMV initiation codon, pGG5S6NTcP1, a MunI-SalI fragment derived from pGG5S6NTcP1Δ54 and a NcoI-MunI fragment of the PCR product amplified from pXb57 with primers C and B (see above) were inserted in pGG5S6N digested with NcoI and SalI. Finally, pGPPVTcP1 was obtained by a triple ligation of MunI-SacI and BsaBI-SacI fragments of pGPPVTmP1 and a MunI-BsaBI fagment derived from pGG5S6NTcP1.

pGPPVR/PS1334esN and pGPPVR/PS1334esNP1Tm were obtained by inserting a BglI-NcoI fragment from pGG5S6N and a NcoI-Bsh1361I fragment derived either from pGG5S6N (pGPPVR/PS1334esN) or from pGPPVP1Tm (pGPPVR/PS1334esNP1Tm) in pGPPVPSE109S232, a full-length clone derived from the PPV-PS isolate, digested with BglI and Bsh1361I.

pICPPVN5´BD GFP was constructed by replacing the BglI-CpoI fragment of pICPPV5´BD GFP, a chimeric clone derived from PPV isolates D and R, with the corresponding fragment of pICPPVN, a derivative of pICPPV (López-Moya and García,

2000b) harboring the mutations of the first AUG codons introduced in pGG5S6N (J.J. López-Moya and J.A.G., unpublished results).

pICPPVNP1Tm5´BD GFP and pICPPVNP1Tc5´BD GFP were constructed by replacing the NcoI-Bpu1102I fragment of pICPPVN5´BD GFP with the corresponding fragments from PCR products amplified using as primers 5´AAAATATAAAAACTCAACAC3´ (PPV nt 1-20) and 5´GGAAG*C*TCAGCATTCCGAG3´ (PPV nt 2284-2266, with a mistmach, in italics, to create a Bpu1102I site, underlined) from pGPPVTmP1 and pGPPVTcP1, respectively.

The accuracy of all the constructions was verified by restriction digestion analysis and DNA sequencing of all regions derived from PCR amplification.

II.9 GFP observation and imaging

Plant leaves were observed using a fluorescence stereomicroscope MZ FLIII (LEICA Microsystems) with excitation an arrest window of 480/40 nm and 510 nm, respectively. Pictures were caught with an OLYMPUS DP 70 digital camera and the software DP Controller and DP manager (OLYMPUS OPTICAL CO., LTD).

III. RESULTS

III.1 Host-specific effects on viral pathogenicity of two amino acid heterogeneities in PPV P1 protein

The replication of plus-stranded RNA genomes is carried out by RNA-dependent RNA polymerases that usually lack proofreading activity (Malpica *et al.*, 2002). This causes high mutation rates that together with the short replication times and high RNA yields give rise to virus populations consisting of complex and dynamic mutant swarms, which are usually interpreted in the terms of the quasispecies theory (Eigen, 1996; Domingo and Holland, 1997; Biebricher and Eigen, 2006), although other conceptual models have also been raised (Jenkins *et al.*, 2001; Holmes and Moya, 2002). The mutant spectrum usually fluctuates around a unique consensus sequence that is, however, able to evolve rapidly towards other consensus sequence in response to changes in the selective pressure. Moreover, several dynamic virus populations, each one centered on a consensus sequence, may coexist in single individuals. In these cases, the pathogenic features of the mixed infection depend on complex intra- and inter-population interactions, and may differ from those of single infections of each separated virus population.

So far, little information is available concerning the molecular mechanisms involved in PPV pathogenicity and host range definition. The PPV-PS isolate belongs to the M strain and was originally obtained from an infected peach in Yugoslavia. Virus propagation in an herbaceous host, *N. clevelandii*, showed the complexity of the original virus population (Sáenz *et al.*, 2001). Several subisolates, which showed high sequence conservation (approximately 99,9% identity) but differed largely in pathogenicity in herbaceous hosts and infectivity in woody plants, were segregated from the original isolate by local lesion cloning in *C. foetidum* (Sáenz *et al.*, 2001). Sequence analysis of the subisolates and site directed mutagenesis of an infectious PPV-PS cDNA clone demonstrated that a single amino acid change (K109E) in the HCPro protein caused a drastic effect on virus symptoms in *N. clevelandii* and *N. occidentalis*. However, the mild subisolates 1.3.1 and 2.1.1 resembled the severe subisolates in having E at position 109 of HCPro, indicating that undiscovered attenuating change(s) should exist in genome regions of these PPV-PS mild subisolates not sequenced yet (Sáenz *et al.*, 2001). We have now completed the sequencing of PPV-PS 1.3.1 genome and identified two new amino acid changes in the P1 protein involved in symptom attenuation in herbaceous hosts and lost of infectivity in peach.

III.1.1 Two new amino acid changes in mild PPV-PS 1.3.1 and 2.1.1 subisolates compared to strong PPV-PS 4.1.4 subisolate

The genome of PPV-PS 1.3.1 differed from the PPV-PS cDNA sequence of the previously reported pGPPVPS clone (Sáenz *et al.*, 2000) in seven nucleotides. In addition to the change causing the K109E substitution in HCPro previously described, there were two nucleotide changes that caused amino acid substitutions in P1 (W29R and V139E) and four silent changes in the 5´ NCR and in the coding regions of CI, NIaVPg, and NIb (Table 1). The possible relevance in pathogenicity of the P1 amino acid changes was highlighted by the observation that R29 and E139, the residues present in PPV-PS 1.3.1, were also present in the second mild isolate (2.1.1), which as 1.3.1 is non-infectious in peach, whereas amino acids W and V occupied P1 positions 29 and 139 in two severe isolates, 4.1.4 and 10_7, which are able to infect peach (Table 1).

Table 1. Genome localization in different PPV-PS subisolates of nucleotide differences between the subisolate 1.3.1 and the cDNA clone PS-MCl and PSes

	Genome-nucleotide position (amino acid position in the protein)[a]									
Virus[a]	**5´NCR 40**	**P1 231 (29)**	**P1 562 (139)**	**HC 1395 (109)**	**HC 1764 (232)**	**CI 4250 (200)**	**NIaVPg 6200 (162)**	**NIb 7565 (181)**	**NIb 7793 (257)**	**CP 8711 (45)**
PS-MCl	**G**	**T (W)**	**T (V)**	**A (K)**	**G (G)**	**G**	**G**	**A**	**A**	**C (F)**
PSes	**G**	**T (W)**	**T (V)**	**G (E)**	**A (S)**	**G**	**G**	**A**	**A**	**C (F)**
4.1.4	**G**	**T (W)**	**T (V)**	**G (E)**	**A (S)**	**G**	**G**	**A**	**C**	**A (K)**
1.3.1	**A**	**C (R)**	**A (E)**	**G (E)**	**G (G)**	**A**	**A**	**G**	**A**	**C (F)**
2.1.1	**ND**	**C (R)**	**A (E)**	**G (E)**	**G (G)**	**ND**	**ND**	**ND**	**A**	**C (F)**
10_7	**G**	**T (W)**	**T (V)**	**G (E)**	**A (S)**	**G**	**G**	**A**	**C**	**C (F)**

[a] Grey and white rows indicates severe and mild viruses, respectively

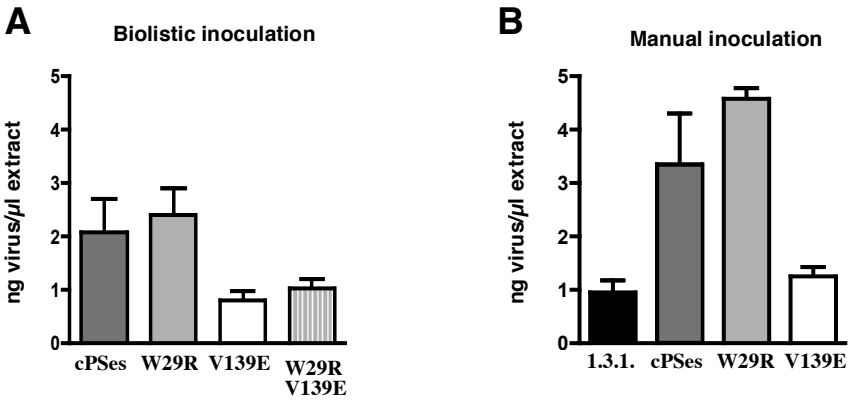
III.1.2 The amino acid change V139E affects symptoms and viral accumulation in N. clevelandii and N. occidentalis

In order to assess the significance of the P1 heterogeneities, they were incorporated, independently or together, in a cDNA clone of PPV-PS. Since the virus progeny of the original pGPPVPS clone produces only mild symptoms in herbaceous hosts, as a consequence of the presence of K at HCPro position 109 (Sáenz *et al.*, 2001), this clone is not suitable to study the attenuating effect of 1.3.1-specific sequences. A new PPV-PS cDNA clone, pICPPVPSes (cPSes), was constructed containing the HCPro coding sequence of the severe

subisolate 4.1.4 (differing from that of pGPPVPS in having E109 and S232 (Sáenz *et al.*, 2001). In addition, in cPSes, the virus sequence is under the control of the CaMV 35S promoter, which allows the production *in planta* of infectious transcripts after inoculation with plasmid DNA (López-Moya and García, 2000b). Inoculation with cPSes caused in *N. clevelandii* and *N. occidentalis* severe symptoms that were indistinguishable from those induced by PPV-PS 4.1.4 (Figs. 2 and 3, and Sáenz *et al.*, 2001). cPSes was modified by site-directed mutagenesis to express P1 proteins incorporating either of the two amino acid changes associated with the mild PPV-PS subisolates, and the resulting clones pICPPVPSes W29R (W29R) and pICPPVPSes V139E (V139E) were tested for infectivity in *N. clevelandii* plants (Fig. 2). Both clones infected 100% of the plants inoculated, either biolistically o by hand rubbing, in seven independent experiments. However, whereas W29R caused severe symptoms similar to those induced by cPSes, V139E resembled the natural subisolate 1.3.1 in causing a mild infection (Fig. 2C). In addition, levels of virus accumulation of PSes and W29R were comparable and significantly higher than those of PPV PS 1.3.1 and V139R (Fig. 2A and B). In order to test the effect of both 1.3.1-specific P1 changes in combination in a cPSes background, the clone pICPPVPSes W29RV139E (W29RV139E) was also constructed. Viral accumulation of W29RV139E was similar to that of V139E (Fig. 2A). Surprisingly, symptoms observed in W29RV139E-infected plants were slightly, but reproducibly, more prominent than those caused by V139E, although they were still much milder than those induced by cPSes and W29R (Fig. 2C).

Virus variants segregated from the original PPV-PS isolate also differed largely in the symptoms induced in another herbaceous host, *N. occidentalis*. In this host, severe subisolates, such as 4.1.4 and 10_7, or virus progeny of the clone cPSes caused very conspicuous local necrotic lesions, which were not able to prevent systemic spread of the virus, while mild subisolates, such as 1.3.1, caused systemic infections without apparent symptoms in the inoculated leaves (Sáenz *et al.*, 2001 and Fig. 3). Necrotic lesions similar to those induced by cPSes were observed in *N. occidentalis* leaves inoculated with the W29R mutant, but not in those inoculated with the 1.3.1 subisolate or the V139E mutant (Fig. 3B and C). The absence of lesions in the inoculated leaves correlated with lower viral accumulation (Fig. 3A). Interestingly, the W29R change appeared to have a compensatory effect over the V139E substitution, since the W29RV139E double mutant induced necrotic lesions and accumulated to similar levels to those of cPSes in the *N. occidentalis* inoculated leaves (Fig. 3). The fact that the subisolate 1.3.1 and the W29RV139E mutant have quite

different phenotypes in *N. occidentalis* in spite of sharing identical P1 sequences, suggests that differences in the 1.3.1 and PSes background outside the P1 region, for instance the presence of S or G at position 232 of HCPro (Table 1), could also affect virus pathogenicity in this host.



C

Virus	Symptoms	
1.3.1.	Very mild chlorotic mottling	+/–
cPSes	Strong chlorotic mottling	+++
W29R	Strong chlorotic mottling	++
V139E	Very mild chlorotic mottling	+/–
W29RV139E	Mild chlorotic mottling	+

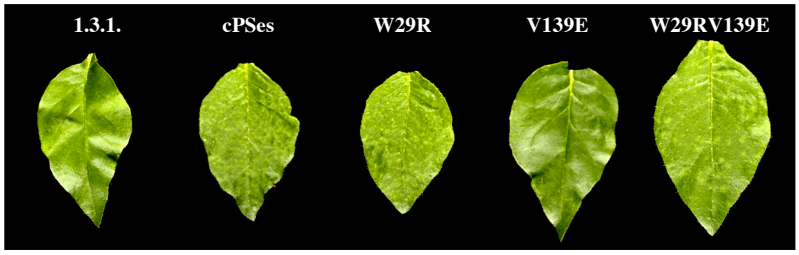
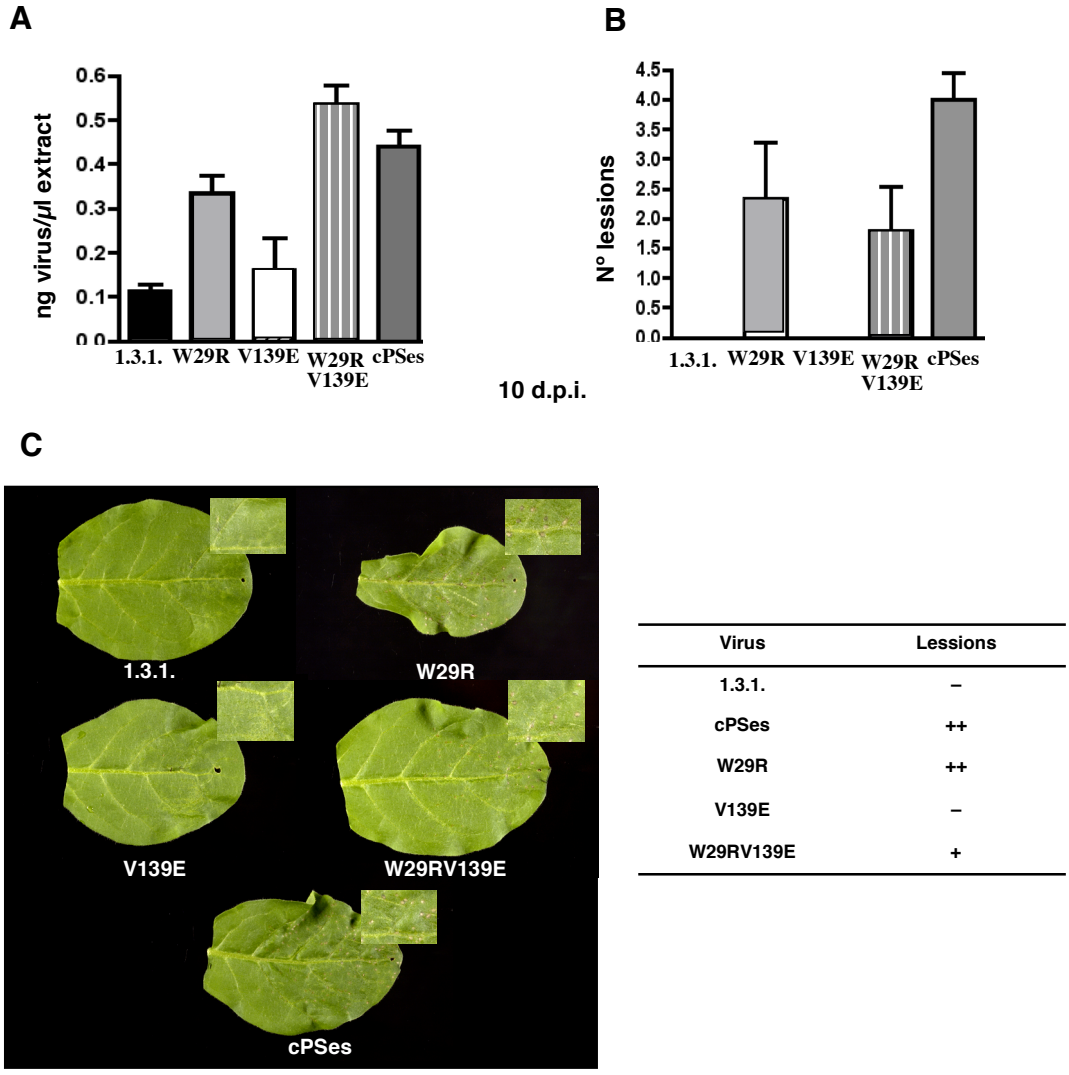


Fig. 2. Infectivity of PPV-PS mutants in *N. clevelandii*. Virus accumulation in young systemically infected leaves of biolistically inoculated (A) or hand inoculated (B) *N. clevelandii* plants (21 d.p.i.) determined by DASI-ELISA. Bars in A and B represent average values and standard deviations of four different plants. (C) Symptoms induced by the different viruses. The severity of the symptoms is ranked from almost not detectable (+/-) to the most intense chlorotic mottling (+++). Pictures of systemically infected leaves taken at 21 d.p.i. are shown at the bottom panel.

All these results clearly indicate that an E at position 139 of P1 protein is an attenuating factor for PPV-PS infection in *N. clevelandii* and *N. occidentalis*, but the amino acid present in position 29 of P1, and other factors outside P1, might modulate the effect of the amino acid 139 on virus pathogenesis in these plants.



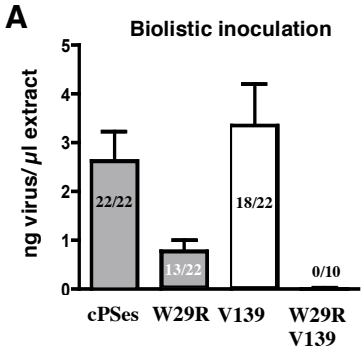
Virus	Lessions
1.3.1.	–
cPSes	++
W29R	++
V139E	–
W29RV139E	+

Fig. 3. Infectivity of PPV-PS mutants in *N. occidentalis*. (A) Virus accumulation in hand inoculated leaves of *N. occidentalis* plants (10 d.p.i.) determined by DASI-ELISA. (B) Number of necrotic lesions in hand inoculated leaves of *N. occidentalis* plants (10 d.p.i.). Bars in A and B represent average values and standard deviations of six different plants. (C) Symptoms induced by the different viruses. The intensity of the local lesions is ranked from not detectable (-) to very prominent (++). Pictures of inoculated leaves taken at 10 d.p.i. are shown at the left panel.

III.1.3 The amino acid change W29R affects viral infectivity and accumulation in GF305 peach

PPV-PS was originally isolated from a naturally infected peach tree. However, some virus variants segregated from the PPV-PS isolate by local lesion cloning in *C. foetidum*, such as 1.3.1, were unable to infect peach seedlings (Sáenz *et al.*, 2002). In order to assess the relevance of the amino acid present at positions 29 and 139 for PPV pathogenicity in its natural host, GF305 peach seedlings, were biolistically inoculated with cPSes and its derivatives. cPSes and the V139E mutant showed high infectivity, 100% and 82%,

respectively, on GF305 peach seedlings, and both viruses caused similar severe symptoms and accumulated to comparable levels in the infected plants (Fig. 4). The infectivity on peach of the W29R mutant (58%), as well as its accumulation level and symptom severity, were significantly lower than those of cPSes and V139E. Interestingly, although the single V139E mutation did not significantly affect *Prunus* infection, it enhanced the effect of the W29R mutation, and the W29RV139E double mutant resembled the 1.3.1 subisolate in being completely unable to infect GF305 peach in our experimental conditions (Fig. 4). Thus, although our results show that P1 amino acid 29 is specially relevant for *Nicotiana* infection and so does P1 amino acid 139 for *Prunus* infection, both amino acids appear to play concerted actions in the two hosts.



B

Virus	Symptoms	
1.3.1.	–	–
cPSes	Strong vein clearing and leaf distortion	++
W29R	Mild vein clearing and leaf distortion	+
V139E	Strong vein clearing and leaf distortion	+++
W29RV139E	–	–

Fig. 4. Infectivity of PPV-PS mutants in *P. persicae* cv GF305. (A) Virus accumulation in young systemically infected leaves of biolistically inoculated GF305 peach seedlings (35 d.p.i.) determined by DASI-ELISA. Bars represent average values and standard deviations of 6 different plants. The number of infected plants/number of inoculated plants ratio in a total of five experiments is indicated inside the bars. (B) Symptoms induced by the different viruses. The severity of the symptoms is ranked from not detectable (-) to the most intense vein clearing and leaf distortion (+++). Pictures of systemically infected leaves taken at 35 d.p.i. are shown at the bottom panel.

III.2 Identification of Plum pox virus pathogenicity determinants in herbaceous and woody hosts

Determinants involved in genome amplification, cell-to-cell and long distance movement, symptom expression, aphid and seed transmission have been identified for several potyvirus species (Revers *et al.*, 1999), however the capacity of PPV to establish local and systemic infections in woody hosts has not been yet accurately located. The construction of hybrid genomes between full-length cDNA clones from viruses showing different phenotypes has proven to be a useful tool for the identification of strain- and isolate-specific pathogenicity and host range determinants. Previous studies using chimeric viruses constructed from two cloned PPV isolates, PPV-Rankovic (PPV-R) and PPV-PS, have led to the identification of complex determinants of pathogenicity in herbaceous (Sáenz *et al.*, 2000) and woody hosts (Dallot *et al.*, 2001). However, the high sequence divergence (12·5% and 4·2% at nucleotide and amino acid level, respectively) between these isolates, which belong to different PPV strains, limited the capacity to map precisely viral pathogenicity determinants.

We have engineered a full-length infectious cDNA clone of the PPV-D isolate, a strain D isolate. Conversely to the previously described cDNA clone of PPV-R (Riechmann *et al.*, 1990), also belonging to the strain D, the PPV-D clone is able to infect efficiently GF305 peach seedlings, but lacks the ability to infect the herbaceous host *N. clevelandii*. Taking advantage of this host range difference and of the low divergence between PPV-R- and PPV-D-derived clones (0·69% and 0·76% at nucleotide and amino acid level, respectively), we engineered recombinant viruses by exchanging fragments of the viral genomes between the infectious cDNA clones to gain insight into the elements of the potyvirus genome that influence host range selection. Furthermore, to complement this approach, the adaptative evolution of a chimeric virus derived from PPV-D to *N. clevelandii* has been studied by serial passaging experiments. The analysis of the mutations introduced in the adapted variants has provided additional information about the viral regions relevant for efficient host infection in the herbaceous plant.

III.2.1 Virus progeny of a PPV-D full-length cDNA clone causes severe symptoms in GF305 peach seedlings but is not infectious in N. clevelandii

A full-length cDNA copy of the genome of a D-type PPV isolate that efficiently infect GF305 peach seedlings (named from here on PPV-D) was cloned and sequenced (Genebank accession EF569214). Although PPV-D derives from the PPV-Dideron isolate sequenced by Teycheney *et al*. (1989.), we have found some sequence divergence (46 nucleotide and 17 amino acid changes) between the PPV-D sequence and the previously published sequence (X16415.1) (Fig. 5A).

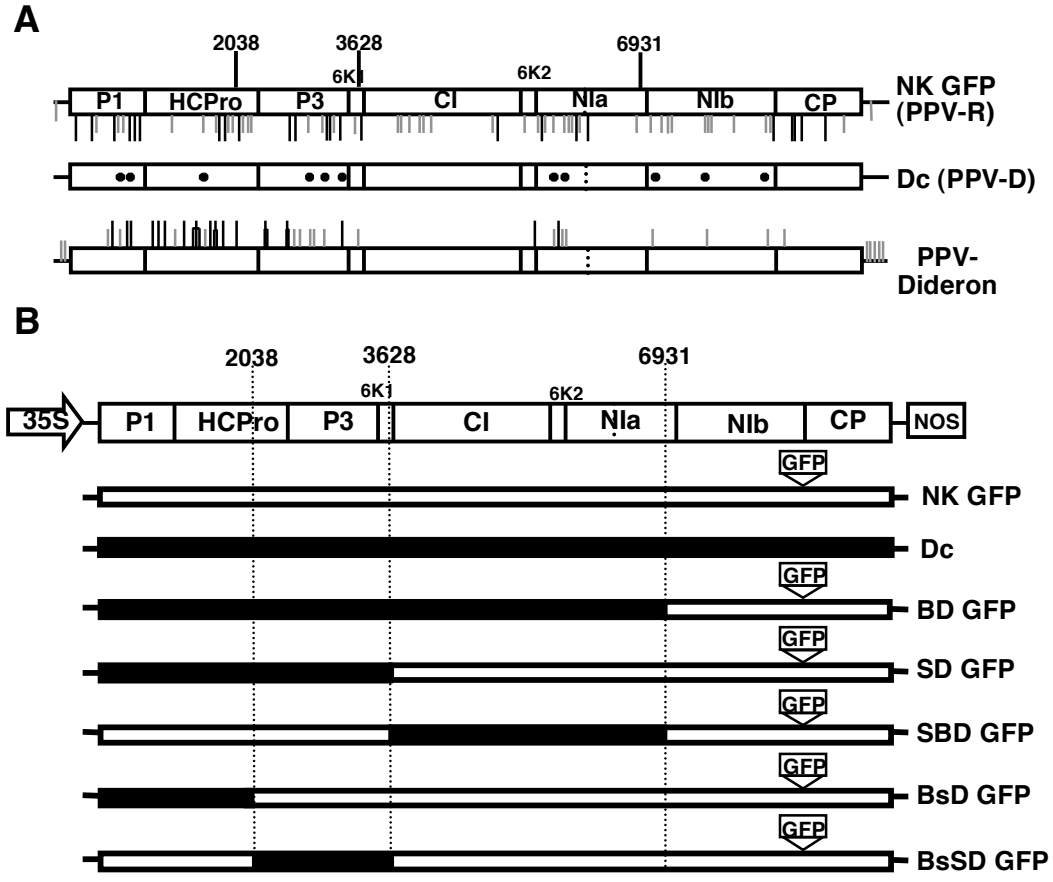
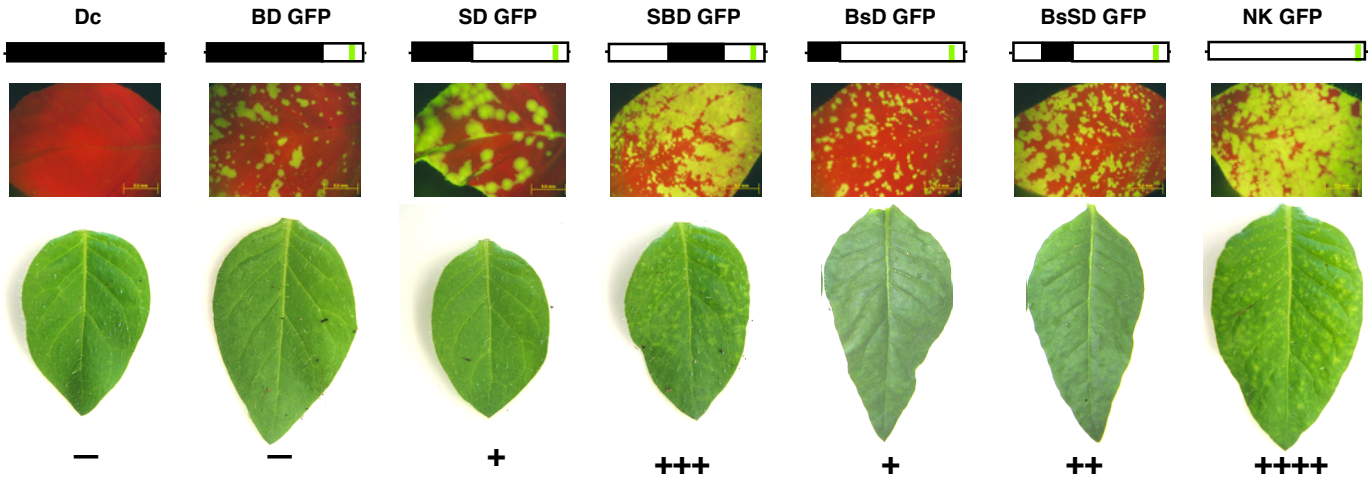


Fig. 5. Comparison of PPV-R, PPV-Dideron and PPV-D sequences and construction of PPV-D/R hybrids. (A) Comparison between the PPV-R and PPV-D sequences cloned in pICPPV-NK GFP (EF569215) and pICPPV-Dc (EF569214), respectively, and the PPV-Dideron sequence (X16415.1). The silent and missense nucleotide changes of PPV-Dideron and PPV-R with respect to PPV-D are represented by short gray and long black vertical lines, respectively. Nucleotide changes shared by both PPV-Dideron and PPV-R sequences are indicated in PPV-D (●). (B) Construction of PPV-D/R hybrids. Schematic representation of PPV cDNA clones NK GFP (in white) and Dc (in black) and of the hybrids constructed between them. GFP sequence is represented with a box between NIb and CP. In both panels, positions of the restriction sites used for the construction of the hybrids are highlighted as vertical lines with the PPV-R nucleotide number indicated above.

The full-length cDNA of the PPV-D isolate was cloned between the 35S promoter from *Cauliflower mosaic virus* and the nopaline syntase termination signal, which allowed the *in planta* expression of the viral RNA. DNA of the resulting plasmid, pICPPV-Dc (Dc) was

biolistically inoculated onto leaves of GF305 peach seedlings and *N. clevelandii* plants. Dc caused in all the inoculated peach plants strong leaf distortion, blotches and vein clearing indistinguishable from those caused by the parental PPV-D isolate (Fig. 7). In agreement with early reports on the host range of PPV-Dideron (Kerlan and Dunez, 1979), none of the 25 *N. clevelandii* plants bombarded with Dc in 7 independent experiments displayed disease symptoms or showed virus accumulation in ELISA or Western-blot analyses (Fig. 6).



Construct	Infectivity	Symptoms
Dc	0/25	—
BD GFP	9/9	Asymptomatic
SD GFP	8/8	Very mild chlorotic mottling
SBD GFP	12/12	Very strong chlorotic mottling
BsD GFP	10/10	Mild chlorotic mottling
BsSD GFP	10/10	Strong chlorotic mottling
NK GFP	6/6	Very strong chlorotic mottling

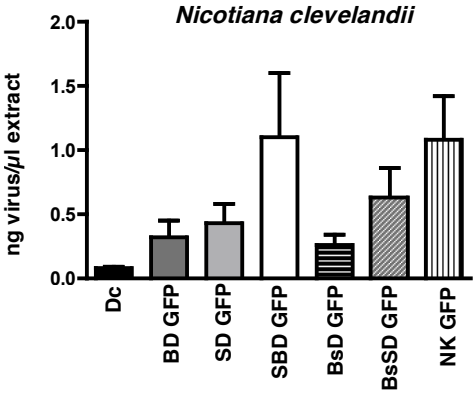


Fig. 6. Phenotypic assessment of the PPV-D/R hybrids in *N. clevelandii*. Pictures show GFP expression (upper row) and symptoms (lower row) in young leaves *N. clevelandii* plants at 21 d.p.i. A schematic representation of each virus genome is shown above the pictures and an estimate of the strength of the symptoms is indicated below. Regions of PPV-Dc and PPV-NK GFP genomes are represented as black and white boxes, respectively. The green box represents the GFP insert. The infectivity of each virus (number of infected plants/number of inoculated plants) and a description of the symptoms induced are indicated in the table. The graph shows the virus accumulation in young leaves of infected *N. clevelandii* plants (21 d.p.i.) determined by DASI-ELISA. Each bar represents the average value and the standard deviation from four different plants.

III.2.2 Specific host range determinants of PPV-D and PPV-R isolates are extensively spread through the virus genome

PPV-R is a previously sequenced D-strain isolate (Laín *et al.*, 1989a) that has lost its ability to infect systemically *Prunus* hosts after being propagated for long time in herbaceous

hosts (Dallot *et al.*, 2001). In agreement with the host range of PPV-R, infection of *N. cleveladii,* but not of GF305 peach seedlings, was achieved after biolistic inoculation with DNA of pICPPV-NK GFP (NK GFP), a plasmid based on the PPV-R sequence and carrying GFP as a reporter gene (Fernández-Fernández *et al.*, 2001) (Fig. 6 and 7).

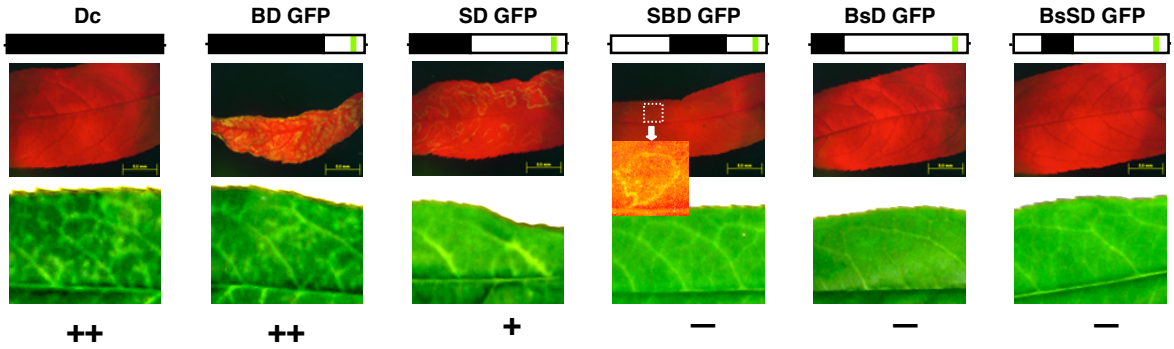
Sequence analysis showed 68 nucleotide changes in NK GFP with respect to Dc, which where translated into 24 amino acid changes (Fig. 5A, 10A). This divergence is slightly higher than that shown between the two related isolates PPV-Dideron and Dc. Interestingly, whereas the differences between Dc and NK GFP were quite evenly distributed through all the genome, Dc and PPV-Dideron were very similar in a great part of the genome (only 14 nucleotide changes, giving rise to 2 amino acid substitutions between nucleotides 3631 and 9786) and most heterogeneities were concentrated in one hot spot region spanning from nucleotide 413 to 3630 (30 nucleotides and 15 amino acid changes). This region comprises P1 C-terminus, HCPro, P3 and 6K1 coding regions, but changes mapped preferentially to the HCPro sequence with 14 nucleotide changes giving rise to 9 amino acid changes between nucleotides 1093 and 2031 (Fig. 5A).

In order to map PPV genomic sequences involved in the host range differences found between the very closely related PPV-R and PPV-D isolates, PPV-R/D chimeras were constructed by exchanging fragments between the parental full-length cDNA clones NK GFP and Dc (Fig. 5B). All the chimeric viruses contain the GFP sequence between the NIb and CP coding regions, which allowed us to easily monitor viral infection.

Young plants of *N. clevelandii* and GF305 peach seedlings were biolistically inoculated with chimeric constructs pICPPV-5'BD GFP (BD GFP), pICPPV-5'SD GFP (SD GFP), and pICPPV-SBD GFP (SBD GFP), which contain PPV-D-derived nucleotides 1-6931, 1-3628, and 3629-6931, respectively. Every construct was assayed in at least 4 different experiments, with consistent results both in symptoms induction and viral accumulation.

GFP monitoring, DASI-ELISA and Western-blot analyses showed that, in contrast with clone Dc, all the hybrids assayed in this study were able to initiate a systemic infection in *N. clevelandii* (Fig. 6). The results of the BD GFP chimera indicated that the substitution of the 3´ terminal third of the genome of PPV-D isolate, including the C-terminus (30 amino acids) of NIa and the complete NIb and CP coding sequences, for that of PPV-R was sufficient to confer infectivity on *N. clevelandii*. Notable differences in symptom severity and viral accumulation were observed among the different chimeric viruses. *N. clevelandii* plants infected with BD GFP were asymptomatic while typical chlorotic mottling was observed in the plants inoculated with SD GFP or SBD GFP. However, whereas plants infected with SBD

GFP displayed severe symptoms similar to those of the plants infected with the parental clone NK GFP, symptoms induced by SD GFP were much milder (Fig. 6).



Construct	Infectivity	Symptoms
Dc	13/13	Strong vein clearing, blotch and distortion
BD GFP	18/18	Strong vein clearing, blotch and distortion
SD GFP	14/14	Mild vein clearing, blotch and distortion
SBD GFP	3/22	–
BsD GFP	0/12	–
BsSD GFP	0/11	–
NK GFP	0/4	–

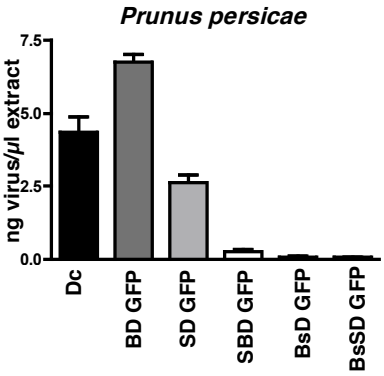


Fig. 7. Phenotypic assessment of the PPV-D/R hybrids in GF305 peach. Pictures show GFP expression (upper row) and symptoms (lower row) in leaves of GF305 peach seedlings at 35 d.p.i. A schematic representation of each virus genome is shown above the pictures and an estimate of the strength of the symptoms is indicated below. Regions of PPV-Dc and PPV-NK GFP genomes are represented as black and white boxes, respectively. The green box represents the GFP insert. The infectivity of each virus (number of infected plants/number of inoculated plants) and a description of the symptoms induced are indicated in the table. The graph shows the virus accumulation in young leaves of infected GF305 peach seedlings (35 d.p.i.) determined by DASI-ELISA. Each bar represents the average value and the standard deviation from five different plants.

In spite of the fact that SD GFP caused very mild chlorotic symptoms in *N. clevelandii* and BD GFP infection is asymptomatic in this host, the accumulation of the progenies of both chimeras were similar, suggesting that genetic determinants for virus accumulation and symptom induction are not exactly overlapping. Accumulation levels of SBD GFP and NK GFP progenies were significantly higher than those of the BD GFP and SD GFP progenies (Fig. 6). The differences in virus accumulation observed by DASI-ELISA were also confirmed by Western-blot analysis (data not shown). The strong chlorotic mottling and high viral accumulation detected in the plants inoculated with SBD GFP showed that the PPV-R region comprising nucleotides 1-3628 (including the sequences coding for P1, HCPro, P3 and part of 6K1) contains important determinants for viral replication and symptom induction in *N. clevelandii*.

The behavior of the different PPV chimeras in GF305 peach mirrored that observed in *N. clevelandii*, the better a chimera infected the herbaceous plant the worse it infected the woody host. Both BD GFP and SD GFP were able to initiate a symptomatic infection in GF305 peach seedlings, but, while BD GFP caused severe symptoms indistinguishable from those of Dc, symptoms of SD GFP-infected trees were milder compared to those of the trees infected with Dc and BD GFP (Fig 7). Moreover, fluorescence derived from GFP expression was stronger and more widespread in BD GFP- than in SD GFP-infected plants (Fig. 7). SBD GFP, which was highly infectious in *N. clevelandii*, was unable to induce symptoms in GF305 peach seedlings (Fig. 7). However, while virus-derived GFP expression was observed in none of the peach seedlings inoculated with NK GFP (data not shown), very faint and extremely erratic fluorescence blotches were detected in the few peach trees infected with SBD GFP (Fig. 7). Virus accumulation assessed by ELISA positively correlated with symptom severity and GFP fluorescence intensity. Accumulation differences between Dc- and BD GFP-infected trees were not statistically significant, however SD GFP accumulation was significantly lower (Fig. 7). Virus accumulation in the rare fluorescence blotches of SBD GFP-infected seedlings was extremely low (Fig. 7).

To further map the genetic determinants on PPV genome conferring the capacity to infect peach, two additional chimeras, pICPPV-5'BsD GFP (BsD GFP, Dc nucleotides 1-2038) and pICPPV-BsSD GFP (BsSD GFP, Dc nucleotides 2039-3628), were constructed by subdividing the PPV-D region included in the SD chimera (Fig. 5B). *N. clevelandii* and GF305 peach plants were biolistically inoculated with these constructs and symptom development and GFP expression were monitored. GFP expression was observed at 21 d.p.i. in *N. clevelandii* plants inoculated with either construct. However, while BsD GFP caused mild chlorotic mottling in *N. clevelandii* upper non-inoculated leaves, BsSD GFP infected plants showed strong systemic chlorotic mottling (Fig. 6). Accumulation of both hybrids in *N. clevelandii* correlated with symptomatology, as BsSD GFP virus titers were two times higher than those of BsD GFP (Fig. 6). Neither symptoms nor GFP were detected by 35 d.p.i. in GF305 peach seedlings inoculated with either of these chimeras (Fig. 7). Thus, it can be concluded that both PPV-D regions included in BsD GFP and BsSD GFP are required for peach infectivity, and the 5'-third of the PPV-D genome contained in SD GFP appears to be the minimal region conferring the ability to infect peach.

All together, the results obtained with the different PPV R/D chimeras point out that the determinants of PPV pathogenicity in *N. clevelandii* and *P. persicae* are not localized in a limited region of the viral genome but are extensively spread.

III.2.3 Species-specific outcompetition of less fitted PPV-R/D chimeras in mixed inoculations

In order to verify the host-specific fitness differences between PPV Dc, NK GFP and the R/D chimeras suggested by single infections, coinoculation by particle bombardment of GF305 peach seedlings and *N. clevelandii* plants with different pairs of constructs was performed. The DNA of the partners that yielded higher viral accumulation in the single infections were diluted three times with respect to the competing constructs that produced lower viral levels. Persistence of the competing viruses after 30 d.p.i. was assessed by IC-RT-PCR from young infected leaves and sequence analyses of the amplified virus cDNA fragments (Table 2).

Table 2. Output of mixed infections with PPV-D/R hybrids.

	Inoculum 3:1[a]							
	Nicotiana clevelandii				***Prunus* GF305**			
Recovered virus[b]	**BD GFP: NK GFP[c]**	**BD GFP: SD GFP[c]**	**SD GFP: SBD GFP[c]**	**SBD GFP: NK GFP[c]**	**BD GFP: Dc[c]**	**BD GFP: Dc[c]**	**SD GFP: BD GFP[c]**	**SBD GFP: SD GFP[c]**
Dc					**4/4[d]**	**4/4[d]**	—	—
BD GFP	**0/3[d]**	**4/4[d]**	—	—	—	—	**4/4[d]**	—
SD GFP	—	**4/4[d]**	**0/4[d]**	—	—	—	—	**4/4[d]**
SBD GFP	—	—	**4/4[d]**	**3/3[d]**	—	—	—	—
NK GFP	**3/3[d]**	—	—	**0/3[d]**				

[a]DNA ratio in the inocula.
[b]Recovered virus after 30 d.p.i.
[c]DNA mixtures in the inocula
[d]Number of plants infected with the indicated virus/total number of inoculated plants

The NK GFP parental virus and the SBD GFP chimera were confirmed to be the best fitted viruses in *N. clevelandii*. NK GFP and SBD GFP were the only viruses detected in all the plants coinoculated with NK GFP plus BD GFP, and SBD GFP plus SD GFP, respectively, in spite of the fact that NK GFP and SBD GFP were diluted three times in the inocula with respect to their competitors. BD GFP and SD GFP appeared to have similar fitness in *N. clevelandii*, since both viruses were maintained at 30 d.p.i. in plants coinoculated with these constructs. The fact that SD GFP was three times less concentrated in the inoculum than BD GFP could suggest a slight preference for SD GFP in *N. clevelandii*, but both chimeras still coexisted after a second infection round in plants infected with extracts of systemically infected leaves from the coinfected plants (data not shown). Further serial passages might be necessary to reveal minor fitness differences between BD GFP and SD GFP, if there is any, in this host. In agreement with the similar levels of virus accumulation

observed in plants singly infected with SBD GFP and NK GFP, no evidence of preference for the parental NK GFP virus over the SBD GFP chimera was obtained from the coinoculation experiment, as SBD GFP is the virus recovered in the plants coinoculated with SBD GFP plus NK GFP (3 to 1 concentration ratio in the inoculum).

In agreement with the results of single infections, BD GFP showed higher fitness than SD GFP in the mixed infections of GF305 peach, as only BD GFP was recovered in plants coinoculated with BD GFP and SD GFP (Table 2). Similarly, only SD GFP was detected in GF305 peach coinoculated with SD GFP and SBD GFP, as it was expected from the erratic and extremely inefficient behavior of SBD GFP in this host. Interestingly, although symptoms and viral accumulation were similar in GF305 peach seedlings individually infected with Dc and BD GFP (Fig. 7), the Dc parental virus clearly outcompeted the BD GFP chimera in coinoculated plants (Table 2), demonstrating that replacement of the 3'-terminal third of the PPV D genome by the corresponding region from PPV-R caused a drop of fitness in peach.

III.2.4 Natural PPV NAT deletion selectively impairs PPV infection in peach

The ability of Dc and BD GFP to infect efficiently GF305 peach demonstrates that the 3' terminal region of both PPV-R and PPV-D genomes are functional for virus replication and spread in this host (Fig. 7). However, the possibility exists that this genomic region could contain specific determinants for *Prunus* infection that were conserved in both isolates. In this respect, the sequence coding for the N-terminus of the CP is one of the most variable regions of the potyviral genome, and for this reason, it has been suggested that it could be involved in host range selection. Supporting this hypothesis, a deletion of 15 amino acids from this region (Fig. 8A) has been detected after transfer of PPV from woody to herbaceous hosts, by two independent laboratories (Maiss *et al*., 1989; López-Moya *et al*., 1995). The deletion is named NAT because the mutant virus is **N**on **A**phid **T**ransmissible

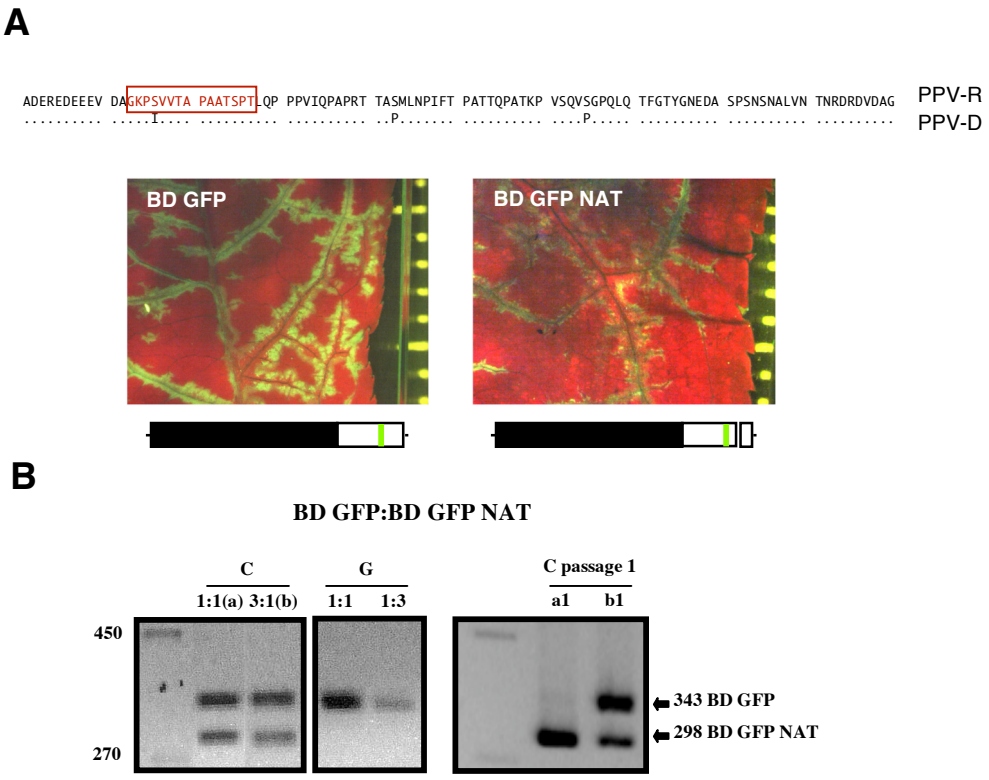


Fig. 8. Effect of NAT deletion on PPV infectivity. (A) Alignment of the CP N terminal sequences of PPV-R and PPV-D. Amino acids removed by the NAT deletion are shown in red. Pictures show GFP expression in leaves of GF305 peach seedlings infected with the indicated viruses. A schematic representation of each virus genome is shown below the pictures. Regions of PPV-Dc and PPV-NK GFP genomes are represented as black and white boxes, respectively. The green box represents the GFP insert. (B) IC-RT-PCR products amplified from pools of four *N. clevelandii* (C) or GF305 (G) plants biolistically inoculated with mixtures of BD GFP and BD GFP NAT DNA and from pools of four *N. clevelandii* plants (C passage 1, samples a1 and b1) inoculated with extracts of the pools of previously coinfected *N. clevelandii* plants (a, b). The DNA ratio of each inoculum is shown above the lanes. Expected positions of the KpnI-digested IC-RT-PCR products for BD GFP (343 nt) and BD GFP NAT (298 nt) are indicated. Standard molecular weight markers are indicated on the left side.

In order to assess the relevance of the NAT deletion for *Prunus* infection, it was introduced in BD GFP. The resulting construct pICPPV-5'BD GFP NAT (BD GFP NAT) was biolistically inoculated into *N. clevelandii* and GF305 peach plants. *N. clevelandii* plants became infected and, as expected, virus derived from BD GFP NAT displayed an asymptomatic infection similar to the BD GFP infection. Local and systemic GFP foci were similar in size and fluorescence intensity in *N. clevelandii* plants infected with either BD GFP or BD GFP NAT (data not shown). All four GF305 peach seedlings inoculated with BD GFP NAT resulted infected, although virus symptoms were delayed in two of the four infected plants and GFP expression was fainter in comparison with that of BD GFP infected trees (Fig. 8A). These results suggest that NAT deletion did not abolish but impaired PPV infection in peach. In order to further confirm this observation, plants of *N. clevelandii* and GF305 peach

were coinoculated with BD GFP and BD GFP NAT by particle bombardment. In *N. clevelandii* both viruses still coexisted after 30 d.p.i. (Fig. 8B), suggesting that NAT deletion had not a drastic effect on viral fitness in this host. Subsequent inoculation of *N. clevelandii* with sap of BD GFP:BD GFP NAT coinfected plants showed that either BD GFP NAT is selected or both viruses coexist after 30 d.p.i. (Fig. 8B). However the only virus recovered from coinoculated peach seedlings after 30 d.p.i. was BD GFP, even when it was diluted three times in the inoculum with respect to BD GFP NAT (Fig. 8B), confirming that the CP sequences lacking in the NAT deletion are somewhat relevant for PPV infection of *Prunus* hosts.

III.2.5 A limited number of nucleotide changes are associated to partial adaptation of the PPV-BD GFP chimera to N. clevelandii

To gain insight into virus factors involved in PPV adaptation to *N. clevelandii,* evolution was forced by serial passages of the BD GFP progeny virus in this host. In a first series of passages, no symptoms were detected in the BD GFP-infected plants during the first rounds. However, a chlorotic mottling similar to that of the control NK GFP-infected plants, but fainter, was observed in the BD GFP-infected plants by the fifth and ensuing passages (symptoms of a plant infected at passage eight, BD GFP A8, are shown in Fig. 9A).

Moreover, GFP fluorescence was stronger and virus accumulation was significantly higher in those plants than in plants directly inoculated with BD GFP progeny virus, although still was much lower than that of NK GFP-infected plants (Fig. 9). The complete genome sequence of BD GFP-derived virus from plants infected at passage six (BD GFP A6) was determined by direct sequencing of virus-derived cDNA fragments amplified from the infected tissue by IC-RT-PCR. BD GFP A6 differed from the original BD GFP by 5 nucleotide substitutions, corresponding to 3 amino acid changes, which were confirmed by sequencing of additional IC-RT-PCR amplification products. All the mutations found in BD GFP A6 were absent in plants directly inoculated with BD GFP and were maintained after two additional passages (BD GFP A8). The changes were located at nucleotide positions 106 (5´ NCR), 1044 (K300Q in P1), 2772 (D876N in P3), 3644 (H1166Q in the 6K1-CI protease recognition site) and 9206 (silent in CP) (Fig. 10B). Interestingly, the 3 amino acid changes affected residues which were conserved in Dc and the original PPV-Dideron isolate, which are not able to infect *N. clevelandii*, but were different in the PPV-R (NK GFP) isolate, which is well adapted to this host (Fig. 10B). Furthermore, the mutation at 6K1 (H1166Q) introduced the same amino acid present in PPV-R.

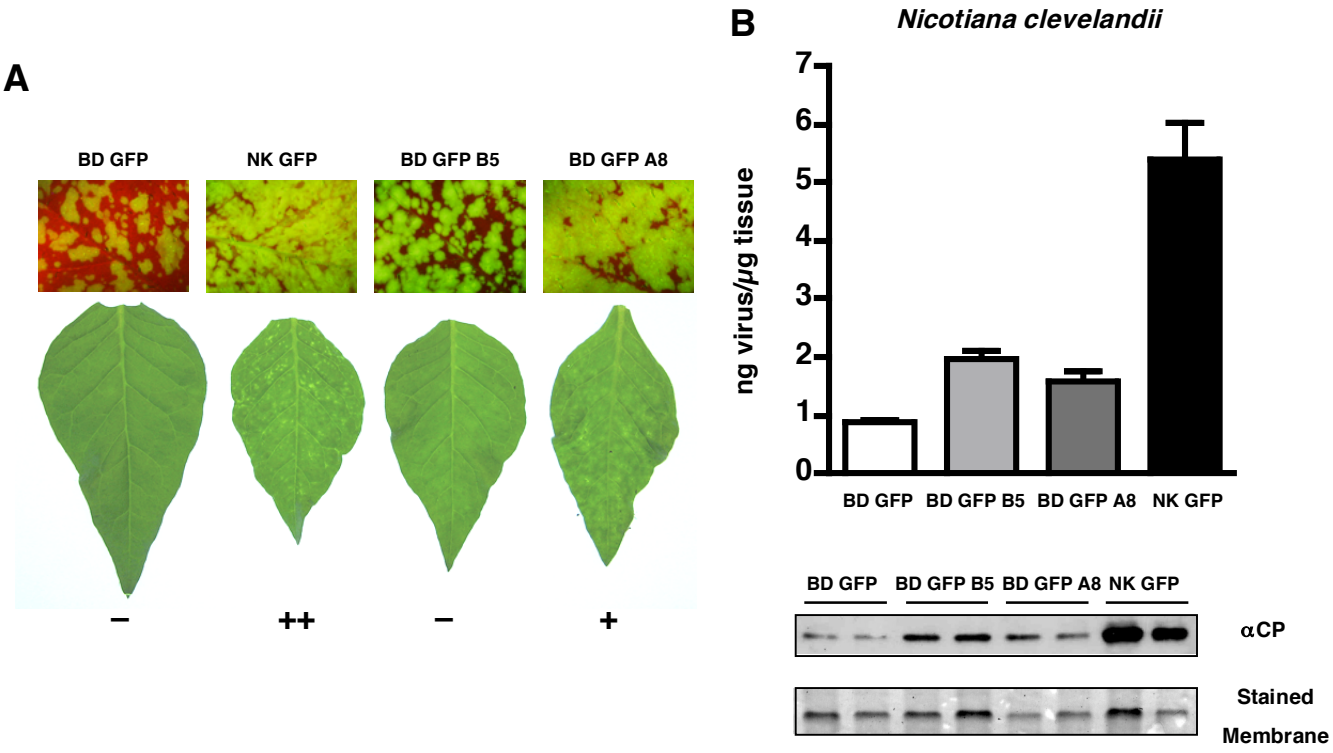
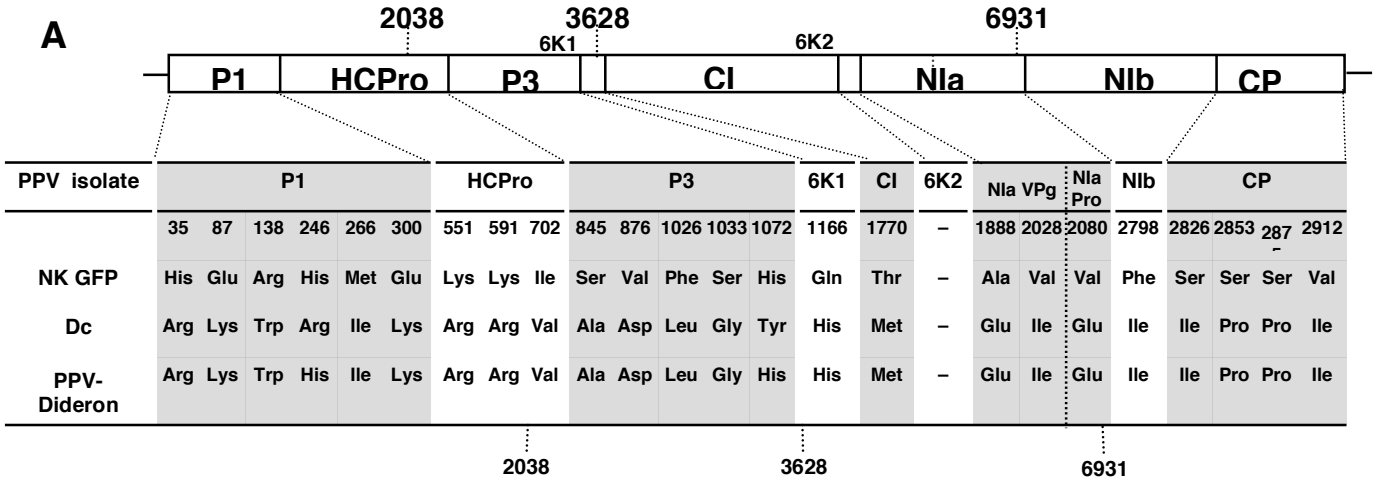


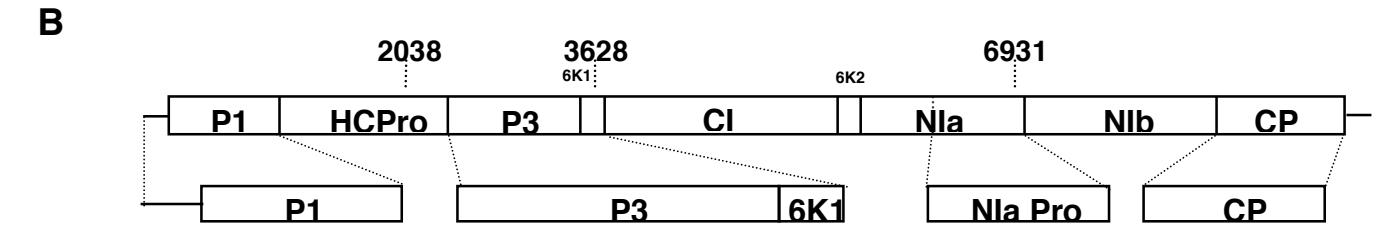
Fig. 9. Phenotypic assessment of BD GFP derivatives partially adapted to *N. clevelandii*. (A) Pictures show GFP expression (upper row) and symptoms lower row) in young leaves of *N. clevelandii* plants at 21 d.p.i. An estimate of the strength of the symptoms is indicated below. (B) Accumulation of each virus in young leaves of infected *N. clevelandii* plants at 15 d.p.i. assessed by DASI-ELISA. Each bar represents the average value and the standard deviation from four different plants. Western-blot analysis of infected plant extracts is shown below the graph. Each lane corresponds to a pool of young leaves of two *N. clevelandii* plants infected with the virus indicated above collected at at 15 d.p.i. Poinceau red staining of the blot is shown at the bottom as a loading control.

In order to assess the specificity of the mutations introduced during the adaptation process, a second series of BD GFP passages was conducted in *N. clevelandii*. Noticeable disease symptoms were not observed after seven passages (a leaf from an infected plant at passage five, BD GFP B5, is shown in Fig. 9A). However, GFP fluorescence was stronger and virus accumulation was higher in BD GFP B5 infected plants than in plants directly inoculated with BD GFP progeny virus, and similar to those of BD GFP A8 (Fig. 10). Sequence analysis of BD GFP B5 detected two mutations, one involving nucleotide 7014 (Y2290H in NIaPro), and the other at nucleotide 2773 in the P3 coding sequence, affecting the same amino acid (D876 in P3) that was concerned by mutation at nucleotide 2772 in BD GFP A6 (Fig. 10B). Interestingly, in BD GFP B5 D876 was replaced by Val, the same residue present at this position in PPV-R (NK GFP).



PPV isolate	P1						HCPro			P3					6K1	Cl	6K2	Nla VPg		Nla Pro	Nlb	CP			
	35	87	138	246	266	300	551	591	702	845	876	1026	1033	1072	1166	1770	–	1888	2028	2080	2798	2826	2853	287 – 2912
NK GFP	His	Glu	Arg	His	Met	Glu	Lys	Lys	Ile	Ser	Val	Phe	Ser	His	Gln	Thr	–	Ala	Val	Val	Phe	Ser	Ser	Ser	Val
Dc	Arg	Lys	Trp	Arg	Ile	Lys	Arg	Arg	Val	Ala	Asp	Leu	Gly	Tyr	His	Met	–	Glu	Ile	Glu	Ile	Ile	Pro	Pro	Ile
PPV-Dideron	Arg	Lys	Trp	His	Ile	Lys	Arg	Arg	Val	Ala	Asp	Leu	Gly	His	His	Met	–	Glu	Ile	Glu	Ile	Ile	Pro	Pro	Ile

2038 3628 6931



Virus	106	1044 (300)	2772 (876) 2773	3644 (1166)	7014 (2290)	9206 (3019)
NK GFP	A	G (Glu)	G (Val) T	G (Gln)	T (Tyr)	A (Pro)
BD GFP	A	A (Lys)	G (Asp) A	T (His)	T (Tyr)	A (Pro)
BD GFP A6	G	C (Gln)	A (Asn) A	G (Gln)	T (Tyr)	G (Pro)
BD GFP B5	A	A (Lys)	G (Val) T	T (His)	C (His)	A (Pro)

Fig. 10. Amino acid heterogeneities in different PPV variants**.** (A) Amino acid differences between PPV-Dc and PPV-NK GFP. The amino acids in PPV-Dideron at the divergent positions are also shown. A schematic representation of PPV genome is depicted above the table. (B) Mutations accumulated in the adapted BD GFP variants obtained by serial passages in *N. clevelandii* plants are shown in the table. A schematic representation of PPV genome is depicted above the table. The regions containing changes are shown above the affected nucleotide positions and the corresponding amino acid residues are indicated in brackets. In both panels, vertical dotted lines indicate the nucleotide position of the restriction sites used for the construction of hybrid viruses between PPV-Dc and PPV-NK GFP.

III.3 Host-specific effect of P1 exchange between two potyviruses

Plum pox virus (PPV) and *Tobacco vein mottling virus* (TVMV) belong to the genus *Potyvirus* in the family *Potyviridae*. The host range of both viruses includes several herbaceous species of the *Nicotiana* genus such as *N. clevelandii* and *N. benthamiana,* however natural hosts of PPV are stone fruit trees of *Prunus* species, which are not included in the host range of TVMV (Pirone and Shaw, 1988; Glasa and Candresse, 2005). In addition, whereas TVMV infects systemically *N. tabacum*, PPV only is able to establish a local infection in this host (Sáenz *et al.*, 2002). Previous studies have attributed possible roles in host range definition for several potyviral proteins. For instance, the restriction of PPV systemic spread in *N. tabacum* was overcame in transgenic tobacco plants expressing HCPro from the tobacco-infecting potyvirus TEV (Sáenz *et al.*, 2002) and a determinant of the ability of TuMV to infect Brassica spp. and/or *Raphanus sativus* has been shown to be in its P3 protein (Suehiro *et al.*, 2004).

As mentioned earlier, P1 is the most variable protein among potyviruses both in sequence and in length (Adams *et al.*, 2005b) and P1 diversification has been suggested to contribute to succesful adaptation of potyviruses to a wide range of host species (Valli *et al.*, 2007). However a direct implication of P1 protein in host range definition has not been demonstrated so far.

Highly coordinated functions involving different viral proteins, non-translated and, possibly, other cis-acting RNA sequences are required for a successful virus infection cycle. The complex network of potyviral protein-protein interactions (Daròs *et al.*, 1999; Merits *et al.*, 1999; Choi *et al.*, 2000; Guo *et al.*, 2001; Kang *et al.*, 2004) makes coevolution a possible constrain for the functionality of heterologous potyviral proteins in divergent viral backgrounds. However, successful interspecies cistron exchanges have been engineered rendering fully infectious viruses within the family *Potyviridae* (Varrelmann *et al.*, 2000b; Tobias *et al.*, 2001; Ullah *et al.*, 2003; Stenger and French, 2004).

Our aim was to investigate the role of the potyviral P1 protein in host range specificity. To address this question regions of the PPV P1 cistron have been replaced with the corresponding regions of TVMV genome and the infectivity of the hybrid viruses has been assessed in common hosts for both viruses and in PPV exclusive hosts.

III.3.1 PPV/TVMV chimeric viruses carrying partial or complete TVMV P1 coding sequence are infectious in herbaceous hosts

P1 is, together with the N-terminus of CP, the most variable potyviral protein. TVMV P1 has 34 amino acids less than PPV P1, and they share 24,1% identity. The length difference derives mainly from large gaps at the N-terminal region of TVMV P1, and the most conserved region is the C-terminal serine proteinase domain, although conserved motifs are also detected at the N-terminus (FGSFT) and in an internal region ($AKAx_4VEx_1Ix_2KRV$) (Fig. 11B and Valli *et al*., 2007). In the first series of chimeric clones that we engineered, the P1 coding sequence of PPV-R isolate (pGPPV) was partially (5'-terminal 183 codons, pGPPVTmP1, RTm), or totally (pGPPVTcP1, RTc), replaced by that of TVMV (Fig. 11A). The exchanged PPV P1 sequence starts at a NcoI site engineered around the second AUG of the large viral ORF, which has been shown to be the codon used for translation initiation in PPV infection (Riechmann *et al*., 1991); in addition, the first AUG of the ORF was distorted in the chimeric viruses. Regardless of the high divergence between the interchanged sequences, *in vitro* synthesized transcripts of the hybrid clones were infectious in *N. clevelandii* plants (Fig. 12). Only minor symptom differences were observed between PPV-R and the chimeras, both in *N. clevelandii* and *N. benthamiana*, where symptoms induced by RTc hybrid were somewhat more severe than those of PPV-R and RTm (Fig. 12). ELISA analysis at 15 d.p.i. showed that both hybrid viruses appeared to accumulate to slightly lower levels than PPV-R in *N. clevelandii* and *N. benthamiana* leaves (Fig. 12). RTm and RTc caused necrotic lesions indistinguishable from those of PPV-R in *C. foetidum*, a local host of PPV, which is not susceptible to TVMV infection. Another plant that displays different responses against PPV and TVMV is *N. tabacum*. TVMV infects systemically tobacco, but PPV only is able to establish a local infection in this host (Sáenz *et al*., 2002). Interestingly, RTm and RTc resembled wild type PPV-R in being unable to infect systemically tobacco. The three viruses caused similar ring-shaped symptoms (not shown) and accumulated to comparable levels (Fig. 12) in the inoculated leaves of this plant. All together these results show that the presence of TVMV P1 sequences does not drastically affect the herbaceous host range of PPV, although it has minor effects on symptoms and viral accumulation.

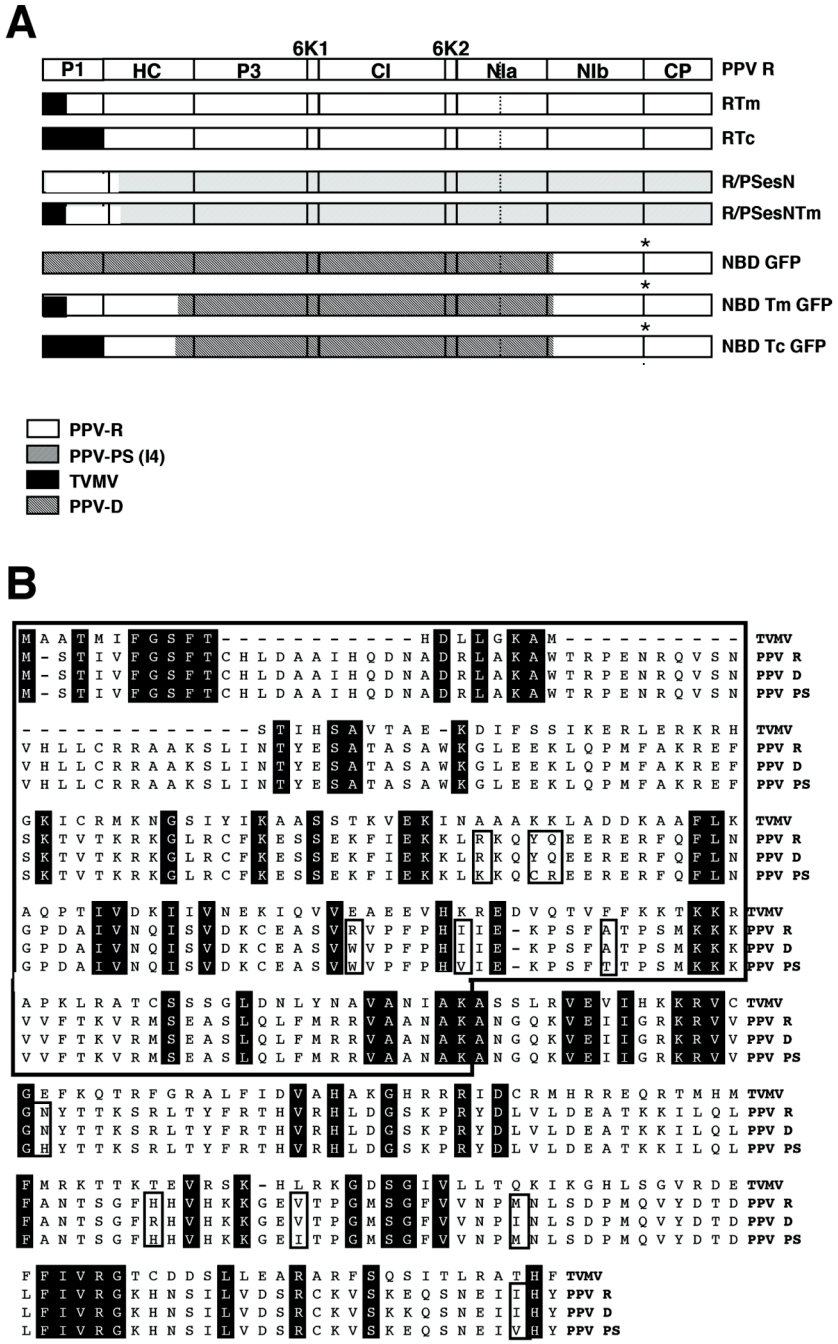


Fig. 11. Schematic representation of the different virus constructs used in this work (A) and sequence alignment of PPV and TVMV P1 proteins (B). The pattern assigned to each parental virus is depicted below the constructs. GFP sequence is indicated with an asterisk. Conserved amino acid positions in the sequence alignment are shadowed in black. Open boxes indicate amino acid differences between the PPV isolates.

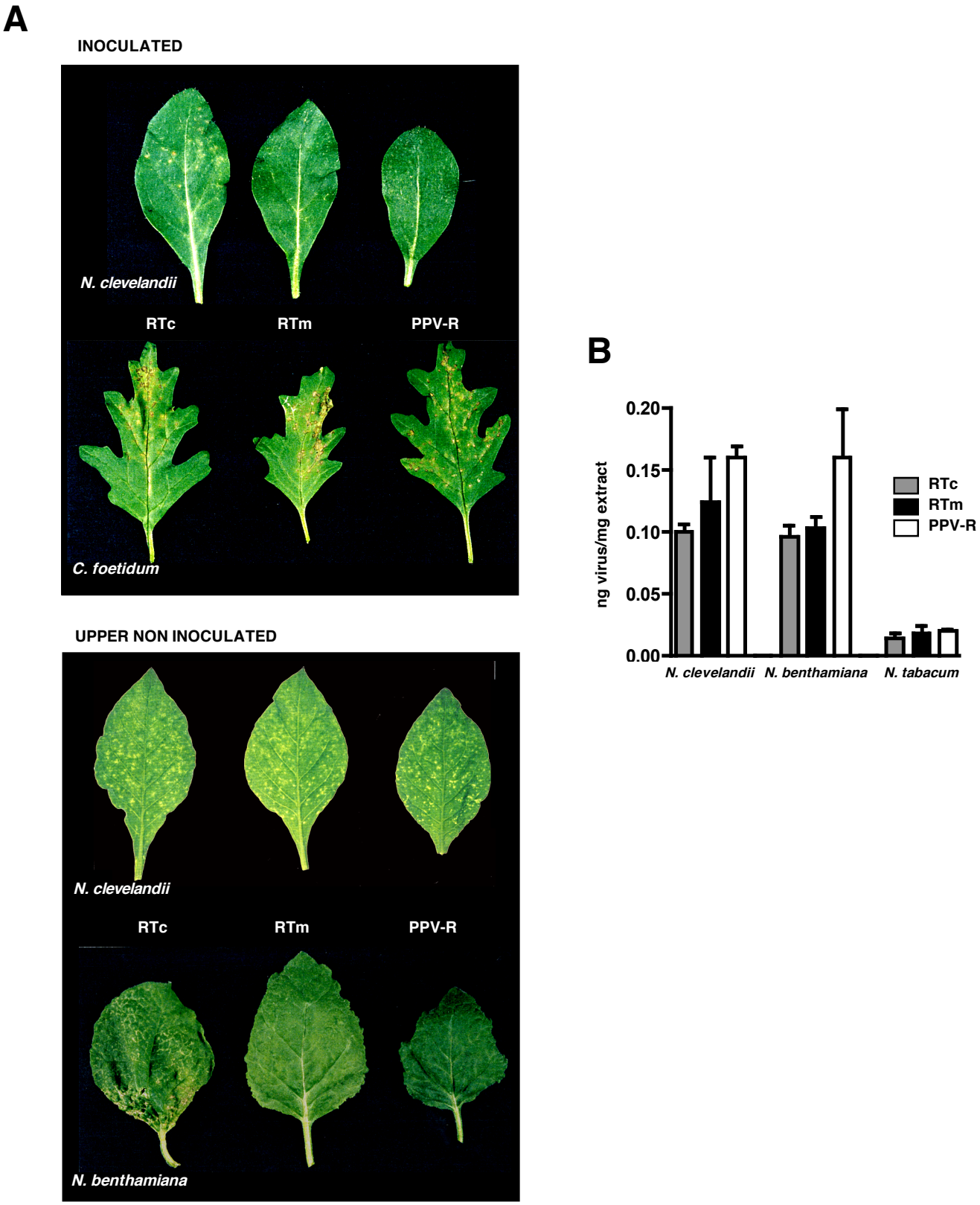


Fig. 12. Analysis of the infection of PPV-R/TVMV chimeras in different herbaceous hosts. (A) Symptoms in inoculated leaves of *N. clevelandii* at 15 d.p.i. and *C. foetidum* at 7 d.p.i. and in upper noninoculated leaves of *N. clevelandii* and *N. benthamiana at 15 d.p.i.* (B) Virus accumulation in upper noninoculated leaves of *N. clevelandii* and *N. benthamiana* and in inoculated leaves of *N. tabacum at 15 d.p.i.estimated by ELISA.* Bars represent the average values and standar deviations of 8, 6 and 2 different plants of *N. clevelandii, N. benthamiana* and *N. tabacum* respectively.

III.3.2 TVMV P1 sequence impairs PPV ability to infect peach trees

RTm and RTc chimeras are not useful to assess the effect of P1 substitution in the natural woody hosts of PPV because they derive from PPV-R, a PPV isolate that has lost the ability to infect *Prunus* species after extensive propagation in herbaceous plants (Dallot *et al.*, 2001). Consequently, new PPV chimeras based on pGPPV-PSE109S232 (PSes), a full-length cDNA clone derived from the PPV-PS isolate that is infectious in both herbaceous and woody hosts (Sáenz *et al.*, 2001), were constructed. In pGPPVR/PS1334esN (R/PSesN) and pGPPVR/PS1334esNP1Tm (R/PSesNTm), the first 1334 nucleotides of pGPPVPSE109S232 were replaced by the corresponding sequences of PPV-R and RTm, respectively (Fig. 11A). This fragment includes, in addition to the P1 coding sequence, the sequence coding for the the first 88 amino acids of HCPro, but there are only 4 nucleotide differences, none of them causing an amino acid change, between PPV-R and PPV PS in this HCPro region. *In vitro* synthesized transcripts of both chimeras were infectious in common PPV and TVMV hosts (*N. clevelandii* and *N. benthamiana*) (data not shown). GF305 peach seedlings were manually inoculated with extract from *N. benthamiana* plants infected with RTm, RTc, R/PSesNTm, R/PSesN or PSes. The presence of virus was analyzed by ELISA at approximately 30 d.p.i. in inoculated leaves and 60 d.p.i. in upper noninoculated leaves in at least two different experiments (data summarized in table 3). Most of the PSes-inoculated trees were systemically infected, as shown by faint mottling symptoms and virus accumulation in upper noninoculated leaves. As expected, no tree was infected with the PPV/TVMV hybrids RTm and RTc as PPV-R is not infectious in this host. No symptoms were observed in R/PSesN-inoculated GF305 seedlings. However, ELISA data showed that at least almost 40% of the seedlings were infected with this hybrid, although levels of virus accumulation were considerably lower than in the PSes-infected plants (data not shown). This indicates that the frgment of PPV-R (unable to infect peach) present in R/PSesN reduces but not abolishes viral infectivity in peach. However, when the coding sequence of the N-terminal region of TVMV P1 was included in the chimera (R/PSesNTm, Fig. 11), the infectivity was completely abolished, pointing out that although this TVMV region is fully functional for infection of herbaceous hosts, it is unable to support PPV infection in a natural woody host.

Manual inoculation of GF305 trees is a method with low efficiency of infection, for that reason we decided to use biolistic delivery of viral cDNA to be transcribed *in planta*, which is a more efficient inoculation method, to further asses the effect of P1 exchange in *Prunus* infectivity. New PPV/TVMV chimeras were constructed from pICPPVN5´BD GFP (NBD

GFP), a derivative of pICPPV5´BD GFP (described in section II) that includes the same NcoI site and mutations of the first two AUGs present in chimeric viruses used before. NBD GFP expresses a hybrid full-length cDNA made up of sequences of PPV-R and PPV-D isolates under the control of the CaMV 35S promoter (Fig. 11A), and is highly infectious in GF305 peach (Fig. 13B). NBD-GFP is also infectious in *N. clevelandii* although causes no symptoms in this herbaceous host (Fig. 13A). pICPPVN5´BD GFP Tm (NBD Tm GFP) and pICPPVN5´BD GFP Tc (NBD Tc GFP) are the result of replacing nucleotides 146 to 2276 (coding sequence of P1 and the first 402 amino acids of HCPro) of NBD GFP with the corresponding region of RTm and RTc, respectively (Fig. 11A). Micro gold particles coated with DNA of the different chimeras were prepared for biolistic inoculation and three different inoculation experiments were performed using both GF305 peach and *N. clevelandii* plants. GFP expression could be observed in 100% of inoculated *N. clevelandii* plants, although fluorescence intensity was higher in plants infected with NBD Tm GFP and, specially, NBD Tc GFP compared to the control NBD GFP (Fig. 13A). ELISA analysis showed significantly higher viral accumulation in *N. clevelandii* infected leaves of both PPV/TVMV chimeras with respect to NBD GFP, and NBD Tc GFP accumulated to higher titers than NBD Tm GFP (Fig. 13B). The higher GFP expression and virus accumulation correlated with the appearance of symptoms in both NBD Tm GFP and NBD Tc GFP infected plants, which showed extensive mild or strong chlorotic mottling in upper noninoculated leaves, respectively (Fig. 13). These data indicate that, in the case of the NBD GFP-derived chimeras, increase of the extent of TVMV P1 sequence enhances virulence in *N. clevelandi*.

Table 3. Infectivity of PPV/TVMV hybrids in GF305 trees.

Virus [a]	Inoculated leaves[b]	Upper noninoculated leaves[b]
PSes	45 / 48	47 / 48
RTm	0 / 24	0 / 24
RTc	0 / 24	0 / 24
R/PSesN	9/ 24	11 / 24
R/PSesNTm	0 / 48	0 / 48

[a] Inoculated virus
[b] Number of PPV infected trees determined by ELISA/total number of inoculated trees.

In contrast, whereas 100% of the sixteen GF305 peach seedlings inoculated with NBD GFP showed GFP expression and strong vein clearing and distortion (Fig. 13), none of the twenty two GF305 plants shot with the NBD Tm GFP or NBD Tc GFP chimeras carrying part or the complete TVMV P1 sequence, showed symptoms or GFP expression. ELISA analysis confirmed virus accumulation in the NBD GFP-inoculated peach seedlings and its absence in those inoculated with NBD GFP Tc or NBD GFP Tm (Fig. 13B). These results further support the conclusion that TVMV P1 is not functional in the PPV-specific host *P. persicae*.

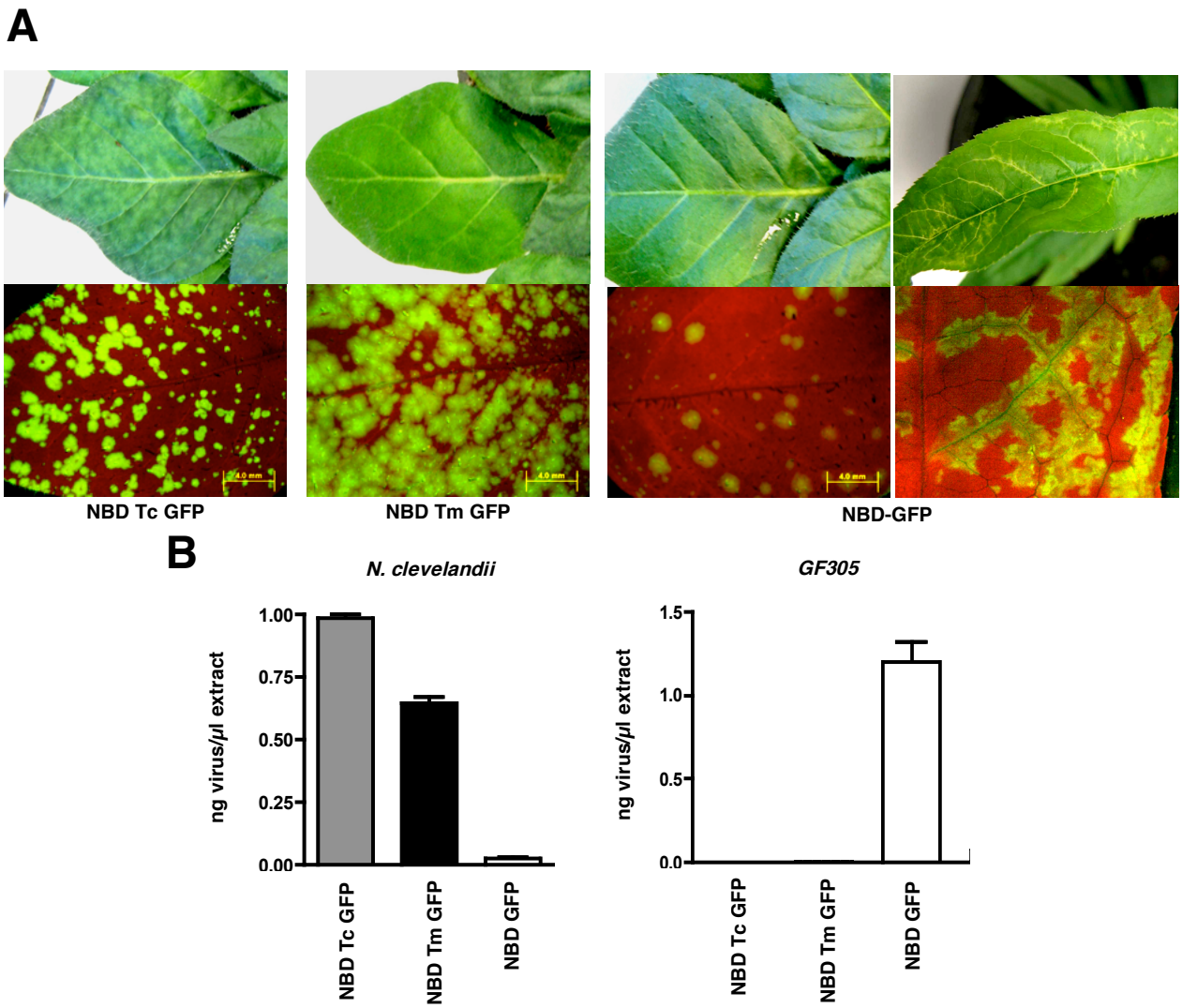


Fig. 13. Analysis of the infection of NBD GFP, NBD Tm GFP and NBD Tc GFP in *N. clevelandii* and GF305 peach. (A) Symptoms and GFP expression observed under visible or UV light, respectively at 21 d.p.i. for *N.clevelandii* and at 35 d.p.i. for GF305 peach. (B) ELISA data of virus accumulation in upper noninoculated infected leaves of *N. clevelandii* (21 d.p.i.) and GF305 peach (35 d.p.i.) plants. Each graph bar represents the average virus accumulation of 4 *N. clevelandii* and 8 peach plants, respectively.

IV. DISCUSSION

The worldwide spread of sharka disease is a stated and worrisome fact due to the high economic losses that it causes in important crops. Hence the efforts, at different levels are aimed to restrain the advance of the disease and to eradicate it from the countries where it is established. The causal agent of sharka disease, *Plum pox virus* or PPV has been the object of the study in this thesis. In addition to the scientific interest itself, knowledge of molecular biology of the virus and of its behavior in different hosts is one of the necessary approaches to desing effective strategies to fight against the disease. In particular, this work has been focused on the study of the virus determinants for pathogenicity and host range taking advantage of the phenotypic differences between PPV subisolates, between isolates from the same viral strain and between PPV and other potyviruses.

IV.1 Contribution of P1 protein to differences in pathogenicity between PPV-PS subisolates

Previously reported data indicated that single amino acid changes in HCPro between different virus variants segregated from the PPV-PS isolate caused a drastic effect on virus symptoms in herbaceous hosts and affected virus infectivity in peach seedlings (Sáenz *et al.*, 2001). The two new amino acids changes affecting the pathogenicity of PPV-PS subisolates that we identify now affect the P1 protein. Data on P1 protein functions are restricted to its proteinase activity (Verchot *et al.*, 1991; Verchot and Carrington, 1995a), its involvement in genome amplification (Verchot and Carrington, 1995b), its ability to bind single-stranded RNA (Brantley *et al.*, 1993; Soumounou *et al.*, 1994) and its capacity to enhance the silencing suppression activity of HCPro (Pruss *et al.*, 1997; Rajamäki and Valkonen, 2003; Valli *et al.*, 2006). In addition, sequence comparison analysis suggested that P1 protein could be especially relevant for host adaptation (Adams *et al.*, 2005b; Valli *et al.*, 2007). The marked difference in the effects of W29R and V139E mutations in PPV-PS pathogenicity in *Nicotiana* and *Prunus* plants supports the existence of species-specific interactions between P1 and host factors that influence the ability of the virus to infect a certain host and to determine its pathogenic properties. Although our data do not shed light on the nature of these interactions, the fact that both P1 and HCPro changes affected the pathogenicity of PPV-PS subisolates could suggest that the P1 function as an enhancer of the HCPro silencing suppression activity might be involved, directly or indirectly, in the role of P1 protein in pathogenesis and host adaptation. Nevertheless, the

involvement of P1 and HCPro in two independent mechanisms affecting virus pathogenesis cannot be ruled out.

The segregation of different virus variants from the original PPV-PS isolate propagated in *N. clevelandii*, indicates that this isolate consists of a complex virus population (Sáenz *et al.*, 2001). The very low divergence between the different PPV-PS subisolates could be in agreement with they being derived from a quasispecies distribution of the PPV-PS population. However, as it has been previously discussed, the amino acid changes observed in the PPV-PS subisolates do not appear to follow the random distribution expected for a single quasiespecies (Sáenz *et al.*, 2001). In addition, the population complexity of the original PPV-PS isolate was not regained after further propagation of the segregated subisolates in *N. clevelandii*, which also argues against the possibility that all PPV-PS subisolates derive from an original single quasispecies. Three different phenotypes were observed in the segregated PPV-PS subisolates: mild, severe and highly severe (Sáenz *et al.*, 2001). The complete genome sequencing of the highly severe subisolate 10_7 revealed a divergence between this subisolate and the severe subisolate 4.1.4 similar to that found between 4.1.4 and the mild subisolate 1.3.1 (10 nucleotide differences in each case) (Beatriz Salvador, Juan Antonio García and Carmen Simón-Mateo, unpublished results). Sequence divergence between 10_7 and 1.3.1 was a bit larger (18 nucleotide changes), in agreement with the higher symptom differences observed between these two isolates. Comparisons of the complete genome sequences of subisolates 1.3.1 and 4.1.4 with the partial ones of subisolates 2.1.1 (mild) and 5.1.3 (severe) showed that, the sequences of subisolates with the same phenotype were almost, although not completely identical, suggesting that they could belong to mutant clouds of single quasiespecies.

The presence of virus variants expressing R29E139 P1 proteins contrast with the absolute conservation of amino acids W29 and V139 in P1 proteins from 24 isolates from 6 different PPV strains (data not shown), which suggests the existence of a strong selective pressure to preserve the W29V139 sequence. We do not know whether the R29E139 PPV variant was already present in the naturally infected peach or it was selected after transfer to the herbaceous host. Although P1 protein has been shown to be active in trans (Verchot and Carrington, 1995b) it is difficult to explain how R29E139 PPV, which is unable to infect peach by itself and would be dependent on a putative helper P1 activity, could survive in nature unless this mutant contribute somehow to the fitness of the global PPV-PS population. A similar collaborative effect of R29E139 P1

would be necessary to explain the selection of mild subisolates such as 1.3.1 and 2.1.1 after transfer of PPV-PS to *N. clevelandii*, since the infection efficiency of the segregated mild subisolates in this host does not appear to be higher than that of subisolates carrying the typical W29V139 P1 sequence. In summary, our results supports a relevant role of potyviral P1 protein in host-specific pathogenicity and points out that further research is needed to unravel not only the molecular basis of P1 function but also the relationships governing complex populations of virus variants differing in P1 sequence.

IV.2 Genetic determinants of host-specific differences in pathogenicity between two isolates of the same PPV strain

Although many plant viruses have very restricted host ranges, others are able to infect a large variety of plant species. This is the case of PPV, which, in addition to infect several woody hosts from the genus *Prunus* (van Oosten, 1975), it has shown the capacity to infect different herbaceous hosts (van Oosten, 1970). However, some PPV isolates have lost the ability to infect their natural woody hosts after extensive propagation in herbaceous plants, and the molecular basis of this deadaptation is poorly understood (Dallot *et al.*, 2001).

Here, we show that an infectious cDNA clone derived from the PPV-R isolate (NK GFP), which is unable to infect systemically GF305 peach, differs from the infectious clone derived from PPV-D (Dc), which infects very efficiently GF305 peach, by only 68 nucleotide changes translated into 24 amino acid substitutions (Fig. 5A, 9A). Interestingly, Dc is also at odds with NK GFP over infectivity on *N. clevelandii*, since this plant is not susceptible to Dc (Fig. 6). NK GFP/Dc chimeras were infectious in at least one of the two hosts tested, *N. clevelandii* and GF305 peach, but gain of performance in one host is always accompanied by lost of fitness in the other host (Fig. 6, 7 and Table 2).

The replacement of the 3' terminal region of the genome, including the coding sequences for the last 30 amino acids of NIa and the complete NIb and CP proteins and the 3′ NCR, of Dc clone by that of NK GFP provided the resulting hybrid virus, BD GFP, with the ability to infect *N. clevelandii* plants, although with low efficiency. There is one nucleotide difference between Dc and BD GFP in the 3' NCR. In addition, there are 11 nucleotide differences in the NIb coding sequence, however all but one are silent and the A8538T change causes a quite conservative I2798F substitution (Fig. 5 and Fig.

9A). In contrast, four out of the five nucleotide changes of the CP coding sequence give rise to amino acid substitutions, and three of them are in the highly variable N-terminus of the protein (Fig. 5 and Fig. 9A). Interestingly, the I2826S heterogeneity lays on the region affected by the natural NAT deletion, which has been found to be associated with PPV transfer from woody to herbaceous hosts (Maiss *et al.*, 1989; López-Moya *et al.*, 1995), and BD GFP NAT was able to prevail over BD GFP in mixed infection *in N. clevelandii.* It is tempting to speculate that a host-specific defect of plant-virus interactions involving the N-terminus of CP might be the cause of the inability of Dc to infect *N. clevelandii*, but further research is required to verify this hypothesis. Both Dc and BD GFP are able to establish efficient infection in GF305 peach, however the competition data clearly showed that Dc has a higher fitness than BD GFP in this host (Table 2). This indicates that the 3' terminal region of the PPV genome also contains specific determinants for PPV infection in *Prunus*. The fact that BD GFP NAT appeared to infect GF305 peach seedlings less efficiently than BD GFP and was outcompeted by BD GFP in a mixed infection further supports the hypothesis that some of these host-specific pathogenicity determinants lie on the CP N-terminus.

BD GFP and SD GFP differ in 21 nucleotides, and this difference is translated into one amino acid substitution in 6K1, CI and NIaPro proteins and two amino acid substitutions in NIaVPg (Fig. 5 and Fig. 9A). These changes appear not to be very important for PPV fitness in *N. clevelandii*, since BD GFP and SD GFP accumulated to similar levels in single infections (Fig. 6) and coexisted in mixed infection (Table 2). However, the changes might affect regions relevant for symptom induction in *N. clevelandii*, since weak, but appreciable, symptoms were produced by SD GFP, in contrast with the asymptomatic infection of BD GFP. Amino acid changes at the P3-6K1 cleavage site modifying symptom severity with no detectable effects on virus accumulation have been reported previously (Riechmann *et al.*, 1995). Interestingly, H1166 in 6K1 of BD GFP was changed to Q, the same amino acid present in NK GFP and SD GFP, in the symptomatic virus BD GFP A6 derived from serial passages of BD GFP in *N. clevelandii*. In contrast, BD GFP B5 derived from an independent series of passages, which reached similar accumulation levels than those of the virus derived from the first series but did not cause symptoms, conserved H1166. These results suggest that the amino acid present at position 1166 in 6K1 could be relevant for symptom induction in *N. clevelandii*, although a Q at this position is not the essential for PPV causing symptoms in this host, since SBD GFP, whose nucleotides 3628-6931

derive from Dc and which has H1166, caused severe symptoms similar to those of NK GFP (Fig. 6).

Sequence changes between PPV-R and PPV-D in 6K1-CI-6K2-NIa region appears to be more relevant for adaptation to the *Prunus* host, since, although both BD GFP and SD GFP were able to infect GF305 peach, virus accumulation was lower in plants infected with SD GFP than in those infected with BD GFP (Fig. 7), and BD GFP completely outcompeted SD GFP in competition experiments (Table 2). However, main species-specific determinants for *Prunus* adaptation lie in the 5' terminal third of the PPV genome, since SBD GFP, which contains the first 3628 nucleotides from the PPV-R genome only showed very rare and isolated infection foci in GF305 peach (Fig. 7). This region contains, in addition to the 5'NCR, the coding regions for P1, HCPro, P3 and part of 6K1, and shows 30 nucleotide heterogeneities between Dc and NK GFP, which are translated in six, three and five amino acid changes in P1, HCPro and P3, respectively (Fig. 5 and Fig. 9A). Evidence connecting these proteins with virus pathogenicity has been previously reported. HCPro is a multifunctional protein involved in aphid transmission and in suppression of RNA silencing (Syller, 2005), and it has been shown to contain specific symptom determinants (Gal-On *et al.*, 2000) and to be involved in the host-specific defect of PPV for systemic infection in *N. tabacum* (Sáenz *et al.*, 2002).

Together with the N-terminus of CP, P1 and P3 are the most variable potyviral proteins, which suggest that they could be involved in virus-host interactions. This suggestion is supported in the case of P1, as previously said, by the identification of recombination events affecting its coding sequence that appeared to be linked to host adaptation (Valli *et al.*, 2007). Little is known about the function of the potyviral P3 protein. P3 is required for genome amplification (Klein *et al.*, 1994). In addition, this protein is the avirulent factor for some resistance genes (Johansen *et al.*, 2001; Jenner *et al.*, 2003; Hajimorad *et al.*, 2006) and a pathogenicity determinant relevant for symptom severity (Sáenz *et al.*, 2002) and host range definition in *Prunus* (Dallot *et al.*, 2001) and other plants (Suehiro *et al.*, 2004; Tan *et al.*, 2005). Interestingly, an RNA element in the P3 coding sequence has also shown to be important for virus replication and movement (Choi *et al.*, 2005). The fact that neither BsD GFP nor BsSD GFP were able to infect GF305 peach seedlings (Fig. 7) demonstrates that specific determinants for *Prunus* infection exist at both sides of the 5'NCR-P1-HCPro-P3 region, further illustrating the complexity of virus-plant interactions that define host range in PPV.

Replacement of the Dc-derived first 3628 nucleotides of BD GFP by the corresponding ones from NK GFP, resulting in SBD GFP, caused a drastic increase in virus accumulation in *N. clevelandii* (Fig. 6), which contrasts with the lost of infectivity in GF305 peach (Fig. 7). This demonstrates that the 5'NCR-P1-HCPro-P3 region of PPV also contains specific determinants for *N. clevelandii* infection, and corroborates that the 6K1-CI-6K2-NIa region of Dc allows an efficient infection not only of *Prunus* but also of *N. clevelandii*.

The comparison of the infection of *N. clevelandii* caused by NK GFP, SD GFP, BsD GFP and BsSD GFP clearly shows that specific determinants for PPV infection in this host also lie at both ends of the 5'NCR-P1-HCPro-P3 region, since accumulation of neither BsD GFP nor BsSD GFP did reach the levels of NK GFP (Fig. 6). The fact that the symptoms of the BsSD GFP-infected plants were more severe than those of the plants infected with SD GFP or BsD GFP highlights the relevance of the P1 and/or N-terminus of HCPro in symptom induction (Fig. 6).

In support of the influence of P3 in host adaptation, a non-conservative mutation D876V in this protein was fixed in virus population derived from a series of passages of BD GFP in *N. clevelandii* (BD GFP B5) (Fig. 9). The evolved virus accumulated in this host at levels significantly higher than those of the original BD GFP (Fig. 10). Interestingly, Val was the amino acid present at this position in the *N. clevelandii*-adapted PPV-R isolate (Fig. 9), and another mutation at this position, D876N was also fixed in an independent series of passages of BD GFP in *N. clevelandii* (BD GFP A6) (Fig. 9). All these data evidence the relevance for *N. clevelandii* adaptation of the amino acid present at position 876 in the P3 protein. A second mutation, Y2290H in NIaPro, was fixed in BD GFP B5, but since it lays in the genome region of BD GFP that derives from the *N. clevelandii*-adapted PPV-R isolate, probably it is not very relevant for the adaptation process. Whereas D876V and Y2290H were the only mutations observed in BD GFP B5, BD GFP A6 showed four mutations in addition to D876N. Two of these mutations were silent and affected positions with identical sequence in NK GFP and Dc, and probably are not very relevant for the adaptation, although the A106G mutation lays in a region of the 5'NCR that has been previously shown to affect PPV competitiveness in *N. clevelandii* (Simón-Buela *et al.*, 1997b). In contrast, the other two mutations caused non-conservative amino acid substitutions, K300Q (P1 protein) and H1166Q (6K1 protein), at positions displaying also sequence divergence between Dc and NK GFP. An acidic (Glu) and a basic (Lys) amino acids are present at position 300 of P1 in

NK GFP and Dc, respectively. The selection of a mutation at this position (K300Q) also supports the role in host adaptation proposed for P1. In agreement with this role are the amino acid heterogeneities in P1 affecting virus accumulation in *N. clevelandii* that we have observed in subpopulations of PPV-PS and again the host-specific effect of PPV P1 substitution by that of TVMV. Although virus derived from each series of passages showed similar accumulation levels, they differed in the ability to produce symptoms in *N. clevelandii*, being asymptomatic the infection caused by BD GFP B5 while mild symptoms were observed in the plants infected with BD GFP A8 (Fig. 10). These data confirm that there is not an absolute correlation between symptom development and virus accumulation and indicate that additional mutation(s) to that affecting position 876 (see above for discussion about H1166Q in 6K1), contribute to enhance virus replication and to facilitate the induction of symptoms in *N. clevelandii*.

Levels of viral accumulation of BD GFP A8 and BD GFP B5 were lower than those of NK GFP, which indicate that they are only partially adapted to *N. clevelandii*. No further increase in levels of virus accumulation has been observed after four additional passages in *N. clevelandii* of these viruses. This suggests that quite common and simple mutations are involved in first steps of PPV adaptation to *N. clevelandii*, but thorough adaptation requires the coordinated action of a number of mutations that only takes place after long, and perhaps singular, evolutionary lines.

Our results show that a number of changes that have been introduced in the genome of PPV-R during its long-term replication in *N. clevelandii* have caused important drawbacks in their interactions with its natural woody hosts. However other PPV isolates have shown to be able to infect both *N. clevelandii* and *Prunus* species (Dallot *et al.*, 2001), demonstrating that PPV infection abilities in these hosts are not mutually exclusive. In agreement with this, partially adapted BD GFP A8 and BD GFP B5 are still able to infect GF305 peach, although competition experiments would be required to ascertain whether the adaptation to *N. clevelandii* has aroused a lost of fitness in the woody plant. Further study of forced evolution of BD GFP in *N. clevelandii* would unravel at what extent adaptation to a host causes unfitting to other ones, and will provide valuable insight on plant virus interactions governing virus infection.

IV.3 Host-specific effects in pathogenicity of P1 exchange between two potyviruses

Although abundant information has been gathered in recent years about the molecular biology of potyviruses in general and of PPV in particular, progress in relation to knowledge of the role of some mature proteins, such as P1 and P3, has been rather modest (reviewed by (Urcuqui-Inchima *et al.*, 2001and Salvador *et al.*, 2006)). Gene functions appear to be more conserved among plant viruses that gene sequences, as it is illustrated by the viability of chimeric viruses derived from cistron replacements between non-related viruses even from different families (for instance De Jong and Ahlquist, 1992). These chimeric viruses can help to clarify protein functions and to define specific regions that are involved in them. We have followed this approach as an additional way to explore the possible role of the potyviral P1 protein in host specificity.

Potyviral P1 was initially suggested to be a movement protein (Domier *et al.*, 1987). However, it has been demonstrated that P1 functions as an accessory factor for genome amplification and that it plays little, if any, role in virus movement (Verchot and Carrington, 1995b). The relevance of P1 for potyvirus infection is highlighted by the fact that although chimeric viruses with foreign sequences cloned between P1 and HCPro cistrons of PPV (Guo *et al.*, 1998) and TEV (Dolja *et al.*, 1993), undergo deletions in the infected plants, even affecting the N-terminal region of TEV HCPro, these deletions never removed P1 coding sequences. As previously mentioned, the high divergence of P1 sequence among different potyviruses (Adams *et al.*, 2005b; Valli *et al.*, 2007) and the observation of recombination events affecting P1 sequences that could be linked to host adaptation (Valli *et al.*, 2007) have prompted the suggestion that P1 function could depend on precise interactions with species-specific host factor(s). Also supporting this hypothesis, insertions in PVA P1 coding sequence were shown to have a host-specific effect in virus accumulation and symptom severity (Rajamäki *et al.*, 2005).

In this work we show that, in spite of the high divergence between P1 proteins of PPV and TVMV, all PPV hybrids with TVMV P1 sequences efficiently infect PPV/TVMV common herbaceous host plants (*N. benthamiana, N. clevelandii*). This indicates that structural and/or sequence determinants involved in intramolecular P1 organization and in interactions of P1 with other virus or plant factors are conserved in PPV and TVMV, allowing heterologous contacts to be functional. Interestingly, whereas substitution of sequences of the *N. clevelandii*-adapted PPV-R isolate for those of P1 TVMV (chimeric clones RTm and RTc) had little effect on virus virulence in this

plant (Fig. 12), the replacement of TVMV P1 sequences in a PPV-D-derived chimera that infects poorly herbaceous hosts, NBD GFP, appeared to contribute to the enhancement of infection efficiency in *N. clevelandii,* being more effective the substitution of the complete TVMV P1 sequence (NBD Tc GFP) than part of it (NBD Tm GFP) (Fig. 13). Thus, appropriate P1 sequences, such as those of PPV-R or TVMV are required for efficient infection of *N. clevelandii* plants.

Sequencing of the *Prunus* adapted PPV-D isolate showed that it differs in 68 nucleotides and 24 amino acids from the PPV-R isolate, which is unable to infect *Prunus* trees. Interestingly, nine of the nucleotide changes, six of them causing amino acid substitutions, laid in the P1 coding region and the analysis of the R/D chimeric clones remarked the especial relevance of the 5' third of the genome for *Prunus* infectivity. However, although the 5′ terminal 3628 nucleotides of the SBD GFP genome derive from PPV-R (Fig. 5B), this chimeric virus still was sligthly infectious in GF305 peach (Fig. 7), indicating that P1 from PPV-R is able to support *Prunus* infection. In contrast, neither NBD Tm GFP nor NBD Tc GFP were able to infect GF305 peach in our experimental conditions (Fig. 13), in spite of the high efficiency of the biolistic inoculation method. This result is in perfect agreement with the inability to infect GF305 peach of R/PsesNTm, a PPV PS/R chimera including the coding sequence for the N-terminal region of TVMV P1 (Table 3), and both results together strongly suggest that TVMV P1 is not functional in *Prunu*s trees further highlighting the relevance of potyviral P1 in host adaptation.

The fact that the RTc and RTm chimeras, in spite of having either TVMV or a hybrid TVMV/PPV P1 protein, resembled PPV rather than TVMV in causing necrotic local lesions in *C. foetidum* and an infection localized to the inoculated leaves in *N. tabacum*, demonstrates that, as it could be expected, P1 is not the only viral factor involved in host specificity. This is also in agreement with the extensive spread in the PPV genome of host adaptation factors of PPV-R and D isolates (see above).

Our results do not unravel the nature of the host-specific functions of potyviral P1. P1 protease activity catalyzes self-cleavage at the P1/HCPro junction, and this cleavage rather than P1 proteolytic activity itself is essential for viral infectivity, either helping to a correct folding of the mature proteins or facilitating their correct subcellular localization (Verchot and Carrington, 1995b). The existence of a plant cofactor required for P1 proteolytic activity was hypothesized because in vitro self-cleavage at the C-end of TEV P1 took place in a wheat germ system but not in a reticulocyte lysate (Verchot

et al., 1992). PPV and TVMV P1 proteins also are not able to cleave themselves in a rabbit reticulocyte lysate, but the PPV/TVMV chimeric P1 of RTm is active in this system (Pilar Sáenz and Juan Antonio García, unpublished results), suggesting that a mammalian protein, possibly a chaperone, can substitute the plant cofactor depending on particular features of the potyviral P1 protein. The possibility exists that specific coevolution of the plant cofactor and the P1 protein could play an important role in host adaptation of potyviruses. Moreover, some reports have suggested that P1 is able to enhance, directly or indirectly, the silencing suppression activity of HCPro (Pruss *et al.*, 1997; Kasschau *et al.*, 1998; Rajamäki *et al.*, 2005; Valli *et al.*, 2006). Compatible interactions between P1 and host factors that ensure a correct polyprotein processing and/or facilitate the P1 role in silencing suppression may be essential for overcoming antiviral defense mechanisms and thereby for host susceptibility to the virus. In the future, the identification of plant factor(s) interacting with P1 protein will be a valuable key to shed light on the molecular basis governing a plant virus infection.

V. CONCLUSIONS

V. Conclusions

1. Host-specific pathogenicity determinants are largely spread in the PPV genome.
2. Analyses of chimeras between PPV-D and PPV-R isolates showing different adaptation to *Nicotiana clevelandii* and *Prunus persicae* revealed that gain of performance in one host was always accompanied by lost of fitness in the other host. However, other PPV isolates have shown to be able to infect efficiently both hosts, demonstrating that requirements for PPV infection in herbaceous and woody hosts are not mutually exclusive.
3. Although the 3´ terminal region of the PPV-R genome is functional in both herbaceous and *Prunus* hosts, the host-specific effect of the natural NAT deletion in PPV pathogenicity in peach and *Nicotiana clevelandii* suggests that relevant host specificity determinants lie on the CP N-terminus.
4. Important determinants for host-specific pathogenicity of PPV are located in the 5´ terminal third of the genome including the 5´ NCR and the coding regions of P1, HCPro and P3-6K1.
5. Quite common and simple mutations are involved in first steps of PPV adaptation to *N. clevelandii*, but thorough adaptation appears to require the coordinated action of a number of mutations that only takes place after long, and perhaps singular, evolutionary lines.
6. In spite of their large sequence divergence, TVMV and a hybrid PPV/TVMV P1 protein are functionally equivalent to PPV P1 protein in herbaceous hosts, suggesting that sequence and structural determinants involved in the intramolecular organization of P1 protein and in interactions with other viral or host factors should be conserved in different potyvirus species, allowing functional heterologous contacts.
7. TVMV P1 appears to be functionally incompetent in peach, a plant species that is not susceptible to TVMV infection, further highlighting the relevance of potyviral P1 in host adaptation
8. PPV-PS isolate consists of a complex mixture of virus variants including mutants with changes in P1 residues strictly conserved in all sequenced PPV isolates, which cause host-specific defects on virus infectivity and pathogenicity. The significance of the maintenance in the virus population of these low-fitness mutants is presently a challenging question.

Conclusiones

1. Los determinantes de patogenicidad de PPV específicos de huésped se encuentran ampliamente distribuidos a lo largo de su genoma.
2. El estudio de quimeras derivadas de los aislados PPV-D y PPV-R, que presentan distinta adaptación a *Nicotiana clevelandii* y a *Prunus persicae*, han revelado que la mejora en la adaptación a un huésped va siempre acompañada de una perdida de eficacia biológica en el otro. Sin embargo, la existencia de otros aislados de PPV que infectan eficientemente ambos huéspedes demuestra que los requerimientos para la infección de huéspedes herbáceos y leñosos por PPV no son mutuamente excluyentes.
3. Aunque la región 3´ terminal del genoma de PPV-R es funcional tanto en huéspedes herbáceos como en *Prunus,* el efecto que hemos observado de la deleción natural NAT en la patogenicidad de PPV en melocotonero y en *Nicotiana clevelandii* sugiere que la región N-terminal de la proteína CP contiene algún elemento importante para la especificidad de huésped.
4. El tercio 5´ terminal del genoma de PPV, que incluye la región 5´ no codificante y las regiones codificantes de las proteínas P1, HCPro y P3-6K1, contiene elementos importantes para la patogenicidad viral específicos de huésped.
5. Pocas y repetitivas mutaciones están implicadas en las primeras etapas de adaptación de PPV a *Nicotiana clevelandii*, sin embargo una adaptación más completa parece requerir la acción coordinada de varias mutaciones que sólo se producirían tras largos, y quizás singulares, procesos evolutivos.
6. A pesar de la alta divergencia de sus secuencias, tanto la proteína P1 de TVMV como la proteína P1 híbrida PPV/TVMV son funcionalmente equivalentes a la proteína P1 de PPV en huéspedes herbáceos. Esto sugiere que los determinantes estructurales y de secuencia implicados en la organización intramolecular de la proteína P1 y en las interacciones con otras proteínas virales o de la planta están conservados en diferentes especies de potyvirus y permiten contactos heterólogos funcionales.
7. El hecho de que la proteína P1 de TVMV no sea funcional en melocotonero, una especie vegetal no susceptible a TVMV, de nuevo apunta a P1 como un factor relevante en la adaptación al huésped de los potyvirus.

8. El aislado PPV-PS está formado por una mezcla compleja de variantes virales que incluye mutantes con cambios en residuos muy conservados de la proteína P1, que causan defectos en la infectividad y en la patogenicidad específicos de huésped. El mecanismo de subsistencia en la población viral de estos mutantes de menor eficiencia biológica y la relevancia de su conservación para la patogenicidad viral, pueden ser objetos de investigación futura de gran interés.

VI. REFERENCES

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Education

20 July 2007 **Ph.D. degree**. National Centre of Biotechnology, Madrid, Spain.
1994-1999 **B.Sc. degree in Biology**. Autonoma University, Madrid, Spain.

Experience

2000- **Ph.D. student**, National Centre of Biotechnology, Madrid, Spain.
Investigated Plum Pox Virus pathogenicity and the host defense mechanism based on RNA silencing.

2002 **Ph.D. student**, Scottish Crop Research Institute, Dundee, United kingdom.
Investigated Plum Pox Virus movement through its plant host using fluorescence labeling and confocal and electron microscopy.

1998-1999 **B.Sc. student**, Centre of Molecular Biology "Severo Ochoa", Madrid, Spain.
Investigated ß-amyloid peptide toxicity in primary culture of neurons from rat embryos and its consequence in NF-κB transcription factor nuclear translocation.

1996-1998 **B.Sc. student**, Hospital "12 de Octubre", Madrid, Spain.
Sample preparation and analysis of human kariotypes

Summary Statement

I possess skills in numerous Molecular biology techniques such as protein analysis ("Western-blot", ELISA, inmunofluorescence); purification and analysis of RNA("Northern-blot", siRNA detection, "in vitro" transcription) purification and analysis of DNA (PCR, Inmunocapture-RT-PCR, cloning); "in vitro" culture of animal cells, protoplast preparation, transitory expression through agroinfiltration. DNA inoculation by pressure shooting of gold microspheres in mice for antibody production, and in plants for viral cDNA inoculation. Tissue preparation with or without fixing for ultra-thin cuts for confocal and electron microscopy, vibrotome and microtome handling. Preparation and analysis of human kariotypes.

Language skills

Spanish: mother language.
English: Proficiency level, fluent oral and writing skills.
French: intermediate level.

Computer skills

OS: MacOS X and Windows XP
Software: Office 2004, Adobe Photoshop, EndNote.

Publications

B. Salvador, P. Sáenz, M.O. Delgadillo, J.A. García and C. Simón-Mateo
Identification of *Plum pox virus* pathogenicity determinants in herbaceous and woody hosts. Submitted for publication

B. Salvador, P. Sáenz, E. Yangüez, J.A. García and C. Simón-Mateo
Host-specific effect of P1 exchange between two potyvirus.
Manuscript in preparation

B. Salvador, P. Sáenz, J.A. García and C. Simón-Mateo
Host-specific effects on viral pathogenicity of two amino acid heterogeneities in Plum pox virus P1 protein.
Manuscript in preparation

B. Salvador, J. A. García and C. Simón-Mateo.
Molecular characterization of plum pox potyvirus. (2006) EPPO Bulletin, 36(2), 229-238.

L. Ion-Nagy, M. Lansac, J.P. Eyquard, **B. Salvador**, J.A. García, O. Le Gall, M. Hernould, V. Schurdi-Levraud, V. Decroocq. (2006).
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Delgadillo, M.O., Sáenz, P., **Salvador, B**., García, J.A. and C. Simón-Mateo (2004).
Human influenza virus NS1 protein enhances viral pathogenicity and acts as an RNA silencing suppressor in plants. J. Gen. Virol. 85, 993-999.

P. Sáenz, **B. Salvador**, C. Simón-Mateo, K. D. Kasschau, J. C. Carrington and J. A. García (2002).
Host-specific involvement of the HC protein in the long-distance movement of potyviruses. J. Virol. 76, 1922-1931.

Contributions to congress

C. Simón-Mateo, **B. Salvador**, O. Delgadillo, J.J. López-Moya, H.S. Guo, E. González and J.A. García
SUPRESSOR ACTIVITY OF POTYVIRAL HCPro AND CUCUMOVIRAL 2b IN POTYVIRUSINDUCED TRANSGENE SILENCING, 7th International Congress of Plant Molecular Biology.

J.A. García, C. Simón-Mateo, M.O. Delgadillo, **B. Salvador**, I. Jiménez, J. Ortuño, P. Sáenz and J.M. Alamillo
RNA SILENCING AND SALICYLIC EFFECTS ON PLUM POX POTYVIRUS INFECTION, EMBO Workshop on Genomic approaches in Plant Virology. Keszthely (Hungría), May 2003.

B. Salvador, P. Sáenz, J.A. Garcia and C. Simón-Mateo
PLUM POX VIRUS PATHOGENICITY DETERMINANTS IN NICOTIANA AND PRUNUS HOSTS, 19th International Symposium on Virus and Virus-Like Diseases of Temperate Fruit Crops. 10th. International Symposium on Small Fruit Virus Diseases, Valencia, Spain 2003.

J.A. García, **B. Salvador**, J.M. Alamillo, P. Sáenz, M.O. Delgadillo, S. Dallot, L. Quiot, J.B.Quiot and C. Simón-Mateo
PATHOGENICITY DETERMINANTS IN PLUM POX VIRUS INFECTION, 19th International Symposium on Virus and Virus-Like Diseases of Temperate Fruit Crops. 10th International Symposium on Small Fruit Virus Diseases, Valencia, Spain 2003.

Carmen Simón-Mateo, **Beatriz Salvador**, M. Otilia Delgadillo, Juan José López-Moya, Hui Shan Guo, Elena Gónzalez and Juan Antonio García
ESTUDIO DEL SILENCIAMIENTO GENICO POST-TRANSCRIPCIONAL EN PLANTAS TRANSGENICAS QUE CONTIENEN SECUENCIAS DE DNA DEL VIRUS DE LA SHARKA, VIII National Virology Congress, Barcelona, Spain 2003.

M. Otilia Delgadillo, , Pilar Sáenz, **Beatriz Salvador**, Juan A. García and Carmen Simón-Mateo
LA PROTEINA NS1 DEL VIRUS DE LA GRIPE TIENE ACTIVIDAD SUPRESORA DEL SILENCIAMIENTO EN PLANTAS, VIII National Virology Congress, Barcelona, Spain 2003

Salvador, B., J.A. García, R.M. Pérez-Clemente, M.A., Pérez-Sanjuan, L.A. Cañas Clemente, P. Sáenz, M.O. Delgadillo, C. Simón-Mateo.
VIRUS INDUCED GENE SILENCING IN PLUM POX VIRUS INFECTED GFP TRANSGENIC TREES, IInd European Congress of Virology, Madrid, Spain 2004.

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SUPPRESSOR ACTIVITY OF POTYVIRAL HCPRO AND CUCUMOVIRAL 2B IN POTYVIRUS-RESISTANT TRAMSGENIC PLANTS, Keystone symposium on siRNAs and miRNAs. 2004.

J.A. García, A. Valli, J.M. Alamillo, M.O. Delgadillo, **B. Salvador** and C. Simón-Mateo
RNA SILENCING IN THE DEFENSE AND COUNTER-DEFENSE STRATEGIES OF PLANT AND VIRUSES, 1st Workshop on "Advances in sources, mechanisms and durability of resistance against plant viruses and vectors" Alicante, Spain 2006.

A.Valli, **B. Salvador**, P. Sáenz, E. Yángüez, A. M. Martín-Hernández, J. J.López-Moya, C. Simón-Mateo and J. A. García
P1 PROTEIN OF PLUM POX POTYVIRUS AND CUCUMBER VEIN YELLOW IPOMOVIRUS, ENHANCES OR SUBSTITUTES, RESPECTIVELY, THE SILENCING SUPPRESSION ACTIVITY OF HCPro. EMBO Workshop on "Suppression and circumvention of host defense by plant viruses" Haikko (Finlandia) 2006.