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Broad bean wilt virus 2 encoded VP53, VP37 and large capsid protein orchestrate suppression of RNA silencing in plant

Lingfang Kong[a,1], Yaqin Wang[a,1], Xiuling Yang[a], Garry Sunter[b], Xueping Zhou[a,*]

[a] State Key Laboratory of Rice Biology, Institute of Biotechnology, Zhejiang University, Hangzhou 310029, People's Republic of China
[b] Department of Biology, University of Texas at San Antonio, San Antonio, TX 78249, USA

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ABSTRACT

Viruses encode RNA silencing suppressors to counteract host RNA silencing-mediated defense responses. In this study, we demonstrate that VP53, VP37 and LCP encoded by RNA2 of broad bean wilt virus 2 (BBWV-2), a member of the genus *Fabavirus*, are strong suppressors of RNA silencing triggered by single-stranded sense RNA. They, however, had no effect on suppression of RNA silencing induced by double-stranded RNA. We provide evidence that these three suppressors can significantly limit the accumulation of small interfering RNAs (siRNAs) in tissues where the *GFP* gene has been silenced, and prevent the long distance spread of the induced silencing signal. Gel mobility shift assays showed that all three suppressors could bind ssRNA in a size-specific manner. Interestingly, VP37 and LCP, but not VP53, could reverse the silencing of a *GFP* gene in leaf tissue. Furthermore, these three proteins are capable of enhancing pathogenicity of potato virus X. Collectively, our findings indicate that viruses employ a more sophisticated strategy to overcome the host defense response mediated through suppression of RNA silencing during virus infection. As far as we are aware, this is the first report of RNA silencing suppressors encoded by a virus in the genus *Fabavirus*.

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1. Introduction

Viruses are causal agents of many important plant and animal diseases (Wang et al., 2012). To combat these diseases, plants and animals have evolved diverse defense mechanisms, including RNA silencing, to suppress virus replication at both the cellular and organismal levels. RNA silencing is a sequence-specific RNA degradation mechanism (Ding and Voinnet, 2007) and is triggered by double-stranded (ds) RNAs derived from virus replication or single-stranded (ss) RNAs with highly structured regions (Dalmay et al., 2000; Molnar et al., 2005). Dicer (a dsRNA-specific RNase) processes the dsRNAs that accumulate during virus infection into short dsRNAs of 21–24 nucleotides (nt) in length, designated small interfering RNAs (siRNAs) (Deleris et al., 2006; Hamilton and Baulcombe, 1999; Xie et al., 2004). The siRNAs generated are then recruited by an Argonaute (AGO) protein, a component of the RNA-induced silencing complex (RISC), to initiate the sequence-specific degradation of targeted host mRNA or viral RNAs (Ding and Voinnet, 2007; Hammond et al., 2000). Once RNA silencing is induced, a sequence-specific silencing signal spreads into neighboring cells and then throughout the entire plant via the vasculature to confer autonomous silencing of homologous targets (Dunoyer et al., 2010; Kalantidis et al., 2008; Voinnet and Baulcombe, 1997). Hence, siRNAs generated at the site of initial virus infection reduce not only the amount of viral RNAs locally, but also promote systemic silencing of viral RNA leading to the recovery of plants from virus infection (Mlotshwa et al., 2008; Tatineni et al., 2012).

To counteract virus-induced RNA silencing, plant viruses have evolved to encode diverse, yet functionally conserved proteins (i.e. viral suppressors of RNA silencing, VSRs) that are capable of suppressing RNA silencing (Li and Ding, 2006). To date, more than 50 VSRs have been identified and many of the VSRs are multifunctional, playing important roles in viral replication, encapsidation, cell-to-cell and/or systemic movement or pathogenesis (Csorba et al., 2009). Evidence suggests that VSRs suppress RNA silencing via two general mechanisms. The most accepted mechanism is the interaction of a VSR with siRNA leading to a block of siRNA activity (Lakatos et al., 2006). The tombusvirus-encoded P19 protein is the most commonly studied VSR and forms a head-to-tail homodimer that specifically binds siRNA duplexes (Vargason et al., 2003; Ye et al., 2003). The other commonly proposed mechanism for VSR activity is the direct interaction of a VSR with host specific proteins that are either a component of the RNA silencing machinery or

* Corresponding author. Tel.: +86 57186971680; fax: +86 57186971498.
E-mail address: zzhou@zju.edu.cn (X. Zhou).
[1] These two authors contributed equally to this work.

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regulators of the silencing pathway. For example, cucumber mosaic virus encoded 2b protein inhibits the cleavage function of AGO1 and turnip crinkle virus (TCV) encoded P38 protein blocks the activities of DCL2 and DCL4 in *Arabidopsis thaliana* (Deleris et al., 2006; Zhang et al., 2006). Identification of the functions of virus encoded silencing suppressors is important for our understanding of the mechanism of virus infection in plants and for dissection of the molecular pathway involved in RNA silencing (Yaegashi et al., 2007).

Broad bean wilt virus 2 (BBWV-2) is a member of the genus *Fabavirus*, subfamily *Comovirinae*, family *Secoviridae*, and is transmitted by aphids in a non-persistent manner (Lee et al., 2000). The incidence of BBWV-2 has been reported worldwide and causes significant damage to many economically important horticultural and ornamental crops (Qi et al., 2000). The BBWV-2 genome consists of two positive sense genomic ssRNAs of 6.0 and 3.6 kb long, which are packaged in 25 nm icosahedral virion. Both RNAs are translated into single polyprotein precursors from which individual functional proteins are produced through proteolytic cleavage. RNA1 encodes proteins involved in genome replication and expression, and RNA2 encodes the 44 kDa large coat protein (LCP), 22 kDa small CP (SCP) and 53 kDa/37 kDa (designated VP53/VP37) proteins, and the VP53 and VP37 proteins overlap at the C-terminus and are translated from two potential translation initiation sites (Fig. 1A). The VP37 protein is a multifunctional protein that binds single-strand nucleic acids, interacts with viral coat protein (CP) and potentiates the virus cell-to-cell movement in its host plant by inducing tubule-like structures (Liu et al., 2009, 2011). But the function of VP53 is still unclear. Within the subfamily *Comovirinae*, the SCP encoded by cowpea mosaic virus (CPMV, a member of the genus *Comovirus*) has been previously identified as a suppressor of RNA silencing (Liu et al., 2004). To date, little is known about the VSRs encoded by fabaviruses.

Here we present evidence showing that VP53, VP37 and LCP of BBWV-2 can effectively suppress both local and systemic RNA silencing triggered by ssRNA, through blocking systemic spread of the RNA silencing signal in plants. BBWV-2 VP53 can bind 24-nt ssRNA and dramatically enhances the pathogenicity of potato virus X (PVX) in *Nicotiana benthamiana*. In addition, BBWV-2 VP37 and LCP, but not V53, can reverse RNA silencing in plants and bind long ssRNA, indicating that these VSRs may function differently when counteracting the host antiviral defense response.

2. Materials and methods

2.1. Source of virus and plant growth conditions

The source of BBWV-2 isolate B935 has been previously reported (Qi et al., 2000). The virus was maintained in *Chenopodium quinoa* through mechanical inoculation. BBWV-2-infected plants were maintained in growth chambers set at 24 °C.

2.2. Plasmid construction

We amplified each open reading frame (ORF) encoded by BBWV-2 RNA2 from total RNA extracted from BBWV-2-infected leaf tissue by reverse transcription-PCR (RT-PCR) using specific primers (Table S1, Supplementary Table). The resulting RT-PCR products were individually cloned into the pGEM-T vector (Promega, Madison, WI, USA), digested with specific enzymes, and cloned downstream of the cauliflower mosaic virus (CaMV) 35S promoter contained within the binary vector pCHF3 (Cai et al., 2007) to generate: p35S-VP53, p35S-VP37, p35S-LCP, p35S-SCP and p35S-VPN128, respectively. A mutant version of VPN128 was generated that contained the N-terminal 128 aa sequence of VP53 (Fig. 1A). A plasmid expressing the cymbidium ring spot virus (CymRSV) P19 gene (p35S-P19) (Lakatos et al., 2004) was used as a control plasmid in various experiments. An inverted repeat sequence of GFP (p35S-dsGFP) was constructed as previously reported (Xiong et al., 2009). Constructs were individually electroporated into *Agrobacterium* strain C58C1 with a Gene Pulser II system as described (Bio-Rad, Hercules, CA, USA). *Agrobacterium tumefaciens* harboring a binary Ti plasmid capable of expressing full length GFP (p35S-ssGFP) was kindly provided by Professor D.C. Baulcombe.

Supplementary Table 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2014.08.010.

Various ORFs of BBWV-2 RNA2 were also individually cloned into the PVX-based pGR107 vector, kindly provided by Professor D.C. Baulcombe, as previously described to produce PVX-VP53, PVX-VP37, PVX-LCP, PVX-SCP and PVX-VPN128. The tobacco etch virus HC-Pro gene was amplified using the plasmid 35S-HC-Pro (from Dr. J.C. Carrington, Oregon State University, USA) and cloned into the pGR107 vector (PVX-HC-Pro) used as a positive control. The resulting plasmids were individually transformed into *Agrobacterium* strain GV3101 through electroporation.

2.3. Agroinfiltration and GFP imaging

Leaves of wild type *N. benthamiana* or *N. benthamiana* line 16c transgenic for green fluorescent protein (GFP) (Baulcombe, 2004) were infiltrated with *Agrobacterium* cultures carrying different plasmids. For co-agroinfiltration, equal volumes of two different *Agrobacterium* cultures (optical density at 600 nm = 1 each) were mixed and then infiltrated into leaves using needle-less syringes. GFP fluorescence in the agroinfiltrated plants was observed under a hand-held UV lamp and photographed using a 400D Cannon digital camera.

2.4. RNA analysis

Total RNA was extracted from *N. benthamiana* leaf tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The accumulation of GFP mRNA and PVX genomic and subgenomic RNAs in sampled leaves was determined by Northern blot analysis. Total RNA (10 μg each) was separated on a 1% formaldehyde agarose gel, transferred to nylon membranes, and probed with [α-32^{P}]dCTP-labeled RNA probes specific for GFP mRNA or PVX RNA as described previously (Xiong et al., 2009). For Northern blot analysis of GFP siRNAs, low-molecular-weight RNAs were enriched from total RNA samples by eliminating high-molecular-weight RNAs using a 5% polyethylene glycol (MW 8000)/0.5 M NaCl solution. The siRNAs were separated in 15% polyacrylamide gels containing 7 M urea, transferred onto nylon membranes, and then probed with a [α-32^{P}]dCTP-labeled probe specific for the GFP sequence.

2.5. Western blot analysis

Agroinfiltrated leaf tissue was harvested and each sample homogenized in extraction buffer [50 mM Tris–HCl (pH 6.8), 9 M urea, 4.5% sodium dodecyl sulfate (SDS) and 7.5% β-mercaptoethanol] at a ratio of 100 mg tissue per 200 μL buffer. The extracts were centrifuged at 12,000 × g for 15 min, separated in 12.5% SDS-PAGE gels by electrophoresis followed by transfer to nitrocellulose membranes. The membranes were then probed with a rabbit anti-GFP monoclonal antibody (Epitomics, Burlingame, CA, USA). Secondary antibody was goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma, St. Louis, MO, USA). Detection was achieved through incubation of the membranes in NBT-BCIP solution as instructed (Promega).

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