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BRIEF REPORT

Multiple interspecies recombination events within RNA2 of *Grapevine fanleaf virus* and *Arabis mosaic virus*

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Abstract Sequence alignments and SISCAN analyses inferred multiple interspecies recombination events within RNA2 of strains GHu of *Grapevine fanleaf virus* (GFLV) and Ta of *Arabis mosaic virus* (ArMV), two closely related subgroup A nepoviruses in the family *Comoviridae*. Interspecies recombination events were identified in the 5′ untranslated region, the putative homing protein and movement protein genes but not in the coat protein gene and 3′ untranslated region. These findings suggest a dynamic relationship between GFLV and ArMV, and a differential selection pressure on RNA2-encoded proteins with constraints in terms of function and co-adaptation that limit interspecies recombination to certain gene segments.

Grapevine fanleaf virus (GFLV) and *Arabis mosaic virus* (ArMV), two closely related members of the genus *Nepovirus,* family *Comoviridae* [16], are responsible for fanleaf degeneration, the most severe viral disease of grapevines [1, 5]. These two viruses often occur in mixed infection in central European vineyards [5]. The bipartite genome of GFLV and ArMV encodes replicase and protein maturation functions in RNA1, and a putative homing protein ($2A^{HP}$), the movement protein ($2B^{MP}$) and coat protein ($2C^{CP}$) in RNA2 (Fig. 1a). The same arrangement of protein domains within the RNA2-encoded polyprotein is conserved in picorna-like viruses [12]. The RNA2 of GFLV and ArMV have 60–66% nucleotide identity in the 5′ untranslated region (UTR), 68–78% in gene $2A^{HP}$, 76–78% in gene $2B^{MP}$, 67–68% in gene $2C^{CP}$ and 72–73% in the 3′UTR [14, 21, 28, 29].

RNA recombination is a natural mechanism involved in genetic variation and evolution of plant virus populations [7, 15, 30]. It commonly occurs when the replicase complex switches template after encountering secondary RNA structures like hairpins and/or stretches of substantial similarity between donor and acceptor RNA strands during virus replication [18, 22, 23, 27, 31]. Intraspecies recombination has been documented for various GFLV strains with crossover sites distributed all along the open reading frame of RNA2 [19, 25, 26]. No information is available on interspecies recombination between GFLV and other viruses, in particular ArMV [16], albeit protein $2A^{HP}$ of ArMV strain NW has higher amino acid similarity with GFLV than with other ArMV strains [28]. In this study, we determined the sequence of RNA2 of GFLV strain GHu (GFLV-GHu) and ArMV strain Ta (ArMV-Ta), and identified interspecies recombination events in the 5′ UTR, genes $2A^{HP}$ and $2B^{MP}$ but not in gene $2C^{CP}$ and the 3′ UTR. Our findings suggest a dynamic relationship between GFLV and ArMV, and a distinct evolutionary diversification of their RNA2-encoded proteins.

GFLV-GHu [9] and ArMV-Ta [13] were isolated from *Vitis vinifera* cvs. Gloriae Hungariae and Tannat, respectively. Their RNA2 was characterized from infected grapevine leaves by reverse transcription-polymerase chain

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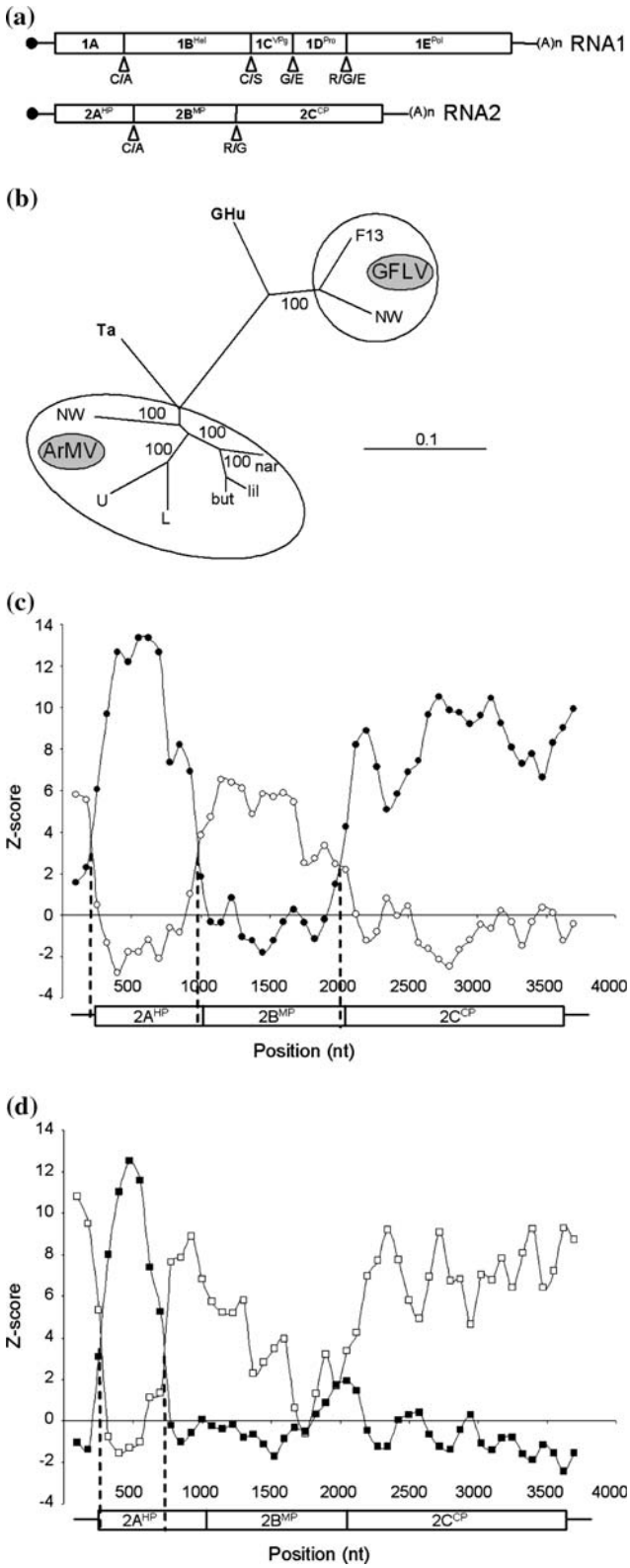

Fig. 1 **a** Schematic representation of the GFLV and ArMV genome. The ORF of each genomic RNA is represented by an *open rectangle*, the flanking 5′ and 3′ UTRs by a *black line*, the VPg by a *closed circle*, and poly-A tails by $A_{(n)}$. Processed proteins are indicated within each polyprotein (1A: putative proteinase cofactor; $1B^{Hel}$: putative helicase; $1C^{VPg}$: VPg; $1D^{Pro}$: proteinase; $1E^{Pol}$: putative RNA-dependent RNA polymerase; $2A^{HP}$: homing protein; $2B^{MP}$: movement protein; and $2C^{CP}$: coat protein). Cleavage sites are indicated below polyproteins. **b** Phylogenetic relationship within RNA2 of GFLV-GHu (GHu: EF426852), ArMV-Ta (Ta: EF426853) and other GFLV (F13: NC_003623, NW: AY017338) and ArMV (but: AB279739, L: X81815, lil: AB279741, nar: AB279740, NW: NC_006056, U: X81814) strains for which full-length sequences are available in databases. NJ unrooted phylogenetic trees were reconstructed from RNA2 nucleotide sequences. *Ellipses* correspond to GFLV and ArMV groups. *Numbers* below critical branches are significant bootstrap values (%). *Scale bars* represent a genetic distance of 0.1. **c** SISCAN analysis of aligned nucleotide sequences of RNA2 from recombinant GFLV-GHu with GFLV-F13 (*filled circle*) and ArMV-U (*open circle*). The window covered 200 bp positions and moved through the alignment with a step size of 75 bp. Graph is based on *Z* values using the total nucleotide identity scores. Recombination crossover sites are shown by a *dotted line*. **d** SISCAN analysis of aligned nucleotide sequences of RNA2 from recombinant ArMV-Ta with GFLV-F13 (*filled square*) and ArMV-U (*open square*). Parameters are identical to (A)

reaction (RT-PCR) using total RNA extracted with the RNeasy Plant Mini Kit (Qiagen) and degenerate primers selected in conserved regions [14, 19, 21, 25, 26, 28] (Table 1). The 5′ and 3′ RNA2 extremities of GFLV-GHu and ArMV-Ta RNA2 were determined with the rapid amplification of cDNA ends (RACE) technology (Roche). All GFLV-GHu and ArMV-Ta cDNAs were cloned in plasmid pGEM-T (Promega), transformed into *Escherichia coli* JM109 cells, and sequenced by the dideoxynucleotide chain termination method with primers hybridizing to viral RNA2 regions (Table 1) as well as primers SP6 and T7. The RNA2 sequence of GFLV-GHu, ArMV-Ta and other GFLV and ArMV strains was compared using CLUSTAL W [24]. Phylogenetic relationships determined with neighbour-joining [20] inferred two distinct groups, as expected (Fig. 1b). Interestingly, GFLV-GHu and ArMV-Ta had an intermediate position between the GFLV and ArMV groups (Fig. 1b).

SISCAN analysis [8] of the 3,806 bp long RNA2 indicated that GFLV-GHu is an interspecies recombinant between GFLV and ArMV with multiple crossover sites mapping to the 5′ UTR between nucleotides (nts) 137 and 160, the 36 part of gene $2A^{HP}$ between nts 880 and 921, and the 3′ part of gene $2B^{MP}$ between nts 1,969 and 2,031 (Fig. 1c). The chimeric nature of GFLV-GHu RNA2 was further supported by the size of its 5′ UTR (264 bp) which is similar to most ArMV strains [14, 28], including ArMV-Ta (260 bp), but larger than other GFLV strains (231–232 bp) [21, 26, 28]. To the best of our knowledge, this is the first report of recombination in the 5′ UTR of a nepovirus genomic RNA. Recombination was suspected previously in this region but empirical evidence was lacking [16]. An overall high AU content (63–72%), a predicted stem loop structure (GAGTTAAGAAACTC) conserved between GFLV and ArMV in positions 145–159

Table 1 Oligonucleotides used in this study

Primer	Purpose	GFLV position[a]	ArMV position[b]	Sequence 5′–3′[c]
395	RT-PCR	298–320 (+)	294–316 (+)	GCCTGYTGGGCTGCTGGDAAGAA
396	RT-PCR	2419–2444 (−)	2412–2437 (−)	TTRAACCACATDGCRTGDCCRCACAA
397	RT-PCR	3736–3755 (−)	3708–3727 (−)	GGCAAGTGTGTCCAAAGGAC
398	RT-PCR	1957–1975 (+)	1956–1974 (+)	TGGGARARYRTNGAGGAAC
225	5′ RACE and sequencing	705–722 (−)	692–709 (−)	AGTGCCGCAGCTCTCGAC
446	5′ RACE	807–827 (−)	NA	TCATACCACTTCCTCCAAGTG
447	5′ RACE	NA	797–817 (−)	TCATACCATCTCCTCCAGGTG
490	3′ RACE	2632–2649 (+)	NA	TGCCCTCCCATATTCTTT
77	3′ RACE	NA	2058–2074 (+)	ATGAGCACTACTACGCG
449	Sequencing	374–392 (−)	370–388 (−)	ACAGTTTGGCGGAAGGAGG
443	Sequencing	NA	940–958 (−)	GCCCTAGCACAAGACTGCC
400	Sequencing	NA	1235–1254 (+)	TGCATATCTTGGTGCTGCTG
429	Sequencing	NA	1548–1566 (−)	CCATATTGCGCTCGTGCCC
445	Sequencing	NA	2269–2290 (−)	GCGTCAAAACTCATAACCCACG
110	Sequencing	NA	2191–2217 (+)	AGTGGATTACTGCAGGACTTGTTATGC
140	Sequencing	NA	2795–2812 (+)	TAGCCCTTGCACTTATGG
438	Sequencing	NA	3369–3388 (+)	GGCATGGATGGAGCATCCCG
8	Sequencing	886–904 (−)	NA	GAGGATTTTGGATTGGGGG
32	Sequencing	1221–1238 (−)	NA	GCATTCCTGGCCTGCTCA
28	Sequencing	1302–1323 (+)	NA	TTACGCCCCTAGGGGTTTGTGG
13	Sequencing	1609–1627 (−)	NA	AAATGGCTCTAGCTAACCC
115	Sequencing	2012–2032 (+)	NA	CTGTGAGGATTGATAGAAACG
78	Sequencing	2109–2129 (−)	NA	GCCTGGCAATCCTTGGGAATG
16	Sequencing	2269–2289 (+)	NA	GGATTGACATGGGTGATGAGC
423	Sequencing	2421–2439 (−)	NA	CCACATDGCRTGACCACACA
432	Sequencing	2629–2650 (+)	NA	ACTTGCCCTCCCATATTCTTTG
424	Sequencing	3082–3101 (−)	NA	AAAGAGAGATCTGGGCGCAC
431	Sequencing	3178–3200 (+)	NA	GGCTCTCGTTTCTTTGATTTYAC
122	Sequencing	3328–3348 (+)	NA	AGCGGGAGCGTTACCATCACG
29	Sequencing	3491–3512 (−)	NA	TGATCCAATTTAATTGCCATCC

[a] GFLV positions are given for strain GHu (EF426852). Hybridization on minus (−) and plus (+) RNA strand is indicated; *NA* not applicable

[b] ArMV positions are given for strain Ta (EF426853)

[c] D: A or G or T; R: A or G; Y: C or T; N: A or C or G or T

of GFLV-GHu RNA2 and an AU-rich region (76%) sequence downstream of the recombination site could account for template switching of the replicase complex during negative strand synthesis of viral RNA2. Similar contexts have been shown to facilitate polymerase pausing at AU-rich folded sequences and association between acceptor and donor RNA strands in other viruses [18, 22, 27, 31].

Gene $2A^{HP}$ of GFLV-GHu is more related to GFLV than ArMV (86–90% vs. 57–64% nt identity), except the ultimate 117 nts at the 3′ end which are closer to ArMV than GFLV (81–86% vs. 68–72% nt identity), suggesting a recombination event between nts 880 and 921 (Fig. 1c). Amino acid alignments confirmed a mosaic structure at the C-terminus of protein $2A^{HP}$, notably with a characteristic ArMV motif LQ/RAAL in positions 220–224 (Fig. 2c). For gene $2A^{HP}$ of ArMV-Ta, the 5′ end is more closely related to GFLV than ArMV (82–87% vs. 44–58% nt identity) whereas the core region and 3′ end are closer to ArMV than GFLV (85–88% vs. 72–77% nt identity). SISCAN analysis of the 3,780 bp long RNA2 of ArMV-Ta predicted interspecies recombination crossover sites between nts 255 and 269 and between 570 and 599 nts (Fig. 1d). The chimeric structure of protein $2A^{HP}$ of ArMV-Ta was confirmed with characteristic GFLV motifs WAAGKNP in positions 14–20, SFRQTVKEDVQ in positions 38–48, RMFSWK in positions 58–63, and WHFVLTGER in positions 77–85 at the N-terminus (Fig. 2a) as well as a conserved ArMV motif LQ/RAAL in position 221–225 at the C-terminus (Fig. 2c). Similar

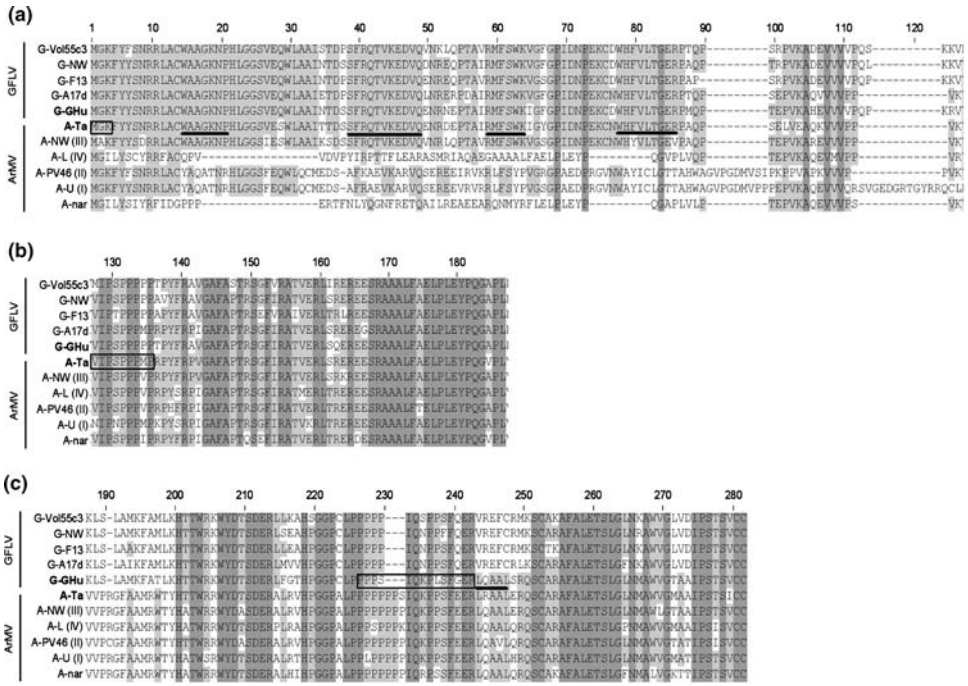

Fig. 2 Alignment of the **a** N-terminus, **b** core region, and **c** C-terminus of protein $2A^{HP}$ of several representative GFLV (GHu, F13, NW, A17d: AY780901, Vol55c3: DQ922673) and ArMV (Ta, L, nar, NW, U, PV46: AY090003) strains for which sequences are available in databases. *White zones* indicate nonsimilar or weakly similar amino acids, clear *grey zones* indicate conservative amino acids, and *dark grey zones* indicate identical amino acids. Residues conserved in **a** GFLV at the N-terminus and in **c** ArMV at the C-terminus are *underlined*. The location of the putative recombination sites in protein $2A^{HP}$ of GFLV-GHu and ArMV-Ta are *boxed*

results were reported previously for gene $2A^{HP}$ of other ArMV strains although no reference to recombination was made [28, 29]. A distinct degree of relatedness between different parts of a protein is an indicator of extensive recombination events in its past evolution, as shown for *Picornaviridae* [23]. Gene $2A^{HP}$ of GFLV and ArMV is the only RNA2 encoded-gene showing variability in size and high amino acid diversity [19, 26, 29], suggesting an evolutionary divergence relative to genes $2B^{MP}$ and $2C^{CP}$.

Further evidence of interspecies recombination in GFLV-GHu was found in gene $2B^{MP}$. This gene is more closely related to ArMV than GFLV (81–83% vs. 78–79% nt sequence identity). Accordingly, protein $2B^{MP}$ is of ArMV origin, except the final 17 C-terminal residues, which are of GFLV origin and conserved among GFLV strains [19, 21, 26, 28]. Within this stretch, the final nine amino acids (EPRLSSTVR) are strictly conserved and critical for a functional interaction between chimeric GFLV/ArMV proteins $2B^{MP}$ and GFLV protein $2C^{CP}$ to achieve systemic plant infection [3]. These data inferred that the natural recombination patterns of GFLV-GHu in gene $2B^{MP}$ mirror almost perfectly the functionality of synthetic chimeric GFLV/ArMV RNA2 constructs. Similar results were reported for a plant DNA virus [15]. Recombination in gene $2B^{MP}$ was previously documented between *Grapevine chrome mosaic virus* (GCMV) and *Tomato black ring virus* (TBRV) [11], two nepoviruses of subgroup B [16]. The high nucleotide sequence homology in gene $2B^{MP}$ of nepoviruses [17] may have favored homologous recombination and the emergence of not only functional chimeric proteins but also functional hybrid viruses between GFLV and ArMV from nepovirus subgroup A, and GCMV and TBRV from nepovirus subgroup B [16].

Interspecies recombination was not detected in gene $2C^{CP}$ of GFLV-GHu and ArMV-Ta. It is possible that genetic exchange within the capsid gene is less likely to generate viable viral offsprings than elsewhere in RNA2. Synthetic GFLV/ArMV recombinants produced in our laboratory confirm this prediction because the exchange of partial gene $2C^{CP}$ segments results mainly in nonfunctional chimeric RNA2 whereas exchange of full-length gene $2C^{CP}$ results in infectious chimeric RNA2 if the biological compatibility between proteins $2B^{MP}$ and $2C^{CP}$ is maintained [2–4]. Important selection pressure notably due to structural constraints imposed on protein $2C^{CP}$ for subunit assembly into particles and vector transmission could account for a limited evolution of gene $2C^{CP}$. A similar differential selection pressure on structural and nonstructural proteins is reported for *Picornaviridae* [30].

No recombination was detected in the 3′ UTR of GFLV-GHu and ArMV-Ta in spite of recombination-promoting signals [18, 22, 27, 31], including a high AU content (67%), a predicted hairpin loop (AAAAAGAKTTTBH(Y/-)T(W/-)TCTTTTT) in positions 3,767–3,788 of GFLV-GHu and 3,739–3,761 of ArMV-Ta RNA2, and a lower nucleotide sequence identity at the 5′ end than at the 3′ end with other GFLV and ArMV strains (28–42% vs. 85–91%). A transfer of the 3′ UTR between GCMV and TBRV was reported in pseudorecombinant isolates consisting of GCMV RNA1 and TBRV RNA2 [10].

The closely related GFLV and ArMV have a great potential for creating hybrid RNA molecules because they can co-exist in grapevines [5] over extended time, i.e. 30–40 years, their RNA2 have moderate to high sequence identity (60–78%), and their replication mechanism is error-prone due to a lack of proofreading mechanism associated with their RNA-dependent RNA polymerase [7, 30].

Our observations with GFLV-GHu and ArMV-Ta are consistent with a differential selection pressure exerted on structural and nonstructural genes with constraints in terms of gene function and co-adaptation within RNA2 that limit interspecies recombination to certain gene segments, as shown for other viruses [6, 15, 23]. Although rare, interspecies recombination can impact the evolution of virus populations and lead to the emergence of new viruses and eventually new diseases [7, 30]. It will be interesting to determine the biological properties and fitness characteristics of GFLV-GHu and ArMV-Ta, and examine if their reduced virulence [9, 13] is associated with any of the interspecies recombination events identified in this study.

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