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Tandem leader proteases of Grapevine leafroll-associated virus-2: Host-specific functions in the infection cycle

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Introduction

Diverse RNA and DNA viruses of eukaryotes rely on proteases for the needs of genome expression and virus–host interactions (Barrett and Rawlings, 2001; Dougherty and Semler, 1993; Koonin and Dolja, 1993; Lindner, 2007). Several viruses in the genus Closterovirus including Grapevine leafroll-associated virus-2 (GLRaV-2), encode a tandem of papain-like leader proteases (L1 and L2) whose functional profiles remained largely uncharacterized. We generated a series of the full-length, reporter-tagged, clones of GLRaV-2 and demonstrated that they are systemically infectious upon agroinfection of an experimental host plant Nicotiana benthamiana. These clones and corresponding minireplicon derivatives were used to address L1 and L2 functions in GLRaV-2 infection cycle. It was found that the deletion of genome region encoding the entire L1–L2 tandem resulted in a ~100-fold reduction in minireplicon RNA accumulation. Five-fold reduction in RNA level was observed upon deletion of L1 coding region. In contrast, deletion of L2 coding region did not affect RNA accumulation. It was also found that the autocatalytic cleavage by L2 but not by L1 is essential for genome replication. Analysis of the corresponding mutants in the context of N. benthamiana infection launched by the full-length GLRaV-2 clone revealed that L1 or its coding region is essential for virus ability to establish infection, while L2 plays an accessory role in the viral systemic transport. Strikingly, when tagged minireplicon variants were used for the leaf agroinfiltration of the GLRaV-2 natural host, Vitis vinifera, deletion of either L1 or L2 resulted in a dramatic reduction of minireplicon ability to establish infection attesting to a host-specific requirement for tandem proteases in the virus infection cycle.

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Virus host, grapevine, compared to an experimental herbaceous host, the interaction of L1 and L2 into virus infection is much more critical in a natural inoculated cells and systemic transport. Strikingly, overall contributions of L1 and L2 into virus infection is much more critical in a natural virus host, grapevine, compared to an experimental herbaceous host, Nicotiana benthamiana, suggesting that the tandem of leader proteases evolves to facilitate an expansion of the Closterovirus host range.

**Results**

Generation of GLRaV-2 replicons tagged by insertion of the fluorescent, enzymatic, and epitope reporters

Because the infectious cDNA clones provide powerful tools for investigation of the RNA virus gene functions (Peremyslov and Dolja, 2007; Pogue et al., 2002), we generated such clone for GLRaV-2 to determine functional profiles of L1 and L2. The entire, 16,486 nt-long GLRaV-2 genome was sequenced (Genbank accession no. FJ436234) and compared to the other isolates of this virus to reveal the closest relationship (99.6% nt identity) to the isolate 94/970 (Meng et al., 2005). The initial full-length clone was assembled using a binary vector and primarily conventional cDNA cloning to avoid introduction of the PCR-generated mutations, and sequenced to confirm its correspondence to the consensus nucleotide sequence of the viral genome. To facilitate launching of viral infection by agroinoculation, 35S RNA polymerase promoter of Cauliflower mosaic virus (CaMV) and a ribozyme sequence were inserted upstream and downstream of the GLRaV-2 sequence, respectively, as described earlier for BYV (Peremyslov and Dolja, 2007; Prokhnevsky et al., 2002).

The resulting full-length GLRaV-2 clone was further modified to accommodate a reporter gene expression cassette immediately upstream of the CP open reading frame. This cassette contained GFP open reading frame followed by the BYV CP subgenomic RNA promoter. As a result, the latter promoter directed expression of the GLRaV-2 CP, while the authentic GLRaV-2 CP promoter expressed the GFP reporter. This tagged full-length GLRaV-2 replicon was designated LR-GFP (Fig. 1A, middle diagram).

As was shown for BYV, deletion of the genes that are not required for the viral RNA amplification in the individual cells facilitates experimentation with the remaining genes that code for the leader protease, RNA replicase and RNAi suppressor (Chiba et al., 2006; Hagiwara et al., 1999; Peng and Dolja, 2000). In the case of GLRaV-2, such minireplicon was generated by deletion of the gene block spanning genome region from p6 to p19 open reading frames and retention of the reporter gene. The reporter expression cassette was further modified to express a fusion of GFP with β-glucuronidase to result in the tagged GLRaV-2 minireplicon designated mLR-GFP/GUS (Fig. 1A, bottom diagram).

To permit immunochemical detection of L2, both LR-GFP and mLR-GFP/GUS were modified by an insertion of the triple hemagglutinin epitope (HA) tag into the N-terminal domain of L2 (L2p6 in the Figs. 1B and 2). Infectivity of the full-length and minireplicon variants was tested using leaf agroinfiltration of N. benthamiana, a systemic experimental host of GLRaV-2 (Goszczynski et al., 1996). For mLR-GFP/GUS, such agroinfiltration resulted in minireplicon RNA accumulation and efficient expression of the fluorescent and enzymatically-active GFP/GUS reporter in the initially inoculated cells (Fig. 1B and data not shown). Importantly, the level of GUS activity in a HA-tagged variant was ~85% of that in the original mLR-GFP/GUS. Because this modest reduction was only marginally statistically significant (p value ~0.001), we concluded that the insertion of HA tag into L2 did not significantly affect viral genome amplification. Our attempts to insert an HA tag into L1 resulted in non-infectious replicons and were abandoned.

Both the original and HA-tagged variants of the full-length LR-GFP were systemically infectious in N. benthamiana; typical symptoms of the viral infection and GFP fluorescence were detected in the upper non-inoculated leaves by 3 weeks post-agroinfiltration of the bottom leaves (Fig. 1A and data not shown). Therefore, we successfully generated a series of the infectious tagged GLRaV-2 replicons that can
Fig. 1. (A) Diagrams of GLRaV-2 genome (top); full-length, GFP-tagged cDNA clone of GLRaV-2 (LR-GFP, middle) and GFP/GUS-tagged minireplicon (mLR-GFP/GUS, bottom). Functions of viral genes are shown above and below the diagram. L1 and L2, leader proteases; MET, HEL, and POL, methyltransferase, RNA helicase, and RNA polymerase domains, respectively; p6, 6-kDa movement protein; Hsp70h, Hsp70 homolog; pδ3, 63-kDa protein; CPm, minor capsid protein; CP, major capsid protein; p19, 19-kDa protein; p24, 24-kDa protein; GFP, green fluorescent protein; GFP/GUS, GFP fusion with β-glucuronidase; 35S, 35S RNA polymerase promoter of Cauliflower mosaic virus; RZ, ribozyme. (B) Diagrams of the mutations introduced into L1 and L2 (left) and corresponding phenotypes indicating processing activity, levels of GUS expression, and systemic infectivity. L2HA, insertion of the triple hemagglutinin epitope into L2 (HA, white strip); M1, replacement of the L1 catalytic Cys residue with Ala (C493A); M2, replacement of the L2 catalytic Cys residue with Ala (C767A); ΔL1, deletion of the entire L1 coding region; ΔL2, deletion of the entire L2 coding region; ΔL1/2, deletion of the entire L1 and L2 coding regions; ΔNT1, deletion of the region encoding N-terminal, non-proteolytic domain of L1; ΔPro1, deletion of the region encoding C-terminal, proteolytic domain of L1.

Fig. 2. L1- and L2-mediated processing of the N-terminal part of the GLRaV-2 polyprotein generated in vitro. Lanes correspond to mutant variants shown in the Fig. 1B except for NC, no RNA control. (A) Immunoblot analysis of the in vitro translation products using HA-specific antibody (α-HA) to detect L2. Arrows at the right mark the following processing products: L1–L2, unprocessed fusion of L1 and L2; L2+, L2 fused to a part of MET; L2HA, fully processed, HA-tagged L2. Numbers at the left show the mol. mass (kDa) of the protein markers. (B) Analysis of the 35S-methionine-labeled in vitro translation products. Designations are as in (A).
be launched to *N. benthamiana* and used to address L1 and L2 functions in the viral infection cycle.

**Mutation analysis of the L1 and L2 functions in protein processing and RNA accumulation in the initially inoculated cells of *N. benthamiana***

To address L1 and L2 functions, seven point mutations and deletions were introduced into corresponding coding region (Fig. 1B). In particular, to determine the requirements for the self-processing at the respective C-termini of L1 and L2, the predicted catalytic cysteine residues of the each protease (CyS493 and CyS767) (Peng et al., 2001) were replaced by alanine residues to result in M1 and M2 variants, respectively (Fig. 1B). The processing competence of each variant was investigated using *in vitro* translation of the capped mRNAs encompassing the 5′-terminal untranslated region, the entire L1–L2 open reading frame and a short downstream region that encodes a part of the methyltransferase domain (Fig. 1B). The resulting translation products were analyzed using either immunoblotting and HA-specific antibody (Fig. 2A) or 35S-methionine labeling (Fig. 2B). As expected, a tagged non-mutant variant produced single HA-positive band corresponding to the fully-processed, HA-tagged L2 (Fig. 1B and Fig. 2A, lane L2ha) and, in addition, isoIope-labeled, fully processed L1 (Figs. 1B and 2B, lane L1ha).

In contrast, translation of the M1 variant resulted in accumulation of a single major product corresponding to a L1–L2 fusion (Fig. 1B; Figs. 2A and B, lanes M1). Analogously, mutational replacement of the predicted catalytic cysteine in L2 resulted in a lack of L2 self-processing, but did not affect the autocatalytic release of L1 (Fig. 1B; Figs. 2A and B, lanes M2). Because mutation of the predicted active site residues did inactivate autoproteolysis by each leader protease, we concluded that L1 and L2 are indeed the catalytically active, papain-like proteases.

To determine if the processing by L1 and L2 is required for viral RNA amplification, we used M1 and M2 variants of mLR-GFP/GUS to agroinfiltrate *N. benthamiana* leaves and to determine the resulting GUS activity. As shown previously for BYV minireplicon, GUS activity provides a reliable surrogate marker for measuring accumulation of the viral RNAs in the infected cells (Peng and Dolja, 2000). Using this marker, we found that, unexpectedly, inactivation of the L1 cleavage resulted in more efficient GUS expression; almost 2-fold increase in GUS activity was detected in three independent experiments (Fig. 1B). The former of these minireplicon variants exhibited ~3-fold reduction in accumulation of GUS, while the latter produced even more GUS than the parental variant (Fig. 1B). These data indicated that the non-proteolytic rather than the protease domain of L1 provides a major contribution to viral RNA accumulation in *N. benthamiana* cells. It should be emphasized that the observed requirement for NTD1 for optimal RNA accumulation can reflect either a role of a protein domain, or of a corresponding coding region at the RNA level, or both.

**Roles of L1 and L2 in the virion infectivity and systemic spread of GLRaV-2 in *N. benthamiana***

To define the potential functions of L1 and L2 in the viral cell-to-cell movement and long-distance transport, the ΔL1 and ΔL2 deletions were introduced into the background of the full-length LR-GFP variant. Following agroinfiltration, viroses were isolated from the inoculated leaves and the viroid suspensions of the equal concentrations were used to manually inoculate *N. benthamiana* leaves and to characterize the resulting infection foci using GFP fluorescence at 8 days post-inoculation (Peremyslov et al., 1999). For the parental LR-GFP variant, inoculation yielded 9.9±5.6 infection foci per leaf with the mean diameter of 4.3±1.4 cells. Very similar results (8.2±4.8 foci per leaf; mean diameter of 4.1±1.3 cells) were obtained for the LR-GFPΔL2 variant indicating that L2 is dispensable for both the infectivity and cell-to-cell movement of the GLRaV-2 in *N. benthamiana*. Strikingly, deletion of L1 resulted in a dramatic, 25-fold reduction in the specific infectivity of the LR-GFPΔL1 variant (0.4 cells per leaf). Furthermore, the very few detected GFP-positive foci were unicellular suggesting that either L1 or the corresponding coding region is essential for the virion ability to establish infection in the initially inoculated cells and to move to the neighboring cells.

To determine if L1 and L2 are involved in the systemic transport of GLRaV-2, we tested six replication-competent variants in a context of the full-length LR-GFP launched to *N. benthamiana* plants using agroinfiltration. The inoculated plants were screened for the symptom, GFP, and CP expression at 3, 4, and 5 weeks post-inoculation. Interestingly, most or all of the plants inoculated with M1 and ΔL2 variants became systemically infected indicating that neither L2 nor the cleavage between L1 and L2 is required for the long-distance transport of the virus in *N. benthamiana* (Figs. 1B and 3A). Similar competence for the systemic spread was found in the case of ΔPro1 mutant. However, deletion of the L1 or its N-terminal domain resulted in complete loss of the replicon ability to establish systemic infection (Figs. 1B and 3C).

Observation of the systemically infected leaves revealed apparent differences in the GFP accumulation between the experimental variants (Fig. 1A). To further assess these differences, we evaluated GLRaV-2 CP accumulation in the non-inoculated upper leaves. Conspicuously, it was found that only the ΔPro1 mutant accumulated to the levels comparable to those of the parental L2Ha variant (Fig. 3B). The remaining two mutant variants, M1, and especially ΔL2, each accumulated to the significantly lower levels than that of the parental LR-GFP variant both at 3 and 4 weeks post-inoculation (Fig. 3B). Collectively, these results demonstrated that the L2 per se, and the cleavage between L1 and L2 are required for optimal systemic spread of GLRaV-2 in *N. benthamiana*. In addition, L1 and its N-terminal non-proteolytic domain or the corresponding coding regions are essential for the ability of GLRaV-2 to establish systemic infection since neither GFP nor viral CP were detectable in the upper leaves of the plants inoculated with the ΔNTD1 or ΔL1 variants even at 5 weeks post-inoculation (Fig. 3C and D).

In BYV, both p20 and L-Pro are involved into viral systemic spread (Peng et al., 2003; Prokhnevsky et al., 2002). Of these, p20 is an integral component of the virion tail (Peremyslov et al., 2004a, 2004b).
while it is not known if L-Pro is present in the virions due to unavailability of the L-Pro-specific antibody. Because we generated functional, HA-tagged variant of L2, we were interested to determine if this protease is associated with the virions. The GLRaV-2 virions were isolated from systemically infected leaves and fractionated using sucrose density gradient. The peak of virions was detected in fractions 12–14 using CP-specific antibody (Fig. 4). However, the immunoblot analysis of the same gradient fractions using HA-specific antibodies showed the peak of L2 in fractions 15–17, suggesting that L2 present in the virion suspension is not physically associated with the virions (Fig. 4). This conclusion was further supported by the immunogold-specific electron microscopy used to detect HA epitopes present in L2. Indeed, only very weak gold labeling was found in the fractions 12–14 that contained bulk of the virions. Furthermore, a few gold microspheres detected in these fractions were not directly associated with the virions (Fig. 4, upper inset). The L2 peak fractions 15–17 contained much larger numbers of gold microspheres, but virtually no virions (Fig. 4, bottom inset) suggesting that L2 is not directly associated with GLRaV-2 virions.

L1 and L2 are critical for minireplicon infection of the Vitis vinifera

It is generally accepted that N. benthamiana is, perhaps, the most promiscuous host for a great variety of plant viruses (Goodin et al., 2008). To determine if the seemingly non-essential and largely redundant roles played by L1 and L2 in GLRaV-2 infection in this experimental host do faithfully reflect their roles in a grapevine infection, we agroinfiltrated four minireplicon variants to V. vinifera (Grenache) leaves (Table 1). At 8 days post-inoculation with the parental mL-GFP/GUS variant, up to ~300 unicellular, GFP-
flourescent infection foci per leaf were observed. Strikingly, infiltration using ΔL1 and ΔL2 variants resulted in a ~100-fold and ~7-fold reduction in the foci numbers, respectively, indicating that each of the leader proteases is required for the ability of minireplicon to establish infection in the initially inoculated grapevine leaf cells (Table 1). However, similar to what was observed in N. benthamiana, infectivity of the M1 variant was not significantly different from that of the parental variant.

Remarkably, measurements of GUS activity in the infiltrated leaves correlated well with the data on the numbers of the infected cells (Table 1) suggesting that the principal function of the leader proteases is to aid the establishment of viral infection rather than to increase accumulation of viral RNA in the infected cells. Because the effects of L1 and L2 deletion in V. vinifera were much more dramatic compared to those in N. benthamiana, we suggest that each protease provides a significant and specific contribution into establishment of GLRaV-2 infection in its natural host plant. It should be noted, however, that our experimental design is based on agroinfiltration and targets the leaf epidermal and mesophyll cells whereas in the natural grapevine infections, GLRaV-2 is generally limited to the phloem.

Discussion

Previous work suggested that the tandems of the leader proteases in different clustroviruses emerged via independent gene duplication events (Peng et al., 2001). It was also proposed that the evolution of L1 and L2 involved functional divergence (neofunctionalization) that resulted in the erosion of sequence similarity in the corresponding N-terminal domains. By and large, an experimental analysis presented here corroborated these assumptions and allowed us to delineate three major functions of L1 and L2 in the GLRaV-2 infection cycle: i) polyprotein processing; ii) virus accumulation in the initially infected cells; iii) systemic transport of the infection.

In particular, we found that both L1 and L2 are the active proteases with the conserved catalytic cysteines (Figs. 1B and 2). Similar to BYV, the cleavage upstream from the methyltransferase domain of the viral polyprotein is essential for GLRaV-2 viability (Fig. 1B) (Peremyslov et al., 1998). Surprisingly, although L1 does cleave at its own C-terminus both in vitro (Fig. 2) and in vivo (Fig. 4), neither this cleavage nor the L1 protease domain per se are essential for systemic infection in N. benthamiana as evident from the phenotypes of M1 and ΔPro1 variants (Figs. 1B and 3). However, slower virus accumulation in the non-inoculated leaves in these mutants (Figs. 3A and B) suggests that the L1-mediated cleavage is required for the optimal development of systemic infection.

Our deletion analysis indicated that L1 and L2 play partially overlapping roles in the viral RNA accumulation. When viral minireplicon was launched by agroinfiltration, complete deletion of L1 resulted in a ~5-fold reduction in RNA levels. Similar effect was observed upon deletion of the N-terminal domain of L1 indicating its principal role in L1 function (Fig. 1B). Although the deletion of L2 did not affect RNA accumulation, combined deletion of L1 and L2 resulted in a virtually nonviable minireplicon attesting to a significant contribution of L2 into viral infectivity in the absence of L1.

Interestingly, when isolated virions containing full-length genome were used for plant inoculation, the infectivity and cell-to-cell movement of the ΔL2 variant were indistinguishable from those of the parental variant, while the virions of ΔL1 variant have lost their infectivity. Because both in BYV and in CTV, the 5'-terminal, ~700 nucleotide-long region is involved in the assembly of virion tails (Peremyslov et al., 2004a, 2004b; Satyanarayana et al., 2004), we assume that this region plays a similar role in GLRaV-2. If so, the deletion of L1 coding region could affect virion structure, stability, and infectivity. Therefore, we propose that in addition to L1 involvement in RNA accumulation, the corresponding coding region also functions at the RNA level to facilitate formation of the tailed virions capable of the local and systemic transport.

In accord with the latter assumption, ΔL1 and ΔNTD1 mutants were unable to establish a systemic infection upon agroinfiltration using full-length replicons (Fig. 3C). In contrast, deletion of the protease domain in ΔPro1 variant did not affect systemic infectivity indicating that virion tail formation was likely unaffected. The deletion of L2 resulted in a systemically infectious ΔL2 variant, which, however, exhibited slower accumulation in the upper leaves (Figs. 2A and B). This result indicated that L2 is required for the efficient systemic spread of GLRaV-2 in N. benthamiana.

Perhaps the most significant results of this study were obtained when the minireplicon variants were agroinoculated to the grapevine leaves. In a sharp contrast to N. benthamiana where L2 was superfluous for minireplicon infectivity, ΔL2 variant exhibited a ~10-fold reduction in RNA accumulation upon agroinfiltration into V. vinifera leaves (Table 1). The specific infectivity of the ΔL2 variant measured as a mean number of the GFP-fluorescent infected cells per leaf was also reduced ~10-fold. This correlation in the RNA accumulation and the numbers of infected cells points to a role of L2 in the virus invasiveness, i.e., the ability to establish infection in the inoculated cells. Such role in GLRaV-2 invasiveness in grapevine is even more dramatic in the case of L1 where L1 deletion resulted in ~100-fold reduction in the RNA accumulation and specific infectivity (Table 1). We concluded that both L1 and L2 are essential for the ability of GLRaV-2 to establish infection in the initially inoculated grapevine cells, at least upon the conditions of agroinfection.

Because M1 variant was identical to the parental minireplicon variant in its infectivity in grapevine, it appears that, similar to results obtained in N. benthamiana, cleavage between L1 and L2 is not essential for virus infection of the initially inoculated cells. Interestingly, a similar pattern of cleavage requirements was found in a human coronavirus where the proteolytic activity of the papain-like protease PL2pro was essential for virus infection while that of PL1pro was not (Ziebuhr et al., 2007). Taken together with partial functional overlap between the BYV L-Pro and arteriviral nsp1 (Peng et al., 2002), these striking analogies among diverse viruses suggest parallel requirements for evolution of the large RNA genomes (Dolja et al., 2006; Gorbalenya et al., 2006; Koonin and Dolja, 2006).

What is a functional significance of duplication of the leader proteases in GLRaV-2? We hypothesize that the answer, at least in part, lies in the host-specific effects of L1 and L2 whose functional cooperation is more important for the infection of grapevine than N. benthamiana. A tandem of viral proteases could have evolved to boost the function of a single protease in order to subvert a perennial woody host potentially recalcitrant to virus infection. This hypothesis is compatible with the fact that in addition to GLRaV-2, protease duplication is found in CTV (Karasev et al., 1995), Raspberry mottle virus (Tzanetakis et al., 2007), and Strawberry chlorotic fleck virus (Tzanetakis and Martin, 2007), each of which infects woody and/or perennial hosts, but not in BYV or Mint virus 1 (Tzanetakis et al., 2005) that infect herbaceous annual hosts. Another example of a viral protein that apparently evolved to allow the viral infection of the woody or

| Experiment | Variant | Mean number of the infection foci (% of that in parental variant) | Mean GUS activity (% of the level in parental variant) |
|------------|---------|---------------------------------------------------------------|-------------------------------------------------|
| 1          | mLR-GFP/GUS  | 100.00                                                        | 100.00                                          |
| 1          | ΔL1      | 1.03                                                          | 4.16                                            |
| 1          | ΔL2      | 14.96                                                         | 10.03                                           |
| 1          | M1       | 104.44                                                        | 103.11                                          |
| 2          | mLR-GFP/GUS  | 100.00                                                        | 100.00                                          |
| 2          | ΔL1      | 1.58                                                          | 2.93                                            |
| 2          | ΔL2      | 10.44                                                         | 10.87                                           |
| 2          | M1       | 133.43                                                        | 118.08                                          |
perennial hosts is provided by the AlkB demethylase found primarily in a subset of flexiviruses (Martelli et al., 2007; van den Born et al., 2008).

The generation of the GLRaV-2 variants tagged via insertion of the reporter genes or epitopes highlights a potential of this virus as a gene expression vector for the grapevine. In general, Closterovirus-derived vectors provide advantages of relatively large genetic capacity and stability (Dolja et al., 2006; Folimonov et al., 2007). Utility of closteroviral vectors is further enhanced by a dramatic increase in the vector infectivity by co-expression of the homologous RNAi suppressors with p24 of GLRaV-2 being among the strongest (Chiba et al., 2006). Obviously, full realization of the GLRaV-2 vector potential requires development of the efficient inoculation technique for the grapevine.

Materials and methods

Generation of the GFP-tagged, full-length cDNA clone of GLRaV-2

The GLRaV-2 isolate obtained from a local Oregonian vineyard was propagated on N. benthamiana plants as described earlier (Goszczynski et al., 1996). Virions were isolated (Napuli et al., 2000) and the viral RNA was obtained using TRizol reagent (Invitrogen) according to the manufacturer’s protocol. A strategy for nucleotide sequencing of the viral genome and the generation of the intermediate and full-length viral cDNA clones was as described for BTV (Peremyslov and Dolja, 2007). The resulting sequence of GLRaV-2 RNA was deposited to Genbank (accession no. XXXX). The sequences of the numerous primers used in cloning procedures are available upon request.

In brief, a full-length cDNA clone of GLRaV-2 was assembled using pCB301 mini-binary vector (Xiang et al., 1999), while the cDNA cloning was done using reverse transcription and either conventional or reverse transcriptase. These fragments were inserted into p35S5Δnos-POL. To add a CaMV 35S promoter, a megaprimer (nt 2034) to produce p35S5′-fragment of the viral cDNA (nts 1–2034), a PCR-mediated DNA splicing technique was used. Separate PCRs were done to amplify the 3′ promoter and the 5′ end of GLRaV2 cDNA and to generate products with overlapping ends. These products were combined and used as templates for another round of PCR using primers complementary to the 5′- and 3′-ends of the full-length product. The latter product was cloned into pCB301-NOS-PL using Sac I (added to the 5′-end of 35S promoter) and BamHI I (nt 2034) to produce p35S5′-1R.

To add a riboyme to the 3′-end of the viral cDNA, a megaprimer with a virus-specific part complementary to the 3′- and 5′-ends of the viral cDNA followed by a riboyme sequence designed as described (Prokhnevsky et al., 2002) and the genome was used in combination with a regular primer to amplify the 3′-terminal region of the GLRaV-2 cDNA (nts 14,842–16,486).

To characterize in vitro the replicase activity of the mutated proteases, the resulting capped RNA transcripts were translated using the wheat germ extracts (Promega) and [35S]-Met (Amersham/Pharmacia Biotech) or a non-labeled amino acid mixture. After 1 h of incubation at 25°C, the products were separated by PAGE, electroblotted onto a PROTRAN nitrocellulose membrane and used for autoradiography or for immunoblotting using anti-HA rat monoclonal antibody (Roche) as first antibody and goat anti-rat-peroxidase as secondary antibody.

Mutation analysis of the L1 and L2 roles in RNA accumulation

The pGEM-SP6LR-L2HA variants were used to analyze the proteolytic activity of the mutated proteases in vitro. The DNA fragments from the mutant derivatives of pGEM-35S-LR-L2HA were cloned into mLR-GFP/GUS using Sfi I (located in the vector part of the plasmid) and Nco I (nt 3063) sites. The DNA fragments from mutant derivatives of p35S-miniV94-GFPGUS were also cloned into the full-length cDNA clone LR-GFP using Sfi I (located in the vector part of the plasmid) and Bbv CI (nt 6282).

Generation of the modified and mutant GLRaV-2 variants

The minireplicon variant mLR-GFP/GUS was engineered by modifying the LR-GFP cDNA via deletion of the cDNA fragments from the start codon of the p6 ORF (Fig. 1A) to nt 14,185 and from the Fse I site at the 3′-end of the GFP ORF to nt 15,285 (nt numbers correspond to the original GLRaV-2 cDNA). As a result, GLRaV-2 ORFs encoding p6, Hsp70h, p63, CPM, CP and p19 were deleted (Fig. 1A). The GFP ORF was then replaced with a hybrid GFP/GUS ORF described earlier (Peng et al., 2002) using Pac I at the 5′-terminus of the GFP ORF and Fse I at the 3′-terminus of the GUS ORF.

Two plasmids, pGEM-35S-LR-Pro and pGEM-SP6LR-Pro, containing the whole L1 and L2 coding region and a fragment of the methyltransferase caging region (nts 1–3071) were generated by cloning the corresponding PCR-amplified fragments (Fig. 1B) into pGEM-32zf(+). Both pGEM-35S-LR-Pro and pGEM-SP6LR-Pro were used to generate pGEM-35S-LR-L2HA and pGEM-SP635S-LR-L2HA by inserting three copies of the hemagglutinin epitope (HA) tag (YPYDVPDYA) coding sequence downstream from codon 663 within L2 coding region. Each of these plasmids was used to introduce the following mutations into the L1 or L2.

Mutation 1 (M1) was generated by replacing the catalytic Cys493 residue of L1 with Ala using site-directed mutagenesis. Analogously, mutation 2 (M2) was obtained via substitution of Ala for Cys579 of L2. In ΔL2 mutation, the entire L2-coding region was deleted and 85Residue downstream from L2 scissile bond was replaced with Gly to regenerate an authentic L1 cleavage site. Mutation ΔL1 was made by deleting the entire L1 coding region except for the 5′-terminal start codon. In mutation ΔNTD1, the entire N-terminal, non-proteolytic region of L1 was deleted, again except for the start codon. In mutation ΔPro1, the C-terminal proteinase domain of L1 was deleted while the N-terminal region of L1 was fused to the N-terminal region of L2. In the last mutation ΔL1/2, both L1 and L2 were deleted except for the start codon that was fused with the first Lys codon of the GLRaV-2 replicase, resulting in the formation of a replicase that differed from the proteolytically processed, wild-type replicase only by the presence of the N-terminal Met. The diagrams of all mutations are shown in Fig. 1B.

The pGEM-SP6LR-L2HA variants were used to analyze the proteolytic activity of the mutated proteases in vitro. The DNA fragments from the mutant derivatives of pGEM-35S-LR-L2HA were cloned into mLR-GFP/GUS using Sfi I (located in the vector part of the plasmid) and Nco I (nt 3063) sites. The DNA fragments from mutant derivatives of p35S-miniV94-GFPGUS were also cloned into the full-length cDNA clone LR-GFP using Sfi I (located in the vector part of the plasmid) and Bbv CI (nt 6282).

Mutation analysis of the proteolytic activity of L1 and L2

The pGEM-SP6LR-L2HA variants were linearized using Sma I and the corresponding in vitro RNA transcripts were generated using mMessage Machine kit (Ambion). To assay the proteolytic activity of the leader proteases, the resulting capped RNA transcripts were translated using the wheat germ extracts (Promega) and [35S]-Met (Amersham/Pharmacia Biotech) or a non-labeled amino acid mixture. After 1 h of incubation at 25°C, the products were separated by PAGE, electroblotted onto a PROTRAN nitrocellulose membrane and used for autoradiography or for immunoblotting using anti-HA rat monoclonal antibody (Roche) as first antibody and goat anti-rat-peroxidase as secondary antibody.

Mutation analysis of the L1 and L2 roles in RNA accumulation

Agrobacterium tumefaciens strain C58 GV2260 was transformed by each of the mLR-GFP/GUS variants by electroporation. Corresponding cultures were grown overnight at 28°C with shaking. spun down and resuspended in a buffer containing 10 mM MES-KOH (pH 5.85), 10 mM
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