Virus Research 152 (2010) 30–40

Contents lists available at ScienceDirect

Virus Research

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

Genetic structure and molecular variability of *Grapevine fanleaf virus* populations

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ARTICLE INFO

Article history:
Received 17 March 2010
Received in revised form 27 May 2010
Accepted 31 May 2010
Available online 9 June 2010

Keywords:
Arabis mosaic virus
Genetic structure
Grapevine fanleaf virus
Phylogenetic analysis
Recombination
Selection pressure

ABSTRACT

To gain insights into the evolutionary mechanisms of *Grapevine fanleaf virus* (GFLV) from the genus *Nepovirus*, family *Secoviridae*, the sequences of the complete coding region of RNA2, including genes $2A^{HP}$, $2B^{MP}$ and $2C^{CP}$, and partial sequence from the RNA1-encoded gene $1E^{Pol}$ of 14 GFLV isolates from three naturally infected California vineyards were characterized. Phylogenetic analyses suggested two to three evolutionarily divergent lineages that did not reflect the vineyard origin of the isolates or an association with rootstock genotype or scion cultivar. Examination of the genetic variability of the California isolates alongside isolates worldwide, for which three RNA1 and 44 RNA2 coding sequences are available, revealed similar patterns of molecular evolution for the different regions within the GFLV genome but distinct selection constraints with the strongest pressure exerted on genes $2C^{CP}$ and $2B^{MP}$, an intermediate level of pressure exerted on gene $1E^{Pol}$, and the weakest pressure exerted on gene $2A^{HP}$. Some of the California isolates resulted from interspecies recombination events between GFLV and *Arabis mosaic virus* with crossover sites suspected in gene $1E^{Pol}$ and identified in genes $2A^{HP}$ and $2B^{MP}$; and intraspecies recombination events inferred in the four target genes but most frequently observed within gene $2C^{CP}$. This study suggested that purifying selection and recombination are important evolutionary mechanisms in the genetic diversification of GFLV.

1. Introduction

Grapevine fanleaf virus (GFLV) causes fanleaf degeneration, one of the most devastating viral diseases of grapevines worldwide (Andret-Link et al., 2004a). GFLV is specifically transmitted by the ectoparasitic nematode species *Xiphinema index* and can be very difficult to control due to the extended persistence of viruliferous vectors in vineyard soils even in the absence of host plants (Demangeat et al., 2005). The virus causes problems on nearly every continent and in every region where grapes are grown and the nematode vector is present. In the United States, though GFLV-infected vines have been identified in California (Martelli and Hewitt, 1963; McKenry et al., 2001; Taylor and Hewitt, 1964), Washington (Mekuria et al., 2009), and Missouri (Lunden et al., 2010), the nematode vector *X. index* has been documented only in California (Hewitt et al., 1958; McKenry et al., 2001).

GFLV is a member of the genus *Nepovirus* in the family *Secoviridae* (Sanfaçon et al., 2009). It has a bipartite plus-sense RNA genome (Andret-Link et al., 2004a). RNA1 consists of 7342 nts and codes for a M_r 253K polyprotein, which is cleaved by the virally encoded proteinase (also found on RNA1) into five individual proteins, including a protein of unknown function (1A), a putative helicase ($1B^{Hel}$), a viral protein genome-linked or VPg ($1C^{Vpg}$), a proteinase ($1D^{Pro}$) and a putative RNA-dependent RNA polymerase (RdRp) ($1E^{Pol}$) (Andret-Link et al., 2004a). RNA2 consists of 3774 nts and codes for a polyprotein of M_r 122K, which is cleaved by the RNA1-encoded viral proteinase into three individual proteins, including a homing protein ($2A^{HP}$) necessary for RNA2 replication, a movement protein ($2B^{MP}$) and a coat protein ($2C^{CP}$) (Andret-Link et al., 2004a).

As with other RNA viruses, the GFLV RdRp has no proofreading mechanism. Therefore, its replication is error prone and GFLV exists as a quasispecies (Naraghi-Arani et al., 2001). Based upon genetic variation studies conducted on isolates from France, Germany, Iran, Slovenia, South Africa, Tunisia and the United States, divergence of up to 17% and 9% at the nucleotide and amino acid levels, respectively, has been observed within GFLV gene $2C^{CP}$ (Bashir et al., 2007; Boulila, 2007; Fattouch et al., 2005; Liebenberg et al., 2009; Mekuria et al., 2009; Naraghi-Arani et al., 2001; Pompe-Novak et al., 2007; Pourrahim et al., 2007; Vigne et al., 2004; Wetzel et al., 2001). While several studies have examined the genetic diversity of gene $2C^{CP}$ and to a lesser extent gene $2B^{MP}$ – with over 300 complete or partial sequences of the two genes available in GenBank – sequence information from other parts of the genome is relatively limited.

Recombination can be an important factor in viral evolution (Garcia-Arenal et al., 2001; Moury et al., 2006); and in the case of GFLV, recombination has been reported to occur within RNA2

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doi:10.1016/j.virusres.2010.05.017

both between distinct genetic variants (Boulila, 2007; Mekuria et al., 2009; Pompe-Novak et al., 2007; Vigne et al., 2004, 2005, 2009), and between GFLV and other closely related viruses from the genus *Nepovirus*, including *Arabis mosaic virus* (ArMV) (Jawhar et al., 2009; Mekuria et al., 2009; Vigne et al., 2008) and *Grapevine deformation virus* (GDefV) (Mekuria et al., 2009).

Characterizing the genetic structure of viral populations and the factors that contribute to their evolution may help improve understanding of new epidemics, phylogenetic relationships amongst isolates from various geographical origins, and pathogenicity changes that may result from variations between host genotypes. To characterize GFLV isolates in naturally infected vineyards in California and gain further insights into the mechanisms of GFLV evolution, we analyzed the genetic variation and phylogenetic relationships amongst isolates in four genomic regions, i.e. the RNA1-encoded $1E^{Pol}$ gene and the RNA2-encoded $2A^{HP}$, $2B^{MP}$ and $2C^{CP}$ genes, and investigated recombination between divergent sequence variants. Our analyses suggested that the RNA2 and $1E^{Pol}$ sequences from the GFLV variants from California that were examined in this study segregate into two evolutionarily divergent lineages for RNA2 and three lineages for $1E^{Pol}$ irrespective of host scion cultivar, rootstock genotype, or vineyard origin. In addition, they showed evidence of variable selection pressures exerted on different genes and frequent recombination events.

2. Materials and methods

2.1. Vineyard and leaf sample collection

Three vineyards in Lodi, California with a long history of GFLV infection and presence of the nematode vector *X. index* were selected for this study. These vineyards vary in age (10–40 years) and were established from source materials originating in California. Typical GFLV symptoms, such as foliar mosaic, yellowing and distortion as well as shot berries and reduced yield were observed on the majority of vines in these vineyards. No readily observable differences were recognized among vines or vineyards regarding the severity and types of symptoms present, or between vineyards in terms of disease prevalence. Vineyard A (~2 ha) was established ~20 years ago and consisted of *Vitis vinifera* cv. Zinfandel grafted onto the rootstock Freedom (1613 Couderc [*V. solonis* × Othello] × *V. champinii*). Vineyard B (~1.5 ha) was established ~10 years ago and consisted of *V. vinifera* cv. Cabernet Sauvignon grafted onto the rootstock Freedom. Vineyard C (~3 ha) was established ~40 years ago and consisted of *V. vinifera* cv. Cabernet Sauvignon grafted onto the rootstock Dog Ridge (*V. champinii*). Vineyard A and vineyard B were located adjacent to one another with vineyard C positioned approximately one mile away. GFLV transmission via *X. index* has been determined to be occurring in these vineyards (Fuchs, unpublished).

Leaf samples (8–10 per vine) were collected from the tip of symptomatic shoots on October 16th, 2007 for GFLV detection by double antibody sandwich (DAS)-enzyme linked immunosorbent assay (ELISA) and immunocapture (IC)-reverse transcriptase (RT)-polymerase chain reaction (PCR). We considered a GFLV isolate to be a viral culture from a single vine.

2.2. Virus detection by DAS-ELISA

GFLV was detected by DAS-ELISA in crude leaf extracts with specific antibodies (Bioreba, Reinach, Switzerland). A portion of 8–10 stacked leaves was torn and ground in 200 mM Tris–HCl pH 8.2, 140 mM NaCl, 2% polyvinylpyrrolidone 40, and 0.05% Tween 20 at a 1:5 ratio (w/v) using a semi-automated ball-bearing HOMEX tissue homogenizer (Bioreba, Reinach, Switzerland). Test conditions were according to the manufacturer's instructions (Bioreba, Reinach, Switzerland). Substrate hydrolysis was recorded at 405 nm with an absorbance BioTek® ELx808™ microplate reader (BioTek, Winooski, VT). Samples were considered positive if their optical density ($OD_{405\,nm}$) readings were at least twice those of healthy controls.

2.3. GFLV RNA1 and RNA2 characterization by IC-RT-PCR

GFLV was detected by IC-RT-PCR in plant sap from leaf samples that reacted positively with GFLV antibodies in DAS-ELISA. The immunocapture step was carried out using a 96-well microplate coated with specific GFLV antibodies (Bioreba, Reinach, Switzerland). Leaf material (250 mg) was crushed in the extraction buffer used for DAS-ELISA (2.5 ml) and crude sap (100 μl) was incubated in coated microtiter plates overnight at 4 °C. After four washes with PBS (1×) and Tween 20 (0.05%), sterile water (10 μl) was added to each well before incubation at 70 °C for 10 min followed by 5 min at 4 °C. The RT step was carried out using the AMV RT enzyme (Promega Corporation, Madison, WI), 50 pmol of reverse RNA1 primer 5′-GTTATCCCAGTACCAAGAAT-3′ and reverse RNA2 primer 5′-GAGGATCCCAGTAAAAAGAAAGGAAAA-3′ for 1 h at 42 °C, followed by 5 min at 99 °C and 5 min on ice. Reverse primers were designed based on the full-length genomic sequences of GFLV strain F13 (Ritzenthaler et al., 1991; Serghini et al., 1990) to hybridize to nts 7106–7125 and nts 3742–3760 of RNA1 and RNA2, respectively.

PCR was carried out using the GoTaq DNA polymerase and 20 pmol of specific primers (Supplementary Table 1) in a 50 μl final volume according to the manufacturer's protocol. PCR used a 2 min heating step at 94 °C followed by 30 cycles of 1 min melting at 94 °C, 1 min annealing at 50 °C, and 2 min elongation at 72 °C with a final extension of 7 min at 72 °C. The reaction products were resolved by electrophoresis in 1.5% agarose gels in 90 mM Tris–borate, 2 mM EDTA, pH 8.0, stained with ethidium bromide and subsequently visualized under UV light.

2.4. GFLV sequence determination and analyses

Overlapping viral cDNA amplicons obtained for each isolate by IC-RT-PCR were extracted from agarose gels with the QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA) and cloned in pCR4®-TOPO® (Invitrogen, Carlsbad, CA). TOPO plasmids containing GFLV inserts were extracted from *E. coli* competent cells using the Promega Wizard® *Plus* SV Minipreps DNA Purification System and sequenced bidirectionally using M13F and M13R primers with the Big Dye Terminator Kit, AmpliTaq-FS DNA polymerase and an Applied Biosystem Automated 3730xl DNA Analyzer at the Cornell University DNA Sequencing Facility in Ithaca, NY.

Sequences were analyzed and compared using the DNASTAR Lasergene® v7.2 software package. The algorithm CLUSTAL W was used for alignment of nucleotide sequences and the program SeaView (Galtier et al., 1996) was used for hand editing and construction of contigs. Fragments were assembled only if they had at least 99% nucleotide identity in the overlapping regions. Additional clones (one to three) of each fragment were characterized to confirm sequence integrity. Phylogenetic relationships were determined with neighbour-joining using the GFLV and nepovirus isolate sequences from GenBank listed in Supplementary Table 2 (Ghanem-Sabanadzovic et al., 2005; Loudes et al., 1995) along with ArMV strain NW (Wetzel et al., 2001, 2004) that was used as an outgroup [RNA1: AY303786; RNA2: AY017339]. Six Slovenian isolates (Vol45c1, Vol55c3, Vol47c1, Vol50c2, Vol52c1, and Vol51c3) were chosen to represent the three clades into which 28 Slovenian isolates were previously reported to cluster (Pompe-Novak et al., 2007). The robustness of the inferred evolutionary relationships was assessed by 1000 bootstrap replicates. Branches with boot-