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Detection of members of the *Tombusviridae* in the Tallgrass Prairie Preserve, Osage County, Oklahoma, USA

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ABSTRACT

Viruses are most frequently discovered because they cause disease in organisms of importance to humans. To expand knowledge of plant-associated viruses beyond these narrow constraints, non-cultivated plants of the Tallgrass Prairie Preserve, Osage County, Oklahoma, USA were systematically surveyed for evidence of the presence of viruses. This report discusses viruses of the family *Tombusviridae* putatively identified by the survey. Evidence of two carmoviruses, a tombusvirus, a panicovirus and an unclassifiable tombusvirid was found. The complete genome sequence was obtained for putative TGP carmovirus 1 from the legume *Lespedeza procumbens*, and the virus was detected in several other plant species including the fern *Pellaea atropurpurea*. Phylogenetic analysis of the sequence and partial sequence of a related virus supported strongly the placement of these viruses in the genus *Carmovirus*. Polymorphisms in the sequences suggested existence of two populations of TGP carmovirus 1 in the study area and year-to-year variations in infection by TGP carmovirus 3.

1. Introduction

Many species of plant viruses remain to be discovered due to relative lack of attention to viruses of plants from non-cultivated areas (Cooper and Jones, 2006; Wren et al., 2006). A project (Plant Virus Biodiversity and Ecology, PVBE) to uncover such viruses in plants of the Tallgrass Prairie Preserve (TPP, The Nature Conservancy, Oklahoma; Allen et al., 2009; Hamilton, 2007) was initiated (Melcher et al., 2008) and shown to result in the discovery of evidence of several novel viruses (Muthukumar et al., 2009; Roossinck et al., 2010). We report here the results of searches of the recovered sequences for evidence of members of the family *Tombusviridae*.

Known family *Tombusviridae* members are divided into eight genera (*Dianthovirus*, *Tombusvirus*, *Aureusvirus*, *Avenavirus*, *Carmovirus*, *Necrovirus*, *Panicovirus* and *Machlomovirus*) (Fauquet et al., 2005). Their particles are icosahedral and contain genomes consisting of one or two strands of positive-sense RNA. Their RNAs are uncapped (Kneller et al., 2006) and lack 3′ poly(A) tails (Fauquet et al., 2005). The viruses of the family *Tombusviridae* do not encode a viral helicase, and their RNA-dependent RNA polymerases (RdRPs) are expressed via suppression of a stop codon, or, for dianthoviruses, by a frameshift. Open reading frames (ORFs) initiating in the 3′ half of the genomes are expressed through production, depending on genus, of one (Castaño and Hernández, 2005; Castaño et al., 2009; Kinard and Jordan, 2002) or two subgenomic RNAs (sgRNAs). The number of sgRNAs produced has been proposed as a characteristic useful in taxonomic assignment within the genus *Carmovirus* (Castaño et al., 2009). Those producing only one sgRNA have been proposed to be placed in a new genus, designated "pelarspovirus". Although members of the family *Tombusviridae* are thought to infect plants exclusively, recently a complete viral sequence related to members of the family *Tombusviridae* and umbraviruses was assembled (Culley et al., 2007) from a coastal water metagenomic study of the Straits of Georgia. This virus lacked genes for a coat protein (CP) or movement protein (MP) suggesting it does not infect higher plants. The PVBE project uncovered evidence from plants of two carmoviruses (for discussion purposes named TGP carmoviruses 1 and 3), one tombusvirus (TGP tombusvirus 1), one unclassifiable tombusvirid (TGP tombusvirid 1) described here, and one panicovirus (Scheets, in preparation).

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2. Materials and methods

2.1. Sample collection and tissue processing

Collection, identification, and archiving of plant samples were described earlier (Melcher et al., 2008). Sample identification numbers specify collection year with the first two digits (ex. 05TGP00369 was collected in 2005). Processing of samples by preparation of viral particle fractions from which nucleic acid was isolated (VLP-VNA) and randomly amplified was also described therein. For the samples presented here, sequences from the randomly amplified cDNA were obtained by the 454 pyrosequencing method using a panel of 96 four-nucleotide tags as described by Roossinck et al. (2010). This method was also used for sequencing of DNA derived from dsRNA.

For analysis of bulked plant material, a geographically identified square of land 30 cm × 30 cm was denuded of above-ground plant material which was stored in bags for transport to the laboratory where it was frozen at −80 °C. For preparation of virus like particles, 10 g of mixed plant sample was transferred to a metallic flask and homogenized by using a homogenizer (Biospec, Bartlesville, OK). The homogenate was poured into 250 ml centrifuge bottles, and 75 ml of citrate buffer (Melcher et al., 2008) and 0.63 ml of 0.25 M iodoacetamide were added. After 10 min on ice, the bottles were centrifuged for 15 min at 12,000 × *g*. Of the supernatant, 40 ml were transferred to a 50 ml plastic tube, to which was added 3.8 ml of Triton X-100. Then, 43 ml of the mixture were transferred to a Beckman ultracentrifuge tube (94 ml tubes) and underlaid with 13 ml of 20% sucrose solution in citrate buffer. Centrifugation proceeded at 70,000 × *g* for 11 h at 21 °C in a Beckman Ti 45 rotor. The supernatant was decanted slowly and the pellet resuspended in 200 μL of 0.5× citrate buffer by pipetting. After centrifugation at 8000 × *g* for 10 min, 150 μl of the supernatant was transferred to an ultracentrifuge tube and underlaid with 50 μl of 20% sucrose solution in 0.5× citrate buffer. Final ultracentrifugation was at 150,000 × *g* for 65 min at 21 °C in the Beckman Ti 42.2 rotor. The supernatant was removed by a pipettor and the pellet was resuspended in 250 μl of viral resuspension buffer. The VLP fraction was treated for virus nucleic acid isolation as previously described (Melcher et al., 2008). The procedure was repeated for a total of three 10 g aliquots of the mixed plant sample.

2.2. Sequence processing

All contigs assembled by the 454 process pipeline were used as BLASTn and tBLASTx (Altschul et al., 1997) queries of the nr/nt database and as BLASTx queries of the nr protein database. All contigs assembled by the 454 process pipeline in the project were formatted for BLAST searching by formatdb. This database was queried by BLASTn using viral contigs identified from the initial screening as queries and by tBLASTn using protein sequences encoded by reference genomes of members of the family *Tombusviridae* as queries. The complete contigs from the TPP project metagenome are available at the MG-RAST (Meyer et al., 2008) server (http://mg-rast.mcs.anl.gov/mg-rast/FIG/linkin.cgi?metagenome=4444009.3). Assembled sequences of putative viruses inferred from the contig data were used to identify additional sequences of these viruses among unassembled sequences. The unassembled sequences were also screened with GenBank sequences for Maize necrotic streak virus (MNeSV, NC_007729) and *Cucumber Bulgarian latent virus* (CBLV, NC_004725). These singleton sequences were used in building the consensus sequences for the putative viruses. Because of the chance that an occasional read was assigned mistakenly to the wrong plant sample in a sequencing pool, only those plants belonging to species for which more than 0.5% of specimen reads and more than 8 reads belonged to the virus were considered as positive for the virus, unless it could be established that the sequences were unlikely to have arisen from contamination by a virus source in the pool. Assembled viral sequences were deposited in GenBank (IDs: NC_015227, JF437881, JF437885, HM640931, HM640936, and three pending submissions JN122349–51). Shorter sequences and a consensus sequence of TGP carmovirus 3 are provided in Supplementary files. An internal gap in the assembled sequence of TGP carmovirus 3 was filled by sequencing PCR amplicons made using primers from sequences flanking the gap with cDNA template prepared from VLP-VNA from specimen 08TGP00078 and primers 5′-AAA TGT TAT TCT ATG GCG ACT ACT-3′ and 5′-TAA TGC ACA CTA CCA CTA TTT CCT-3′.

2.3. RNA secondary structure analysis

RNA secondary structure predictions were performed with mfold version 2.3 at 28 °C (http://mfold.rna.albany.edu/?q=mfold) (Zuker, 2003) on TGP carmovirus 1 and the 17 carmoviruses with complete genomes in GenBank: Angelonia flower break virus (AnFBV, NC_007733), Calibrachoa mottle virus (CbMV, GQ244431), *Carnation mottle virus* (CarMV, NC_001265), *Cardamine chlorotic fleck virus* (CCFV, NC_001600), *Cowpea mottle virus* (CPMoV, NC_003535), *Hibiscus chlorotic ringspot virus* (HCRSV, NC_003608), Honeysuckle ringspot virus (HoRV, NC_014967), *Japanese iris necrotic ring virus* (JINRV, NC_002187), *Melon necrotic spot virus* (MNSV, NC_001504), Nootka lupine vein-clearing virus (NLVCV, NC_009017), *Pea stem necrosis virus* (PSNV, NC_004995), Pelargonium chlorotic ring pattern virus (PCRPV, NC_005985), *Pelargonium flower break virus* (PFBV, NC_005286), Pelargonium line pattern virus (PLPV, NC_007017), *Saguaro cactus virus* (SgCV, NC_001780), Soybean yellow mottle mosaic virus (SYMMV, NC_011643), *Turnip crinkle virus* (TCV, NC_003821). Three regions of the 5′ ends of viral RNA and the large sgRNA were folded for each genome: to the first AUG, the second AUG, and the first 120 nt. The complete 3′ UTRs were also folded. The recombinant necrovirus *Galinsoga mosaic virus* (GaMV, NC_001818) was also compared since it was misleadingly listed as a carmovirus (Fauquet et al., 2005).

2.4. Phylogenetic analysis of carmovirus coat proteins

Phylogenetic analysis of CP amino acid sequences of carmoviruses was performed on the CPs of carmoviruses with complete genomes, GenBank accessions of Elderberry latent virus (ELV, AAK74061.1), Pelargonium ringspot virus (PelRSV, AAK74063.1), and the non-carmoviral CP most similar to TGP carmovirus 1, Pelargonium necrotic spot virus (PeNSV, NP_945116.1). Aligned sequences were adjusted manually in a progressive fashion (Melcher, 1990) using parsimony trees generated by Protpars of the Phylip 3.66 package (Felsenstein, 1989) as guide. The adjusted alignment was used in Prottest (Abascal et al., 2005) to generate a maximum likelihood distance tree using PhyML and the LG scoring matrix (Le and Gascuel, 2008). The aligned sequences were also used to generate bootstrap confidence levels using Seqboot, Protdist and Consense of the Phylip package.

3. Results

3.1. Sequence identification and distribution

Contigs were identified with best similarity to sequences from three *Tombusviridae* genera: *Tombusvirus, Panicovirus* and *Carmovirus*. A single contig from the grass *Digitaria cognata* (fall witchgrass) and thus a single virus could be assigned to the *Tombusvirus* genus. For discussion purposes, we designate it TGP tombusvirus 1. The panicovirus Thin paspalum asymptomatic