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Transcriptional mapping of the messenger and leader RNAs of orchid fleck virus, a bisegmented negative-strand RNA virus

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ABSTRACT

The transcriptional strategy of orchid fleck virus (OFV), which has a two-segmented negative-strand RNA genome and resembles plant nucleorhabdoviruses, remains unexplored. In this study, the transcripts of six genes encoded by OFV RNA1 and RNA2 in the poly(A)-enriched RNA fraction from infected plants were molecularly characterized. All of the OFV mRNAs were initiated at a start sequence 3′-UU-5′ with one to three non-viral adenine nucleotides which were added at the 5′ end of each mRNA, whereas their 3′ termini ended with a 5′-AUUUAAA(U/G)AAAA(A)*n*-3′ sequence. We also identified the presence of polyadenylated short transcripts derived from the 3′-terminal leader regions of both genomic and antigenomic strands, providing the first example of plus- and minus-strand leader RNAs in a segmented minus-strand RNA virus. The similarity in the transcriptional strategy between this bipartite OFV and monopartite rhabdoviruses, especially nucleorhabdoviruses (family *Rhabdoviridae*) is additional support for their close relationship.

Introduction

Orchid fleck virus (OFV) is distributed world-wide on several orchid species and transmitted by false spider mites, *Brevipalpus californicus* (Blanchfield et al., 2001; Kondo et al., 2003; Peng et al., 2013). The OFV has unique, non-enveloped bullet-shaped or bacilliform particles about 100–150 nm long and about 40 nm in diameter (Chang et al., 1976; Doi et al., 1977). In infected cells, OFV induces the formation of intranuclear electron-lucent inclusion bodies (viroplasm), which are believed to be the site of viral replication (Kitajima et al., 2001; Kondo et al., 2013). The OFV genome consists of two negative-strand (−)RNAs of 6413 and 6001 nucleotides (nt) (Kondo et al., 2006). In the 3′- and 5′-terminal regions of the OFV genomic RNAs, there are putative extragenic regions called the 3′ leader and 5′ trailer, respectively. RNA1 encodes N (nucleocapsid protein/ORF1), P (candidate for phosphoprotein/ORF2), ORF3 (candidate for cell-to-cell movement protein/ORF3), M (candidate for matrix protein/ORF4) and G (candidate for glycoprotein/ORF5) proteins, whereas RNA2 encodes an RNA-dependent RNA polymerase L (ORF6) (Kondo et al., 2006, 2009). Analysis of amino acid (aa) sequences showed that the N, G and L proteins of OFV have sequence similarity (21% to 38% aa sequence identity) to those of plant nucleorhabdoviruses (genus *Nucleorhabdovirus* in the family *Rhabdoviridae*, order *Mononegavirales*), which have a non-segmented (−)RNA genome. In addition, a phylogenetic analysis based on L core module sequences suggested that OFV and nucleorhabdoviruses form sister taxa that share a common ancestor (Kondo et al., 2006). Based on these properties of OFV, Dietzgen et al. (2014) have recently proposed the creation of the new free-floating genus "Dichorhavirus", which would replace a previous proposal of the genus "Dichorhabdovirus" (Kondo et al., 2006). This newly proposed genus contains OFV and two other *Brevipalpus* mite-transmitted viruses: coffee ringspot virus and *Clerodendron* chlorotic spot virus (Kitajima et al., 2008, 2011).

Rhabdoviruses are ecologically diverse and have a broad range of hosts including plants, invertebrates and vertebrates (Dietzgen et al., 2011). Plant-infecting rhabdoviruses are divided into two genera depending on the site of their replication. Members of the genus *Nucleorhabdovirus* multiply in the nuclei, whereas members of the genus *Cytorhabdovirus* are similar to animal rhabdoviruses, which replicate in the cytoplasm (Dietzgen et al., 2011; Jackson

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et al., 2005). The rhabdoviral (−)RNA genome carries at least five basic genes arranged in the same linear order; 3′-*N*-*P*-*M*-*G*-*L*-5′ (Dietzgen et al., 2011). These genes are sequentially transcribed by a complex of viral N protein, polymerase L and cofactor P into monocistronic mRNAs with 5′-terminal cap-structures and 3′-poly (A) tails, presumably through a "stop-start" mechanism (Easton and Pringle, 2011; Rahmeh et al., 2010; Whelan et al., 2004). The presence of the intergenic and gene stop/start sequences in the genome is a distinct characteristic feature of viruses in the order *Mononegavirales*, including rhabdoviruses (Whelan et al., 2004). The gene junction sequences of rhabdoviruses are broadly conserved and commonly retain three distinct elements: (1) the sequence complementary to the 3′ ends of mRNAs (gene end, GE), which represents the transcription termination/polyadenylation signal, (2) the nontranscribed intergenic sequence (IG) that is not included in either 5′- or 3′-terminal end of mRNAs, and (3) the sequences of the transcription initiation site complementary to the 5′ end of mRNAs (gene start, GS) (Banerjee, 1987; Conzelmann, 1998; Dietzgen et al., 2011). In addition, the 3′-terminal region of the genome is transcribed into a short RNA (plus-strand leader RNA) that is immediately terminated upstream of the *N* gene (Conzelmann, 1998). However, there are only a few examples where minus-strand leader RNA (or short RNA) is transcribed from the 5′-extragenic trailer region of the rhabdoviral antigenomic strand (Leppert and Kolakofsky, 1980; Leppert et al., 1979; Wagner and Jackson, 1997).

All aforementioned ORFs of OFV except for G have been shown to be expressed in OFV-infected plants (Kondo et al., 2009 and H Kondo, unpublished data) possibly from the as-yet-undetected corresponding monocistronic mRNAs. This notion is supported by the fact that OFV has a conserved sequence [3′-UAAAUUUAUU-UUGU(U/A)(G/A)UU-5′] in the gene junction regions (Kondo et al., 2006), although its regulatory role in the transcription of OFV mRNAs has not been characterized. To understand the transcriptional regulation of the OFV genome, we conducted a fine mapping of the transcription initiation and termination sites in the OFV genome by sequencing the 5′- and 3′-terminal sequences of OFV mRNAs. Furthermore, similar analyses showed that the polyadenylated plus- and minus-strand short RNA transcripts are generated from the 3′-terminal regions of both the OFV genomic and antigenomic RNAs. Overall, our results confirm that OFV employs a similar transcription strategy to that of rhabdoviruses and further underline a close relationship between OFV (dichorhavirus) and nucleorhabdoviruses.

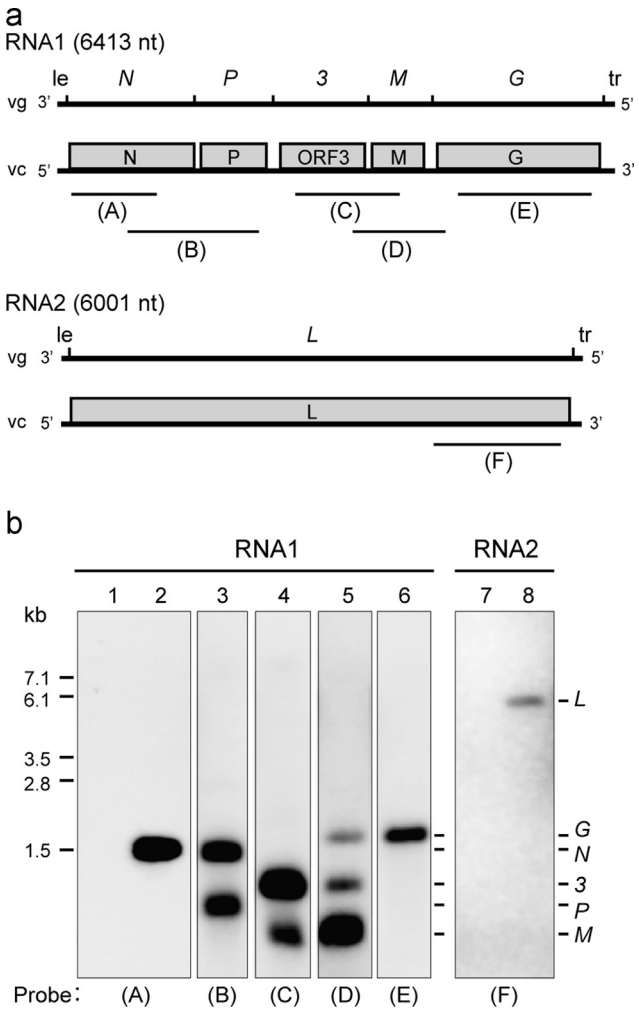

Fig. 1. Accumulation of OFV transcripts in infected plants. (a) A schematic representation of the genomic organization of OFV and the map positions of probes in the genome. Thick horizontal lines vg and vc indicate negative-strand genomic and positive strand anti-genome RNAs, respectively. RNA1 and RNA2 respectively contain five ORFs and a single long ORF in the virus complementary RNA strands (vc). The regions (A)–(F) are covered by the probes used for detection of OFV transcripts. Abbreviations: le, leader region; tr, trailer region. (b) Northern blot analysis of OFV mRNAs. The blots were hybridized with strand-specific DIG-labelled riboprobes (A)–(F) described in (a) (lanes 2–6, 8). Lanes 1 and 7 are RNA samples isolated from mock-inoculated plants. The size of RNA marker (Gibbco BRL) is shown on the left.

Results and Discussion

Detection of OFV mRNAs

To detect OFV mRNAs, we carried out Northern blot analysis using poly(A)-enriched RNA fractions extracted from OFV-infected *Tetragonia expansa* plants. For hybridization, six probes (A to F) that recognize either single or multiple gene coding regions were used (see Fig. 1a for the map positions in the genome). The results showed that all probes consistently detected mRNA species corresponding to the gene coding regions that they cover. Probes (A), (E) and (F) hybridized to mRNAs of the *N*, *G* and *L* genes (~1.4, ~1.7 and ~6.0 kb, respectively) (Fig. 1b, lanes 2, 6 and 8); probe (B) hybridized to mRNAs of the *N* and *P* genes (~1.4 and ~0.8 kb, respectively) (Fig. 1b, lane 3); probe (C) hybridized to mRNAs of the *3* and *M* genes (~1.1 and ~0.6 kb, respectively) (Fig. 1b, lane 4) and probe (D) hybridized to mRNAs of the *3*, *M* and *G* genes (approximately ~1.1, ~0.6 and ~1.7 kb, respectively) (Fig. 1b, lane 5). In a control experiment using non-infected *T. expansa* plants, no signal was obtained by any of the probes (Fig. 1b, lanes 1 and 7, and data not shown). These results suggest that in infected plants, OFV expresses six independent monocistronic mRNAs with A-enriched regions. A novel fly rhabdovirus, *Culex tritaeniorhynchus* rhabdovirus, requires RNA splicing for viral mRNA maturation (Kuwata et al., 2011), but no other rhabdoviruses have been reported to contain an intron in their genome. RT-PCR analysis showed that the DNA fragments (ORF region) amplified from the OFV mRNAs and OFV genomic RNAs were similar in size, thus it appears that OFV genes, like the genes of most rhabdoviruses, do not contain introns (Supplementary Fig. S1).

Sequence analysis of the 5′ and 3′ ends of OFV mRNAs

To determine the 5′ end of OFV mRNAs (see Fig. 2), we performed 5′ RACE (5′ Rapid Amplification of cDNA Ends) analysis. From the sequencing of seven dG-tailed 5′ RACE clones of the OFV N mRNA, three types of 5′-terminal sequences, 5′-(C)nAAAAGUG-3′, 5′-(C)nAAAGUG-3′ and 5′-(C)nAAGUG-3′ were identified (Fig. 2b). By comparing these with the corresponding genomic sequence in

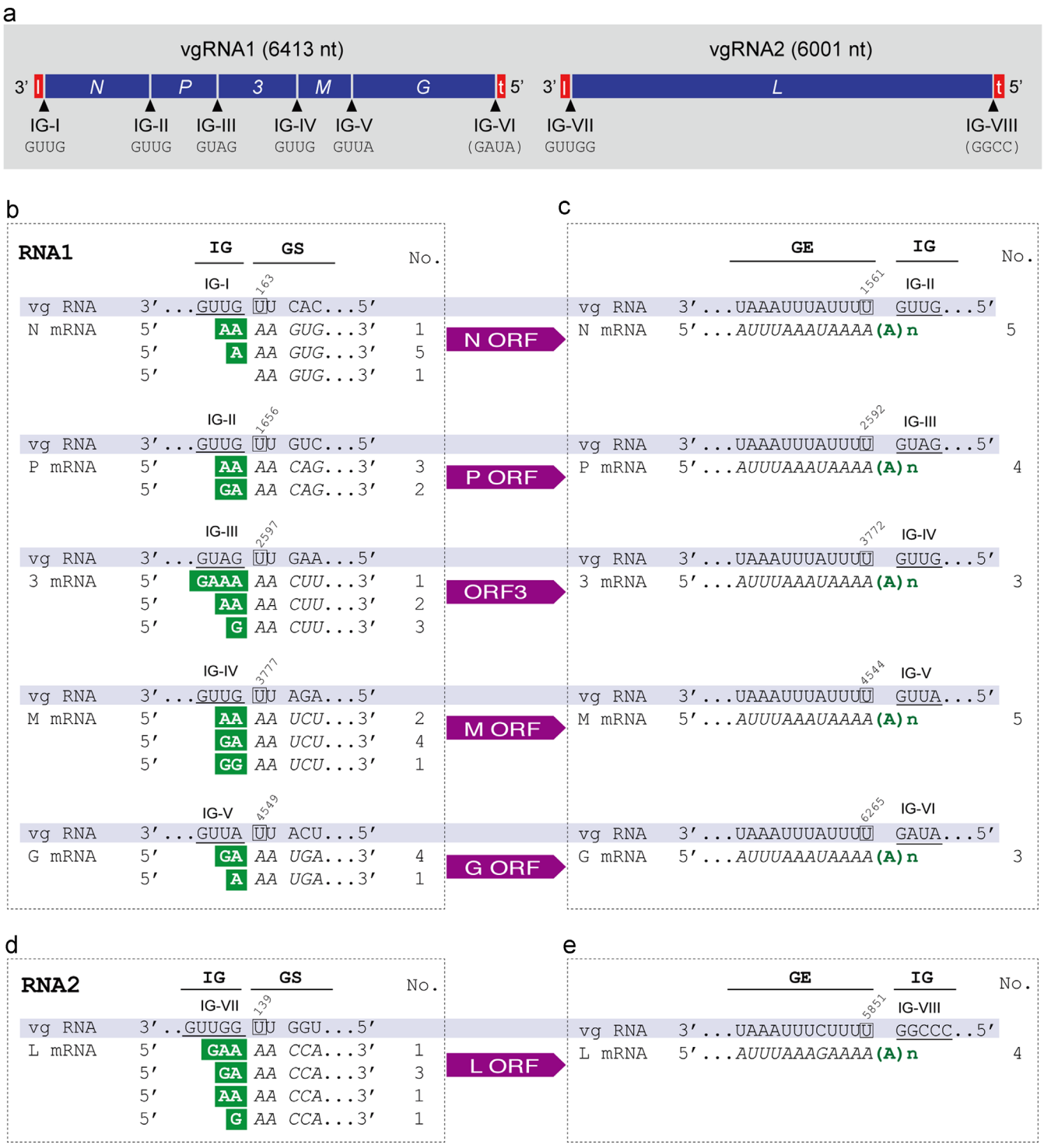

Fig. 2. The 5′- and 3′-terminal sequences of OFV mRNAs. (a) A schematic representation of the OFV genome. Arrowheads indicate the putative gene junction regions and potential junctions between leaders, trailers and adjacent genes. The non-transcribed intergenic sequences (IG) between genes and between leader and first gene (IG-I–IG-V and VII) are specified below (see also Fig. 5a). The 3′ sequences of the 5′-trailer sequence (IG-VI and VIII) are also shown below in parenthesis. ((b)–(e)) The results of the 5′ and 3′ RACE analyses of *N*, *P*, *3*, *M* and *G* mRNAs ((b) and (c)) and *L* mRNA ((d) and (e)), are shown below the complementary sequences in the gene junction regions (written in italic). The regions complementary to the 5′ end of mRNA (gene start: GS), the 3′ end of mRNA (gene end: GE) and their flanking IG sequence (or 5′-trailer RNA) are indicated above the viral genome sequences (vg RNA). The first and last nucleotides of the GS and GE sequences, respectively are marked with rectangles and their nucleotide positions are indicated. IG sequences are underlined. The non-viral sequences present at the 5′ end of viral transcripts are highlighted with boxes. The numbers of the cDNA clone sequenced is shown (column No.).

the gene junction region, it appears that *N* gene transcription was initiated at the genomic sequence 3′-UUCAC-5′ (Fig. 2b, the vgRNA, complementary sequence was underlined in the above sentence) and non-viral extra-nucleotide(s) (AA or A) were present at the 5′ terminus. Similarly, the transcription of other genes (*P*, *3*, *M*, *G* and *L*) was also initiated at the genomic sequence 3′-UUXXX-5′ (X, non-conserved sequence) and their 5′ termini contained extra non-viral nucleotides (Fig. 2b and d). These additional non-viral nucleotides were as follow: *P* (AA or GA), *3* (GAAA, AA or G), *M* (AA, GA or GG), *G* (GA or A) and *L* (GAA, GA, AA or G). These results show

Table 1
Transcription initiation and termination sites of OFV transcripts in the viral genome.

Gene (ORF) or transcription unit	Gene or leader/trailer			UTR		ORF		
	Start (nt)	End (nt)	Length (nt)	5′ UTR (nt)	3′ UTR [a] (nt)	Start (nt)	Stop (nt)	Protein (aa)
RNA1 [b]								
Leader	1	158	158	–[c]	–	–	–	–
N (ORF1)	163	1651	1489	65	71	228	1580	450
P (ORF2)	1656	2592	937	72	151	1728	2441	237
3(ORF3)	2597	3772	1176	85	83	2682 [d]	3689	335
M (ORF4)	3777	4544	768	53	163	3830	4381	183
G(ORF5)	4549	6265	1717	31	57	4580 [d]	6208	543
Trailer	6266	6413	148	–	–	–	–	–
RNA2 [b]								
Leader	1	133	133	–	–	–	–	–
L(ORF6)	139	5851	5713	44	35	183	5816	1877
Trailer	5852	6001	150	–	–	–	–	–

[a] Poly(A) tail was excluded.
[b] Nucleotide position of intergenic sequence: RNA1, 159–162 (GUUG), 1652–1655 (GUUG), 2593–2596 (GUAG), 3773–3776 (GUUG), 4545–4548 (GUUA), RNA2, 134–138 (GUUGG). Trailer regions (RNA1 6266–6413; RNA2 5282–6001) appear to be untranscribed.
[c] –, Absent.
[d] The first transcription initiation codons of the *3* (2577) and *G* (4550) genes are located at the upstream gene or the putative gene junction region (Kondo et al., 2006); therefore, they are probably non-functional, and the second AUG codons at positions 2682 (gene *3*) and 4580 (*G*) might be the actual translation initiation site.

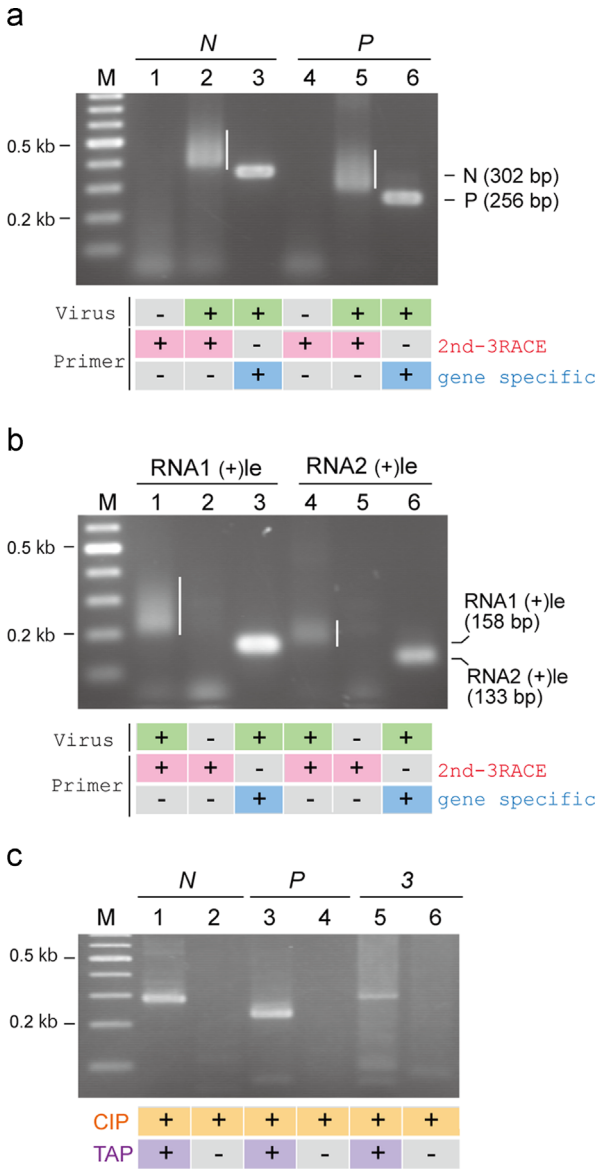

Fig. 3. The RLM-RACE analyses of OFV transcripts. ((a) and (b)) Agarose gel electrophoresis of 3′ RLM-RACE products derived from *N* and *P* mRNAs (a) and plus-strand (+)leader RNAs (b) using the poly(A) RNA-enriched fraction of OFV-infected plants (virus: +) with 2nd3RACE primer or the OFV gene-specific reverse primers. A mock-inoculated sample was used as a template for the negative control (virus: −). Vertical white lines indicate the length distribution of poly(A)-tail amplified by 3′ RLM-RACE. (c) 5′ RACE (Oligo-cap analysis) products derived from *N*, *M* and *3* mRNAs in the CIP-treated (negative control) or CIP plus TAP (a de-capping enzyme)-treated sample. A 100 bp DNA ladder (MBI Fermentas) was used as a size marker (lane M).

that one to four non-viral G or A nucleotides exist at the 5′-terminal end of all OFV mRNAs and that A was the most frequent nucleotide preceding the common starting 5′-AA-3′ dinucleotides, which are complementary to a putative GS sequence 3′-UU-5′ in the gene junction region (Fig. 2b and d).

The 3′-terminal sequences of OFV mRNAs were determined by 3′ RACE method using the poly(A)-enriched RNA fraction isolated from infected plants. Three to five 3′ RACE clones derived from each OFV mRNA were randomly selected and sequenced. The 3′-terminal sequences of OFV RNA1-derived mRNAs (*N*, *P*, *3*, *M* and *G*) were all 5′-AUUUAAAUAAAA(A)*n*-3′, whereas those of RNA2-derived mRNA (*L*) were 5′-AUUUAAAGAAAA(A)*n*-3′ (Fig. 2a, c and e). Thus, there is only one nucleotide change (U/G, underlined) between these two 3′-terminal sequences. The start and end positions in the genome and the estimated length of each OFV transcript excluding the poly(A) tail are summarized in Table 1. To determine the length of the poly(A) tails of OFV mRNAs, we performed 3′ RNA-ligase-mediated (RLM)-RACE analysis (Lin et al., 2012; Sasaya et al., 2004). In this experiment, the poly(A) tail of mRNA was amplified via PCR using gene- and 3′ adaptor-specific primers (Supplementary Fig. S2a). Amplification by PCR using forward primers specific for the *N* or *P* gene and the common reverse primer (2nd3RACE) produced slower-migrating bands than that using reverse primers specific for the 3′-proximal region of each gene (Fig. 3a, compare lanes 2 and 3 or lanes 5 and 6), showing the amplification of the poly(A) tail containing fragments for the former primer set by 3′ RLM-RACE. From the direct sequencing of 3′ RLM-RACE products, at least 147 and 128 A nucleotides were identified at the 3′ end of the *N* and *P* transcripts, respectively (Supplementary Fig. S2b and c). Together, these results suggest that transcription of the six OFV genes uses almost identical termination/polyadenylation signals.

We examined whether the OFV mRNAs have a cap structure at their 5′ ends, using the oligo-capping (5′ RLM-RACE) method (Maruyama and Sugano, 1994; Suzuki et al., 2004). In analyses of the 5′ ends of *N*, *P*, and *3* mRNAs, PCR products with the expected size were generated from samples treated with calf intestinal phosphatase (CIP) and tobacco acidpyrophosphatase (TAP, a de-capping enzyme, selective for capped RNA substrates), whereas no DNA fragment was obtained from samples treated with CIP alone (selective for noncapped RNA substrates) (Fig. 3c). These results suggest that OFV mRNAs have a cap structure at their 5′ termini. From direct sequencing of 5′ RLM-RACE products, two exogenous A nucleotides were identified at the 5′ end of *N* and *P* transcripts, whereas one or two A nucleotides were found in those of gene *3* transcripts (Supplementary Fig. S3). Therefore, an additional G nucleotide shown by the classic 5′ RACE analysis (Fig. 2b and d) is probably due to the copying of a 5′-end cap structure during cDNA synthesis, as is the case for lettuce necrotic yellows virus (LNYV), the type species of the genus *Cytorhabdovirus* (Wetzel et al., 1994).

Similar to that observed for OFV mRNAs, the presence of the exogenous A and also some other nucleotides was identified at the 5′ end of mRNAs of two monocotyl nucleorhabdoviruses; rice yellow stunt virus (RYSV) and maize fine streak virus (MFSV), and A always preceded the common virus-derived nucleotides 5′-AA-3′ of their 5′ mRNA termini (Luo and Fang, 1998; Tsai et al., 2005). This additional nucleotides at the 5′ mRNA terminus has not been observed for animal rhabdoviruses or for other dicot plant rhabdoviruses including sonchus yellow net virus (SYNV; *Nucleorhabdovirus*), potato yellow dowarf virus (PYDV; the type species of the genus *Nucleorhabdovirus*), LNYV and lettuce yellow mottle virus (LYMoV; *Cytorhabdovirus*) (Bandyopadhyay et al., 2010; Heaton et al., 1989; Heim et al., 2008; Wetzel et al., 1994). Recently, Ogino and Banerjee (2007), Ogino et al. (2010) reported an unconventional capping mechanism of the 5′ end of mRNA used by vesicular stomatitis Indiana virus (VSIV, formerly vesicular stomatitis virus Indiana serotype, a prototype of the animal rhabdovirus, *Vesiculovirus*), and also possibly by other mononegaviruses, via RNA:GDP polyribonucleotidyltransferase (PRNTase) and guanine-N7 and ribose 2′-*O* methyltransferase (MTase) activities of the L protein. Intriguingly, a bioinformatic analysis of the L protein of selected animal and plant rhabdoviruses showed that the histidine–arginine (HR) domain required for the PRNTase activity is conserved in all rhabdoviruses, dichorhaviruses and related varicosaviruses, but the MTase motif is absent in both dichorhaviruses and nucleorhabdoviruses (except SYNV) (Supplementary Fig. S4). Thus, it is tempting to speculate that dichorhaviruses and most of nucleorhabdoviruses employ a capping strategy (or enzyme) different from that of the majority of rhabdoviruses and other mononegaviruses. Possibly, the addition of extra-nucleotides (As) at the 5′ ends of the viral mRNAs of OFV and some monocotyl nucleorhabdoviruses might be mediated by the host MTase with other unknown nuclear-enzyme activity and/or unique enzymatic properties of their L proteins. A situation similar to the first alternative was suspected for an MTase-defective mutant (*hr1*) of VSIV with a single amino acid change, D1671V in domain VI of the L protein. This *hr1* mutant is only infectious to particular organisms whose MTase activity (higher in permissive cells) can complement viral MTase deficiency and directly methylate the VSIV mRNA (Grdzelishvili et al., 2005 Horikami and Ferra, 1984), however, it is still unclear whether the *hr1* mRNA possesses additional nucleotides of non-viral origin.

Sequence analysis of plus- and minus-strand leader RNAs of OFV

It is generally known that short non-coding RNAs (leader RNAs) are generated from the 3′ end of rhabdoviral genomes during infections. We investigated whether such RNA species are generated together with the OFV mRNAs (see Fig. 4a). First, the 3′ RACE analysis revealed that polyadenylated leader RNAs [referred to as plus-strand (+)leader RNAs] were synthesized from the OFV 3′-terminal regions of RNA1 and RNA2 with the 3′-terminal sequences: 5′-AUGUAAACAAAA(A)*n*-3′ and 5′-GAUCAUAAAAAA (A)*n*-3′, respectively (Fig. 4b and data not shown). The 3′ RLM-RACE and sequencing analyses further confirmed the polyadenylation of the (+)leader RNAs from RNA1 (Fig. 3b, compare lanes 1 and 3) and RNA2 (Fig. 3b, compare lanes 4 and 6) with at least 134 and 35 A nucleotides were added at the 3′ end, respectively (Supplementary Fig. S2d and e). No 3′ RLM-RACE fragments could be amplified from mock-infected samples (Fig. 3b, lanes 2 and 5). Preliminarily 5′ RACE analysis indicated that (+)leader RNA transcription might initiate from the 3′-end of the OFV genomic RNAs (data not shown). Thus, the length of the (+)leader RNAs of RNA1 and RNA2, excluding the poly(A) tail, was estimated to be 158 nt and 133 nt, respectively (Table 1). Similar to the gene junction sequences, the junctions between the leader and the first gene of RNA1 and RNA2 (*N* and *L* genes, respectively) regions, consist of GE sequences containing U-stretches, four- or five-nucleotides IG intervals and GS sequences for *N* or *L* gene transcription (Fig. 4b).

The (+)leader RNAs of prototypical animal rhabdoviruses, VSIV (47 nt and 48 nt) and rabies virus (RABV; *Lyssavirus*) (56 nt and 58 nt) have been reported to not possess a poly(A) tail (Colonno and Banerjee, 1978; Kurilla et al., 1984). This appears to be consistent with the lack of the consensus polyadenylation signal sequences between the leader and *N* gene coding regions (Conzelmann, 1998) (see Supplementary Fig. S5,A). A similar situation might also exist for some plant cytorhabdoviruses, even though their (+)leader RNAs have not been identified (Wagner et al., 1996) (see also Supplementary Fig. S5),C). For some nucleorhabdoviruses, the existence of polyadenylated (+)leader RNAs of SYNV (approximately 140 nt) and RYSV (undetermined) is consistent with the similarity between the leader-*N* gene junction and other gene junction sequences, namely, the presence of a poly(A) signal (Supplementary Fig. S5, N) (Wagner et al., 1996; Wang et al., 1999; Zuidema et al., 1986).

There are two poly(U) stretches at the 3′ non-coding region of antigenomic OFV RNA2: 3′-ACACUUGUUUUU-5′ and 3′-UAAAUUU-GUUUUU-5′, which are similar to the termination/polyadenylation sequences in the gene junction regions determined in the above experiments (Fig. 4d, RNA2 vcRNA). Therefore, the polyadenylated (−)leader RNAs (same strand with OFV genome) covering the 5′-trailer sequence *in vivo* might be present (see Fig. 4c). To confirm this, we performed 3′ RACE experiments using the Not I/(dT) primer and the OFV-specific primer complementary to the genomic strands in the virus-infected samples (Supplementary Table S1). The small DNA fragments amplified by the 3′ RACE and most RACE clones had a 5′-UGUGAACAAAAA(A)*n*-3′ terminal sequence that was complementary to one of the two poly(U) stretches, thus, the 3′-ACACUUGUUUUU-5′ sequence in the antigenomic strand is considered to function as a transcription termination/polyadenylation site (Fig. 4d, RNA2 vcRNA; one shortened version of GE involved, and data not shown). Likewise, in the 3′ non-coding region of the antigenomic OFV RNA1, two short poly(U) stretches were found and 3′ RACE revealed two alternative terminal sequences for (−)leader RNAs: 5′-GUUUUAUUUAAA(UU)(A)*n*-3′ or 5′-UAUAUAUAAAAA(A)*n*-3′ (Fig. 4d, RNA1 –le). This observation show that polyadenylated (−)leader RNA species of OFV [a possible range of 159–188 nucleotides, excluding the poly (A) tail], are transcribed *in vivo*. The A/U- or U-rich stretches at the 3′-terminus of antigenomic OFV RNAs together with the above-mentioned GE sequences might serve as a polyadenylation signal, possibly because viral polymerases stutter (also known as transcript slippage) at this sequence (Schubert et al., 1980). Various sizes of (−)RNAs [considered as (−)leader RNAs] were identified from the *in vitro* products of the SYNV polymerase (approximately 200 nt and 250 nt) or from the *in vivo* products of the VSIV defective interfering strain (45–48 nt); however, these short RNAs are not polyadenylated (Leppert et al., 1979; Wagner and Jackson, 1997; Wilusz et al., 1983). The sequence following the transcriptional termination sites for OFV (−)leader RNAs is not similar to that of gene junction sequences (the first residue is A or C instead of G) (compare IG in Fig. 4d with Fig. 2c, e and Fig. 4b, and also see below). This might cause transcriptional readthrough and result in alternative transcription ends of (−)leader RNAs.

The function of the (+) and (−)leader RNAs is still unclear, but their unique small size and transcriptional map position in the 3′-terminal region of the viral genome may suggest that leader RNAs play a crucial role in the control of viral replication and/or genome transcription (Banerjee, 1987). In Chandipura virus (CHAV; *Vesiculovirus*), a specific interaction between the P protein and the (+) leader RNA transcript was observed (Basak et al., 2003, 2007).

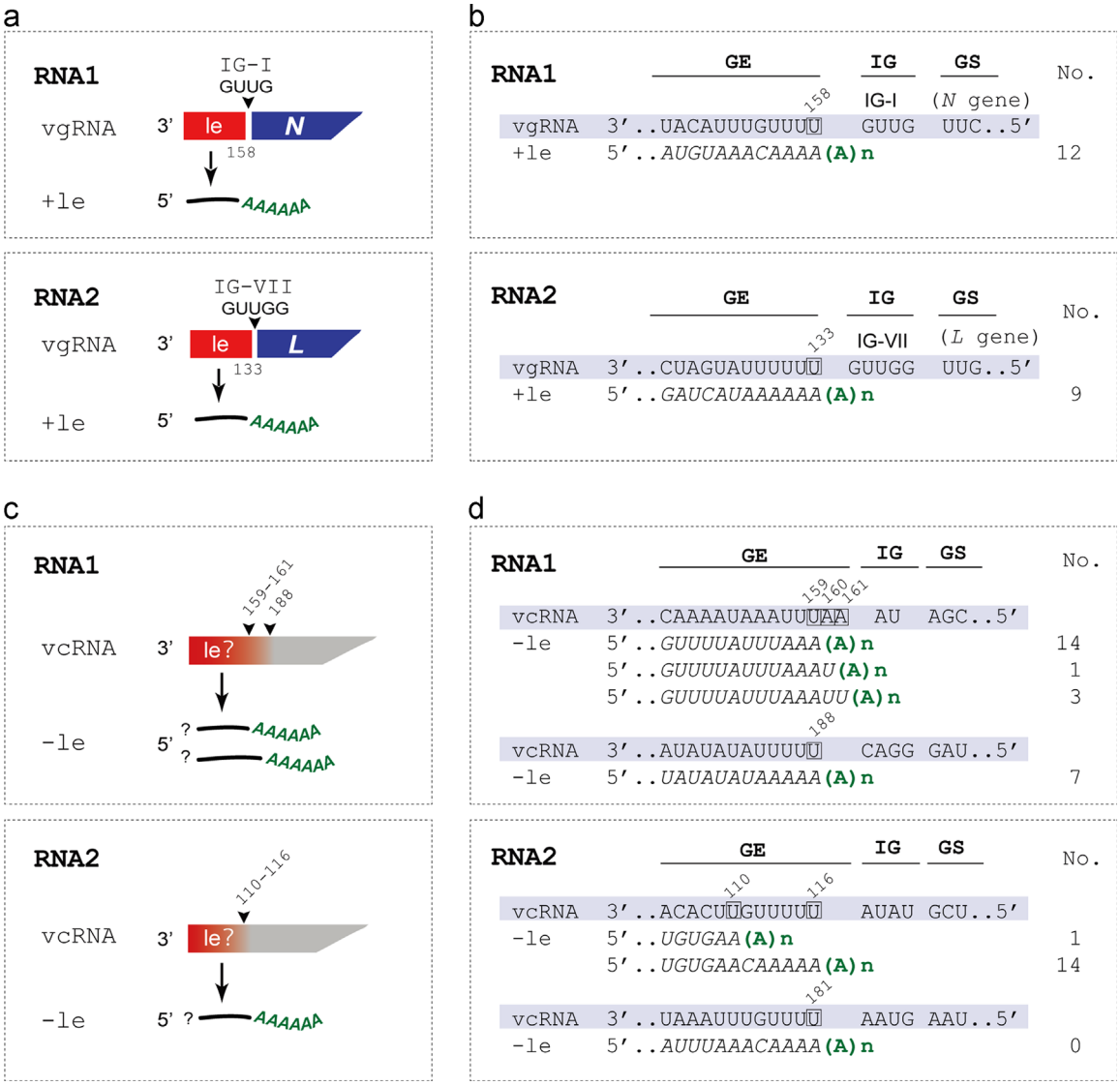

Fig. 4. Leader RNAs of OFV. ((a) and (c)) A graphic illustration depicting the transcription of plus-strand (+) (a) and minus-strand (−)leader RNAs (c) from the 3′-terminal regions of OFV genomic (vg) or antigenomic (vc) RNA. Arrowheads indicate the putative junction regions in the viral genome (IG-I, -VII) or termination/polyadenylation signals in the viral antigenome. Abbreviations: le, leader region; +le, (+)leader RNA; −le, (−)leader RNA. ((b) and (d)) The results of 3′ RACE analysis of the 3′-terminal sequences of the plus-strand (b) and minus-strand (d) RNA transcripts derived from the 3′-leader region of OFV vg or vcRNAs. The regions complementary to the 3′ end of (+)leader or (−)leader RNAs (GE) and their flanking sequences (IG and GS) are indicated above the vg or vcRNA sequences. The last nucleotide of each GE sequence is marked with rectangles and their nucleotide positions are indicated. The numbers of the cDNA clone sequenced is shown (column No.).

It was proposed that this P-leader RNA complex might play a role in switching of L polymerase activity from genomic transcription to replication (Basak et al., 2003; Roy et al., 2013). Furthermore, a recent study on the influenza A virus (a multi-segmented negative-strand RNA virus, *Orthomyxoviridae*)-generated small RNAs (22–27 nt) revealed a unique mechanism for the switching of the viral RNA genome from the transcription to replication modes through the interactions of 5′ end-derived small RNAs with the viral polymerase machinery (Perez et al., 2010). These findings may imply an evolutionarily conserved role for the leader RNA transcripts (including 5′ end small RNAs) in regulating the transcription and replication process of (−)RNA viruses (Roy et al., 2013).

Comparison of the gene junction region sequences of OFV and other negative-strand RNA viruses

The RACE analysis revealed that the conserved 18 nt stretch [3′-UAAAUUUAUUUUGU(U/A)(G/A)UU-5′] in the gene junction sequences of OFV RNA1 consists of a 12 nt GE (3′-UAAAUUUAUUUU-5′), a tetra-nucleotide IG [3′-GU(U/A)(G/A)-5′] and a di-nucleotide GS (3′-UU-5′) sequences (Fig. 5a). Similar semi-conserved sequences are present in the leader -*L* and -*N* junctions, but the GE sequence in the leader -*L* junction contains an additional G nucleotide (3′-GUUGG-5′, underlined) (Fig. 5a). The gene junction sequence of the OFV genome is similar to that of nucleorhabdoviruses, especially MFSV (Fig. 5b). The GS sequence of OFV is identical to that of nucleorhabdoviruses and animal rhabdoviruses (3′-UU-5′), but not to that of cytorhabdoviruses, which is 3′-CU-5′. In contrast, the IG sequence of OFV is not identical to that of any rhabdoviruses, except MFSV. However, the first residue of the IG sequence of OFV and most rhabdoviruses is commonly G (Fig. 5b, IG), suggesting that this residue might be important for the efficient termination of the upstream transcript, as demonstrated by previous reports (Barr et al., 1997; Whelan et al., 2000).

It should be noted that a termination/initiation strategy for mRNA synthesis has been suggested for lettuce big-vein associated virus (LBVaV, floating genus *Varicosavirus*), which has a bipartite (−)RNA genome similar to OFV (Kormelink et al., 2011; Sasaya et al., 2004) (Fig. 5b). However, unlike OFV and other rhabdoviruses, LBVaV lacks

the 3′-terminal regions corresponding to the (+)leader RNAs (Supplementary Fig. S5, V). The differences in the 3′-terminal sequences as well as in their virion morphology (naked rod-like particles) and mode of transmission (soil-borne fungal vector), might reflect a more distant evolutionary relationship between LBVaV and rhabdoviruses than between OFV and rhabdoviruses (Kondo et al., 2006; Sasaya et al., 2001, 2002).

In conclusion, this study strongly suggests that the transcription strategies of OFV and plant nucleorhabdoviruses are very similar, with both having the well-conserved gene-junction sequences and generating monocistronic mRNAs and polyadenylated (+)leader RNAs. These data provide further evidence that OFV and nucleorhabdoviruses might have evolved from a common ancestor, even though OFV has a bipartite genome. Recently, a reverse genetic system for a nucleorhabdovirus (SYNV) has been reported (Ganesan et al., 2013). This newly developed system will facilitate further studies on the transcriptional regulation of plant (−)RNA viruses, especially OFV and nucleorhabdoviruses, which have adapted to the nuclear environment for replication, assembly and transcription.

Methods

Virus propagation

The So isolate of OFV (GenBank accession number AB244417 and AB244418) (Kondo et al., 1995, 2006) was propagated in *T. expansa* plants grown in the greenhouses. Locally infected leaves of *T. expansa* were harvested approximately 3–4 weeks after inoculation and stored at −80 °C until use.

RNA extraction and Northern blot analysis

For detection of OFV mRNAs, the poly(A)-enriched RNA fraction was extracted from OFV-infected *T. expansa* leaves as described previously (Andika et al., 2006). The poly(A)-enriched RNA fractionation was separated on 1.4% agarose horizontal submarine gels in MOPS/EDTA buffer (pH 7.0) under denaturing conditions and then transferred onto a nylon membrane (Hybond-N$^+$; Amersham). The RNA gel blots were hybridized with the strand-specific digoxigenin (DIG)-labelled riboprobes (Roche) transcribed from six cDNA clones (Kondo et al., 2006) (Fig. 1a). The strand-specific riboprobes were synthesized by *in vitro* run-off transcription with T7 or SP6 polymerase as described previously (Kondo et al., 2006). The poly(A)-enriched RNA fraction isolated from healthy *T. expansa* leaves was used as a control.

3′ RACE of OFV transcripts

The 3′ RACE procedure was performed as described by Fang et al. (1994). The poly(A)-enriched RNA fraction isolated from OFV-infected plants was subjected for cDNA synthesis using M-MLV reverse transcriptase (Invitrogen) with a Not I/(dT) primer [5′-AACTGGAAGAATTCGCGGCCGCAGGAA$(T)_{17}$V-3′]. The resulting cDNAs were used for PCR amplification with the Not I/(dT) primer and the OFV-specific primer complementary to the 3′-proximal end of each mRNA (Supplementary Table S1). The DNA fragments were cloned into pGEM-T Vector (Promega) and the plasmids were introduced into competent *Escherichia coli* strain DH5 alpha (Takara Bio). Plasmid DNA was sequenced by the dideoxynucleotide chain-termination method using the DNA sequencer 377 or 3100 models with a PRISM Dye Dideoxi Terminator Cycle Sequencing kit (Applied Biosystems). The resulting sequence trace files obtained from 3′ RACE and other methods (see below) were analyzed using Auto Assembler™ DNA Sequence Assembly Software (Applied Biosystems) or 4Peaks v1.7.2 software (Mek&Tosj; http://nucleobytes.com/index.php/4peaks).

a

OFV RNA1	GE	IG	GS
3′-le/ N	UACAUUUGUUUU	GUUG	UUC
N / P	UAAAUUUAUUUU	GUUG	UUG
P / 3	UAAAUUUAUUUU	GUAG	UUG
3 / M	UAAAUUUAUUUU	GUUG	UUA
M / G	UAAAUUUAUUUU	GUUA	UUA
G /5′-tr	UAAAUUUAUUUU	GAUA	GAG
OFV RNA2	**GE**	**IG**	**GS**
3′-le/ L	CUAGUAUUUUUU	GUUGG	UUG
L /5′-tr	UAAAUUUCUUUU	GGCCC	AUA

b

		GE	IG	GS
D	OFV* RNA1	UAAAUUUAUUUU	GU UG AA	UUN
	OFV* RNA2	UAAAUUUCUUUU	GUUGG	UUG
N	MFSV*	UUUAUUUU	GUAG	UUG
	RYSV*	AUUAUUUUU	GGG	UUGUG
	MMV*/TaVCV*	AAUUCUUUUU	GGG	UUG
	PYDV/EMDV	AAUUAUUUUU	GGG	UUG
	SYNV	AUUCUUUUU	GG	UUG
C	LNYV	CUAAAUUCUUUU	GN(n)	CUU
	LYMoV	AUUCUUUU	GN(n)	CUN
	NCMV*	AUUCUUUUU	GACU	CUAGU
A	VSIV	AUACUUUUUUU	G C U	UUGUC
	RABV	ACUUUUUUU	CN(n)	UUGU
V	LBVaV Type A	UAUNCAUUUUUUU	G	CUCU
	LBVaV Type B	AUAAUCUUUUUU	G	CUCU

Fig. 5. Gene junction sequences in the genomes of OFV and related (−)RNA viruses. (a) Alignment of gene junction sequences in the OFV genome. The sequences between OFV genes or genes and leader/trailer on the genomic RNA strands (3′-to-5′ orientation) are compared. (b) Consensus sequences of gene junction regions in rhabdoviral genomes. D, dichorhavirus; N, plant nucleorhabdoviruses; C, plant cytorhabdoviruses; A, animal rhabdoviruses (vesiculovirus and lyssavirus); V, varicosavirus. Abbreviations: 3′-le, 3′-leader region; 5′-tr, 5′-trailer region; (N)*n*, variable number of nucleotides. Asterisks mark the monocot-infecting rhabdoviruses (including OFV). The gene junction sequences compared here are derived from previous reports (Bandyopadhyay et al., 2010; Heaton et al., 1989; Heim et al., 2008; Sasaya et al., 2004; Tsai et al., 2005; Wetzel et al., 1994). The GS and GE sequences of EMDV, MMV, TaVCV and NCMV (plant rhabdoviruses) have been predicted from sequence homologies.

5′ RACE of OFV transcripts

The 5′ RACE was performed using the 5′ RACE system (Gibco-BRL) according to the manufacturer's instruction with the poly(A)-enriched RNA fraction isolated from OFV-infected plants as a template (Luo and Fang, 1998; Wetzel et al., 1994). The cDNAs were synthesized with reverse primers specific for the 5′-proximal sequence of each OFV mRNA (Supplementary Table S1), and then purified, and dG-tailed with terminal deoxynucleotidyl transferase (Toyobo Co.). PCR amplification was performed using the primer 5′-GTGAAGCTTGAATT$(C)_{17}$-3′ and the nested primer of each OFV mRNA (Supplementary Table S1). The PCR products were cloned into the pGEM-T vector. To obtain a representative set of sequences, five to seven clones were randomly selected and sequenced.

RLM-RACE of OFV transcripts

The 5′ RLM-RACE (Oligo-capping analysis) was performed using the Ambion FirstChoice™ RLM-RACE kit as previously described

(Maruyama and Sugano, 1994; Suzuki et al., 2004). The poly(A)-enriched RNA fractions from OFV-infected plants were sequentially treated with calf intestinal phosphatase (CIP) and tobacco acidpyrophosphatase (TAP, a de-capping enzyme) prior to ligation to a synthesized RNA oligonucleotide adaptor (supplied by the kit) and cDNA synthesis with random decamers. After two rounds of nested PCR amplification using primers specific for the selected OFV mRNAs (Supplementary Table S1), the PCR products were sequenced by direct sequencing.

To estimate the length of the poly(A) tail, we used 3′ RLM-RACE as previously described (Lin et al., 2012; Sasaya et al., 2004) (see Supplementary Fig. S2). The poly(A)-enriched RNA fraction from OFV-infected plants was ligated to the 5′-end phosphorylated oligodeoxynucleotide (5′-PO_4-CAATACCTTCTGACCATGCAGTGA-CAGTCAGCATG-3′) (3RACE-adaptor) using T4 RNA ligase (Takara Bio). The ligated sample was used as a template for first-strand cDNA synthesis with the first 3RACE primer (5′-CATGCTGACTGA-CACTGCAT-3′), the sequence of which was complementary to the 3′ half of the 3RACE-adaptor. The resulting reaction products were then used for the PCR amplifications with the second 3RACE primer (5′-TCGATGGTCAGAAGGTATTG-3′) and each of OFV mRNA-specific forward primer (Supplementary Table S1). The DNA fragments were gel electrophoresed and sequenced by direct sequencing methods.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.01.007.

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