Virology 460-461 (2014) 154–164

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

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

Jesus Agüero, María del Carmen Vives, Karelia Velázquez, José Antonio Pina, Luis Navarro, Pedro Moreno, Jose Guerri *

Centro de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Ctra. Moncada-Náquera Km. 4.5, Moncada, 46113 Valencia, Spain

A R T I C L E I N F O

Article history:
Received 23 January 2014
Returned to author for revisions
12 February 2014
Accepted 12 April 2014
Available online 4 June 2014

Keywords:
CLBV
VIGS
Silencing
Nicotiana benthamiana
Citrus
Viral vector

A B S T R A C T

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.

© 2014 Elsevier Inc. All rights reserved.

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

* Corresponding author. Tel.: +34 96 342 4068; fax: +34 96 342 4001.
E-mail address: jguerri@ivia.es (J. Guerri).

http://dx.doi.org/10.1016/j.virol.2014.04.017
0042-6822/© 2014 Elsevier Inc. All rights reserved.

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

Download English Version:

https://daneshyari.com/en/article/3424048

Download Persian Version:

https://daneshyari.com/article/3424048

Daneshyari.com