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Effectiveness of gene silencing induced by viral vectors based on *Citrus leaf blotch virus* is different in *Nicotiana benthamiana* and citrus plants

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ABSTRACT

Virus induced gene silencing (VIGS) is an effective technology for gene function analysis in plants. We assessed the VIGS effectiveness in *Nicotiana benthamiana* and citrus plants of different *Citrus leaf blotch virus* (CLBV)-based vectors, using insets of the *phytoene desaturase* (*pds*) gene. While in *N. benthamiana* the silencing phenotype was induced only by the construct carrying a 58-nt *pds* hairpin, in citrus plants all the constructs induced the silencing phenotype. Differences in the generation of secondary small interfering RNAs in both species are believed to be responsible for differential host–species effects. The ability of CLBV-based vectors to silence different endogenous citrus genes was further confirmed. Since CLBV-based vectors are known to be stable and induce VIGS in successive flushes for several months, these vectors provide an important genomic tool and it is expected that they will be useful to analyze gene function by reverse genetics in the long-lived citrus plants.

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 hindered 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* (Pan et al., 2003; Sessions et al., 2002), but implementation of this technique in citrus plants is more complicated. Another approach that has been successfully used in model plants is reverse genetics suppressing gene expression by RNA interference (RNAi) after stable genetic transformation (Harmon and Kay, 2003; Senthil-Kumar et al., 2010), 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 host 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), recognize and cleave the cognate single-stranded RNA (ssRNA). Additionally, siRNAs prime new dsRNA synthesis from the ssRNA template by one or more host RNA-dependent RNA polymerases (RDRs). The newly synthesized dsRNA is then processed by Dicer to produce secondary siRNAs that help maintaining silencing (Baulcombe, 2004). During the course of viral infections, dsRNA replicative intermediates or highly structured single-stranded RNA trigger the PTGS mechanism that degrades the genomic RNA (gRNA) as an antiviral defense. 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 gRNA 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 gene tested (Burch-Smith et al., 2004; Senthil-Kumar and Mysore, 2011). VIGS is a particularly useful tool

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for plant functional genomics. Contrasting with mutagenesis and transformation, this technology allows to knockdown genes of interest and observes 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 (Agüero et al., 2012). CLBV is the type member of the genus *Citrivirus*, family *Betaflexiviridae* (Adams et al., 2012), and it has a single-stranded, positive-sense gRNA of 8747 nucleotides with three open reading frames (ORFs) and untranslated regions (UTRs) at the 5′ and 3′ ends of the gRNA (Galipienso et al., 2001; Renovell et al., 2010; Renovell et al., 2012; Vives et al., 2001; Vives et al., 2002a,2002b). Although *Citrus tristeza virus* (CTV) has also been used to express foreign proteins in citrus (Folimonov et al., 2007), potential advantages of CLBV-based viral vectors are: (i) CLBV causes a symptomless infection in most citrus species and cultivars (Galipienso et al., 2000), 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 (Agüero et al., 2013), and (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 in target tissues to a level sufficient to initiate the PTGS (Lacomme et al., 2003). In this study we assessed the VIGS effectiveness of different CLBV vectors in *Nicotiana benthamiana* and citrus plants by cloning host gene fragments of different sizes or inverted repeat sequences in these vectors. Since protein expression by CLBV-based vectors was higher in *N. benthamiana* than in citrus (Agüero et al., 2012), 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 silencing trigger threshold to induce efficient VIGS is different in both species.

Results

VIGS in N. benthamiana plants

Different viral vectors based on a full-genome infectious cDNA clone of CLBV (CLBV-IC) (Vives et al., 2008a) were previously obtained by (i) introducing a unique *Pml* I restriction site at two different positions, at the 3' UTR downstream of the coat protein (CP) encoded by ORF 3 (*clbv3′* vector), or at the intergenic region between the movement protein (MP) and the CP genes (*clbvIN* vector), and (ii) introducing a duplicate of the CP subgenomic RNA (sgRNA) promoter in the two previous vectors restoring the *Pml* I 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 (Agüero et al., 2012; Vives et al., 2008b) (Fig. 1). Previously we tested *clbv3′pr* and *clbvINpr* vectors for their capacity to induce VIGS in citrus plants using linear inserts (Agüero et al., 2012). 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 (Goodin et al., 2008; Senthil-Kumar and Mysore, 2011). Since CLBV replicates in most *N. benthamiana* tissues (Agüero et al., 2013), 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 more laborious.

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 of the same gene (157PDS) 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. 1), were agroinoculated in *N. benthamiana* plants and viral infection was assessed by RT-PCR and northern blot analyses. All the experiments were repeated at least twice, with five to eight plants being agro-infiltrated in each assay. 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. 2).

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. 3). 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. 3A). 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 (Agüero et al., 2013). 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. 3B). 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. 2). On the other hand, no recombination events were detected by RT-PCR analysis with primers encompasing the insertion site of CLBV genome in the plants agroinoculated with these constructs at 40 days post inoculation. 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 an7d *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 [Agüero et al., (2012) and Fig. 2] was likely below the threshold necessary for photo-bleaching to appear.

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. 1), but harboring 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

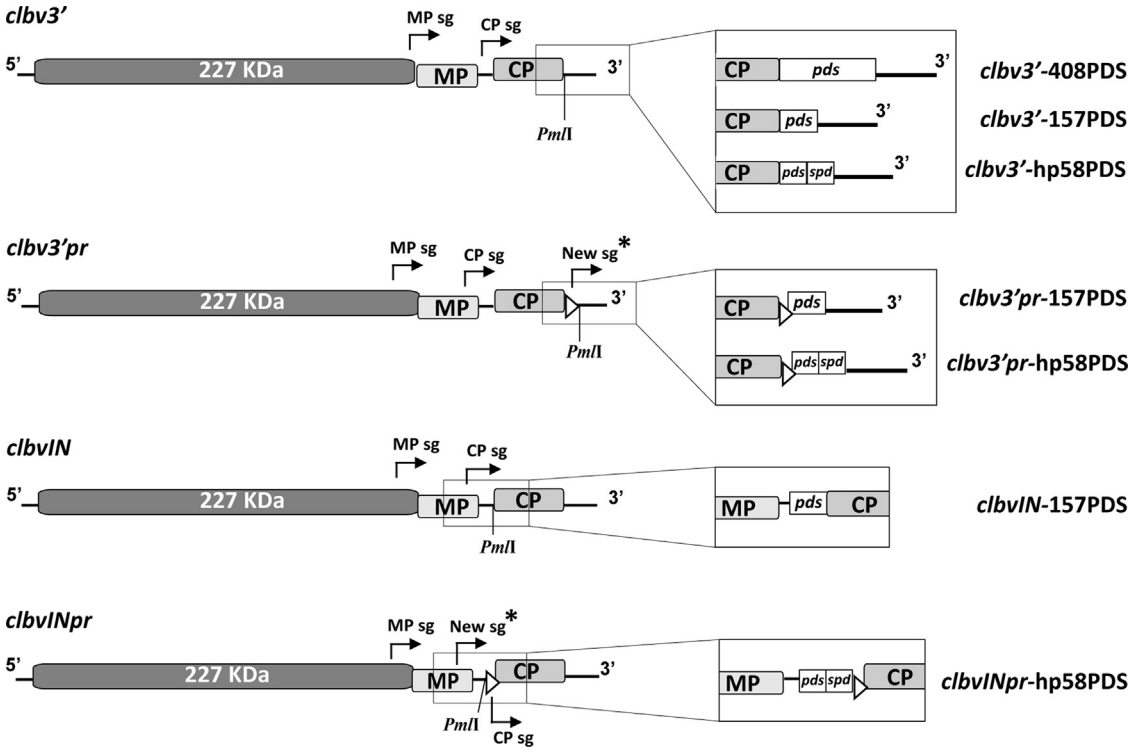


Fig. 1. Outline of the *Citrus leaf blotch virus* (CLBV)-based vectors used in this work. CLBV constructs generated by cloning different fragments of the *Nicotiana benthamiana phytoene desaturase* (*pds*) gene in each CLBV-based vector. Shaded boxes represent the predicted open reading frames in the 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 (MP sg, CP sg, and New sg*). White triangles represent the duplicated CP sgRNA promoter. *Pml*I, restriction site added for cloning. White boxes represent the different *pds* fragment sequences.

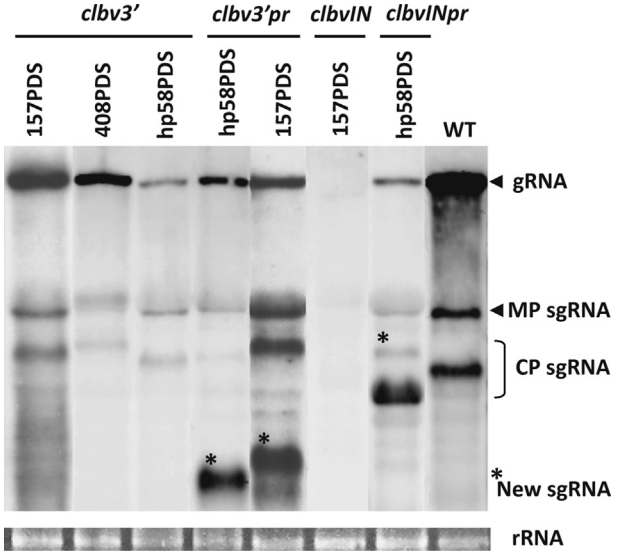


Fig. 2. Viral RNA accumulation in *Nicotiana benthamiana* plants agroinoculated with several *Citrus leaf blotch virus* vectors. Northern blot analysis of total RNA extracts from non inoculated upper leaves of *N. benthamiana* plants agroinoculated with the WT CLBV or with the CLBV constructs carrying different fragments of the *phytoene desaturase* (*pds*) gene at 25 days postinoculation. The membrane was hybridized with a digoxigenin-labeled riboprobe specific for the CLBV 3′ UTR. Arrowheads indicate positions of the CLBV genomic (gRNA) and MP and CP subgenomic (sgRNA) RNAs, and asterisk indicates the new sgRNA generated in some constructs. GelRed staining of rRNA was used as loading control.

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, albeit 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, but white spots or irregular patches in leaves, petioles, thorns and stems were occasionally observed (Fig. 4A–D). This uneven distribution of the virus could be a potential problem to study genes whose silencing phenotype is not visible. 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. The silencing phenotype was similar in all citrus species except in Pineapple sweet orange, which only displayed mild to moderate vein photo-bleaching in a few leaves of plants inoculated with the *clbv3′*-157PDS or the *clbv3′*-408PDS contructs. These results confirm previous observations indicating that in this host CLBV shows low accumulation and uneven distribution (Galipienso et al., 2000; Galipienso et al., 2004; Ruiz-Ruiz et al., 2009).

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 *C. excelsa* plants originally infected with the latter constructs after 2–3 years, depending on the 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

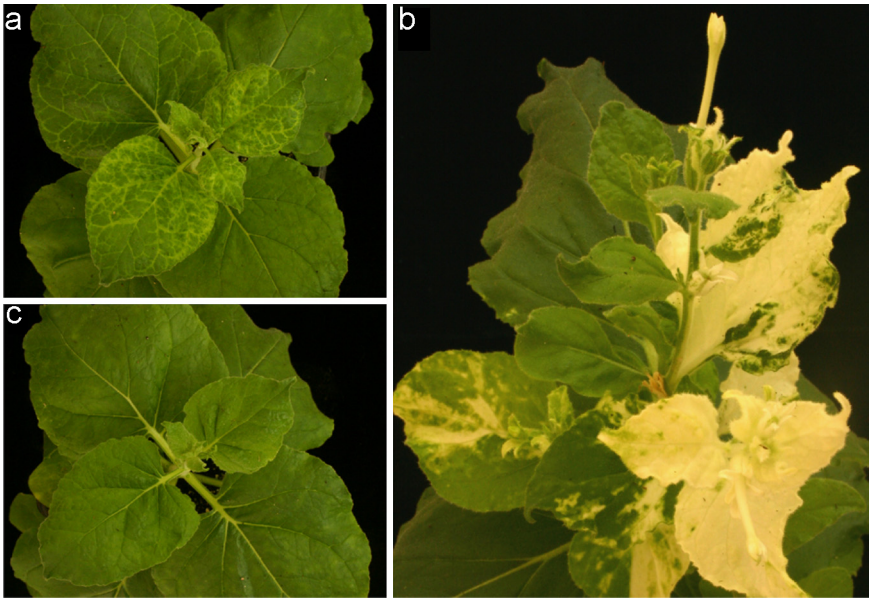


Fig. 3. Virus induced gene silencing in *Nicotiana benthamiana* plants. Photo-bleaching phenotype of *N. benthamiana* plants agroinoculated with the *Citrus leaf blotch virus* (*clbv*)*3′pr*-hp58PDS construct carrying a 58-bp inverted repeat sequence from the *pds* gene at 28 (a) or 90 (b) days post inoculation (dpi), or inoculated with the wild type CLBV (WT) infectious clone at 28 dpi (c).

Fig. 4. Virus induced gene silencing of the *phytoene desaturase* (*pds*) gene in citrus plants. Photo-bleaching phenotype in citrus plants after silencing the *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.

sufficient to trigger PTGS. Therefore, to select the most effective construct for VIGS in citrus we evaluated reduction of the *pds* mRNA accumulation by real time RT-PCR (Agüero et al., 2012), and the photo-bleaching intensity using an effectiveness index (see Experimental procedures), in two successive flushes of five rough lemon plants graft inoculated with the constructs *clbv3′*-157PDS, *clbv3′*-408PDS, *clbv3′pr*-157PDS and *clbv3′pr*-hp58PDS (Table 1). The silencing effectiveness was higher in plants inoculated with

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.

Constructs	Second flush		Third flush	
	E ± SD	*pds* reduction* % ± SD	E ± SD	*pds* reduction* % ± SD
clbv3′-157PDS	1.6 ± 0.6	34.0 ± 4.8	1.5 ± 0.4	49.6 ± 11.2
clbv3′-408PDS	2.2 ± 0.7	41.9 ± 9.1	2.8 ± 0.2	54.9 ± 5.3
clbv3′pr-157PDS	0.5 ± 0.3	30.2 ± 10.6	1.1 ± 0.3	31.6 ± 16.8
clbv3′pr-hp58PDS	1.5 ± 0.4	40.1 ± 12.0	0.9 ± 0.4	31.4 ± 8.9
WT	–	0	–	0

(E) Effectiveness index calculated as described in Experimental procedure section. (SD) standard deviation.

* *pds* mRNA reduction respect to WT-inoculated plants.

clbv3′-based vectors than in those inoculated with *clbv3′pr*-based vectors, with the most intense phenotype being incited by the construct *clbv3′*-408PDS (Table 1). 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 (Faivre-Rampant et al., 2004; Lacomme et al., 2003; Ratcliff et al., 2001).

siRNA 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. 5A). 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 at detectable levels. 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. 5A). 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 (Faivre-Rampant et al., 2004), we also assesed the accumulation of CLBV based vectors in *N. benthamiana* and citrus plants by quantitative real time RT-PCR (Fig. 5B). 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.

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 the magnesium chelatase, an enzyme involved in the chlorophyll biosynthesis pathway, were selected for analysis.

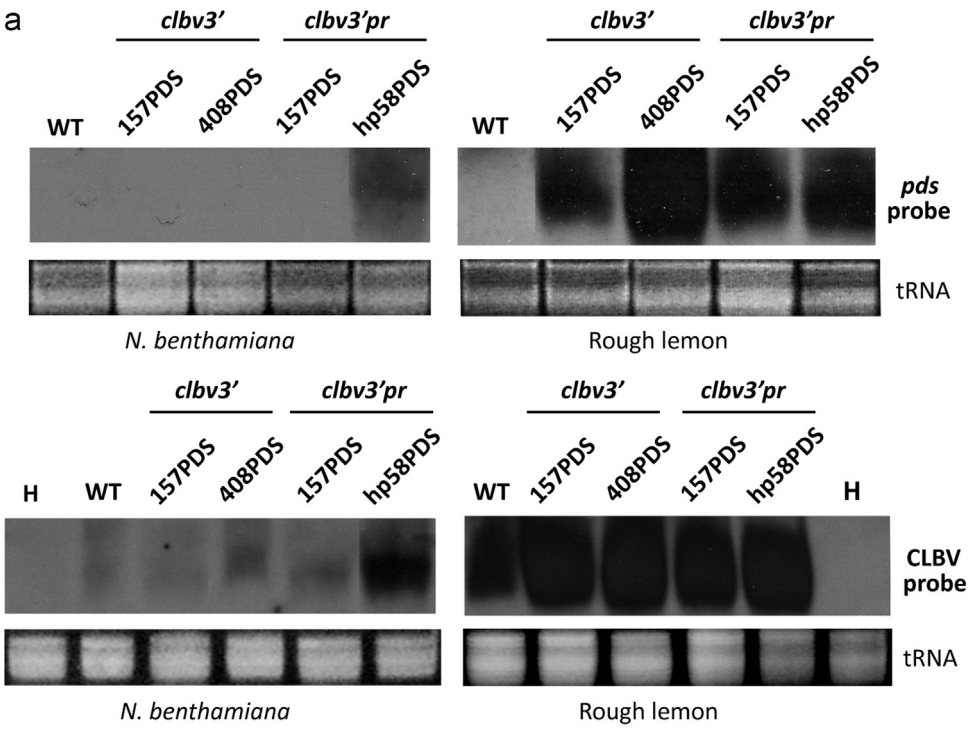
For this purpose, a 269-bp fragment of the *actin* gene from Valencia late sweet orange was cloned into the *clbv3′* vector (*clbv3′*-269ACT). This construct was agroinoculated in *N. benthamiana* plants and the resulting recombinant virions were extracted and slash inoculated on two *C. excelsa* plants. 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. 6A). Moreover, the latter plants showed bent mishapen leaves probably due to differential growth between silenced and not silenced leaf areas (Fig. 6B).

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 two rough lemon plants. Both plants inoculated with *clbv3′*-241SU construct showed large yellowing patches in the leaves and stems (Fig. 6C, E), indicating chlorophyll deficiency. This silencing phenotype was more widespread in the plants than the *pds*-silencing phenotype.

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 three rough lemon plants graft-inoculated at the same time with *clbv3′*-241SU or with CLBV WT. As control, three sour orange seedlings were graft inoculated with the same two inoculum sources. All sour orange seedlings or bud propagated plants inoculated with *clbv3′*-241SU showed the expected silencing phenotype, whereas the control plants inoculated with CLBV WT remained symptomless. However, while the sour orange seedlings inoculated with *clbv3′*-241SU showed in the first flush the 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. 6D). 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. 6E). 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.

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



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

Fig. 5. Detection of *pds*- and CLBV-derived siRNAs and absolute quantitation of CLBV gRNA. (a) Northern blot analyses of the small RNA fraction extracted from *Nicotiana benthamiana* (left panels) and rough lemon (right panels) plants infected with the CLBV infectious clone (WT) or the constructs *clbv3′*-157PDS, *clbv3′*-408PDS, *clbv3′pr*-157PDS, and *clbv3′pr*-hp58PDS at 28 and 120 dpi, respectively. Membranes were hybridized with digoxigenin-labeled 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.

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* (Vives et al., 2008a), 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 (Agüero et al., 2012). In the *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 (Agüero et al., 2012). Indeed virus accumulation was higher with *clbv3′*- than with *clbv3′pr*-based vectors (Figs. 2 and 5B). 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 limiting 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 (Lacomme et al., 2003).

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 (Agustí et al., 2008; Alós et al., 2008; Ancillo et al., 2007; Aprile et al., 2011; Cercós et al., 2006; Gandía et al., 2007; Huerta et al., 2008) and extensive EST collections (Forment et al., 2005) 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 (Cervera et al., 2008). Once a viral vector has systemically infected a citrus plant, it can be easily graft inoculated to a large number of other citrus varieties at either the juvenile or mature stage. A viral vector based on the CTV genome has been developed and used to express foreign proteins in citrus (Folimonov et al., 2007), but 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

Fig. 6. Virus induced gene silencing of endogenous *actin* and *sulfur* genes in citrus plants. (a) Stunting of a Mexican lime plant inoculated with *Citrus leaf blotch virus* (*clbv*)3′-269ACT, expressing a 269-nt fragment of the *actin* gene (left), compared with a CLBV WT-inoculated control plant (right). (b) Misshapen 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.

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 enough titer to trigger VIGS. This suggestion is supported by finding that CLBV accumulates less in the old than in the young leaves (Ruiz-Ruiz et al., 2009), and that in old leaves the silencing phenotype did not increase in comparison with fully expanded young 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 may reflect different response of their silencing machinery to viral infection or different silencing suppresor activity of the CLBV MP protein in both hosts. Of the two steps in the PTGS process, production of primary and secondary siRNAs, the first is dependent on dsRNA formation by the viral RdRp during virus replication or on the presence of highly structured regions in the gRNA, 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). This new dsRNA formation might 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 photobleaching phenotype and *pds*-derived siRNA accumulation, suggests that CLBV replication by itself did not produce in this host enough dsRNA to trigger PTGS and that secondary siRNAs are crucial for maintaining RNA silencing (Dalmay et al., 2000, Di Serio et al., 2010, Himber et al., 2003). In *N. benthamiana* the RDR1 is non functional due to the presence of two premature stop codons in the mRNA coding sequence (Yang et al., 2004), whereas this gene was shown to be up-regulated in citrus after CTV infection (Gandía et al., 2007). RDR1 has been involved in antiviral defense because RDR1-knockout mutant plants of *N. tabacum* and *Arabidopsis thaliana*

accumulated virus RNA to significantly higher levels than the wild type (Xie et al., 2001, Yu et al., 2003). Additionally, RDR1 has been associated to the production of viral siRNAs in *Arabidopsis* plants inoculated with *Tobacco rattle virus* (TRV) (Donaire et al., 2008) and with a mutant of *Cucumber mosaic virus* (CMV) (Díaz-Pendón et al., 2007). Although differences in the silencing pathway triggered by CLBV-based vectors in *N. benthamiana* and citrus are unknown, it is conceivable that primary siRNAs 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 (Vaistij and Jones, 2009). Reduced accumulation of viral siRNAs and increased accumulation of CLBV gRNA and sgRNAs in *N. benthamiana* plants support the potential 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 (Kumagai et al., 1995, Pignatta et al., 2007). Generation of secondary siRNAs in *N. benthamiana* has been associated to RDR6 (Mourrain et al., 2000, Qu et al., 2005, Vaistij and Jones, 2009), originally identified as the polymerase required for the silencing of single-stranded RNA of transcribed transgenes in *Arabidopsis* (Dalmay et al., 2000). 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 (Schwach et al., 2005). 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) (Dalmay et al., 2000, Mourrain et al., 2000). 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 (Donaire et al., 2008). Moreover, Wang and co-workers demonstrated that in *Arabidopsis* plants antiviral defense against CMV required RDR1 or RDR6 function to produce virus-derived secondary siRNAs (Wang et al., 2010).

An alternative explanation for the higher viral gRNA and lower siRNAs accumulation in *N.benthamiana* in comparison with citrus plants would be differences in the RNA silencing suppressor activity of the CLBV MP protein in both hosts. However, this hypothesis seems less likely because the MP suppressor activity in *N. benthamiana* is weak compared to other characterized viral suppressor proteins (Renovell et al., 2012).

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 (Goodin et al., 2008, Kumagai et al., 1995, Thomas et al., 2001) and many viral vectors have been assayed for VIGS in it, in addition to their natural hosts (Muruganantham et al., 2009). 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 (Agüero et al., 2013) would enable studying genes involved in organ development. Since these vectors were stable and induced the silencing phenotype in successive flushes over 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 and fungi or invertebrate pest as they can be potential targets for VIGS.

Experimental procedures

CLBV-based constructs

The infectious CLBV clone CLBV-IC (here named wild type, WT) described previously (Vives et al., 2008a) 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 positions: 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) (Agüero et al., 2012). The four developed CLBV-based vectors were used to clone foreign gene fragments at the *Pml*I site by using standard techniques (Sambrook et al., 1989) and appropriate primers (Table 2). All insertions performed in these plasmids were confirmed by sequencing.

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 were cloned into the *clbv3′* vector to obtain the *clbv3′*-241SUand *clbv3′*-269ACT constructs, respectively.

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 18/26 °C (night/day), using 2-liter plastic containers filled with 50% sand and 50% peat moss and a standard fertilizing procedure (Arregui et al., 1982). *C. excelsa*, Mexican lime, rough lemon, Dweet tangor, Cleopatra mandarin, Pineapple sweet orange and alemow were grown as seedlings. Etrog citron was propagated on a rough lemon rootstock and sour orange was grown either as seedling or propagated on a 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 (Vives et al., 2008a).

Semipurified virion extracts from systemically infected *N. benthamiana* plants (Galipienso et al., 2000) were inoculated to *C. excelsa* and rough lemon plants by stem slashing (Garnsey et al., 1977) with scalpel blades dipped in the virion extracts (Galipienso et al., 2000, Vives et al., 2008a). Bark pieces from both citrus plants infected with the recombinant or the WT CLBV were used to graft inoculate other citrus species.

Table 2
Primers used in this work.

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

[a] Phosphorylated at the 5′ end.
[b] Nt positions are indicated on the sequence of the *Citrus sinensis pds* mRNA (GenBank accession number DQ235261).
[c] Nt positions are indicated on the sequence of the *N. benthamiana pds* mRNA (GenBank accession number DQ469932).
[d] Nt positions are indicated on the coding sequence of the *C. sinensis* homolog of tobacco *sulfur* gene (Phytozome locus name orange1.1 g; 014510 m).
[e] Nt positions are indicated on the sequence of the *C. sinensis* homolog of *actin11* gene (Citrus Functional Genomic database: aCL563Contig1).
[f] Nt positions are indicated on the sequence of the CLBV isolate SRA-153 (EMBL accession number AJ318061).
[g] Nt positions are indicated on the sequence of the putative *C. clementina* actin mRNA (Phytozome locus name clementine0.9_013110 m).

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 (Ancillo et al., 2007). 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 preparations enriched in small RNAs (sRNAs), 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 (Yaegashi et al., 2007). 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 Vives et al. (2002a) and Galipienso et al. (2004), 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 94 °C for 5 min, separated by electrophoresis in 15% polyacrylamide gels containing 7 M urea in 0.5 × TBE buffer (50 mM Tris, 45 mM Boric acid, 0.5 mM EDTA) and electroblotted onto positively charged nylon membranes (Roche Applied Science) at 25 V for 1 h using 0.5 × TBE buffer.

For hybridizations, DIG-labeled riboprobes specific for the CLBV 3′ UTR, CLBV CP (Vives et al., 2002a) and 408PDS fragment of the *N. benthamiana* and citrus *pds* gene were used. The cDNA of the 408PDS fragments were obtained by RT-PCR using RNAt extracts from both species and specific primers (Table 2). PCR products were cloned into the pGEM-T plasmid (Promega Corporation, Madison, WI, USA). DIG-labeled negative-stranded RNA transcripts were synthesized from the tobacco or citrus 408PDS cDNA clones by incorporation of DIG-UTP using the T7 or SP6 RNA polymerase, respectively, according to manufacturer's instructions (Roche Applied Science).

After UV cross-linking, membranes were pre-hybridized in 0.02% sodium dodecyl sulfate (SDS), 50% formamide, 5 × SSC (750 mM NaCl, 75 mM sodium citrate, pH 7), 2% blocking reagent (Roche Applied Science) 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 0.1 × 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) 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).

RT-PCR Detection and real-time RT-PCR quantitation

The sequences inserted in CLBV viral vectors were detected by conventional RT-PCR (Vives et al., 2002b) 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) 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 (Ruiz-Ruiz et al., 2009). 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 homolog of the *actin11* gene as described previously (Agüero et al., 2012). 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 (Livak and Schmittgen, 2001).

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 this 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.

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