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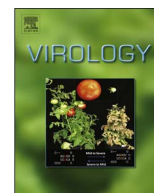

Nicotiana benthamiana plants asymptomatically infected by *Pelargonium line pattern virus* show unusually high accumulation of viral small RNAs that is neither associated with DCL induction nor RDR6 activity

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A B S T R A C T

Pelargonium line pattern virus (PLPV, *Tombusviridae*) normally establishes systemic, low-titered and asymptomatic infections in its hosts. This type of interaction may be largely determined by events related to RNA silencing, a major antiviral mechanism in plants. This mechanism is triggered by double or quasi double-stranded (ds) viral RNAs which are cut by DCL ribonucleases into virus small RNAs (vsRNAs). Such vsRNAs are at the core of the silencing process as they guide sequence-specific RNA degradation Host RNA dependent-RNA polymerases (RDRs), and particularly RDR6, strengthen antiviral silencing by promoting biosynthesis of secondary vsRNAs. To approach PLPV-host relationship, here we have characterized the vsRNAs that accumulate in PLPV-infected *Nicotiana benthamiana*. Such accumulation was found unprecedented high despite DCLs were not induced in infected tissue and neither vsRNA generation nor PLPV infection was apparently affected by RDR6 impairment. From the obtained data, triggers and host factors likely involved in anti-PLPV silencing are proposed.

1. Introduction

RNA silencing is a general term that embraces a series of pathways leading to transcriptional or post-transcriptional gene downregulation in a sequence-specific manner. These pathways are essentially conserved in most eukaryotes and are involved in developmental control, maintenance of genome stability and stress-induced responses (Baulcombe, 2005; Pickford and Cogoni, 2003). The silencing process is triggered by double-stranded (ds) and highly structured single-stranded (ss) RNAs which serve as substrates for Dicer-like ribonucleases (DCL) to produce small RNAs (sRNAs) of 20–25 nt, the key elements of the process (Ghildiyal and Zamore, 2009; Margis et al., 2006; Moazed, 2009; Vaucheret, 2006; Xie and Qi, 2008). These small duplexes are unbound by an RNA helicase and one strand is loaded into an RNA-induced silencing complex (RISC), whose essential catalytic component is an Argonaute (AGO) protein. Once incorporated, the sRNA strand guides RISC to target and inactivate their cognate RNAs (post-transcriptional gene silencing) or DNAs (transcriptional gene silencing), the former through cleavage or translation inhibition and the latter through methylation (Baulcombe, 2005; Vaucheret, 2008).

In plants, RNA silencing is recognized as a major immune system against viruses (Ding, 2010; Hull, 2014). In the case of RNA viruses, the viral molecules that elicit the RNA silencing machinery may correspond to dsRNA replication intermediates (generated by the action of the virus-encoded RNA dependent-RNA polymerase, RDR) or to self-complementary regions of the viral genome (Ding and Voinnet, 2007). The DCL activities that target the inductor molecules leading to production of viral sRNAs (vsRNAs) may be diverse. *Arabidopsis thaliana* encodes four distinct DCL, three of which, DCL4, DCL2 and DCL3, function in a hierarchical fashion to yield vsRNAs of 21, 22 and 24 nt, respectively (Blevins et al., 2006; Deleris et al., 2006; Liu et al., 2009). Concerning RISC effector proteins, AGO1 seems to be particularly relevant in antiviral silencing, at least in *A. thaliana* that contains 10 distinct AGO genes, although other AGO proteins may also have a significant role in this defense mechanism (Carbonell and Carrington, 2015; Harvey et al., 2011; Morel et al., 2002; Qu et al., 2008; Scholthof, et al., 2011). Cellular RDR enzymes, such as RDR6 and, to lesser extent, RDR1 and RDR2 in *A. thaliana* (Díaz-Pendón et al., 2007; Donaire et al., 2008; Mourrain et al., 2000; Qi et al., 2009), could be involved in the generation of viral dsRNA triggers. In addition, they can amplify the primary antiviral response by

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using vsRNAs to prime synthesis of complementary strands of cleaved viral ssRNAs. This will result in the generation of new dsRNA substrates for DCL enzymes, leading to secondary vsRNA production and promoting the spread of the silencing signal from cell to cell (Díaz-Pendón et al., 2007; Donaire et al., 2008; García-Ruiz et al., 2010; Qi et al., 2009).

In order to survive in hosts employing RNA silencing, viruses have evolved a counterdefense by encoding proteins that disrupt the RNA silencing pathway, termed viral suppressors of RNA silencing (VSRs) (Li and Ding, 2006). VSRs from plant viruses display high sequence diversity and can be endowed with additional function(s) (Li and Ding, 2006; Pumplin and Voinnet, 2013). VSRs may block the antiviral silencing at various stages. For instance, many of these proteins sequester sRNAs which will impair both RISC loading and amplification of silencing, thus debilitating host defense. VSRs can also target long dsRNAs or protein components of the silencing machinery such as DCL, AGO, RDR or auxiliary proteins, which may as well have an impact on vsRNA profiles (Csorba et al., 2015; Pumplin and Voinnet, 2013).

Pelargonium line pattern virus (PLPV) is a member of the family *Tombusviridae* with a sole genomic RNA (gRNA) that is ss and of plus polarity. This gRNA is 3,883 nt in length, lacks a 5´ cap and a poly(A) tail and contains five open reading frames (ORFs) flanked by untranslated regions of 6 and 246 nt at the 5′ and 3′ end, respectively. From 5′ to 3′, the ORFs encode two replication proteins (p27 and its read-through product p87), two small movement proteins (MP1 or p7 and MP2 or p9.7) and a coat protein (CP or p37) that also functions as VSR (Castaño and Hernández, 2005; Castaño et al., 2009; Pérez-Cañamás and Hernández, 2015). Among the sixteen genera that currently embraces family *Tombusviridae* (Adams et al., 2016), PLPV shows the closest resemblances with members of genus *Carmovirus* at both genomic and protein level. However, its gene expression strategies differ from members of that genus, particularly because it produces a single subgenomic RNA (sgRNA) for expression of the internal (p7 and p9.7) and 3′-proximal (p37) ORFs (Castaño and Hernández, 2005; Castaño et al., 2009; Blanco-Pérez and Hernández, 2016; Blanco-Pérez et al., 2016) in contrast with carmoviruses that generate two sgRNAs. This characteristic together with other genomic traits (that facilitate translation of three genes from the sgRNA though leaky scanning processes) are shared by four structurally and phylogenetically related viruses. These resemblances have prompted the proposal of the inclusion of all five viruses into a prospective new genus, *Pelarspovirus*, within the family *Tombusviridae*, with PLPV recommended as type species (Castaño and Hernández, 2005; Kinard and Jordan, 2002; Scheets et al., 2015).

As commonly observed in its natural hosts (*Pelargonium* ssp.), PLPV establishes systemic, low-titered and asymptomatic infections in a variety of experimental hosts, including *Nicotiana benthamiana* (Alonso et al., 2005; Ivars et al., 2004 Castaño et al., 2007), a model plant species to study plant-pathogen interactions (Goodin et al., 2008). Like in other plant-virus interactions, the particularities of the PLPV infection process may be largely determined by the silencing-based host defense and the corresponding viral counterdefense, with the vsRNAs being a main component of the interplay between the plant and the infectious agent. To get new insights into this relationship, here we have analyzed the composition and molecular nature of the vsRNAs that are present in PLPV-infected *N. benthamiana* plants and that have been found to accumulate at extraordinarily high levels. In the light of the latter observation, we have assessed whether infection by the virus induces DCL expression or it is influenced by RDR6 activity. The results have provided relevant clues on the likely properties of the templates for PLPV sRNA production and on the array of host factors involved in anti-PLPV silencing.

2. Material and methods

2.1. Plant material and viral inoculation

N. benthamiana plants, either wild type (wt) or a transgenic line in which RDR6 was silenced by RNA interference (RNAi) with a hairpin construct (RDR6i plants; Schwach et al., 2005), were grown from seeds until two-to-four leaf stage and then mock or virus-inoculated (two leaves per plant) with *in vitro* transcripts synthesized from an infectious PLPV clone as previously described (Castaño et al., 2007). Inoculated plants were kept under greenhouse conditions (16 h days at 24 °C, 8 h nights at 20 °C) and systemic leaves were harvested at 30 days post-inoculation (d.p.i.). At this time point, plants had about 12–14 systemic leaves at different developmental stages on the main stem and flowering was just starting. To standardize sample collection, systemic leaves number 5, 6 and 7 (numbering starting from local leaves) from at least two plants were bulked to make up each sample. For certain experimental purposes, local leaves (two leaves per plant from at least two plants for each sample) were collected at 7 d.p.i.

2.2. RNA extraction, tissue printing and Northern blot analysis

Total nucleic acid preparations were obtained from leaf material by phenol-chloroform extraction according Verwoerd et al. (1989), though the lithium chloride precipitation step of the original protocol was omitted and, instead, nucleic acids were recovered from the aqueous phase by ethanol precipitation. For Northern blot analysis, 4 µg of total RNA were denatured by glyoxal-dimethyl sulfoxide treatment and electrophoresed in 1% agarose gels (for PLPV gRNA and sgRNA detection) or 20 µg of total RNA were fractionated by denaturing PAGE on 20% gels (for sRNA detection). In the latter case, oligodeoxyribonucleotides of known lengths (18–27 nt) were loaded in the external lanes to be used as size markers. After ethidium bromide staining, nucleic acids were transferred from the gels to nylon membranes (Hybon+, GE Heathcare). In some experiments, tissue printing was performed for virus detection. To this aim, stems or leaf blades were directly imprinted onto nylon membranes. After UV-crosslinking, the membranes were hybridized with ^{32}P-labeled PLPV-specific RNA probes of plus or minus polarity, generated by *in vitro* transcription from suitable constructs, using conditions previously described (Pérez-Cañamás and Hernández, 2016). Alternatively, the membranes were hybridized with DNA oligonucleotides radioactively labeled at the 5′ end through T4 polynucleotide kinase (Thermo Fisher Scientific)-mediated reaction in the presence of γ-^{32}P-ATP. The specific sequences of the employed DNA oligonucleotides were: 5′-TAGTTCTACGTTGTACCTCGG-3′, complementary to positions 1,234–1,254 of PLPV genome, and 5′-TAGAGCTCCCTTCAATCCAAA-3′, complementary to miR159. When using DNA oligonucleotides as probes, hybridization was performed at room temperature in the presence of 6xSSC, 0.2% SDS, 5xDenhart solution and 50 µg/ml salmon sperm DNA. Specific activity of all probes was measured prior use as reported earlier (Blanco-Pérez and Hernández, 2016). Hybridization signals were visualized by autoradiography or subjected to PhosphorImager (Fujifilm FLA-5100) analysis.

2.3. Quantitative reverse transcription PCR (RT-qPCR)

To perform RT-qPCR measurement of *N. benthamiana* DCL mRNAs, primers were designed using Primer-Express 2.0 software (Applied Biosystems) and the following criteria: melting temperature of 50–60 °C, PCR amplicon lengths of 100–200 bp, length of primer sequences ranging from 19 to 25 nucleotides, and guanine-cytosine content of 40–60%. Specifically, DCL1 primers corresponded to DCL1-FW 5′- TGTGGGTGATGCAGTATT-3′ and DCL1-RV 5′- TGAAAC-CTGGTTTTGATAGT-3′, DCL2 primers to DCL2-FW 5′-TACC-

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