AN ABSTRACT OF THE DISSERTATION OF

Yu-Ping Liu for the degree of Doctor of Philosophy in Botany and Plant Pathology presented on February 24, 2009.

Title: Generation of Full-length cDNA Clone and Functional Analysis of Leader Proteases of *Grapevine Leafroll-associated Virus-2*

Abstract approved:_____

Valerian V. Dolja

Papain-like leader proteases are found in diverse families of human, animal, plant, and fungal positive-strand RNA viruses. In addition to autocatalytic processing, these proteases play a variety of roles in the virus life cycle. In particular, the leader protease (L-Pro) of a prototype member of the *Closterovirus* genus, *Beet yellows virus* (BYV), was implicated in autoproteolytic processing, genome amplification, and long-distance transport. The genetic organization of *Grapevine leafroll-associated virus-2* (GLRaV-2) is similar to that of BYV, however, GLRaV-2 codes for two leader proteases, L1 and L2. Previous work suggested that the tandems of leader proteases in GLRaV-2 and other closteroviruses emerged via independent gene duplication events. 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 N-terminal non-proteolytic domains. This study was designed to characterize the functional profiles of GLRaV-2 leader proteinases.

Because the infectious cDNA clones of the RNA viruses are indispensable for investigation of viral gene functions, we generated such cDNA clone for GLRaV-2. To facilitate observation and quantification of each phase of the virus infection cycle, cDNA clones were tagged via insertion of fluorescent, enzymatic, and epitope reporters. The tagged clones were used to study the functions of GLRaV-2 L1 and L2. We found that the autoproteolytic processing by L2 but not by L1 is critical for virus viability. It was also revealed that L1 and L2 have complementary and overlapping functions in the establishment of the viral infection in the initially inoculated cells, and, to different extent, in the systemic transport of GLRaV-2. Strikingly, we have demonstrated that the overall contributions of L1 and L2 into virus infection are much more critical in a natural virus host, grapevine, compared to an experimental herbaceous host *Nicotiana benthamiana*, suggesting that the tandem of leader proteases evolved to facilitate an expansion of the *Closterovirus* host range into woody plants.

Generation of Full-length cDNA Clone and Functional Analysis of Leader Proteases of *Grapevine Leafroll-associated Virus-2*

by
Yu-Ping Liu

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented February 24, 2009
Commencement June 2009

Doctor of Philosophy dissertation of Yu-Ping Liu presented on February 24, 2009.

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Yu-Ping Liu, Author

ACKNOWLEDGMENTS

This work was supported by a contract with Growers Research Group, L.L.C. (Soledad, California), BARD award No. IS-3784-05 to Valerian V. Dolja, and teaching assistantships provided by the Department of Botany and Plant Pathology.

First and foremost, I would like to thank my major advisor, Valerian V. Dolja, for giving me an opportunity to enter the program and providing me with scientific, editorial, and financial support through this process. Most importantly, I would like to thank him for teaching me how to keep a good attitude toward research and adjust myself in a challenging and competitive environment.

I would like to thank all of my committee members, Dr. Carol Mallory-Smith, Dr. James C. Carrington, Dr. John E. Fowler, Dr. Robert R. Martin, and Dr. Thomas J. Wolpert for their time, advice, and support. Without their commitments, I could not have possibly come this far.

Thanks to all the past and present members of the Dolja lab for their help, and support throughout the years of my work at the lab. Special thanks to Valera Peremyslov who provided invaluable help with generation of the viral cDNA clones and with many experiments, and helped me to feel comfortable with everything in the lab when I first came here. Thanks to Dr. Alexey Prokhnevsky for his stimulating intellectual conversations and for providing information when I was looking for a job.

Finally, I would like to give my sincere appreciation to my parents and brother in Taiwan for loving me, encouraging me, and supporting me through all my life. Thanks to my former professors, friends in Taiwan, and friends in Corvallis for all the support I have received during my graduate study. And many special thanks to Betty Hill, who is my family in Corvallis, for all of her support and affection, and for helping me to overcome the challenges in my study and life.

CONTRIBUTION OF AUTHORS

Dr. Valerian V. Dolja was involved in the major experimental design of the research and provided scientific inspiration, interpretation, and editorial advice on all chapters.

Valera V. Peremyslov contributed to the design, construction, and sequencing of the full-length cDNA clone of GLRaV and was responsible for the Western blot analysis in chapter two.

Chapter three was co-authored with Valera V. Peremyslov, and Vicente Medina. Valera V. Peremyslov was involved in the construction of GLRaV-2 variants. Vicente Medina was responsible for the immunogold electron microscopy analysis (IGEM) of GLRaV-2 virions.

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Chapter 1

Introduction and Literature Review

1.1 Life cycles of plant viruses

Along with fungi and bacteria, viruses are important pathogens of various crop plants (Takanami, 2006). Plant viruses were discovered over a century ago when the science of virology was born (van der Want and Dijkstra, 2006). The International Committee on the Taxonomy of Viruses (ICTV) established a list of about 2000 recognized virus species as of 2005; of these, more than 1000 viruses infect plants (Fauquet *et al*., 2005).

After plant viral particles entry into a cell via incidental cell wounding or vector transmission, the nucleic acid is uncoated and viral information becomes accessible. The life cycle of a typical virus within the infected plant can be divided into phases of virus accumulation, cell-to-cell movement, and systemic transport. For both DNA and RNA viruses, the accumulation of progeny virus involves translation and replication of viral genomes (Nelson and Citovsky, 2005). In the case of positive-strand RNA viruses, the viral genome serves as a messenger RNA (mRNA) for translation. In other cases, the viral genome needs to be transcribed into mRNA before translation. During translation, the viral mRNAs express their information in proteins, some of which are required for the genome replication. In addition to virus-coded proteins such as RNA-dependent RNA polymerase (RdRp) and RNA helicase, RNA viruses usually recruit host factors and endomembranes to aid their replication (Nelson and Citovsky, 2005; Thivierge *et al*., 2005). Large-scale screening for the host factors that affect RNA amplification has been undertaken using yeast as an alternative host, distinguished by a wealth of well-characterized mutants (Kushner *et al*., 2003; Panavas *et al*., 2005). Data from these experiments showed that host genes involved in viral accumulation could differ between viruses. However, RNA replication complexes of the most, if not all, positive-strand RNA viruses are assembled with the involvement of endomembranes such as ER (Ahlquist, 2006).

Viral cell-to-cell movement can be subdivided into stages of intracellular and intercellular movement (Carrington *et al*., 1996; Beachy and Heinlein, 2000). During the intracellular stage, assembled virions or viral genomes are transported from their

replication sites to plasmodesmata (PD) at the cell periphery. Plasmodesmata are specialized organelles interconnecting the cells and providing means for intercellular communications (Carrington *et al*., 1996; van der Want and Dijkstra, 2006). During the second, intercellular stage of cell-to-cell movement, virions or viral genomes pass through PD and enter adjacent cells. Following additional cycles of replication, viruses gradually move from initially inoculated epidermal or trichome cells into adjacent epidermal or mesophyll cells.

Most plant viruses encode specialized nonstructural proteins, termed movement proteins (MP), which are essential for the transport of viral genomes (Waigmann *et al*., 2004; Lucas, 2005). Some viruses encode not just one, but two, or even three MPs. Several distinct MP families, including the "30K" family (Melcher, 2000), triple gene block proteins (TGBs), tymoviral MPs, and several others, have been identified.

One of the best-characterized viruses, *Tobacco mosaic virus* (TMV), encodes a 30-kDa MP (P30), which is necessary and sufficient for the local spread of this virus. P30 was demonstrated to bind single-stranded RNA in a sequence nonspecific manner to form a thread-like RNP complexes (Citovsky *et al*., 1992), to autonomously localize to PD and modify their permeability at the leading edge of TMV infection (Oparka *et al*., 1997), to interact with ER and cytoskeleton (Guenoune-Gelbart *et al*., 2008; Heinlein *et al*., 1995; McLean *et al*. 1995), and to mediate the cell-to-cell trafficking of viral RNA (Lucas, 2005). Although ER and cytoskeletal elements were implicated in viral cell-to-cell transport more than a decade ago, their exact mechanistic contributions are yet to be determined. On one hand, cell-to-cell transport of TMV RNA was functionally correlated with the MP association with microtubules using temperature-sensitive MP mutants (Boyko *et al*., 2008). On the other hand, evidence for the involvement of actomyosin motility system was also provided (Fujiki *et al*., 2006; Wright *et al*., 2007). Furthermore, it was recently proposed that MP targeting to PD occurs via passive diffusion in the ER membrane (Guenoune-Gelbart *et al*., 2008).

For some icosahedral RNA viruses, such as *Cowpea mosaic virus* (CPMV), both MPs and capsid proteins (CPs) are required for the cell-to-cell translocation. Strikingly, these viruses induce formation of the tubular structures interconnecting adjacent cells,

apparently, via modified PD (van Lent *et al*., 1990). MP is the major constituent of the tubules; it is both essential and sufficient for tubule formation. In the infected cells, tubules provide a conduit through which the assembled virions move from cell to cell (Pouwels *et al*., 2003). It was shown that proper targeting and assembly of tubules involves a complex interplay of the cellular endomembrane and cytoskeletal machineries (Laporte *et al*., 2003).

In contrast to the relatively slow movement from cell to cell, viruses move much faster with the flow of photoassimilates upon their entry into the phloem. With the exception of phloem-restricted viruses (e.g., luteoviruses), most plant viruses reach the phloem via translocation from the initially inoculated epidermal or mesophyll cells to bundle sheath cells to phloem parenchyma cells to companion cells, and, finally, to the sieve elements through which the long-distance transport occurs (Carrington *et al*., 1996). Phloem-restricted viruses appear to move locally only between the companion and phloem parenchyma cells, and from leaf to leaf through the sieve elements (Astier *et al*., 2007).

The mechanism of phloem-dependent long-distance movement is different from that of cell-to-cell movement. Most viruses that move cell to cell in a non-virion form (e.g., TMV), require the CPs for long-distance movement (Carrington *et al*., 1996; Ding *et al*., 1996). Whether MPs are also involved in the phloem-dependent movement is currently uncertain, although there are some genetic evidence indicating that MPs may perform specific long-distance movement functions (Carrington *et al*., 1996; Fenczik *et al*., 1995). Proteins other than the CPs and the MPs, such as the potyviral HC protein (HC-Pro), the genome-linked protein (VPg), the cucumoviral 2b, the tombusviral p19, the closteroviral p20 and leader protease, or the umbraviral ORF3, are also proved to be involved in long-distance movement (Dolja, 2003; Kim *et al*., 2007; Saenz *et al*., 2002). Several endogenous phloem proteins, namely different phloem lectins (CmPP2, CsPP2, CmmLec17) and the phloem protein CmPP16, have been proposed to interact with the viral RNAs and could thus be involved in virus import or translocation within the phloem stream (Kehr and Buhtz, 2007). Recent study on the capsid-less umbravirus has also shown that a major nucleolar protein,

fibrillarin, directly interacts with umbravirus ORF3 protein and is required for the formation of viral RNPs and systemic virus infection (Kim *et al*., 2007).

In order to survive, the viral population must maintain itself either by transmission to the progeny of the infected plant, or by infection of the new host plants. In the nurseries of fruit trees and ornamentals, grafting is routinely used to propagate cultivars vegetatively, and viruses can be easily transmitted in this way. In many cases, viruses can be transmitted through seeds or pollen. In rare cases of the highly stable viruses (*Tobamovirus, Potexvirus, Hordeivirus*), mechanical transmission by direct contacts between neighboring plants or through animal or human activities is a prevalent way of dissemination (Astier *et al*., 2007; van der Want and Dijkstra, 2006). However, for the majority of plant viruses, transmission requires a third agent, the vector organism that specifically acquires viruses from the infected plant and transmits them to the healthy plants. Vectors are found among the diverse, mobile organisms: arthropods (insects, mites), fungi, and nematodes.

The Hemipterans including aphids, whiteflies, leafhoppers, planthoppers, and thrips are the most common vectors and transmit the great majority of plant viruses. There are currently four described mechanisms of insect transmission of plant viruses (Hogenhout et al., 2008). These four mechanisms are nonpersistent, semipersistent, persistent circulative and persistent propagative transmissions. For viruses that are transmitted in a nonpersistent manner, two strategies, the capsid and the helper strategies, are employed. In the capsid strategy (as exemplified by *Cucumber mosaic cucumovirus*), virion binding within the insect mouthparts is mediated by the capsid protein. In the helper strategy (as exemplified by *Potyvirus*), the binding is mediated by a nonvirion protein, HC-Pro, which is suggested to bind the aphid stylet and the virion (Froissart *et al*., 2002; Ng and Falk, 2006). The mechanisms of semipersistent transmission are not well understood with the exception of *Cauliflower mosaic caulimovirus* (CaMV). The helper-aided transmission of CaMV involves two proteins, p2 and p3, plus the major virion CP (Drucker *et al*., 2002; Ng and Falk, 2006). Virus species of the *Luteoviridae*, *Geminiviridae,* and *Nanoviridae* families are transmitted in a persistent circulative manner. All these viruses have icosohedrical particles CPs of

which interact with insect-derived components, including proteins involved in endocytosis and exocytosis pathways in insect gut and salivary gland cells, as well as GroEL homologues produced by the bacterial endosymbionts (Hogenhout *et al*., 2008). All enveloped plant viruses (e.g., tospoviruses and rhabdovirus) are transmitted in a persistent propagative manner that involved virus replication in the vectoring arthropod. The glycoproteins of enveloped viruses are important for invasion of vector tissues and hence for the successful infection of the salivary glands and subsequent introduction of virus into plants (Hogenhout *et al*., 2008).

Viral infection triggers an array of plant defense mechanisms, and viruses have evolved to counteract these defenses to various extents. One of the best-characterized mechanisms of plant antiviral defense is mediated by the resistance (R) genes. In the last decade, several R genes that confer resistance to unrelated plant viruses have been identified. Strikingly, they all belong to the NB-ARC-LRR superfamily of plant R gene (Soosaar *et al*., 2005). The nucleotide-binding (NB) site domain and adjacent sequences of *R* genes are similar to the equivalent regions of the metazoan cell-death genes *Apaf-1* and *CED4* and are therefore referred to as the NB-ARC domains. Leucine-rich repeats (LRRs) are imperfect repeats that are involved in protein-protein interactions and protein – ligand interactions. The NB-ARC-LRR R proteins can be further subdivided into Toll-interleukin-1 receptor (TIR)-NB-ARC-LRR or Coiled-coil (CC)-NB-ARC-LRR based on the structure of their N termini (Soosaar *et al*., 2005). One of the well-characterized R genes is the N gene from *Nicotiana sp* which confers hypersensitivity response to TMV (Whitham *et al*., 1994). Mutations in all three domains of the N protein compromise resistance to TMV, indicating that each domain has an important role in pathogen recognition and/or signaling (Dinesh-Kumar *et al*., 2000).

Each R gene confers resistance to a specific pathogen. For example, the *Arabidopsis thaliana* RCY1 gene confers resistance to the Y strain, but not the O strain of *Cucumber mosaic virus*. When the Y strain of CMV infects *RCY1*-containing plants, a defense response is initiated, which restricts the virus to the infection site and prevents disease. The virus is an avirulent pathogen on these resistant plants and this is

termed an incompatible interaction. The pathogen molecule that specifically elicits R-protein-mediated responses is the avirulence (Avr) determinant (Soosaar *et al*., 2005). Any protein component of a virus can function as the specific Avr determinant to elicit resistance mediated by a given R gene.

R proteins can recognize a pathogen's Avr determinant either indirectly (guard hypothesis) or by direct association (Padmanabhan *et al*., 2009). However, the latter model does not apply to any viral R-Avr pair examined to date (Martin *et al*., 2003). The "guard hypothesis", originally proposed by Van der Biezen and Jones in 1998, postulates that R proteins (guards) are constitutively associated with host cellular proteins (guardees) that are required for pathogen infection. One of the greatest supports to the guard hypothesis in the viral R-Avr system is the HRT-*Turnip crinkle virus* (TCV) pair. The TCV coat protein is the Avr determinant for *HRT*-mediated resistance response, and it interacts with a host transcription factor, TCV-interacting protein (TIP). This interaction is required for *HRT*-elicited defense responses, although a direct interaction between HRT and TIP has not been reported (Ren *et al*., 2000). A conserved molecular chaperone, heat shock protein 90 has also been found to bind to the LRR domain of tobacco N protein and to be required for the resistance that is mediated by the tobacco N protein (Padmanabhan *et al*., 2009).

Another plant innate immune response system that targets viruses was discovered in the early 1990s (Lindbo and Dougherty, 1992). This system termed RNA silencing or RNA interference (RNAi) was also found in animals and fungi. The beauty of this response lies in its ability to adapt to all sorts of different viruses, because its specificity is dictated by the sequence of the viral genome itself. Another salient feature of RNAi is that a signal that triggers silencing is not restricted to individual plant cells, but can spread from the site of infection, generating a response in the distant tissues (Carrington, 2000).

RNAi involves production of the virus-derived small (21-23 nucleotides) interfering RNAs (viRNAs) that guide viral RNA degradation by multiprotein effector complexes. viRNAs are processed as duplexes from double-strand RNA precursors by an RNaseIII enzyme called Dicer (Hammond, 2005). For RNA viruses, plant antiviral

Dicers are probably stimulated by both dsRNA replication intermediates and imperfect RNA hairpins present in the viral RNAs. Other plant viRNA sources include dsRNA segments formed by overlapping sense-antisense transcripts produced by circular geminivirus ssDNA genomes (Ding and Voinnet, 2007). Effector complexes called RNA-induced silencing complexes (RISCs) are assembled upon loading of one selected small RNA strand into one member of the Argonaute (Ago) protein family (Ding and Voinnet, 2007; Tolia and Joshua-Tor, 2007). Ago proteins are often named Slicer proteins because they cleave target ssRNAs at the duplex formed with the guide-strand small RNA (Ding and Voinnet, 2007; Tolia and Joshua-Tor, 2007).

Early evidence that viruses encode RNAi suppressor proteins came from experiments in which silenced transgenes in plants were reactivated after virus infection or after introduction of genes encoding candidate suppressor proteins (Carrington *et al.*, 2001). More than 35 unrelated suppressors have been identified from virtually all plant virus types, unraveling a ubiquitous counterdefensive strategy in virus evolution (Ding and Voinnet, 2007; Li and Ding, 2006). Viral suppressors are often considered to be 'novel' evolutionary inventions that act via a range of distinct mechanisms. Many suppressors bind and sequester ds forms of viRNA (Lakatos et al., 2006). In contrast, the *Cucumber mosaic virus* 2b protein physically interacts with viRNA-loaded AGO and inhibits slicing (Ding and Voinnet, 2007).

1.2 Grapevine viruses

Grapevine is an ancient human food plant known for several millennia. Grapevine crop production has constantly increased because the fresh fruit is quite nutritious and also useful as an industrial raw material for developing other food products, juices, and beverages. Sustained efforts to ensure high, reliable yields are continuously threatened due to damage caused by numerous diseases, among which viral diseases are ranked at the top (Sutic *et al*., 1999). In contrast to most fungal and bacterial diseases, viruses seriously disrupt the structure and all functions of infected grapevine plants. Once infected, the canes remain systemically infected for life with

no prospect for a cure. Most viruses that infect grapevine are graft-transmissible, which means they are transmitted from vine to vine through the process of grafting. In addition, insects such as mealybugs and nematodes have also been found to transmit certain grapevine-infecting viruses. Therefore, overall sanitation and good cultural practices are needed to maintain a healthy and productive vineyard (Monis, 2005).

Commonly, viral diseases have been named to reflect the symptoms observed in infected plants (e.g., leafroll, fanleaf, stem pitting, yellow vein, grape decline, etc.) (Monis, 2005; Monis, 2006). With the advent of molecular tools for the characterization of different causal agents, it has been shown that grapevines often carry mixed infections, whereas the same symptoms could be caused by more than one virus. Furthermore, symptoms of the disease can be influenced by the seasonal and climatic conditions (Monis, 2005; Monis, 2006).

1.2.1 Leafroll disease

This disease can be found wherever grapevines are grown. Several different viruses, called *Grapevine leafroll-associated viruses* (GLRaV-1 through -9), have been reported to be associated with leafroll disease. These viruses fit within two closely related virus groups: *Closterovirus* (from "clostero," Greek for thread-like) and *Ampelovirus* (from "ampelo," Greek for grapevine). Plants infected with leafroll viruses are slightly smaller than the healthy ones, and their grape clusters are smaller and have a lower content of sugar than normal. Symptoms include downward rolling and interveinal reddening or yellowing of leaves, depending on the grape variety, but often the main vein of the leaf remains green. One of the grapevine leafroll-associated viruses, GLRaV-2, has also been implicated in graft incompatibility symptoms (Monis, 2005).

1.2.2 Rugose wood complex

The rugose wood (RW) complex, one of the major disease complexes affecting grapevines, occurs worldwide and is especially common in Italy (Credi, 1997). RW is a generic term adopted to designate a group of distinct disorders, mainly characterized

by the development of pitting and grooving in the woody cylinder. Four RW syndromes were identified by graft-inoculation of healthy indicator vines, but only three of them have been associated with viral infection: rupestris stem pitting (RSP) is caused by the *Rupestris stem pitting associated virus* (RSPaV, a *Foveavirus*, from "fovea," Latin for small pits); Kober stem grooving (KSG) is caused by *Grapevine virus A* (GVA, a *Vitivirus*, from "Vitis," the Latin name for grape); corky bark (CB) is caused by *Grapevine virus B* (GVB, a *Vitivirus*). The different rugose wood complex syndromes are difficult to distinguish in the field. Typical symptoms in grafted vines include swelling above the graft union and wood with pits or grooves that can only be seen after the bark is removed. The severity of symptoms ranges from delayed budburst to vine decline and death, varying with the rootstock or scion variety and the virus type (Monis, 2005).

1.2.3 Fleck disease

This disease is characterized by clearing of veinlets and translucent spots in the third- and fourth-order veins of young and medium-aged leaves, wrinkling and upward curling of the leaves. An icosahedral virus, *Grapevine fleck virus* (GFkV, a *Maculavirus*), and an additional, phloem-limited, non mechanically-transmissible virus have been associated with this disease (Monis, 2005; Walter and Cornuet, 1993).

1.2.4 Grapevine decline, yellow vein, and fanleaf degeneration disease

Fanleaf disease, one of the most serious and devastating grapevine virus diseases, is caused by *Grapevine fanleaf virus* (GFLV, a *Nepovirus*). Sensitive cultivars show rapid decline, low quality of fruit and low yield. Symptoms include fan-like distortions of leaves, chlorotic yellowing, ringspots, vein banding, and mottling or mosaic patterns. Fruit set can be reduced up to 80% in some varieties. Affected clusters contain both large and small berries. The small berries are called 'shot berries' and are seedless. Affected vines may be smaller than healthy ones, particularly if the nematode vector is present. The canes and foliage appear clustered because of stunting (Monis, 2006; Weber *et al*., 2002).

Symptoms associated with *Arabis mosaic virus* (ArMV) are similar to fanleaf decline, and ArMV can be present in a mixed infection with GFLV. Infected grapevines show symptoms that include leaf chlorosis, necrosis, and distortion, shortened internodes, reduced growth, and overall decline (Monis, 2006; Weber *et al*., 2002).

Yellow vein disease, also known as Tomato ring spot decline and Tomato ring spot disease, is caused by *Tomato ringspot virus* (TomRSV, a *Nepovirus*). Symptoms include shot berries, shoot stunting, distortion, mottling, and reduced leaf size. The symptoms of yellow vein resemble those described for fanleaf, and they can be easily confused (Monis, 2006; Weber *et al*., 2002). Tobacco ring spot decline, caused by *Tobacco ringspot virus* (TRSV, a *Nepovirus*), induces decline in grapevines. Symptoms are similar to those caused by TomRSV (Monis, 2006)

Raspberry ringspot virus, Tomato black ring virus, Grapevine chrome mosaic, Strawberry latent ring spot, and Grapevine bulgarian latent virus, can also infect grapevines (Monis, 2006). These diseases have been studied to varying degrees, but have never been demonstrated to be common or severe. Many other poorly characterized graft transmissible diseases, including asteroid mosaic, vein necrosis, and vein mosaic, are also likely caused by viruses. Continuing research is necessary to identify important new diseases and to develop diagnostic tools to help minimize their future impact.

1.3 Positive-strand RNA viruses

Viruses could be divided into seven classes on the basis of differing strategies for storing and replicating their genomes through RNA and/or DNA intermediates (Koonin et al., 2006). The virus classes with double-strand (ds) DNA or single-strand (ss) DNA packaged in virions replicate their genomes solely through DNA intermediates. Two classes of retroid viruses replicate by interconverting their genomes between RNA and DNA; some of these package RNA genomes (retroviruses) and some - DNA genomes (hepadnaviruses and pararetroviruses). A wide number of

viruses replicate their genomes solely through RNA intermediates. These RNA viruses are divided into three classes on the basis of whether their virions package mRNA-sense (positive-strand) ssRNA, antisense (negative-strand) ssRNA, or dsRNA (Ahlquist, 2006). Most plant viruses and many viruses of vertebrates have positive-strand RNA genomes, the virions of which deliver immediately translatable messenger-sense RNAs, encapsidate their RNAs without a polymerase and form non-virion RNA-replication and mRNA transcription complexes.

With the accumulation of many positive-strand RNA viral genome sequences of bacteria, plants, fungi, animals, and various unicellular eukaryotes, it is now possible to compare even distant viruses. Because of the high mutation rate of RNA genomes (10^{-3} to 10^{-5}) (Domingo and Holland, 1997) and recombination frequency, it is probable that the conservation of even relatively short, albeit functionally important, protein sequence motifs, and their arrangement on the genetic map are evolutionarily significant. Comparative analysis of the positive-strand RNA viral genomes established that the RdRp is the only viral protein conserved throughout this class of viruses. Furthermore, it is broadly accepted that comparison of the RdRp sequences is the most straightforward methodology for phylogenetic analysis of this virus class (Koonin, 1991; Dolja *et al.*, 1992; Koonin and Dolja, 1993, Koonin *et al*., 2008). Thus, through a combination of phylogenetic analysis of conserved protein sequences, primarily those of RdRp, and comparison of diagnostic features of genome organization that are linked to replication and expression strategies, positive-strand RNA viruses were classified into three superfamilies: picornavirus-like, alphavirus-like and flavivirus-like (Table 1.1) (Koonin and Dolja, 1993; Koonin *et al*., 2008). These superfamilies include most of the known positive-strand RNA viruses, although the classification of nidoviruses and RNA bacteriophages remain uncertain.

The first superfamily, picornavirus-like superfamily, includes animal picorna- and caliciviruses, and plant sobemo-, luteo-, como-, nepo-, poty-, and bymoviruses. Viruses in this superfamily are characterized by a partially conserved set of genes that consists of the RdRp, a chymotrypsin-like protease (3CPro), a superfamily 3 helicase and a genome-linked protein. This set is not found in other characterized RNA viruses

Table 1.1. Superfamilies of positive-strand RNA viruses. Based on the conserved modules of the RdRp, most positive-stand RNA viruses can be subdivided into three superfamilies, including Picorna-, Alpha-, and Flavi-like superfamilies.

Superfamily	Plant viruses	Common Characteristics
Picorna-like	Comoviruses; Luteoviruses Nepoviruses; Bymoviruses Sobemoviruses	5'-VPg Polyprotein processing Set of conversed genes (helicase, protease, RdRp)
Alpha-like	Bromoviruses; Furoviruses Tobamoviruses; Tymoviruses Potexviruses; Hordeoviruses Closteroviruses; Ilarviruses Cucumoviruses, Tobraviruses	5'-cap Subgenomic mRNAs, frequent readthrough Set of conserved genes (methyl transferase, helicase, RdRp)
Flavi-like	Carmoviruses Tomobusviruses Dianthoviruses Barley yellow dwarf viruses (PAV strain)	For plant viruses: Conserved RdRp No methyltransferase, helicase For animal flavi-, pesti-, and hepaciviruses: Set of conversed genes (helicase, protease, RdRp)

with the exception of the distinct 3CPro-like proteases of nidoviruses (Koonin and Dolja, 1993; Koonin *et al*., 2008).

The second, alphavirus-like superfamily, includes animal alpha-, rubi, and Hepatitis E viruses, and at least 10 groups of plants viruses (Table 1.1; Koonin, 1991; Dolja and Koonin, 1993). Viruses in this superfamily are characterized by genomes that encode a type 1 methyltransferase, a superfamily 1 helicase, and a particular lineage of RdRp. Despite conservation of these domains within the superfamily, they may be expressed as parts of a single protein or as separate entities distributed over two or three proteins. Other features in the superfamily are the capped genomic RNAs and the production of one or more (capped) subgenomic RNAs (sgRNAs) encoding

the structural protein(s) (van der Heijden and Bol, 2002).

Viruses in the third, flavivirus-like superfamily, have two very different types of genome organization. The first type is found in plant carmo-, tomobus-, and diathoviruses. Genomes of these viruses encode a conserved RdRp domain, but no methyltransferase domain, helicase domain, or protease. Instead, these viruses encode a small protein which is required for replication, but its amino acid sequence does not contain any conserved motifs (Scholthof *et al*., 1995; van der Heijden and Bol, 2002). The polymerase protein is produced as a C-terminal fusion with this small protein by readthrough of a leaky stop codon or by a ribosomal frameshift. The second type of genome organization is found in the animal flavi-, pesti-, and hepaciviruses. The RdRp sequence is part of a polyprotein, together with a superfamily 2 helicase, and a serine-type proteinase. A group 2 methyltransferase domain has also been detected in flaviviruses (Koonin, 1993; Koonin and Dolja, 1993).

1.4 Family *Closteroviridae*

A large and diverse family *Closteroviridae* includes filamentous plant viruses with either mono- or bipartite positive-strand RNA genomes. More than 40 viruses have currently been classified as definitive or tentative closteroviruses (Martelli *et al*., 2002; Tzanetakis *et al*, 2007; Tzanetakis and Martin, 2007). Closterovirus genomes are the largest among all plant viruses (15.5-19.5 kb); these genomes encode 10-14 proteins (Dolja *et al*., 2006; Tzanetakis *et al*., 2005). Comparative analysis of the closteroviruses revealed three levels of gene conservation (Dolja *et al*., 1994; Karasev, 2000; Dolja *et al*., 2006). The most conserved gene module includes replication-associated genes (RdRp, RNA helicase, and methyltransferase) shared with the animal and plant viruses within the alphavirus-like superfamily. In addition, most of the closteroviruses possess one or two papain-like leader proteases encoded upstream from the replicational module (see below). The second, quintuple gene module is unique to closteroviruses. It encodes a small transmembrane protein p6, a homologue of Hsp70 molecular chaperones (Hsp70h) and three diverged copies of the capsid protein designated as a major capsid protein (CP), minor capsid protein (CPm),

and a 64 kDa protein (p64). Each of these proteins is conserved throughout the family *Closteroviridae*. The gene order in this module is also conserved except for the varying relative position of CP and CPm among distinct genera.

The remaining genes show dramatic variation in their numbers, function, and origins between and often within the three family genera. Proteins encoded by these genes include suppressors of RNA silencing, RNAse III, the AlkB domain implicated in RNA repair, Zn-ribbon-containing protein, and a variety of proteins with no detectable homologues in the current databases (Dolja *et al*., 2006).

A prototype closterovirus, BYV, was first described in 1936; now it is the best characterized member of the family (Dolja, 2003). BYV is geographically wide spread, transmitted by aphids, and infects sugar beet, red beet, and spinach (Dolja *et al*., 1994). The ~15,500 nt BYV genome (Fig. 1.1) is 5'-capped, has no 3'-poly(A) tail, and encodes at least 10 viral proteins (Agranovsky *et al*., 1994; Peremyslov *et al*., 1998). Expression of the ORFs 1a and 1b occurs via translation of the virion RNA and yields all proteins essential for RNA replication, including a core replicase with the methyltransferase, RNA helicase, and RdRp domains and a L-Pro (Agranovsky *et al*., 1994; Peng and Dolja, 2000; Peremyslov *et al*., 1998). L-Pro is encoded by the 5'-terminal region of ORF 1a and is proteolytically separated from the replicase (Peng and Dolja, 2000). The expression of RdRp involves an unusual +1 frameshift event (Agranovsky *et al*., 1994). The expression of the ORFs 2-8 occurs by synthesis of seven sgRNAs (Dolja *et al*., 1990; Peremyslov *et al*., 2002). These ORFs include a quintuple gene block present in all closteroviruses and two less conserved ORFs. These latter ORFs encode a 20-kDa protein required for the systemic transport of BYV (p20) (Prokhnevsky *et al*., 2002), and a 21-kDa protein (p21) that is expressed early in infection (Hagiwara *et al*., 1999). It was demonstrated that p21 is a suppressor of RNAi specific for the genus *Closterovirus* (Reed *et al*., 2003). This protein acts via binding and sequestering small interfering RNAs therefore inhibiting antiviral RNAi response and inadvertently causing developmental defects in BYV-infected plants (Chapman *et al*., 2004; Lakatos *et al*., 2006).

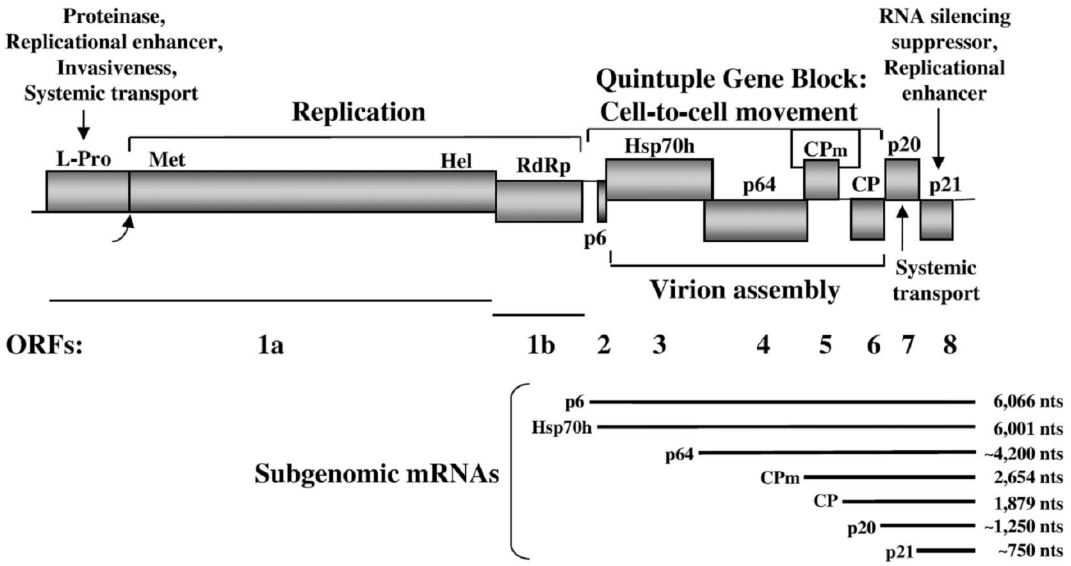

Fig. 1.1. Map of the BYV genome. The ORFs are shown as boxes with the numbering shown below the diagram. The lines under or inbetween boxes correspond to non-coding regions. L-Pro, leader proteinase; Met, Hel, and RdRp, methyltransferase, RNA helicase, and RNA-dependent RNA polymerase domains of the replicase, respectively; p6, a 6-kDa protein; Hsp70h, a Hsp70-homologue; p64, a 64-kDa protein; CPm and CP, the minor and major capsid proteins, respectively; p20 and p21, the 20-kDa and 21-kDa proteins, respectively. The functions of the encoded proteins are indicated. The subgenomic (sg) mRNAs are shown at the bottom. The proteins encoded by each sgRNA are indicated at the left, whereas the sizes of these RNAs are shown at the right. Because the exact 5'-termini of three sgRNAs are not known, their lengths were determined arbitrarily. (Dolja, 2003)

BYV possesses very long and flexuous filamentous virions (Kassanis *et al*., 1977) in which the RNA molecule is encapsidated by several structural proteins. The 1300 nm-long main virion 'body' consists of CP, whereas CPm is a principal component of a 100 nm-long virion 'tail' (Fig 1.2; Agranovsky *et al*., 1995; Peremyslov *et al*., 2004a). This tail has a smaller diameter than the body and exhibits a three-segment structure with the pointed end (Fig 1.2; Peremyslov *et al*., 2004a). This architecture has also been confirmed for other members of the family *Closteroviridae*, such as

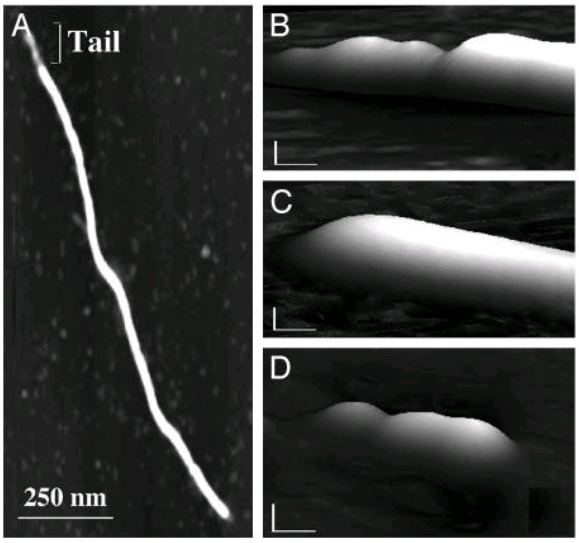

Fig. 1.2. AFM images of the BYV virions. (*A*) A full-length virion. (*B*) A three-segmented virion tail. (*C*) An opposite blunt end of the virion. (*D*) Three-segmented tail isolated after sonication of the virions. *B-D* are 3D images; horizontal bars = 25 nm and vertical bars = 5 nm. (Peremyslov *et al*., 2004)

CTV and LYIV (Febres *et al*., 1996; Tian *et al*., 1999). In addition to CPm, p64, Hsp70h, and p20 were shown to be the integral tail components (Napuli *et al*., 2000; Napuli *et al*., 2003; Prokhnevsky *et al*., 2002; Peremyslov *et al*., 2004a). Of these, p64, and Hsp70h are each required for the assembly of 100 nm tails by CPm (Alzhanova *et al*., 2001; Alzhanova *et al*., 2007). In contrast, p20 is not required for the assembly of tailed virions, but likely binds to preassembled virions to form a pointed tip (Prokhnevsky *et al*., 2002; Peremyslov *et al*., 2004a). Inactivation of CPm, p64, Hsp70h, and p20 does not prevent formation of the BYV virion body suggesting that the body can be assembled independently of the tail (Alzhanova *et al*., 2001; Alzhanova *et al*., 2007; Napuli *et al*., 2003).

Genetic analysis has shown that the cell-to-cell movement of BYV requires five proteins encoded by the quintuple gene block, p6, Hsp70h, p64, CPm, and CP (Alzhanova *et al*., 2000). Each of these proteins is essential, but not sufficient for the

movement, suggesting that these proteins function in a coordinated and interactive fashion. Among these, p6 is a dedicated MP characterized as a type III transmembrane protein that is specifically targeted to the ER (Peremyslov *et al*., 2004b), whereas the remaining four proteins are the components of the BYV virion. An additional tail protein p20 is not required for the cell-to-cell movement, but is essential for the viral transport through the phloem (Prokhnevsky *et al*., 2002). The involvement of all tail proteins in the cell-to-cell or long-distance transport of BYV suggested that a closterovirus tail evolved as a specialized movement device (Dolja, 2003).

Interestingly, Hsp70h is the only BYV movement-associated protein that was found in PD upon ectopic expression or virus infection (Medina *et al*., 1999; Prokhnevsky *et al*., 2005). As shown recently, an autonomous plasmodesmatal targeting of Hsp70h requires the actomyosin motility system, in particular, class VIII myosins, suggesting that these myosins function in protein delivery to plasmodesmata and can be used by viruses for the needs of their intracellular and perhaps intercellular translocation (Avisar *et al.*, 2008; Prokhnevsky *et al*., 2005).

1.5 Grapevine leafroll-associated virus-2

Grapevine leafroll-associated virus-2 (GLRaV-2; genus *Closterovirus*, family *Closteroviridae)*, was first isolated from a corky bark-affected grapevine (Boscia *et al*., 1995; Ling *et al*., 2008; Namba *et al*., 1991; TOMAZIC *et al*., 2008). GLRaV-2 is an important component of the leafroll disease complex in grapevine; it causes eponimous symptoms and a widespread graft-incompatibility condition or quick decline of newly planted vineyards (Bertazzon and Angelini, 2004; Pirolo *et al*., 2006).

GLRaV-2 virions are ~1,600 nm in length and encapsidate the ~16,500 nt RNA genome that encodes at least 11 proteins (Fig. 1.3). Phylogenetic analysis comparing different genes of GLRaV-2, such as RdRp, MP, CP, and CPm with other viruses in closteroviruses family demonstrated the closest relationships with BYV (Zhu *et al*, 1998). However, in contrast to BYV, GLRaV-2 contains two tandemly organized

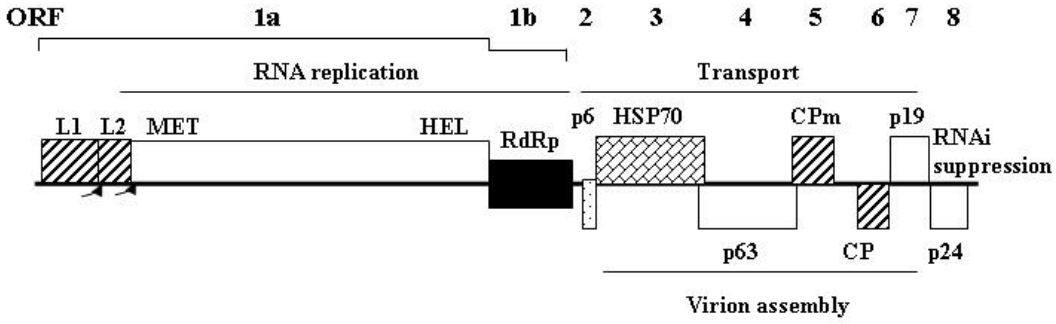

Fig. 1.3. Genome organization of *Grapevine leafroll-associated virus-2* (GLRaV-2).

papain-like proteases (L1 and L2). Accordingly, gene functions of GLRaV-2 can be deduced from information available for BYV. In the case of a 24-kDa protein (p24) that is an ortholog of BYV p21, function in suppression of RNA silencing has been experimentally confirmed (Chiba *et al*., 2006).

1.6 Viral papain-like leader proteases

Proteases are enzymes that catalyze the hydrolysis of peptide bonds. The protease involvement in virus gene expression was first suggested for the poliovirus and the DNA bacteriophage T4 in the mid-70s (Dougherty and Semler, 1993). During the following 30 years, it was shown that many viruses encode one or more proteases that are often essential for infectivity (Byrd and Hruby, 2006; Dougherty and Semler, 1993; Tong, 2002). Viral proteases appear to fulfill one of two functions during the replication cycle: processing viral polyproteins into functional proteins or processing of the structural proteins necessary for assembly during morphogenic proteolysis. Recent studies have shown that in some viruses, such as adenovirus and *Severe acute respiratory syndrome coronavirus* (SARS-CoV), their proteases function as deubiquitinating enzymes aiding in stabilization of either cellular or viral proteins in order to overcome host cell defenses (Balakirev *et al*., 2002; Lindner *et al*., 2005).

Depending on the structure and/or catalytic mechanism, all known proteases have been divided into 7 classes including aspartatic, cysteine, glutamic, metallo, serine, threonine, and unknown (Rawlings *et al*., 2008). Four of these classes contain viral proteases (asparatic, cysteine, serine, and metalloproteases) (Rawlings *et al*., 2008).

Cysteine proteases have been categorized as papain-like, interleukin-1 beta-converting enzyme (ICE)-like, or picornaviral, or 3C-like (which are similar to seine proteases but with a cysteine instead of serine) (Byrd and Hruby, 2006). Viral papain-like cysteine proteases is one of the major types of viral proteolytic enzymes similar to cellular papain-like proteases (Peng, 2002a). The essential catalytic dyad consisting of Cys and a downstream His and variants of α+β fold were characterized in this class of proteases, such as potyviral HC-Pro, closteroviral L-Pro, and alphaviral nsp2 (Gorbalenya *et al*., 1991; Oh and Carrington, 1989; Peng, 2002a; Strauss and Strauss, 1994; Ziebuhr *et al*., 2000). Viral papain-like proteases can be further subdivided into two classes: "main" and "accessory" or "leader" proteases. The main proteases, responsible for the processing of nonstructural polyprotein and for RNA replication, are located internally in the polyprotein and can possess both *cis* and *trans* cleavage activities. In contrast, the leader proteases, typically responsible for a single autocatalytic cleavage at their C-terminus, usually encompass the N-terminal part of the polyprotein and normally cleave only *in cis* (Peng *et al*, 2001; Peng *et al*., 2002b; Ziebuhr *et al*, 2000).

In the case of BYV, efficient amplification of the BYV RNA requires the activity of L-Pro, a 66-kDa multifunctional protein (Fig. 1.1; Agranovsky *et al*., 1994; Peng and Dolja, 2000; Peremyslov *et al*., 1998). Mutation of the variable N-terminal domain of L-Pro results in a dramatic reduction of RNA accumulation suggesting that this domain plays a prominent role in RNA amplification (Peng and Dolja, 2000; Peremyslov *et al*., 1998). Alanine-scanning mutagenesis was used to complete functional analysis of the entire L-Pro molecule throughout the virus life cycle. This analysis indicated that the conserved, papain-like C-terminal domain of L-Pro is not only essential and sufficient for autocatalytic processing at the Gly-Gly scissile dipeptide, but also executes a replication-related function that is separable from the

self-cleavage (Peng *et al*., 2002b, Peng *et al*., 2003). Examination of the L-Pro role in BYV cell-to-cell movement revealed that none of the replication-competent mutants was movement-defective implying that the L-Pro is not directly involved in a local spread. However, several L-Pro mutations affected the long-distance transport of BYV to various degrees, or completely abolished the transport suggesting that L-Pro also serves as the BYV long-distance transport factor (Peng *et al*., 2003).

By and large, papain-like leader proteases of the plant and animal viruses have been implicated in autoproteolytic processing, genome amplification, virus invasiveness, pathogenicity, transport within the infected organism, and suppression of the host defense responses (Peng and Dolja, 2000; Peng *et al*, 2001; Peng *et al*, 2002b; Peng *et al*, 2003; Tijms *et al*, 2001; Tijms *et al*., 2007). Therefore, the viral leader proteases play a variety of roles in the life cycles of different RNA viruses showing a high degree of the functional and evolutionary plasticity.

Chapter 2

Generation of the full-length cDNA clone of Grapevine leafroll-associated virus-2

2.1 Abstract

Because the infectious cDNA clones of the positive-strand RNA viruses are indispensable for investigation of the functions of viral genes and control elements, we set to generate an infectious cDNA clone GLRaV-2. A full-length cDNA copy of the genomic RNA of GLRaV-2 was constructed under the control of the 35S RNA polymerase promoter of *Cauliflower mosaic virus* (CaMV), the NOS terminator, and a ribozyme inserted downstream from the GLRaV-2 sequence. The resulting full-length GLRaV-2 clone was further modified to accommodate a reporter gene expression cassette containing GFP ORF followed by the BYV CP subgenomic RNA promoter cloned upstream of the GLRaV-2 CP ORF. After agroinfiltration of *N. benthamiana* with the modified full-length cDNA clone, GFP expression could be visualized using epifluorescent stereoscope, and GLRaV-2 CP could be detected using Western blot analysis. At 3 weeks postinoculation, the whole inoculated plants developed severe disease symptoms and the vein clearing and wilting symptoms were found on the 'systemic' noninfiltrated leaves. Also, GFP-fluorescent cells were detectable on the systemic leaves, stems, and petioles. These results demonstrated that the GFP-tagged, full-length, cDNA clone of GLRaV-2 was indeed infectious.

2.2 Introduction

Grapevine leafroll is a world-wide graft-transmitted virus disease which adversely affects grape yields and fruit quality. At least 8 serologically unrelated ampelo- and closteroviruses have been found in LR-infected grapevines. GLRaV-2 is one of the viruses associated with this disease (Bertazzon and Angelini, 2004; Pirolo *et al*., 2006). Because the development of infectious full-length cDNA clones of plant viruses provides useful tools for studying viral gene function, we aimed at generating such clone for GLRaV-2 in order to determine functional profiles of L1 and L2. Although previous bioinformatics and indirect experimental studies provided some insight into origins and functions of the tandem leader proteases of closteroviruses

(Peng *et al*., 2001), these functions were not directly addressed using a reverse genetic approach.

Construction of the full-length cDNA clone of an RNA virus genome from which infectious RNA can be transcribed *in vitro* or *in vivo*, is a crucial step towards mapping the viral genetic determinants. Even though engineering of such clones for 4-10 kb viral RNAs has became a routine, it remains to be a challenging task for the 15-20 kb RNA genomes of closteroviruses. BYV was the first closterovirus for which an infectious cDNA clone has been made (Peremyslov *et al*., 1998; Peremyslov and Dolja, 2007). A wealth of information on BYV gene functions and mechanisms has been accumulated since then (e.g., Chapman *et al*., 2004; Peremyslov *et al*., 2004a; Peremyslov *et al*., 2004b; Prokhnevsky *et al*., 2002; see Dolja *et al*., 2006 for a review). *Citrus tristeza virus* (CTV) was the only other member of genus *Closterovirus* for which an infectious cDNA clone has been available. Although, due to a host range restricted to *Citrus* spp., CTV is a less versatile model virus (Satyanarayana *et al*., 2001), an important progress in understanding CTV RNA synthesis and gene functions has been achieved (Ayllon *et al*., 2003; Satyanarayana *et al*., 2002; Satyanarayana *et al*., 2004).

The strategic considerations and techniques that were used to engineer the infectious cDNA clone of BYV have been described (Peremyslov and Dolja, 2007). Following this experience, we engineered a full-length cDNA clone of GLRaV-2 (Fig. 2.1) and demonstrated that this cDNA clone was infectious after agroinfiltration of *N. benthamiana.*

2.3 Materials and Methods

2.3.1 Virus and RNA

GLRaV-2 was obtained from an Oregonian vineyard and propagated on *Nicotiana benthamiana* plants in the greenhouse under standard conditions as described earlier (Goszczynski *et al*., 1996). We are grateful to Prof. Robert Martin for providing us with the virus-infected cuttings. Virus was purified as described by

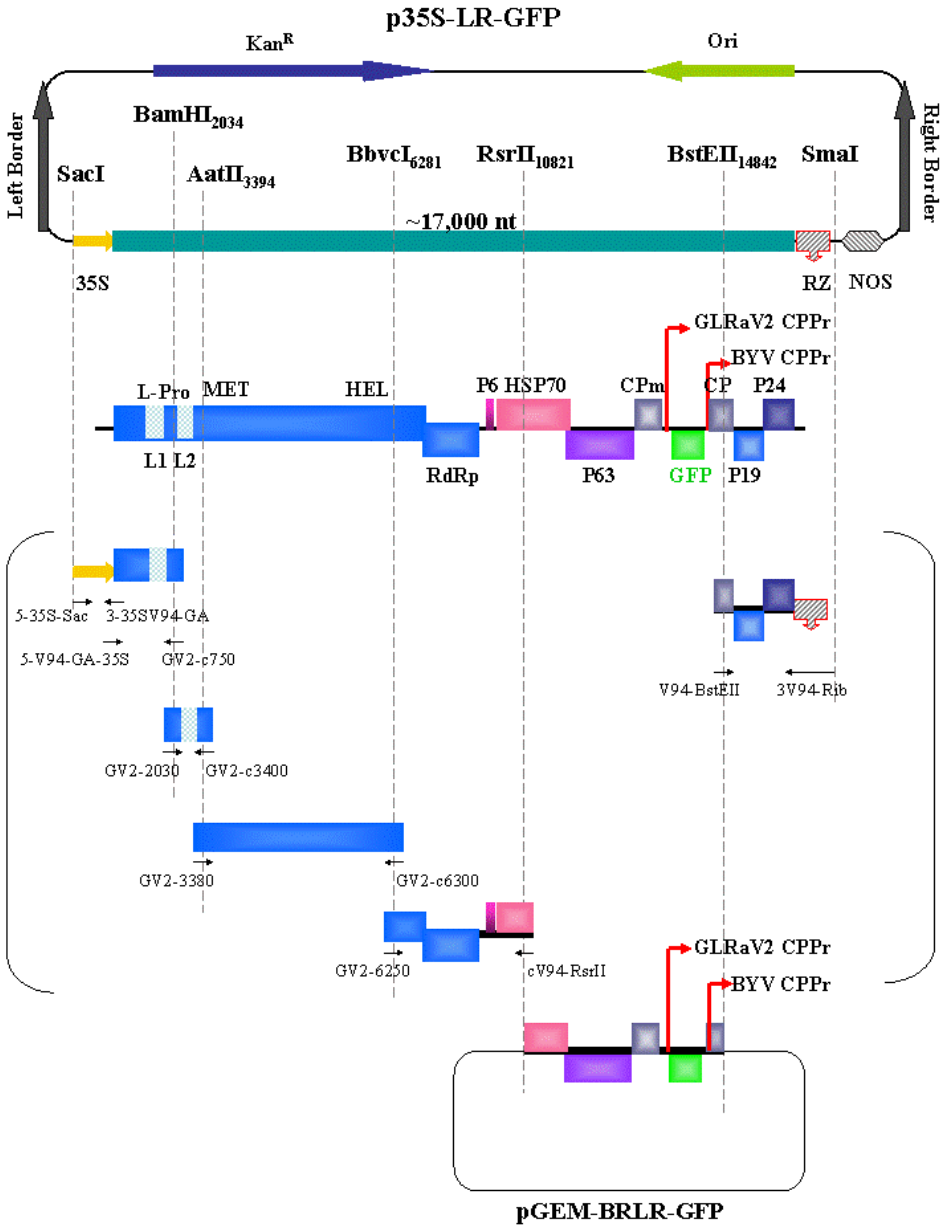

Fig. 2.1. Strategy for constructing the full-length cDNA clone of GLRaV-2 (p35S-LR-GFP; shown at the top). A cistron map of GLRaV-2 is shown below the diagram of p35S-LR-GFP. The intermediate vector pGEM-BRLR-GFP, used to accommodate a reporter gene expression cassette immediately upstream of the CP open reading frame, is shown at the bottom. All the primers are depicted as small arrows; their RT-PCR products shown in the middle. Restriction sites used for cloning are indicated together with their position in the genome.

Klaassen *et al*. (1994). Total RNA from this virus was purified by 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 cDNA clones was as described for BYV (Peremyslov and Dolja, 2007). The published partial sequence of the GLRaV-2 isolate 94/970 facilitated the characterization of clones and the design of primers. The resulting sequence of the entire 16,486 nt-long GLRaV-2 genome was deposited to Genbank (accession no. FJ436234).

2.3.2 Plasmid vectors and bacterial cells

For constructing a full-length cDNA clone of GLRaV-2, we used a mini-binary vector pCB301 (Xiang *et al*., 1999). This plasmid has a relatively small size of ~3.5kb, accumulates to a low level in *E. coli*, carries a convenient polylinker, and can be used for agroinfection. The cDNA cloning was done using reverse transcription and either conventional synthesis of a dsDNA or PCR amplification. A sequence of a NOS terminator required for transcription termination was first added to the pCB301 by regular cloning using unique sites *Sac* I and *Kpn* I to produce a pCB301-NOS. NOS terminator is routinely used in combination with the CaMV 35S promoter as a natural transcriptional stop signal. Multiple restriction sites, *Sac* I, *Bam* HI, *Aat* II, *Bbv* CI, *Rsr* II, *Bst* EII, and *Sma* I were then inserted between *Sac* I site and NOS terminator to produce a pCB301-NOS-PL. pGEM-3Zf(+) was used to construct an intermediate vector with partial GLRaV-2 cDNA inserted with ERGFP reporter gene followed by a BYV CP promoter. *E. coli* strains XL1-Blue and/or DH10B were used for cloning. *Agrobacterium tumefaciens* strain C58 GV2260 was used for agroinfiltration with the modified full-length cDNA clone.

2.3.3 Adding a sequence of RNA polymerase promoter to the 5'-end of the viral cDNA

The CaMV 35S promoter is widely used for RNA transcription in a variety of plants including *N. benthamiana*. To add a sequence of this promoter fused to the

5'-end of the viral genome, a PCR-mediated DNA splicing technique was used. Separate RT-PCR using the pairs of primers 5-35S-Sac and 3-35SV94-GA, and 5-V94-GA-35S and GV2-c750 (Table 2.1) was used to amplify the 35S promoter and the 5'-end of GLRaV-2 cDNA and to generate products with overlapping ends. These products were combined and used as template for another round of PCR using primers 5-35S-Sac and GV2-c750 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 *Bam* HI (nt 2034) to produce p35S5'LR.

2.3.4 Adding ribozyme to the 3'-end of the viral cDNA

To generate a nearly authentic 3'-terminus of the nascent RNA following transcription of viral cDNA in a plant nucleus, we added a hammerhead ribozyme sequence between the viral cDNA and NOS terminator. The ribozyme RNA is capable of *in-cis* cleavage at a defined position. Design of the ribozyme was based on the minimal sequence described by Haseloff and Gerlach (1988) (Prokhnevsky *et al*., 2002). The secondary structure of the newly engineered ribozyme was checked and analyzed using the web-based RNA secondary structure prediction program (http://bioinfo.hku.hk/Pise/mfold.html). To add the ribozyme sequence to the 3'-end of the viral cDNA, a megaprimer 3V94-Rib with a virus-specific part complementary to the 3'-end of the viral cDNA followed by a ribozyme sequence and a *Sma* I site was used in combination with a regular primer V94-BstEII to amplify the 3'-terminal region of the GLRaV-2 cDNA (nts 14,842-16,486). Resulting PCR product was cloned into P35S5'LR using restriction sites *BstE* II (nt 14,842) and *Sma* I (added at the 3'-terminal of the megaprimer) to produce a p35S5'3'LR-Rib.

2.3.5 RT-PCR amplification and cloning of the internal part of viral RNA

The procedures described above resulted in generation of the cassette that contained the 35S promoter, 5'-terminal and 3'-terminal regions of the GLRaV-2 cDNA, ribozyme, and NOS-terminator in a binary vector. This cassette was then used for assembly of the full-length clone by stepwise addition of large internal cDNA

Table 2.1. Primers used for constructing full-length cDNA clone to GLRaV-2

Name	Sequence	Purpose
5-35S-Sac[a]	TAT**GAGCTC***GCATGCCTGCAGGTCAAC*	5' primer to PCR 35S promoter w/dual enhancer
3-35SV94-GA[b]	CCAACAGAATAT**TC***CTCTCCAAATGAAA TGAACTTCC*	Reverse primer to splice 35S end to 5' GLRaV-2
5-V94-GA-35S[c]	CATTTGGAGAG**GA***ATATTCTGTTGGCTT TCATCTGTGC*	Forward primer to splice 35S end to 5' GLRaV-2
GV2-c750	CGGCTCGAGCGGCGCCACACCAGC	GLRaV-2 positions 2249-2226 for cDNA synthesis
V94-BstEII[d]	TATA**GCGGCCGC***AG**GGTGACCT**TAAGA AGG*	GLRaV-2 positions 15941-15957 for cDNA synthesis
3V94-Rib[e]	TAT**CCCGGG***AAAAGAAGCTTTCGTCCTC ACGGACTCATCAGAAGACATGTGAATCAT GTCTTGA*GCTTCTTTTTTTTTTTTTTAT TT	Reverse PCR primer to add Ribozyme and Sma I to 3' end GLRaV-2
GV2-2030	AACGGGCGGCCGGCGGATCCG	GLRaV-2 positions 2015-2035 for cDNA synthesis
GV2-c3400	ATTACGACGTCCGCGTGCACA	GLRaV-2 positions 3404-3384 for cDNA synthesis
GV2-3380	GTGCACGCGGACGTCGTAATG	GLRaV-2 positions 3385-3405 for cDNA synthesis
GV2-c6300	AAGCGACGCGCTGAGGACACGA	GLRaV-2 positions 6296-6275 for cDNA synthesis
GV2-6250	CTTCGTGCGCATCGTAGGAGC	GLRaV-2 positions 6237-6257 for cDNA synthesis
cV94-RsrII[f]	TATA**GCGGCCGC***AAG**CGGACCG**AGTA ATGAGG*	GLRaV-2 positions 10830-10811 for cDNA synthesis
GV2-SacI-RsrII[g]	TATA**GAGCTC***C**CTCGGTCCG**CTTGGTTA AGATC*	GLRaV-2 positions 10819-10840 for cDNA synthesis
cGV2-PstI-BstEII[h]	TATA**CTGCAG***AA**GGTCACC**CTCTGTTA CTAC*	GLRaV-2 positions 15931-15951 for cDNA synthesis

[a] Boldface letters, added *Sac* I site; italicized letters, 5'-terminal 18 nucleotides of 35S promoter.
[b] Boldface letters, added GA between 35S and GLRaV-2; italicized letters, 23 nucleotides complementary to the 3'-terminal of 35S promoter; underlined letters, 11 nucleotides complementary to the 5'-terminal of GLRaV-2.
[c] Boldface letters, added GA between 35S and GLRaV-2; italicized letters, 5'-terminal 25 nucleotides of GLRaV-2; underlined letters, 3'-terminal 11 nucleotides of 35S promoter.
[d] Boldface letters, *Not* I (added) and *Bst* EII (in genome) sites; italicized letters, GLRaV-2 positions 15941-15957.
[e] Boldface letters, added *Sma* I site; italicized letters, Ribozyme.
[f] Boldface letters, *Not* I (added) and *Rsr* II (in genome) sites; italicized letters, GLRaV-2 positions 10830-10811.
[g] Boldface letters, *Sac* I (added) and *Rsr* II (in genome) sites; italicized letters, GLRaV-2 positions 10819-10840.
[h] Boldface letters, *Pst* I (added) and *Bst* EII (in genome) sites; italicized letters, GLRaV-2 positions 15951-15931.

fragments using appropriate restriction sites naturally preset in the viral cDNA. For cloning the internal region (nt 2029-10827), three partially overlapping fragments of ds cDNA were obtained using conventional cDNA cloning and Gibco-BRL protocol for SuperScript II reverse transcriptase. Separate RT-PCR using the pairs of primers GV2-2030 and GV2-c3400, GV2-3380 and GV2-c6300, and GV2-6250 and cV94-RsrII (Table 2.1) were used to amplify these three partially overlapping fragments. Resulting RT-PCR fragments were inserted into p35S5'3'LR-Rib using restriction sites *Bam* HI (nt 2029) and *Ata* II (nt 3394), *Ata* II and *Bbv* CI (nt 6281), and *Bbv* CI and *Rsr* II (nt 10,821), respectively, to generate p35S-5'BR3'LR-Rib.

2.3.6 Adding a reporter gene expression cassette

In order to modify the full-length clone to accommodate a reporter gene expression cassette upstream of the CP ORF, the remaining part of the viral cDNA (nt 10,821-14,848) was PCR-amplified and cloned into an intermediate vector pGEM-3Zf(+). This part was PCR-amplified using primers GV2-SacI-RsrII and cGV2-PstI-BstEII (Table 2.1) and inserted into pGEM-3Zf(+) using unique sites *Sac* I (added at the 5'-terminal of the primer GV2-SacI-RsrII) and *Pst* I (added at the 5'-terminal of the primer cGV2-PstI-BstEII) to generate pGEM-RBLR. A polylinker containing restriction sites *Pac* I, *Fse* I, and *Sda* I was inserted upstream from the 5'-end of GLRaV-2 CP ORF to generate pGEM-RBLR-PFS. A PCR fragment containing a nucleotide sequence encoding an endoplasmic reticulum-targeted GFP (Haseloff *et al*., 1997) followed by a BYV CP promoter was inserted into pGEM-RBLR-PFS using restriction sites *Pac* I and *Sda* I to generate pGEM-RBLR-GFP. As a result, the BYV CP promoter directed the expression of the GLRaV-2 CP, while the authentic GLRaV-2 CP promoter expressed the GFP reporter. The resulting cDNA fragment in the pGEM-RBLR-GFP was then cloned back into p35S-5'BR3'LR-Rib using *Rsr* II (nt 10,821) and *Bst* EII (nt 14,842) sites to generate the full-length GRLaV-2 clone p35S-LR-GFP or LR-GFP for the brevity.

2.3.7 Agrobacterium-mediated delivery of the viral cDNA clone to plants

To investigate the infectivity of the viral full-length cDNA clone in *N. benthamiana*, *A. tumefaciens* strain C58 GV2260 was transformed with p35S-LR-GFP by electroporation. *Agrobacterium*-mediated delivery of the viral cDNA is the method of choice for launching large viral RNAs. Its major advantage over mechanical inoculation with *in vitro* RNA transcripts is much more efficient delivery of the viral cDNA to cells including phloem tissue that is preferentially colonized by closteroviruses. Transformed bacterial cultures were grown overnight at 28℃ with shaking, spun down and resuspended in a buffer containing 10 mM MES-KOH (pH 5.85), 10 mM $MgCl_2$, and 150 mM acetosyringone. Bacterial suspensions were then mixed with corresponding culture transformed to express an RNAi suppressor P1/HC-Pro from *Turnip mosaic virus* to enhance infectivity (Chiba *et al*., 2006). The induced bacterial cultures were infiltrated into leaves of young *N. benthamiana* (6-8 leaf stage) plants. The GFP-fluorescent cells were visualized using epifluorescent stereomicroscope Leica MZ 16F (Deerfield, IL) at 8 days in the infiltrated leaf and at 3 weeks in the systemic leaves, stems and petioles.

2.4 Results

2.4.1 Construction of the full-length cDNA clone of GLRaV-2

The entire, 16,486 nt-long GLRaV-2 cDNA cloned into pCB301 as described above 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. The resulting full-length GLRaV-2 clone was further modified to accommodate GFP ORF as described to result in LR-GFP (Fig. 2.1).

2.4.2 Infectivity assays using LR-GFP

Our objective for producing LR-GFP was to artificially extend the viral infection cycle by adding a DNA phase. This approach permits plant infection via launching virus to *N. benthamiana* plants using LR-GFP clone and agroinfiltration. The infiltrated leaves were screened for the GFP and CP expression at 8 days post-inoculation. The inoculated plants were then screened for the symptom appearance and the vascular tissues of inoculated plants were screened for the GFP expression at 3 weeks post-inoculation.

At 8 days post-inoculation, the green fluorescent cells were observed under epifluorescent stereomicroscope on the LR-GFP infiltrated leaves indicating the expression of GFP reporter (Fig. 2.2A). SDS-PAGE and Western blot using the sap of the LR-GFP infiltrated leaves and antiserum to GLRaV-2 CP as a probe showed the presence of a single protein band corresponding to GLRaV-2 CP (Fig. 2.2B) demonstrating the expression of GLRaV-2 CP in the infiltrated leaves.

At 3 weeks postinoculation, severe disease symptom started to develop on the plants inoculated with LR-GFP (Fig. 2.3A, plant on the left). Vein clearing and wilting symptoms could be found on the noninfiltrated leaves on the top of LR-GFP inoculated plants (Fig. 2.3B, leaf on the left). In contrast, the pCB301-empty vector inoculated control plants remained asymptomatic thought the test and were able to develop to normal maturity (Fig. 2.3A, plant on the right; Fig. 2.3B, leaf on the right).

To further confirm the replication and transportation of GLRaV-2 throughout the whole inoculated plants, the expression of the GFP reporter was checked using epifluorescent stereomicroscope. The GFP-fluorescent cells could be visualized on the systemic leaves, stems, and petioles of LR-GFP inoculated *N. benthamiana* (Fig. 2.4), but not in the control plants. These results suggested that the LR-GFP clone was indeed infectious, and the infiltrated LR-GFP could not only replicate in the infiltrated site but also spread systemically throughout the plant, primarily in the phloem tissue.

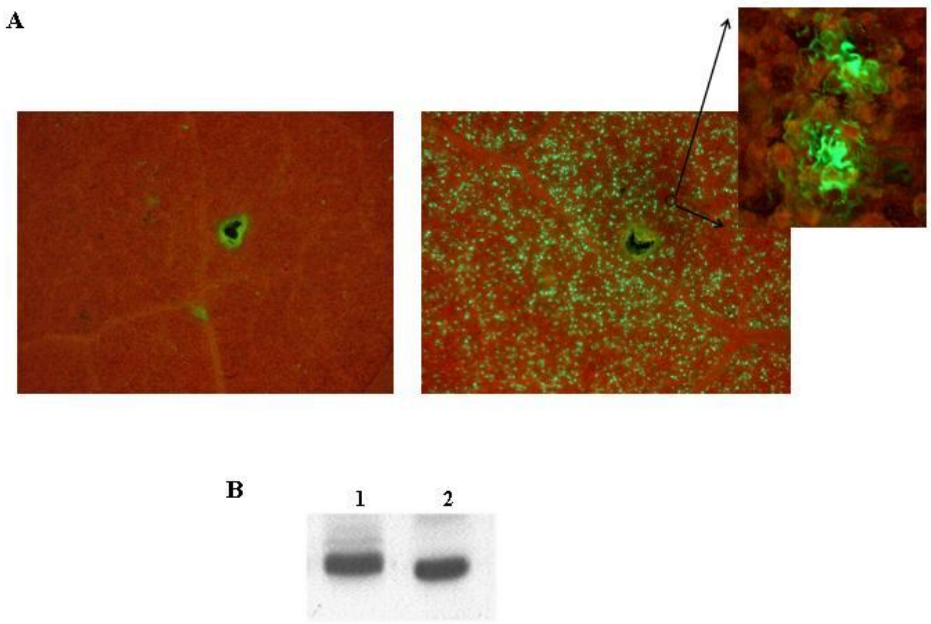

Fig. 2.2. Replication of LR-GFP following agroinoculation of *N. benthamiana*. (A) Images of the leaves inoculated with pCB301-empty vector (left) or p35S-LR-GFP (right) at 8 days post-inoculation under epifluorescent stereoscope, showing the GFP expression in the LR-GFP inoculated leaf. Black spots in the middle are infiltration sites. (B) Western blot analysis using antiserum to GLRaV-2 coat protein, showing detection of the coat protein in plants inoculated with cDNA clone under the control of the 35S promoter. 1, purified virus; 2, leaves inoculated with LR-GFP clone.

2.5 Discussion

In order to investigate the functions of GLRaV-2 leader proteases and to generate a virus-based gene expression vector for the grapevine, we have obtained a biologically active full-length cDNA clone (Fig. 2.1) that launches a virus capable of replication, systemic infection, and disease induction (Figs. 2.2, 2.3 and 2.4). Despite successful generation of the cDNA clones of BYV (Peremyslov *et al*., 1998), and CTV (Satyanarayana *et al*., 2001), making such clones for closteroviruses still remains technologically challenging because of their large genome sizes. One critical issue is

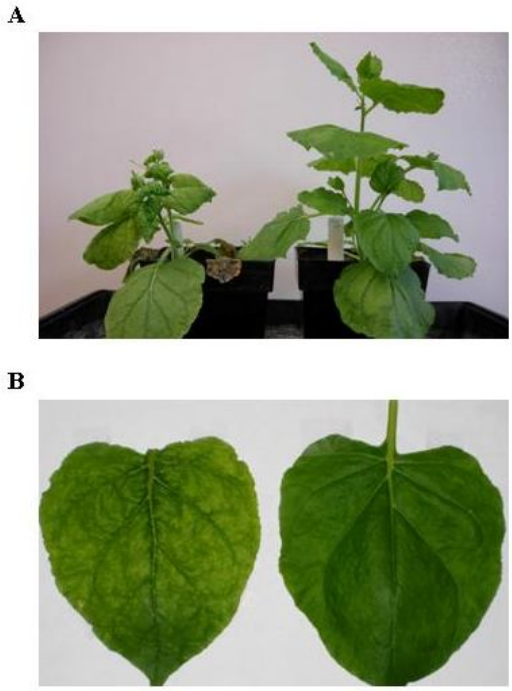

Fig. 2.3. *N. benthamiana* plants agroinoculated with LR-GFP were systemically infected and showed disease symptom on almost whole plant (A, plant on the left) at 3 weeks postinoculation. Vein clearing and wilting symptoms showed on the systemic leaf of LR-GFP inoculated plant (B, leaf on the left). *N. benthamiana* plant agroinoculated with pCB301-empty vector didn't show any symptoms (A, plant on the right; B, leaf on the right)

the ability to generate cDNA that is as close to authentic viral genome as possible. To achieve this, the design of junctions between the RNA polymerase promoter and ribozyme with the 5'- and 3'-ends of viral cDNA need to result in an efficient RNA transcription and a few if any non-viral nucleotides at the transcript ends. Another issue that is beyond the control of a researcher, is incidental toxicity of large foreign DNAs for *E. coli*, which may result in a slow growth of transformed cells and selection for bacterial strains that possess mutant, rearranged, or lost cDNAs

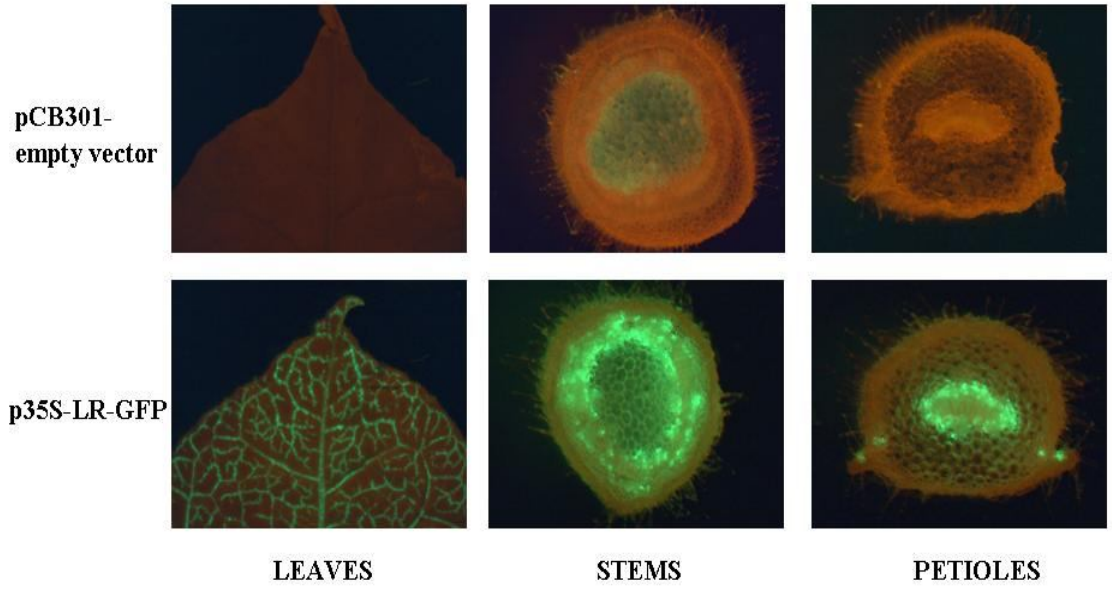

Fig. 2.4. LR-GFP expresses reporter in vascular tissues throughout the plant**.** Images of the systemic leaves, stems, and petioles of *N. benthamiana* inoculated with pCB301-empty vector (top) as control or p35S-LR-GFP (bottom) at 3 weeks post-inoculation under epifluorescent stereoscope.

(Satyanarayana *et al*., 2003). To mitigate this problem, we used a low copy number binary vector, pCB-301, which could also be used to infect plants via agroinfiltration.

The ability of our LR-GFP clone to efficiently produce GFP highlights a potential of this virus as a gene expression vector for the grapevine. In general, *Closterovirus*-derived vectors provide advantages of a 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 RNAi suppressors with p24 of GLRaV-2 being 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.

Another limitation for the utility of GLRaV-2-derived vector is its restriction to the phloem tissue of the grapevine. However, producing proteins with secretion signal peptides to export the protein out of the cell into the intercellular space where the protein or peptide is dispersed in the liquid films between cells could probably reduce this limitation (Folimonov *et al*., 2007) and make the vector useful for controlling fungal or bacterial pathogens. Furthermore, phloem itself plays critical roles in grapevine physiology (transport of metabolites) and pathology (a target of viral infections).

It should be emphasized that the addition of an extra gene expression cassette had no obvious effect on the virus infectivity or spread. Although the maximal genetic capacity of the GLRaV-2 vector is not known, our experience with a similar BYV vector indicated that at least three foreign proteins could be expressed using this vector (Peremyslov and Dolja, unpublished). Thus, it seems likely that a GLRaV-2 vector could be designed to express multiple foreign genes.

The ability to propagate and manipulate the viral genome in *E. coli* is a major advance for examination of GLRaV-2 genome. As was previously shown for BYV (Hagiwara *et al*., 1999), generation of the mini-replicon-based vector in which only the replication-associated genes are retained, has even greater utility for studying functions of these genes. Because of that, we have also generated a tagged mini-LR replicon that we used to address the functions of the GLRaV-2 leader proteases (see below).

Chapter 3

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

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Virology (2009) **383**, 291-299

3.1 Abstract

Several viruses in the genus *Closterovirus* including 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-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.

3.2 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). The several distinct classes of the viral proteases appear to have evolved independently following acquisition of the related enzymes of the host cells with the chymotrypsin-like protease of picona-like viruses being a prime example (Gorbalenya *et al*., 1989).

Another class of widespread viral proteases is papain-like protease present in diverse families of the positive-strand RNA viruses (Gorbalenya *et al*., 1991). Independent of their evolutionary origins, viral proteases can be subdivided into the categories of main and leader proteases. The main proteases typified by the picornaviral 3CPro are responsible for multiple cleavages within the viral polyprotein. In contrast, leader proteases are typically located at the N-terminus of the polyprotein and cleave *in-cis* only at their own C-termini. A well-studied example of a leader protease is an eponymous protease of the animal aphtoviruses (de los Santos *et al*., 2006). Interestingly, several diverse viral groups, e.g., animal nidoviruses (Ziebuhr *et al*., 2000) and plant potyviruses (Urcuqui-Inchima *et al*., 2001) possess two leader proteases arranged in a tandem.

In addition to self-processing, many leader proteases are required for efficient amplification or transcription of the viral genome. In particular, nsp1 papain-like protease of arteriviruses regulates synthesis of the subgenomic mRNAs (Tijms *et al*., 2007), while the potyviral papain-like protease HC-Pro is required for efficient genome accumulation (Kasschau *et al*., 1997). Another common theme in the functions of leader proteases is virus-host interactions. Thus, aphtoviral leader protease facilitates viral infection by interfering with regulation of the host protein synthesis and innate immune response (de los Santos *et al*., 2006). The papain-like proteases of the potyviruses and fungal hypoviruses are the potent suppressors of the antiviral RNAi response (Ding and Voinnet, 2007; Lakatos *et al*., 2006; Segers *et al*., 2006). The fact that in different potyviruses either papain-like or chymotrypsin-like protease can assume the RNAi suppression function (Valli *et al*., 2006) highlights the evolutionary plasticity of the leader proteases. Indeed, many of these proteases contain additional domains such as insect transmission (Ng and Falk, 2006) or RNA demethylase (Susaimuthu *et al*., 2008; van den Born *et al*., 2008) domains found in papain-like and chymotrypsin-like proteases, respectively, of the distinct potyviruses.

Closteroviruses possess the largest genomes among all known plant viruses and belong to the alphavirus-like superfamily of the positive-strand RNA viruses (Koonin and Dolja, 1993). The family *Closteroviridae* contains three genera defined on the

basis of phylogenetic analysis, genome organization, and the type of the vectoring insects (Dolja *et al*., 2006; Karasev, 2000). In particular, genus *Closterovirus* with BYV as a prototype member (Dolja, 2003) contains the aphid-transmissible viruses with the 15-20 kb, monopartite genomes. Similar to other family members, BYV encodes a L-Pro, RNA replicase (Peng *et al*., 2001; Peremyslov *et al*., 1998), and a quintuple block of genes responsible for virion assembly and cell-to-cell movement (Alzhanova *et al*., 2000; Alzhanova *et al*., 2001; Alzhanova *et al*., 2007; Peremyslov *et al*., 1999; Peremyslov *et al*., 2004b). In addition, BYV encodes a RNAi suppressor that is conserved in this genus (Chiba *et al*., 2006; Lu *et al*., 2004; Reed *et al*., 2003), and a 20-kDa long-distance transport factor (p20) (Prokhnevsky *et al*., 2002).

Perhaps, the most conspicuous feature of closteroviruses is the complex molecular architecture of their exceptionally long filamentous virions. These virions contain an over 1000 nm-long "body" assembled by the major CP and a ~100 nm-long "tail" whose major component is CPm (Agranovsky *et al*., 1995) evolutionary related to CP (Boyko *et al*., 1992). Tails encapsidate ~700 nt-long, 5'-terminal region of the virion RNA (Alzhanova *et al*., 2007; Peremyslov *et al*., 2004a; Peremyslov *et al*., 2004b; Satyanarayana *et al*., 2004). In addition to CPm, tails incorporate the Hsp70 homolog and ~60-kDa protein (Napuli *et al*., 2003; Napuli *et al*., 2000; Peremyslov *et al*., 2004a; Peremyslov *et al*., 2004b; Satyanarayana *et al*., 2000; Tian *et al*., 1999) that facilitate tail assembly by CPm and define the tail length (Alzhanova *et al*., 2007; Satyanarayana *et al*., 2004). In BYV, tail exhibits a three-segment structure with the tip segment likely formed by the long-distance transport factor p20 (Peremyslov *et al*., 2004a). Because the mutant tail-less virions formed by CP alone are capable of encapsidating the entire genome (Alzhanova *et al*., 2001; Satyanarayana *et al*., 2004), and because each tail component is essential for virus transport (Alzhanova *et al*., 2000; Peremyslov *et al*., 1999; Prokhnevsky *et al*., 2002), tail apparently evolved as a device for facilitating *Closterovirus* spread in the infected plants (Dolja *et al*., 2006). Strikingly, tail-like structures potentially involved in virion transport were also discovered in potyviruses (Gabrenaite-Verkhovskaya *et al*., 2008; Torrance *et al*., 2005).

Our previous work showed that the BYV L-Pro is required for efficient RNA amplification and virus long-distance transport (Peng *et al*., 2003; Peng and Dolja, 2000). Interestingly, replacement of L-Pro by the proteases from other closteroviruses (Peng *et al*., 2001) or even from an animal arterivirus (Peng *et al*., 2002b) can rescue the RNA amplification, but not the transport function of the leader protease. It seems likely that the role of L-Pro in systemic transport is dual. First, it has a specific, protein-mediated role that was revealed by the site-directed mutagenesis (Peng *et al*., 2003). Second, because tail encapsidates the 5'-terminal one third of the protease-coding region, mutations in this area can interfere with the tail assembly and virus transport.

The GLRaV-2 is a close BYV relative in the *Closterovirus* genus whose genetic organization is almost identical to that of BYV (Zhu *et al*., 1998). However, unlike BYV that possesses one leader protease, GLRaV-2 codes for two leader proteases, L1 and L2 (Meng *et al*., 2005; Peng *et al*., 2001) (Fig. 3.1A, top diagram). Here we demonstrate that L1 and L2 have complementary functions in establishment of the GLRaV-2 infection in the initially inoculated cells and systemic transport. Strikingly, overall contribution 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 evolved to facilitate an expansion of the *Closterovirus* host range.

3.3 Materials and Methods

3.3.1 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. 3.1A) 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. 3.1A). The GFP ORF was then replaced with a hybrid GFP/GUS ORF described

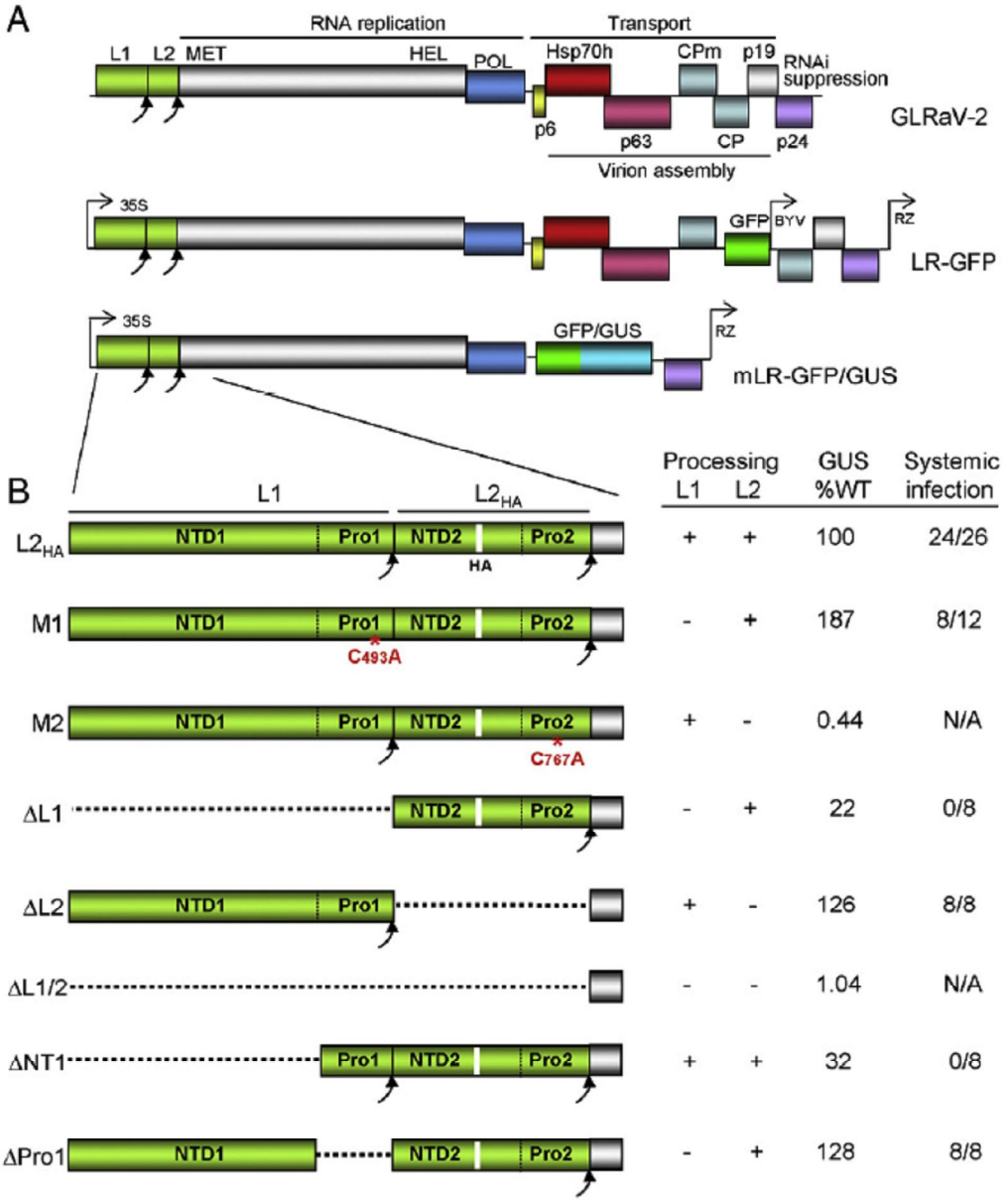

Fig. 3.1. (A) Diagrams of GLRaV-2 genome (top), full-length, GFP-tagged cDNA clone of GLRaV-2 (LR-GFP, middle) and GFP/GUS-tagged minireplicon (mLR-GFP/GUS, bottom). Functions of viral genes are shown above and below the diagram. L1 and L2, leader proteases; MET, HEL, and POL, methyltransferase, RNA helicase, and RNA polymerase domains, respectively; p6, 6-kDa movement protein; Hsp70h, Hsp70 homolog; p63, 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. $L2_{HA}$, insertion of the triple hemagglutinin epitope into L2 (HA, white strip); M1, replacement of the L1 catalytic Cys residue with Ala ($C_{493}A$); M2, replacement of the L2 catalytic Cys residue with Ala ($C_{767}A$); 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.

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-35SLR-Pro and pGEM-SP6LR-Pro, containing the whole L1 and L2 coding region and a fragment of the methyltransferase coding region (nts 1-3071) were generated by cloning the corresponding PCR-amplified fragments (Fig. 3.1B) into pGEM-3Zf(+). Both pGEM-35SLR-Pro and pGEM-SP6LR-Pro were used to generate pGEM-35SLR-$L2_{HA}$ and pGEM-SP635SLR-$L2_{HA}$ 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 Cys_{493} residue of L1 with Ala using site-directed mutagenesis. Analogously, mutation 2 (M2) was obtained via substitution of Ala for Cys_{767} of L2. In ΔL2 mutation, the entire L2-coding region was deleted and Lys_{848} residue 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 mutationΔPro1, 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. 3.1B.

The pGEM-SP6LR-L2$_{HA}$ variants were used to analyze the proteolytic activity of the mutated proteases in vitro. The DNA fragments from the mutant derivatives of pGEM-35SLR-L2$_{HA}$ were cloned into mLR-GFP/GUS using *Sbf* I (located in the vector part of the plasmid) and *Stu* 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).

3.3.2 Mutation analysis of the proteolytic activity of L1 and L2

The pGEM-SP6LR-L2$_{HA}$ 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 [^{35}S]-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.

3.3.3 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 $MgCl_2$, and 150 mM acetosyringone. Bacterial suspensions of each variant were mixed with corresponding cultures transformed to express an RNAi suppressor P1/HC-Pro from *Turnip mosaic virus* to enhance minireplicon infectivity (Chiba *et al*., 2006). The final bacterial concentrations were

1.0 OD_{600} for minireplicon-expressing variants and 0.1 OD600 for the P1/HC-Pro-expressing variant. The induced bacterial cultures were infiltrated into lower surface of the *N. benthamiana* leaves using a syringe without a needle or vacuum infiltrated into the grapevine leaves. The GFP-fluorescent leaf cells were visualized using epifluorescent stereomicroscope Leica MZ 16F (Deerfield, IL) at 8 days post-infiltration. Samples for GUS assays were prepared and GUS activity was measured using Hoefer TKO100 DNA fluorimeter (Hoefer Scientific Instruments) as described (Dolja *et al*., 1992).

3.3.4 Analysis of the local and systemic virus transport

To assay the cell-to-cell movement of the GFP-tagged virus variants, virions were isolated from the agroinfiltrated leaves of *N. benthamiana* at 2 weeks post-inoculation (Napuli *et al*., 2000), resuspended in a buffer containing 20 mM sodium phosphate (pH 7.4) and 1 mM Na_2-EDTA and inoculated manually to leaves of *N. benthamiana*. The fluorescent infection foci were analyzed using the epifluorescent stereomicroscope at 8 days post-inoculation.

To investigate the systemic spread in *N. benthamiana,* plasmids carrying the corresponding variants in a context of the LR-GFP were mobilized into *A. tumefaciens*, the resulting bacterial suspensions were mixed with those engineered to express P1/HC-Pro as described above, and infiltrated into leaves of young *N. benthamiana* (6-8 leaf stage) plants. After 3, 4, or 5 weeks, the upper leaves of these plants were screened for the symptom development, whereas epifluorescence microscopy and a spot camera MicroPublisher3.3 RTV (QImaging) were used to document accumulation of the virus-expressed GFP. Immunoblotting and custom-made GLRaV-2-specific antiserum in 1:5000 dilution were used to document accumulation of CP.

3.3.5 Virion analyses

To determine if HA-tagged L2 was associated with the virions, the sucrose gradient fractionation followed by immunoblotting was used. Virions isolated as

described above were resuspended in a buffer containing 20mMNa-phosphate (pH 7.4) and 1mMNa_2-EDTA, loaded to the top of 10-40% sucrose gradients prepared in the same buffer, and centrifuged at 25,000 RPM for 4 h in a Beckman SW40 rotor at 4°C. Gradients were separated into 25 fractions and the immunoblot analysis was done using anti-HA rat monoclonal antibody (Roche) and GLRaV-2-specific antibody to detect $L2_{HA}$ and CP, respectively. The immunogold-specific electron microscopy to detect $L2_{HA}$ was done essentially as described (Medina *et al*., 1999).

3.4 Results

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

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. 3.1A, 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 ($L2_{HA}$ in the Figs. 3.1B and 3.2). 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

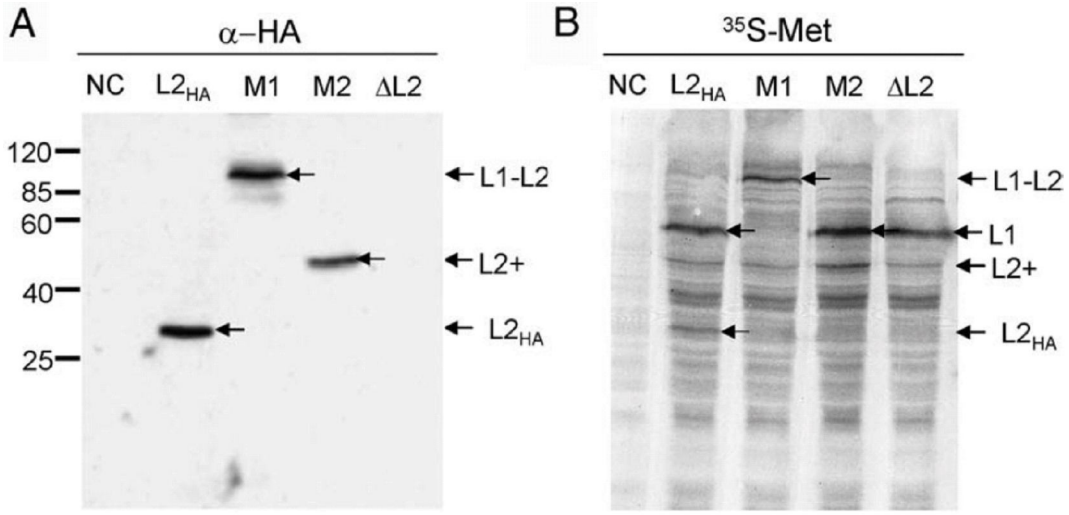

Fig. 3.2. L1- and L2-mediated processing of the N-terminal part of the GLRaV-2 polyprotein generated *in vitro*. Lanes correspond to mutant variants shown in the Fig. 3B except for NC, no RNA control. (A) Immunoblot analysis of the in vitro translation products using HA-specific antibody (α-HA) to detect 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; $L2_{HA}$, fully processed, HA-tagged L2. Numbers at the left show the mol. mass (kDa) of the protein markers. (B) Analysis of the ^{35}*S*-methyonine-labeled in vitro translation products. Designations are as in (A).

reporter in the initially inoculated cells (Fig. 3.1B 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. 3.1A and data not shown). Therefore, we successfully generated a series of the infectious tagged GLRaV-2 replicons that can be launched to *N. benthamiana* and used to address L1 and L2 functions in the

viral infection cycle.

3.4.2 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. 3.1B). 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 (Cys_{493} and Cys_{767}) (Peng *et al*., 2001) were replaced by alanine residues to result in M1 and M2 variants, respectively (Fig. 3.1B). 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. 3.1B). The resulting translation products were analyzed using either immunoblotting and HA-specific antibody (Fig. 3.2A) or ^{35}S-methionine labeling (Fig. 3.2B). As expected, a tagged non-mutant variant produced single HA-positive band corresponding to the fully-processed, HA-tagged L2 (Fig. 3.1B and Fig. 3.2A lane $L2_{HA}$) and, in addition, isotope-labeled, fully processed L1 (Figs. 3.1B and 3.2B, lane $L2_{HA}$).

In contrast, translation of the M1 variant resulted in accumulation of a single major product corresponding to a L1-L2 fusion (Fig. 3.1B; Figs. 3.2A 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. 3.1B; Figs. 3.2A 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. 3.1B). In contrast, inactivation of L2 cleavage virtually abolished minireplicon infectivity: the corresponding GUS expression level was less than 0.5% of that of the parental mLR-GFP/GUS (Fig. 3.1B). This result is in complete agreement with the strict requirement for the cleavage by L-Pro for BYV minireplicon infectivity (Peremyslov *et al*., 1998); indeed fusion of either L-Pro or L2 with the replicase is likely to interfere with the synthesis of viral RNAs.

To further define the roles of L1 and L2 in RNA accumulation, we have generated the mutants in which the coding regions of L1, L2, or both, were deleted. Interestingly, the L1 null mutant ΔL1 was capable of replication, although a corresponding level of GUS activity was ~5-fold lower than that in the parental mLR-GFP/GUS variant (Fig. 3.1B). Unexpectedly, deletion of L2 in the ΔL2 variant resulted in a slight increase in GUS expression suggesting that L2 is not essential for minireplicon accumulation in the *N. benthamiana* cells (Fig. 3.1B). However, simultaneous deletion of L1 and L2 yielded the minireplicon ΔL1/2 that expressed only ~1% of the GUS activity observed in a parental mLR-GFP/GUS variant (Fig. 3.1B). Taken together, these results indicated that although the role of L1 in viral RNA amplification is more prominent than that of L2, the latter protease can rescue RNA accumulation of the L1-deficient mutant, and therefore L1 and L2 have partially overlapping functions in this process.

Both L1 and L2 possess the C-terminal papain-like protease domains (Pro1 and Pro2, respectively) and the N-terminal domains (NTD1 and NTD2, respectively; Fig. 3.1B). To determine the relative contributions of NTD1 and Pro1 in the L1 function, we generated ΔNTD1 and ΔPro1 variants in which these domains were deleted (Fig. 3.1B). 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. 3.1B). 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.

3.4.3 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, virions were isolated from the inoculated leaves and the virion 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. 3.1B and 3.3A). Similar competence for the systemic spread was

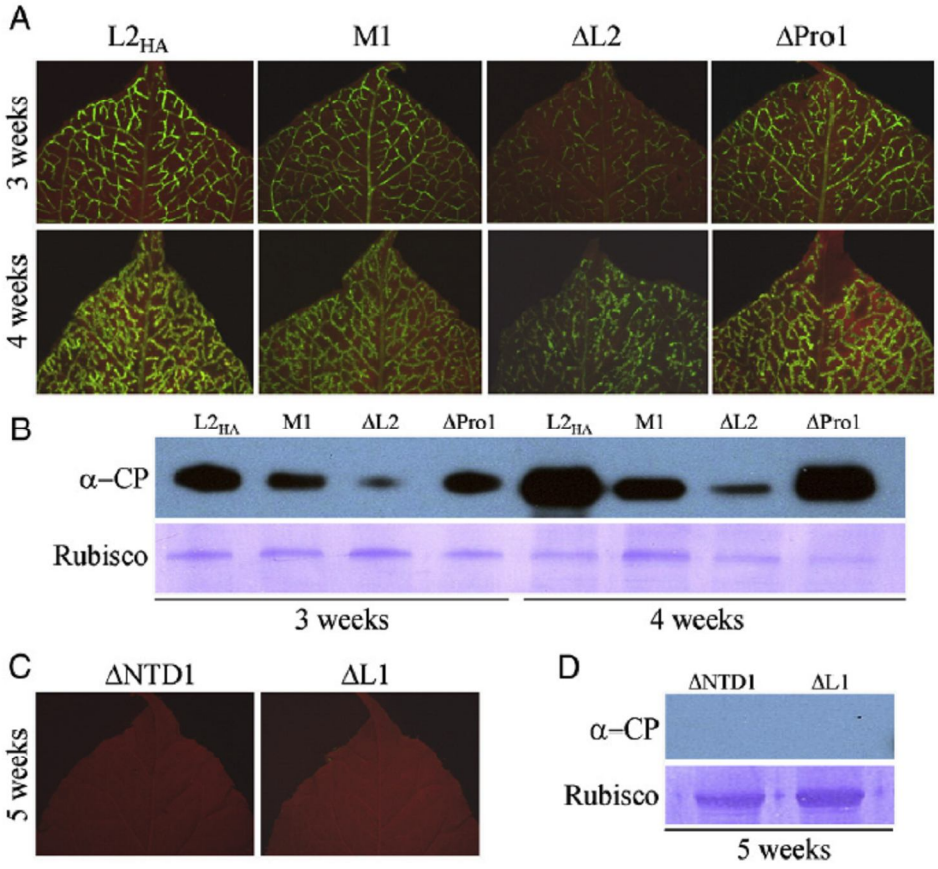

Fig. 3.3. Systemic transport of the LR-GFP variants harboring HA-tagged L2 following agroinoculation of N. benthamiana. Variants are marked as in Fig. 3B. (A) Images of the upper, noninoculated leaves under epifluorescent stereoscope showing GFP expressed in veins. (B) Immunoblot analyses using anti-CP antibody (α-CP, top). Bottom panel, loading control showing Coomassie-stained Rubisco band on the membrane used for immunoblotting. (C and D) Lack of systemic infection in the plants agroinoculated using ΔNTD1 and ΔL1 variants revealed using epifluorescent microscopy (C) or immunoblotting (D).

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. 3.1B and 3.3C).

Observation of the systemically infected leaves revealed apparent differences in

the GFP accumulation between the experimental variants (Fig. 3.1A). 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 $L2_{HA}$ variant (Fig. 3.3B). 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. 3.3B). 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 3.3C 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. 3.4). 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. 3.4). 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. 3.4, upper inset). The L2 peak fractions 15-17 contained much larger numbers of gold microspheres, but virtually no virions (Fig. 3.4, bottom inset) suggesting that L2

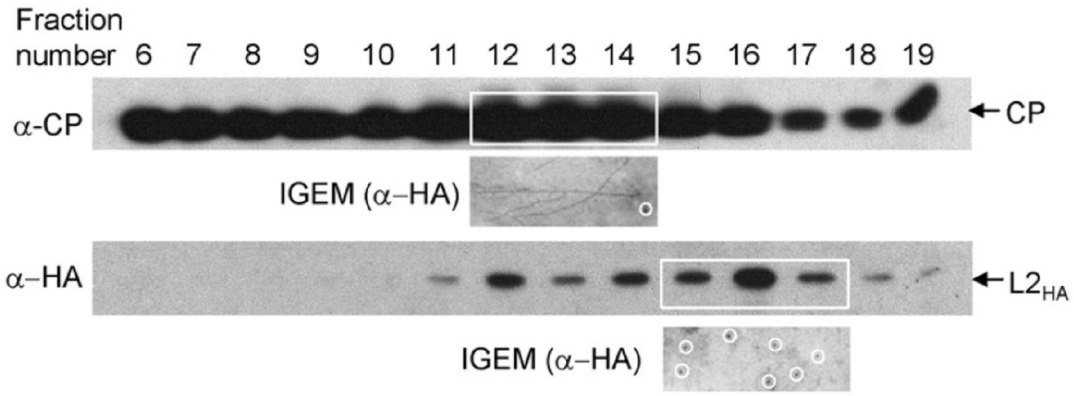

Fig. 3.4. Sucrose density gradient separation and immunoblot analysis of the GLRaV2 virions using anti-CP antibody (α-CP, top) or anti-HA antibody (α-HA, bottom). Gradient fractions were numbered from the bottom of the gradient. The insets show HA-specific immunogold electron microscopy analysis (IGEM) of the combined fractions marked by white boxes; white circles highlight gold microspheres. Arrows mark positions of L2-Pro and CP.

is not directly associated with GLRaV- 2 virions.

3.4.4 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 3.1). At 8 days post-inoculation with the parental mLR-GFP/GUS variant, up to ~300 unicellular, GFP-fluorescent 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 3.1). However, similar to what was observed in

Table 3.1. Infectivity and GUS expression by mLR-GFP/GUS minireplicon variants in *V. vinifera*

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/GFU	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/GFU	100.00	100.00
2	ΔL1	1.58	2.93
2	ΔL2	10.44	10.87
2	M1	133.43	118.08

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 3.1) 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.

3.5 Discussion

Previous work suggested that the tandems of the leader proteases in different closteroviruses 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. 3.1B and 3.2). Similar to BYV, the cleavage upstream from the methyltransferase domain of the viral polyprotein is essential for GLRaV-2 viability (Fig. 3.1B) (Peremyslov *et al*., 1998). Surprisingly, although L1 does cleave at its own C-terminus both *in vitro* (Fig. 3.2) and *in vivo* (Fig. 3.4), 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. 3.1B and 3.3). However, slower virus accumulation in the non-inoculated leaves in these mutants (Figs. 3.3A 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. 3.1B). 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. 3.3C). 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. 3.2A 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 2.1). 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 3.1). 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 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).

3.6 Acknowledgments

The authors are thankful to Robert R. Martin and Karen Keller (USDA-ARS, Corvallis, Oregon), Baozhong Meng (University of Guelph, Ontario, Canada), Dariusz E. Goszczynski (Plant Protection Research Institute, Pretoria, South Africa), and Alexey I. Prokhnevsky (Oregon State University) for their help and advice at the initial phase of this work. Presented research was supported by contract with Growers Research Group, L.L.C. (Soledad, California) and BARD award No. IS-3784-05 to V.V.D.

Chapter 4

General Conclusion

Because the infectious cDNA clones provided powerful tools for investigation of the RNA virus gene functions (Peremyslov and Dolja, 2007; Pogue *et al*., 2002), the major objective of this thesis was to generate the full-length GLRaV-2 cDNA clone and use this clone to investigate the functional profiles of two papain-like leader proteases (L1 and L2) in the GLRaV-2 infection cycle.

In general, conversion of RNAs into faithful cDNA clones is a challenging task especially for the viruses with large genomes. As described in chapter 2, a full length cDNA copy of the genomic RNA of GLRaV-2 was constructed with a CaMV 35S RNA polymerase promoter and a ribozyme sequence inserted upstream and downstream of the GLRaV-2 sequence, respectively, in a binary vector. The resulting full-length GLRaV-2 clone was further modified to accommodate a GFP reporter gene expression cassette immediately upstream of the CP open reading frame. After agroinfiltration of *N. benthamiana* with the modified full-length cDNA clone LR-GFP, plants developed typical symptoms, GFP expression was observed throughout the plant, and viral CP could be detected by immunoblot analysis, demonstrating that the GFP-tagged viral cDNA clone was fully biologically active.

Virus-derived vectors based on viruses that infect herbaceous plants are widely used in plant molecular biology and genomics for expressing or silencing genes in plants. Yet there is a pressing need for viral vectors for woody plants, which demand greater genetic stability because of the longer time required for systemic infection in these plants. The generation of full-length GLRaV-2 cDNA clone tagged by a reporter gene highlights a potential of this cDNA clone as a gene expression vector for the grapevine. In general, *Closterovirus*-derived vectors provide advantages of unmatched genetic stability (Folimonov *et al*., 2007) and ability to accommodate multiple expression cassettes and efficiently produce reporters or other beneficial proteins in the infected plants (Dolja *et al*., 2006). However, full realization of the GLRaV-2 vector potential requires development of the efficient inoculation technique for the grapevine.

Although most viruses in *Closterovirus* genus possess one leader protease, CTV, GLRaV-2, and some other closteroviruses code for two leader proteases, L1 and L2

(Peng *et al*., 2001). Previous phylogenetic analysis suggested that the tandems of the leader proteases emerged via independent gene duplication events (Peng *et al*., 2001). However, the exact functions of L1 and L2 in a context of viral infection remained unexplored. The experimental analysis presented in the chapter 3 corroborated previous 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 chapter 3, we describe generation of a mini-GLRaV-2 variant that possessed only the genes required for optimal RNA replication and was tagged by insertion of a GFP/GUS reporter that allowed both visual detection of the infected cells and sensitive measurements of RNA accumulation and expression (mLR-GFP/GUS). This mini-replicon was used to facilitate investigation of the roles of L1 and L2 in genome amplification (Hagiwara *et al*., 1999; Peng and Dolja, 2000). To permit immunochemical detection of L2, both LR-GFP and mLR-GFP/GUS were further modified by an insertion of the triple hemagglutinin epitope tag (HA) into the N-terminal domain of L2. In the first part of the chapter 3, we demonstrated that insertion of HA-tag did not significantly affect viral genome amplification, and that HA-tagged LR-GFP was systemically infectious upon agroinfection of an experimental host plant *N. benthamiana*. Therefore, we successfully generated a series of the infectious, tagged, GLRaV-2 replicons that can be launched to *N. benthamiana*. To examine L1 and L2 functions, several point mutations and deletions were introduced into corresponding coding region. We found that both L1 and L2 are the active proteases with conserved catalytic cysteines, and that the cleavage upstream from the methyltransferase domain of the viral polyprotein is essential for GLRaV-2 viability. Complete deletion of L1 resulted in a ~5-fold reduction in RNA level, whereas deletion of L2 did not affect RNA accumulation. However, 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. L1 or its coding region was also proved to be essential for virus ability to establish infection, while L2 plays an accessory role in the viral systemic transport.

The most intriguing results in the chapter 3 were obtained when the tagged minireplicon variants were agroinfiltrated to the GLRaV-2 natural host, *Vitis vinifera*. Both L1 and L2 were proved to be important for the ability of GLRaV-2 to establish infection in the initially inoculated grapevine cells. It was hypothesized that the functional significance of duplication of the leader proteases in GLRaV-2 lies in the host-specific effects of L1 and L2 whose functional cooperation is more important for the infection of grapevine than of *N. benthamiana*. In order to subvert a perennial woody host potentially recalcitrant to virus infection, a tandem of viral proteases could have evolved to boost and diversify the functions of a single protease.

What is the possible mechanism by which L1 and L2 facilitate GLRaV-2 infection? The fact that each of these leader proteases acts in a host-specific manner to aid viral infectivity suggests that L1 and L2 could be involved in a suppression of an antiviral defense response. One possibility is that L1 and L2 are involved in suppression of RNA interference (RNAi) either independently from or in cooperation with the RNAi suppressor p24 (Chiba *et al*., 2006). However, our efforts to identify effects of GLRaV-2 L1 and L2 or BYV L-Pro on the RNAi response in several model systems invariably failed (Liu and Dolja, unpublished data). Moreover, we found that the ectopic co-expression of L-Pro with the reporter reduced accumulation of the latter (Peremyslov and Dolja, unpublished data) suggesting possible involvement of the leader proteases in gene regulation at the RNA or protein level.

By analogy to papain-like proteases of coronaviruses (Lindner, 2007), it can be hypothesized that closteroviral proteases act as deubiquitination enzymes (DUBs) whereby affecting regulation of the plant innate immunity defense. The only fact that is in a disagreement with the DUB hypothesis is that inactivation of the L1 proteolytic activity in M1 variant has little effect on the viral infection. This apparent discrepancy can be explained if the L1-mediated binding rather than cleavage of the host defense-related proteins is sufficient to exert L1 function. Determination of the actual mechanism by which closteroviral proteases counteract antiviral defenses is a promising area for the future research into virus-plant interactions.

Bibliography

Agranovsky, A.A., Koonin, E.V., Boyko, V.P., Maiss, E., Frotschl, R., Lunina, N.A., and J.G. Atabekov. (1994) Beet yellows Closterovirus: complete genome structure and identification of a leader papain-like thiol proteases. Virology 198: 311-324.

Agranovsky, A.A., Lesemann, D.E., Maiss, E., Hull, R., and J.G. Atabekov. (1995) "Rattlesnake" structure of a filamentous plant RNA virus built of two capsid proteins. Proc. Natl. Acad. Sci. U.S.A. 92 (7): 2470-2473.

Ahlquist, P. (2006) Parallels among positive-strand RNA viruses, reverse-transcribing viruses and double-stranded RNA viruses. Microbiology. 4: 371-382.

Alzhanova, D.V., Hagiwara, Y., Peremyslov, V.V., and V.V. Dolja. (2000) Genetic analysis of the cell-to-cell movement of beet yellows Closterovirus. Virology 268 (1): 192-200.

Alzhanova, D.V., Napuli, A., Creamer, R., and V.V. Dolja. (2001) Cell-to-cell movement and assembly of a plant Closterovirus: roles for the capsid proteins and Hsp70 homolog. EMBO J. 20: 6997-7007.

Alzhanova, D.V., Prokhnevsky, A.I., Peremyslov, V.V., and V.V. Dolja. (2007) Virion tails of Beet yellows virus: coordinated assembly by three structural proteins. Virology 359: 220-226.

Astier, S., Albouy, J., Maury, Y., Robaglia, C., and H. Lecoq. (2007) Principles of Plant Virology: Genome, Pathogenicity, Virus Ecology. Enfield, NH: Science Publishers.

Avisar, D., Prokhnevsky, A.I., and V.V. Dolja. (2008) Class VIII myosins are required for plasmodesmatal localization of a closterovirus Hsp70 homolog. J Virol. 82: 2836-2843.

Ayllon, M.A., Gowda, S., Satyanarayana, T., Karasev, A.V., Adkins, S., Mawassi, M., Guerri, J., Moreno, P., and W.O. Dawson. (2003) Effects of modification of the transcription initiation site context on citrus tristeza virus subgenomic RNA synthesis. J. Virol. 77: 9232-9243.

Balakirev, M.Y., Jaquinod, M., Haas, A.L., and J. Chrobozek. (2002) Deubiquitinating function of adenovirus proteinase. J. Virol. 76: 6323-6331.

Barrett, A.J., and N.D. Rawlings. (2001) Evolutionary lines of cysteine peptidases. Biol. Chem. 382: 727-733.

Beachy, R.N., and M. Heinlein. (2000) Role of P30 in replication and spread of TMV. Traffic. 7: 540-544.

Bertazzon, N., and E. Angelini. (2004) Advances in the detection of grapevine leafroll-associated virus 2 variants. J. Plant Path. 86(4): 283-190.

Boscia, D., Greif, C., Gugerli, P., Martelli, G.P., Wlater, B., and D. Gonsalves. (1995) Nomenclature of grapevine leafroll-associated putative closteroviruses. Vitis. 34: 171-175.

Boyko, V.P., Karasev, A.V., Agranovsky, A.A., Koonin, E.V., and V.V. Dolja. (1992) Coat protein gene duplication in a filamentous RNA virus of plants. Proc. Natl. Acad. Sci. U.S.A. 89: 9156-9160.

Boyko, V., Hu, Q., Seemanpillai, M., Ashby, J., and M. Heinlein. (2008) Validation of microtubule-associated Tobacco mosaic virus RNA movement and involvement of microtubule-aligned particle trafficking. Plant J. 51: 589-603.

Byrd, C.M., and D.E. Hruby. (2006) Viral proteinases: targets of opportunity. Drug Develop. Res. 67: 501-510.

Carrington, J.C., Kasschau, K.D., Mahajan, S.K., and M.C. Schaad. (1996) Cell-to-cell and long-distance transport of viruses in plants. Plant Cell 8: 1669-1681.

Carrington, J.C. (2000) Moving targets. Nature 408:150-151.

Carrington, J.C., Kasschau, K.D., and L.K. Johansen. (2001) Activation and suppression of RNA silencing by plant viruses. Virology 281: 1-5.

Chapman, E.J., Prokhnevsky, A.I., Gopinath, K., Dolja, V.V., and J.C. Carrington. (2004) Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. Genes Dev. 18: 1179-1186.

Chiba, M., Reed, J.C., Prokhnevsky, A.I., Chapman, E.J., Mawassi, M., Koonin, E.V., Carrington, J.C., and V.V. Dolja. (2006) Diverse suppressors of RNA silencing enhance agroinfection by a viral replicon. Virology 346: 7-14.

Citovsky, V., Wong, M.L., Shaw, A.L., Prasad, B.V., and P. Zambryski. (1992) Visualization and characterization of tobacco mosaic virus movement protein binding to single-stranded nucleic acids. Plant Cell 4(4): 397-411.

Credi, R. (1997) Characterization of grapevine rugose wood disease sources from Italy. Plant Disease. 81 (11): 1288-1292.

Ding, S.-W., and O. Voinnet. (2007) Antiviral immunity directed by small RNAs. Cell 130: 413-426.

de los Santos, T., de Avila Botton, S., Weiblen, R., and M.J. Grubman. (2006) The leader proteinase of foot-and-mouth disease virus inhibits the induction of beta interferon mRNA and blocks the host innate immune response. J. Virol. 80: 1906-1914.

Dinesh-Kumar, S. P., Tham, W.-H. and B. Baker. (2000) The structure–function analysis of the tobacco mosaic resistance gene *N*. Proc. Natl Acad. Sci. USA 97: 14789-14794.

Ding, B., Kwon, M.-O., and L. Warnberg. (1996) Evidence that actin filaments are involved in controlling the permeability of plasmodesmata in tobacco mesophyll. Plant J. 10: 157–164.

Ding, S.W., and O. Voinnet. (2007) Antiviral immunity directed by small RNAs. Cell 130: 413-426.

Dolja, V.V., Karasev, A.V., and A.A. Agranovsky. (1990) Organization of the beet yellows clostervirus genome. In *New Aspect of Positive Strand RNA viruses*, pp31-35. Editied by R. Rueckert and M. Brinton. Wachinton, DC: Am. Soc. Micrebiol.

Dolja, V.V., McBride, H.J., and J.C. Carrington. (1992) Tagging of plant potyvirus replication and movement by insertion of beta-glucuronidase into the viral polyprotein. Proc. Natl. Acad. Sci. U.S.A. 89: 10208-10212.

Dolja, V.V., Karasev, A.V., and E.V. Koonin. (1994) Molecular biology and evolution of closteroviruses: sophisticated build-up of large RNA genomes. Annu. Rev. Phytopathol. 32: 261-285.

Dolja, V.V., (2003) Beet yellows virus: the importance of being different. Mol. Plant Pathol. 4: 91-98.

Dolja, V.V., Kreuze, J.F. and J.P.T. Valkonen. (2006) Comparative and functional genomics of closteroviruses. Virus Res. 117: 38-51.

Domingo, E., and J.J. Holland. (1997) RNA viruses mutations and fitness for survival. Annu. Rev. Phytopathol. 32: 151-178.

Dougherty, W.G., and B.L. Semler. (1993) Expression of virus-encoded proteinases:

functional and structural similarities with cellular enzymes. Microbiol. Rev. 57: 781-822.

Drucker, M., Froissart, R., Hebrard, E., Uzest, M., and M. Ravallec. (2002) Intracellular distribution of viral gene products regulates a complex mechanism of *Cauliflower mosaic virus* acquisition by its aphid vector. Proc. Natl. Acad. Sci. USA 99: 2422-2427.

Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., and L.A. Ball editors. (2005) Virus taxonomy: VIIIth report of the International Committee on Taxonomy of Viruses. San Diego: Elsevier Academic Press.

Febres, V.J., Ashoulin, L., Mawassi, M., Frank, A., Bar-Joseph, M., Manjunath, K.L., Lee, R.F., and C.L. Niblett. (1996) The p27 protein is present at one end of citrus tristeza virus particles. Phytopathology 86: 1331-1335.

Fenczik, C.A., Padgett, H.S., Holt, C.A., Casper, S.J., and R.N. Beachy (1995) Mutational analysis of the movement protein of Odontoglossum ringspot virus to identify a host-range determinant. MOI. Plant-Microbe Interact. 8: 666-673.

Folimonov, A.S., Folimonova, S.Y., Bar-Joseph, M., and W.O. Dawson. (2007) A stable RNA virus-based vector for citrus trees. Virology 368(1): 205-216.

Froissart, R., Michalakis, Y., and S. Blanc. (2002) Helper component-transcomplementation in the vector transmission of plant viruses. Phytopathology 92: 576-579.

Fujiki, M., Kawakami, S., Kim, R.W., and R.N. Beachy. (2006) Domains of tobacco mosaic virus movement protein essential for its membrane association. J. Gen. Virol. 87: 2699–2707.

Gabrenaite-Verkhovskaya, R., Andreev, I.A., Kalinina, N.O., Torrance, L., Taliansky, M.E., and K. Mäkinen. (2008) Cylindrical inclusion protein of potato virus A is associated with a subpopulation of particles isolated from infected plants. J. Gen. Virol. 89: 829-838.

Goodin, M.M., Zaitlin, D., Naidu, R.A., and S.A. Lommel. (2008) Nicotiana benthamiana: its history and future as a model for plant-pathogen interactions. Mol. Plant Microbe. Interact. 21: 1015-1026.

Gorbalenya, A.E., Donchenko, A.P., Blinov, V.M., and E.V. Koonin. (1989) Cysteine proteases of positive strand RNA viruses and chymotrypsin-like serine proteases. A distinct protein superfamily with a common structural fold. FEBS Lett. 243: 103-114.

Gorbalenya, A.E., Koonin, E.V., and M.M.-C. Lai. (1991) Putative papain-related thiol proteases of positive-strand RNA viruses. Identification of rubi- and aphthovirus proteases and delineation of a novel conserved domain associated with proteases of rubi-, alpha, and coronaviruses. FEBS. 288: 201-205.

Gorbalenya, A.E., Enjuanes, L., Ziebuhr, J., and E.J. Snijder. (2006) Nidovirales: evolving the largest RNA virus genome. Virus Res. 117 (1): 17-37.

Goszczynski, D.E., Kasdorf, G.G.F., Pietersen, G., and H. Van Tonder. (1996) Grapevine leafroll-associated virus-2 (GLRaV-2)-mechanical transmission, purification, production and properties of antisera, detection by ELISA. S. Afr. J. Enol. Vitic. 17: 15-26.

Guenoune-Gelbart, D., Elbaum, M., Sagi, G., Levy, A., and B.L. Epel. (2008) *Tobacco mosaic virus* (TMV) replicase and movement protein function synergistically in facilitating TMV spread by lateral diffusion in the plasmodesmal desmotubule of *Nicotiana benthamiana*. MPMI 21: 335-345.

Hagiwara, Y., Peremyslov, V.V., and V.V. Dolja. (1999) Regulation of closterovirus gene expression examined by insertion of a self-processing reporter and by northern hybridization. J. Virol. 73: 7988-7993.

Hammond, S.M. (2005). Dicing and slicing: the core machinery of the RNA interference pathway. FEBS Lett. 579: 5822-5829.

Haseloff, J., and W.L. Gerlach. (1988) Simple RNA enzymes with new and highly specific endoribonuclease activities. Nature 334: 585-591.

Haseloff, J., Siemering, K.R., Prasher, D.C., and S. Hodge. (1997) Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly. Proc. Natl. Acad. Sci. U.S.A. 94 (6): 2122-2127.

Heinlein, M., Epel, B.L., Padgett, H.S., and R.N. Beachy (1995) Interaction of tobamovirus movement proteins with the plant cytoskeleton. Science 270: 1983–1985.

Hogenhout, S.A., Ammar, E.-D., Whitfield. A.E., and M.G. Redinbaugh. (2008) Insect vector interactions with persistently transmitted viruses. Annu. Rev. Phytopathol 46: 327-359.

Karasev, A.V., Boyko, V.P., Gowda, S., Nikolaeva, O.V., Hilf, M.E., Koonin, E.V., Niblett, C.L., Cline, K., Gumpf, D.J., Lee, R.F., Garnsey, S.M., Lewandowski, D.J., and W.O. Dawson. (1995) Complete sequence of the citrus tristeza virus RNA genome. Virology 208: 511-520.

Karasev, A.V. (2000) Genetic diversity and evolution of Closteroviruses. Annu. Rev. Phytopathol. 38: 293-324.

Kassanis, B., Carpenter, J.M., White, R.F., and R.D. Woods. (1977) Purification and som properties of beet yellows virus. Virology 77: 95-100.

Kasschau, K.D., Cronin, S., and J.C. Carrington. (1997) Genome amplification and long-distance movement functions associated with the central domain of tobacco etch potyvirus helper component-proteinase. Virology 228 (2): 251-262.

Kehr, J., and A. Buhtz. (2007) Long distance transport and movement of RNA through the phloem. J. Experimental Botany 59: 85-92.

Kim, S.H., MacFarlane, S., Kalinina, N.O., Rakitina, D.V., Ryabov, E.V., Gillespie, T., Haupt, S., Brown, J.W.S. and M. Taliansky. (2007) Interaction of a plant virus-encoded protein with the major nucleolar protein fibrillarin is required for systemic virus infection. Proc. Natl. Acad. Sci. U.S.A. 104: 11115-11120.

Klaassen, V.A., Boeshore, M., Dolja, V.V., and B.W. Falk. (1994) Partial characterization of the lettuce infectious yellows virus genomic RNAs, identification of the coat protein gene, and comparison of its amino acid sequence with those of other filamentous RNA plant viruses. J. Gen. Virol. 75: 1525-1533.

Koonin, E.V. (1991) The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. J. Gen. Virol. 72: 2197-2206.

Koonin, E.V. (1993) Computer-assisted identification of a putative methyltransferase domain in NS5 protein of flaviviruses and lambda 2 protein of reovirus. J Gen Virol. 74: 733–740.

Koonin, E.V., and V.V. Dolja. (1993) Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. Crit. Rev. Biochem. Mol. Biol. 28 (5): 375-430.

Koonin, E.V., and V.V. Dolja. (2006) Evolution of complexity in the viral world: the dawn of a new vision. Virus Res. 117: 1-4.

Koonin, E.V., Wolf, Y.I., Nagasaki, K., and V.V. Dolja. (2008) The Big Bang of picorna-like virus evolution antedates the radiation of eukaryotic supergroups. Nat. Rev. Microbio. 6: 925-939.

Kushner, D.B., Lindenbach, B.D., Grdzelishvili, V.Z., Noueiry, A.Z., Paul, S.M., and P. Ahlquist. (2003) Systematic, genome-wide identification of host genes affecting

replication of a positive-strand RNA virus. Proc. Natl. Acad. Sci. USA 100: 15764-15769.

Lakatos, L., Csorba, T., Pantaleo, V., Chapman, E.J., Carrington, J.C., Liu., Y.P., Dolja, V.V., Calvino, L.F., Lopez-Moya, J.J., and J. Burgyan. (2006) Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. EMBO J. 25: 2768-2780.

Laporte, C., Vetter, G., Loudes, A.M., Robinson, D.G., Hillmer, S., Stussi-Garaud, C., and C. Ritzenthaler. (2003) Involvement of the secretory pathway and the cytoskeleton in intracellular targeting and tubule assembly of grapevine fanleaf virus movement protein in tobacco BY-2 cells. Plant Cell 15: 2058-2075.

Li, F., and S.W. Ding. (2006). Virus counterdefense: diverse strategies for evading the RNA-silencing immunity. Annu. Rev. Microbiol. 60: 503-531.

Lindbo, J.A., and W.G. Dougherty. (1992) Untranslatable transcripts fo the tobacco etch virus protein gene sequence can interfere with tobacco etch replication in transgenic plants and protoplasts. Virology 189: 725-733.

Linder, H.A., Forouhi-Ardakani, N., Lytvyn, V., Lanchance, P., Sulea, T., and R. Menard. (2005) The papain-like protease from the severe acute respiratory syndrome coronavirus is a deubiquitinating enzyme. J. Virol. 79(24): 15199-15208.

Lindner, H.A., (2007) Deubiquitination in virus infection. Virology 362: 245-256.

Ling, K.-S., Zhu, H.-Y., and D. Gonsalves. (2008) Resistance to grapevine leafroll associated virus-2 is conferred by post-transcriptional gene silencing in transgenic *Nicotiana benthamiana*. Transgenic Res. 17:733-740.

Lu, R., Folimonov, A.S., Shintaku, M., Li,W.X., Falk, B.W., Dawson,W.O., and S.W. Ding. (2004) Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome. Proc. Natl. Acad. Sci. U.S.A. 101: 15742-15747.

Lucas, W.J. (2005) Plant viral movement proteins: Agents for cell-to-cell trafficking of viral genomes. Virology 344: 169-184.

Martelli, G..P., Agranovsky, A.A., Bar-Joseph, M., Boscia, D., Candresse, T., Coutts, R.H.A., Dolja, V.V., Falk, B.W., Gonsalves, D., Jelkmann, W., Karasev A.V., Minafra, A., Namba, S., Vetten, H.J., Wiseler, G.C., and N. Yoshikawa. (2002) The family Closteroviridae revised. Arch. Virol. 147(10): 2039-2044.

Martelli, G.P., Adams,M.J., Kreuze, J.F., and V.V. Dolja. (2007) Family Flexiviridae: a case study in virion and genome plasticity. Annu. Rev. Phytopathol. 45: 73-100.

Martin, G.B., Bogdanove, A.J., and G. Sessa. (2003) Understanding the functions of plant disease resistance proteins. Annu. Rev. Plant Biol. 54: 23-61.

Medina, V., Peremyslov, V.V., Hagiwara, Y., and V.V. Dolja. (1999) Subcellular localization of the HSP70-homolog encoded by beet yellows Closterovirus. Virology 260 (1): 173-181.

Melcher, U. (2000) The "30K" superfamily of viral movement proteins. J. Gen. Virol. 81: 257-266.

McLean, B.G., Zupan, J., and P.C. Zambryski. (1995) Tobacco mosaic virus movement protein associates with the cytoskeleton in tobacco plants. Plant Cell 7: 2101–2114.

Meng, B., Li, C., Goszczynski, D.E., and D. Gonsalves. (2005) Genome sequences and structures of two biologically distinct strains of Grapevine leafroll-associated virus 2 and sequence analysis. Virus Genes 31: 31-41.

Monis, J. (2005) Pinpointing grapevine virus disease. Wine Business Monthly 7 (11): 37-39.

Monis, J. (2006) Pinpointing grapevine virus disease: part II. Wine Business Monthly 8 (3): 39-41.

Namba, S., Boscia, D., Azzam, O., Maixner, M., Hu, J.S., Golino, D., and D. Gonsalves. (1991) Purification and properties of closterovirus-like particles associated with grapevine corky bark diseases. Phytopathology 81: 964-970.

Napuli, A.J., Falk, B.W., and V.V. Dolja. (2000) Interaction between HSP70 homolog and filamentous virions of the Beet yellows virus. Virology 274 (1): 232-239.

Napuli, A.J., Alzhanova, D.V., Doneanu, C.E., Barofsky, D.F., Koonin, E.V., and V.V. Dolja. (2003) The 64-kDa capsid protein homolog of beet yellows virus is required for assembly of virion tails. J. Virol. 77: 2377-2384.

Nelson, R.S., and V. Citovsky. (2005) Plant viruses. Invaders of cells and pirates of cellular pathways. Plant Physiology 138: 1809-1814.

Ng, J.C.K., and B.W. Falk. (2006) Virus-vector interactions mediating nonpersistent and semipersistent transmission of plant viruses. Annu. Rev. Phytopathol. 44: 183-212.

Oh, C.S., and J.C. Carrington. (1989) Identification of essential residues in potyvirus

proteinase HC-Pro by site-directed mutagenesis. Virology 173: 692-699.

Oparka, K.J., Prior, D.A.M., Santa Cruz, S., Padgett, H.S., and R.N. Beachy. (1997) Gating of epidermal plasmodesmata is restricted to the leading edge of expanding infection sites of tobacco mosaic virus. Plant J. 12: 781-789.

Padmanabhan, M., Cournoyer, P., and S.P. Dinesh-Kumar. (2009) The leucine-rich repeat domain in plant innate immunity: a wealth of possibilities. Cell. Micro. 11: 191-198.

Panavas, T., Serviene, E., Brasher, J., and P.D. Nagy. (2005) Yeast genome-wide screen reveals dissimilar sets of host genes affecting replication of RNA virus. Proc. Natl. Acad. Sci. USA 102: 7326-7331.

Peng C.-W., and V.V. Dolja. (2000) Leader proteinase of the beet yellows closterovirus: mutation analysis of the function in genome amplification. J. Virol. 74(20): 9766-9770.

Peng C.-W., Peremyslov, V.V., Mushegian, A.R., Dawson, W.O., and V.V. Dolja. (2001) Functional specialization and evolution of leader proteinases in the family Closteroviridae. J. Virol. 75(24): 12153-12160.

Peng, C.-W. (2002a) Multiple functions of a proteinse in closterovirus life cycle. PdD dissertation, Oregon State University.

Peng C.-W., Peremyslov, V.V., Snujder, E.J., and V.V. Dolja. (2002b). A replication-competent chimera of plant and animal viruses. Virology 294: 75-84.

Peng C.-W., Napuli, A.J., and V.V. Dolja. (2003) Leader proteinase of beet yellows virus functions in ling-distance transport. J. Virol. 77(5): 2843-2849.

Peremyslov, V.V., Hagiwara, Y., and V.V. Dolja. (1998) Genes required for replication of the 15.5-kilobase RNA genome of a plant Closterovirus. J. Virol. 72: 5870-5876.

Peremyslov, V.V., Hagiwara, Y., and V.V. Dolja. (1999) HSP70 homolog functions in cell-to-cell movement of a plant virus. Proc. Natl. Acad. Sci. U.S.A. 96: 14771-14776.

Peremyslov, V.V., and V.V. Dloja (2002) Identification of the subgenomic mRNAs that encode 6-kDa movement protein and Hsp70 homologue of beet yellows virus. Virology 295: 299-306.

Peremyslov, V.V., Andreev, I.A., Prokhnevsky, A.I., Duncan, G.H., Taliansky, M.E., and V.V. Dolja. (2004a) Complex molecular architecture of beet yellows virus

particles. Proc. Natl. Acad. Sci. U.S.A. 101: 5030-5035.

Peremyslov, V.V., Pan, Y.-W., and V.V. Dolja. (2004b) Movement protein of a Closterovirus is a type III integral transmembrane protein localized to the endoplasmic reticulum. J. Virol. 78: 3704-3709.

Peremyslov, V.V., and V.V Dolja. (2007) Cloning of large positive-strand RNA viruses. Curr. Protocols Microbiol. (Suppl. 7), 16F.1.1–16F.1.26.

Pirolo, C., Boscia, D., La Notte, P., Campanele, A., Savino, V., and G.P. Martelli. (2006) Further evidence of the involvement of Grapevine leafroll associated virus 2 in graft incompatibility. In: Proc. 15th Meeting ICVG, Stellenbosch, South Africa, April 3-7: 242-243.

Pogue, G.P., Lindbo, J.A., Garder, S.J. and W.P. Fitzmaurice. (2002) Making an ally from an enemy: plant virology and the new agriculture. Annu. Rev. Phytopathol. 40: 45-74.

Pouwels, J., Kornet, N., van Bers, N., Guighelaar, T., van Lent, J., Bisseling, T., and J. Wellink. (2003) Identification of distinct steps during tubule formation by the movement protein of cowpea mosaic virus. J. Gen. Virol. 84: 3485-3494.

Prokhnevsky, A.I., Peremyslov, V.V., Napuli, A.J., and V.V. Dolja. (2002) Interaction between long-distance transport factor and Hsp70-related movement protein of beet yellows virus. J. Virol. 76: 11003-11011.

Prokhnevsky, A.I., Peremyslov, V.V., and V.V. Dolja. (2005) Actin cytoskeleton is involved in targeting of a viral Hsp70 homolog to the cell periphery. J Virol. 79:14421-14428.

Rawlings, N.D., Morton, F.R., Kok, C.Y., Kong, J., and A.J. Barrett. (2008) *MEROPS*: the peptidase database. Nucleic Acids Res. 36: D320-D325.

Reed, J.C., Kasschau, K.D., Prokhnevsky, A.I., Gopinath, K., Pogue, G.P., Carrington, J.C., and V.V. Dolja. (2003) Suppressor of RNA silencing encoded by beet yellows virus. Virology 306: 203-209.

Ren, T., Qu, F., and T.J. Morris. (2000) *HRT* gene function requires interaction between a NAC protein and viral capsid protein to confer resistance to turnip crinkle virus. Plant Cell 12: 1917-1926.

Saenz, P., Salvador, B., Simon-Mateo, C., Kasschau, K.K., Carrington, J.C., and J.A. Garcia. (2002) Host-Specific Involvement of the HC Protein in the Long-Distance Movement of Potyviruses. J. Virol. 76: 1922-1931.

Satyanarayana, T., Gowda, S., Mawassi, M., Albiach-Marti, M.R., Ayllon, M.A., Robertson, C., Garnsey, S.M., and W.O. Dawson. (2000) Closterovirus encoded HSP70 homolog and p61 in addition to both coat proteins function in efficient virion assembly. Virology 278 (1): 253-265.

Satyanarayana, T., Bar-Joseph, M., Mawassi, M., Albiach-Marti, M.R., Ayllon, M.A., Gowda, S., Hilf, M.E., Moreno, P., Garnesy, S.M., and W.O. Dawson. (2001) Amplification of Citrus tristeza virus from a cDNA clone and infection of citrus trees. Virology 280(1): 87-96

Satyanarayana, T., Gowda, S., Ayllon, M.A., Albiach-Marti, M.R., and W.O. Dawson. (2002) Mutational analysis of the replication signals in the 3'-nontranslated region of citrus tristeza virus. Virology 300: 140-152.

Satyanarayana, T., Gowda, S., Ayllon, M.A., and W.O. Dawson. (2003) Frameshift mutatios in infectious cDNA clones of Citrus tristeza virus: a strategy to minimize the toxicity of viral sequences to Escherichia coli. Virology 313: 481-491.

Satyanarayana, T., Gowda, S., Ayllon, M.A., and W.O. Dawson. (2004) Closterovirus bipolar virion: evidence for initiation of assembly by minor coat protein and its restriction to the genomic RNA 5' region. Proc. Natl. Acad. Sci. U.S.A. 101: 799-804.

Scholthof, K.-BG., Scholthof, H.B., and A.O. Jackson. (1995) The tomato bushy stunt virus replicase proteins are coordinately expressed and membrane associated. Virology 208: 365–369.

Segers, G.C., van Wezel, R., Zhang, X., Hong, Y., and D.L. Nuss. (2006) Hypovirus papin-like protease p29 suppresses RNA silencing in the natural fungal host and in a heterologous plant system. Eukaryot. Cell 5: 896-904.

Soosaar, J.L.M., Burch-Smith, T.M., and S.P. Dinesh-Kumar. (2005) Mechanisms of plant resistance to viruses. Nature Rev. 3: 789-798.

Strauss, J.H., and E.G. Strauss. (1994) The Alphaviruses: gene expression, replication, and evolution. Microbiol. Rev. 58: 491-562.

Susaimuthu, J., Tzanetakis, I.E., Gergerich, R.C., and R.R. Martin. (2008) A member of a new genus in the Potyviridae infects Rubus. Virus Res. 131: 145-151.

Sutic, D.D., Ford, R.E., and M.T. Tosic. (1999) HADNBOOK OF PLANT VIRUS DIESASES. Boca Raton, FL: CRC Press LLC. 447p- 496p.

Takanami, Y. (2006) Contributions of plant virus research to the biological sciences. J.

Gen. Plant. Pathol. 72: 393-395.

Thivierge, K., Nicaise, V., Dufresne, P.J., Cotton, S., Laliberte, J.-F., Le Gall, O., and M.G. Fortin. (2005) Plant virus RNAs coordinated recruitment of conserved host functions by (+) ssRNA viruses during early infection events. Plant Physiol. 138: 1822-1827.

Tian, T., Rubio, L., Yeh, H.-H., Crawford, B., and B.W. Falk. (1999) Lettuce infectious yellows virus: in vitro acquisition analysis using partially purified virions and the whitefly, Bemisia tabaci. J. Gen. Virol. 80: 1111-1117.

Tijms, M.A., Rubio, L., Yeh, H.H., Crawford, B., and B.W. Falk. (2001) A zinc-finger-containing papain-like protease couples subgenomic mRNA synthesis to genome translation in a positive-strand RNA virus. Proc. Natl. Acad. Sci. U.S.A. 98: 1889-1894.

Tijms, M.A., Nedialkova, D.D., Zevenhoven-Dobbe, J.C., Gorbalenya, A.E., and E.J. Snijder. (2007) Arterivirus subgenomic mRNA synthesis and virion biogenesis depend on the multifunctional nsp1 autoprotease. J. Virol. 81: 10496-10505.

Tolia, N.H., and L. Joshua-Tor. (2007). Slicer and the Argonautes. Nat. Chem. Biol. 3: 36-43.

TOMAZIC, I., MAVRIC PLESKO, I., PETROVIC, N., RAVNIKAR, M.,and Z. KOROSEC-KORUZA. (2008) Introduction of grapevine virus B and grapevine leafroll-associated virus 2 testing in sanitary selection of grapevine. Acta agriculturae Slovenica 91: 75-85.

Tong, L. (2002) Viral proteases. Chem Rev. 102: 4609-4626.

Torrance, L., Andreev, I.A., Gabrenaite-Verhovskaya, R., Cowan, G., Makinen, K., and M.E. Taliansky. (2005) An unusual structure at one end of potato potyvirus particles. J. Mol. Biol. 357: 1-8.

Tzanetakis, I.E., Reed, J., and R.R. Martin. (2005) Nucleotide sequence, genome organization and phylogenetic analysis of strawberry pallidosis associated virus, a new member of the genus *Crinivirus*. Arch. Virol. 150: 273-286.

Tzanetakis, I.E., and R.R. Martin. (2007) Strawberry chlorotic fleck: identification and characterization of a novel Closterovirus associated with the disease. Virus Res. 124: 88-94.

Tzanetakis, I.E., Halgren, A., Mosier, N., and R.R. Martin. (2007) Identification and characterization of Raspberry mottle virus, a novel member of the Closteroviridae.

Virus Res. 127: 26-33.

Urcuqui-Inchima, S., Haenni, A.L., and F. Bernardi. (2001) Potyvirus proteins: a wealth of functions. Virus Res. 74: 157-175.

Valli, A., Martín-Hernández, A.M., López-Moya, J.J., and J.A. García. (2006) RNA silencing suppression by a second copy of the P1 serine protease of Cucumber vein yellowing ipomovirus (CVYV), a member of the family Potyviridae that lacks the cysteine protease HCPro. J. Virol. 80: 10055-10063.

van den Born, E., Omelchenko, M.V., Bekkelund, A., Leihne, V., Koonin, E.V., Dolja, V.V., 811 and P.O. Falnes. (2008) Viral AlkB proteins repair RNA damage by oxidative demethylation. Nucleic Acids Res. 36: 5451-5461.

Van der Biezen, E.A., and J.D. Jones. (1998) Plant disease-resistance proteins and the gene-for-gene concept. Trends Biochem. Sci. 23: 454-456.

van der Heijden, and J.F. Bol. (2002) Composition of alphavirus-like replication complexes: involvement of virus and host encoded proteins. Arch. Virol. 147: 875-898.

van der Want, J.P.H., and J. Dijkstra. (2006) A history of plant virology. Arch. Virol. 151: 1467-1498.

Van Lent, J.W., Wellink, J., and R.W. Goldbach. (1990) Evidence for the incolvement of 58K and 48K proteins in the intracellular movement of cowpea mosaic virus. J. Gen. Virol. 71: 219-223.

Waigmann, E., Ueki, S., Trutnyeva, K., and V. Citovsky (2004) The ins and outs of nondestructive cell-to-cell and systemic movement of plant viruses. Crit. Rev. Plant Sci. 23: 195–250.

Walter, B. and P. Cornuet. (1993) Elisa detection of grapevine fleck virus (GFkV). Agronomie. 13: 651-657.

Weber, E., Golino, D.A. and Rowhani, A. (2002) Laboratory testing for grapevine virus diseases. Practical Winery and Vineyard 12(2): 13.26.

Whitham, S., Dinesh-Kumar, S.P., Choi, D., Hehl, R., Corr, C., and B. Baker. (1994) The product of the tobacco mosaic virus resistance gene *N*: similarity to toll and the interleukin-1 receptor. Cell 78: 1101-1115.

Wright, K.M., Wood, N.T., Roberts, A.G., Chapman, S., Boevink, P., Mackenzie, K.M., and K.J. Oparka. (2007) Targeting of TMV movement protein to plasmodesmata

requires the actin/ER network; Evidence from FRAP. Traffic 8: 21–31.

Xiang, C., Han, P., Lutziger, I., Wang, K., and D.J. Oliver. (1999) A mini binary vector series for plant transformation. Plant Mol. Biol. 40: 711-717.

Zhu, H.-Y., Ling, K.-S., Goszczynski, D.E., McFerson, J.R., and D. Gonsalves. (1998) Nucleotide sequence and genome organization of grapevine leafroll-associated virus-2 are similar to beet yellows virus, the closterovirus type member. J. Gen. Virol. 79: 1289-1298.

Ziebuhr, J., Snijder, E.J., and A.E. Gorbaleya. (2000) Virus-encoded proteinases and proteolytic processing in the Nidovirales. J. Gen. Virol. 81: 853-879.

Ziebuhr, J., Schelle, B., Karl, N.,Minskaia, E., Bayer, S., Siddell, S.G., Gorbalenya, A.E., and V. Thiel. (2007) Human coronavirus 229E papain-like proteases have overlapping specificities but distinct functions in viral replication. J. Virol. 81: 3922-3932.