DESARROLLO DE VECTORES VIRALES BASADOS EN EL VIRUS DEL MANCHADO FOLIAR DE LOS CÍTRICOS (CLBV)

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Departamento de Biotecnología

Desarrollo de vectores virales basados en el virus del manchado foliar de los cítricos (CLBV)

Tesis doctoral

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¿Y AHORA QUÉ?

Nacido en Santander, viví en Guardo durante más de 8 años, jugué con el *Micronova* y tras irradiar mi cerebro con episodios de Erase una Vez la Vida y El Hombre y la Tierra, nos trasladamos a Burgos, tierra del Cid y de Félix Rodríguez de la Fuente, ¿Qué más hace falta para acabar estudiando Biología?, pues una cosa más, que te des cuenta a tiempo de que la medicina no es para ti. Una fabulosa estancia en León de donde me llevé una Licenciatura, buenos amigos y un tesoro, y después avión a Edimburgo para descubrir que todo el inglés que había estudiado hasta la fecha, no había sido suficiente.

Sonaban gaitas en Oban, un pequeño pueblo en la costa oeste de Escocia, cuando el ministerio me concedió una beca para hacer la tesis. Hicimos las maletas y llenos de ilusión, nos vinimos a Valencia. Por lo tanto, es a ti Ruth a quien más tengo que agradecer, pues has estado apoyándome y animándome, desde el primer día.

A mis padres, gracias por estar ahí y aquí cuando os he necesitado. A mi hermano, a mi cuñada y a Alejandro, quien me iba a decir cuando empecé esta tesis que mi ahijado tendría 3 años cuando la leyera. A mis "suegros". Y a mi niña, a todos os dedico este trabajo.

A toda la gente que me ha ayudado y con la que he compartido tan buenos momentos, con las que he compartido laboratorio, los del piso de abajo, los de más abajo, los de al lado, los de enfrente, los de allá, los de acá, maestros de fotografía, compañeros de máster, de congresos, de invernaderos, de cafés, de almuerzos, de descansos en la escalera de incendio, de horas en la sala de becarios, de comidas, de celebraciones, de paellas, de escapadas a esquiar, los que ya sois doctores y los que lo seréis, los que dejasteis el IVIA, y los que los dejaréis, los que aguantaréis, los venezolan@s, francés@s, tunecin@s, canadienses, cuban@s, brasileñ@s, italian@s, asturian@s, valencian@s … y todos los que me habéis aguantado en algún momento, espero que a todos os haya dedicado en algún momento un gracias y una sonrisa y si no lo hice, aquí lo tenéis. GRACIAS y ☺.

A mis directores de tesis, gracias por la oportunidad que me habéis dado, sin vosotros no habría sido posible llevar a cabo este trabajo del que estoy muy orgulloso, pero que es fruto del trabajo anterior, del trabajo del equipo y que espero que dé muchos frutos en el futuro.

¿Y ahora qué? Me apasiona el trabajo de investigación y me apasiona la biotecnología. Estoy seguro que la biotecnología va a ser uno de los motores de la economía y de hecho está presente en cualquiera de los ámbitos de nuestra vida: alimentos funcionales, detección de enfermedades, medicamentos, vacunas, cosmética, nanotecnología, bio-combustibles, agricultura… El hecho de haber realizado una tesis con resultados aplicables y dirigidos a solucionar problemas, me ha despertado un gran interés por la biotecnología y su aplicación. Lo que toca ahora es no dejar de aprender y de descubrir, investigar y aplicar lo investigado, y no olvidarnos de divulgar, pues no hacemos ciencia para nosotros, sino para a hacer un mundo mejor.

RESUMEN

Los cítricos son el cultivo frutal económicamente más importante tanto en España como en el resto de los países productores. La clave para mantener la competitividad de este sector consiste en obtener material vegetal de alta calidad, para lo cual son indispensables los programas de mejora. La mejora de cítricos por métodos clásicos es muy complicada, por lo que hay que recurrir a las nuevas tecnologías para intentar acelerar y optimizar el procedimiento. La reciente secuenciación del genoma de dos especies de cítricos ha permitido identificar una larga lista de genes candidatos a participar en determinados procesos biológicos. Sin embargo, son necesarios nuevos análisis para asociar cada gen a un fenotipo específico o función biológica.

El empleo de vectores virales para determinar la función de genes mediante silenciamiento génico inducido por virus (VIGS) ha demostrado ser una herramienta muy útil para los estudios de genética reversa realizados en plantas. Este sistema presenta ventajas respecto a los métodos tradicionales para estudiar la función de genes como son la mutagénesis o la transformación genética, ya que permite ensayar la función de numerosos genes en un corto periodo de tiempo. Esto es especialmente crítico en el caso de los cítricos, que poseen largos periodos juveniles de entre 6 y 8 años y donde la transformación de plantas adultas es muy difícil. Además, permite estudiar la función de genes que son esenciales para el crecimiento o el desarrollo de la planta y cuyo análisis es inviable con los métodos tradicionales.

Al comienzo de la tesis se había desarrollado un vector viral para cítricos basado en el virus de la tristeza de los cítricos (CTV) con el que se pueden expresar proteínas pero que no se ha ensayado para estudiar la función de genes mediante VIGS. En el laboratorio disponíamos de un clon infeccioso de cDNA del genoma completo del virus del manchado foliar de los cítricos (CLBV), un virus que infecta a todas las especies y variedades de cítricos ensayadas y es asintomático en la mayoría de ellas. Este clon infeccioso se ha modificado para obtener vectores virales basados en el genoma de CLBV que pueden servir tanto para expresar proteínas como para silenciar mediante VIGS genes de cítricos para la mejora genética de este cultivo. Para ello, se ha introducido un punto de corte único *Pml*I en dos zonas del genoma de CLBV: en el extremo 3' no traducible (vector *clbv3'*) o en la zona intergénica localizada entre los genes de las proteínas de movimiento y cápsida (CP) (vector *clbvIN*). Para la expresión de secuencias foráneas mediante la formación de un nuevo RNA subgenómico (sgRNA) se delimitó la secuencia mínima promotora del

sgRNA CP mediante clonación de fragmentos de distinta longitud en torno al origen de transcripción de dicho sgRNA en el vector *clbv3´.* El fragmento de 92 bases localizado entre los nt -42 y +50 respecto al inicio de transcripción del sgRNA CP contenía todos los elementos necesarios para la promoción de un nuevo sgRNA *in vivo*. Esta secuencia mínima promotora se clonó en los 2 vectores virales previamente desarrollados para generar los vectores *clbv3'pr* y *clbvINpr*, respectivamente. Ambos vectores fueron capaces de producir un nuevo sgRNA y de expresar proteínas recombinantes.

Para determinar la estabilidad de los vectores obtenidos se clonaron en ellos fragmentos de secuencias lineales de distinto tamaño, o en tándem invertido para la formación de una estructura en horquilla, y se inocularon en plantas de *N. benthamiana* y cítricos. Todas las construcciones derivadas del vector *clbv3´* se mostraron estables a lo largo de las diferentes brotaciones analizadas durante al menos 3 años, comprobándose la replicación viral e integridad del inserto. Sin embargo, no se detectó multiplicación viral con ninguna de las construcciones derivadas del vector *clbvIN.* La estabilidad de las construcciones derivadas de los vectores con el promotor duplicado dependía del tamaño del inserto. Con todas ellas se detectó replicación viral pero se observaron eventos de recombinación cuando se clonaban fragmentos superiores a 720 nt en el vector *clbvINpr* o 408 nt en el vector *clbv3´pr.*

Un factor importante para determinar la eficiencia y funcionalidad de los vectores desarrollados es conocer cómo se mueve y se distribuye el virus en los distintos tejidos de la planta. Para ello se inocularon plantas de *N. benthamiana* y cítricos con la construcción *clbv3'pr*-GFP, que expresa GFP en los tejidos donde se localiza el virus. En *N. benthamiana*, la observación de GFP permitió detectar la presencia de CLBV en la mayoría de tejidos, acumulándose preferentemente en óvulos y regiones meristemáticas. En cítricos no se pudo visualizar GFP pero el virus se detectó en regiones meristemáticas mediante RT-PCR a tiempo real e hibridación molecular. La acumulación de CLBV en tejidos meristemáticos explicaría la dificultad de eliminar este virus mediante microinjerto.

Para evaluar la capacidad de los vectores *clbv3´pr* y *clbvINpr* para expresar proteínas se clonó en ellos la secuencia completa del gen *gfp* y se cuantificó la cantidad de proteína GFP sintetizada en las plantas infectadas. En *N. benthamiana* la cantidad de GFP estimada para el vector *clbv3´pr* fue de 16 µg de proteína por gramo de peso fresco, cantidad que resultó entre 5 y 6 veces superior a la estimada

para el vector *clbvINpr.* Sin embargo, en cítricos, debido a la inestabilidad del vector *clbv3´pr*, sólo se pudo cuantificar la proteína expresada por la construcción del vector *clbvINpr,* estimándose en 0.6 µg de GFP por gramo de peso fresco.

La efectividad de los vectores *clbv3´, clbv3´pr* y *clbvINpr* para silenciar genes mediante VIGS se ensayó clonando fragmentos de genes tanto endógenos de plantas (*pds*, *actina*, *sulfur*) como el gen *gfp* introducido experimentalmente en plantas transgénicas. En cítricos todas las construcciones de los tres vectores indujeron fenotipo de silenciamiento del gen ensayado, aunque el vector *clbv3´* fue el más efectivo para el estudio de VIGS en este huésped. Sin embargo, en *N. benthamiana* sólo se desencadenó el silenciamiento en las plantas inoculadas con la construcción *clbv3'pr*-hp58PDS, que expresa una horquilla de doble cadena de un fragmento de 58 nt del gen *pds*. En todas las plantas silenciadas se detectó una disminución del correspondiente mRNA del gen ensayado y una acumulación de siRNAs derivados tanto del mRNA del gen insertado como del RNA genómico del virus. Por otro lado, el fenotipo de silenciamiento de los genes ensayados se observó en sucesivas brotaciones, lo que confirma la gran estabilidad de los vectores basados en el genoma de CLBV.

Los vectores virales desarrollados en esta tesis constituyen una herramienta eficiente para el estudio de la función de genes mediante genética reversa utilizando la técnica VIGS. También pueden ser útiles para estudio de genética directa mediante expresión de proteínas o para la protección del cultivo frente a enfermedades producidas por virus, bacterias y hongos o frente a plagas de invertebrados.

ABSTRACT

Citrus represent the main fruit crop in Spain and in the world. Breeding programs are needed in order to obtain high quality competitive varieties. Traditional breeding of citrus is a slow and complicated process due to their complex reproductive biology and long juvenile periods; therefore, new technologies are necessary to speed up this process. The availability of citrus genome sequences and gene-expression patterns may help identifying candidate genes potentially involved in a particular biological process; however, further analyses are required to associate each gene with a specific phenotype or biological function.

Virus induced gene silencing (VIGS) using viral vectors has been shown to be a helpful tool to evaluate plant gene function by reverse genetics. This technology has advantages respect to traditional methods like mutagenesis and genetic transformation, because it allow us to study the function of many genes in a short time. This is important in the case of citrus, whose juvenile period is often more than 6 years and genetic transformation of adult plants is difficult. Besides, VIGS allows studying genes whose function is essential for plant viability, as these are silenced after the plant has already grown.

At the beginning of this thesis, a viral vector based on the *Citrus tristeza virus* (CTV) genome was available to express foreign proteins, but it was not tested for VIGS analysis. In our laboratory, a cDNA infectious clone was developed of the *Citrus leaf blotch virus* (CLBV) genome, a virus that causes asymptomatic infection in most of the citrus varieties tested. This infectious clone has been modified in order to get viral vectors that can be used for expressing foreign proteins or for citrus gene silencing. For this purpose, a *Pml*I restriction site was engineered at two different positions in the CLBV genomic (g)RNA: at the 3' untranslated region (UTR) (vector *clbv3'*) or at the intergenic region between the movement (MP) and the coat (CP) protein genes (vector *clbvIN*). In order to express foreign sequences by formation of a new subgenomic (sg)RNA, the minimum promoter sequence of the CP sgRNA was delimited by cloning different sized fragments in the *clbv3*' vector. A minimum 92-base fragment between positions -42 and +50 around the transcription start site of the CP sgRNA was shown to contain all the elements required for full promoter activity *in vivo* out of its natural context. This minimum sequence was cloned in the *clbv3'* and *clbvIN*

vectors and both were able to produce a new sgRNA an express recombinant proteins.

The efficiency and stability of these vectors was assessed by inserting different sized linear sequences, or inverted repeats that upon transcription fold as an RNA hairpin, and then inoculating *N. benthamiana* and citrus plants with these constructs. Analyses of viral replication and insert integrity showed that all the constructs based on *clbv3'* were stable in successive flushes for at least three years. However, viral replication was not detected for any of the constructs based on *clbvIN*. Stability of the constructs based on vectors with the duplicated promoter depended on the insert size. Viral replication was always detected but recombination events were observed when the cloned fragments were longer than 720 nt for the *clbvINpr* vector or 408 nt for the *clbv3'pr*.

An important factor to determine efficiency and functionality of the developed vectors is to know their movement and spread patterns in the plant tissues. For this purpose, *N. benthamiana* and citrus plants were inoculated with *clbv3'pr*-GFP, a construct expressing the green fluorescent protein (GFP) in the tissues where it replicates. In *N. benthamiana* plants, observation of GFP allowed CLBV detection in most of tissues, the virus accumulating preferentially in ovules and meristematic regions. GFP could not be observed in citrus but the virus was detected in meristematic regions by real time RT-PCR and molecular hybridization. These results explain in part the difficulty to eliminate CLBV by shoot-tip grafting *in vitro.*

In order to evaluate the ability of *clbv3'pr* and *clbvINpr* to express proteins, the complete ORF of the *gfp* gene was cloned and the amount of synthesized GFP was quantified in the infected plants. In *N. benthamiana* plants, the amount of GFP produced by the *clbv3'pr* vector was 16 µg·g-1 of fresh tissue, about 5 to 6 times more than that produced by the *clbvINpr* vector. In citrus, this latter vector produced only 0.6 µg of GFP per gram of fresh tissue, whereas no GFP was detected with *clbv3'pr-GFP* due to the instability of the construct in this host.

The VIGS silencing effectiveness of the vectors *clbv3'*, *clbv3'pr* and *clbvINpr* was assayed by cloning endogenous gene fragments (*pds, actin, sulfur*) as well as the *gfp* gene, experimentally introduced in transgenic citrus plants. In citrus, all the constructs based on the three vectors induced a silencing phenotype for the genes studied, with*clbv3'* vector being the most effective for VIGS analyses

in this host. However, in *N. benthamiana*, silencing was induced only in plants inoculated with the *clbv3'pr*-hp58PDS construct, which expresses a hairpin with a 58-nt inverted repeat of the *pds* gene. All the silenced plants showed a reduction of the cognate mRNA in the plant and an accumulation of siRNAs derived from the inserted gene and from the CLBV gRNA. On the other hand, the silencing phenotype of the studied genes was observed in successive flushes, which confirms the great stability of the CLBV-based vectors.

Viral vectors developed in this thesis provide an efficient tool for gene function studies through reverse genetics using the VIGS technique. They can also be used in forward genetic studies through protein expression or in plant protection against virus, bacteria or fungal diseases or invertebrate pests.

RESUM

Els cítrics són el cultiu fruiter econòmicament més important tant a Espanya com a la resta de països productors. La clau per a mantindre la competitivitat d'aquest sector està basat en obtindre material vegetal d'una alta qualitat, per la qual cosa són indispensables els programes de millora. La millora dels cítrics per mètodes clàssics és molt complicada, per tant cal recórrer a les noves tecnologies per a intentar accelerar i optimitzar el procediment. La recent seqüenciació del genoma de dues espècies de cítrics ha permès identificar una llarga llista de gens candidats a participar en determinats processos biològics. No obstant això, són necessàries noves anàlisis per a associar cada gen a un fenotip específic o funció biològica.

L'ús de vectors virals per a determinar la funció de gens per mitjà de silenciament gènic induït per virus (VIGS) ha demostrat ser una ferramenta molt útil per als estudis de genètica reversa realitzats en plantes. Aquest sistema presenta avantatges respecte als mètodes tradicionals per a estudiar la funció de gens, com són la mutagènesi o la transformació genètica, en tant que permet assajar la funció de nombrosos gens en un curt període de temps. Això és especialment crític en el cas dels cítrics, que presenten llargs períodes juvenils d'entre 6 i 8 anys i on la transformació genètica de plantes adultes és molt complicada. A més, permet estudiar la funció de gens que són essencials per al creixement o el desenvolupament de la planta, l'anàlisi dels quals és inviable amb els mètodes tradicionals.

Al començament de la tesi s'havia desenvolupat un vector viral per a cítrics basat en el virus de la tristesa dels cítrics (CTV), amb el qual es poden expressar proteïnes, però que no s'ha assajat per a estudiar la funció de gens per mitjà de VIGS. Al laboratori disposàvem d'un clon infecciós de cDNA del genoma complet del virus del tacat foliar dels cítrics (CLBV), un virus que infecta a totes les espècies i varietats de cítrics assajades, i que és asimptomàtic en la majoria d'elles. Aquest clon infecciós s'ha modificat per a obtindre vectors virals basats en el genoma de CLBV que hi poden servir tant per a expressar proteïnes com per a silenciar, mitjançant VIGS, gens de cítrics per a la millora genètica d'aquest cultiu. Per a dur-ho a terme, s'ha introduït un punt de tall únic *Pml*I en dues zones del genoma de CLBV: a l'extrem 3' no traduïble (vector *clbv3'*) o en la zona del intergènica localitzada entre els gens de les proteïnes de moviment i càpsida (CP) (vector *clbvIN*). Per a l'expressió de seqüències foranies mitjançant la formació d'un nou RNA subgenòmic

(sgRNA) es va delimitar la seqüència mínima promotora del sgRNA CP mitjançant la clonació de fragments de diferent longitud al voltant de l'origen de transcripció del sgRNA al vector *clbv3´*. El fragment de 92 bases localitzat entre els nucleòtids -42 i +50 respecte a l'inici de la transcripció del sgRNA CP contenia tots els elements necessaris per a la promoció d'un nou sgRNA *in vivo*. Aquesta seqüència mínima promotora es va clonar als dos vectors virals prèviament desenvolupats per a genera els vectors *clbv3'pr* i *clbvINpr* respectivament. Ambdós vectors van ser capaços de produir un nou sgRNA i expressar proteïnes recombinants.

Per a determinar l'estabilitat dels vectors obtinguts, es clonaren en ells fragments de seqüències lineals de diferent grandària o en tàndem invertit, per a la formació d'una estructura en forquilla, i s'inocularen plantes de *N. benthamiana* i cítrics. Totes les construccions derivades del vector *clbv3´* es mostraren estables al llarg de les diferents brotacions analitzades durant al menys 3 anys, comprovant-ne la replicació viral e integritat de l'inserit. No obstant això, no es va detectar multiplicació viral amb cap de les construccions derivades del vector *clbvIN*. L'estabilitat de les construccions derivades dels vectors amb el promotor duplicat depenia de la grandària de l'inserit. Amb totes elles es va detectar replicació viral, però es van observar esdeveniments de recombinació quan es clonaven fragments superiors a 720 nt en el vector *clbvINpr* o 408 nt en el vector *clbv3´pr*.

Un factor important per a determinar l'eficiència i funcionalitat dels vectors desenrotllats és conéixer com es mou i es distribuïx el virus en els distints teixits de la planta. Per a això, es van inocular plantes de *N. benthamiana* i cítrics amb la construcció *clbv3'pr-GFP*, que expressa GFP en els teixits on es localitza el virus. En *N. benthamiana*, l'observació de GFP va permetre detectar la presència de CLBV en la majoria de teixits, acumulant-se preferentment en òvuls i regions meristemàtiques. En cítrics no es va poder visualitzar GFP però el virus es va detectar en regions meristemàtiques per mitjà de RT-PCR a temps real i hibridació molecular. L'acumulació de CLBV en teixits meristemàtics explicaria la dificultat d'eliminar este virus per mitjà de microempelt. Per a avaluar la capacitat dels vectors *clbv3´pr* i *clbvINpr* per a expressar proteïnes es va clonar en ells la seqüència completa del gen *gfp* i es va quantificar la proteïna GFP sintetitzada en les plantes infectades.

En *N. benthamiana*, la quantitat de GFP estimada per al vector *clbv3´pr* va ser de 16 µg de proteïna per gram de pes fresc, quantitat que va resultar entre 5 i 6 vegades superior a l'estimada per al vector *clbvINpr*. No obstant això, en cítrics, a causa de la inestabilitat del vector *clbv3´pr*, només es va poder quantificar la

proteïna expressada per la construcció del vector *clbvINpr*, estimant-se en 0.6 µg de GFP per gram de pes fresc.

L'efectivitat dels vectors *clbv3´*, *clbv3´pr* i *clbvINpr* per a silenciar gens per mitjà de VIGS es va assajar clonant fragments de gens tant endògens de plantes (*pds, actina, sulfur*) com el gen *gfp* introduït experimentalment en plantes transgèniques. En cítrics, totes les construccions dels tres vectors van induir fenotip de silenciament del gen assajat, encara que el vector *clbv3´* va ser el més efectiu per a l'estudi de VIGS en este hoste. No obstant això, en *N. benthamiana* només es va desencadenar el silenciament en les plantes inoculades amb la construcció *clbv3'pr-hp58PDS*, que expressa una forquilla de doble cadena d'un fragment de 58 nt del gen *pds*. En totes les plantes silenciades es va detectar una disminució del corresponent mRNA del gen assajat i una acumulació de siRNAs derivats tant del mRNA del gen inserit com del RNA genòmic del virus. D'altra banda, el fenotip de silenciament dels gens assajats es va observar en successives brotacions, la qual cosa confirma la gran estabilitat dels vectors basats en el genoma de CLBV.

Els vectors virals desenrotllats en esta tesi constituïxen una ferramenta eficient per a l'estudi de la funció de gens per mitjà de genètica reversa utilitzant la tècnica VIGS. També poden ser útils per a estudis de genètica directa per mitjà d'expressió de proteïnes o per a la protecció del cultiu enfront de malalties produïdes per virus, bacteris i fongs o enfront de plagues d'invertebrats.

ÍNDICE

INTRODUCCIÓN GENERAL

1. El cultivo de los cítricos: Origen, distribución e importancia económica

El origen de los cítricos se sitúa en el sudeste asiático y actualmente su cultivo se extiende por la mayor parte de las regiones con clima tropical, subtropical y en el límite entre subtropical y templado. Los responsables de la introducción de las primeras especies cultivadas de cítricos en España fueron los árabes y hoy en día este cultivo se extiende principalmente por las zonas costeras del Este y del Sur de la península, localizándose sobre todo en lugares próximos al litoral y en los valles de los ríos.

Los cítricos con interés comercial pertenecen a la familia *Rutaceae,* subfamilia *Aurantioideae.* Dentro de esta subfamilia se incluyen las tribus *Clauseneae* y *Citreae*. La tribu *Citreae* se divide a su vez en tres subtribus, una de las cuales, la *Citrinae*, contiene todos los géneros donde se engloban los cítricos cultivados: *Citrus, Fortunella* y *Poncirus* [1].

Las especies cítricas cultivadas más importantes se pueden englobar en cuatro grandes grupos: los naranjos, que incluyen naranjos dulces (*Citrus sinensis* (L.) Osb.) y amargos (*C. aurantium* L.); los mandarinos, que incluyen satsumas (*C. unshiu* (Mak.) Marc.), clementinos (*C. clementina* Hort. *ex* Tan.) e híbridos; los limoneros (*C. limon* (L.) Burm. f.) y limeros (*C. aurantifolia* (Christm.) Swing.); y los pomelos (*C. paradisi* Macf.), todos pertenecientes al género *Citrus.* El género *Fortunella* incluye los kumquats, caracterizados por sus frutos pequeños de corteza dulce y comestible. El interés agronómico de la única especie del género *Poncirus* (*P. trifoliata* (L.) Raf.) viene dado por su uso como portainjertos.

Los cítricos son el principal cultivo frutal en el mundo, con una producción estimada en el año 2011 de más de 128 millones de toneladas (faostat.fao.org, FAO 2012). En España también constituyen el principal frutal, con una superficie total de cultivo de unas 291.000 Ha y una producción superior a los 5 millones de

toneladas (FAO 2012). Nuestro país es el sexto productor del mundo después de China, Brasil, EEUU, India y México.

España es el principal exportador de cítricos para consumo en fresco a nivel mundial. El valor medio de las exportaciones ha supuesto el 55% de la producción media en la última década, siendo una de las principales fuentes de entrada de divisas. Al valor de la producción hay que añadir el de una fuerte actividad económica secundaria dependiente de este cultivo generada por las empresas de viveros, fertilizantes, confección de la fruta, etc. La importancia económica de los cítricos en España también viene dada por el valor social, ya que tanto el cultivo como la comercialización generan una fuerte demanda de mano de obra. Dada la importancia de este cultivo para la economía española, es imprescindible disponer de material vegetal de alta calidad, tanto en variedades como en patrones para garantizar el mantenimiento de la producción y la competitividad de este sector.

2. La mejora genética clásica de cítricos

La mayoría de las variedades de cítricos utilizadas comercialmente en la actualidad provienen de la selección clonal de mutaciones espontáneas aparecidas con relativa frecuencia en el campo y la propagación vegetativa de las mismas. Con el fin de mejorar la calidad y cantidad de la producción de cítricos, en muchos países se han desarrollado diversos programas de mejora genética. Entre los métodos de mejora tradicional en cítricos destacan la mutagénesis inducida y la hibridación sexual.

La mutagénesis inducida es una técnica con la cual se pretende cambiar uno o varios caracteres de forma artificial mediante un tratamiento mutagénico

que se aplica a las semillas o a las yemas. La radiación gamma es el método más común que se emplea para inducir mutagénesis.

La hibridación sexual entre individuos diploides es la técnica que se usa tradicionalmente en los programas de mejora, sin embargo el número de genotipos de valor comercial que se ha obtenido con esta técnica es muy limitado. Además, tiene el inconveniente de que la mayoría de los híbridos diploides que se obtienen son fértiles y por lo tanto producen semillas, un carácter no deseado para la comercialización.

Los programas de mejora clásica de cualquier especie leñosa tienen unos requerimientos de tiempo y espacio muy elevados y en el caso de los cítricos, además presentan limitaciones inherentes a este cultivo como son: 1) Su biología reproductiva es muy compleja, la mayoría de los genotipos presentan apomixis o embrionía nucelar, un tipo de reproducción asexual mediante el cual se producen embriones nucelares idénticos a la planta madre que impiden o dificultan el desarrollo de los embriones sexuales. 2) Muchas variedades comerciales presentan esterilidad parcial o total del polen y/o de los óvulos, así como autoincompatibilidad e incompatibilidad entre genotipos distintos, lo que representa una barrera para su empleo en mejora clásica por hibridación. 3) Tienen una elevada tasa de heterozigosis, las progenies presentan una gran segregación de los caracteres y resulta muy difícil obtener híbridos con los caracteres deseados de los parentales. Otro problema es el desconocimiento del modo de herencia de la mayoría de caracteres de interés agronómico. 4) Los cítricos tienen periodos de juvenilidad largos de entre 5 y 8 años, durante los cuales no se produce floración ni fructificación. Todo esto, así como la escasez de marcadores moleculares ligados a caracteres de interés, complica la mejora de los cítricos por métodos clásicos.

3. Biotecnología y genómica en la mejora de cítricos

La utilización de la biotecnología en la mejora genética de variedades vegetales ha permitido acelerar el proceso y mejorar el rendimiento de la mejora clásica. Técnicas como el cultivo de tejidos *in vitro*, la hibridación somática y la citometría de flujo han facilitado notablemente la obtención de híbridos triploides para su empleo como variedades cultivadas, ya que son estériles y producen frutos sin semillas, además de no inducir la producción de semillas en otras variedades. Algunos de los híbridos obtenidos se han protegido y se están explotando comercialmente [2].

Otra aplicación biotecnológica para la mejora de variedades es la transformación genética, que permite incorporar uno o varios genes en un genotipo sin alterar su fondo genético. La transformación genética puede ser empleada para mejorar los genotipos actualmente cultivados introduciendo genes que confieran caracteres deseables sin los inconvenientes de la hibridación sexual. La transformación de material vegetal adulto se ha desarrollado para acortar el periodo entre transformación y floración, pero es un proceso largo y difícil [3]. La transformación genética se ha aplicado en la mejora de cítricos introduciendo distintos caracteres de interés agronómico [4], aunque tiene el inconveniente del rechazo social de los alimentos obtenidos utilizando esta técnica.

3.1 Genómica funcional

Una forma de acelerar la mejora de cítricos es la aproximación genómica, cuyo objetivo es identificar genes implicados en caracteres de interés agronómico. En los últimos años el número de secuencias de distintos organismos disponibles en las bases de datos se ha incrementado exponencialmente debido al desarrollo

de plataformas de secuenciación masiva. En el marco del Consorcio Internacional del Genoma de Cítricos (ICGC, www.citrusgenome.ucr.edu) se han obtenido más de 550.000 secuencias de DNA complementario (cDNA) del transcriptoma de cítricos (*Expressed Sequences Tags*, ESTs) que se encuentran disponibles en una de las bases de datos del NCBI (www.ncbi.nlm.nih.gov/dbEST/). Esta colección incluye una amplia representación de secuencias procedentes de genotecas de cDNA obtenidas de diferentes órganos, tejidos y estadios del desarrollo, tratamientos hormonales y de plantas sometidas a distintos estreses bióticos y abióticos (*Phytophtora*, tristeza (*Citrus tristeza virus*, CTV), sequía, salinidad, etc.). El 80% de estas secuencias provienen principalmente de 4 especies: *C. sinensis* (38%), *C. clementina* (22%), *C. reticulata* Blanco (9.5%) y *P. trifoliata* (10.5%).

Con estas colecciones de ESTs se han fabricado varias micromatrices; en Japón [5, 6], EEUU [7] y España [8, 9], esta última con 21081 unigenes, que se han usado para determinar patrones de expresión génica en distintos tejidos y en diferentes estadios del desarrollo [10-13], en varios genotipos [14, 15], o en respuesta a distintos estreses bióticos o abióticos [16-18].

Además, en el ICGC también se ha obtenido la secuencia completa de dos especies de cítricos, un clementino haploide (*C. clementina* cv. Nules) y un naranjo dulce diploide (C. *sinensis* cv. Ridge Pineapple). El clementino haploide, libre de patógenos y procedente de una línea del IVIA [19], se seleccionó para minimizar las complicaciones del ensamblaje de un genoma con alta heterozigosis y se secuenció con el método Sanger. Mediante el genotipado con marcadores moleculares se obtuvo un mapa genético de referencia que se usó para facilitar el ensamblaje de la secuencia [20]. Esta secuencia sirve como genoma de referencia para todas las secuencias de cítricos obtenidas por otros métodos. La secuencia del genoma del naranjo dulce diploide se ha obtenido utilizando la tecnología de pirosecuenciación 454. Ambas secuencias ensambladas y con una anotación

preliminar, están disponibles en el portal de internet www.phytozome.net, desarrollado por el *Joint Genome Institute*, departamento de energía de EEUU.

La disponibilidad de secuencias y patrones de expresión pueden ayudar a identificar los genes candidatos a participar en un determinado proceso biológico, sin embargo, la asociación entre un gen y un fenotipo o una función biológica requiere de posteriores análisis y actualmente constituye el principal desafío de la era postsecuenciación.

Para estudiar la función de genes en plantas se han empleado diversas aproximaciones entre las que destacan la transformación estable con genes o fragmentos de genes [21], el etiquetado mediante activación transcripcional [22], la inserción de DNA de transferencia (T-DNA), conocido como trampas génicas [23], o de elementos transponibles [24] y la obtención de mutantes utilizando sustancias químicas o radiaciones ionizantes [25, 26]. Estas técnicas se han aplicado con éxito en plantas modelo, pero en cítricos, debido al tamaño de los árboles, la capacidad de caracterización de muchas líneas obtenidas mediante los métodos mencionados es muy limitada. Además, el largo período juvenil y la compleja biología reproductiva de los cítricos aún entorpecen más futuros avances en genómica funcional utilizando dichos métodos. En *Populus* se han desarrollado aproximaciones de mutagénesis insercional y etiquetado mediante activación transcripcional [27, 28], aunque con escaso éxito. Otro inconveniente de los métodos anteriores es que no permiten estudiar la función de genes esenciales para el crecimiento o desarrollo de las plantas ya que la falta de función de este tipo de genes hace que éstas puedan morir durante la germinación o la regeneración.

Una estrategia alternativa que se ha desarrollado para estudiar la función de genes en plantas sin los inconvenientes anteriores es el silenciamiento génico inducido por virus (*Virus-Induced Gene Silencing*, VIGS) mediante el uso de vectores virales.

4. Vectores virales

El conocimiento de los mecanismos moleculares e interacciones con el hospedador de multitud de virus de plantas ha permitido transformarlos en importantes herramientas biomédicas y biotecnológicas. Diferentes virus con una amplia gama de hospedadores se han modificado genéticamente para silenciar genes de plantas mediante VIGS y estudiar así su función mediante genética reversa, o para expresar proteínas de interés.

Las estrategias que se emplean para infectar las plantas con vectores virales son la agroinoculación, la inoculación mecánica de transcritos de RNA o la biolística, que consiste en el bombardeo de tejidos vegetales con diferentes tipos de partículas que llevan adheridos transcritos del virus o plásmidos que contienen el cDNA del virus. La agroinoculación consiste en introducir el virus modificado en el T-DNA de un plásmido binario que se puede replicar tanto en *Escherichia coli* como en *Agrobacterium tumefaciens.* Al inocular las plantas con un cultivo de *A. tumefaciens* transformado con el plásmido, el T-DNA se transfiere a las células vegetales, el cDNA del virus se transcribe a RNA y éste comienza a expresarse y replicarse [29, 30]. El empleo de este sistema para iniciar la infección viral supuso un gran avance en el desarrollo de los vectores virales. La agroinoculación se puede hacer en hojas con una jeringa o en la planta entera por inmersión e infiltración al vacío, de tal forma que el vector infecta toda la planta. En plantas leñosas donde la agroinoculación puede ser difícil o imposible, primero se agroinoculan plantas de un huésped herbáceo para que el virus se multiplique, y posteriormente, a partir de estas plantas se obtiene un extracto purificado de viriones que se inocula mecánicamente en plantas del huésped leñoso. Otro método que ha resultado eficiente para inocular las plantas consiste en sumergir las raíces en una suspensión de *Agrobacterium* [31]. Por otro lado, mediante la agroinoculación directa de frutos se han obtenido mejores resultados de

silenciamiento en estos órganos que con la inoculación tradicional de hojas [32, 33].

4.1 Silenciamiento génico inducido por virus (VIGS)

El término VIGS se usó por primera vez para describir el fenómeno por el cual una infección viral induce la resistencia de las plantas a posteriores infecciones por el mismo virus [34]. Hoy en día el término VIGS se utiliza para definir una metodología que sirve para estudiar la función de genes mediante genética reversa. Para ello el genoma del virus se utiliza como un vector en el que se introducen genes o fragmentos de genes de la planta. Cuando se inocula el virus, el procesamiento de los RNAs bicatenarios (dsRNAs) o de zonas monocatenarias altamente estructuradas del genoma viral por una nucleasa específica da lugar a la degradación tanto del genoma del virus como del gen insertado en el mismo, originando la formación de RNAs pequeños (sRNAs). Estos sRNAs dirigen la degradación de los RNAs mensajeros (mRNA) del gen homólogo o conjunto de genes homólogos a la secuencia que se ha clonado en el virus y la planta muestra el fenotipo de silenciamiento o pérdida de función del gen ensayado. El efecto del VIGS se basa en un fenómeno epigenético que se conoce como silenciamiento génico post-transcripcional (*post-transcriptional gene silencing*, PTGS).

4.1.1 Mecanismo del silenciamiento génico

El silenciamiento génico se produce por inhibición de la transcripción (*transcriptional gene silencing*, TGS) y por degradación del RNA o inhibición de su traducción (PTGS). Se conocen distintas funciones en las que está implicado el silenciamiento génico a través de diferentes rutas. La primera función biológica

conocida es el mecanismo de defensa antiviral en plantas [35-37]. Otras funciones incluyen la defensa contra elementos de DNA transponibles a nivel transcripcional y postranscripcional, la regulación de la estabilidad de la cromatina o el control del desarrollo y de la expresión de genes [38-44].

La represión de la expresión génica está mediada por sRNAs de 21 a 30 nucleotidos (nt). Estos sRNAs están bien caracterizados e incluyen micro RNAs (miRNAs) y sRNAs interferentes (siRNAs, *short interfering* RNAs). A su vez, dentro del grupo de los siRNAs también se han descrito diferentes tipos que intervienen en varias de las rutas del silenciamiento de RNA en animales y plantas [45, 46]. Los diferentes tipos de sRNAs se diferencian entre sí por su función biológica y por su génesis, ya que proceden de distintos transcritos primarios o precursores.

En todos los casos los sRNAs provienen del procesado de moléculas de dsRNA, o de RNA de cadena sencilla (ssRNA) altamente estructurado, por enzimas de tipo *Dicer* (*Dicer like*, DCL) [47], que poseen dominios de tipo RNasa III. Las enzimas DCL cortan las moléculas dsRNA y se producen siRNAs de entre 21 y 24 nt de longitud que contienen dos nt protuberantes en los extremos 3' [48-50]. Para asegurar su estabilidad, las ribosas de los extremos 3' de estos siRNAs son metilados en el grupo hidroxilo por la metil-transferasa "*Hua Enhancer 1"* (*HEN1)* [51]. Una de las hebras de los siRNA (hebra guía) se asocia con proteínas específicas de la familia Argonauta (AGO) [52], que forman parte del complejo efector llamado RISC (*RNA-induced silencing-complex*), y la otra hebra queda liberada. Es la hebra guía de los siRNA la que dirige el RISC al RNA diana mediante complementariedad de bases bloqueando su traducción o degradándolo [53]. En el silenciamiento génico transcripcional, el complejo efector es guiado hasta el DNA de secuencia complementaria al que se une, induciendo su metilación y bloqueando su transcripción. En este último caso, los siRNAs deben entrar en el núcleo de las células [54, 55] (Fig. 1).

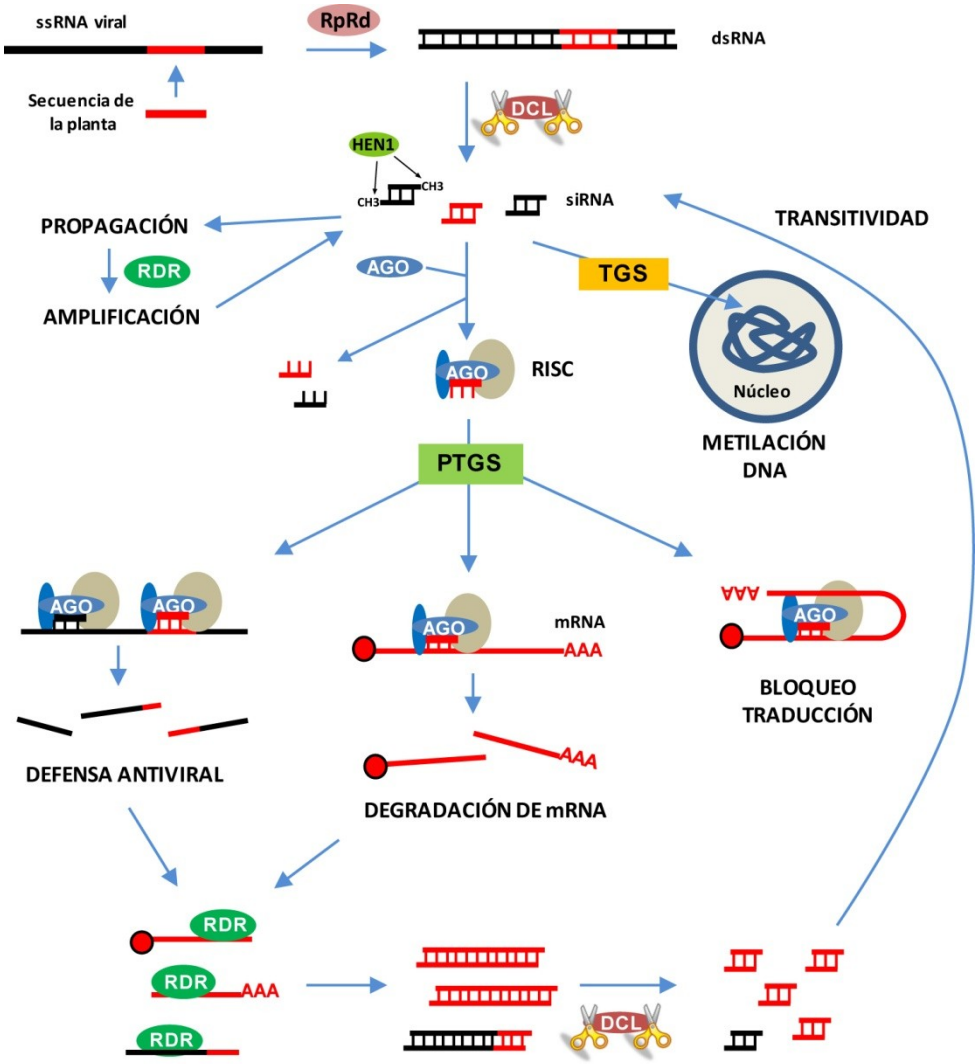

Figura 1. Mecanismo del silenciamiento génico inducido por virus (VIGS). El proceso comienza con la formación de dsRNA a partir de un vector viral que lleva un fragmento de un gen de la planta. Los dsRNAs son procesados por enzimas de restricción (DCL) para formar los siRNAs, que son metilados en sus extremos 3' protuberantes por HEN1. Estos inducen la metilación de DNA en el núcleo o se incorporan a un complejo proteico (RISC) desencadenando el bloqueo de la traducción, la degradación de mRNA o la defensa antiviral. La señal del silenciamiento puede propagarse célula a célula y a larga distancia y puede ser amplificada por la acción de polimerasas de RNA celulares (RDR) y DCLs.

Los dsRNAs, que son la clave para la activación del silenciamiento de RNA, se pueden formar directamente como consecuencia de las características del transcrito (altamente estructurado) o del proceso de replicación de virus y transposones. Los miRNAs se forman a partir de transcritos primarios que forman moléculas de dsRNA de tipo horquilla por auto-complementariedad [56]. Al igual que los miRNAs, los siRNAs se pueden formar a partir de estructuras en bucle, pero éstas requieren una complementariedad más precisa que los precursores de miRNAs. Por otro lado, durante el proceso de replicación de los virus de ssRNA, que representa el grupo más abundante entre los virus de plantas, se forman intermediarios replicativos de dsRNA. Estas moléculas híbridas contienen una copia de la cadena positiva y otra de la cadena negativa de su RNA genómico (gRNA) o subgenómicos (sgRNA) y su formación está mediada por la acción de una RNA polimerasa RNA dependiente codificada por el virus (RdRp). El procesado de estas formas replicativas y de zonas con alta estructura secundaria del genoma viral por las enzimas DCL son la base de la defensa antiviral y por lo tanto del VIGS [57, 58]. En el caso del VIGS los siRNA*s* se forman tanto del virus como del gen introducido en el vector viral, lo cual hace que los complejos RISC dirijan la hidrólisis tanto del RNA viral como del mRNA diana (Fig. 1). Se ha comprobado que la introducción de repeticiones invertidas del gen diana hace que se formen estructuras de dsRNA de tipo horquilla que inducen el VIGS más eficientemente que las secuencias únicas [59].

4.1.2 Amplificación y diseminación de la señal

Al igual que en hongos o en *Caenorhabditis elegans* y a diferencia de insectos y vertebrados, la formación en plantas de dsRNAs derivados de transgenes, virus, transposones y elementos repetitivos depende también de la acción de RNA polimerasas celulares (RDRs) que usan ssRNA como molde [60]

(Fig. 1). En *Arabidopsis* se ha demostrado que RDR6 regula la acumulación de algunos virus [61] y en *Nicotiana benthamiana* también se ha visto que esta polimerasa está implicada en la amplificación de la señal de VIGS [62]. La actividad de RDR6 es necesaria para que las células infectadas respondan a la señal inicial de silenciamiento que reciben para generar siRNAs secundarios, los cuales activan una respuesta VIGS antiviral más eficiente. Los dsRNAs producidos por las RDRs son reconocidos y procesados por las enzimas DCL dando lugar a nuevos siRNAs secundarios y amplificando así el proceso de PTGS [63].

En plantas y *C. elegans* se ha descrito el fenómeno de la transitividad del silenciamiento [64, 65]. Cuando se induce el silenciamiento de un gen diana mediante la introducción de un fragmento de ese gen, en el proceso de amplificación de la señal de silenciamiento, dependiente de RDR6, del transcrito y de la hebra negativa de los siRNAs primarios, se producen nuevos siRNAs secundarios de las regiones 5' y 3' respecto de la secuencia que activó la señal inicial, además de los producidos en esa zona. Los genes endógenos, a diferencia de los trangenes, suelen escapar a la transitividad [64-68], excepto cuando son expresados como transgenes [69], posiblemente debido a que éstos últimos están regulados por promotores mucho más fuertes, o cuando presentan altos niveles de expresión [70]. El nivel de expresión de un gen y el tamaño del fragmento introducido son factores importantes en la susceptibilidad de dicho gen al silenciamiento. Cuanto mayores son estos factores, más efectivo resulta el silenciamiento del gen diana [71, 72].

4.1.3 Supresores del silenciamiento

Como se ha indicado anteriormente, una de las funciones del silenciamiento en plantas es la defensa antiviral. Sin embargo, los virus han evolucionado y desarrollado estrategias para evadir o contrarrestar este

mecanismo de defensa del huésped mediante proteínas codificadas en su genoma, denominadas supresores de silenciamiento [73, 74]. Estas proteínas no tienen motivos estructurales o secuencias en común entre diferentes grupos de virus y además, interfieren en diferentes pasos de la ruta de silenciamiento de RNA. Algunas, como la proteína p19 del virus del enanismo arbustivo del tomate (*Tomato bushy stunt virus*, TBSV), se unen a siRNAs interfiriendo en su incorporación a RISC [75, 76] y otras se unen a dsRNAs largos o interactúan con componentes proteicos de la maquinaria de silenciamiento [77-79].

4.1.4 Vectores VIGS desarrollados

El primer vector que se usó en VIGS se desarrolló a partir del genoma de un virus de RNA, el virus del mosaico del tabaco (*Tobacco mosaic virus*, TMV), y se utilizó para silenciar el gen de la fitoeno desaturasa (*pds*) en plantas de *N. benthamiana*. Las plantas silenciadas mostraron un fenotipo de blanqueamiento de los distintos tejidos tras la infección con transcritos del vector viral [80]. Desde entonces, son muchos los virus que se han modificado para desarrollar vectores VIGS (Tabla 1). Algunos de estos vectores pueden silenciar genes en distintos huéspedes, lo que permite utilizar el mismo vector para el estudio de genes en distintas especies.

Mediante VIGS se ha estudiado la función de numerosos genes, principalmente relacionados con la respuesta de defensa a patógenos, estreses abióticos o con el crecimiento y desarrollo (Tabla 1). Hay varias revisiones que analizan la mayoría de los vectores desarrollados, genes estudiados y especies en las que es posible realizar experimentos de VIGS [81-85].

Uno de los factores que influye en la eficiencia de los vectores virales para silenciar el gen homólogo es el tamaño del fragmento de gen que se introduce en el vector. Tamaños entre 150 y 800 pb son los más utilizados, aunque también se

han utilizado con éxito fragmentos de hasta 23 pb. Los fragmentos que tienen entre 200 y 350 pb suelen ser los que muestran una mayor eficiencia para inducir silenciamiento [84].

Tabla 1. Resumen de vectores VIGS y plantas utilizadas en silenciamiento génico

Vector viral	Plantas utilizadas en VIGS	Genes silenciados	Citas
Virus RNA			
Apple latent spherical virus (ALSV)	Manzano, Peral, Tomate, *Nicotiana* spp., *A. thaliana*, cucurbitáceas, leguminosas	*pds, su, pcna, ch42, rcy1, ifs2, N*	[86-88]
Barley stripe mosaic virus (BSMV)	Cebada, trigo, avena, maíz, espinaca, jengibre, *Brachypodium distachyon*, *Haynaldia villosa*	*pds*, *Lr21, rar1, sgt1, hsp90, blufensin1, HvLrr, coi1, dmc1, gbss*	[89-96]
Bean pod mottle virus (BPMV)	Soja, *Phaseolus vulgaris*	*pds, mp4k, sgt1, rps6, rps13*	[97-99]
Brome mosaic virus (BMV)	Cebada, arroz, maíz	*pds, actina 1, rubisco activasa*	[100]
Cucumber mosaic virus (CMV)	Soja	*Chs, sf3'h1*	[101]
Cymbidium mosaic virus (CymMV)	Orquídeas (*Phalaenopsis* spp.)	*pds, mads5/6, ufgt3*	[102, 103]
Grapevine virus A (GVA)	Vid, *N. benthamiana*	*pds*	[104]
Pea early browning virus (PEBV)	*Pisum sativum, Medicago trunculata, Lathyrus odorata*	*pds, uni, kor*	[105, 106]
*Pepino mosaic virus (*PepMV)	*N. benthamiana*	*pds*	[107]
Poplar mosaic virus (PopMV)	*N. benthamiana*	*gfp*	[108]
Potato virus A (PVA)	*N. benthamiana*	*gfp*	[109]
Potato virus X (PVX)	Patata, tomate, *N. benthamiana*, *A. thaliana*	*pds, cry2, gfp, gus, ssu, nfl, lfy, cyp51*	[110-114]
Tobacco mosaic virus (TMV)	*N. benthamiana, N. tabacum*	*pds, psy, gfp*	[59, 80]
Tobacco rattle virus (TRV)	Solanaceas, algodón, opio, *N. benthamiana, A. thaliana*, *Jatropha curcas, Aquilea vulgaris, Thalictrum spp.*	*pds, rbcS, GhNdr1, GhMkk2, eds1, leafy, pb7, chlH, ctr1, ein2, AqAns, pistillata, rar1, npr1, agamous*	[31, 32, 115-125]
Tomato bushy stunt virus (TBSV)	*N. benthamiana*	*pds, chlH, gfp*	[126, 127]
Turnip yellow mosaic virus (TYMV)	*A. thaliana*	*Pds, lfy*	[128]
Satellite tobacco mosaic virus (STMV)	*Nicotiana glauca, N. tabacum*	*pds, chsA,rbcS/L, cesA, als, rpII, cat-1, npk-1, parp,gln1-5, ppx-1*	[129, 130]
Virus DNA			
African cassava mosaic virus (ACMV)	Yuca, *N. benthamiana*	*pds, su, cyp79d2*	[131]
Cabbage leaf curl virus (CaLCuV)	*A. thaliana*	*gfp, ch42, pds, su, cla1, sgt1*	[132, 133]

Vector viral	Plantas utilizadas en VIGS	Genes silenciados	Citas
Virus DNA			
Tobacco curly shoot virus (TCSV) *alphasatellite*	Tomate, *Petunia x hybrida*, *Nicotiana* spp.	*gfp, su, chs, pcna*	[134, 135]
Tomato golden mosaic virus (TGMV)	*N. benthamiana*	*su, luc, rbr1, pcna*	[136-138]
Tomato yellow leaf curl China virus (TYLCCNV)	Tomate, *Nicotiana* spp.	*pcna, pds, su, gfp, fro1*	[139-141]

La tecnología VIGS puede contribuir a la mejora de cultivos que son recalcitrantes a la transformación genética mediada por *Agrobacterium*, silenciando genes que actúan como represores [142].

Una de las aplicaciones que se ha desarrollado más recientemente para este tipo de vectores virales consiste en introducir miRNAs artificiales o de la planta para determinar su función [133].

La utilización de la técnica VIGS presenta ventajas sobre la mutagénesis o la transformación genética ya que permite ensayar la función de numerosos genes en un corto periodo de tiempo. Esto es especialmente crítico en el caso de los cítricos, que poseen largos periodos juveniles (normalmente 6-8 años), donde la transformación de plantas adultas es muy difícil y son necesarios largos periodos de tiempo entre la transformación y la fructificación. Otra ventaja es que permiten estudiar la función de genes que son esenciales para el crecimiento o el desarrollo de la planta; mientras que en los estudios mediante mutagénesis o transformación genética, la falta de función hace que la planta pueda morir durante la germinación o la regeneración, con el vector viral el RNA diana sólo se silencia cuando el virus infecta la planta.

Por otro lado, esta tecnología también presenta algunos inconvenientes. Por ejemplo, en la mayoría de los casos no se puede inhibir por completo la expresión del gen y la expresión residual puede ser suficiente para llevar a cabo su función. En segundo lugar la eficiencia y el fenotipo pueden variar entre plantas o

réplicas de experimentos, por lo que es importante el uso de genes marcadores de silenciamiento con un fenotipo visible. Algunos de los genes más utilizados como marcadores son la fitoeno desaturasa (*pds*), una enzima implicada en la biosíntesis de carotenoides que protegen a la clorofila de la foto-oxidación; el gen *sulfur* (*su*), que codifica para una subunidad de la quelatasa de magnesio necesaria para la producción de clorofila; o la chalcona sintetasa (*chs*), que es una enzima de la ruta de biosíntesis de pigmentos flavonoides.

4.2 Vectores virales para la expresión de proteínas

Entre los sistemas que se usan para expresar proteínas recombinantes en plantas se encuentran la transformación estable de plantas completas, de microalgas o de cultivos *in vitro* de células o tejidos de plantas y el uso de vectores virales [143] (Fig. 2).

La transformación estable desencadena una expresión constitutiva de los genes transformados. El proceso consiste en introducir el gen de interés en el T-DNA de un plásmido binario. Al inocular las plantas con un cultivo de *A. tumefaciens* transformado con el plásmido, el T-DNA se transfiere y se integra en el genoma nuclear o cloroplástico de las células vegetales, expresándose la proteína de interés [144].

Los vectores virales se han desarrollado para aprovechar la capacidad que tienen los virus de dirigir los recursos de la célula huésped para la expresión en grandes cantidades de las proteínas codificadas en su genoma, y convertir esta habilidad en una herramienta para producir proteínas de interés.

Los métodos de expresión basados en la transformación estable presentan limitaciones, como el tiempo necesario para obtener líneas

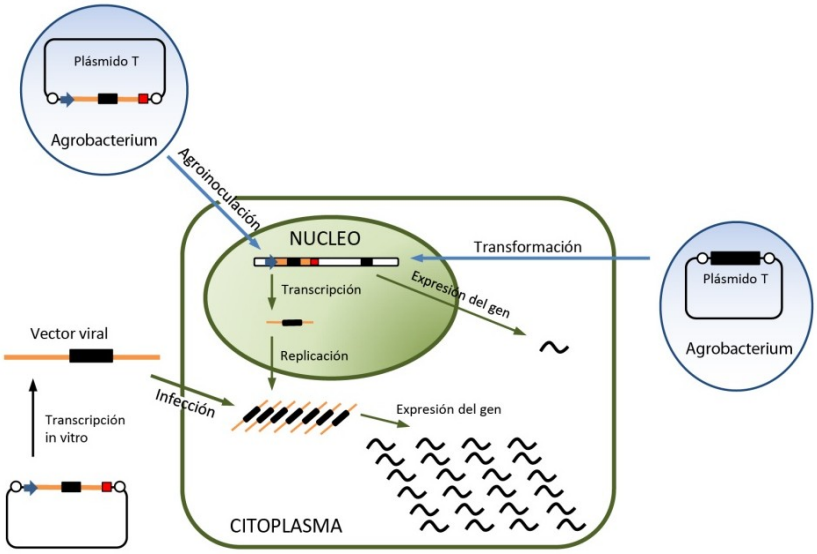

Figura 2. Esquema de las diferentes estrategias para expresar proteínas en plantas. Comparación de los niveles de expresión (línea curva) del gen introducido (rectángulo negro), de un transgén (rectángulo negro en el cromosoma blanco) con la expresión desde un vector viral bien por inoculación de transcritos o bien mediante agroinoculación. Adaptado de Scholthof y col. [145] y ampliado.

transgénicas o el bajo rendimiento en la expresión de la proteína de interés, a diferencia de los altos niveles de expresión que se obtienen mediante el uso de vectores virales (Fig. 2). Otra ventaja de los virus es que se puede inocular rápidamente un gran número de plantas de uno o varios huéspedes diferentes. Además, el máximo nivel de expresión de la proteína heteróloga desde el vector se produce en un corto periodo de tiempo, normalmente en la primera o segunda semana después de la inoculación. Sin embargo, el rendimiento de la expresión del gen introducido puede verse disminuido por el efecto del PTGS de la planta en respuesta a la infección viral. Este inconveniente se puede solventar coinoculando las plantas con un plásmido que exprese un supresor de silenciamiento viral. Se ha demostrado que la expresión simultánea del supresor p19 de TBSV en los tejidos

agroinoculados previene el PTGS y permite obtener niveles de expresión mayores [146].

El primer vector viral de expresión en plantas se construyó a partir del genoma del virus del mosaico de la coliflor (*Cauliflower mosaic virus*, CaMV), el único virus de plantas con un genoma de DNA de doble hebra. Este vector presentaba limitaciones en la capacidad de empaquetado y no admitía secuencias exógenas mayores de 250 pb [147]. Los avances en la biología molecular, como la posibilidad de sintetizar DNA complementario (cDNA) a partir de un molde de RNA, permitieron buscar nuevos modelos de expresión en el grupo de virus de plantas más abundante en la naturaleza, los virus de RNA de cadena sencilla. La construcción del primer clon infectivo de un virus de RNA de cadena sencilla y positiva marcó el comienzo del desarrollo de los vectores virales [148].

Los vectores virales de expresión se han utilizado para obtener proteínas de interés biomédico, como anticuerpos [149-151] y antígenos para fabricar vacunas para humanos y animales [152-160]. En el ámbito de la agricultura los vectores virales se emplean para expresar genes que controlan caracteres de interés de la planta [161-163]. Una de las aplicaciones más recientes es en el campo de la bionanotecnología, donde los vectores se utilizan para expresar biopolímeros y nanopartículas [164-167].

4.2.1 Tipos de vectores de expresión

Existen varios tipos de vectores virales que se diferencian en la estrategia que emplean para expresar las proteínas de interés. Los vectores de primera generación son vectores de inserción y se caracterizan por el uso de un virus completamente funcional. Los vectores de segunda generación se pueden clasificar en vectores de sustitución, modulares y de fusión [159, 168].

a. Vectores de inserción

Los vectores de primera generación o de inserción están formados por virus completos a los que se les añade un marco de lectura abierta suplementario (*Open* Reading Frame, ORF). Para su formación se requiere la duplicación del promotor de un sgRNA del virus dentro de su genoma seguida de la inserción de la secuencia del gen candidato, lo que da lugar a la formación de un nuevo sgRNA a partir del cual se expresa la nueva proteína. Otra estrategia es la fusión del gen de interés a un ORF del virus y la duplicación de una zona de corte entre ambos que es reconocida por una proteasa liberando la proteína insertada. En ambos casos la funcionalidad del virus se mantiene intacta pero tiene el inconveniente de aumentar el tamaño del genoma, lo que puede dificultar la multiplicación y encapsidación de los viriones. Además, pueden surgir recombinaciones no homólogas causando la pérdida del gen introducido, un problema que se puede reducir utilizando promotores de otros virus del mismo género.

Siguiendo esta estrategia se han obtenido vectores de inserción, entre otros, con TMV [169, 170], con el virus X de la patata (PVX) [157, 171], con el virus A de la vid (GVA) [172], el virus del moteado de la vaina de la judía (BPMV) [97] o el virus del mosaico amarillo del calabacín (*Zucchini yellow mosaic virus*, ZYMV), éste último construido sobre un virus atenuado con una mutación en el supresor de silenciamiento para obtener un vector no transmisible a otras plantas de forma natural [173].

b. Vectores de sustitución

Con el fin de solventar los inconvenientes que presentan los vectores de primera generación se desarrollaron nuevas estrategias para expresar proteínas desde la secuencia de un virus. Los vectores de sustitución o reemplazamiento se

basan en el cambio de una secuencia endógena del virus por un gen de interés. Esta aproximación se usó por primera vez con éxito para el CaMV [174] y posteriormente con un vector basado en el TBSV, donde la sustitución del ORF de la proteína de la cápsida (CP) por un gen heterólogo permitió el movimiento y la infección del vector viral [175]. Sin embargo, la CP normalmente es necesaria para el movimiento célula a célula y/o a larga distancia y la infección del vector viral [176, 177]. Así, en un vector basado en el virus del mosaico del bromo (BMV) en el que se reemplazó la CP por un gen heterólogo, se perdió la capacidad de movimiento célula a célula en la hoja inoculada [178], y en otros casos, aunque los vectores conservaban la movilidad célula a célula, no podían moverse sistémicamente [169, 179]. En ocasiones se ha eliminado la proteína de movimiento (MP) y transmisión, lo que disminuye el riesgo ambiental de escape del virus modificado a otras plantas dentro o fuera del invernadero [180]. Esta estrategia se ha empleado, por ejemplo, con el virus del mosaico del tabaco (TMV) [181].

Los vectores virales se pueden emplear para expresar varias proteínas diferentes en una misma célula pero este proceso puede ser ineficiente, probablemente debido a la competencia entre los virus homólogos que llevan en su secuencia los genes de interés. Este problema se puede resolver utilizando dos sistemas basados en diferentes virus que no compitan entre ellos, por ejemplo TMV y PVX, expresando una proteína distinta desde cada uno de ellos [182]. Otra estrategia para expresar dos proteínas en la misma célula con el mismo virus se empleó con un sistema de dos componentes virales basado en TMV, uno era defectivo de la replicasa y la MP y el otro era defectivo de la CP. En este sistema un virus depende del otro y ambos expresan proteínas heterólogas diferentes en sustitución de sus componentes [183].

c. Vectores modulares

Otra estrategia empleada se basa en separar los componentes del virus e insertarlos en distintos plásmidos binarios que se agroinoculan simultáneamente en la planta mediante distintas cepas de *Agrobacterium*. Los elementos menos importantes para la expresión del virus se aportan en *trans* y el gen de interés se clona en uno de los módulos del virus. Posteriormente los distintos componentes se ensamblan dentro de la célula con la ayuda de una recombinasa específica de secuencia [184-186].

El primer vector modular de un virus de plantas está basado en el virus del mosaico del guisante pinto (*Cowpea mosaic virus*, CPMV) [187]. Recientemente, se ha publicado una revisión de la construcción de vectores virales de CPMV mediante esta y otras estrategias y sus aplicaciones [188]. Otro virus muy utilizado en este tipo de vectores es TMV [184, 185]. También se ha usado esta estrategia con el virus del mosaico de la alternanthera (*Alternanthera mosaic virus,* AltMV) [186].

En ocasiones, alguno de los genes del virus se expresa como un transgén. Por ejemplo, un vector basado en PVX requiere de una planta transgénica que expresa la MP del virus para que el vector infecte sistémicamente y exprese la proteína introducida en lugar de la MP [189]. De esta forma se reduce el riesgo medioambiental de dispersión del virus modificado.

d. Vectores de fusión

Los vectores virales de fusión están diseñados para proporcionar un anclaje molecular a diversos tipos de péptidos que se fusionan a la CP del virus y así quedan expuestos en la superficie de los viriones quiméricos [190, 191]. Es importante que estas fusiones sean compatibles con la funcionalidad del virus. El

aprovechamiento de la geometría repetitiva de las cápsidas de virus de plantas ha incrementado el potencial de estos vectores para su uso como vacunas ya que se pueden anclar multitud de copias de secuencias de antígenos. Distintos virus han sido utilizados como plataformas para la fusión y exposición de péptidos en su CP, como CPMV [187, 192], TMV [193], PVX [194], el virus del mosaico del tomate (*Tomato mosaic virus,* ToMV) [195], los virus del mosaico del pepino (*Pepino mosaic virus*, PepMV [107] y CMV [196]) o el virus A de la necrosis del tabaco (*Tobacco necrosis virus A*, TNV-A) [197].

5. Movimiento de los virus en la planta

Para que un virus infecte sistémicamente una planta tiene que replicarse en las células infectadas, moverse a células contiguas y alcanzar zonas lejanas de la planta a través de los haces vasculares.

El movimiento célula a célula de los virus se realiza a través de unos canales simplásticos, los plasmodesmos, que cruzan la rígida pared celular y proporcionan continuidad entre células a lo largo de toda la planta. Dado que el tamaño de exclusión molecular de estos canales es muy inferior al tamaño de los virus, éstos han evolucionado produciendo unas proteínas, llamadas proteínas de movimiento (MPs), que interaccionan con los plasmodesmos para permitir el paso de la partícula viral y/o de su genoma [198]. En algunos virus además de la proteína de movimiento son necesarias otras proteínas codificadas por el mismo virus como la CP.

Para que un virus se mueva a larga distancia debe alcanzar el tejido vascular. Es bien sabido que los virus siguen el mismo camino que las sustancias sintetizadas en las hojas para ser transportadas al resto de la planta. Las venas de las hojas son funcionalmente distintas dependiendo del estado fisiológico y de

desarrollo. Trabajos realizados con TMV y con CPMV han demostrado que las venas mayores y menores son puntos de entrada de los virus al floema para establecer la infección en las hojas superiores [199, 200].

Para estudiar el movimiento de los virus dentro de las plantas se han utilizado distintos métodos como la microscopía electrónica o técnicas de detección *in situ* del RNA viral o de proteínas codificadas por el virus [201-203]. Estas técnicas son destructivas y no permiten la observación del movimiento del virus a tiempo real. La utilización de virus marcados con la proteína verde fluorescente de medusa (*Green Fluorescent Protein*, GFP) permite estudiar cómo se mueven los viriones por la planta mediante observación al microscopio [204]. Recientemente se ha desarrollado otro sistema que consiste en expresar desde el virus el factor de transcripcion MYB *Rosea1* de *Antirrhinum majus*, que activa la ruta de los antocianos. La expresión de este factor de transcripción induce una coloración roja donde se está multiplicando el virus, lo que permite estudiar el movimiento viral a simple vista [205].

6. El virus del manchado foliar de los cítricos

6.1 Sintomatología, gama de huéspedes, transmisión e incidencia

El virus del manchado foliar de los cítricos (*Citrus leaf blotch virus*, CLBV) se detectó por primera vez en un kumquat Nagami (*Fortunella magarita* (Lour.) Swing.) que procedía de Córcega (clon SRA-153). Este clon mostraba síntomas de mala unión de injerto sobre el patrón citrange Troyer (*Citrus sinensis* (L.) Osb. x *P. trifoliata* (L.) Raf.), pero no sobre limonero rugoso (*C. jambhiri* Lush) [206]. Se realizaron ensayos para caracterizar biológicamente esta nueva enfermedad mediante la inoculación por injerto de fragmentos de corteza del clon SRA-153 en

distintas especies de cítricos. Los síntomas observados fueron acanaladuras en la madera en cidro Etrog (*C. medica* L.), clorosis nervial en las hojas de naranjo amargo, pomelo Marsh, tangelo Orlando (*C. paradisi* x *C. reticulata*), naranjo dulce Pineapple, citrange Troyer y tangor Dweet (*C. reticulata* x *C. sinensis*), y manchado clorótico en hojas de tangor Dweet [206, 207].

Con el objetivo de saber si este patógeno producía mala unión patrón/injerto en otras variedades comerciales de cítricos se inocularon plantas de clementino de Nules, limonero Eureka, pomelo Marsh y naranjo dulce Pineapple con el clon SRA-153 y se propagaron sobre citrange Troyer. Las plantas de clementino y limonero mostraron mala unión con el patrón a los seis meses de la propagación mientras que en pomelo y naranjo dulce la unión era normal [207]. Además, las variedades que mostraban síntomas de mala unión también inducían clorosis nervial en naranjo dulce Pineapple, manchado foliar en tangor Dweet y acanaladuras en la madera en cidro Etrog, mientras que las variedades que no presentaban mala unión sólo inducían manchado foliar en tangor Dweet y acanaladuras en la madera en cidro Etrog. Estos resultados y otros sugieren que en el kumquat existían dos virus o cepas del mismo virus y que uno/a de ellos/as se eliminaba al pasar por naranjo dulce o pomelo.

En los años previos al inicio de esta tesis se desarrolló un clon infeccioso del genoma completo de CLBV (IC-CLBV) a partir del aislado SRA-153. Dicho clon induce acanaladuras en la madera en cidro Etrog y manchado foliar en hojas de tangor Dweet (Fig. 3), pero no induce clorosis nervial en hojas de naranjo dulce Pineapple ni mala unión patrón/injerto al propagarse sobre citrange. Estos resultados indican que CLBV sólo es responsable de la inducción de los síntomas en cidro Etrog y tangor Dweet, y que la mala unión sobre citrange y la clorosis nervial en hojas de naranjo dulce están causadas por otro patógeno o por la interacción de CLBV y otro factor biótico [208].

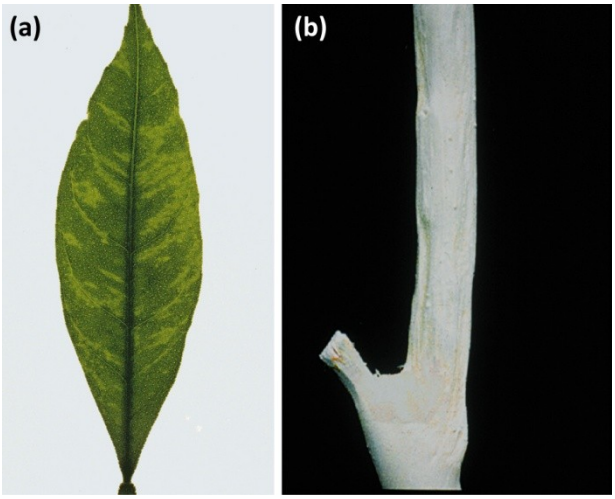

Figura 3. Síntomas causados por el aislado SRA-153 de CLBV. (a) Manchado foliar en hojas de tangor Dweet. (b) Acanaladuras en la madera en cidro Etrog. Adaptada de Galipienso y col. [207].

CLBV infecta todas las especies y variedades de cítricos ensayadas pero sólo induce síntomas en cidro Etrog y tangor Dweet, especies que no son comerciales en España. Además, se ha conseguido transmitir a los huéspedes experimentales *N. benthamiana*, *N. occidentalis* y *N. cavicola* [209], en los que tampoco induce síntomas.

El virus se trasmite de forma natural por injerto, al utilizar yemas o fragmentos de corteza infectados y también por semilla, aunque en una proporción muy baja, de poco más del 2% [210]. Experimentalmente puede transmitirse a cítricos sanos mediante cortes en el tallo realizados con una cuchilla mojada con extracto de viriones semipurificados de planta infectada. No se tienen evidencias de su trasmisión por vectores [208].

CLBV se ha detectado en España [207, 211, 212], Japón, Florida, Australia, Italia [213] y Nueva Zelanda [214], en árboles que presentaban mala unión del

injerto con patrones trifoliados. Se desconoce la importancia económica del virus ya que no se ha determinado su incidencia en las distintas regiones geográficas donde se ha localizado y tampoco se ha podido demostrar su participación, junto a otros patógenos, en la incompatibilidad de ciertas variedades de cítricos sobre patrones trifoliados [215].

6.2 Estructura y características moleculares

CLBV pertenece a la familia *Betaflexiviridae* [216, 217] y es la especie tipo del género *Citrivirus* [218]. Los viriones tienen morfología filamentosa, con una longitud media de 960 nm y un diámetro de 14 nm. Están formados por una molécula de RNA monocatenario de polaridad positiva, de 8747 nt y unidades de la proteína de cubierta de 41 KDa (Fig. 4).

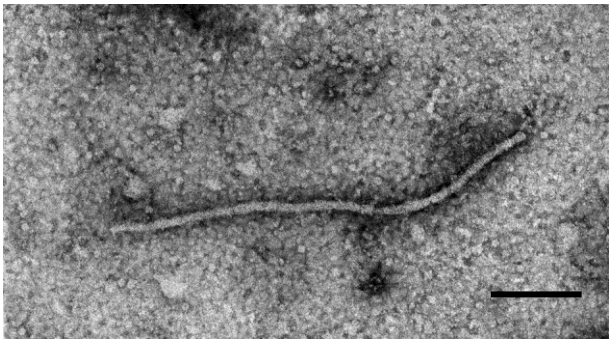

Figura 4. Partícula viral de CLBV purificada a partir de kumquat SRA-153. Tinción con acetato de uranilo al 1%. La barra corresponde a 200nm. Adaptada de Galipienso y col. [211].

El genoma del virus contiene 3 ORFs y dos regiones no codificantes (*UnTraslated Regions*, UTRs) en los extremos 5' (73nt) y 3' (541 nt). El ORF 1,

codifica una poliproteína de 227KDa que contiene los dominios metil-transferasa, AlkB y OTu peptidasas, proteasa, helicasa y RNA polimerasa dependiente de RNA (RdRp), conservados en el grupo de los alfavirus. El ORF 2 codifica la MP de 40 KDa, que tiene similitud de secuencia a las proteínas de movimiento de la "superfamilia 30K", y al ser mutada el virus pierde la capacidad de infectar sistémicamente la planta sin que se vea afectada la replicación [177]. Por último, el ORF 3, separado del ORF 2 por una zona intergénica de 64 nt, codifica la CP de 41 KDa [218]. En los mutantes con CP truncada el virus no se replica más allá de 12 días después de la inoculación, probablemente debido a que el RNA viral queda más expuesto a la maquinaria de silenciamiento [177].

El ORF 1 se traduce directamente a partir del gRNA, mientras que los genes de MP y CP se expresan a partir de sendos sgRNA 3' coterminales que se transcriben desde la cadena negativa del sgRNA por acción de la RdRp viral. Además, el virus sintentiza 2 sgRNA 5' coterminales cuya función es desconocida [219] (Fig. 5). Los sgRNA 3' MP y CP se encapsidan, tiene en su extremo 5' una UTR de 123 y 284 nt, respectivamente, y al igual que el gRNA, comienzan con el hexanucleótido GAAAAG [219]. Mediante experimentos de mutaciones puntuales se confirmó que el inicio de transcripción del sgRNA 3' CP está situado en la primera G del hexanucleótido (+ 1) y debe estar implicada en el reconocimiento por la RdRp viral [177]. En este mismo trabajo se mapeó la zona promotora del sgRNA 3' CP, quedando delimitada entre los nucleótidos -67 y +50 respecto al inicio de trascripción.

La MP del virus se ha identificado como un supresor del silenciamiento débil en comparación a otros supresores de virus bien caracterizados. Esta proteína es capaz de suprimir el silenciamiento intracelular pero no a larga distancia. Aunque no se conoce el mecanismo por el cual interfiere en la ruta del silenciamiento, se sabe que actúa después de la generación de los siRNAs [220].

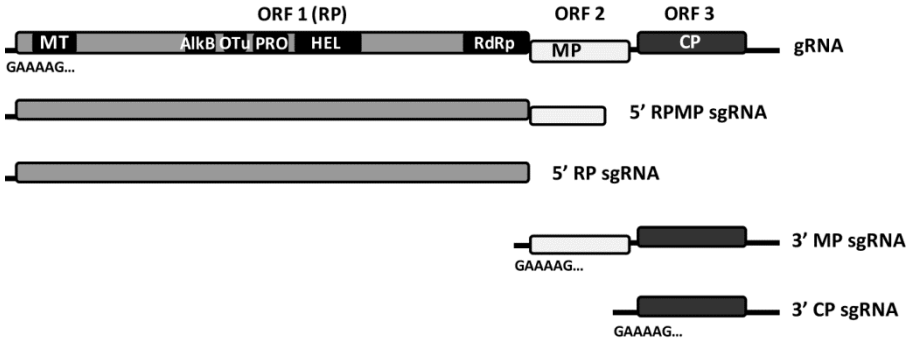

Figura 5. Representación esquemática de la organización genómica del gRNA y de los sgRNAs de CLBV. Los rectángulos representan los ORFs, indicándose en su interior la proteína o dominios que codifican: RP, replicasa; MT, metiltransferasa; AlkB y OTu, peptidasas del tipo AlkB y OTu; PRO, protesasa; HEL, helicasa; RdRp, RNA polimerasa dependiente de RNA; MP, proteína de movimiento; CP, proteína de cápsida. Se representan los sgRNA 5' coterminales (5'RPMP y 5'RP) y los sgRNA 3' coterminales (3'MP y 3'CP).

6.3 Técnicas de diagnóstico

Para detectar el virus en plantas infectadas se han desarrollado métodos biológicos y métodos moleculares. Como se ha indicado anteriormente, CLBV induce síntomas en tangor Dweet y cidro Etrog, por lo que estas dos especies se pueden utilizar para el diagnóstico biológico del virus mediante su inoculación con material infectado. Debido a que el síntoma de moteado clorótico en tangor Dweet es más fácil de observar y aparece más pronto que las acanaladuras en la madera en cidro Etrog, este ensayo es el que se utiliza para el diagnóstico rutinario del virus [207].

Los métodos moleculares desarrollados se basan en la detección de la CP o del RNA viral. A partir de la CP expresada en bacterias se preparó un suero y el virus se pudo detectar mediante Western blot utilizando extractos semipurificados de viriones, aunque no se detectó mediante ELISA utilizando

extractos vegetales, por lo que su uso para el diagnóstico rutinario de la enfermedad es muy limitado [218].

La disponibilidad de la secuencia del genoma de CLBV permitió el desarrollo de técnicas de diagnóstico basadas en la detección del RNA viral mediante hibridación molecular (HM) o mediante transcripción reversa y amplificación mediante la reacción en cadena de la polimerasa (RT-PCR) [221]. La HM con sondas de cDNA marcadas con digoxigenina permite la detección del virus en plantas utilizando improntas de tejido o extractos de RNA total o de dsRNA de brotes jóvenes. La técnica de RT-PCR es más sensible que la HM y permite la detección a partir de cualquier tejido infectado. Estos métodos permiten el diagnóstico fiable del virus de numerosas variedades de cítricos, sin embargo, en otras, como el naranjo dulce Pineapple, el virus se distribuye irregularmente dentro de la planta y sólo se detecta en un 20% de las muestras analizadas, por lo que es necesario utilizar un mínimo de diez muestras individuales por árbol para un diagnóstico fiable. Además, en condiciones de campo la detección de CLBV es menos consistente que en invernadero debido a que el título viral es más bajo y la distribución más errática. Así, en variedades como el naranjo dulce cv. Navelina, el virus se detecta en la mayoría de las muestras analizadas de plantas cultivadas en invernadero tanto por HM como RT-PCR, pero en condiciones de campo sólo dan reacción positiva entre el 10 y el 20% de las muestras analizadas de un mismo árbol infectado. Por este motivo se puso a punto la técnica de RT-PCR a tiempo real para la detección del virus, usando sondas *Taqman*. Esta técnica resulta 1000 veces más sensible que la RT-PCR convencional y es la técnica que se está empleando actualmente para la detección rutinaria de CLBV [222].

JUSTIFICACIÓN Y OBJETIVOS

Los cítricos constituyen el principal cultivo frutal en España, con una superficie total cultivada de unas 291.000 Ha y una producción superior a los 5 millones de toneladas. Actualmente, más del 50% de la producción se dedica a la exportación, lo que convierte a los cítricos en una de las principales fuentes de entrada de divisas. Sin embargo, la competencia en los mercados está aumentando de forma espectacular en los últimos años debido a distintos factores, entre los que se incluyen la mejora de la calidad en la producción de países terceros, la mayor eficacia y rapidez de los medios de transporte marítimo a largas distancias y la apertura de los mercados como consecuencia de los acuerdos sobre globalización y la P.A.C. de la Unión Europea.

El mantenimiento de la producción y competitividad de este sector depende en gran medida de la disponibilidad de material vegetal de alta calidad, tanto en variedades como en patrones, por lo que estos aspectos tienen un carácter estratégico para nuestra citricultura. La mayoría de las variedades de cítricos que se cultivan actualmente se han obtenido a lo largo de la historia a partir de mutaciones espontáneas y sólo unas pocas mediante cruzamientos. La compleja biología reproductiva de los cítricos, su elevada heterozigosis, su dilatado período juvenil (hasta 6-8 años) y la falta de marcadores moleculares apropiados complican la mejora de cítricos por métodos clásicos.

Para acelerar el proceso de mejora de cítricos se puede emplear la aproximación genómica, cuyo objetivo es la identificación de genes responsables de caracteres de interés agronómico. En los últimos años el número de secuencias disponibles de distintos organismos se ha incrementado exponencialmente debido al desarrollo de plataformas de secuenciación masiva. En cítricos se dispone de una colección de 550.000 ESTs (Expressed sequence tags) (http://www.citrusgenome.ucr.edu/) y de la secuencia completa de dos especies

(*Citrus clementina* y *C. sinensis*) (http://www.phytozome.net/). La posible función de algunos genes se ha inferido por homología con genes de otros organismos, pero muchos de ellos no presentan homología con otros genes conocidos. Con estas secuencias se han fabricado varias micromatrices de cDNAs que se están usando para definir patrones de expresión génica en distintos tejidos, estadios del desarrollo y como respuesta a diversos tratamientos de estrés biótico (virus, viroides u hongos) o abiótico (salinidad, sequía, etc.).

La disponibilidad de secuencias y de perfiles de expresión génica permite la identificación de genes candidatos a participar en un determinado proceso biológico, pero no aporta información sobre su función ni identifica cuáles son los genes responsables de un determinado proceso. El objetivo último de cualquier proyecto de genómica es asociar cada gen del genoma con un fenotipo o función. Aquellos genes que se consiga identificar como responsables de caracteres de interés agronómico se podrían utilizar para expresarlos en plantas mediante transformación genética o mediante el uso de vectores virales, y además se podrían utilizar como marcadores en programas de mejora genética clásica.

En los últimos años se ha desarrollado una estrategia muy atractiva para determinar la función de genes que consiste en el empleo de vectores virales. Esta tecnología se basa en la capacidad que tienen los virus para inducir el mecanismo de silenciamieno génico postranscripcional (VIGS). La utilización de vectores virales tiene ventajas sobre la mutagénesis o la transformación genética, ya que permite ensayar la función de numerosos genes en un corto periodo de tiempo. Esto es especialmente crítico en el caso de los cítricos, que poseen largos periodos juveniles (normalmente 6-8 años) y donde la transformación de plantas adultas es muy difícil. Otra ventaja es que permite estudiar la función de genes que son esenciales para el crecimiento o el desarrollo de la planta. En los ensayos clásicos mediante mutagénesis o transformación genética, la falta de función de estos genes hace que la planta pueda morir durante la germinación o la regeneración.

Sin embargo, con los vectores virales, el RNA diana sólo se silencia cuando el virus infecta la planta.

Los vectores virales también se pueden usar para expresar genes de interés para la alimentación (vitaminas, compuestos cardioprotectores, etc.), para producir compuestos farmacéuticos (vacunas, anticuerpos, etc.) y materiales que se pueden emplear en nanotecnología, para obtener plantas resistentes frente a patógenos o herbicidas y para estudiar la función de una proteína.

El objetivo general de esta tesis es el desarrollo de vectores virales eficientes para la expresión o el silenciamiento de genes en cítricos, basados en el virus del manchado foliar de los cítricos (CLBV) que se puedan utilizar como una herramienta genética para determinar la función de genes útiles para la mejora de este cultivo.

Al comienzo de la tesis, Folimonov y colaboradores [223] habían desarrollado un vector viral para cítricos basado en el virus de la tristeza de los cítricos (CTV), que permitía expresar proteínas desde su genoma pero que no servía para estudiar la función de genes mediante VIGS. Por este motivo se analizaron las ventajas que presentaría un vector basado en CLBV sobre el vector de CTV: 1) CLBV infecta a todas las especies y variedades de cítricos ensayadas sin inducir síntomas en la mayoría de ellas, lo que evitaría interferencias con la expresión fenotípica del gen o genes silenciados. 2) CLBV multiplica en todos los tejidos de la planta, al contrario de lo que ocurre con CTV, que está limitado a células del floema. 3) CLBV es capaz de invadir meristemos, lo que lo hace especialmente interesante para el estudio de la expresión de genes implicados en el desarrollo de hojas y frutos. 4) CLBV no se transmite por vectores, por lo que podría ser usado sin peligro en futuros experimentos de campo.

La eficiencia de un vector viral depende principalmente de la capacidad que tiene el virus para invadir un huésped y acumularse hasta alcanzar un nivel

umbral que sea suficiente para inducir la expresión o el silenciamiento del gen a estudiar. Para que un vector viral pueda ser útil en cítricos tiene que ser lo suficientemente estable como para que mantenga su funcionalidad a lo largo de una brotación o del ciclo reproductivo de la planta, procesos que suelen durar desde algunos meses hasta varios años. Los vectores virales tienen tendencia a ser inestables, produciéndose eventos de recombinación que inducen la pérdida de la secuencia insertada y por lo tanto de su función. Por ello, para optimizar la eficiencia de un vector es necesario conocer los factores que afectan a su estabilidad. Así mismo, es necesario estudiar los niveles de multiplicación del vector en los distintos tejidos de la planta.

En consecuencia, los objetivos establecidos para la tesis doctoral fueron los siguientes:

1) Desarrollo de vectores virales basados en el genoma del virus del manchado foliar de los cítricos para la expresión o silenciamiento de genes en cítricos.

2) Estudio del movimiento y distribución de CLBV en plantas de *N. benthamiana* y cítricos.

3) Ensayo de la eficiencia de los vectores desarrollados para el silenciamiento de genes en *N. benthamiana* y cítricos.

CAPÍTULO 1:

Desarrollo de vectores virales basados en CLBV para la expresión de proteínas o silenciamiento de genes en cítricos.

Adaptado del artículo:

Agüero J, Ruiz-Ruiz S, Vives MC, Velázquez K, Navarro L, Peña L, Moreno P, and Guerri J (2012) Development of viral vectors based on *Citrus leaf blotch virus* to express foreign proteins or analyze gene function in citrus plants. Mol.Plant-Microbe Interact. **25,** 1326-1337.

1. Introduction

Viral vectors have been used to express valuable proteins in plants [159, 168, 224, 225], but they can also be a helpful tool to study the function of host genes by reverse genetics. When a viral vector carries a sequence of an endogenous plant gene, virus infection triggers degradation of the cognate plant mRNA through an homology-dependent RNA degradation mechanism known as virus-induced gene silencing (VIGS), that results in a loss of function phenotype of the host [110]. VIGS technology has been used to knock down expression of either transgenes or endogenous genes in reverse genetic studies of a variety of plant biology processes [81, 82, 226, 227].

Citrus is a major commodity in fruit crop production in the world. The complex reproductive biology of citrus trees due to apomixis and sexual incompatibility between varieties, long juvenile period (often more than 6 years) and the lack of knowledge on genes regulating different functions, have hindered genetic improvement by traditional breeding methods. This might be speeded up by genomic approaches aimed at identifying genes responsible for relevant agronomic characters that could be used for genetic transformation or as molecular markers in conventional breeding programs. In the last few years, genome-wide expressed sequence tag (EST) collections have been generated [8] and microarray analysis has been used to identify gene expression patterns in different tissues or at different developmental stages [10-13], in various genotypes [15], or in response to abiotic or biotic stress situations [16-18]. Availability of sequences and gene-expression patterns may help identify candidate genes potentially involved in a particular biological process; however, further analyses are required to associate each gene with a specific phenotype or biological function, the basic information necessary for any improvement program. While confirmation of gene function often requires extensive genetic

screening or costly experiments of plant transformation and transgenic line characterization, this process can be simplified by the use of VIGS.

The use of viral vectors to evaluate plant gene functions is particularly attractive for woody species like citrus, in which analysis of certain traits like flowering and fruiting by conventional breeding is hampered by their long juvenile period, and genetic transformation of adult plants is difficult [3].

Citrus leaf blotch virus (CLBV), the type member of the genus *Citrivirus*, family *Betaflexiviridae* [216, 217], has filamentous virions about 960x14 nm in size. Virions are composed of a single-stranded, positive-sense genomic RNA (gRNA) of 8,747 nucleotides (nt) and a 41-kDa coat protein (CP) [211, 218]. The CLBV gRNA has three open reading frames (ORFs) and untranslated regions (UTRs) at the 5´ (73 nt) and 3´ (541 nt) ends. The ORF1, encoding a ~227-kDa polyprotein with methyl-transferase, AlkB-like, OTu-like peptidase, papain-like protease, helicase and RNA-dependent RNA polymerase motifs, is translated directly from the gRNA. The ORF 2, encoding a ~40-kDa protein with a motif characteristic of cell-to-cell movement proteins (MP) of the 30K superfamily, and the ORF3, encoding the CP, are translated from two 3' co-terminal subgenomic RNAs (MP sgRNA and CP sgRNA, respectively). ORFs 2 and 3 are separated by a 64-nt intergenic region [177, 218, 219].

The objective of this work was developing efficient and stable CLBV-based viral vectors for either gene silencing or protein expression that could be used to identify gene functions helpful for citrus genetic improvement. We developed different viral vectors by engineering a *Pml*I restriction site at two different positions in the CLBV gRNA: at the 3' UTR downstream of the stop codon of the CP gene, or at the intergenic region between the MP and the CP ORFs. For this purpose we used a full–length cDNA clone of the CLBV genome (CLBV-IC) that was shown to be infectious upon agroinoculation to *Nicotiana benthamiana* or citrus

plants [208]. We also duplicated a minimum 92-bp promoter derived from the native promoter of the CP sgRNA in both cloning sites to express foreign genes or gene fragments by formation of new sgRNAs. In the 3' UTR position, transcription of the new sgRNA was induced by the duplicated promoter, whereas in the MP-CP intergenic region transcription of the new sgRNA was induced by the native promoter of the CP sgRNA and CP expression was controlled by the duplicated promoter. The efficiency and stability of these vectors was assessed by inserting the green fluorescent protein (GFP) gene or fragments of the phytoene desaturase (*pds*), a subunit of the magnesium chelatase (*su*) or *gfp* genes in the corresponding cloning site, agroinoculating *N. benthamiana* plants with the new constructs and using the virions produced in this host to slash inoculate citrus plants. While constructs carrying the *pds* or *su* fragments triggered RNA silencing of the cognate genes and incited a silencing phenotype in normal citrus tissues, the vectors carrying *gfp* or fragments thereof incited loss of fluorescence in GFP-expressing transgenic citrus plants.

2. Results

2.1 Infectivity of CLBV-based vectors

Although citrus is the natural host of CLBV, this virus also infects *N. benthamiana* plants, thus enabling use of this herbaceous host for preliminary testing the efficiency and stability of CLBV-based vectors before their application on citrus plants. Using CLBV as viral vector requires insertion of foreign sequences into the CLBV genome at positions that do not compromise viral infectivity. We first tried to insert these sequences at the 3' UTR immediately downstream of the CP gene, and for this purpose a unique *Pml*I restriction site was added just after the stop codon of the ORF 3 in the CLBV-IC clone to create the *clbv3'* vector. Real time RT-PCR analysis [222] of *N. benthamiana* plants agroinoculated with this

construct showed that CLBV gRNA accumulation was similar to that observed in plants agroinoculated with the CLBV-IC clone (hereafter referred to as wild type, WT), indicating that insertion of a *Pml*I restriction site at this position did not affect viral infectivity.

In order to obtain a viral vector able to express foreign genes efficiently, a duplicate of the CP sgRNA promoter was inserted at the *Pml*I site of *clbv3´*. Previously we mapped the 5´ end of the CP sgRNA at position 6,831 of the gRNA, 284 nt upstream of the start codon of the CP gene [219], and delimited boundaries of the CP sgRNA promoter in its natural context at positions -67 and +50 around the transcription start site (+1) [177]. To further confirm these results and determine the minimal sequence able to promote RNA transcription in the new genomic context we cloned fragments of different size around the transcription start site of the CP sgRNA into the *Pml*I site of *clbv3´*, keeping a new *Pml*I site downstream of the duplicated promoter. Then a 154-nt fragment of the *gfp* gene was inserted into the new *Pml*I restriction site of each construct (Fig. 1A). In a first group of constructs, the 5´ boundary of the duplicated promoter was mapped using a constant 3´ terminus at position +284 and variable 5´ termini between positions -136 and -25 (Fig 1B, left panel). These potential promoter sequences were PCR-amplified from the clone CLBV-IC using appropriate primers, cloned into the *Pml*I site of *clbv3'* and, after adding the *gfp* fragment (Fig.1A), the new vectors were agroinoculated in *N. benthamiana* leaves. At 16 days post-inoculation (dpi), northern blot analysis of total RNA (RNAt), using a DIG riboprobe specific for the CLBV 3' UTR, revealed the presence of a new 3' co-terminal sgRNA of the expected size in leaves agroinoculated with vectors containing duplicated promoter fragments with their 5' terminus between positions -136 and -42, but not in leaves agroinoculated with the vector carrying the -25/+284 duplicated promoter (Fig. 1B, left panel). Therefore, the 5´ boundary of a fully active CP sgRNA promoter in the new genomic context was located between positions -42

and -25 upstream of the transcription start site. Due to the location of the inserted fragments, the gRNA and the two natural sgRNAs (MP sgRNA and CP sgRNA) showed increased size in comparison with the cognate molecules in leaves agroinoculated with the WT clone (Fig. 1B, left panel). Deletions between positions -136 and -42 resulted in comparable accumulation of the new sgRNA, indicating that this region does not contain regulatory elements affecting sgRNA transcription and it is not needed to promote sgRNA synthesis.

In a second group of constructs the 3' boundary of the duplicated promoter was mapped using a constant 5' terminus at position -42 and variable 3´ termini at positions +206, +114, +50, +28 and +6 downstream of the CP sgRNA transcription start site (Fig.1B, right panel). Again, these fragments were PCR amplified from CLBV-IC, cloned into *clbv3'* and, after inserting the *gfp* gene at the *Pml*I site downstream of the duplicated promoter (Fig. 1A), the new vectors were agroinoculated in *N. benthamiana*. Northern blot analysis of RNAt from leaves at 16 dpi showed that increasing deletions between positions +206 and +50 induced higher accumulation of the new sgRNA (Fig. 1B, right panel), confirming that this genomic region is an inhibitory sequence that modulates CP sgRNA transcription [177]. However, deletions until positions +28 and +6 decreased the amount of the new sgRNA, indicating that the 3´ border of a fully active CP sgRNA promoter is located between nucleotides +50 and +28.

CLBV was detected by RT-PCR and northern blot analysis in non-inoculated upper leaves of *N. benthamiana* plants agroinoculated with all constructs described (data not shown), indicating that the resulting viruses retained their capacity to systemically infect *N. benthamiana* plants. The vector with a duplicated promoter in the 3' UTR comprising the sequence between positions -42 and +50 around the CP sgRNA transcription start site was named *clbv3'pr* and the same vector expressing GFP was named *clbv3'pr*-GFP.

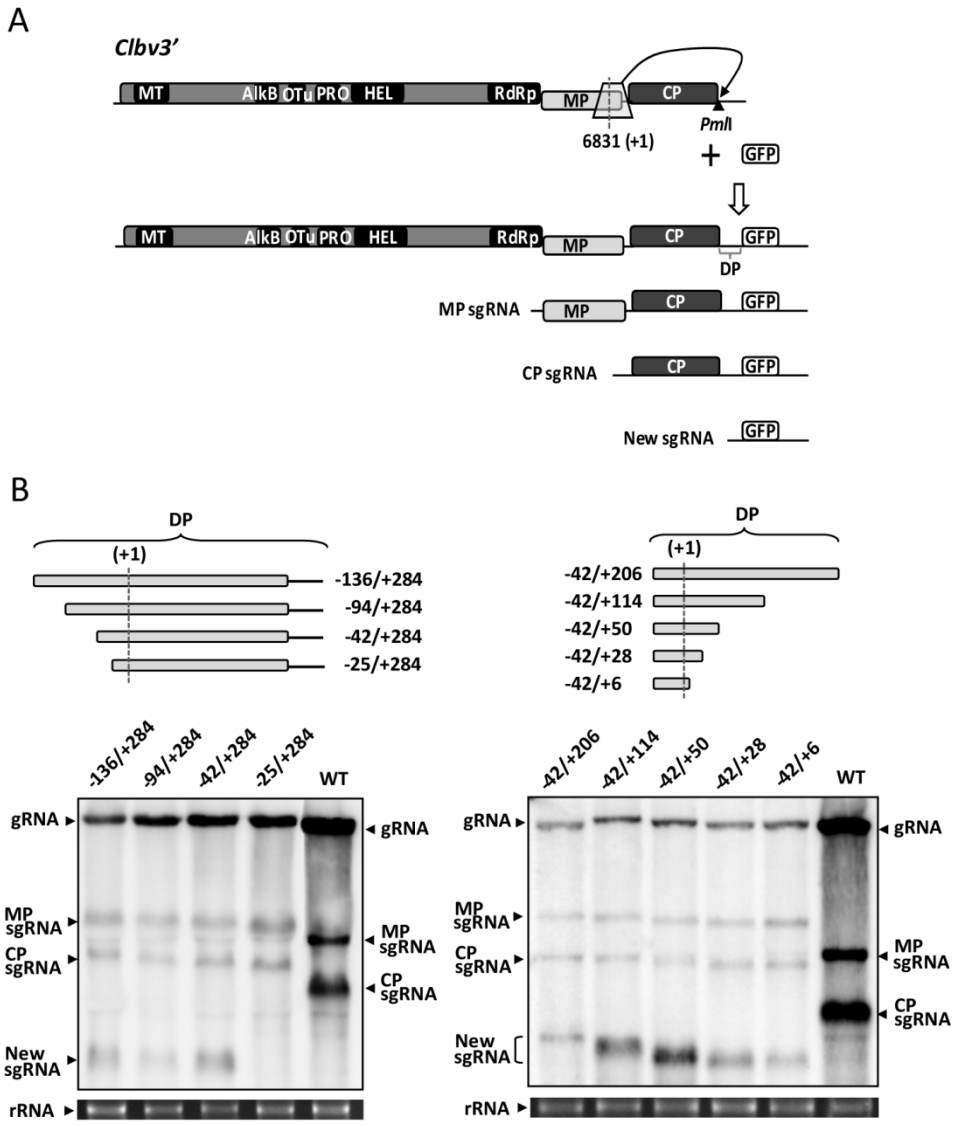

Figure 1. Delineation of the boundaries of the CP subgenomic RNA (CP sgRNA) duplicate promoter used in CLBV-based vectors. (A) Outline of the strategy followed to map the 5´ and 3´ borders of the duplicate promoter (DP) by inserting fragments of different size around the CP sgRNA transcription start site into the *Pml*I site of the *clbv3´* vector and the expected viral RNAs. Dotted line indicates the transcription start site of the CP sgRNA (+1) located at position 6831 in the CLBV genome; shaded boxes represent the predicted open reading frames (ORFs) and functional domains in the CLBV genome (MT methyl-transferase, AlkB AlkB-like peptidase, OTu OTu-like peptidase, PRO protease, HEL helicase, RdRp RNA-dependent RNA polymerase, MP movement protein and CP coat protein); solid black lines represent untranslated

regions and the white box represents the *gfp* gene or the 154-bp fragment thereof cloned downstream of the DP. (B) Schematic diagram showing the 5´and 3´end of DP fragments used to map the 5´ (left panel) or the 3´ (right panel) borders, and northern blot analyses of total RNA extracts (RNAt) from *N. benthamiana* leaves agroinoculated with the wild type CLBV-IC clone (WT) or with different mutant constructs at 16 dpi. The membrane was hybridized with a DIG-riboprobe specific for the CLBV 3' UTR. The numbers indicate nucleotide positions relative to the CP sgRNA transcription start site (+1). Arrowheads and bracket indicate positions of the gRNA (genomic RNA) and sgRNAs in plants infected with the WT virus (right) or with the mutants (left). The increased size of the MP and CP sgRNAs of the mutant vectors causes reduced mobility in comparison with the cognate WT sgRNAs. Size differences among the new sgRNAs from different mutants can be observed in the right panel. GelRed-staining of rRNA (ribosomal RNA) was used as loading control.

The CLBV gRNA and the two natural sgRNAs accumulated in *N. benthamiana* leaves agroinoculated with the different vectors to lower level than in those agroinoculated with the WT clone (Fig. 1B), suggesting that expression of the new sgRNA could compromise transcription of the gRNA and the MP and CP sgRNAs. Since transcription of the new sgRNA from the *clbv3'pr*-based vectors was much higher than that of the CP sgRNA and the CP is required for viral accumulation [177], we reasoned that CP synthesis and thus viral accumulation might be increased by putting the CP gene under the control of the duplicated minimum promoter and the foreign sequences under the control of the natural CP sgRNA promoter. To test this hypothesis a unique *Pml*I restriction site was added in the intergenic region between ORFs 2 and 3, just upstream of the CP translation start codon, thus obtaining the *clbvIN* vector. Viral gRNA accumulated at similar level in *N. benthamiana* leaves agroinoculated with *clbvIN* or with the WT construct as detected by real time RT-PCR (data not shown), indicating that this modification did not affect virus infectivity. The promoter fragment -42/+50 around the transcription start site of the CP sgRNA, carrying a *Pml*I restriction site in its 5' end, was cloned in the *Pml*I site of *clbvIN* to obtain the *clbvINpr* vector.

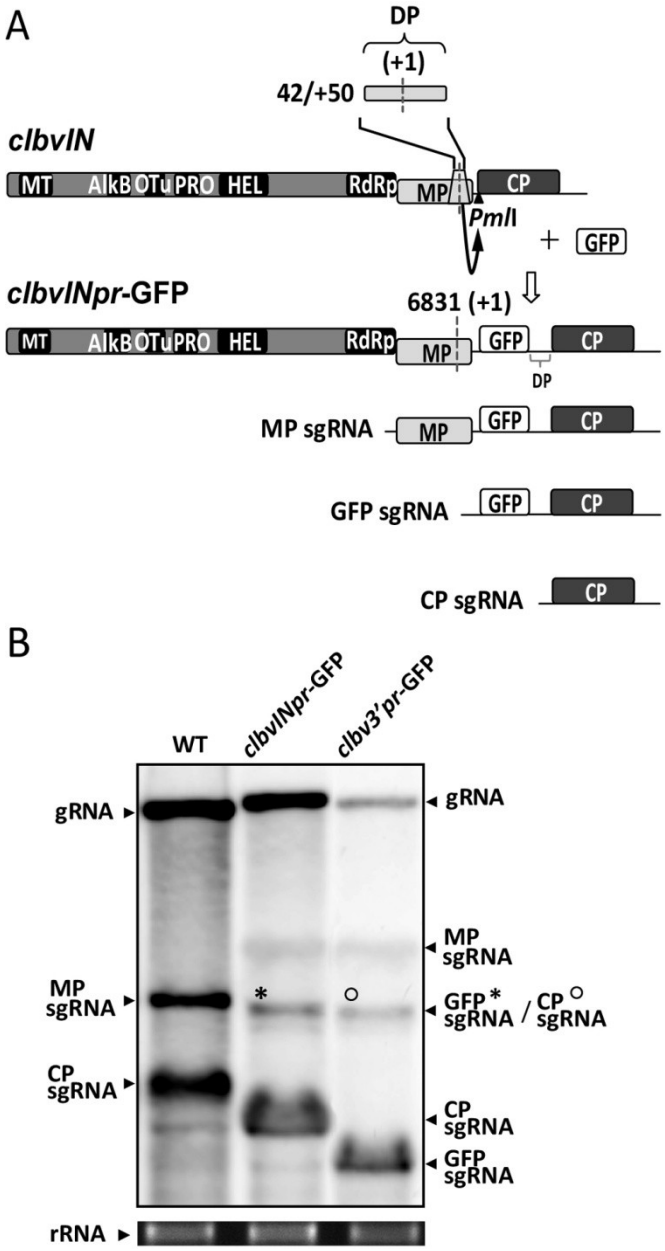

Figure 2. Viral RNA accumulation in *Nicotiana benthamiana* plants agroinoculated with the wild type *Citrus leaf blotch virus* (CLBV)-IC clone (WT) or with the *clbvINpr* or *clbv3'pr* vectors expressing the green fluorescent protein (GFP) (*clbvINpr*-GFP or *clbv3'pr*-GFP, respectively). (A) Cloning strategy for duplication of the -42/+50 fully active minimum promoter to obtain the *clbvINpr*-GFP vector and the expected

RNAs derived. Dotted line indicates the transcription start site of the CP sgRNA (+1); shaded boxes represent the predicted open reading frames and functional domains (MT methyl-transferase, AlkB AlkB-like peptidase, OTu OTu-like peptidase, PRO protease, HEL helicase, RdRp RNA-dependent RNA polymerase, MP movement protein and CP coat protein); solid black lines represent untranslated regions; DP the duplicated promoter and GFP the new gfp gene inserted. (B) Northern blot analysis of total RNA extracts from *N. benthamiana* leaves agroinoculated with the CLBV-IC clone (WT), or with the *clbvINpr*-GFP or *clbv3'pr*-GFP constructs at 16 dpi. The membrane was hybridized with a DIG-riboprobe specific for the CLBV 3' UTR. Arrowheads indicate positions of the genomic RNA (gRNA) and sgRNAs in plants infected with WT CLBV (left) or with the mutants (right). GelRed-staining of rRNA was used as loading control.

The *gfp* gene was then inserted in the new *Pml*I site upstream of the duplicated promoter to obtain the *clbvINpr*-GFP clone, in which *gfp* was expressed under the control of the native CP sgRNA promoter and the CP gene under the duplicated minimum promoter (Fig. 2A). Agroinoculation of *clbvINpr*-GFP in *N. benthamiana* leaves resulted in efficient replication and movement of the viral progeny. Northern blot hybridization of RNAt from agroinoculated and systemically infected leaves revealed an extra 3´ co-terminal sgRNA (GFP sgRNA) of the expected size between the MP and the CP sgRNAs, with the MP sgRNA showing slower electrophoretic mobility due to its size increase and the CP sgRNA faster mobility due to the smaller size of its 5'UTR (Fig. 2B).

To compare viral replication and foreign gene expression with the two CLBV vector variants (location of the foreign gene at the 3' UTR or between ORFs 2 and 3), *N. benthamiana* plants were agroinoculated with *clbv3'pr*-GFP or *clbvINpr*-GFP clones and the level of gRNA and GFP sgRNA accumulation and fluorescence emission were analyzed at 25 dpi in systemically infected leaves. While gRNA accumulation estimated by real time RT-PCR analysis [222] was about 10 times higher in plants agroinoculated with *clbvINpr*-GFP than in plants agroinoculated with *clbv3'pr*-GFP (data not shown), the amount of GFP sgRNA

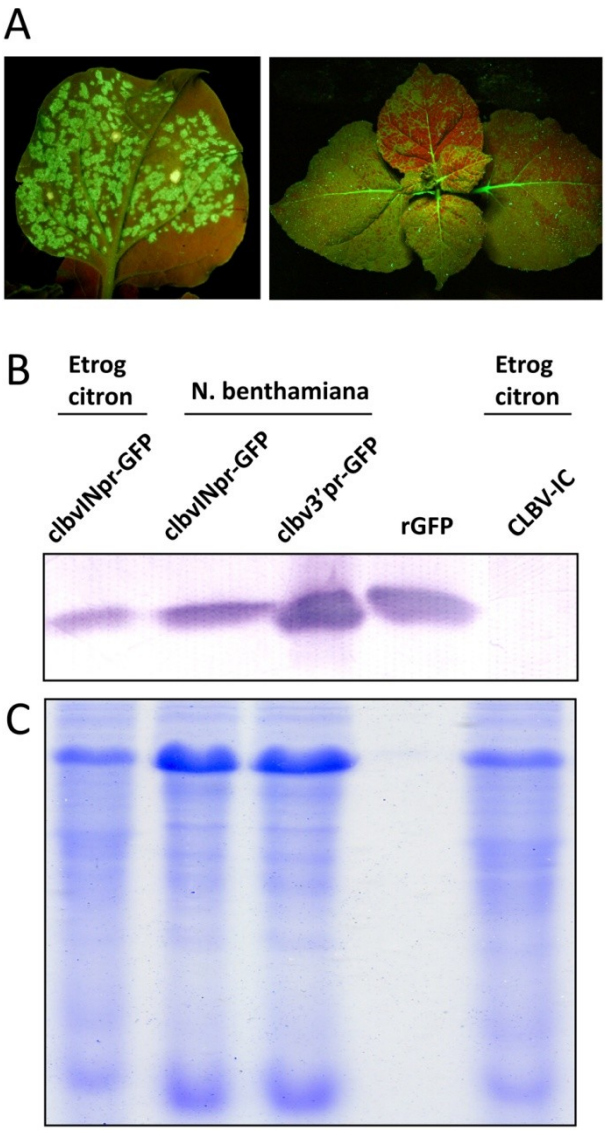

Figure 3. Green fluorescent protein (GFP) detection in plants inoculated with C*itrus leaf blotch virus* (CLBV)-based vectors expressing GFP. (A) Fluorescence observed in *Nicotiana benthamiana* plants agroinoculated with the *clbv3'pr*-GFP clone: agroinoculated leaves at 16 dpi (left) and systemically infected leaves at 20 dpi (right). Photographs were taken under illumination with a handheld UV lamp. (B) Western blot analysis of leaf proteins from *N. benthamiana* plants agroinflitrated with *clbvINpr*-GFP or *clbv3'pr*-GFP vectors and from Etrog citron inoculated with *clbvINpr*-GFP or CLBV-IC-derived virions, using a mixture of two anti-GFP monoclonal antibodies. A recombinant GFP protein (rGFP) was used as control. (C) Equal protein loading was estimated by staining a parallel gel with Coomassie blue.

estimated in northern blots using a Luminescent Image Analyzer LAS-3000 (FujiFilm) was about 10 times lower in the first than in the second plants (Fig. 2B). This comparison was repeated twice using five plants per assay. GFP expression in plants agroinoculated with *clbv3'pr*-GFP was first observed at 11 dpi as small fluorescent spots on inoculated leaves (Fig. 3A, left panel) that increased in size with time. At about 15 dpi fluorescence was observed in systemically infected leaves, first in the veins and then in other tissues (Fig. 3A, right panel). The amount of GFP in these leaves was estimated as about 16 μg g^{-1} of fresh tissue by western blot analysis. Contrastingly, fluorescence was not observed in plants agroinoculated with *clbvINpr*-GFP, although western blot analysis showed GFP accumulation of about 3 μg g^{-1} of fresh tissue in systemically infected leaves of these plants (Fig. 3B).

2.2 Genetic stability of CLBV-based vectors in *N. benthamiana* plants

Viral vectors are often unstable and tend to lose the inserted sequence by recombination with subsequent loss of its function. Therefore, elucidation of factors that favor those recombination events is critical to improve vector stability. We first examined the effect of the duplicated promoter size on the stability of the *clbv3´pr*-derived vectors by agroinoculating five *N. benthamiana* plants with the constructs used to map its 5' boundary and then examining the gRNA integrity of the virus progeny in inoculated and systemically infected leaves at 13 and 20 dpi, respectively, by RT-PCR analysis with primers encompassing the promoter insertion site in the CLBV genome. While no recombination event was detected in agroinoculated leaves, in systemically infected leaves larger duplicated promoters resulted in higher recombination rates. Thus, in 3 of the 5

plants agroinoculated with the construct carrying the largest duplicated promoter (-136/+284), the size of the RT-PCR amplified product was smaller than expected, whereas in 4 out of 5 plants inoculated with the construct carrying the -42/+284 promoter a unique DNA band of the expected size was amplified (data not shown).

To assess the influence of the foreign sequence size on vector stability, five different DNA fragments from 1,645 to 154 nt were cloned into the *clbv3'pr* or the *clbvINpr* vectors and the resulting constructs were agroinoculated in *N. benthamiana* plants. These fragments corresponded to the RNA 2 of *Citrus psorosis virus* (CPsV) (1,645 nt), the complete *gfp* gene (720 nt) and different fragments thereof (414, 253 and 154 nt). While all constructs cloned in *clbv3´pr* retained the insert in agroinoculated leaves at 13 dpi, in systemically infected leaves the vector expressing the CPsV RNA 2 had lost the insert in the five agroinoculated plants at 20 dpi, the vector expressing the complete *gfp* gene showed recombination events in 3 out of 32 plants at 20 dpi and in 18 plants at 40 dpi, and only constructs carrying fragments smaller than the *gfp* gene retained the insert for at least 60 dpi. In some plants showing reorganization of the viral genome, RT-PCR analysis with primers flanking the promoter insertion site showed a DNA band of the size expected for the complete inserted sequence and others of smaller size (see example in Fig. 4A, lane 2), suggesting a viral population with two or more different genomic sizes after appearance of recombinant sequences, whereas in other plants a unique band corresponding to the reorganized virus genome was observed (see examples in Fig. 4A, lanes 3 and 4). Sometimes the most conspicuous PCR-amplified DNA band co-migrated with that amplified from the WT clone, suggesting precise deletion of the insert by non-homologous recombination. However, cloning and sequencing DNA products of this mobility, PCR-amplified from two different plants, revealed deletion of the

inserted sequence plus 8 additional nt of the 3' UTR. Similar analysis of the DNA band migrating between those of the WT and the complete insert (Fig. 4A, lane 3)

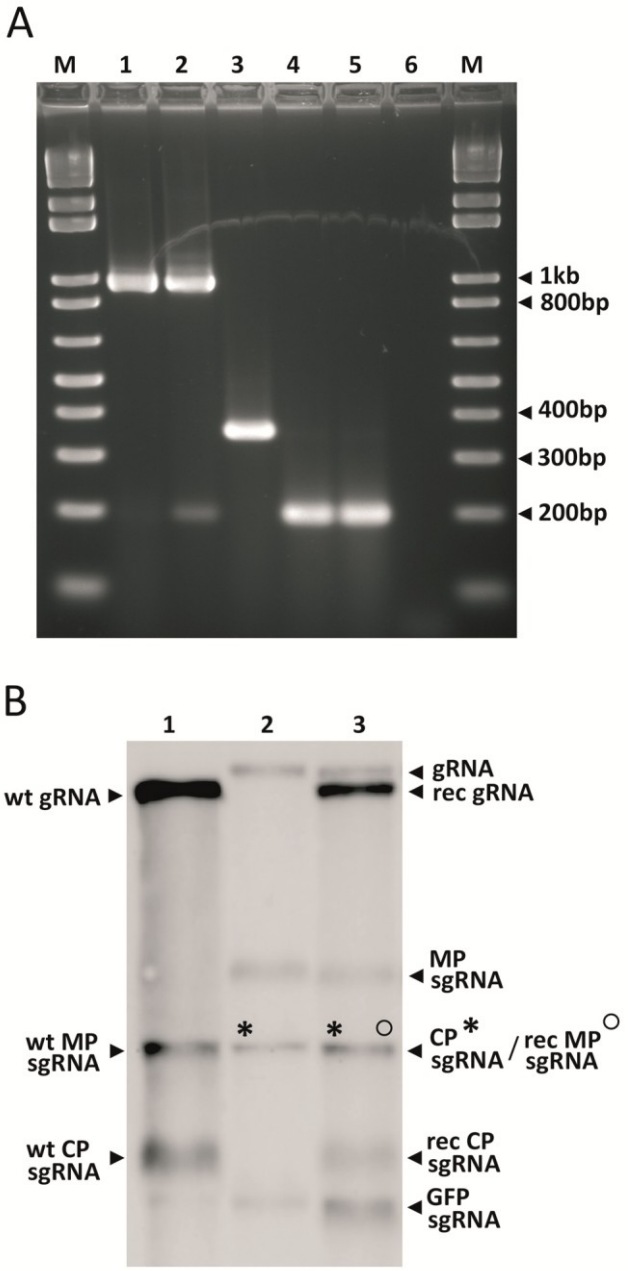

Figure 4. Recombination analysis of *Citrus leaf blotch virus* (CLBV) mutants. (A) Reverse-transcription polymerase chain reaction analysis of total RNA from *Nicotiana benthamiana* agroinoculated with Replace with the *clbv3'pr* vector expressing the green fluorescent protein (GFP) (*clbv3'pr*-GFP) using primers KU17L-KU7L flanking the promoter insertion site: lane 1, mutant keeping the whole *gfp* insert; lane 2, mixed viral population with some mutants carrying the whole insert and others that lost a *gfp* fragment after recombination; lane 3, a uniform recombinant population lacking the *gfp* fragment between positions 90 and 703; lane 4**,** a uniform recombinant population lacking the whole *gfp* fragment plus the first 8 nt of the 3' UTR; lane 5, wild type (WT) CLBV; lane 6, healthy plant ; M, 1 Kb Plus molecular size ladder. (B) Northern blot analysis of total RNA from systemically infected leaves of *N. benthamiana* plants agroinoculated with the wild type CLBV-IC clone (1), with *clbv3'pr* carrying the -42/+114 duplicate promoter (2), or with the cognate *clbv3'pr*-GFP vector (3) at 25 dpi. The latter contains a mix of viral RNAs carrying the whole *gfp* insert and others that lost it by recombination. The recombinant movement protein subgenomic RNA (rec MP sgRNA) and the nonrecombinant coat protein (CP sgRNA) show the same electrophoretic mobility. The membrane was hybridized with a DIG-riboprobe specific for the CLBV 3' UTR.

showed the loss of a *gfp* fragment between nucleotide positions 90 and 703 of this gene. Recombination events were also detected by northern blot analysis (Fig. 4B).

When the same DNA fragments were inserted in *clbvINpr*, recombination events were detected only in systemically infected leaves of *N. benthamiana* plants agroinoculated with the vector carrying the CPsV RNA2 (data not shown).

2.3 Genetic stability of CLBV-based vectors in citrus plants

To examine the stability of the *clbvINpr* vector in citrus plants, purified virions from *N. benthamiana* plants agroinoculated with the *clbvINpr*-GFP clone were slash inoculated on the trunk bark of two Etrog citron (*Citrus medica* L) plants. Both plants became systemically infected, as detected by RT-PCR using RNAt from the first flush at 45 dpi. A unique band of the size expected for the construct carrying the full *gfp* sequence was PCR amplified using primers flanking the promoter insertion site, suggesting stable replication of this viral vector not

only in *N. benthamiana* but also in citrus plants. Although intensity of the GFP fluorescence in citrus was too weak to be directly observed, western blot analysis of leaf extracts using a specific antibody showed that these plants accumulated about 0.6 µg of GFP per gram of fresh tissue (Fig. 3B).

Genetic stability of the *clbvINpr* vector was also examined in other citrus species by graft-inoculating *C. excelsa* Wester, Mexican lime [*C. aurantifolia* (Christm.) Swing.], Eureka lemon [*C. limon* (L.) Burm. f.], rough lemon (*C. jambhiri.* Lush.), Dweet tangor [*C. tangerina* Hort. ex Tan. x *C. sinensis* (L.) Osb.] and *C. macrophylla* Wester plants with bark pieces from infected Etrog citron. Vector stability in these plants and in Etrog citron was monitored along 21 months (at least 6-7 consecutive flushes) by RT-PCR analysis with primers flanking the promoter insertion site. A unique band of the size expected for the construct carrying the full *gfp* insert was obtained with all samples, demonstrating long-term stability of this vector in different citrus species and hybrids.

Mechanical inoculation of two Etrog citron plants with virions purified from *N. benthamiana* plants agroinoculated with the *clbv3'pr*-GFP clone yielded successful infection in only one of the plants, that showed low virus titer and loss of the insert by recombination as revealed by RT-PCR analysis with primers flanking the promoter insertion site. However, in Mexican lime and Eureka lemon plants slash inoculated with virions from *N. benthamiana* carrying a 253-nt fragment of the *gfp* gene (agroinoculation with the *clbv3'pr*-253GFP vector) the insert has remained stable in the virion progeny for at least 10 months. These results confirm that, as observed in *N. benthamiana* plants, vectors carrying smaller inserts tend to be more stable.

2.4 CLBV-based vectors expressing GFP trigger RNA silencing of a *gfp* transgene in citrus plants

Transgenic Mexican lime and Eureka lemon plants constitutively expressing GFP were graft inoculated with bark pieces from an Etrog citron plant infected with virions derived from the *clbvINpr*-GFP vector and the onset of *gfp* silencing was monitored by fluorescence observation under UV light. While non transgenic citrus plants appeared dark red due to chlorophyll auto-fluorescence, transgenic citrus expressing GFP appeared green (Fig. 5C, F and J). Transgenic citrus plants inoculated with virions derived from the *clbvINpr*-GFP construct developed silenced foci in leaf primordia, young and old leaves, thorns, and stems (Fig. 5). A remarkable decrease of GFP fluorescence was observed in shoot tips of these plants (Fig. 5G) in comparison with equivalent shoot tips from plants inoculated with the WT virions (Fig. 5H), thus confirming that CLBV is able to invade meristematic regions. The silencing pattern was different in both transgenic citrus species. While Mexican lime showed scattered non-fluorescent dark spots on the leaves (Fig. 5A, B), the silenced areas in Eureka lemon leaves appeared along the veins and then spread to a wider area (Fig. 5D, E), suggesting different viral accumulation patterns in both citrus hosts. Occasionally, the silencing phenotype was uniform and affected large leaf areas. The GFP silencing phenotype has been observed in successive flushes for at least 21 months, being more intense in the second and following flushes than in the first. In silenced plants CLBV could be detected by RT-PCR in most leaf regions regardless the silencing phenotype and in all cases it contained the *gfp* insert, suggesting that a threshold level of virus accumulation may be required to induce efficient VIGS in citrus plants, as observed with other plant viruses [113].

Slash inoculation of *gfp*-transgenic Mexican lime and Eureka lemon plants with virions derived from the *clbv3'pr*-GFP vector induced the silencing phenotype

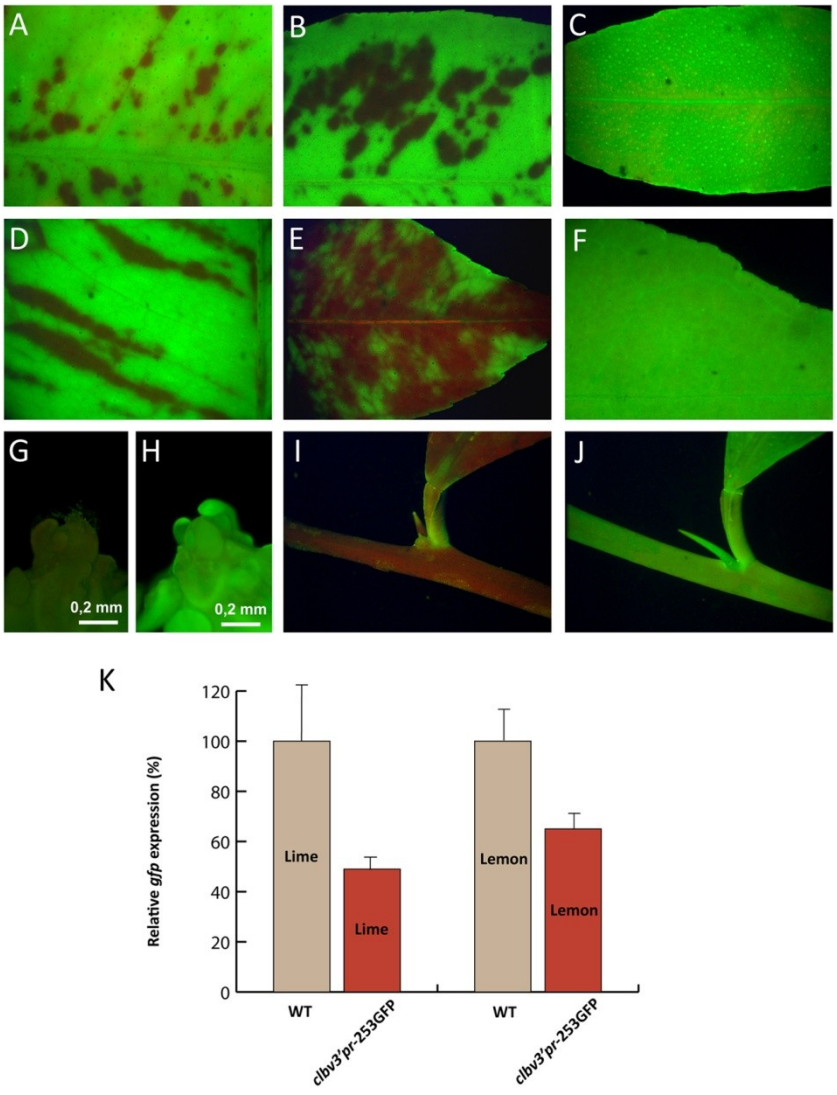

Figure 5. Green fluorescent protein (GFP) silencing in transgenic citrus plants after inoculation with mutant *Citrus leaf blotch virus* (CLBV) virions containing an ectopic *gfp* gene. Photographs show silencing phenotypes on Mexican lime young (A) and mature (B) leaves and shoot tip (G), and Eureka lemon young (D) and old (E) leaf blades or stem, thorn, and young leaf petiole (I) after inoculation with mutant virions derived from the *clbvINpr*-GFP clone. Similar tissues from Mexican lime (C and H) and Eureka lemon (F and J) plants inoculated with wild type CLBV (WT) virions show GFP fluorescence. (K) Relative accumulation of *gfp* transcripts in *gfp*-transgenic Eureka lemon and Mexican lime plants slash-inoculated with WT or with

virions derived from the *clbv3'pr*-253GFP vector. The amount of *gfp* transcripts was normalized to that of the *actin* gene in the same plants. The average expression in control plants infected with WT CLBV was considered 100, and the normalized expression in plants infected with the mutant virions was relative to the average of the control plants. Bars represent standard deviation values.

only in the first flush of some plants, suggesting that the silencing phenotype developed only in plants where the CLBV vector retained the foreign insert. To confirm this hypothesis transgenic Mexican lime and Eureka lemon plants were inoculated with virions derived from the clbv3'pr-253GFP vector. All these plants have shown a silencing phenotype identical to that observed in plants inoculated with clbvINpr-GFP-derived virions in successive flushes for at least 10 months.

We examined if the silencing phenotype was associated with low trangene expression by comparing GFP mRNA accumulation in *gfp*-transgenic Eureka lemon and Mexican lime plants inoculated with WT CLBV or with virions derived from the *clbv3'pr*-253GFP vector by quantitative real time RT-PCR. Leaves showing the silencing phenotype had less than 65 % GFP mRNA in comparison with their counterparts from plants inoculated with WT CLBV (Fig. 5K).

2.5 CLBV-based vectors can trigger silencing of endogenous citrus genes

The ability of CLBV-derived vectors to silence endogenous citrus genes was tested using as targets the *pds* and a citrus homologue of the tobacco *su,* required for chlorophyll production [228], because lack of function of both genes incite a visible phenotype. [80, 86, 110, 115]. For this purpose, a 157-bp fragment of the *pds* gene from Valencia late sweet orange [*C. sinensis* (L.) Osb.] was cloned into the *clbv3'pr* vector (*clbv3'pr*-157PDS) that was then agroinoculated in *N. benthamiana* plants and the resulting recombinant virions were slash inoculated onto citrus plants. PDS is an enzyme required for biosynthesis of carotenoid

pigments protecting chlorophyll from photo-oxidation and *pds* silencing causes photobleaching. While no silencing phenotype was observed in *N. benthamiana* plants agroinoculated with *clbv3'pr*-157PDS vector, probably due to low nucleotide identity between *N. benthamiana* and citrus *pds* genes (80%), mechanical inoculation of four *C. excelsa* plants with virions purified from those *N. benthamiana* plants induced photobleaching in the leaves of the first flush. Photobleaching was first confined to leaf veins and later developed vein banding (Fig. 6A). The silencing phenotype was usually observed in fully expanded leaves and rarely in young developing leaves, with the discolored areas remaining in the old leaves. The photo-bleaching phenotype was detectable in leaves of successive flushes for at least 8 months.

Inoculation of *C. excelsa* plants with a *clbv3'pr* vector carrying a 241-nt fragment of the *su* gene from Valencia late sweet orange (*clbv3'pr*-241SU) showed a yellowing phenotype in leaves indicating chlorophyll deficiency (Fig. 6B). This silencing phenotype appeared in younger leaves and was more widespread than the *pds* silencing phenotype. The yellowing phenotype remained in old leaves. Quantification of *pds* and *su* mRNAs by real-time RT-PCR showed that citrus leaves with a silencing phenotype had lower mRNA accumulation than similar leaves from plants infected with WT CLBV (Fig. 6C). Silencing with the vector carrying the *pds* fragment was less efficient than with the vector carrying the *su* fragment.

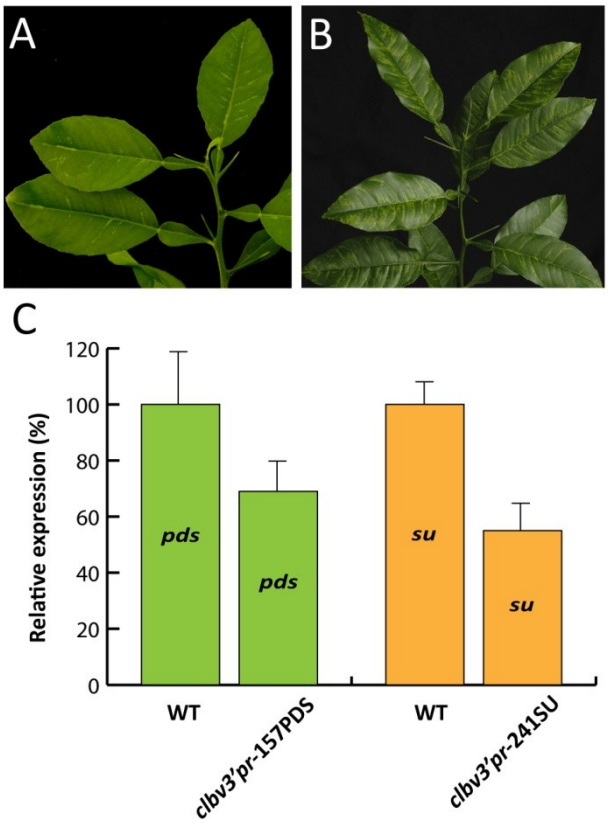

Figure 6. Silencing phenotype of *Citrus excelsa* plants inoculated with mutant *Citrus leaf blotch virus* (CLBV) virions carrying (A) a 157-bp fragment of the *phytoene desaturase* gene (*pds*), or (B) a 241-bp fragment of the homologue of the sulfur gene (*su*) from Valencia sweet orange. (C) Relative accumulation of the *pds* and *su* mRNAs in *C. excelsa* plants inoculated with wild type CLBV (WT) or with virions derived from the *clbv3'pr*-157PDS and *clbv3'pr*-241SU vectors, respectively. For each gene the amount of mRNA was normalized to that of the *actin* gene in the same plants. The average expression in control plants infected with WT CLBV was considered 100, and the normalized expression in plants infected with the mutant virions was relative to the average of the control plants. Bars represent standard deviation values.

3. Discussion

We have developed CLBV-based vectors useful to express foreign genes and to trigger VIGS in citrus plants. A viral vector derived from *Citrus tristeza virus* (CTV) was used to express a foreign protein in citrus [223], but this is the first report of VIGS in these woody plants. In comparison with the CTV-based vector, the CLBV-based vectors developed here offer the following potential advantages: i) CLBV causes a symptomless infection in most citrus species and cultivars [206-208], therefore, phenotypic expression of gene silencing would not be masked, ii) in contrast with CTV, CLBV is not phloem limited, thus the vectors described here would be appropriate for gene expression or silencing in non-phloem tissues including meristematic regions, iii) contrary to CTV, CLBV is not transmitted by vectors and therefore it could be safely used in future field experiments.

In a previous work we delimited the boundaries of the CP sgRNA promoter in its natural context between nucleotides -67 and +50 around the transcription start site [177], but it was not tested if this reduced sequence was sufficient to promote the ectopic synthesis of a new sgRNA. Here we addressed this issue and delimited the minimal functional promoter by duplicating variable sequence fragments around the CP sgRNA transcription start site at the 3' UTR of the CLBV gRNA. A 92-base fragment of the CP sgRNA promoter (-42/+50) duplicated in the CLBV genome led to the synthesis of a new sgRNA in infected plants, indicating that this fragment contains all the elements required for full promoter activity *in vivo*. Although deletions between positions -520 and -202 upstream of the transcription start site reduced the amount of CP sgRNA in its natural context [177], in the duplicated promoter deletions upstream of the nucleotide -42 did not affect accumulation of the new sgRNA, suggesting that reduction in the amount of CP sgRNA observed previously might be caused by reduced stability of viral RNAs due to the large deletion within the MP ORF that

could affect the silencing suppressor activity of the MP [220]. Increasing deletions between positions +206 and +50 downstream of the CP sgRNA transcription start site induced higher accumulation of the new sgRNA, supporting previous suggestion that this region might be an inhibitory sequence to modulate CP sgRNA transcription [177]. However, further deletions between positions +50 and +6 resulted in reduced accumulation of the new sgRNA, indicating that this region must be necessary for efficient sgRNA transcription.

Remarkably, the new sgRNA synthesized after duplication of the minimum promoter at the 3' UTR of the CLBV genome (*clbv3'pr*-GFP clone*)* accumulated to higher level than the CP sgRNA synthesized under the control of the wild promoter in its natural context. This higher accumulation of the ectopic sgRNA might be due to i) an enhanced promoter activity derived from the lack of the inhibitory sequence downstream of position +50 present in the wild promoter, and/or ii) the preference of virus replicase for a sgRNA promoter closer to the 3' end of the gRNA, as reported for other plant viruses [229-232]. However, since the ratio between the ectopic 3'-terminal sgRNA and the gRNA in plants agroinoculated with *clbv3'pr*-GFP was higher than the ratio CP sgRNA/gRNA in plants infected with the WT CLBV, as determined with the Multi Gauge v3.0 software (FujiFilm) (data not shown), we believe that elimination of the inhibitory sequence is actually the most important reason for enhanced transcription of the ectopic 3'-terminal sgRNA.

Accumulation of gRNA was significantly lower in *N. benthamiana* plants agroinoculated with *clbv3'pr*-GFP than in plants infected with the WT. This reduction in viral accumulation might be due in part to the increased genome size. Indeed viral accumulation was higher in plants infected with the WT CLBV than in those agroinoculated with a *clbv3´* construct carrying a duplicated -25/+284 promoter fragment (not shown), even if this construct does not promote the synthesis of a new sgRNA. On the other hand, the synthesis of a new 3' terminal

sgRNA compromises transcription of the two natural sgRNAs (MP and CP sgRNAs). Previous work with other viruses clearly showed a competition between different sgRNAs, with increased accumulation of one sgRNA resulting in a decrease of the others [231, 233-235]. In *N. benthamiana* plants agroinoculated with the *clbv3'pr*-GFP clone, expression of the new GFP sgRNA also reduced the amount of MP and CP sgRNAs and thus of MP and CP synthesis. Since CP was shown to be necessary for viral accumulation in cells [177] we decided to increase CP accumulation by putting the CP gene under the control of a duplicated –42/+50 promoter and the *gfp* gene under the control of the wild CP sgRNA promoter in the *clbvINpr*-GFP vector. The gRNA accumulation was higher in plants agroinoculated with this vector than in those agroinoculated with the *clbv3'pr*-GFP vector, albeit slightly lower than in those infected with the WT CLBV, possibly due to higher transcription of the MP sgRNA in the latter. Reduced MP synthesis in the mutant vectors could hinder cell-to-cell and long distance movement during virus infection. Moreover, since MP is the CLBV silencing suppressor [220], reduced expression of this protein may further prevent viral accumulation in infected cells.

GFP fluorescence was observed in *N. benthamiana* leaves agroinoculated with the *clbv3'pr*-GFP clone and in upper non-inoculated leaves, demonstrating that this modified virus was able to move both cell-to-cell and long distance. Contrastingly, no fluorescence was observed in citrus plants infected with the same virions likely due to higher instability or lower GFP accumulation in this host in comparison with *N. benthamiana*. Since fluorescence is a convenient reporter, GFP expression in *N. benthamiana* plants agroinoculated with CLBV mutants derived from the *clbv3´pr*-GFP vector could be a helpful tool to analyze sequence motifs involved in CLBV replication, assembly and movement in this host.

Instability associated to high error rates during RNA replication and frequent recombination events have been limiting factors in the use of plant viral vectors. Genetic variation of CLBV isolates from different geographical origin and

citrus hosts was shown to be very low in comparison with most plant viruses [212], indicating that CLBV is a very stable virus. Virions derived from the *clbvINpr*-GFP construct were more stable than those derived from *clbv3'pr*-GFP in both *N. benthamiana* and citrus plants. In citrus plants, virions derived from the first construct have remained stable for at least 21 months (6-7 consecutive flushes), whereas those derived from the second lost their *gfp* insert after the first flush. However, when a *gfp* fragment of 253 nt was cloned in the *clbv3'pr* vector no recombination events were detected in citrus plants after 10 months (3-4 flushes). Similar results were obtained with *Potato virus X*-based vectors, where insertion of smaller fragments tended to be more stable [236]. Our results suggest that *clbv3'pr*-derived vectors should not be used for protein expression in citrus but they could be used for gene silencing. Indeed expression of gene fragments with this vector in citrus induced silencing of endogenous genes or a transgene.

A major challenge in post-genomic biology is assigning function to gene sequences identified by differential expression or large-scale genome sequencing. Presently there are more than 230,000 ESTs available, derived from different cDNA libraries representing a wide range of citrus organs, developmental stages, genotypes and plants subjected to diverse biotic and abiotic stresses [8]. Furthermore, the genome sequences of two citrus species (*C. clementina* and *C. sinensis*) are also available. In plants, reverse genetics by functional gene knockout has been based on genetic transformation [237]. However, this technique is time consuming and often requires generation and screening of many transgenic lines to obtain a few silenced individuals. As an alternative, reverse genetic approaches based on VIGS have been developed and successfully applied in several plant species [81, 82, 226, 227]. Our results showed that CLBV-based vectors induce reliable silencing of endogenous genes or a transgene in citrus plants. Further experiments aimed at silencing other endogenous citrus genes using the vectors developed here are in course with promising preliminary results.

Plant tissues that can be targeted by VIGS depend mostly on viral vector characteristics. CLBV accumulates in all tissues of citrus plants [222] including meristematic regions as deduced from seed transmission [210] and from the difficulty to recover CLBV-free plants by shoot-tip grafting *in vitro* [206]. CLBV-based vectors carrying *gfp* sequences were able to silence a *gfp* transgene expressed in leaf primordia, young and old leaves, stems, thorns and shoot-tips of transgenic citrus plants. The ability of these vectors to induce VIGS in meristematic regions would enable studying genes involved in organ development pathways. Since these vectors were stable and induced the silencing phenotype in successive flushes along several months, hopefully they could be also used to knock down genes involved in reproductive stages of the long-lived citrus plants.

In summary, it is expected that the CLBV-based vectors developed in this work will enable cost-effective screening of many citrus genes for which a function has yet to be discovered or confirmed, as well as expression of foreign proteins in these woody hosts. Once a viral vector has systemically infected a citrus plant it can be easily graft-inoculated to plants of other citrus varieties, either at the juvenile or at the mature stage.

4. Material and methods

4.1 Plasmid constructs

The infectious CLBV construct pBIN35SRbz-CLBV (CLBV-IC) described previously [208] (here named wild type, WT) contains a full-length cDNA of the CLBV gRNA cloned into the pBIN19 binary plasmid, between the duplicated 35S promoter of *Cauliflower mosaic virus* and the nopaline synthase terminator. This plasmid was mutated in order to generate CLBV-based vectors using standard

techniques [238] and appropriate primers (Table 1). All mutations performed on this plasmid were confirmed by sequencing and restriction analysis.

To introduce a unique *Pml*I restriction site in CLBV-IC, the 2686 nt fragment comprised between restriction sites *Age*I and *Apa*I of this clone, including the 3' half of CP gene, the 3' UTR and a fragment of the pBIN19 plasmid, was PCR amplified with the primer pair KUV3/KUV6 (Table 1), and cloned into the pGEM-T plasmid (Promega) to obtain pGEM-*3'T*. To add a *Pml*I site (CAC GTG) after the stop codon of the CP ORF, this plasmid was PCR amplified with the primer pair VV1/VV2 (Table 1), each containing half of the *Pml*I restriction sequence at their 5´end, and self-ligated to obtain the pGEM-*3'Tpml* plasmid. This clone was digested with *Age*I and *Apa*I and the released fragment was cloned into the CLBV-IC plasmid digested with the same enzymes to obtain the *clbv3'* vector. To introduce a *Pml*I site just before the start codon of the CP ORF we followed the same strategy but using the plasmid pCP [177], that included the 3' half of MP and the 5' end of the CP genes cloned into the pUC19 plasmid. This plasmid was amplified by PCR using primers MP2L and MP8D (Table 1), each containing half of the *Pml*I restriction sequence at their 5´end, and the PCR product was self-ligated to obtain the *pCPpml* plasmid. This clone was digested with *Xho*I and *Bsu*36I and the released fragment was cloned into the CLBV-IC plasmid digested with the same enzymes to obtain the *clbvIN* vector.

The minimum sequence promoting transcription of an additional sgRNA was defined by testing sequence fragments of variable size around the CP sgRNA transcription start site, inserted at the *Pml*I site of the *clbv3'* vector (Fig. 1). To map the 5´ border of this minimum promoter, fragments with a constant 3´ terminus at nt +284 downstream of the transcription start site (position +1) and variable 5´ termini located at the upstream positions -136, -94, -42 and -25 were PCR amplified using forward primers MP1U (-136), MP3U (-94), MP4U (-42) and MP25U (-25) and the reverse primer MP2L (+284) containing GTG at its 5' end to

keep a *Pml*I restriction site downstream of the duplicated promoter (Table 1 and Fig. 1B). After cloning these PCR products into the *Pml*I site of *clbv3´*, a 154 nt fragment of the *gfp* gene, C3 version [239], was PCR amplified from the pCAM9R-GFP plasmid (kindly provided by Dr. W.O. Dawson, University of Florida, Citrus Reseach and Education Center, Lake Alfred) with the primer pair G303D/G456R (Table 1) and cloned into the new *Pml*I restriction site (Fig. 1A).

To map the 3' border of the minimum promoter, the fragments -42/+206, -42/+114 and -42/+50, with a constant 5' terminus (-42) and variable 3' termini downstream of the CP sgRNA transcription start site, were PCR amplified from the CLBV-IC clone using the forward primer MP4U and the reverse primers MP6R (+206), MP7R (+114) and MP4R-P (+50) containing GTG at their 5' end to keep a *Pml*I restriction site downstream of the duplicated promoter as before (Table 1 and Fig. 1B). The fragments -42/+28 and -42/+6 were generated by hybridizing complementary oligonucleotides Pm28D and Pm28R, and Pm6D and Pm6R, respectively (Table 1 and Fig. 1B). Each of these fragments was cloned into the *Pml*I site of *clbv3'* and then the *gfp* gene was PCR amplified from the pCAM9R-GFP plasmid with primers GFPF and GFPL (Table 1) and cloned into the new *Pml*I site of each construct (Fig. 1A). The construct with the duplicated promoter fragment -42/+50 was named *clbv3´pr* and the cognate construct expressing GFP was named *clbv3´pr*-GFP.

The minimum promoter fragment -42/+50 was also inserted between the MP and the CP ORFs using the *clbvIN* construct. For this purpose, the -42/+50 fragment was PCR amplified from the CLBV-IC clone using the forward primer MP11D, containing GTG at its 5' end, and the reverse primer MP4R (Table 1), and the product was cloned into the *Pml*I-digested *pCPpmI* plasmid to obtain the plasmid *pCPpr.* This plasmid was digested with *Xho*I and *Bsu*36I and the released fragment was cloned into the CLBV-IC clone digested with the same enzymes to

obtain the vector *clbvINpr*. Then the *gfp* gene, PCR amplified as before, was cloned into the *Pml*I-digested *clbvINpr* vector to obtain *clbvINpr*-GFP.

Fragments G154, G253 and G414 of the *gfp* gene were PCR-amplified from the pCAM9R-GFP plasmid, and a cDNA of the CPsV RNA2 (1,645 nt) was RT-PCR amplified using RNAt from a CPsV-infected citrus plant and appropriate primers (Table 1). These amplicons were cloned into the *Pml*I-digested *clbv3´pr* and *clbvINpr* vectors. Fragments of 157 and 241 nt of the phytoene desaturase (*pds*) and sulfur (*su*) genes, respectively, were RT-PCR amplified from Valencia sweet orange RNAt and cloned into *clbv3'pr.*

Fragment synthesized	Primer	Sequence 5'-3'[a]	Position (nt)
3'T	KUV3	TCTGA*ACCGGT*ACATGCTGAATCAGCAC	7523-7550[b]
	KUV6	TGATC*GGGCCC*GGCGTCC	
pGEM3'Tpml	VV1[h]	**GTG**TCCCGAATTCTGGCATGG	8207-8224[b]
	VV2[h]	**GTG**CTACATTTCTAAGAGTTTTGCTTTGT	8206-8181[b]
-136/+284	MP1U[h]	AGCGAACACCTTTGATTGGCAGCTTGG	6695-6721[b]
	MP2L[h]	**GTG**GGATGTACACTATCTATATTAATTCAA	7114-7088[b]
-94/+284	MP3U[h]	GACGCAAAGTGATGCCTGTTCGCAGACG	6737-6764[b]
	MP2L[h]		
-42/+284	MP4U[h]	GAAGGATTATGTGTCTCATGTTAAGTCAG	6789-6817[b]
	MP2L[h]		
-25/+284	MP25U[h]	ATGTTAAGTCAGAGACGGCTTCACT	6805-6831[b]
	MP2L[h]		
-42/+206	MP4U[h]		
	MP6R[h]	**GTG**TGTCACTGGCAGTTGAAGACTCTG	7036-7013[b]
-42/+114	MP4U[h]		
	MP7R[h]	**GTG**GGTGCTTTAACCAACTGATGATC	6944-6922[b]
-42/+50 (3')	MP4U[h]		
	MP4R-P[h]	**GTG**ATTTTCTCAATCTATCATTACCCCAATC	6880-6853[b]
-42/+28	Pm28D[h]	GAAGGATTATGTGTCTCATGTTAAGTCAGAGACGGCTTC ACTGAAAAGAAGTCAAAGTGGAAGAGATTGG**CAC**	6789-6858[b]
	Pm28R[h]	**GTG**CCAATCTCTTCCACTTTGACTTCTTTTCAGTGAAGCC GTCTCTGACTTAACATGAGACACATAATCCTTC	6858-6789[b]
-42/+6	Pm6D[h]	GAAGGATTATGTGTCTCATGTTAAGTCAGAGACGGCTTC ACTGAAAAG**CAC**	6789-6836[b]
	Pm6R[h]	**GTG**CTTTTCAGTGAAGCCGTCTCTGACTTAACATGAGAC ACATAATCCTTC	6836-6789[b]
GFPc3 ORF	GFPF[h]	ATGGCTAGCAAAGGAGAAGAACTTTTCACT	1-30[c]
	GFPL[h]	TTATTTGTAGAGCTCATCCATGCCATGTG	720-692[c]
pCPpml	MP8D[h]	**GTG**ATGAAAATCACCAATGATAATGCCGCAACTATC	7115-7147[b]
	MP2L[h]		

-42/+50 (IN)	MP11D[h]	**GTG**GAAGGATTATGTGTCTCATGTTAAGTCAG	6789-6817[b]
	MP4R[h]	ATTTTCTCAATCTATCATTACCCCAATC	6880-6853[b]
G154	G303D[h]	CAAAGATGACGGGAACTACAAGAC	303-326[c]
	G456R[h]	GTATACATTGTGTGAGTTATAGTTGTACTCG	456-426[c]
G253	G239D[h]	AACGGCATGACTTTTTCAAGAGTG	239-262[c]
	G491R[h]	GCTTTGATTCCATTCTTTTGTTTGTC	491-466[c]
G414	G239D[h]		
	G652R[h]	GGTCACGCTTTTCGTTGGG	652-634[c]
RNA2 (CPsV)	Ps69-F[h]	GATACTTTTTTTTGTGATTAAAGCATCACGTTGAC	
	Ps70-R[h]	GGTGTCATCCTGGTGACATTTCCACGCC	
Flanking 3' *Pml*I	KU17L	ATGTAACTTCAAGTCCACTGTACAATCGTGGG	8130-8161[b]
	KU7L	ATCTTGGATTCAGATTCATGAGGCTCCG	8328-8301[b]
Flanking IN *Pml*I	MP3U		
	MpC	GTGTCTCCATGCTCGGCCACTACAGC	7254-7229[b]
Pds 157 pb	PDS1[h]	GGCACTAAACTTCATAAACC	765-784[d]
	PDS5[h]	TGACTGAATGTGTTCAACAAT	921-901[d]
Su 241 pb	Su-F[h]	CGGGCTCTGTTTGCAGTTACC	7-27[e]
	Su-R[h]	CGGGGAGTAAATCTACCAAGGACC	247-224[e]
Pds Q-PCR	QPdsF	AATGCT GACTTGGCCGGAG	597-616[d]
	QPdsR	ATGCCTGTCCGCCAATTATT	667-648[d]
Su Q-PCR	QsuF	GGAGGAGAGAGCTCGATTTG	741-760[e]
	QsuR	GAGAGAACTCCTGGCTGAGG	846-827[e]
Gfp Q-PCR	QsGFPF	ACAAGTTCAGCGTGTCCGGC	77-96[f]
	QsGFPR	GCAGATGAACTTCAGGGTCAGCTT	147-124[f]
	sGFPL	TTACTTGTACAGCTCGTCCATGC	720-698[f]
Actin Q-PCR	CiACTqF	CAGTGTTTGGATTGGAGGATCA	1146-1167[g]
	CiACTqR	TCGCCCTTTGAGATCCACAT	1217-1198[g]

Table 1. Primers used for PCR or RT-PCR amplifications.

[a] Sequences in italics correspond to restriction sites. Nucleotides in bold indicate insertions. Underlined nucleotides indicate the CP sgRNA transcription start site.

[b] Nt positions are indicated on the sequence of the CLBV isolate SRA-153 (EMBL accession number AJ318061).

[c] Nt positions are indicated on the coding sequence of GFP "cycle 3" (GenBank accession number U62637).

[d] Nt positions are indicated on the sequence of the *C. sinensis* PDS mRNA (GenBank accession number DQ235261).

[e] Nt positions are indicated on the coding sequence of the *C. sinensis* homologue of tobacco *sulfur* gene (Phytozome locus name orange1.1g014510m).

[f] Nt positions are indicated on the coding sequence of sGFP (GenBank accession number EF090408).

[g] Nt positions are indicated on the sequence of the *C. sinensis* homologue of *actin11* gene (Citrus Functional Genomic database: aCL563Contig1).

[h] Phosphorylated at the 5' end.

4.2 Plant growth, agroinoculation of *N. benthamiana* leaves and slash- and graft-inoculation of citrus plants

N. benthamiana plants were grown in small pots with an artificial potting mix (50% vermiculite and 50% peat moss) in a plant growth chamber at 20/24°C (night/day), 60% humidity, and a 16/8 h light/dark regime. Citrus plants were grown in a glasshouse at 18/26ºC (night/day), using 2-liter plastic containers filled with 50% sand and 50% peat moss and a standard fertilizing procedure [240]. *C. excelsa* Wester, Mexican lime [*C. aurantifolia* (Christm.) Swing.], Eureka lemon [*C. limon* (L.) Burm. f.], rough lemon (*C. jambhiri* Lush.), Dweet tangor [*C. tangerina* Hort. ex Tan. x *C. sinensis* (L.) Osb.] and *C. macrophylla* Wester were grown as seedlings and Etrog citron (*C. medica* L.) was propagated on a rough lemon rootstock.

Recombinant CLBV clones were transfected to *Agrobacterium tumefaciens* cells, strain COR 308, carrying the helper plasmid pCH32 (kindly provided by Dr. C. M. Hamilton, Cornell Research Foundation) as previously described [208]. In all agroinoculation experiments *N. benthamiana* leaves were co-infiltrated with equal volumes of *A. tumefaciens* cultures carrying the CLBV-derived binary plasmids or the *p19* gen of *Tomato bushy stunt virus* (pBI-19, kindly provided by Dr. J.A. García, Centro Nacional de Biotecnología, CSIC, Madrid) that encodes a strong RNA silencing suppressor protein [241].

Semipurified virion extracts from agroinoculated *N. benthamiana* plants [207] were inoculated to citrus plants by stem slashing [242] with scalpel blades dipped in the viral extracts [207, 243]. Bark pieces from citrus plants infected with the recombinant or the WT CLBV were used to graft inoculate other citrus species.

4.3 RNA extraction, RT-PCR detection and RNA quantification by real time RT-PCR and northern blot analysis

RNAt from inoculated plants was prepared from 500 mg of leaf tissue using (i) TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) following the manufacturer's instructions, or (ii) a standard protocol with two phenol:chloroform:isoamyl alcohol extractions, followed by RNA precipitation with 12 M lithium chloride, and re-suspension in 25 µl of diethyl pyrocarbonate (DEPC)-treated distilled water [14]. RNA content was measured in a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, U.S.A.) and adjusted to the same concentration for northern blot and real time RT-PCR analysis.

The sequences inserted in CLBV viral vectors were detected by conventional RT-PCR [244] with the primer pairs KU17L/KU7L and MpC/MP3U (Table 1) flanking the two insertion sites. The DNA synthesized was visualized by 2% agarose gel electrophoresis and GelRed-staining (Biotium Inc., Hayward, CA, U.S.A.).

Accumulation of viral gRNA in plants inoculated with CLBV-derived constructs was determined by quantitative real-time RT-PCR performed in a Light-Cycler platform (Roche Diagnostics GmbH., Mannheim, Germany) with 20 µl glass capillaries. Reverse transcription and amplification were performed using DNA-free (Turbo DNA-*free* Kit, Ambion, Inc., USA) RNAt (2 µl) adjusted to 10 ng RNA/µl and primers and a TaqMan probe targeted to the ORF1 of the gRNA [222]. Each sample was analyzed in duplicate in two independent assays.

In gene silencing experiments, the mRNA level of endogenous citrus genes or transgenes was estimated by quantitative real-time RT-PCR using SYBR GREEN detection. DNA-free RNAt from plants infected with the WT or mutant CLBV was reverse transcribed using sGFPL primer (Table 1) for *gfp* trangene and oligo (dT)

for *pds, su* and *actin* genes and SuperScript II reverse transcriptase (Invitrogen). PCR amplification was performed with primers QsGFPF, QsGFPR, QPdsF, QPdsR, QsuF and QsuR (Table 1), designed with Primer Express Software (Applied Biosystems, Foster City, CA, USA) to exclude the gene regions cloned into the CLBV vector and thus ensure that only endogenous mRNA was amplified. Each sample was analyzed in triplicate using pooled leaf extracts from five independent plants. The mRNA amount estimated for each gene was normalized to the expression level of the citrus homologue of the *actin11* gene in the same sample with primers CiACT11qF and CiACT11qR (Table 1). The expression of each gene in plants infected with mutant CLBV virions relative to the control plants infected with WT CLBV was determined by the $2^{-\Delta\Delta CT}$ method [245].

Northern blot analysis of CLBV RNAs was performed according to Vives *et al*. [219] and Galipienso *et al.* [221], with minor modifications. Three to five micrograms of RNAt were denatured at 94°C for 5 min in 50% formamide, chilled on ice, separated by electrophoresis in formamide-formaldehyde denaturing 1.2% agarose gels in MOPS buffer, and electroblotted onto positively charged nylon membranes (Roche Diagnostics) at 250 mA for 1 h and 1 A for 15 h, using 25 mM phosphate buffer, pH 6.45. Membranes were hybridized at 68ºC with a digoxigenin (DIG)-labelled RNA probe specific for the CLBV 3´ UTR as in [221] but including two extra washings with 0.1 x SSC and 0.1% SDS at 68°C before incubation with the anti-DIG antibody. The reaction was developed using CPD-Star chemiluminescent substrate (Roche Diagnostics) and visualized with the Luminescent Image Analyzer LAS-3000 (FujiFilm, Tokyo, Japan) and/or with X-Ray films (Carestream Health Inc., Rochester, NY, U.S.A.). Quantification of the CLBV sgRNA in northern blot images taken before saturation was performed with the Multi Gauge v3.0 software (FujiFilm).

4.4 GFP detection

GFP accumulation was monitored by fluorescence observation and by western blot analysis. In *N. benthamiana* plants agroinoculated with GFP-expressing vectors fluorescence was observed under a long-wavelength UV lamp (Black Ray® model B100AP, UV Products, Upland, CA, U.S.A.) and images were taken with a CANON EOS 300D digital camera using a yellow filter (Jos. Schneider Optische Werke, B+W Filter, Bad Kreuznach, Germany). GFP silencing in transgenic citrus plants was observed with a Leica MZ16 stereomicroscope (Leica Microsystems, Heerbrugg, Switzerland) using a high-energy light source and a GFP filter. Images were taken with a Leica DFC490 digital camera using the IM50 software (Leica Microsystems).

For western blot analysis, 1 g of leaf tissue was ground in 4 ml of protein extraction buffer (100 mM Tris-Cl pH 6.8, 0.3% mercaptoethanol, 0.2% PVP) and successively centrifuged 15 min at 2,500 g and 15 min at 10,000 g. Supernatant (20 µl) was boiled with loading buffer [246] and electrophoresed in SDS polyacrylamide gels (14% acrylamide) at 50 V for 1 hour and then at 100 V until the bromophenol blue marker reached the lower gel edge. Proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, U.S.A.) using the appropriate buffer [246]. Serial dilutions (100 to 3,125 ng) of recombinant GFP (Roche Diagnostics) were included as standard to estimate GFP concentration in plant extracts. Equal protein loading was confirmed by staining a parallel gel with Coomassie blue. The membranes were incubated with a 1:2,000 dilution of a mixture of two anti-GFP monoclonal antibodies (Roche Diagnostics), and then with a 1:4,000 dilution of an anti-mouse antibody conjugated with alkaline phosphatase (Sigma-Aldrich Inc., St. Louis, MO, U.S.A.) in TBS-T buffer (20 mM Tris-Cl, pH 7.5, 500 mM NaCl and 0.1% Tween-20). The reaction was developed with the CPD-Star chemiluminescent substrate (Roche

Diagnostics) or with the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (BCIP/NBT).

CAPÍTULO 2:

Estudio del movimiento y distribución de CLBV en plantas de *N. benthamiana* y cítricos

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Adaptado del artículo:

Agüero J, Vives MC, Velázquez K, Ruiz-Ruiz S, Juárez J, Navarro L, Moreno P, and Guerri J (2013) *Citrus leaf blotch virus* invades meristematic regions in *Nicotiana benthamiana* and citrus. Mol. Plant. Pathol. **14(6),** 610-616.

1. Introduction

Systemic infection of plants by viruses requires replication of the viral genome in infected cells, cell-to-cell movement in the inoculated leaf, and long-distance transport to start new infection sites. For cell-to-cell movement viruses cross through plasmodesmatal intercellular connections. Long-distance movement requires entry of the virus into phloem sieve tubes from adjacent companion cells, fast motion through the connected sieve elements and subsequent unloading into new adjacent cells to start again cell-to-cell movement and invade neighbour cells of distal plant tissues [247, 248]. While most viruses use phloem transport to move systemically, some of them have been shown to traffic through the xylem [249-251]. Plant viruses usually encode movement proteins (MPs) that, in cooperation with some host proteins, facilitate their translocation through plasmodesmata and the vascular system [252, 253].

Citrus leaf blotch virus (CLBV), family *Betaflexiviridae,* has a single-stranded, positive-sense genomic RNA (gRNA) of 8,747 nucleotides with three open reading frames (ORFs) and untranslated regions (UTRs) at the 5' and 3' ends of the gRNA as previously described [177, 211, 218-220]. While preliminary data on CLBV accumulation in different plant organs have been reported [222], invasion of growing shoots by CLBV has not yet been examined.

Methods for virus localization in infected cells include electron microscopy or *in situ* techniques for detection of either viral RNA or virus encoded proteins [201-203]. An alternative approach has been to introduce the reporter gene *β-glucuronidase* into the viral genome, thus allowing histochemical localization of virus infected cells [171, 254]. However, all these techniques are invasive and do not allow real-time observation of virus movement. Introduction of the jellyfish *green fluorescent protein* (GFP) gene into viral genomes as an *in vivo* reporter has increased the ability to observe inter and intracellular events

accompanying virus infection by fluorescence observation or confocal microscopy [199, 200, 204, 255-257].

Several reports documented that most viruses and viroids are unable to invade the apical meristems of infected shoots [258-263]. Generally, a variable region of 100 to 1000 µm remains virus-free, a feature that has been exploited to recover virus-free plants from infected varieties by growing excised shoot tips in appropriate tissue culture media [264]. Similarly, shoot-tip grafting *in vitro* has been used to recover virus-free plants from infected citrus cultivars [265]; however, CLBV was difficult to eliminate by this procedure [266], suggesting that they might be able to replicate very close to the meristem.

2. Results and discussion

In this work we examined CLBV spread during systemic infection of *Nicotiana benthamiana* plants in nearly real-time conditions using an infectious cDNA clone engineered to express GFP (*clbv3'pr*-GFP clone) (Fig. 1) [208, 267].

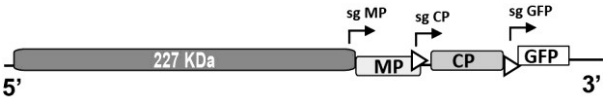


Figure 1. Outline of the infectious CLBV clone *clbv3'pr*-GFP expressing the green fluorescent protein (GFP). Grey boxes represent the CLBV ORFs (227 KDa polyprotein containing the replicase domains; MP, movement protein; CP, coat protein) and the white box the *gfp* gene. Arrows indicate transcription of subgenomic RNAs (sg). White triangle represents the promoter of the CP subgenomic RNA, duplicated to express GFP.

This clone, maintaining all genes and controller regions of the wild virus, was agroinoculated in *N. benthamiana* plants as described in [267]. The viral progeny produced by this construct will be referred to as CLBV-GFP. The infection pathway was initially monitored observing GFP expression in inoculated plants illuminated with a long-wavelength UV lamp (Black RayR model B100AP, UV Products) and capturing images with a CANON EOS 300D digital camera using a yellow filter (Jos. Schneider Optische Werke, B+W Filter). Infection was first detected in agroinoculated leaves at approximately 11 or 12 days post-inoculation (dpi) by the appearance of bright green fluorescent spots (Fig. 2a) that increased in number and size the following days (Fig. 2b). Discontinuous fluorescent foci were later observed in veins of different size including the midrib (Fig. 2c), suggesting that the virus was loaded into the sieve tubes and that minor and major veins may be entry points for long-distance infection of *N. benthamiana* plants, as reported for other plant viruses [199, 200]. Detailed observation with a stereomicroscope (Leica Microsystems) using a high-energy light source and a GFP filter revealed clusters of infected cells beside the vein foci (Fig. 2d), suggesting that CLBV-GFP arrived to veins via cell-to-cell movement. Within cells, confocal laser-scanning microscopy (Leica TCS-SL) showed that fluorescence was located in the cytoplasm and the nucleus, but not in vacuoles of infected epidermal cells, with the most intense fluorescence being observed in the nucleus (Fig. 2e). This GFP accumulation does not mean that the virus replicates in the nucleus.

Systemic viral infection was detected at 18 dpi by the presence of new fluorescent areas in the stem, petiole and veins of upper leaves (Fig. 2f), and then in other leaf tissues (Fig. 2g), indicating that once the virus enters the vascular system it can move long distances in a short time. For other plant viruses cell-to-cell movement has been reported to occur at a rate of approximately 25 $\mu m \cdot h^{-1}$ [268-270], whereas movement through the vascular system occurs at a rate of centimetres per hour [271]. Generally fluorescence was not uniform throughout

stem, petioles or leave veins, with scattered fluorescent spots of variable size and intensity being observed (Fig. 2f). This distribution is consistent with CLBV-GFP

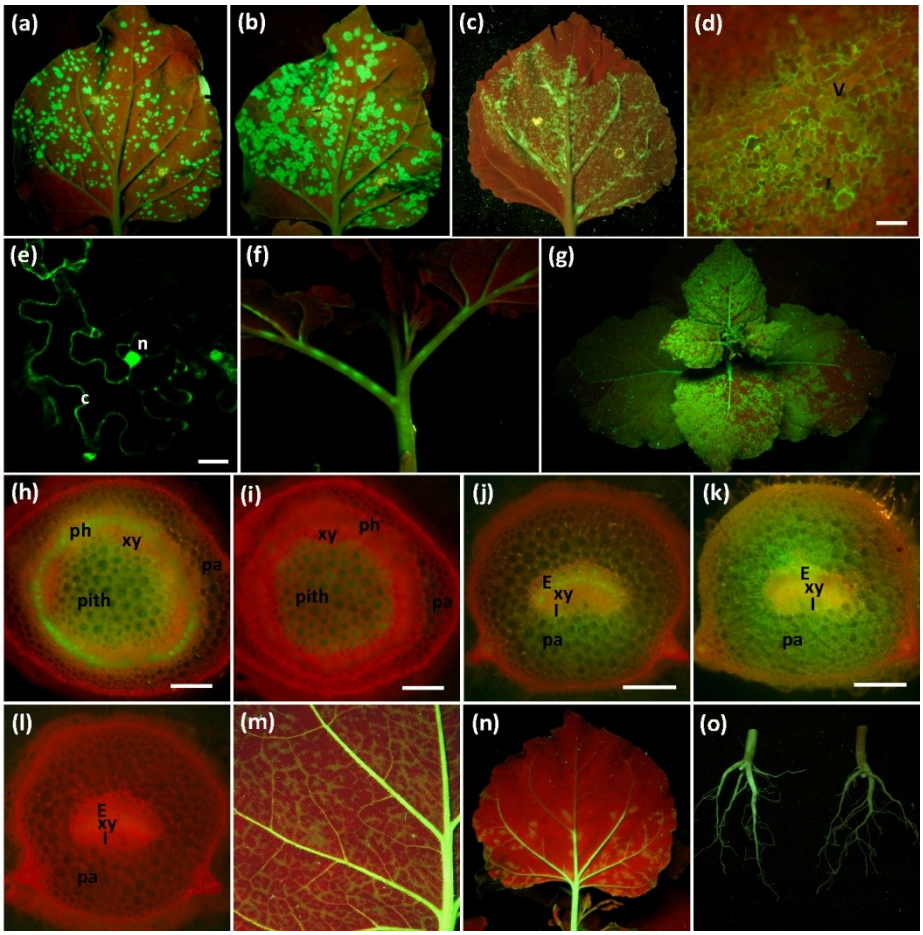


Figure 2. Expression of the green fluorescent protein (GFP) in *Nicotiana benthamiana* plants agroinoculated with the infectious CLBV clone *clbv3'pr*-GFP. (**a** and **b**) Infection foci in agroinoculated leaves at 13 and 16 days post inoculation (dpi), respectively. (**c**) Virus loading into the phloem vessels at different sites of agroinoculated leaves. (**d**) Fluorescence focus beside a vein observed in a fluorescence stereomicroscope. Bar = 80µm (**e**) Epidermal cells observed by confocal microscopy. Bar = 10µm. (n, nucleus; c, cytoplasm) (**f**) GFP accumulation in stem, petiole and veins of upper leaves at 18 dpi. (**g**) Plant systematically infected at 25 dpi. (**h** and **j**) Cross-sections of stem and petiole from an infected plant. **(k)** Similar petiole cross-section from an intense fluorescence spot. (**i and l**) Similar stem and petiole cross-sections from a healthy plant. Bar = 500µm. (ph, phloem; xy,

xylem; pa, parenchyma; E, external phloem; I, internal phloem). (**m**) Upper leaf of infected plants at 20 dpi. (**n**) Leaf undergoing the sink/source transition at 20 dpi. (**o**) Roots of infected (left) and healthy (right) plants. Photographs **a**-**c**, **f**-**g** and **m**-**o** were taken with a digital camera using UV light and a yellow filter, **d** and **h**-**l** with a fluorescence stereomicroscope and **e** with a confocal laser-scanning microscope.

delivery into surrounding cells after long distance transport via the phloem vessels. Observation of stem cross-sections from infected plants with the stereomicroscope showed GFP fluorescence in the phloem and pith, indicating the presence of the virus in these tissues (Fig. 2h). Stem cross-sections from healthy plants showed red fluorescence due to chlorophyll, or a faint yellow fluorescence in xylem and parenchyma cells, probably due to phenolic compounds (Fig. 2i). In the petiole of infected leaves, GFP fluorescence was observed in the phloem and to a lesser extent in parenchyma (Fig. 2j), whereas no fluorescence was observed in identical sections from healthy plants (Fig. 2l). At the most intense fluorescence spots, in either stem (data not shown) or petioles additional GFP fluorescence was detected in parenchyma and epidermal cells (Fig. 2k), probably due to cell-to-cell movement.

After vascular transport of the virus through the stem, it leaves the phloem to establish new infection foci in sink tissues. In young developing leaves, GFP fluorescence was first seen mainly in the midrib -class I vein- and class II veins. The first indication of virus exit to the mesophyll of these leaves was the appearance of disperse fluorescent flecks in the lamina, suggesting that virus unload did not occur uniformly (Fig. 2m). Fluorescent foci associated with virus unload were mainly observed in class III and occasionally in class II veins at later stages of infection, but rarely in smaller veins. Similar results were reported by Roberts *et al*. [272] comparing phloem unload of GFP-tagged *Potato virus X* (PVX) and the fluorescent solute carboxyfluoresceine in *N. benthamiana* plants. Both virus and carboxyfluoresceine were predominantly unloaded from class III veins,

with minor veins -classes IV and V- playing no role in the process. From the initial site of CLBV unload, the infection spread by cell-to-cell movement, as indicated by progressive appearance of GFP fluorescence in the interveinal mesophyll tissue (Fig. 2g). The invasion pattern depends on the developmental stage of the leaf at the time of infection. The three leaves immediately above those agroinoculated did not show any fluorescence, indicating that these leaves were already developed and not at the sink stage when CLBV reached the vascular system. In the next two or three following upper leaves the virus invaded only their basal region but it never reached the leaf apex (Fig. 2n), suggesting that the virus arrived to these leaves when they were at the sink/source transition, as previously described for PVX [272]. The virus would be unable to advance through the veins, but still it would move cell-to-cell from some basal class III veins invading the mesophyll around the unloading point (Fig. 2n). CLBV also invaded and accumulated in *N. benthamiana* roots as detected by real time RT-PCR (rtRT-PCR) with a TaqMan probe targeted to the CLBV ORF1 [222] (data not shown) and by strong green GFP fluorescence observed in infected plants in comparison with the weak yellowish fluorescence shown by healthy plant roots (Fig. 2o).

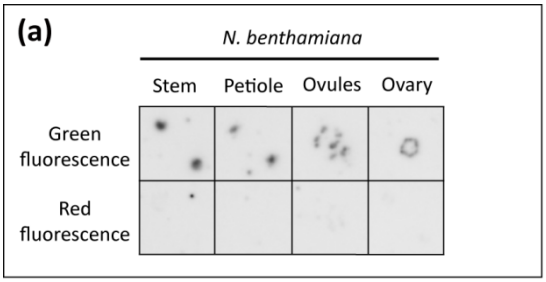
Cell-to-cell and long distance movement of free GFP has been observed in plants after biolistic bombardment of sink leaves with a plasmid encoding the *gfp* gene [273, 274] and in plants expressing the *gfp* gene under the control of the companion cell-specific *AtSUC2* promoter [275]. In order to confirm that GFP fluorescence observed in plants agroinoculated with the *clbv3'pr*-GFP clone is due to the CLBV presence, tissue print hybridization was performed with fresh sections of different plant organs and a DIG-labelled RNA probe specific for the 3' UTR of the gRNA [267]. CLBV was detected in all tissues showing GFP fluorescence, whereas no hybridization signal was obtained in non fluorescent tissues (Fig. 3a). These results confirmed that the GFP fluorescence observed was due to virus spread rather than to independent translocation of the GFP protein.

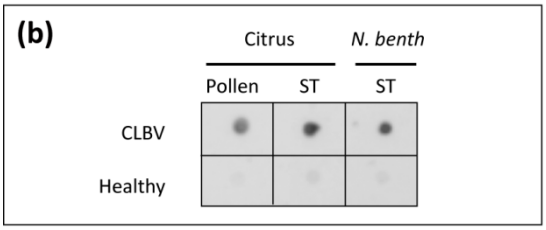
Moreover, GFP fluorescence faded in a couple of weeks, suggesting that the bright fluorescence observed in the initial stages of infection was due to GFP expression during CLBV replication.

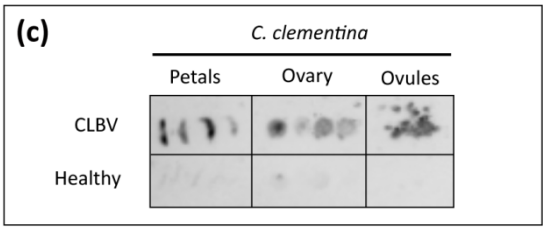
To explore the possibility that CLBV could invade meristems, shoot tips of infected *N. benthamiana* plants were examined by confocal laser-scanning microscopy. Two kinds of meristems were observed: i) Shoot apical meristems (SAM) located at the tip of growing stems and surrounded by the leaf primordia (Fig. 4a). A strong fluorescent signal was observed in both SAM and leaf primordia with GFP fluorescence being located mainly in the cytoplasm (Fig. 4b, c), whereas no GFP fluorescence was observed in shoot tips from non infected plants (data not shown). In these meristems, cells divide actively and different stages of the cell cycle were observed (Fig. 4c). ii) Latent axillary meristems located in leaf axils (Fig. 4d). Cells in these meristems are quiescent and GFP fluorescence appeared mainly in the nuclei (Fig. 4e, f). Because GFP fluorescence expressed by CLBV-GFP was detected in the corpus cells of both meristems, where no vascular tissue is present, the virus likely moves cell-to-cell from the infected protophloem to meristematic cells.

Since Imlau *et al*. [275] showed that GFP can spread by passive diffusion through expanding tissues we tried to confirm the presence of CLBV in SAM cells analyzing total RNA extracts from infected and healthy meristematic tissue (about 0.2 mm shoot tips) by dot-blot hybridization and rtRT-PCR. CLBV was readily detected by both techniques in meristems of infected plants (Fig. 3b and data not shown), confirming that the GFP fluorescence observed was due to virus spread rather than to passive translocation of the GFP protein from neighbour regions.

In CLBV-GFP infected citrus plants GFP fluorescence was not observed, probably due to the instability of the construct [267]. Therefore, to examine CLBV







(d)

Tissue	CLBV		CLBV + CTV		CTV	
	q-clbv	q-ctv	q-clbv	q-ctv	q-clbv	q-ctv
Shoot Tip	2,3*	-	3,4*	-	-	-
Bark	20,1*	-	17,7*	80*	-	72*

* x10^3 RNA copies/ng total RNA (mean of 6 plants)

Figure 3. CLBV detection by molecular hybridization in infected tissues of *Nicotiana benthamiana* and citrus plants. (a) Imprints of different tissues from *N. benthamiana* plants agroinoculated with the CLBV infectious clone *clbv3'pr*-GFP showing green (GFP) or red (chlorophyll) fluorescence. (b) Dot-blot hybridization using total RNA extracted from healthy or CLBV-infected *Citrus clementina* (pollen), Mexican lime (shoot tips, ST) or *N. benthamiana* (ST) plants. (c) Tissue-print hybridization of petals, ovary or ovules from healthy or CLBV-infected *C. clementina* plants. The membranes were hybridized with a DIG-labeled RNA probe specific for the CLBV 3' UTR. (d) Detection and absolute quantification of genomic RNA copies of CLBV (q-clbv) or CTV (q-ctv) in 0.2 mm shoot tips or bark of Mexican lime plants infected with CLBV, CTV or co-inoculated with both viruses. -, not detected.

infection in citrus meristematic regions, six Mexican lime (*Citrus aurantifolia* (Chrism.) Swing.) plants were graft-inoculated with CLBV, with the T318A isolate of *Citrus tristeza virus* (CTV), a phloem-restricted virus that is easily eliminated by shoot-tip grafting *in vitro*, or co-inoculated simultaneously with both viruses. CLBV was detected by dot-blot hybridization (Fig. 3b) and rtRT-PCR [222] (Fig. 3d) in 0.2-mm shoot tips from citrus plants inoculated with CLBV or CLBV plus CTV, whereas CTV was not detected by rtRT-PCR [276] in similar shoot tips from plants inoculated with CTV or CTV plus CLBV. These results explain in part the difficulty to eliminate CLBV by shoot-tip grafting *in vitro*. Most virus and viroids are eliminated by this technique in more than 90% of micrografted plants. However, depending on the citrus genotype, CLBV is usually eliminated in only 10 to 50% of micrografted plants, and in some genotypes no CLBV-free plant was recovered.

In most plant-virus combinations, viruses are not detected in the SAM [261], but it is unknown whether this virus absence is due to inability of viruses to replicate in meristematic tissues or if the presence of some active or passive barrier impairs their entry to the SAM. An attractive hypothesis to explain the ability of meristematic cells to avoid viral infection is that a strong defence mechanism would allow newly differentiated tissues to develop virus-free. It has been reported that an RNA-mediated surveillance system protects the shoot tip from viral infection [277]. The RNA-dependent RNA polymerase 6, which is an integral part of the RNA silencing machinery, is involved in exclusion of viruses [62, 278] and viroids [263] from *N. benthamiana* meristems. To counteract RNA silencing, most plant viruses encode proteins that act as suppressors of the host antiviral defence. *Tobacco rattle virus* (TRV) can invade SAM cells using a 16K suppressor protein encoded by its genome. This protein showed a weak suppressor activity in agroinoculated *N. benthamiana* 16c plants in comparison with the p19 protein of *Tomato bushy stunt virus* [279]. The authors suggested that the weak suppressor activity of 16K might be a crucial evolutionary factor,

since a strong suppressor activity would allow high virus accumulation in meristematic cells probably causing severe damage to infected plants. Similarly, the CLBV MP is a weak silencing suppressor [220] and it could be the factor responsible for viral invasion of meristematic cells without causing important symptoms in most citrus hosts [207]. On the other hand, the 16K protein of TRV is able to act *in trans* whereas the MP of CLBV is not. Thus, while plants co-infected with TRV and PVX accumulated PVX in 53% of the meristems, this virus was excluded from meristems in plants co-infected with PVX and a *16k* mutant of TRV [279]. However, in plants co-inoculated with CLBV and CTV, the latter virus was never detected in meristematic tissues.

Extensive examination of longitudinal sections of *N. benthamiana* flowers with a fluorescence stereomicroscope revealed that CLBV-GFP reaches the flower through phloem channels (Figs. 4 g1 and h), and then it invades the ovary (Fig.4i), style and stigma (Fig. 4j), sepals and petals (Fig. 4k). In mature ovaries strong GFP fluorescence was observed in ovules (Figs.4 l and m), indicating preferential accumulation of the virus in these organs. The ovary, style, stigma and petals of healthy plants showed red fluorescence (Fig 4 g2, p, q and r). The anthers of healthy plants showed a strong green fluorescence, probably due to accumulation of phenolic compounds in the pollen exine that hampered detection of GFP fluorescence in infected plants (data not shown). The presence of CLBV in ovary and ovules was confirmed by tissue-print hybridization (Fig. 3a) and rtRT-PCR (data not shown).

CLBV infection in flower organs of citrus was examined by dot-blot hybridization with total RNA extracted from pollen (Fig. 3b) and by tissue-print hybridization with petals, ovary and ovules (Fig. 3c) from CLBV-infected *C. clementina*. The virus was detected in all these tissues indicating that CLBV invades floral organs including pollen at least in citrus.

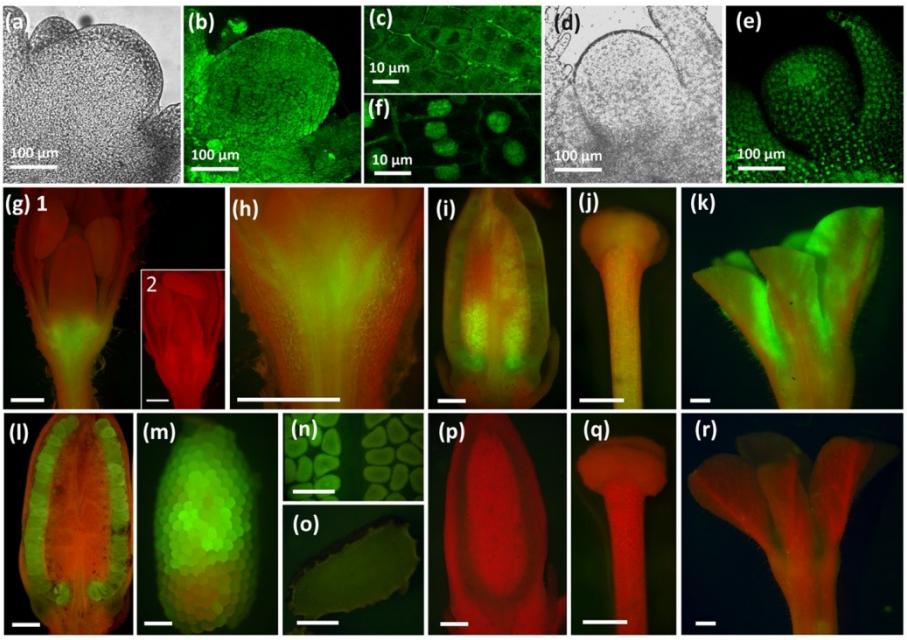


Figure 4. GFP detection in meristems and floral organs of *Nicotiana benthamiana* plants agroinoculated with the infectious CLBV clone *clbv3'pr*-GFP. (**a**-**c**) Shoot apical meristem (SAM). (**d**-**f**) Axillary meristem. (**g**-**h**) Longitudinal sections of flowers from an infected plant at early developmental stages (**g1** and **h)** and a similar section from a healthy plant (**g2**). (**i**-**k**) Developing ovary, style and stigma and corolla from an infected plant and similar organs from a healthy plant (**p**-**r**). (**l**-**m**) Longitudinal section of a mature ovary from an infected plant (**l**) and a similar ovary after eliminating the carpel (**m**). (**n**) Young seeds from infected (left) and healthy (right) plants. (**o**) Mature seed from a healthy plant. (**b**, **c**, **e** and **f**) Images were captured using a confocal laser-scanning microscope, (**a** and **d**) with a light microscope and (**g** through **r**) with a fluorescence stereomicroscope. Bars in the latter images = 500µm.

Finally, GFP fluorescence was also observed in young seeds of CLBV-infected *N. benthamiana* plants (Fig. 4n, left), in comparison with the weak yellowish fluorescence due to the presence of lignin in healthy seeds (Fig. 4n, right), indicating that CLBV-GFP is able to invade the seed coat. However, cross sections of dry seeds from infected or healthy plants showed similar yellowish

fluorescence (Fig. 4o and data not shown). This finding indicates that while CLBV is able to infect maternal seed tissues it would be excluded from the embryo, suggesting that embryos are not symplastically connected with the maternal tissue. Additionally, we did not observe seed transmission in more than 100 seedlings obtained from CLBV-GFP infected *N. benthamiana* plants after fluorescence observation and RT-PCR analyses [267], whereas a low rate of seed transmission (about 2.5%) was previously observed in citrus plants [210].

CAPÍTULO 3:

Ensayo de la eficiencia de los vectores desarrollados para el silenciamiento de genes en *Nicotiana benthamiana* y cítricos

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Adaptado del artículo:

Agüero J, Vives MC, Velázquez K, Pina JA, Navarro L, Moreno P and Guerri J. Effectiveness of gene silencing induced by viral vectors based on *Citrus leaf blotch virus* is different in *Nicotiana benthamiana* and citrus plants. (Submitted to PLOS ONE).

1. Introduction

Citrus represents a major fruit crop commodity in the world. However, the complex reproductive biology of citrus trees due to apomixis and sexual incompatibility between varieties, their long juvenile period (often more than 6 years) and the lack of knowledge on genes regulating different functions, have jeopardized genetic improvement programs by traditional breeding methods. Recently, complete sequencing of the citrus genome (http://www.phytozome.net/citrus) has provided a platform to expedite identification of genes responsible for relevant agronomic characters that could be used for genetic transformation or as molecular markers in conventional breeding programs. However, the genomic sequence by itself does not provide enough information to determine the individual gene functions in an organism. To fully exploit the sequence information and accurately annotate the function of each gene, high throughput screening is required. Mutagenesis programs have provided valuable resources for gene function analyses in model species as *Arabidopsis* [280, 281], but implementation of this technique in citrus plants is more complicated. Another approach that have been successfully used in model plants is reverse genetics suppressing gene expression by RNA interference (RNAi) after stable genetic transformation [282, 283], but this procedure is also inappropriate for high-throughput functional analysis in long life cycle plants as citrus, that have low transformation efficiency and long regeneration time.

In the last two decades, virus induced gene silencing (VIGS) has emerged as an attractive tool to determine gene function. This procedure relies on posttranscriptional gene silencing (PTGS), an RNA-mediated regulatory mechanism in which endogenous or exogenous double stranded RNAs (dsRNAs) are processed by a type III nuclease (Dicer-like) to yield 21-25 nucleotides (nt) small interfering RNAs (siRNAs) that, upon incorporation to an RNA-induced

silencing complex (RISC), recognizes and cleaves the cognate single-stranded RNA (ssRNA). Additionally, siRNAs prime new dsRNA synthesis from the ssRNA template by one or more RNA-dependent RNA polymerases (RDRs), which is then processed by Dicer to produce secondary siRNAs that help maintaining silencing [63]. During the course of viral infections, dsRNA replicative intermediaries or highly structured single stranded RNA trigger the PTGS mechanism that degrades the genomic RNA as an antiviral defence. VIGS technology uses this mechanism to silence plant genes in order to determine their function. When a viral vector carries a plant gene, or a fragment thereof, both the genomic RNA and the inserted sequence are processed and the siRNAs produced lead to the degradation of the mRNAs of the gene (or gene family) homologous to the sequence inserted, causing in the plant a loss-of-function phenotype for the tested gene [83, 226]. VIGS is a particularly useful tool for plant functional genomics. Contrasting with mutagenesis and transformation this technology allows to knockdown genes of interest and observe the elicited phenotype in a short time, including genes whose function is essential for plant viability, as these are silenced after the plant has already grown.

Recently, several *Citrus leaf blotch virus* (CLBV)-based viral vectors have been developed for either gene silencing or protein expression in citrus [267]. CLBV is the type member of the genus *Citrivirus*, family *Betaflexiviridae* [216], and it has a single-stranded, positive-sense genomic RNA (gRNA) of 8,747 nucleotides with three open reading frames (ORFs) and untranslated regions (UTRs) at the 5' and 3' ends of the gRNA [177, 211, 218-220]. Although *Citrus tristeza virus* (CTV) has also been used to express foreign proteins in citrus [223], potential advantages of CLBV-based viral vectors are: i) CLBV causes a symptomless infection in most citrus species and cultivars [207], therefore, phenotypic expression of gene silencing would not be masked, ii) CLBV is not phloem limited, thus these vectors would be appropriate for gene expression or silencing in non-

phloem tissues including meristematic regions [284], iii) contrarily to CTV, CLBV is not transmitted by vectors and therefore it could be safely used in future field experiments.

VIGS efficiency depends mainly on the capacity of the viral vector to invade the host and accumulate to sufficient level in target tissues to initiate the PTGS [59]. In this study we assessed the VIGS effectiveness of different CLBV vectors in *Nicotiana benthamiana* and citrus plants by cloning in these vectors host gene fragments of different sizes or inverted repeat sequences. Since protein expression by CLBV-based vectors was higher in *N. benthamiana* than in citrus [8], we expected that only constructs able to trigger a significant VIGS response in *N. benthamiana* would have the potential for inducing VIGS in citrus. However, we found that some constructs unable to trigger VIGS in *N. benthamiana* plants induced VIGS in citrus, despite showing lower virus accumulation in this latter host, indicating that the threshold virus accumulation to induce efficient VIGS is different in both species. The potential role of a distinct secondary siRNA generation process in both species is discussed.

2. Results

2.1 VIGS in *N. benthamiana* plants

Different viral vectors based on a full-genome cDNA infectious clone of CLBV (CLBV-IC) [208] were previously obtained by i) introducing a unique restriction site in two different positions, at the 3' UTR downstream of the coat protein (CP) encoded by ORF 3 (*clbv*3´ vector), or at the intergenic region between the movement protein (MP) and the CP genes (*clbv*IN vector), and ii) introducing a duplicate of the CP subgenomic RNA (sgRNA) promoter in the two previous vectors restoring the restriction site downstream (*clbv3'pr)* or upstream (*clbvINpr*) of the duplicated CP sgRNA promoter in order to express foreign sequences by

producing an extra sgRNA [208, 267] (Fig. 1a). Previously we tested *clbv3'pr* and *clbvINpr* vectors for their capacity to induce VIGS in citrus plants using linear inserts [267]. In order to improve analysis of gene function by VIGS we assessed the effectiveness of the four different CLBV-based vectors using host gene inserts of different size or inverted repeat sequences.

N. benthamiana is the most widely used experimental host for VIGS assays because its susceptibility to a large number of plant viruses and the rapid appearance of loss-of-function phenotypes [83, 285]. Since CLBV replicates in most *N. benthamiana* tissues [284], we asumed that this herbaceous host could be used for preliminary tests of efficiency and stability of CLBV-based vectors before their application on citrus plants, where experiments are longer and tedious.

The ability of CLBV-based vectors to silence endogenous genes was tested using as target the *phytoene desaturase* (*pds*) gene, an enzyme required for biosynthesis of carotenoid pigments that protect chlorophyll from photo-oxidation, with downregulation of *pds* gene expression leading to a characteristic photo-bleaching phenotype. For this purpose, a 58-nucleotide (nt) inverted repeat (hp58PDS) of *N. benthamiana pds* gene was cloned in *clbv3'*, *clbv3'pr* and *clbvINpr* vectors, a 157-nt linear fragment (157PDS) of the same gene was cloned in *clbv3', clbv3'pr* and *clbvIN* vectors, and a 408-nt *pds* linear fragment (408PDS) was also cloned in *clbv3'* vector. These constructs, labeled with the viral vector name followed by the insert tag (Fig. 1b), were agroinoculated in *N. benthamiana* plants and viral infection was assessed by RT-PCR and northern blot analyses. CLBV was detected by RT-PCR in non inoculated upper leaves of *N. benthamiana* plants inoculated with all constructs except *clbvIN*-157PDS, probably because insertion of an extra sequence in this genomic region disrupts the CP sgRNA synthesis. Northern blot analyses of total RNA from infected plants, using a digoxigenin (DIG)-labeled riboprobe specific for the CLBV 3´ UTR, showed the presence of

bands of the size expected for the viral gRNA and the different sgRNAs. Constructs carrying a duplicated CP sgRNA promoter showed the presence of a new sgRNA (Fig. 1c).

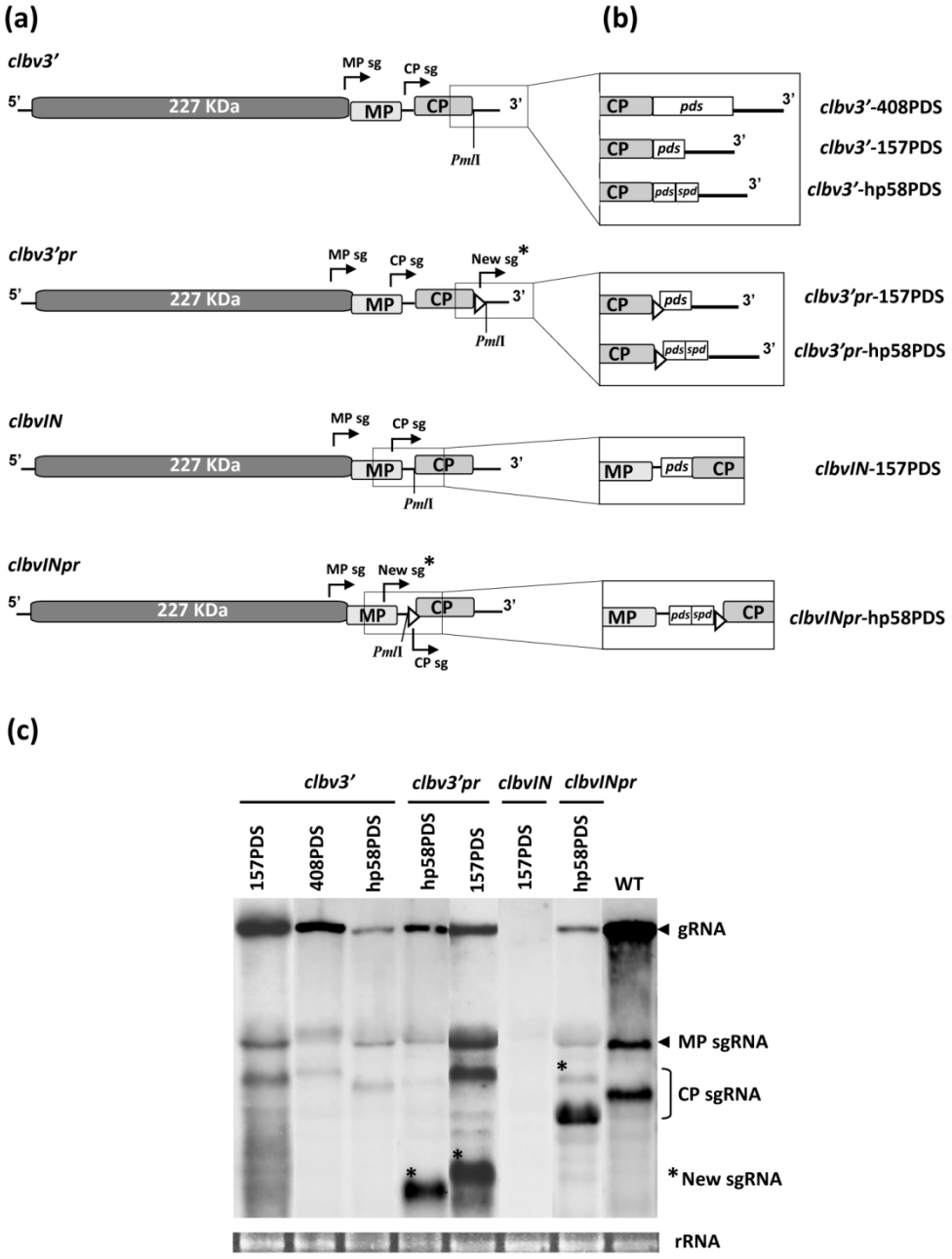


Figure 1. Viral RNA accumulation in *Nicotiana benthamiana* plants agroinoculated with the wild-type *Citrus leaf blotch virus* (CLBV) infectious clone (WT) or with several CLBV-based vectors carrying different fragments of the *phytoene desaturase* (*pds*) gene. (a) Outline of the CLBV-based vectors used in this work. Shaded boxes represent the predicted open reading frames in the CLBV genome and the proteins encoded (227-KDa, polyprotein containing the replicase domains; MP, movement protein; CP, coat protein). Arrows indicate transcription start site of the MP, CP and the new subgenomic RNAs (sg). White triangles represent the duplicated CP sgRNA promoter. *Pml*I, restriction site added for cloning. (b) CLBV constructs generated by cloning different fragments of the *N. benthamiana pds* gene in each CLBV-based vector. White boxes represent the different *pds* fragment sequences. (c) Northern blot analysis of total RNA extracts from non inoculated upper leaves of *N. benthamiana* plants agroinoculated with the WT or with the different CLBV-*pds* constructs at 25 days postinoculation (dpi). The membrane was hybridized with a digoxigenin-riboprobe specific for the CLBV 3' UTR. Arrowheads indicate positions of the CLBV genomic RNA (gRNA) and sgRNAs. GelRed staining of rRNA was used as loading control.

Only the construct *clbv3'pr*-hp58PDS, which transcribes a new sgRNA with a 58-nt hairpin from the *pds* gene, induced photo-bleaching in *N. benthamiana* plants (Fig. 2). The bleaching phenotype appeared in all agroinoculated plants, being first observed in veins of systemically infected leaves, at 22-25 days post-inoculation (dpi) (Fig. 2a). The *pds* silencing phenotype pattern was similar to that observed in *N. benthamiana* plants inoculated with a CLBV-based vector expressing the green fluorescent protein [284]. Often photo-bleaching was unevenly distributed in the plant, with some regions displaying affected whole leaves, sepals, stems and flowers while others were essentially unaffected (Fig. 2b). The other CLBV-*pds* constructs did not induce any obvious photo-bleaching phenotype in agroinoculated *N. benthamiana* plants, even though they showed similar, if not higher, CLBV gRNA accumulation as those agroinoculated with the *clbv3'pr*-hp58PDS construct (Fig. 1c). This result suggests that those constructs do not produce enough dsRNA during virus replication to trigger PTGS. The *pds* inverted repeat sequence expressed by the *clbv3'pr*-hp58PDS and *clbvINpr*-

hp58PDS constructs potentially enabled dsRNA formation, which could trigger the RNA silencing machinery, but lower accumulation of the new sgRNA expressed by the second construct ([267] and Fig. 1c) was likely below the threshold necessary for photo-bleaching to appear.

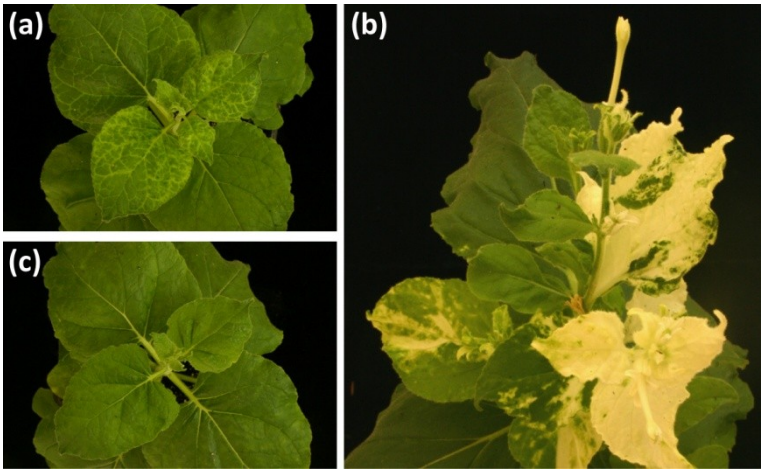


Figure 2. Photobleaching phenotype of *Nicotiana benthamiana* plants agroinoculated with the *Citrus leaf blotch virus* (clbv)*3'pr*-hp58PDS construct carrying a 58-bp inverted repeat sequence from the *pds* gene at 28 (a) or 90 (b) days post inoculation (dpi), or inoculated with the wild CLBV infectious clone at 28 dpi (c).

2.2 VIGS in citrus plants

Since RNA silencing is homology dependent and the *N. benthamiana* and citrus *pds* genes are only about 80% identical, to test the ability of CLBV-derived vectors to induce VIGS in citrus, we prepared constructs equivalent to those used in *N. benthamiana* (Fig. 1a), but harbouring fragments of the *pds* gene from Valencia late sweet orange [*Citrus sinensis* (L.) Osb.]. These constructs were agroinoculated in *N. benthamiana* plants and the resulting recombinant virions were purified and slash inoculated in two *C. excelsa* (Wester) plants. These plants

were later used as inoculum source to graft-inoculate two plants of six different citrus species: Etrog citron (*C. medica* L.), Rough lemon (*C. jambhiri* Lush.), Dweet tangor (*C. tangerina* Hort. ex Tan. × *C. sinensis*), alemow (*C. macrophylla* Wester), Cleopatra mandarin (*C. reshni* Hort. ex Tan.) and Pineapple sweet orange. The *pds* silencing phenotype was observed in three successive flushes and vector stability was monitored at the second flush by RT-PCR analysis using primers encompassing the insertion site in the CLBV genome.

Surprisingly, all the constructs induced the photo-bleaching phenotype in all citrus species tested. However, the pattern and degree of *pds* silencing was different depending on the construct and the citrus species. Photo-bleaching was usually restricted to leaf veins and adjacent areas, albeit white spots or irregular patches in leaves, petioles and stems were occasionally observed, sometimes accompanying white veins (Fig. 3). Generally the silencing phenotype was observed in the first flush after inoculation, but it was more intense in the second and following flushes. Photo-bleaching produced by *clbv3*'-157PDS, *clbv3'*-408PDS, *clbv3'pr*-157PDS and *clbv3'pr*-hp58PDS was more pronounced than that incited by the other constructs, with silencing phenotype being similar in all citrus species but Pineapple sweet orange, which only displayed mild to moderate vein photobleaching in a few leaves of plants inoculated with the clbv3'-157PDS or clbv3'-408PDS contructs. These results confirm previous observations indicating that in this host CLBV shows low accumulation and uneven distribution [207, 221, 222].

Stability analyses showed recombination in 9 of the 12 plants inoculated with *clbv3'*-hp58PDS and in 1 of the 12 inoculated with *clbv3'pr*-hp58PDS, whereas the other constructs were stable, suggesting that the hairpin structure favours recombination events. Moreover, no recombination event was detected in the originally infected *C. excelsa* plants after 2-3 years, depending on the construct.

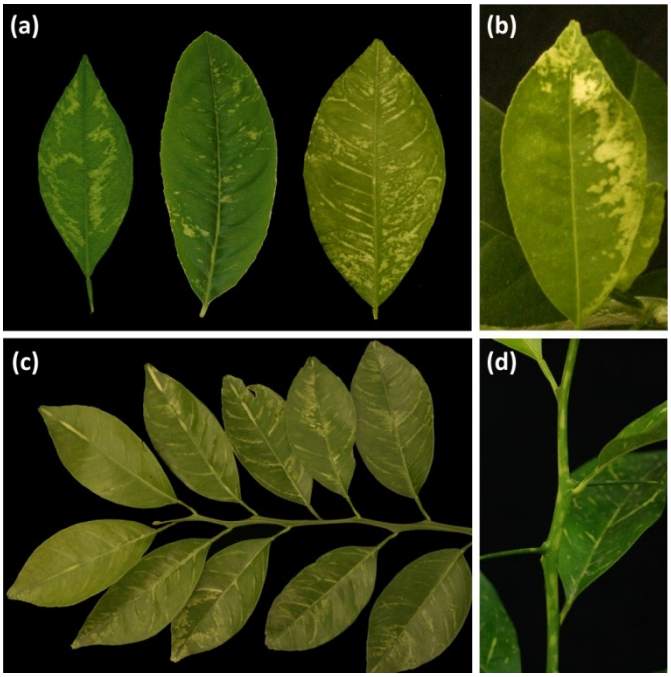


Figure 3. Photo-bleaching phenotype in citrus plants after silencing the *phytoene desaturase* (*pds*) gene with *Citrus leaf blotch virus* (CLBV)-based vectors. (a) From left to right, leaves of Dweet tangor, Etrog citron and Rough lemon plants inoculated with the *clbv3'*-157PDS construct. (b-d). Leaf (b), and stem (d) of Rough lemon and shoot (c) of Cleopatra mandarin plants inoculated with the *clbv3'*-408PDS construct.

VIGS efficiency of a viral vector depends mainly on its ability to invade the host plant and to accumulate in target tissues at a level sufficient to trigger PTGS. Therefore, to select the most effective construct for VIGS in citrus we evaluated reduction of the *pds* mRNA accumulation, using real time RT-PCR [267], and the photo-bleaching intensity, using an effectiveness index (see Materials and Methods), in two successive flushes of five rough lemon plants slash inoculated with the recombinant virions obtained from the constructs *clbv3'*-157PDS, *clbv3'*-408PDS, *clbv3'pr*-157PDS and *clbv3'pr*-hp58PDS (Table 1). The silencing effectiveness was higher in plants inoculated with *clbv3'*-based vectors than in

those inoculated with *clbv3'pr*-based vectors, with the most intense phenotype being incited by the construct *clbv3´*-408PDS (Table1). All silenced plants showed a significant reduction of *pds* mRNA accumulation. However, the photo-bleaching intensity did not correlate with the decrease in *pds* mRNA accumulation (Table 1), in agreement with similar data reported using other viral vectors [59, 113, 115].

Table 1. Effectiveness index (E) of *phytoene desaturase* gene silencing in Rough lemon plants inoculated with different *Citrus leaf blotch virus* (CLBV) vectors or with the wild type CLBV infectious clone (WT) in two successive flushes.

	Second flush		Third flush	
Constructs	**E**	***pds* reduction %***	**E**	***pds* reduction %***
clbv3'-157PDS	1.6	34	1.5	49.6
clbv3'-408PDS	2.24	41.9	2.8	54.9
clbv3'pr-157PDS	0.5	30.2	1.09	31.6
clbv3'pr-hp58PDS	1.45	40.1	0.9	31.4
WT	---	0	---	0

(E) The effectiveness index was calculated considering the number of photo-bleached leaves in each flush and the photo-bleaching intensity as described in the Material and Methods section

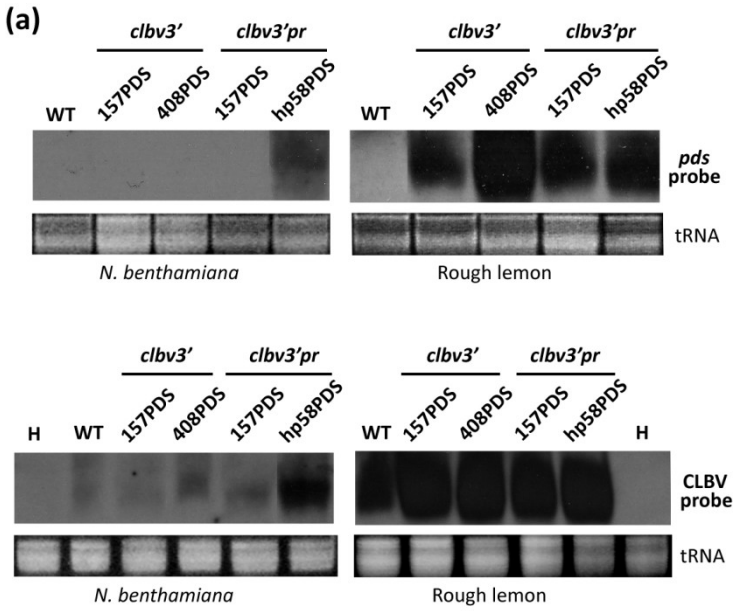
* Relative pds mRNA accumulation was normalized to that of the actin gene in the same plants and expressed as percentage of reduction respect to the *pds* mRNA level of the WT-inoculated plants (mean of 5 plants).

2.3 siRNAs analysis in *N. benthamiana* and citrus plants infected with different CLBV-based vectors

The presence of siRNAs in infected tissues is a hallmark of RNA silencing in plants. To assess the ability of the different CLBV-*pds* constructs to trigger RNA silencing in *N. benthamiana* and rough lemon plants, siRNA-rich extracts from a pool of leaves infected with *clbv3'*-157PDS, *clbv3'*-408PDS*, clbv3'pr*-157PDS,

clbv3'pr-hp58PDS or CLBV WT virions were analyzed by northern blot using DIG-labeled riboprobes specific for a fragment of the *pds* gene not cloned in the vectors or for the *CP* ORF (Fig. 4a). In *N. benthamiana* plants *pds*-derived siRNAs were detected only in plants inoculated with the *clbv3'pr*-hp58PDS construct, but not in equivalent extracts from plants inoculated with the other constructs or with CLBV WT, or from non-inoculated plants. This suggested that only the vector carrying the inverted repeat *pds* sequence was able to trigger RNA silencing in *N. benthamiana* plants. Contrastingly, in citrus plants *pds*-derived siRNAs accumulated to high level in plants inoculated with all the constructs but not in similar extracts from plants inoculated with the CLBV WT or from non-inoculated plants. Northern blot analysis of the same siRNA-rich extracts using the CLBV CP probe showed a strong hybridization signal in RNA extracts from *N. benthamiana* plants inoculated with the *clbv3'pr*-hp58PDS construct and from citrus plants inoculated with all the constructs, indicative of CLBV-derived siRNA formation. A weaker signal was observed in *N. benthamiana* or citrus plants inoculated with CLBV WT and in *N. benthamiana* plants inoculated with *clbv3'*-157PDS, *clbv3'*-408PDS or *clbv3'pr*-157PDS (Fig. 4a). These results suggest that degradation by Dicer of the *pds* endogenous gene inserted in the viral vectors also led to degradation of the CLBV gRNA.

As a threshold level of virus accumulation is required to induce VIGS [113], we also assesed the accumulation of CLBV based vectors in *N. benthamiana* and citrus plants by quantitative real time RT-PCR (Fig. 4b). CLBV accumulation was higher in *N. benthamiana* plants infected with each construct than in citrus plants, despite the observation that accumulation of *pds*- and CLBV- derived siRNAs was lower in *N. benthamiana* than in citrus plants. These results suggest that both species would require a different CLBV accumulation threshold to trigger silencing.



(b)

	WT	*clbv3'* 157PDS	*clbv3'* 408PDS	*clbv3'pr* 157PDS	*clbv3'pr* hp58PDS
N. benthamiana	28.6	5.2	3.3	4.1	2.1
Rough lemon	3.9	1.0	0.6	0.4	0.5

x10^3 RNA copies/ng total RNA

Figure 4. Detection of *pds*- and CLBV-derived siRNAs and absolute quantitation of CLBV gRNA in *N. benthamiana* and Rough lemon plants inoculated with the CLBV infectious clone (WT) or the constructs *clbv3'*-157PDS, *clbv3'*-408PDS, *clbv3'pr*-157PDS, and *clbv3'pr*-hp58PDS. (a) Northern blot analyses of the small RNA fraction extracted from *N. benthamiana* (left panels) and citrus (right panels) infected plants at 28 and 120 dpi, respectively. Membranes were hybridized with digoxigenin (DIG)-labelled RNA probes specific for a segment of the *N. benthamiana* or citrus *phytoene desaturase* gene (upper panels) or for the CLBV *CP* ORF (lower panels). H, non-inoculated healthy plants. Ethidium bromide staining of tRNA was used as loading control. (b) Quantitative real time RT-PCR analysis of CLBV gRNA in total RNA extracts from a pool of leaves from *N. benthamiana* and Rough lemon plants inoculated with the different constructs using primers and a TaqMan probe specific for the CLBV ORF 1.

2.4 VIGS of other citrus genes

To confirm the ability of CLBV-based vectors to silence endogenous citrus genes, the *actin* gene, encoding an ubiquitous protein associated with plant cytoskeleton, and the *sulfur* gene, that encodes a subunit of magnesium chelatase, an enzyme involved in the chlorophyll biosynthesis pathway, were selected for analysis.

For this purpose, a 269-bp fragment of the *actin* gene from Valencia late sweet orange was cloned into the *clbv3'* vector (*clbv3´*-269ACT). This construct was agroinoculated in *N. benthamiana* plants and the resulting recombinant virions were extracted and slash inoculated on two *C. excelsa* plants. These plants showed stunting and leaf cupping compared with non-inoculated healthy plants. To confirm these results 10 Mexican lime (*C. aurantifolia* (Christm.) Swing.) plants were graft inoculated with bark pieces from infected *C. excelsa*. As control, 10 Mexican lime plants were graft-inoculated with CLBV WT. The plants were guided to a single shoot and their height was measured at the end of the second flush. Mexican lime plants inoculated with the *clbv3´*-269ACT construct showed an average height of 38,4 ±3,2 cm, whereas the height of equivalent plants inoculated with CLBV WT was 45,5 ±2,6 cm, indicating size reduction of plants inoculated with the *clbv3´*-269ACT construct (Fig. 5a). Moreover, the latter plants showed bent mishapen leaves probably due to differential growth between silenced and not silenced leaf areas (Fig. 5b).

On the other hand, a construct carrying a 241-bp fragment of the *sulfur* gene from Valencia late sweet orange (*clbv3´*-241SU) was also agroinoculated in *N. benthamiana* plants and the extracted virions were slash inoculated in 2 Rough lemon plants. All plants inoculated with *clbv3´*-241SU construct showed large yellowing patches in the leaves and stems (Fig. 5c-e), indicating chlorophyll deficiency. This silencing phenotype was more widespread in the plants than the *pds*-silencing phenotype.

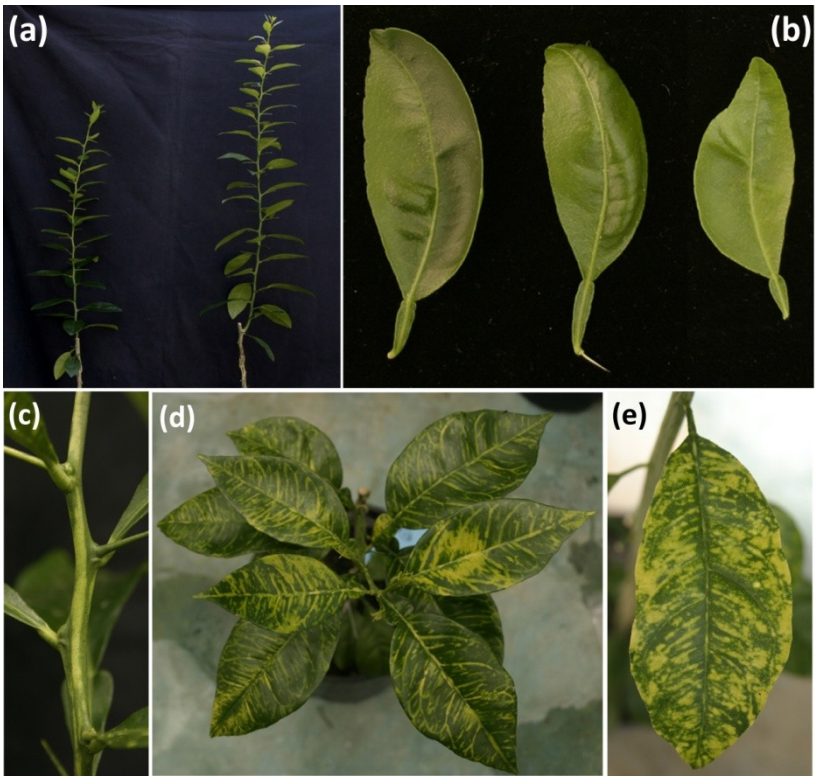


Figure 5. Virus induced gene silencing (VIGS) of endogenous *actin* (upper panels) and *sulfur* (lower panels) genes by *Citrus leaf blotch virus*-based vectors in citrus plants. (a) Stunting of a Mexican lime plant inoculated with *clbv3'*-269ACT (left) compared with a CLBV WT-inoculated control plant (right). (b) Mishapen leaves of the stunted Mexican lime plant. (c,d) Yellowing phenotype in the stem or in the first flush of a sour orange plant inoculated with *clbv3'*-241SU. (e) Idem in an old leaf of a rough lemon plant inoculated with the same recombinant virions.

Since commercial citrus varieties are usually bud propagated on rootstocks we compared the silencing phenotype in seedling or budlings of the same variety. For this purpose, we propagated sour orange (*C. aurantium* L.) buds on 3 rough lemon plants graft-inoculated at the same time with bark pieces from infected rough lemon, and as control, 3 sour orange seedlings were graft inoculated with the same inoculum source or with CLBV WT. While the sour orange seedlings inoculated with the *clbv3´*-241SU construct showed in the first

flush a silencing phenotype confined to the veins of some leaves, and irregular yellowing patches in leaves, petioles and stems in the second and successive flushes, the sour orange/rough lemon plants displayed strong yellowing in all leaves in the first flush (Fig. 5d). This result suggests that the slower growth of propagated plants in comparison with seedlings allows the virus to reach enough titer to trigger silencing in the first flush. In all cases the silencing phenotype was usually observed in fully expanded leaves and rarely in young developing leaves, with the yellowing areas remaining in old leaves (Fig. 5e). Quantitative real-time RT-PCR analysis of *sulfur* mRNA in total RNA from rough lemon and sour orange seedlings with the yellowing phenotype or infected with CLBV WT, showed in the first a reduction of 74% (rough lemon) and 71% (sour orange) in *sulfur* mRNA accumulation in comparison with the WT-inoculated control plants, thus confirming that yellowing was due to silencing of the endogenous *sulfur* gene.

3. Discussion

Optimizing the silencing efficiency of a viral vector is crucial for successful VIGS experiments. Here we compared different CLBV-based vectors for VIGS in *N. benthamiana* and citrus plants using linear inserts of different sizes or inverted repeat sequences of the *pds* gene to assess visually their effectiveness. Contrasting with other viral vectors CLBV causes symptomless infection in *N. benthamiana* [243], therefore, phenotypic expression of gene silencing is not masked by virus symptoms.

Our results demonstrated that the *clbv3´*vector is more effective for VIGS studies in citrus than the *clbv3'pr* or *clbvINpr* vectors previously tested [267]. In *clbv3'pr*-based vectors the synthesis of a new 3´terminal sgRNA compromises transcription of the two natural sgRNAs, and therefore movement and accumulation of the virus [267]. Indeed virus accumulation was higher with *clbv3'*-

than with *clbv3'pr*-based vectors (Fig. 1b and Fig.4c). Since VIGS initiation is thought to be triggered by dsRNA intermediates produced during virus replication, reduced dsRNA formation in the latter constructs could be a limitating factor to trigger RNA silencing, despite high accumulation of the new sgRNA. Higher effectiveness of the *clbv3´pr*-58hpPDS in comparison with the *clbv3´pr*-157PDS construct confirms results obtained with other viral vectors in which expression of inverted repeats increases VIGS induction [59].

The CLBV-based vectors developed provide a reliable and efficient tool to evaluate citrus gene function by reverse genetics using VIGS. Availability of the citrus genome sequence (http://www.phytozome.net/citrus), microarray data sets [10-16] and extensive EST collections [8] provide a long list of candidate genes that might be associated with interesting agronomic traits for breeding programs. The use of CLBV-based vectors to evaluate plant gene function is particularly attractive for citrus, in which analysis of genes involved in certain biological processes like flowering and fruiting by conventional breeding programs is hampered by their long juvenile period, and the difficulty for genetic transformation of adult plants [3]. Once a viral vector has systemically infected a citrus plant, it can be easily graft inoculated to a large amount of plants of other citrus varieties at either the juvenile or mature stage. Although a viral vector based on the CTV genome has been developed [257], it has been used only to express foreign proteins in citrus. So far, CLBV-based vectors are the first developed to induce VIGS in this host.

VIGS is generally considered a transient assay system. However, most of the constructs derived from CLBV-based vectors were able to induce a silencing phenotype in successive flushes for at least 36 months. The mechanism allowing persistent VIGS in citrus is unknown, but at least two factors might contribute to it: i) the high stability of CLBV-based vectors, and ii) the sympodial growth of citrus, which implies a growth arrest of at least one month between the end of a

flush and the induction of a new bud. Although virus replication and VIGS may be less effective during this period and a long-distance silencing signal might not be produced, virions escaping plant silencing in the old tissues actively replicate and spread systemically in the new flush and reach again titer enough to trigger VIGS. This suggestion is supported by finding that CLBV accumulates less in the old than in the young leaves [222], and that in old leaves the silencing phenotype did not increase in comparison with young fully expanded leaves.

The constructs *clbv3'*-157PDS, *clbv3'*-408PDS and *clbv3'pr*-157PDS triggered RNA silencing in citrus, but not in *N. benthamiana* plants. Northen blot analyses of siRNA-rich citrus extracts with riboprobes specific for the *pds* or the CLBV *cp* genes revealed accumulation of *pds*- and CLBV-derived siRNAs that were not observed in equivalent extracts from *N. benthamiana* plants, in spite of detecting lower virus titer in citrus than in *N. benthamiana*. Distinct accumulation patterns of CLBV gRNA and CLBV-derived siRNAs in *N. benthamiana* and citrus plants likely reflect differences in the response of their silencing machinery to viral infection. Of the two steps in PTGS process, production of primary and secondary siRNAs, the first is dependent on dsRNA formation by the viral RdRp during virus replication, whereas the second depends on new dsRNA synthesis primed by the negative strand of primary siRNAs using RNA dependent RNA polymerases of the host (RDRs). It is possible that this new dsRNA formation may be a limiting factor to further increase RNA silencing in *N. benthamiana* plants. Finding that only *N. benthamiana* plants inoculated with the *clbv3'pr*-58hpPDS construct, expressing a dsRNA hairpin structure, induced the photo-bleaching phenotype and *pds*-derived siRNA accumulation, suggests that CLBV replication by itself did not produces in this host enough dsRNA to trigger PTGS and that secondary siRNAs are crucial for maintaining RNA silencing [67, 263, 286]. In *N. benthamiana* the RDR1 is non functional due to the presence of two premature stop codons in the mRNA coding sequence [287], whereas this gene was shown to be up regulated in citrus after

CTV infection [16]. RDR1 has been involved in antiviral defence because RDR1-knockout mutant plants of *N. tabacum* and *Arabidopsis thaliana* accumulated virus RNA to significantly higher levels than the wild type [288, 289]. Additionally, RDR1 has been associated to the production of viral siRNAs in *Arabidopsis* plants inoculated with *Tobacco rattle vi*rus (TRV) [50] and with a mutant of *Cucumber mosaic virus* (CMV) [290]. Although differences in the silencing pathway triggered by CLBV-based vectors in *N. benthamiana* and citrus are unknown, it is conceivable that primary siRNAs produced in *N. benthamiana* and may not be good silencing effectors and that the RDR1 may be required to generate secondary siRNAs that would drive a more effective antiviral response [291]. Reduced accumulation of viral siRNAs and increased accumulation of CLBV gRNA and sgRNAs in *N. benthamiana* plants support the importance of RDR1 activity in the formation of secondary siRNAs. However, silencing of endogenous genes by VIGS in *N.benthamiana* plants using different viral vectors has been reported [80, 127].

Generation of secondary siRNAs in *N. benthamiana* has been associated to RDR6 [61, 278, 291], originally identified as the polymerase required for the silencing of single-stranded RNA of transcribed transgenes in *Arabidopsis* [286]. However, previous studies showed that silencing RDR6 in *N. benthamiana* enhanced susceptibility to PVX, *Potato virus Y* (PVY) and CMV but not to TRV and TMV [62]. In *Arabidopsis* the RDR6 mutants were also found to be hyper-susceptible to CMV but not to TMV, TRV, *Turnip mosaic virus* (TuMV) or *Turnip vein clearing virus* (TVCV) [61, 286]. This variable reaction of RDR6-knockdown *N. benthamiana* plants against different viruses could be due to different RDRs being involved in the secondary siRNA generation. Indeed in *Arabidopsis* TRV-derived siRNA production is strongly dependent on the combined action of RDR1, RDR2 and RDR6 [50]. Moreover, Wang and co-workers demonstrated that in

Arabidopsis plants antiviral defence against CMV required RDR1 or RDR6 function to produce virus-derived secondary siRNAs [95].

Data obtained here demonstrate that the ability of CLBV-based vectors to trigger VIGS can be different depending on the host used and the results obtained in a host cannot be extrapolated to the other. *N. benthamiana* has been one of the model plants for VIGS studies [80, 285, 292] and many viral vectors have been assayed for VIGS in it, in addition to their natural hosts [104]. However, there must be aware that the results obtained may be conditioned by its lack of a functional RDR1.

The VIGS capacity of CLBV-based vectors provides an important genomic tool for the citrus research community, since rapid functional studies by reverse genetics are now possible. The ability of CLBV to invade and to induce VIGS in meristematic tissues [284] would enable studying genes involved in organ development. Since these vectors were stable and induced the silencing phenotype in successive flushes along several months, they might be also used to knockdown genes involved in reproductive stages of the long-lived citrus plants, or to perform a wide range of assays in studies focused on assessing biotic and abiotic stress or senescence related genes. The developed vectors also could be used in disease protection against pathogens as viruses, bacteria and fungi or invertebrate pest as they can be potential targets for VIGS.

4. Material and methods

4.1 CLBV-based constructs

The infectious CLBV clone CLBV-IC (here named wild type, WT) described previously [208] contains a full-length cDNA of the CLBV gRNA cloned into the pBIN19 binary plasmid, between the duplicated 35S promoter of *Cauliflower mosaic virus* and the nopaline synthase terminator. This plasmid was mutated in

order to generate CLBV-based vectors by introducing a unique *Pml*I restriction site in two different sites: at the 3' UTR (*clbv3'* vector) or at the intergenic region between the MP and CP ORFs (*clbvIN* vector), and then introducing a duplicate of the minimum CP sgRNA promoter in the two previous vectors restoring the *Pml*I restriction site (*clbv3'pr* and *clbvINpr* vectors, respectively) [267]. The four developed CLBV-based vectors were used to clone foreign gene fragments in *Pml*I site by using standard techniques [238] and appropriate primers (Table 2). All insertions performed in these plasmids were confirmed by sequencing.

Table 2. Primers used in this work.

Fragment synthesized	Primer	Sequence 5'-3'	Position (nt)
157PDS	PDS1[g]	GGCACTAAACTTCATAAACC	765-784[a]
	PDS5[g]	TGACTGAATGTGTTCAACAAT	921-901[a]
408PDS	PDS1[g]		765-784[a]
	PDS2[g]	CTTCAGTTTTCTGTCAAACC	1173-1154[a]
hp58PDS	Nhp58PDS[g]	AATGGCCTTTTTAGATGGTAACCCTCCTGAGAGACTTTGCA TGCCGATTGTGGAA*CACGTG*TTCCACAATCGGCATGCAAA GTCTCTCAGGAGGGTTACCATCTAAAAAGGCCATT	945-1002-945[b]
hp58PDS	Chp58PDS[g]	GATGGCATTCTTAGATGGCAACCCCCCAGAGAGACTTTGCT TGCCTATTGTTGAA*CACGTG*TTCAACAATAGGCAAGCAAAG TCTCTCTGGGGGGTTGCCATCTAAGAATGCCATC	855 -912-855[a]
241SU	Su-F[g]	CGGGCTCTGTTTGCAGTTACC	7-27[c]
	Su-R[g]	CGGGGAGTAAATCTACCAAGGACC	247-224[c]
269ACT	ActinF[g]	GCAAAGACCAGCTCAGCTGTGG	694-715[d]
	ActinR[g]	GCAGTGATCTCCTTGCTCATTCTGTC	962-937[d]
Flanking 3' *Pml*I	KU17L	ATGTAACTTCAAGTCCACTGTACAATCGTGGG	8130-8161[e]
	KU7L	ATCTTGGATTCAGATTCATGAGGCTCCG	8328-8301[e]
Flanking IN *Pml*I	MP3U	GACGCAAAGTGATGCCTGTTCGCAGACG	6737-6764[e]
	MpC	GTGTCTCCATGCTCGGCCACTACAGC	7254-7229[e]
Pds Q-PCR	QPdsF	AATGCTGACTTGGCCGGAG	597-616[a]
	QPdsR	ATGCCTGTCCGCCAATTATT	667-648[a]
Su Q-PCR	QsuF	GGAGGAGAGAGCTCGATTTG	741-760[c]
	QsuR	GAGAGAACTCCTGGCTGAGG	846-827[c]
Actin Q-PCR	CiACTqF	CAGTGTTTGGATTGGAGGATCA	1146-1167[f]
	CiACTqR	TCGCCCTTTGAGATCCACAT	1217-1198[f]

[a] Nt positions are indicated on the sequence of the *Citrus sinensis pds* mRNA (GenBank accession number DQ235261).

[b] Nt positions are indicated on the sequence of the *N. benthamiana pds* mRNA (GenBank accession number DQ469932).

[c] Nt positions are indicated on the coding sequence of the *C. sinensis* homologue of tobacco *sulfur* gene (Phytozome locus name orange1.1g014510m).

[d] Nt positions are indicated on the sequence of the *C. sinensis* homologue of *actin11* gene (Citrus Functional Genomic database: aCL563Contig1).

[e] Nt positions are indicated on the sequence of the CLBV isolate SRA-153 (EMBL accession number AJ318061).

[f] Nt positions are indicated on the sequence of the putative *C. clementina* actin mRNA (Phytozome locus name clementine0.9_013110m).

[g] Phosphorylated at the 5' end.

To create the constructs used to induce silencing of the *phytoene desaturase* (*pds*) gene, fragments 157PDS and 408PDS of the *pds* gene were RT-PCR amplified from *N. benthamiana* and Valencia sweet orange total RNA (RNAt) extracts using appropriate primers (Table 2). To generate the hairpin insert hp58PDS of *N. benthamiana* and citrus *pds* genes, the 116 bp oligonucleotide containing a 58-nt inverted repeat sequence was synthesized based on the *pds* sequence of the cognate host (Table 2). The 157PDS and the hp58PDS fragments were cloned into the *clbv3'*, *clbv3'pr* and *clbvIN* vectors, and the 408PDS fragments were cloned into the *clbv3'* vector in order to generate the constructs *clbv3'*-157PDS, *clbv3'*-408PDS, *clbv3'*-hp58PDS, *clbv3'pr*-157PDS, *clbv3'pr*-hp58PDS, *clbvIN*-157PDS and *clbvINpr*-hp58PDS.

Fragments 241SU and 269ACT of the *sulfur* and *actin* genes, respectively, were RT-PCR amplified from Valencia sweet orange RNAt using appropriate primers (Table 2). These fragments was cloned into *clbv3'* vector to obtain *clbv3'*-241SU *and clbv3'*-269ACT constructs, respectively.

4.2 Plant growth and inoculations

N. benthamiana plants were grown in small pots with an artificial potting mix (50% vermiculite and 50% peat moss) in a plant growth chamber at 20/24°C

(night/day), 60% humidity, and a 16/8 h light/dark regime. Citrus plants were grown in a glasshouse at 18/26ºC (night/day), using 2-liter plastic containers filled with 50% sand and 50% peat moss and a standard fertilizing procedure [240]. *C. excelsa*, Mexican lime, rough lemon, Dweet tangor, Cleopatra mandarin, Pineapple sweet orange and alemow were grown as seedlings. Etrog citron was propagated on rough lemon rootstock and sour orange was grown either as seedling or propagated on rough lemon rootstock.

Recombinant CLBV clones were transfected to *Agrobacterium tumefaciens* cells, strain COR 308 (kindly provided by Dr. C. M. Hamilton, Cornell Research Foundation) and these cultures were agroinfiltrated on *N. benthamiana* leaves as described previously [208].

Semipurified virion extracts from infected *N. benthamiana* plants [207] were inoculated to *C. excelsa* and rough lemon plants by stem slashing [242] with scalpel blades dipped in the viral extracts [207, 243]. Bark pieces from both citrus plants infected with the recombinant or the WT CLBV were used to graft inoculate other citrus species.

4.3 RNA extraction and northern blot analyses

RNAt from inoculated plants was prepared from 500 mg of leaf tissue using (i) TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, or (ii) a standard protocol with two phenol:chloroform:isoamyl alcohol extractions, followed by RNA precipitation with 12 M lithium chloride, and re-suspension in 25 µl of diethyl pyrocarbonate (DEPC)-treated distilled water [14]. RNA content was measured in a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and adjusted to the same concentration for northern blot and real time RT-PCR analyses.

To obtain small RNA (sRNA)-enriched preparations, RNAt from 1 g of infected tissue was extracted with TRI-Reagent and 1-bromo-3-chloro-propane (Sigma-Aldrich Inc., St. Louis, MO, USA), precipitated with isopropanol and re-suspended in 150 µl of RNase-free water. High-molecular mass RNAs were precipitated with 1 M NaCl and 10% polyethyleneglycol (PEG 8000), and the sRNAs were ethanol precipitated and re-suspended in 50 µl of RNase-free water [293]. RNA concentration was measured in duplicate using a NanoDrop™ spectrophotometer and adjusted to approximately 250 ng/µl to normalize the different extractions. Aliquots were stored at -20°C until use. Northern blot analysis of CLBV RNAs was performed according to [219] and [221], with minor modifications. Three to five micrograms of RNAt were denatured at 94°C for 5 min in 50% formamide, chilled on ice, separated by electrophoresis in formamide-formaldehyde denaturing 1.2% agarose gels in MOPS buffer, and electroblotted onto positively charged nylon membranes (Roche Applied Science, Mannheim, Germany) at 250 mA for 1 h and 1 A for 15 h, using 25 mM phosphate buffer, pH 6.45. To analyze CLBV- and *pds*-derived siRNAs, 5 µg of sRNAs were mixed with an equal volume of formamide, heated at 94ºC for 5 min, separated by electrophoresis in 15% polyacrylamide gels containing 7 M urea in 0.5X TBE buffer (50 mM Tris, 45 mM Boric acid, 0·5 mM EDTA) and electroblotted onto positively charged nylon membranes (Roche Applied Science, Mannheim, Germany) at 25 V for 1 h using 0.5X TBE buffer.

For hybridizations, DIG-labeled riboprobes specific for the CLBV 3' UTR, CLBV CP [219] and 408PDS fragment of the *N. benthamiana* and citrus *pds* gene were used. The cDNA of 408PDF fragments were obtained by RT-PCR using RNAt extracts from both species and specific primers (Table 2). PCR products were cloned in the pGEM-T vector (Promega Corporation, Madison, WI, USA). DIG-labeled negative-stranded RNA transcripts were synthesized from the tobacco or citrus 408PDF cDNA clones by incorporation of DIG-UTP using the T7 or SP6 RNA

polymerase, respectively, according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany).

After UV cross-linking, membranes were pre-hybridized in 0.02% sodium dodecyl sulphate (SDS), 50% formamide, 5X SSC (750 mM NaCl, 75 mM sodium citrate, pH 7), 2% blocking reagent (Roche Applied Science, Mannheim, Germany) and 0.1% lauryl sarcosine for 1 h at 68°C (for CLBV RNAs) or 42°C (for CLBV and *pds* siRNAs) and then hybridized overnight in ULTRAhyb™ Hybridization Buffer (Applied Biosystems, Carlsbad, CA, USA) at the same temperatures. Membrane washing was done with 2X SSC and 0.1% SDS at room temperature, and then with 0.1X SSC and 0.1% SDS at 68°C (for CLBV RNAs) or 50°C (for CLBV and *pds* siRNAs). Hybridization reactions were developed using CPD-Star chemiluminescent substrate (Roche Applied Science, Mannheim, Germany) and visualized with the Luminescent Image Analyzer LAS-3000 (FujiFilm, Tokyo, Japan) and/or with X-Ray films (Carestream Health Inc., Rochester, NY, USA). Quantification of the CLBV sgRNAs in northern blot images taken before saturation was performed with the Multi Gauge v3.0 software (FujiFilm, Tokyo, Japan).

4.4 RT-PCR detection and real-time RT-PCR quantitation

The sequences inserted in CLBV viral vectors were detected by conventional RT-PCR [244] with the primer pairs KU17L/KU7L and MpC/MP3U (Table 2) flanking the two insertion sites. The DNA synthesized was analyzed by 2% agarose gel electrophoresis and GelRed-staining (Biotium Inc., Hayward, CA, USA).

Accumulation of viral gRNA in plants inoculated with CLBV-derived constructs was determined by quantitative real-time RT-PCR performed in a Light-Cycler platform (Roche Applied Science, Mannheim, Germany) with 20 µl glass capillaries. Reverse transcription and amplification were performed using DNA-

free (Turbo DNA-*free* Kit, Ambion, Inc., USA) RNAt (2 µl) adjusted to 10 ng RNA/µl and primers and a TaqMan probe targeted to the ORF1 of the CLBV gRNA [222]. Each sample was analyzed in duplicate in two independent assays.

In gene silencing experiments, the mRNA level of endogenous citrus genes was estimated by quantitative real-time RT-PCR using SYBR GREEN detection. DNA-free RNAt from plants infected with the WT or the different CLBV constructs was reverse transcribed using oligo (dT) and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). PCR amplifications were performed with specific primers (Table 2) and normalized to the expression level of the citrus homologue of the *actin11* gene as described previously [267]. The expression of each gene in plants inoculated with the different CLBV constructs relative to the control plants infected with WT CLBV was determined by the $2^{-\Delta\Delta CT}$ method [245].

4.5 Effectiveness of gene silencing

The effectiveness index of *pds* gene silencing (E) in Rough lemon plants inoculated with *clbv3'*-157PDS, *clbv3'*-408PDS, *clbv3'pr*-157PDS or *clbv3'pr*-hp58PDS constructs was calculated in the second and third flushes of infected plants using the following formula:

E = number of leaves showing silencing phenotype in a flush× i **/** total number of leaves in a flush.

In which i was the photo-bleaching intensity estimated as: 0, no photo-bleaching; 1, photo-bleaching restricted to veins; 2, photo-bleaching in veins and adjacent areas; 3, photo-bleaching also in petioles and stem.

DISCUSIÓN GENERAL

Los cítricos representan el cultivo frutal de mayor impacto económico tanto a nivel mundial como en España. El mantenimiento de la producción y competitividad de este sector depende en gran medida de la disponibilidad de material vegetal de alta calidad, tanto en variedades como en patrones. Sin embargo, la compleja biología reproductiva de los cítricos, su elevada heterozigosis, su dilatado período juvenil (hasta 6-8 años) y la falta de marcadores moleculares apropiados complican la mejora de este cultivo por métodos clásicos.

Para acelerar el proceso de mejora de cítricos se puede emplear una aproximación genómica, cuyo objetivo es la identificación de genes responsables de caracteres de interés agronómico. La disponibilidad de la secuencia completa del genoma de un naranjo dulce y un clementino (www.phytozome.net/), de una amplia colección de ESTs (www.ncbi.nlm.nih.gov/dbEST/) y de perfiles de expresión génica en distintos tejidos, estadios de desarrollo o bajo diferentes estreses [10-17], ha permitido identificar una larga lista de genes candidatos a participar en determinados procesos biológicos. Sin embargo, son necesarios nuevos análisis para asociar cada gen a un fenotipo específico o una función biológica.

En los últimos años se ha desarrollado una estrategia muy atractiva para determinar la función de genes mediante genética reversa basada en el silenciamiento génico postranscripcional inducido por virus (VIGS, *Virus Induced Gene Silencing*). Para ello se utiliza el genoma de un virus como vector para introducir genes o fragmentos de genes de la planta. Cuando se inocula el virus, el procesamiento del genoma viral que se produce como defensa de la planta frente a la infección conduce también a la degradación del gen insertado, lo que desencadena la degradación del mRNA homólogo de la planta, mostrándose el fenotipo de pérdida de función del gen ensayado. Los vectores virales también se han utilizado para expresar proteínas de interés en la planta.

Al comienzo de esta tesis sólo estaba disponible un vector viral para cítricos basado en el virus de la tristeza de los cítricos (CTV), que permite expresar proteínas desde su genoma pero que nunca ha sido ensayado para estudiar la función de genes mediante VIGS [223]. Por lo tanto, el objetivo principal de esta tesis ha sido el desarrollo de vectores virales eficientes basados en el genoma del virus del manchado foliar de los cítricos (CLBV), que sirvan tanto para expresar proteínas como para silenciar genes de cítricos, lo que permitiría utilizarlos como herramienta para determinar la función de genes útiles para la mejora genética de este cultivo. Los vectores basados en el genoma de CLBV podrían tener las siguientes ventajas sobre el vector basado en el genoma de CTV: 1) CLBV infecta todas las especies y variedades de cítricos ensayadas sin inducir síntomas en la mayoría de ellas [207], lo que evitaría interferencias con la manifestación fenotípica del silenciamiento de los genes analizados. 2) CLBV se multiplica en todos los tejidos de la planta, al contrario de lo que ocurre con CTV, que está limitado a las células del floema. 3) CLBV es capaz de invadir regiones meristemáticas, lo que lo hace especialmente interesante para el estudio de genes implicados en el desarrollo de hojas y frutos. 4) CLBV no se transmite por vectores, a diferencia de CTV, por lo que podría ser usado sin peligro en futuros experimentos de campo.

Para desarrollar los vectores virales basados en el genoma de CLBV se empleó un clon infeccioso del virus obtenido previamente en nuestro laboratorio [208], al que se le introdujo un punto de corte único *Pml*I en dos zonas del genoma: en el extremo 3' no traducible adyacente al gen de la proteína de cápsida (CP) (vector *clbv3'*), o en la zona intergénica entre los genes de las proteínas de movimiento (MP) y CP (vector *clbvIN*). Con el objetivo de obtener un vector viral para la expresión de secuencias foráneas mediante la formación de un nuevo sgRNA, se delimitó la secuencia mínima promotora del sgRNA de la CP mediante clonación de fragmentos de distinta longitud en torno a su origen de

transcripción en el vector *clbv3´.* Demostramos que un fragmento de 92 nt (-42/+50) alrededor del inicio de transcripción del sgRNA CP contenía todos los elementos necesarios para la síntesis de un nuevo sgRNA *in vivo*. A este nuevo vector con la secuencia mínima promotora del sgRNA CP duplicada en el extremo 3´ se le denominó *clbv3'pr*. La acumulación en plantas de *N. benthamiana* del nuevo sgRNA sintetizado por la progenie viral derivada de una construcción que contiene la proteína verde fluorescente (GFP) clonada en este vector (*clbv3´pr*-GFP) fue mucho mayor que la de los sgRNAs naturales del virus debido a que el promotor mínimo duplicado carece de una secuencia represora presente en el promotor completo [177]. Sin embargo, la acumulación de gRNA en las plantas inoculadas con *clbv3'pr*-GFP fue inferior a la obtenida con el clon infeccioso original, probablemente debido a que la alta expresión del nuevo sgRNA compromete la expresión de los otros sgRNAs. Se sabe que la presencia de CP es necesaria para la acumulación del virus y que hay una correlación entre la cantidad de sgRNA CP y la de gRNA [177]. Para aumentar la acumulación de gRNA se desarrolló un nuevo vector mediante la clonación de la secuencia mínima promotora en el vector *clbvIN,* dando lugar al vector *clbvINpr.* La acumulación de gRNA en plantas de *N. benthamiana* inoculadas con una construcción que contiene GFP clonada en este vector (*clbvINpr*-GFP) fue ligeramente inferior a la del clon infeccioso original de CLBV y hasta 10 veces superior a la de *clbv3'pr*-GFP.

Para que un vector viral sea eficiente en cítricos debe ser suficientemente estable para permitir evaluar los caracteres a lo largo de una brotación o del ciclo reproductivo de la planta, procesos que en cítricos pueden tardar desde algunos meses a varios años. Los vectores virales tienen tendencia a ser inestables, produciéndose eventos de recombinación que inducen la pérdida de la secuencia insertada y por lo tanto de su funcionalidad. Por ello, para optimizar un vector viral es necesario conocer los factores que afectan a su estabilidad. Con este fin, en los distintos vectores desarrollados se clonaron secuencias de diferente

tamaño o en tándem invertido y se estudió su estabilidad. Todas las construcciones derivadas del vector *clbv3´* fueron estables durante el periodo analizado (hasta 3 años). Sin embargo, la introducción de cualquier secuencia foránea en el vector *clbvIN* impidió la multiplicación del virus, probablemente porque está afectada la transcripción del sgRNA CP [177]. Los vectores con el promotor duplicado resultaron en general más inestables, aunque la estabilidad dependía del tamaño del inserto. El vector *clbvINpr* se mostró más estable que el vector *clbv3´pr*, ya que no se produjeron eventos de recombinación con insertos menores de 720 nt, mientras que para el vector *clbv3´pr* este límite fue de 408 nt.

Para un uso apropiado de los vectores virales como herramienta de silenciamiento génico o de expresión de proteínas es importante conocer la distribución del virus en los distintos tejidos de la planta. Por lo tanto, para estudiar el movimiento y distribución de CLBV en plantas de *N. benthamiana* se empleó la construcción *clbv3'pr*-GFP, que expresa GFP desde el promotor duplicado en la región 3' no traducible, La observación de GFP permitió detectar la presencia de CLBV en la mayoría de los tejidos de las plantas infectadas, destacando una gran acumulación del virus en los óvulos. El virus se acumuló en altas proporciones en los meristemos, por lo que los vectores basados en el genoma de CLBV resultan especialmente interesantes para el estudio de genes implicados en el desarrollo de hojas, flores y frutos. Debido a que esta construcción no es muy estable no se pudo realizar el estudio del movimiento del virus en plantas de cítricos mediante observación de la fluorescencia de GFP. Sin embargo, CLBV se detectó mediante RT-PCR e hibridación molecular en regiones meristemáticas apicales de plantas de cítricos inoculadas con CLBV o CLBV+CTV, mientras que CTV, un virus que no invade meristemos, no se detectó en estas regiones. El hecho de que CLBV se acumule en regiones meristemáticas explicaría la dificultad de obtener plantas libres de este virus mediante microinjerto [266].

La capacidad de los vectores derivados de CLBV para expresar proteínas se evaluó mediante la cuantificación de GFP producida por las progenies virales derivadas de las construcciones *clbv3'pr*-GFP y *clbvINpr*-GFP. En plantas de *N. benthamiana* inoculadas con *clbv3'pr*-GFP se estimó una producción de 16 µg de GFP por gramo de peso fresco, cantidad que resultó entre 5 y 6 veces superior a la producida en plantas inoculadas con *clbvINpr*-GFP. En cítricos, debido a la inestabilidad de la construcción *clbv3'pr*-GFP, sólo se pudo cuantificar la proteína expresada por *clbvINpr*-GFP, estimándose en 0.6 µg de GFP por gramo de peso fresco.

La efectividad de los vectores desarrollados para silenciar genes en plantas de cítricos se ensayó inicialmente silenciando el transgén *gfp* en plantas transgénicas. En todos los casos se observó el fenotipo de silenciamiento de *gfp* al inocular las plantas transgénicas con los vectores *clbv3'pr* y *clbvINpr* portando el gen o fragmentos del gen *gfp*. El fenotipo de silenciamiento se observó en hojas, peciolos, espinas, tallo e incluso meristemos, lo que confirma que CLBV es capaz de invadir la región meristemática de cítricos. Se observaron diferencias en el patrón de silenciamiento desencadenado en las distintas especies de cítricos analizadas, probablemente debido a diferencias en las pautas de acumulación del virus en distintas especies. Para confirmar que el fenotipo de silenciamiento observado estaba asociado con la degradación del mRNA expresado por el transgen de *gfp*, dicho mRNA se cuantificó mediante RT-PCR en tiempo real. En las plantas inoculadas con los vectores virales se observó una disminución en la acumulación del mRNA de *gfp* que oscilaba entre el 35 y el 50% respecto a la de las plantas inoculadas con el clon infeccioso original de CLBV.

Para evaluar la efectividad que presentan los vectores basados en el genoma de CLBV para el estudio de la función de genes endógenos mediante VIGS, se clonaron fragmentos de distinta longitud o en tandem invertido del gen de la fitoeno desaturasa (*pds*) de *N. benthamiana* o cítricos, según la especie a

estudiar, en los vectores desarrollados. Sólo la construcción *clbv3'pr*-hp58PDS, que expresa una secuencia de 58 nt en tandem invertido mediante la formación de un nuevo sgRNA, fue capaz de inducir el fenotipo de fotoblanqueamiento típico del silenciamiento del gen *pds* en plantas de *N. benthamiana,* mientras que en cítricos todas las construcciones ensayadas fueron capaces de inducir el silenciamiento de dicho gen mostrando el fenotipo esperado de fotoblanqueamiento. En las plantas de *N. benthamiana* y de cítricos que expresaron el fenotipo de silenciamiento se observó una acumulación de RNAs pequeños (siRNAs) procedentes del gen insertado y del genoma del virus. Estos siRNAs no se observaron en las plantas infectadas con construcciones que no indujeron el fenotipo de silenciamiento o con el clon infeccioso de CLBV, ni en las plantas sanas. En *N. benthamiana,* la RNA polimerasa dependiente de RNA 1 (RDR1) no es funcional debido a la presencia de dos codones de parada en su secuencia [287], mientras que este gen se sobreexpresa al inocular plantas de cítricos con CTV [16]. Aunque no se conocen las diferencias en el mecanismo de silenciamiento que desencadenan los vectores virales basados en el genoma de CLBV en *N. benthamiana* y cítricos, es posible que los siRNAs primarios producidos en *N. benthamiana,* en ausencia de RDR1, no sean unos buenos efectores y que la RDR1 presente en cítricos sea necesaria para generar siRNAs secundarios que son capaces de producir una respuesta eficiente frente al virus. *N. benthamiana* ha sido el huésped experimental más utilizado para el estudio de VIGS. Sin embargo, los datos presentados en esta tesis indican que los resultados obtenidos en un huésped no siempre se pueden extrapolar a otros.

El vector *clbv3´* fue el más efectivo para el estudio de VIGS en cítricos. La mayor efectividad de la construcción *clbv3´pr*-58hpPDS respecto a *clbv3´pr*-157PDS confirma que la expresión de secuencias en tándem invertido incrementa la inducción de VIGS. En todos los casos donde se observó la aparición del fenotipo de silenciamiento también se detectó una disminución del mRNA

mediante RT-PCR a tiempo real. El fenotipo de silenciamiento se observó en distintas especies de cítricos y en sucesivas brotaciones, lo que confirma la gran estabilidad de los vectores basados en el genoma de CLBV.

Para confirmar la capacidad de los vectores desarrollados para silenciar otros genes endógenos de cítricos se seleccionó el gen que codifica la actina, una proteína asociada al citoesqueleto, y el gen *sulfur*, que codifica una subunidad de la magnesio quelatasa, enzima asociada a la síntesis de clorofila. El silenciamiento del gen de la actina dio lugar a plantas enanas y a deformaciones en las hojas, probablemente debido a diferencias en el crecimiento entre las zonas silenciadas y las no silenciadas. El silenciamiento del gen *sulfur* dió lugar a amplias zonas amarillas en hojas, tallos y peciolos debido a la deficiencia en las síntesis de clorofila en las hojas.

Los vectores desarrollados en esta tesis proporcionan una herramienta eficiente para el estudio de la función de genes mediante genética reversa utilizando la técnica VIGS. También podrían ser útiles, y experimentos actualmente en curso así lo demuestran, para el estudio de la función de genes mediante expresión de proteínas. La utilización de vectores virales tiene ventajas sobre otros métodos de estudio de la función de genes, como la mutagénesis o la transformación genética, ya que permite ensayar la función de numerosos genes en un corto periodo de tiempo. Esto es especialmente crítico en el caso de los cítricos, que poseen largos periodos juveniles (normalmente 6-8 años) y donde la transformación de plantas adultas es muy difícil. Otra ventaja es que permite estudiar la función de genes que son esenciales para el crecimiento o el desarrollo de la planta. En los ensayos clásicos mediante mutagénesis o transformación genética, la falta de función de estos genes hace que la planta pueda morir durante la germinación o la regeneración, mientras que con los vectores virales, el RNA diana sólo se silencia cuando el virus infecta la planta. Cuando CLBV infecta una planta de cítrico el virus puede ser fácilmente transmitido a otras plantas de

diferentes especies de cítricos, juveniles o adultas, mediante injerto. La capacidad que tiene CLBV de invadir y de inducir VIGS en tejidos meristemáticos lo convierte en una herramienta idónea para el estudio de genes asociados al desarrollo de hojas o frutos. Como los vectores desarrollados son estables y las construcciones derivadas de ellos inducen el fenotipo de silenciamiento en sucesivas brotaciones durante al menos 3 años, que es el tiempo estudiado hasta la fecha, dichos vectores se podrían utilizar para silenciar genes asociados a los estados reproductivos o para realizar ensayos de genes relacionados con estreses bióticos o abióticos o con senescencia. Los vectores desarrollados en esta tesis también podrían ser útiles para inducir protección frente a enfermedades producidas por virus, hongos o invertebrados mediante VIGS o frente a factores del huésped necesarios para la infección o expresando péptidos microbicidas sintéticos, así como frente a insectos u otros invertebrados expresando moléculas tóxicas o repelentes frente a éstos o pequeños RNAs interferentes frente a la expresión de genes críticos para su desarrollo o supervivencia.

CONCLUSIONES

- Se han desarrollado vectores virales basados en el virus del manchado foliar de los cítricos (CLBV) a partir de un clon infeccioso de cDNA del genoma completo del virus, introduciendo un sitio de restricción *Pml*I en la zona 3' no traducible (vector *clbv3'*) o en la zona intergénica localizada entre los genes de las proteínas de movimiento y cápsida (CP) (vector *clbvIN*).

- Para expresar secuencias foráneas mediante la formación de un nuevo RNA subgenómico (sgRNA) se ha mapeado la secuencia mínima promotora del sgRNA CP, quedando delimitada en 92 nt localizados entre las posiciones -42/+50 respecto del inicio de transcripción de dicho sgRNA. Esta secuencia se ha clonado en el sitio de restricción *Pml*I de los vectores anteriores para obtener los vectores *clbv3'pr* y *clbvINp,* respectivamente. Ambos vectores fueron capaces de producir un nuevo sgRNA y de expresar proteínas recombinantes en plantas infectadas de *Nicotiana benthamiana* y cítricos.

- Se ha analizado la estabilidad de los distintos vectores obtenidos en plantas de *N. benthamiana* y cítricos clonando insertos de diferente tamaño. Todas las construcciones derivadas del vector *clbv3´* se mostraron estables a lo largo de las diferentes brotaciones analizadas durante al menos 3 años. Sin embargo, ninguna de las construcciones derivadas del vector *clbvIN* fue funcional. Por otro lado, la estabilidad de las construcciones derivadas de los vectores *clbv3'pr* y *clbvINpr* dependió del tamaño del inserto. Con el vector *clbv3'pr* se observaron eventos de recombinación con insertos superiores a 408 nt, mientras que con el vector *clbvINpr* aparecieron con insertos mayores de 720 nt.

- Se ha estudiado el movimiento y distribución de CLBV en plantas de *N. benthamiana* y cítricos clonando GFP en el vector *clbv3'pr*. El virus se detectó en la mayoría de tejidos y órganos de la planta, acumulándose preferentemente en óvulos y regiones meristemáticas.

- Se ha analizado la efectividad de los vectores *clbv3´, clbv3´pr* y *clbvINpr* para silenciar genes endógenos y foráneos mediante VIGS. En cítricos todas las construcciones de los tres vectores indujeron fenotipo de silenciamiento del gen ensayado, aunque el vector *clbv3´* fue el más efectivo para el estudio de VIGS en este huésped. Sin embargo, en *N.*

benthamiana sólo se indujo silenciamiento cuando se empleó una construcción del vector *clbv3´pr* que expresaba un fragmento de secuencia con repeticiones invertidas formando una horquilla de doble cadena.

- Se ha demostrado que la capacidad de los vectores basados en el genoma de CLBV para inducir VIGS es diferente en *N. benthamiana* y cítricos, por lo que los resultados obtenidos en un huésped no pueden ser extrapolados a otro.

- Los vectores virales basados en CLBV desarrollados en esta tesis son una herramienta idónea para expresar proteínas en cítricos y para determinar mediante VIGS la función de numerosos genes de interés agronómico para la mejora de los cítricos.

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