Arch Virol (2010) 155:1859–1863
DOI 10.1007/s00705-010-0770-5

BRIEF REPORT

A novel monopartite dsRNA virus from rhododendron

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Received: 31 March 2010 / Accepted: 23 July 2010 / Published online: 19 August 2010

Abstract A dsRNA molecule of 3.4 kbp was extracted from two great rhododendron samples from Great Smoky Mountains National Park. Sequencing of this molecule suggests that it represents the genome of an undescribed virus, for which the provisional name rhododendron virus A (RhVA) is proposed. In phylogenetic analyses, this virus clustered together with southern tomato virus and related viruses, forming a coherent and distinct clade among dsRNA viruses. RhVA likely belongs to a yet-to-be-established taxon of viruses with a non-segmented dsRNA genome.

Introduction

Double-stranded (ds) RNAs, usually present in detectable amounts in plants infected with RNA viruses (either as a genomic molecule or a product of viral replication), are increasingly becoming preferred templates for detection and characterization of these viruses. They are more stable than single-stranded molecules and, combined with the proper choice of downstream approaches (cloning, sequencing), represent ideal starting material for detection/identification of novel plant viruses.

During study on viruses in non-agronomic ecosystems, carried out in Great Smoky Mountains National Park (GSMNP), leaf samples of great rhododendron (*R. maximum* L.), one of the major components of the park flora, were included in the survey and tested in the laboratory at Mississippi State University. The general procedure involved dsRNA analysis as described by Valverde et al. [21]. The extracts were further purified by selective enzymatic (DNase and RNAse) digestions as described by Saldarelli et al. [16]. The number and relative sizes of extracted dsRNAs were estimated by 1% agarose and/or 6% polyacrylamide gel electrophoreses (PAGE) and compared with dsRNAs of known plant viruses: peanut stunt virus (PSV), tobacco mosaic virus (TMV), grapevine leafroll-associated virus 2 (GLRaV-2) and southern tomato virus (STV).

Purified dsRNA preparations were heat denatured and reverse transcribed using a slight modification of the protocol described by Froussard [7]. Synthesized cDNAs were size-selected with columns and cloned into pGEM-T Easy plasmid (Promega, USA). The resulting recombinant plasmids were transferred in *Escherichia coli* Top 10 competent cells (Invitrogen, USA) and sequenced. Primary sequence data were assembled and mapped using the DNAStar package (Lasergene Inc, USA). Missing genomic portions were generated by RT-PCR using virus-specific primers designed on preliminary sequences. Terminal sequences were determined by RACE-PCR using commercial kits (Invitrogen, USA). A search for similar sequences in NCBI/GenBank was performed using BLAST resources [2], while pairwise comparisons were performed with ClustalW [19]. Secondary structures of nucleic acids and putative protein products were predicted using RNAfold [8], KnotSeeker [18] and PSIPRED [13]. For phylogenetic studies, amino

Sequence data were deposited in GenBank as accession number HQ128706.

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acid sequences were aligned with the ProbCons software [5], and the tree was reconstructed using the Bayesian inference method implemented in the MrBayes program [10] and visualized with TreeDyn [3].

Northern hybridization was performed on dsRNAs extracted from 10 g of tissue, analyzed in 1% TAE (Tris–Acetate-EDTA) buffer, denatured in 50 mM NaOH and transferred via capillarity onto a positively charged membrane, as described previously [15]. A digoxygenine-labeled DNA probe was synthesized by amplifying the C-terminal portion of ORF2 between nt 2,624 and 2,956 from a reverse-transcribed dsRNA template using a PCR DIG Probe Synthesis Kit (Roche Diagnostics, USA). Hybridization was performed following the manufacturer's instructions.

In order to obtain putative virions, experiments were attempted following two purification procedures. The first one was described for purification of cryptic viruses and involved 0.1 M phosphate buffer and clarification with an equal volume of chloroform prior to alternating cycles of low- and high-speed centrifugation [14]. The second procedure involved a Tris–HCl-buffer-based extraction, low-speed clarification and PEG (MW 6,000) precipitation before ultracentrifugation. Concentrated extracts were placed on carbon-coated grids, stained with 2% uranyl acetate and examined with an electron microscope. These preparations and/or leaf extracts were rubbed onto cellite-dusted leaves of several herbaceous plants belonging to the families *Solanaceae*, *Chenopodiaceae* and *Fabaceae* in order to attempt mechanical transmission of the putative virus.

A 3.4-kbp dsRNA (Fig. 1a) was obtained from samples collected randomly from two asymptomatic great

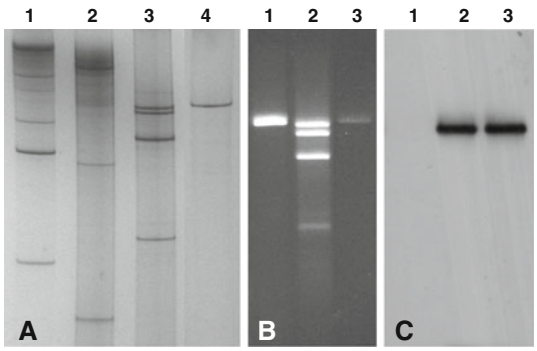

Fig. 1 **a** dsRNA extracted from rhododendron GSM-1 (*lane 4*) containing rhododendron virus A (RhVA) compared with dsRNAs extracted from plants infected with peanut stunt virus (PSV) (*lane 3*), tobacco mosaic virus (*lane 2*) and grapevine leafroll-associated virus 2 (GLRaV-2, lane 1). **b** Agarose gel electrophoresis showing co-migration of dsRNAs extracted from rhododendron (*lane 3*) and southern tomato virus-infected tomato (*lane 1*). *Lane 2* contains dsRNAs isolated from PSV-infected beans. **c** Single signal obtained in northern blot hybridization of dsRNA extracted from two RhVA-containing rhododendron plants (*lanes 2* and *3*). An extract from RhVA-free rhododendron is shown in *lane 1*

rhododendron plants in the GSMNP. This dsRNA was similar in size to the genomic dsRNA of STV [15], as they co-migrated when compared in the same gel (Fig. 1b).

Complete sequencing revealed that dsRNA extracted from rhododendron is 3,427 bp long and contains two partially overlapping putative open reading frames (ORFs; Fig. 2a). This genome organization is similar to that of STV and the related viruses vicia cryptic virus M (VCVM) [11] and blueberry latent virus (BlLV; blueberry fruit drop associated virus) [12, 20], which will be referred to as "STV-like viruses" throughout this paper. The full nucleotide sequences shared 30–37% identical residues with these viruses, with the region between nt 1,200–2,500 being the most conserved part of the genome.

The genome of RhVA starts with a tetranucleotide sequence (GUAU) that is identical to those of BlLV and VCVM. The untranslated (UTR) regions of the genome are 94 (5′UTR) and 98 (3′UTR) nucleotides long, rich in A + U (66 and 63%, respectively) and are capable of forming complex secondary hairpin-like structures (not shown). ORF1, starting at position 95 and terminating at nucleotides 1,307–1,309, encodes a 404-amino-acid (aa)-long protein of a predicted molecular mass of 44.2 kDa (p44) of unknown function. A search for similar proteins in GenBank revealed limited levels (27% identity; 46% similarity) of conservation with the p42 protein of STV. No statistically significant matches were found when this protein was compared with representatives of different protein families available in the Pfam database [6]. Prediction of secondary structure with PSIPRED [13] revealed the dominating presence of long alpha helix and coil structures. The only three short regions predicted to form beta-strands were found localized at the extreme N and C termini (not shown). A secondary structure low in beta strands is one of the major distinctions between the p42 protein encoded by the STV genome and coat proteins encoded by ORF1 of members of the family *Totiviridae* [15].

ORF2 overlaps the first cistron by ca. 400 nt (Fig. 2a). This overlapping genomic portion is rich in pseudoknot structures (7 predicted structures) that also involve the putative stop codon. Along with the presence of some putative "slippery" sequences, this suggests expression of the ORF2-encoded product as a fusion protein involving a +1 ribosomal frameshift phenomenon. This expression strategy has also been hypothesized for STV, BlLV and VCVM [11, 12, 15]. The putative fusion protein is 1,077 aa long and has an estimated molecular mass of 122 kDa (p122). Furthermore, northern blot hybridizations performed on purified dsRNAs and total nucleic extracts from infected rhododendrons did not reveal the presence of any subgenomic molecule as the possible template for expression of ORF2 (Fig. 1c).

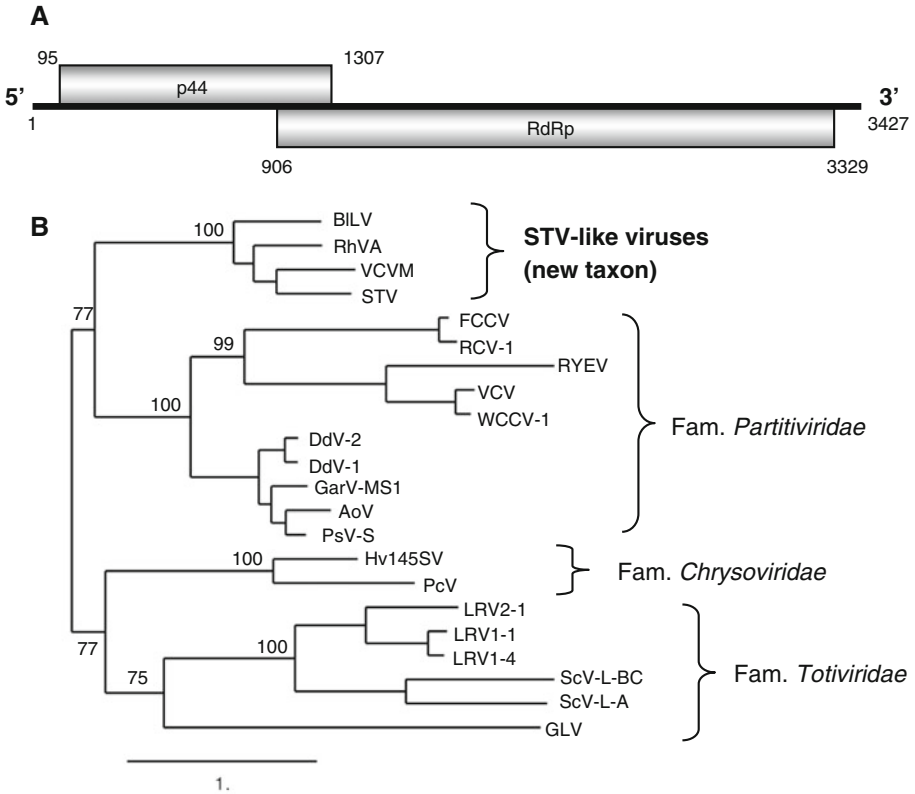

Fig. 2 **a** Diagrammatic representation of the rhododendron virus A (RhVA) genome with nucleotide coordinates. **b** Bayesian-inferred phylograms constructed on whole amino acid sequences of putative viral RNA-dependent RNA polymerases encoded by dsRNA viruses belonging to different taxa. The clade formed by RhVA, southern tomato virus (STV), vicia cryptic virus M (VCVM) and blueberry latent virus (BlLV) is clearly distinct from clusters formed by members of the families *Totiviridae*, *Partitiviridae* and *Chrysoviridae*. Sequences and acronyms used in the analyses are as follows: *Aspergillus* ochraceous virus (AoV; ABC86749), blueberry latent virus (BlLV; EF442779), Discula destructiva virus 1 (DdV-1; NC_002797), Discula destructiva virus 2 (DdV-2; NC_003710), Fragaria chiloensis cryptic virus (FCCV, NC_009519), Giardia lamblia virus (GLV; NC_003555), Gremmeniella abietina RNA virus MS1 (GaRV-MS1, NC_004018), Helminthosporium victoriae 145S virus (Hv145SV, NC_005978), Leishmania RNA virus 1-1 (LRV-1-1, NC_002063), Leishmania RNA virus 1-4 (LRV-1-4, NC_003601), Leishmania RNA virus 2-1 (LRV-2-1, NC_002064), Penicillium chrysogenum virus (PcV, NC_007539), Penicillium stoloniferum virus S (PsV-S; NC_005976); rose cryptic virus 1 (RCV-1, NC_010346), Saccharomyces cerevisiae virus L-A (ScV-L-A, NC_003745), Saccharomyces cerevisiae virus L-BC (ScV-L-BC, NC_001641), southern tomato virus (STV; NC_01191), Vicia cryptic virus (VCV, EF173392), Vicia cryptic virus M (VCVM; EU371896) and white clover cryptic virus 1 (WCCV-1, NC_006275)

However, ORF2 starts at nt position 906, spans 807 codons and potentially codes for a 92-kDa protein, identified as viral RNA-dependent RNA polymerase (RdRp) due to the signature triplet Gly-Asp-Asp (GDD) present almost universally in this class of virus-encoded enzymes. This protein shares significant levels of sequence identity with the corresponding genomic products of VCVM (49%), STV (47%), BlLV (43%) and to lesser extent, with orthologs of some partitiviruses. The central part of this protein (aa 145–628) represents the most conserved part of the genome, with high levels of amino acid conservation (55–60%) with STV-like viruses (Fig. 3a).

Phylogenetic analysis of the entire putative viral RdRp showed that RhVA grouped with STV, VCVM and BlLV, forming a distinct lineage of dsRNA viruses that are evolutionary related to members of the families *Totiviridae* and *Partitiviridae* (Fig. 2b). Separation of the "STV-like" clade from toti- and partitiviruses was confirmed by different methods for inferring phylogenies (not shown).

Repeated attempts to purify and/or visualize virions from infected tissues or to transmit the dsRNA virus mechanically to solanaceous test plants were unsuccessful, which is in agreement with what has been reported for STV and VCVM [11, 15]. Additionally, no putative fungal host of this virus could be identified by culturing surface-sterilized rhododendron specimens or by PCR using universal ITS primers [22].

During an investigation of its distribution in the GSMNP employing a virus-specific primer set, the virus was detected in seven of 25 samples that were randomly collected from great rhododendron plants at geographically distinct locations within the park (i.e. Cataloochee, Sugarland, Deep

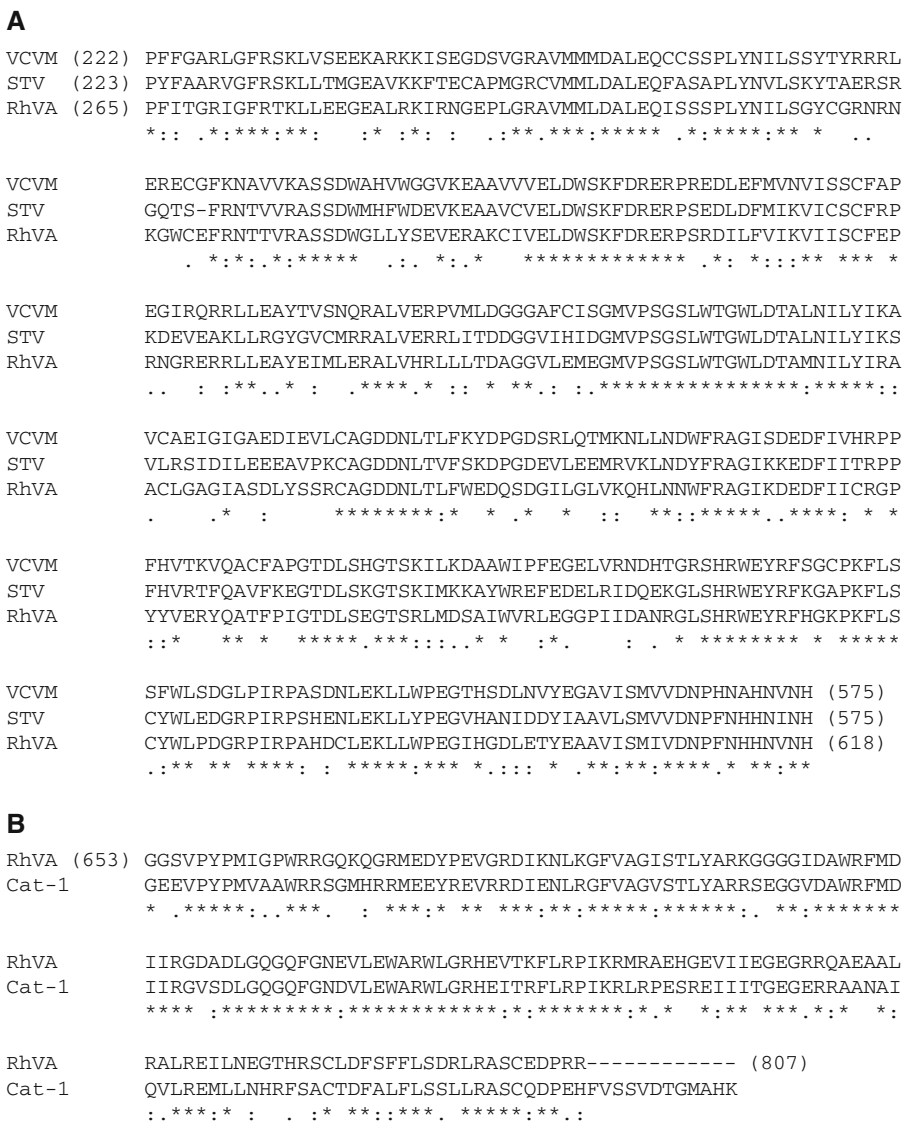

Fig. 3 **a** Alignments of the amino acid sequences of putative RdRps of RhVA, STV and VCVM. *Asterisks* denote identical amino acids in all three proteins used in analysis, and ":" and "." indicate conserved and semi-conserved substitutions, respectively. **b** Comparison between RhVA and partial sequences obtained from rhododendron sample Cat-1 suggesting the presence of another STV-related virus in this plant species

Creek, Cades Cove), indicating that this virus is relatively widespread in the local rhododendron germplasm. No symptoms have been found associated with infections by this virus in tested rhododendrons.

Our data indicate that the dsRNA isolated from great rhododendron represents the genome of a novel virus with a genome structure resembling those of totiviruses. Totiviruses (*sensu lato*) have been reported to infect fungal or protozoan hosts [23]. Although we do not have direct proof of the presence of this virus in rhododendron cells, all of the indirect data generated in this study (i.e. failure to isolate a putative fungal host, negative ITS-based PCR tests, phylogeny) together with the results of previous studies on similar viruses [11, 12, 15] fail to support the hypothesis of its mycoviral nature. Thus, we assume that rhododendron is a natural host for this virus, for which the tentative name rhododendron virus A (RhVA) is proposed. To our knowledge, the only virus previously reported from this plant is rhododendron ringspot virus [4], currently classified as a tentative species in the genus *Potexvirus* [1].

Interestingly, RhVA is apparently not the only STV-like virus in rhododendrons. Partial sequencing of dsRNA isolated from an additional rhododendron sample referred to as Cat-1 suggests the presence of another STV-like virus that shares 65% aa identity with RhVA in a portion of the genome covering the 3′end of ORF2 (Fig. 3b). Furthermore, STV-related sequences have also been obtained from members of other plant species during a recent study on viruses in the GSMNP, indicating the presence of similar viruses in other hosts in natural ecosystems (S. Sabanadzovic, unpublished). These results, together with already published reports on similar viruses in tomato [15], blueberry [12, 20], and *Vicia faba* [11] strongly suggest that this type of virus is relatively common in both cultivated and naturally grown plants. The genomes of these viruses share several traits, such as a strikingly uniform size (3,437 bp for

STV; 3,434 bp for VVM and 3,427 bp for RhVA) and organization (presence of the two partially overlapping genes). Furthermore, they share extensive sequence similarity and close phylogenetic relationships, suggesting that they represent a coherent and still unclassified group of dsRNA viruses. Although the mechanism of expression of viral replicase is not yet experimentally proven, in silico analyses suggest a frameshift-driven "gag-pol" type of fusion protein rather than the internal initiation reported for Helminthosporium victoriae virus 190S [9, 17]. As experimentally verified for STV, it is likely that RhVA is also efficiently seed transmissible. Seed transmission is one of the main characteristics of members of the genera *Alphacryptovirus* and *Betacryptovirus* (family *Partitiviridae),* from which these STV-like viruses clearly differ in genomic organization, but to which they are phylogenetically related.

In conclusion, RhVA is a novel dsRNA virus that has been identified in symptomless specimens of giant rhododendron plants growing naturally in the GSMNP. It is similar to STV and two other viruses recently reported from different crops and not assigned to any of the extant taxa. Available biological, molecular and phylogenetic data on these viruses indicate that they form a coherent group that is clearly distinct from currently known taxa of phyto- and mycoviruses. Therefore, the establishment of a new genus of dsRNA viruses should be considered in order to accommodate this growing class of viruses.

Acknowledgments This work was partially supported by funds provided by Discover Life in America Inc to the first author. The authors thank A. Lawrence for help in electron microscopy and Dr. J. Rock (National Park Services) for help in botanical identification. Approved for publication as Journal Article No J-11851 of the Mississippi Agricultural and Forestry Experiment Station, Mississippi State University.

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