Journal of Virological Methods 155 (2009) 167–174

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Grapevine virus A-mediated gene silencing in *Nicotiana benthamiana* and *Vitis vinifera*

Mookkan Muruganantham [a], Yoni Moskovitz [a], Sabrina Haviv [a], Tamar Horesh [a], Annie Fenigstein [a], Jacques du Preez [b], Dirk Stephan [b], Johan T. Burger [b], Munir Mawassi [a,*]

[a] *The S. Tolkowsky Laboratory, Department of Plant Pathology-The Virology Unit, Agricultural Research Organization, Volcani Center, Bet Dagan 50250, Israel*
[b] *Department of Genetics, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa*

Article history:
Received 12 June 2008
Received in revised form 4 October 2008
Accepted 9 October 2008
Available online 2 December 2008

Keywords:
Agroinoculation
Grapevine
Grapevine virus A
Phytoene desaturase
Virus-induced gene silencing
Virus vector

A B S T R A C T

Virus-induced gene silencing (VIGS) is an attractive approach for studying gene function. Although the number of virus vectors available for use in VIGS experiments has increased in recent years, most of these vectors are applied in annual or herbaceous plants. The aim of this work was to develop a VIGS vector based on the *Grapevine virus A* (GVA), which is a member of the genus *Vitivirus*, family *Flexiviridae*. The GVA vector was used to silence the endogenous phytoene desaturase (PDS) gene in *Nicotiana benthamiana* plants. In addition, an *Agrobacterium*-mediated method for inoculating micropropagated *Vitis vinifera* cv. Prime plantlets via their roots was developed. Using this method, it was possible to silence the endogenous PDS gene in *V. vinifera* plantlets. The GVA-derived VIGS vector may constitute an important tool for improving functional genomics in *V. vinifera*.

1. Introduction

Infection of a plant by a virus often triggers a defense response and activates post-transcriptional gene silencing in the plant, a key mechanism for protecting plants against viral invasion. In this virus-induced gene silencing (VIGS) response, the plant defense system specifically targets RNAs derived from the viral genome for degradation (Bernstein et al., 2001; Nykanen et al., 2001). As a consequence, if the virus is harboring a fragment of a plant gene in its genome, the defense system will trigger the destruction of the corresponding plant mRNA, as well. On this basis, in recent years, genomes of plant viruses have been widely utilized to knock down expression of either transgenes or endogenous genes, and have been proven to be important tools for the analysis of gene function in plants (Atkinson et al., 1998; Baulcombe, 1999; Burton et al., 2000; Lu et al., 2003; Burch-Smith et al., 2004). Although the number of developed VIGS vectors has increased in recent years, most of these vectors are meant to be used in annual or herbaceous plants (Kumagai et al., 1995; Ruiz et al., 1998; Ratcliff et al., 2001; Holzberg et al., 2002; Brigneti et al., 2004). However, new virus vectors are required to expand the application of VIGS to a wider range of plants. The aim of this study was to test whether the *Grapevine virus A* (GVA) could be used as a VIGS vector.

GVA is closely associated with the economically important rugose wood (RW) disease of grapevine, specifically with Kober stem grooving (Garau et al., 1994; Chevalier et al., 1995, 1997). This virus, which is spread through infected propagation plant materials and by mealybugs, is a member of the genus *Vitivirus*, family *Flexiviridae* (Martelli et al., 2007). It is a filamentous particle about 800 nm long, and is considered to be a phloem-associated virus. The GVA genome (~7.4 kb) consists of five open reading frames (ORFs; Galiakparov et al., 1999, Saldarelli et al., 2000; Galiakparov et al., 2003c). ORF1, located at the 5′-terminus of the genome, encodes a 194-kDa polypeptide with conserved motifs of replication-related proteins. ORF2 encodes a ~20-kDa protein whose function is unknown. ORF3 is the movement protein (MP) gene, ORF4 encodes the coat protein (CP) and ORF5 encodes a small protein that exhibits sequence similarities to small RNA binding proteins of various plant viruses (Galiakparov et al., 2003b) and suppresses RNA silencing (Chiba et al., 2006; Zhou et al., 2006).

Recently a GVA-derived expression vector was developed and used to express the beta-glucuronidase (GUS) gene in *Nicotiana benthamiana* plants (Haviv et al., 2006). Infection of *Vitis vinifera* plants with the GVA vector has not been developed so far.

* Corresponding author at: The S. Tolkowsky Laboratory, Department of Plant Pathology-The Virology Unit, Agricultural Research Organization, the Volcani Center, Bet Dagan 50250, Israel. Tel.: +972 3 9683844; fax: +972 3 9604180.
E-mail address: mawassi@volcani.agri.gov.il (M. Mawassi).

0166-0934/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2008.10.010

Infection of this host with a cloned infectious cDNA of GVA is difficult to accomplish using the simple agro-infiltration methods that are used for most plant viruses. The present study describes: (i) the use of the GVA-derived vector for silencing the endogenous phytoene desaturase (PDS) gene in *N. benthamiana*, (ii) an *Agrobacterium*-mediated method developed for inoculating in vitro-propagated *V. vinifera* plantlets with cloned infectious GVA cDNAs, through their roots, and (iii) the use of the method developed for applying the GVA-derived vector for silencing the endogenous PDS gene in *V. vinifera* plantlets.

2. Materials and methods

2.1. Plant material

N. benthamiana and *V. vinifera* cv. Prime were used in this study. *N. benthamiana* plants were grown in pots under greenhouse conditions: 25 °C; a 16-h light cycle and 60% humidity. *V. vinifera* plantlets, which were obtained from plant material treated by cryopreservation to ensure virus elimination, were micropropagated and maintained at a temperature of 26 °C under a 16-h photoperiod, as described by Wang et al. (2003).

2.2. GVA constructs

Fig. 1 presents schematic maps of the GVA-derived constructs used in this study. The vector pGVA-118, which is described by Haviv et al. (2006), contains the GVA cDNA under the promoter of T7 RNA polymerase. The entire cDNA of GVA-118 was PCR-amplified and cloned into the *Stu I* cleavage site of the pCass2 vector (Shi et al., 1997) between the 35S promoter and terminator sequences derived from *Cauliflower mosaic virus* (CaMV). The cassette, consisting of the GVA cDNA and the CaMV 35S promoter and terminator, was next transferred into the pCAMBIA2301 binary vector with the help of *Pvu II* digestion, to generate pGVA-378.

The cDNA fragment corresponding to the nts 1–500 of the *N. benthamiana* PDS gene (NbPDS) (GenBank accession no. DQ469932) was PCR-amplified using Taq DNA polymerase and the primers NbPDSf1 (5′-AATCAT*GCGGCCGC*ATGCCCCAAATCGGACTTGT-3′) (Italics *Not I* cleavage site) and NbPDSr1 (5′-CTCTTA-*GGGCCC*AATATGTGCAACCCAGTCTC-3′) (Italics *Apa I* cleavage site). The PCR product was digested with *Not I* and *Apa I* restriction enzymes and cloned into a similarly digested pGVA-118 vector. Next, the resulting GVA-nbPDS cDNA was inserted into the pCAMBIA2301 vector, under the control of the CaMV 35S promoter and terminator, to generate pGVA-nbPDS-349.

To obtain sequence information about the *V. vinifera* PDS (VvPDS) gene, sequence alignments of at least 16 PDS cDNAs derived from various plants and available in GenBank were performed. Sequences of conserved regions were selected and used to design specific primers. A 304-nts cDNA fragment of the *V. vinifera* cv. Prime PDS gene was PCR-amplified with the primers VvPDSf1 (5′-AATCAT*GCGGCCGC*GGCCTTCTTAGATGGTAATCCT-3′) (Italics *Not* I cleavage site) and VvPDSr1 (5′-CTCTTA-*GGGCCC*CTCAAACCATATATGAACATTGA-3′) (Italics *Apa I* cleavage site). The PCR product (submitted for publication in GenBank under the accession no. EU816356) was digested with *Not I* and *Apa I* restriction enzymes and cloned into a similarly cleaved pGVA-118 vector. The resulting GVA-nbPDS cDNA was next inserted into the pCAMBIA2301, under the control of the CaMV 35S promoter and terminator, to generate pGVA-VvPDS-377.

The green fluorescent protein (GFP) gene was PCR-amplified with primers specific to the 18 nts of the 5′ and the 3′ termini of the gene. The product was cloned into pGVA-118, between the *Not I* and *Apa I* cleavage sites. The resulting recombinant GVA-GFP cDNA was then inserted into the pCAMBIA2301, under the control of the CaMV 35S promoter and terminator, to generate pGFP-GVA-160.

2.3. Agro-inoculation of N. benthamiana and V. vinifera plantlets

Virus infections of *N. benthamiana* were achieved through *Agrobacterium*-mediated transient expression of infectious GVA cDNAs cloned into the binary vector pCAMBIA2301. *Agrobacterium tumefaciens* strain EHA 105, re-suspended to an OD_{600} of 1.0 in 10 mM $MgCL_2$, 10 mM MES and 100 μM acetosyringone, was infiltrated to the underside of leaves of 3-week-old *N. benthamiana* plants using a 2 ml syringe without a needle, as described by Johansen and Carrington (2001).

Virus infections of micropropagated *V. vinifera* plantlets were achieved by inoculating the roots of the plantlets with *Agrobacterium*, using a modified version of the agrodrenching technology described by Ryu et al. (2004). Micropropagated plantlets, with two to four leaves each, were detached from the agar medium together with their roots. The roots were then gently injured with a sterile needle. The plantlets were next transferred onto sterile Whatman No. 1 filter paper immersed in 10 ml half-strength MS liquid medium (Murashige and Skoog, 1962) with BA at 1 mg/l and NAA at 0.5 mg/l. For inoculation, a 1 ml suspension of *Agrobacterium* inoculum, re-suspended in MS medium containing 100 μM acetosyringone to an OD_{600} of 1.0, was added to the media. The plantlets were kept with the *Agrobacterium*-containing media for 10 days and then transferred to fresh MS liquid media, where they were kept for another 14 days. At the end of this period, the plantlets were transferred to fresh media supplemented with cefotaxime at 500 mg/l and maintained for subsequent analyses.

2.4. RNA extraction, RT-PCR, semi-quantitative PCR and Northern blot analyses

Total RNA from *N. benthamiana* was extracted using the Tri-Reagent kit (Sigma–Aldrich, USA) according to the manufacturer's instructions. Total RNA of *V. vinifera* plants was extracted as described by Chang et al. (1993).

For RT-PCR, total RNA extracts were treated with DNAase and used for cDNA synthesis with the primer 18dT composed of poly (T). PCR amplifications were performed using primers that were designed based on the sequences of the *N. benthamiana* PDS gene, the *V. vinifera* PDS gene (obtained as described above) and the GVA genome (GenBank accession no. AY244516).

Semi-quantitative PCR was performed as described by Liu et al. (2002), using the *N. benthamiana* PDS-specific primers NbPDSf2 (5′-GGTTGCAGTGGAAGGAACAT-3′) and NbPDSr2 (5′-AGCGTACACACTGAGCAACG-3′), or the *V. vinifera* PDS-specific primers VvPDSf2 (5′-CTTACCCAAATGTGCAGAACCTGTT-3′) and VvPDSr2 (5′-CCTGGGTTCAAAGCAATCAATATACA-3′). As a control, semi-quantitative PCR was conducted to amplify the host elongation factor (EF)-1 α mRNA from the same cDNA product with the primers rEF1 (5′-GATTGGTGGTATTGGAACTGTC 3′) and fEF-1 (5′-AGCTTCGTGGTGCATCTC-3′). The intensities of the PCR products were analyzed using Gel Doc 2000 (Bio-Rad, USA).

Northern blot and hybridization analyses were performed according to Galiakparov et al. (2003a), with DIG-labeled riboprobes (Roche Molecular Biochemicals). The probes, specific to the *N. benthamiana* PDS and the *V. vinifera* PDS, were made of the same 760- and 420-nts fragments. These fragments were obtained from the semi-quantitative PCR assays described above. The probe used for GVA detection was designed to hybridize with the 5′-1.0 kb of its genome.