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Title: Multiple Functions of a Proteinase in Closterovirus Life Cycle.

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More than half of the recognized genera of positive strand RNA viruses
employ polyprotein processing as one of the strategies for their genome expression.
Normally, this processing is mediated by virus-encoded proteinases that belong to
the trypsin-like or papain-like family. In particular, papain-like, leader proteinases
were found in diverse families of human, animal, plant, and fungal positive strand
RNA viruses. In addition to autocatalytic processing, these proteinases play a
variety of roles in the virus life cycle. In plant potyviruses, a papain-like helper
component-proteinase (HC-Pro) was implicated in genome amplification, cell-to-
cell movement, long distance transport, and suppression of host defense. The p29
proteinase encoded by a fungal hypovirus CHV1 was found to be dispensable for
virus replication, but it was identified as a major determinant of viral pathogenicity.
In an animal equine aterivirus (EAV), a papain-like proteinase nsp1 was
demonstrated to possess a putative zinc finger domain, which functions in

subgenomic RNA synthesis, although it is not essential for virus replication. The Lab proteinase of the foot and mouse disease virus (FMDV) is involved in inhibition of cellular mRNA translation and in virus spread in infected animals. In general, it appears that functional plasticity of the papain-like leader proteinases played an important role in the evolution of viral diversity.

Here, we examined the functions of a papain-like leader proteinase (L-Pro) in the life cycle of the beet yellows closterovirus (BYV). It was found that L-Pro is required for autoproteolytic processing, genome amplification, virus invasiveness and cell-to-cell movementfor BYV. The gene swapping experiments involving several closterviruses, a potyvirus, as well as CHV1, FMDV, and EAV revealed complex functional profiles of the papain-like leader proteinases. The possible mechanisms that underlie L-Pro functions are discussed.

Multiple Functions of a Proteinase In Closterovirus Life Cycle

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CONTRIBUTION OF AUTHORS

Dr. Valerian V. Dolja was involved in the major experimental design of the research project, and provided the funding for this study from his discretionary funds. The vast majority of the thesis work was done in the laboratory of Valerian V. Dolja, and some of the transfection experiments were done in the laboratory of William O. Dawson.

Chapter three was co-authored with Valery V. Peremyslov, Arcady R. Mushegian and William O. Dawson. Valery Peremyslov was involved in the microscopic analysis, Arcady R. Mushegian contributed to phylogeneic analysis, and William O. Dawson provided important technical support regarding protolast transfection experiments.

Chapter four was co-authored with Valery V. Peremyslov and Eric J. Snijder. Valery V. Peremyslov contributed the major part of the microscopic analysis. Eric J. Snijder provided EAV cDNA clones and the experimental data regarding EAV research.

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF TABLES

Multiple Functions of a Proteinase in Closterovirus Life Cycle

Chapter 1

Introduction and Literature Review

1.1 Life cycles of plant viruses

According to a current report, the number of recognized viruses is 3,618, of which ~25% are plant viruses (Van Regenmortel *et al*., 1999). The production of rigid cell walls by plant cells is one of the major differences between plants and animals. Hence, it is not surprising that the basic differences between animal and plant viruses can be traced to this specific structure of plant cells. The plant cell wall is an effective barrier to virus entry and spread within the plant. External surface layers composed of cutin and waxes provide an additional protective barrier for the plant as a whole. Thus, to enter the cell and establish infection, a virus must mechanically breach these barriers (Fig. 1.1). Although a few plant viruses have been reported to be transmitted by plant to plant contact, most plant viruses are transmitted through the action of a vector (Fig. 1.1). In the majority of cases, this involves virus introduction into aerial parts of the plant by the action of insects feeding on leaves and stems (Fig. 1.1; Gray and Rochon, 1999). Viruses that are closely related to each other are transmitted by the same type of vector and in a similar manner (Gray and Banerjee, 1999). Once mechanically introduced into a

Fig. 1.1. General life cycles of plant viruses
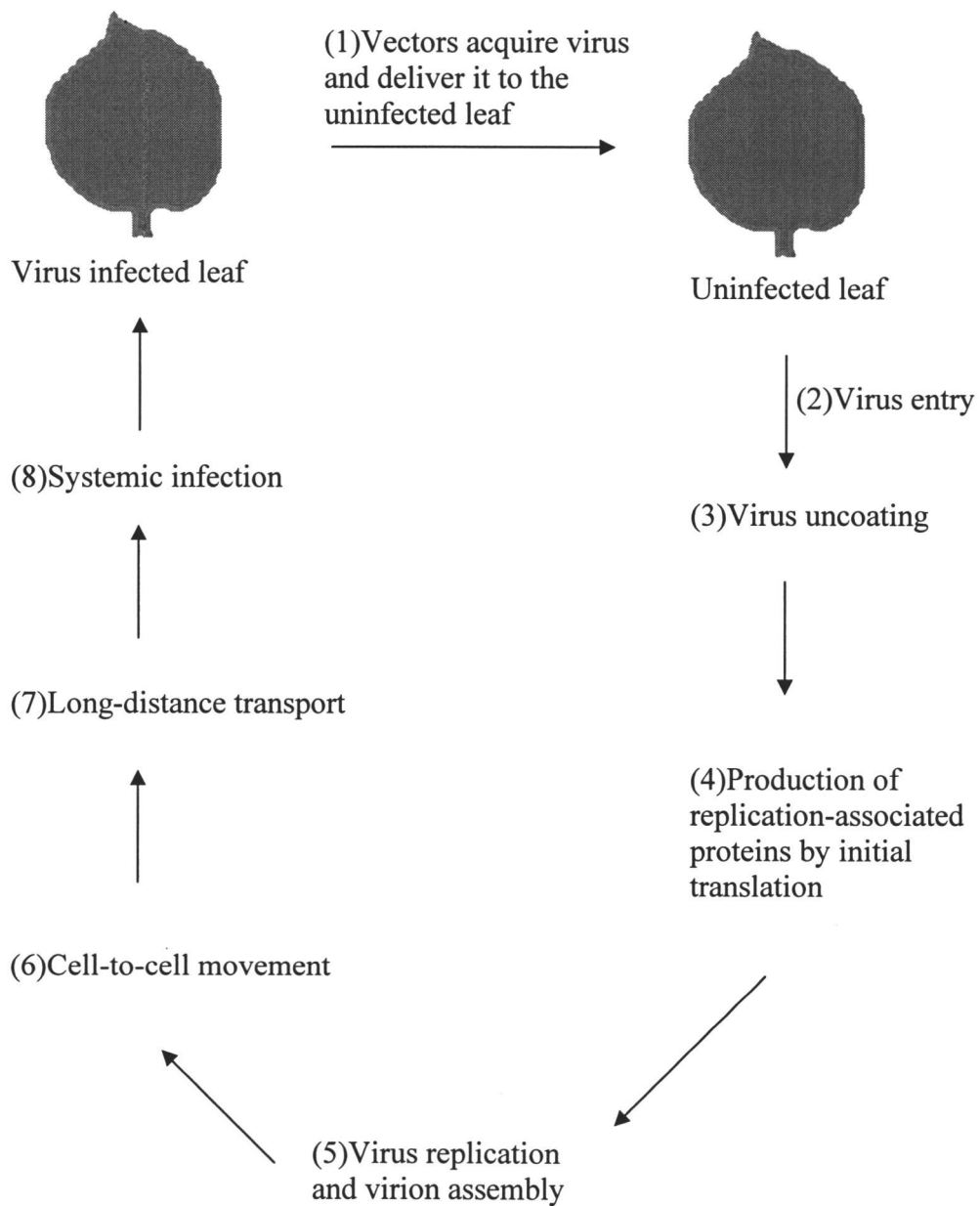
plant, or by insect feeding or other means, plant viruses will initiate infection of the inoculated leaves (Fig. 1.1). For positive-strand RNA viruses, initiation of virus infection includes uncoating of the virus particle (disassembly), initial translation of replication-associated proteins, and genome replication. After initial infection, virus must spread from cell to cell within the inoculated leaf and invade the growing plant, a process termed systemic infection. The insect acquires the virus by feeding on infected plants and then transmits it when feeding on unifected plants, and thereby the virus continues its life cycle.

To successfully establish movement between neighboring cells, plant viruses encode movement proteins (MPs) and/or structural proteins to facilitate this process. The study of virus encoded MPs has revealed how viruses can alter plasmodesmata to move from cell to cell and enter phloem tissue (Fujiwara *et al.*, 1993; Heinlein *et al.*, 1998; Lazarowitz and Beachy, 1999). For virus infection to disseminate throughout the plant, the virus first moves from cell-to-cell within the inoculated leaf and enters the vascular system. With the exception of phloem-restricted viruses such as luteoviruses and geminiviruses, the movement of most plant viruses involves moving from infected epidermal cells to mesophyll cells, and from these to bundle sheath cells. To establish a systemic infection in hosts, virus must move from bundle sheath cells to phloem parenchyma and companion cells, and enter the sieve elements. From the sieve elements, virus can infect additional sites as well as follow photonassimilate flow to move to distant leaves and systemically infect the plant (Nelson *et al.*, 1998; Carrington *et al.*, 1996). Phloem-restricted viruses appear to move locally between companion cells, phloem parenchyma, and bundle sheath cells, and from leaf to leaf through sieve elements.

1.2 Overview of positive strand RNA viruses

Viruses and viroids are the only known organisms that use RNA as their genetic material. Positive strand RNA viruses contain a messenger-sense RNA genome that can be translated into certain functional proteins. Viral RNA genomes can be conceptualized as mosaics of gene modules. The recombinational shuffling of these modules has been proposed as the major force driving large-scale evolution of positive-strand RNA viruses. Comparative analysis of viral genomes established that the RNA-dependent RNA polymerase (RdRp) is the only universally conserved module (Koonin *et al.*, 1991; Koonin and Dolja, 1993). RdRp is essential for RNA replication, is not usually found in uninfected hosts, and must therefore be encoded in the viral genome and expressed during infection. Sequence analyses of RdRp, have revealed striking similarities among the plant and animal RNA viruses (Koonin *et al.*, 1991; Koonin and Dolja, 1993).

Unlike DNA polymerase, RdRp lacks proofreading activity. The resulting high error rates of 10^{-3} to 10^{-5} (Domingo and Holland, 1997) cause high frequencies of point mutation among RNA viruses. Such high mutation rates were proposed as the major factor that limits the sizes of RNA genomes. The largest RNA genomes of ~30kb were found in animal coronaviruses. Among plant RNA viruses, closteroviruses possess the largest genomes of 15-20 kb. In general, most RNA viruses contain genomes that are less than 10 kb in size. Phylogenetic analyses based on multiple alignments of RdRp sequences revealed several large subdivisions of positive-strand RNA viruses and led to the grouping of plant and animal RNA viruses into "superfamilies" (Koonin, 1991; Dolja and Carrington, 1992; Dolja and Koonin, 1993). The first, picornarvirus-like supergroup, includes

Table 1.1: Superfamilies of positive strand RNA viruses. Based on the conserved modules of the RdRp, all positive strand RNA viruses can be subdivided into three superghroups, including Picornavirus-, Flavivirus-, and Sindbis virus-like superfamilies.

Subdivision	Animal viruses	Plant Viruses
Picornavirus-like supergroup	Picornaviruses Caliciviruses	Comoviruses; Luteoviruses Nepoviruses; Bymoviruses Sobemoviruses
Flavivirus-like supergroup	Flaviviruses Pestiviruses Hepativiruses	Carmoviruses Tombusviruses Dianthoviruses Barley yellow dwarf virus, (PAV strain)
Sindbis virus-like supergroup	Alphaviruses Rubiviruses	Tobamoviruses; Furoviruses Bromoviruses; Tobraviruses Potexviruses; Hordeoviruses Closteroviruses; Tymoviruses Cucumoviruses; Ilarviruses

animal picorna- and caliciviruses, and plant como-, nepo-, poty-, and bymoviruses. The second, flavivirus-like supergroup, combines animal flavi-, pesti- and hepatitis C viruses, and plant carmo-, tombus-, and dianthoviruses. The most crowded is the Sindbis virus-like supergroup, or supergroup III, which combines animal alpha-, rubi- and Hepatitis E viruses, and at least 12 groups of plant viruses (Table 1.1; Koonin, 1991; Dolja and Carrington, 1992; Dolja and Koonin, 1993).

1.3 Closteroviruses

Closteroviruses were first recognized in 1975 by the Plant Virus Subcommittee of the International Committee of Taxonomy of viruses (Fenner, 1976; Shepherd *et al.*, 1976). More than 30 plant viruses are currently classified as definitive or tentative members of a *Closteroviridae* family (Martelli *et al.*, 2000; Karasev, 2000). Based on their particle morphology and vector specificities, closteroviruses can be further divided into at least three groups (Dolja *et al.*, 1994; Karasev, 2000). The transmission of closteroviruses that belong to each group is mediated by aphids, whiteflies, or mealybugs, respectively, in a semi-persistent manner (Bar-Joseph *et al.*, 1979). Most closteroviruses are associated with phloem in infected plants and hence are considered as phloem-limited viruses (Esau, 1960; 1971). However, mechanical transmission is also possible for some closteroviruses, such as beet yellow virus (BYV) and grapevine leafroll-associated virus 2 (GLRaV-2) (Kassanis, 1949; Goszczynski *et al.*, 1996). The diseases caused by closteroviruses affect primarily the phloem of the infected plant. The symptoms often resemble nutrition deficiencies and premature senescence, for instance, yellowing, reddening, vein-clearing, stunting, or wilting (Bar-*Joseph et al.*, 1979). The large RNA genomes and lack of mechanical transmission for most of the closteroviruses are two major factors that contributed to a long delay in research into closteroviruses as compared to other plant viruses.

The virions of closteroviruses are formed with one RNA molecule and at least two types of capsid proteins. The "main body" of the particle consists of major capsid protein (CP), whereas the minor capsid protein (CPm) forms a "tail" (Fig. 1.2; Agranovsky *et al.*, 1995; Febres *et al.*, 1996; Tian *et al.*, 1999). Closteroviruses have extremely flexuous filamentous virions (Brandes *et al.*, 1959; 1965). The RNA genomes of closteroviruses vary from 15 to ~ 20 kb (Dolja *et al.*, 1994; Karasev, 2000). In addition, closteroviruses exhibit striking similarities in

genome organization to animal coronavirus-like viruses (Dolja *et al*., 1994). It was speculated that these similarities imply parallel evolution toward large RNA genomes. Although the gene content varies among closteroviruses, two gene blocks are conserved among all members (Fig. 1.3; Dolja *et a*l., 1994; Karasev *et al*., 2000). The first, 5' terminal block is represented by open reading frames (ORFs) 1a and 1b that encode replication-associated proteins. The capping, RNA helicase, and RNA polymerase domains encoded in this block are conserved throughout the supergroup of Sinbis virus-like viruses. Based on phylogenetic reconstructions, closteroviruses were grouped with bromo-, tobamo-, tobra-, and hordeiviruses (Dolja *et al*., 1994; Jelkmann, 1997; Karasev, 1995; 2000). The second gene block is unique for closteroviruses and encompasses five ORFs encoding proteins responsible for virus assembly and cell-to-cell movement. The variable regions of closteroviral genomes may possess three to five genes depending on the virus.

1.3.1 Aphid-transmitted closteroviruses

1.3.1.1 Beet Yellows Virus (BYV)

BYV is a type member of the *Closterovirus* genus. The modal length of the BYV particle is 1,250-1,450 nm (Milne, 1988), and the length of the RNA genome is 15,480 nt (Agranovsky *et al*., 1994). The main agriculturally important hosts infected by BYV include sugar beet, red beet, mangold, and spinach. The RNA genome of BYV is 5' capped and has no poly (A)-tail (Fig. 1.3; Karasev, *et al*., 1989). BYV possesses a filamentous virion (Fig 1.2), and the virus transmission is mediated by aphids. The virion consists of 95% CP and 5% CPm (Fig 1.2). Recently, two additional proteins, Hsp70h and p64, were also implicated in virion

assembly (Napuli *et al*., 1999; Tian *et al*., 1999; Satyanarayana *et al*., 2000). The virion body is responsible for primary genome protection, whereas the tail represents a specialized device for cell-to-cell movement (Alzhanova *et al*., 2001).

Fig. 1.2. Filamentous morphology of BYV virions (Panel A). The rod-shaped and filamentous virions are assembled from two capsid proteins, CP and CPm (Panel B).

Tail
CPm (5%)

Body
CP (95%)

A.

B.
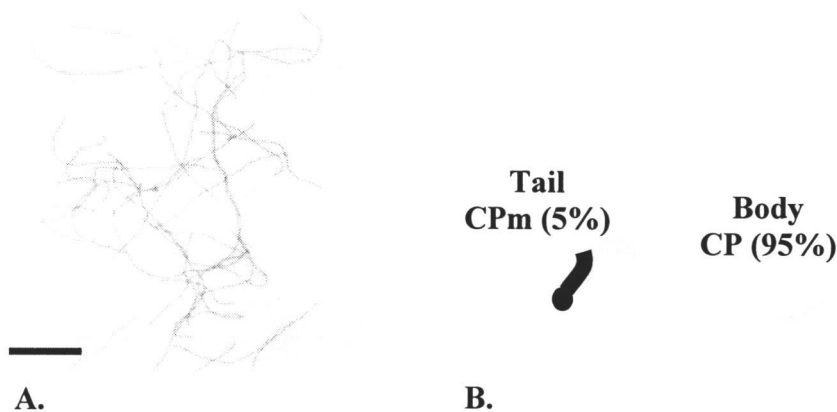
The BYV genome contains nine ORFs. The 5'-terminal part of the BYV genome harbors ORF 1a and 1b, encoding papain-like leader proteinase (L-Pro), methyltransferase (MET), HEL, and RNA polymerase domains. The replication-associated products of ORF 1a are directly translated from the genomic RNA, and the ORF 1b is presumably translated by a frameshift mechanism (Karasev *et al*., 1995; Klaassen *et al*., 1995). The 3'-terminal part of BYV encompasses ORFs 2 through 8 that code for a 6-kDa hydrophobic protein (p6), a homolog of HSP70 protein family (HSP70h), a 64-kDa protein (p64), two capsid proteins; CP and CPm, a 20-kDa protein (p20), and a 21-kDa protein (p21). All ORFs located in the 3'-terminal region are expressed via formation of a nested set of subgenomic RNAs (sgRNAs) (Dolja *et al*., 1990; He *et al*., 1997). It appears that the

mechanism of closterovirus transcription is similar to that of Sindbis virus-like viruses, and distinct from leader priming that was found in coronavirus-like viruses.

The available information concerning the functions of closteroviral proteins was derived using BYV and Citrus tristeza virus (CTV). Our previous work reported that ORF 1a and 1b of BYV encode the main replication-associated genes, whereas both L-Pro and p21 act as replication enhancers. The functional profile of L-Pro partially overlaps that of the helper component proteinase (HC-Pro) of potyviruses (Kasschau *et al.*, 1995; Peremyslov *et al.*, 1998; Peng *et al.*, *2000*). Cell-to-cell movement of BYV requires activities of three movement proteins (p6, HSP70h, and p64), and two structural proteins (CP and CPm). It was also found that assembly of the tailed BYV virions is essential for cell-to-cell movement (Alzhanova *et al.*, 2000). The study of the virion structure revealed that the tail formation of the virion is mediated by HSP70h, and a tailless virion is incapable of translocation (Alzhanova *et al.*, 2001). p20 was shown to interact with HSP70h, and appears to be a major determinants of long distance transport (Prokhnevsky *et al.*, unpublished). In addition to functioning as a replication enhancer, p21 was demonstrated to possess an activity of an RNA silencing suppressor and a major determinant of pathogenicity (Reed et al., unpublished).

1.3.1.2 Citrus tristeza virus (CTV)

CTV is a definitive member of the *Closterovirus* genus, and is the largest known plant virus with a genome of 19,296 nt. CTV has a ~2,000 nm-long particles which are transmitted by aphids or by grafting (Karasev et al., 1995). CTV infects only citrus species, and severe strains of the virus cause serious reduction of citrus production. Stem pitting is the most common symptom of CTV

infection. Also, vein-clearing and seedling yellows were observed in many CTV-infected plants (Bar-Joseph *et al.*, 1979). Complete genomic sequences of four CTV isolates from different geographic areas have been determined. These genomic sequences differ markedly, with 50% to 80% nucleotide identity in much of the genome. The identity of some sequences is nearly uniform throughout the genome, whereas the identity level of other sequences is asymmetrical and progressively decreases toward the 5'-terminus (Mawassi *et al.*, 1996; Vives *et al.*, 1999).

The CTV genome possesses 12 ORFs (Fig. 1.3). The 10 3'-proximal ORFs are translated from the sgRNAs. Most of the CTV encoded ORFs are similar to those of BYV, including the array of L-Pro, HEL, MET and POL, a small hydrophobic protein, an HSP70h, a 61-kDa protein (p61), which is homologous to p64 of BYV, and two CPs. There are also some differences between the genomes of CTV and BYV. In addition to the two conserved gene blocks, CTV has five "extra" ORFs plus a second leader proteinase (L2). A 33-kDa protein (p33; ORF 2), a 13 kDa protein (p13; ORF 9), and a 23 kDa RNA binding protein have no analogs in BYV genome (Dolja *et al.*, 1994; Karasev, 2000).

1.3.2   Whitefly-transmitted closteroviruses

Currently characterized whitefly-transmitted closteroviruses (WTC) belong to the genus *Crinivirus* of the family *Closteroviridae*. Lettuce infectious yellows virus (LIYV) is a type member of the *Criniviruse* (Martelli *et al.*, 1997; Wisler *et al.*, 1998). The significant difference between LIYV and other closteroviruses is that this virus posses two RNAs, RNA1 of 8,118 nucleotides and RNA 2 of 7,193 nucleotides. (Fig. 1.3; Klaassen *et al.*, 1995). Despite the difference in the number of genome components, LIYV resembles monopartite closteroviruses in many

aspects (Fig. 1.3; Dolja *et al*., 1994, Klaassen *et al*., 1996; Karasev, 2000). Comparison of the genome organization of LIYV and monopartite closteroviruses

Fig. 1.3. Genome organization of BYV, CTV, and LIYV.
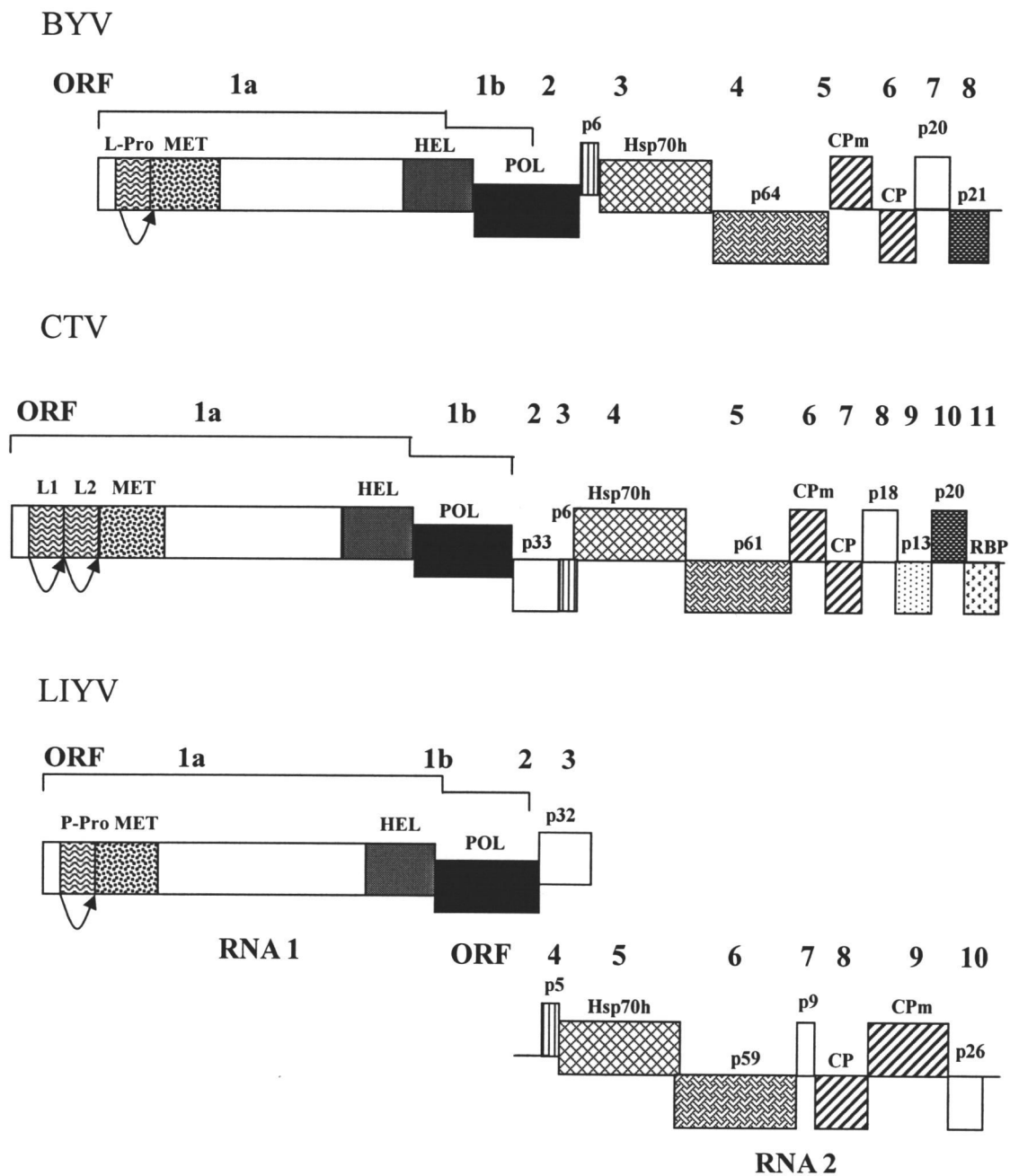
reveals close resemblance, with RNA 1 matching the 5'-proximal ORFs of monopartite viruses and RNA 2 corresponding to the 3'-terminal genes. 10 ORFs are encoded in LIYV. Among these, three ORFs are present in RNA 1 (ORF1a, ORF1b and ORF2), and the rest are encoded by RNA 2. LIYV also has two conserved gene blocks; the replication-associated proteins are encoded in RNA 1, while a set of five genes is present in RNA 2. The polyprotein encoded by ORF 1a presumably possesses a single leader proteinase. In adiition, the order of CP and CPm in LIYV genome is reverse relative to that in BYV and CTV.

1.4 Viral papain-like leader proteinases

Positive-strand RNA viruses employ several strategies for expression of multiple viral proteins, such as polyprotein synthesis and processing, subgenomic mRNA synthesis, readthrough, leaky scanning, and translational frameshifting. In particular, processing of the polyprotein by viral proteinases is found in all major subdivisions of RNA viruses infecting eukaryotes (Bazan and Fletterick, 1989; Strauss 1990, 1992; Gorbalenya *et al.*, 1990). Two mjor classes of virus proteolytic enzymes have been identified based on experimental studies and amino acid sequence comparisons. One of these classes is evolutionarily related to cellular serine-type or trypsin-like enzymes (Bazan *et al.*, 1988; Gorbalenya *et al.*, 1989). Extensive work on characterization of viral trypsin-like proteinases has been carried out using animal picornaviruses and plant potyviruses (Bazan *et al.*, 1988; Blinov *et al.*, 1985; Gorbalenya *et al.*, 1989). The second major type of viral proteolytic enzymes share similar features with cellular papain-like proteinases (Hardy *et al.*, 1989; Oh *et al.*, 1989; Bransom and Dreher, 1994). The essential catalytic residues Cys and His were characterized in this type of proteinase, such as potyviral HC-Pro, tymoviral Pro, closteroviral L-Pro, hypoviral p29, and alphaviral

nsp2 (Oh and Carrington, 1989; Craven *et al.*, 1993; Strauss and Strauss, 1994; Agranovsky *et al.*, 1994).

Viral papain-like proteinases can be further subdivided into main and leader proteinases. It was suggested that the papain-like proteinases represent a series of distinct evolutionary lineages diverged extensively from distant common relatives (Dougherty and Semler, 1993; Strauss and Strauss, 1994; Babe and Craik, 1997). The main proteinases possess both *cis* and *trans* cleavage activities, are located internally in the polyprotein, and thus are responsible for the polyprotein maturation of replication components, and are intimately involved in genome amplification. In contrast, the leader proteinases are located in the N-terminal part of the polyprotein, and normally cleave in *cis* at a single cleavage site. Leader proteinases are implicated in genome amplification, subgenomic RNA synthesis, inhibition of cellular mRNA translation, viral pathogenicity, virus transport within the infected organism, and suppression of the host defense response (Guarne *et al.*, 1998; Kasschau and Carrington, 1998; Chinsangaram *et al.*, 1999; Suzuki *et al.*, 1993; Tijms *et al.*, 2001). In general, the leader proteinases play a variety of roles in the life cycles of different RNA viruses.

1.5 Overview of the thesis

The major objective of this thesis is to investigate the functions of the papain-like leader proteinase (L-Pro) in the closterovirus life cycle. We first examined the function of L-Pro in genome amplification. To facilitate this study, a mini-BYV variant that possesses only the genes required for optimal RNA replication was utilized (Hagiwara et. al., 1999). This mini-BYV was tagged by insertion of a GUS reporter. In the initial phase of the project, we demonstrated that GUS activity provided a sensitive and accurate measure of virus RNA

accumulation. Thus, we were able to use this mini-BYV to study BYV replication in isolated plant protoplasts. A set of deletion and alanine-scanning mutations of L-Pro was generated using site-directed mutagenesis to investigate the role of L-Pro in genome amplification. Characterization of the mutant phenotypes revealed that L-Pro is required for efficient accumulation of BYV RNA (See Chapter 2). Although L-Pro is not essential for basal-level replication, deletion of its N-terminal domain resulted in a 1,000-fold reduction in RNA accumulation. In addition, we identified a ~150 nt-long element in the 5'-terminal region of the L-Pro ORF that is critical for RNA replication. This element presumably functions at the RNA level via forming (part of) the promoter for (+) strand RNA synthesis. To get further insight into function of the C-terminal proteolytic domain of L-Pro, we employed a gene swapping approach (Chapters 3 and 4). In the first part of this study (Chapter 3), we conducted comparative analyses of the leader proteinases derived from three closteroviruses and a potyvirus. Although each of the heterologous proteinases efficiently processed the chimeric polyprotein, only two out of four proteinases were capable of rescuing the replication of chimeric RNA. These results revealed an unexpected degree of the functional specialization of the homologous proteinase domains of different plant viruses. In addition, the gene swapping approach allowed identification of the novel functions of the closteroviral proteinase domain in RNA amplification, virus invasion, and cell-to-cell movement. In the second part of this project (Chapter 4), we expanded the scope of the study by inclusion of the leader proteinases derived from a fungal virus CHV1 and two animal viruses, FMDV and EAV. We found that the proteinase of EAV, but not FMDV or CHV1, provided a function that is critical for genome amplification and that is separable from polyprotein processing. In general, this work revealed complex and partially overlapping functional profiles of the papain-like leader proteinases of the plant and animal viruses. The multifunctional nature and occurrence of the leader proteinases in dissimilar, positive strand RNA viruses illustrate their ability to fulfill important needs in evolution of virus diversity.

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Chapter 2

Leader Proteinase of the Beet Yellows Closterovirus:
Mutation Analysis of the Function in Genome Amplification

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2.1 Abstract

The beet yellows closterovirus (BYV) leader proteinase (L-Pro) is a 66-kDa protein encoded in the 5'-proximal portion of the ORF 1a. L-Pro possesses a C-terminal, papain-like, proteinase domain and a non-proteolytic, N-terminal domain. The function of each domain in RNA amplification was investigated using site-directed mutagenesis and transfection of tobacco protoplasts. Mutations were introduced into a mini-BYV genome that encodes only the proteins required for efficient genome amplification and a reporter protein, β-glucuronidase (GUS). GUS activity was demonstrated to be an accurate measure of the RNA accumulation. The deletion mutant that expressed only a proteinase domain, was replication competent. However, this mutant exhibited a 1000-fold reduction in RNA accumulation, highlighting the importance of the N-terminal domain for efficient genome amplification. Expansion of the deletion to remove the proteinase domain resulted in no further reduction in RNA accumulation. The viability of this null mutant indicated that the L-Pro is not essential for basal levels of genome replication. Analysis of 12 alanine-scanning mutations targeting the N-terminal domain revealed its structural flexibility except for its very N-terminal region. The corresponding 54-codon long, 5'-terminal element in the ORF 1a was found to function in RNA amplification at both RNA and protein levels.

2.2 Introduction

Viral proteases belong to several structural prototypes; some of which are unique, whereas others share structural motifs with cellular enzymes (4). In particular, papain-like cysteine proteinases are found in diverse families of positive-

strand RNA viruses infecting plants, fungi, and animals (9, 13-15, 23, 25, 30). One class of these proteinases exemplified by the nsP2 of animal alphaviruses is responsible for processing the nonstructural polyprotein and is intimately involved in RNA replication (31). Similarly, papain-like proteinases encoded by plant tymoviruses and related viruses are involved in the processing of replication-associated polyproteins (6, 17). Proteinases of another class that typically cleave only *in cis* at their C-termini are called leader proteinases (L-proteinases). Examples of these are found in animal arteriviruses (30) and aphthoviruses (15, 28), as well as in plant potyviruses (7) and fungal hypoviruses (25). In addition to autocatalytic processing, several L-proteinases were reported to function in various processes of virus-host interaction (8, 15, 20, 21, 28, 32).

Members of the *Closteroviridae* family of positive-strand RNA viruses possess 15-20 kb genomes encapsidated into filamentous virions that are the longest so far described (5). Computer-assisted analysis revealed that closteroviruses belong to the Sindbis virus-like superfamily (23). Although the gene content varies among closteroviruses to a remarkable extent, two genome blocks are conserved among all members (11, 33). The first, 5'-terminal block is represented by ORFs 1a and 1b with the latter ORF encoding RNA polymerase that is presumably expressed via a +1 frameshift event (1, 18, 22). In beet yellows virus (BYV), a prototype closterovirus, ORF 1a codes for a replication-associated polyprotein that possesses a papain-like leader proteinase (L-Pro), a putative methyltransferase and an RNA helicase domains, and a large interdomain region which is unique to closteroviruses (Fig. 2.1). The second, quintuple, gene block encompasses ORFs encoding proteins responsible for virus assembly (2) and cell-to-cell movement (3, 27).

The BYV L-Pro provides a dual function in viral genome amplification. The autocatalytic cleavage at the C-terminus of L-Pro is essential for virus viability, whereas the non-proteolytic, N-terminal domain is required for efficient

accumulation of RNA (26). This functional profile is reminiscent of that described for the potyviral leader proteinase HC-Pro (12, 20).

In this study, we expand the functional analysis of L-Pro by using a "mini-BYV" genome that lacks six virus genes which are superfluous for genome amplification in isolated protoplasts (16, 26). This BYV variant retains the ORFs 1a, 1b, and a 3'-terminal ORF encoding a 21-kDa protein (p21), which functions as an activator of genome amplification (Fig. 2.1, ref. 26). In order to provide a simple and sensitive marker to monitor genome replication and expression, a reporter gene encoding bacterial β-glucuronidase (GUS) was engineered into this truncated BYV variant dubbed BYV-GUS-p21 (16).

To further explore structure-to-function relations in the L-Pro molecule, we generated 17 mutants (Fig. 2.2). Analysis of the mutant phenotypes revealed unexpectedly high tolerance to structural changes in most of the N-terminal domain. In contrast, a 54-codon long, 5'-terminal region of the ORF 1a was found to be critical for virus viability. In addition, we demonstrated that although the L-Pro is not essential for basal level genome amplification, its activity results in 1000-fold elevation of this level.

2.3 Materials and Methods

2.3.1 Generation of the BYV mutants

All mutations in the L-Pro coding region were generated using plasmid p5'BYV encompassing BYV nucleotides 1 through 3472 and site-directed mutagenesis as described (24, 26). Each mutation was verified by nucleotide sequencing; the full-length clones of the mutant BYV genomes were engineered by

Fig. 2.1. Genomic map of BYV (top) and a diagram of the cDNA clone of mini-BYV genome, pBYV-GUS-p21, tagged by insertion of the β-glucuronidase gene (bottom). The BYV ORFs from 1a to 8 encoding leader proteinase (L-Pro), replication-associated proteins possessing putative methyltransferase (MET), RNA helicase (HEL), and RNA polymerase (POL) domains, 6 kDa-protein (p6), HSP70-homolog (HSP70h), 64 kDa-protein (p64), minor capsid protein (CPm), major capsid protein (CP), 20 kDa-protein (p20), and 21 kDa-protein (p21) are shown as the boxes. The rounded arrow designates the self-processing site for the BYV L-Pro. The arrows marked CP and p21 on the map of pBYV-GUS-p21 show the approximate positions of the 5'-termini of the subgenomic RNAs expressing GUS and p21 and driven by the CP and p21 promoters, respectively. fs, the frame-shift mutation inactivating BYV replicase (28). Selected restriction endonuclease sites are shown below the pBYV-GUS-p21 diagram. The arrows marked SP6 and T7 show positions and orientation of the corresponding RNA polymerase promoters
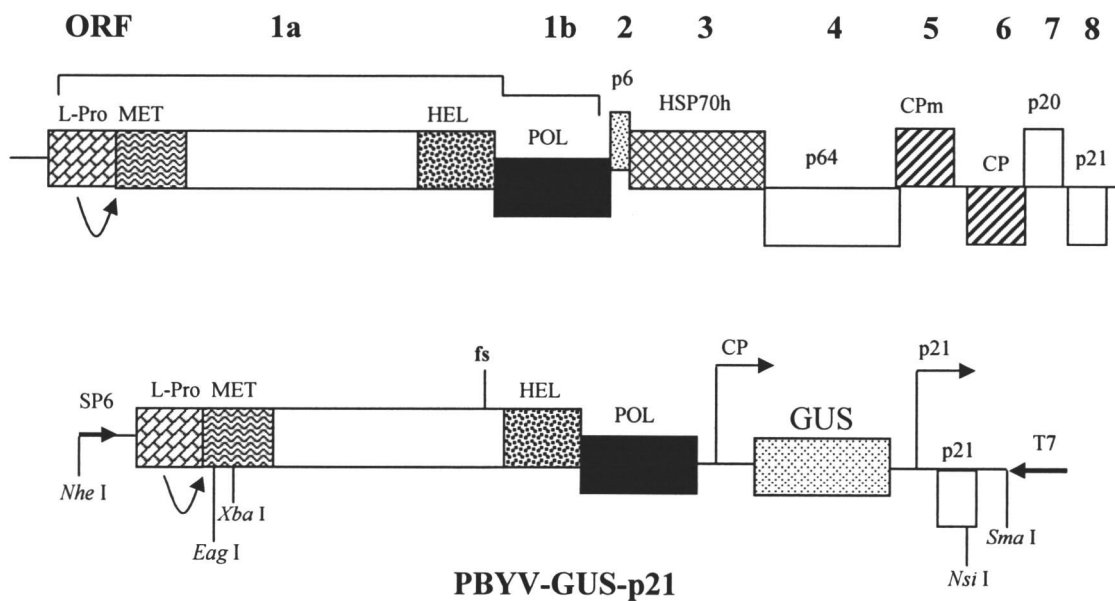
PBYV-GUS-p21

cloning the *Nhe* I - *Eag* I fragments of the modified p5'BYV variants into appropriately digested pBYV-GUS-p21 (Fig. 2.1). The latter plasmid represented the mini-BYV genome in which six viral genes that are superfluous for RNA

amplification in protoplasts were replaced by a reporter β-glucuronidase (GUS) gene (16).

In the DELL mutant, the entire region coding for BYV L-Pro was deleted in-frame via "loop-out" mutagenesis except for the start codon of the ORF 1a. This codon was fused with the first glycine codon of the BYV replicase (Fig. 2.2) to result in the formation of the BYV replicase whose only difference from the proteolytically processed, wild-type replicase would be the presence of an N-terminal methionine. The S-1 mutation resulted in the in-frame deletion of the ORF 1a codons 2 through 54 (Fig. 2.2).

The double mutant N-ATG was generated by simultaneously introducing two site-directed mutations. The first mutation changed the ORF 1a start codon to ACA using the mutagenic oligonucleotide 5'-CTATCGACA**CAC**CATTCTTGAA CG (changed nucleotides are shown in boldface here and thereafter). In addition, the G residue downstream from the start codon was replaced with C to disrupt the context favorable for its recognition as a translational start signal. A new ORF 1a start codon was engineered at codon 57 using the oligonucleotide 5'-CTTCTCTG TCCCG**GA**C**ATG**G**T**CTTTTTGAACGCG (three nucleotides surrounding ATG were changed to ensure the optimal context for translation. The N-ATG-ΔN mutant was derived from the N-ATG variant via deletion of codons 58 through 442 (original numbering); the mutant ORF 1a encoded only the C-terminal, proteinase domain of L-Pro (Fig. 2.2). In another derivative of the N-ATG mutant, N-ATG-ΔALL, the entire region coding for the L-Pro was deleted. In this mutant, the modified ORF 1a produced an unchanged BYV replicase which would be translated from the artificial ATG (Fig. 2.2).

Twelve alanine-scanning mutations (A1 to A12) were introduced throughout the N-terminal domain of L-Pro (Fig. 2.2). In each of these mutants, three consecutive charged or polar amino acid residues were replaced with the three alanine residues (Table 2.2). The nucleotide sequences of the corresponding mutagenic oligonucleotides are available upon request. The replication-deficient

Fig. 2.2. Mutagenic analysis of the function of the N-terminal and proteinase domains of L-Pro in BYV genome amplification. The 5'-terminal part of the BYV genome including non-coding leader region (L), L-Pro coding region (box), and part of the methyltransferase domain (Met; open box) is shown in the middle. The asterisks designate the translation start codons; G-G, a scissile glycine-glycine bond cleaved by the L-Pro. Vertical lines mark sites of the alanine-scanning mutations from A1 to A12. N-ATG, mutant in which the original start codon was replaced with ACA, whereas an artificial start codon was introduced at the indicated position. S-1 and DELL, mutants in which in-frame deletions were introduced in the L-Pro coding region. N-ATG-ΔN and N-ATG-ΔALL, mutants in which deletions were introduced into L-Pro coding region possessing an artificial start codon.
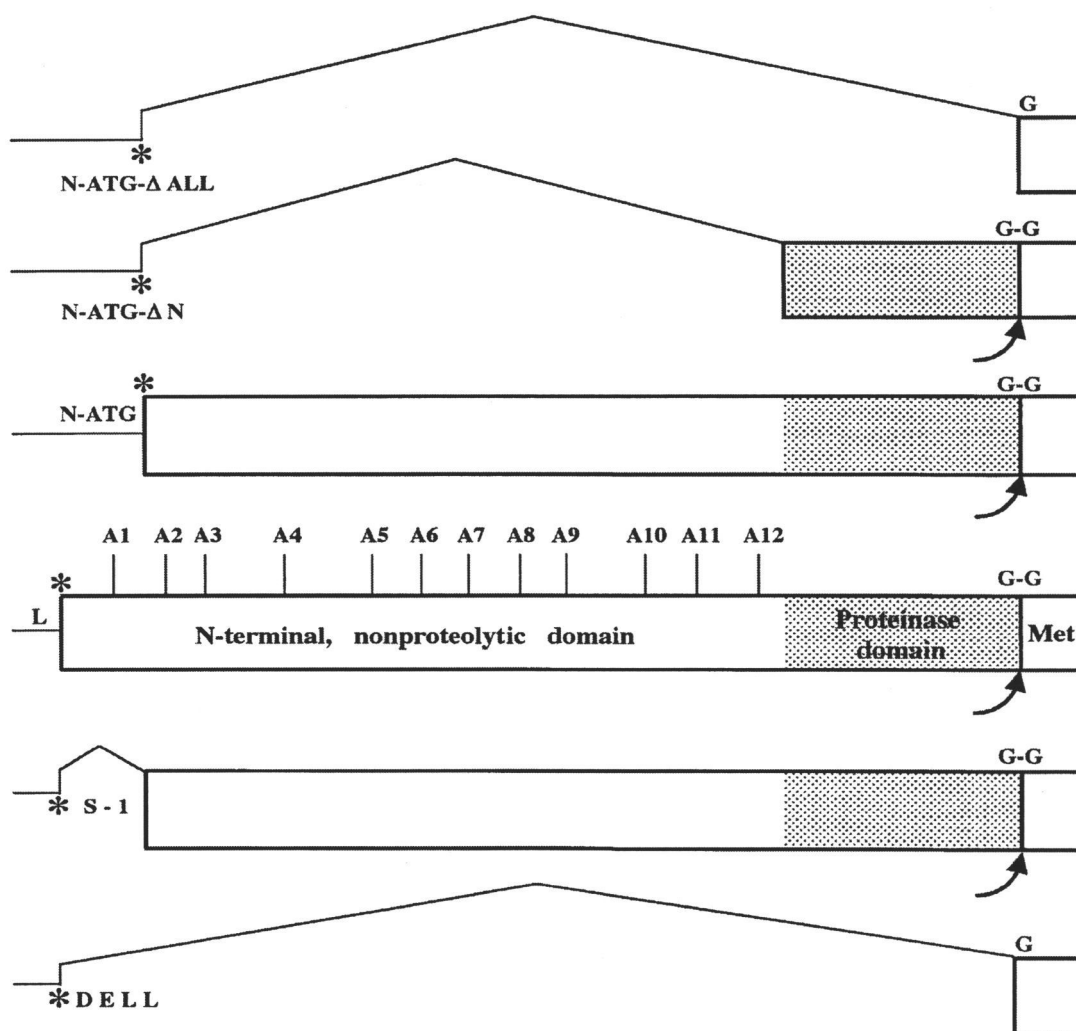
"fs" variant harboring a frame-shift mutation upstream of the RNA helicase domain was described previously (26). The corresponding mutant region of fs was cloned into pBYV-GUS-p21 using unique restriction endonuclease sites *Xba* I and *Sna* BI (Fig. 2.1).

2.3.2   Protoplast transfection and analysis of the mutant phenotypes

The phenotypes of the mutant BYV-GUS-p21 variants were characterized using transfection of the protoplasts isolated from suspension culture of the *Nicotiana tabacum* Xanthi nc line DF as described (12). Each transfection sample contained ~4×10^6 cells. The capped RNA transcripts used for electroporation into protoplasts were derived using SP6 RNA polymerase (Epicentre) and *Sma* I-linearized plasmid DNA (Fig. 2.1). Protoplasts were propagated in the dark and at the room temperature for 86 hrs prior to harvesting. GUS activity was assayed as described (10) and expressed as per cent of the activity found in the protoplasts transfected with the parental BYV-GUS-p21 variant (positive control). The mock-transfected protoplasts were used as a negative control. Each variant was characterized using at least four independent transfections; means and standard deviations were used to compare GUS activity.

The RNA samples were isolated using TRIZOL (Gibco-BRL), and the Northern hybridization analysis was conducted as described (26). The ^{32}P-labeled, single-stranded, negative-polarity RNA probe was generated using T7 RNA polymerase and *Nsi* I-linearized plasmid p3'BYV (Fig. 2.1; ref. 26). This probe was complementary to the ~400 3'-terminal nucleotides of the BYV RNA. The radiolabeled hybridization products were detected and quantified using a PhosphorImager (Molecular Dynamics); the means and standard deviations from four independent experiments were used to characterize each variant. *In vitro*

translations were conducted using wheat germ extracts (Promega), ^{35}S-cysteine, and *Xba I*-linearized variants of p5'BYV exactly as described (26).

2.4 Results

In order to determine the role that each of the L-Pro domains plays in BYV RNA amplification, a series of mutations was introduced into the region of ORF 1a encoding L-Pro. The previously generated cDNA clone encompassing a mini-BYV genome containing GUS ORF was utilized for this purpose (Fig. 2.1; ref. 16). The capped RNA transcripts derived from linearized pBYV-GUS-p21 variants were transfected into tobacco protoplasts. The GUS assays were used as a sensitive surrogate marker for quantification of the levels of genome amplification.

2.4.1 The 5'-terminal region of ORF 1a is critical for RNA replication and L-Pro function

In our previous work we demonstrated that cleavage between L-Pro and the remainder of ORF 1a product is essential for virus viability, whereas the N-terminal, non-proteolytic domain functions as activator of genome amplification (26). However, it was not known if the release of the mature replicase is the only function of L-Pro that is essential for RNA replication, and if the proteinase domain provides any additional activity required for efficient RNA accumulation. To address these questions, we generated a mutant called DELL (for DELetion of the Leader proteinase) in which the complete L-Pro ORF except for the start codon was deleted such that the translation of mutant RNA would result in production of mature, unchanged replicase (Fig. 2.2). Protoplast transfection experiments

revealed that the DELL variant was incapable of producing any detectable GUS-activity (Table 2.1), or accumulating virus-specific RNA (Fig. 2.3, lane DELL). In fact, this mutant was indistinguishable from the replication-deficient, frame-shift (fs) mutant expressing nonfunctional replicase (Table 2.2; Fig. 2.3, lane fs; ref. 26).

Table 2.1. Comparison between the GUS activity and accumulation of the genomic RNA in BYV variants at 4 days post transfection of protoplasts (each expressed as per cent of the level found in the parental variant BYV-GUS-p21).

BYV Variant	GUS Activity	RNA Accumulation
GUS-p21	100	100
Mock	<0.001	UD[a]
fs	<0.001	UD
DELL	<0.001	UD
S-1	<0.001	UD
N-ATG	2.5±1.5	2.3±0.6
N-ATG-ΔN	0.1±0.003	UD
N-ATG-ΔALL	0.1±0.007	UD
A1	1.1±0.7	1.7±0.7

[a]UD, undetectable.

One possible interpretation of the inability of the DELL variant to replicate is that the function of the proteolytic domain is not limited to a single autocatalytic cleavage at the C-terminus of the L-Pro, but may also involve cleavage(s) elsewhere in the replicase. An alternative explanation would be that the non-catalytic, N-

terminal domain is indispensable for virus viability. However, we have shown earlier that a mutant, 1-4, lacking most of the N-terminal domain while retaining its very N-terminal, 54-amino-acid-long peptide was viable, accumulating ~5 times less RNA than the wild-type virus (26). Because of that, complete loss of viability in a DELL mutant could be attributed to either the loss of the 54-codon-long RNA region, or to the loss of a region encoding the proteinase domain.

To test the role of the 5'-proximal region of the ORF 1a in RNA amplification, we generated a mutant (S-1) in which the codons 2 to 54 were deleted in frame to result in expression of the truncated L-Pro possessing most of the N-terminal domain and a complete proteinase domain (Fig. 2.2). Unexpectedly, the S-1 mutant was nonviable (Table 2.1 and Fig. 2.3, lane S-1). This result could be due to the indispensability of the short N-terminal peptide for the L-Pro function, or to a critical role played by the deleted RNA region (e.g., in the RNA folding or interaction with the replicase).

In order to distinguish between these two possibilities, we generated a double point mutant, N-ATG (for the New ATG codon), in which the original start codon of the ORF 1a was replaced with the ACA, and an artificial start codon was engineered in place of codon 57 of ORF 1a (Fig. 2.2). As expected, *in vitro* translation of the N-ATG RNA yielded a truncated L-Pro. This mutant product accumulated *in vitro* to a level similar to that of the nonmutant L-Pro, indicating that no significant changes in the translational and proteolytic activity occurred due to the transfer of the start codon to the downstream location (data not shown).

In protoplast transfection experiments, the N-ATG variant was viable, although it produced only 2.5% of the GUS activity of the parental variant (Table 2.1). Northern hybridization analysis yielded similar results (Table 2.1 and Fig. 2.3, lane N-ATG), once again indicating that the GUS activity accurately reflects RNA accumulation. The phenotype exhibited by the N-ATG mutant is indicative of a major defect in RNA amplification. Comparison of the phenotypes of the S-1 and N-ATG mutants suggests that the 5'-terminal, 54-codon-long region of the ORF 1a

provides a dual function. At the RNA level, this region is indispensable for virus viability, likely due to its role in overall RNA folding or its function as a *cis*-replicational signal. At the protein level, the peptide encoded in this region plays an important role in the L-Pro function in accumulation of viral RNA.

2.4.2 Roles played by each of the L-Pro domains in RNA accumulation

The viability of the N-ATG variant allowed us to revisit the problem of the relative functional importance of the N-terminal and proteinase domains for BYV RNA accumulation. To this end, we engineered two deletion mutants based on the N-ATG variant. In the first mutant, N-ATG-ΔN, an artificial start codon was fused with the proteinase domain to result in expression of L-Pro variant lacking all of its N-terminal domain, but possessing a proteinase domain (Fig. 2.2). *In vitro* translation experiments using the mutant mRNA revealed formation of the expected ~16-kDa proteinase domain that efficiently released itself from the downstream protein product (not shown). This result was in agreement with our previous work demonstrating that the N-terminal domain is not required for the proteolytic activity of the C-terminal domain (26).

In the second mutant, N-ATG-ΔALL, the same artificial start codon was placed immediately upstream of the first codon of the putative methyltransferase domain. This mutant was designed to express intact replicase in a complete absence of L-Pro expression (Fig. 2.2). Protoplast transfection experiments demonstrated that N-ATG-ΔN variant was viable, although it produced only ~0.1% of the GUS activity found in parental variant (Table 2.2.). This result emphasized the importance of the N-terminal domain for RNA amplification: in its absence, only a low, basal level of viral RNA has been produced. The level of GUS activity in protoplasts transfected with N-ATG-ΔALL variant was indistinguishable from that

found in N-ATG-ΔN variant (Table 2.1). This result can be interpreted to mean that in the absence of a need for the proteolytic release of the replicase N-terminus, the proteinase domain provides no other activity in genome amplification. Alternatively, strong debilitation of the genome amplification after deletion of the N-terminal domain could itself be a rate-limiting event masking the need in a proteinase domain.

It should be emphasized that although the GUS activity measured in N-ATG-ΔN and N-ATG-ΔALL variants was only 0.1% of that found in parental BYV-GUS-p21 variant, it was ~100-fold higher than the background GUS activity detected in the replication-deficient fs variant. This result confirmed that low GUS activity detected in N-ATG-ΔN and N-ATG-ΔALL mutants was due to amplification and transcription of the viral RNA rather than to direct translation of the input RNA transcripts.

2.4.3 Alanine-scanning mutagenesis of the N-terminal domain

To further examine the functional significance of the different regions in the N-terminal L-Pro domain, twelve alanine-scanning mutations designated from A1 to A12 and located through the entire domain's length were generated (Fig. 2.2). In each of these mutants, three adjacent codons specifying charged or polar amino acid residues were replaced with alanine codons (Table 2.2). These mutations were expected to affect the L-Pro function in RNA amplification via disrupting the electrostatic and/or hydrophilic interactions within the L-Pro molecule, or between the L-Pro and its putative protein partners. Surprisingly, the effects of eleven out of

Table 2.2. GUS activity in BYV variants with alanine scanning mutations in L-Pro at 4 days after transfection of protoplasts

BYV Variant	Replaced residues[a]	Mean GUS activity (% of level in BYV-GUSp21)±SD
A1	SDD_{39-41}	1.1 ± 0.7
A2	DNP_{72-74}	76 ± 12
A3	NGS_{92-94}	128 ± 19
A4	$SKP_{144-146}$	93 ± 8
A5	$KRK_{196-198}$	105 ± 17
A6	$SRP_{228-230}$	97 ± 3
A7	$RRR_{255-257}$	63 ± 10
A8	$KRK_{288-290}$	94 ± 14
A9	$KEE_{316-318}$	90 ± 9
A10	$RRP_{367-369}$	89 ± 10
A11	$EKK_{395-397}$	91 ± 12
A12	$SER_{434-436}$	87 ± 7

[a]Three consecutive amino acid residues of L-Pro that were replaced with three alanine residues in each mutant are shown along with their corresponding positions (subscript numbers) in the L-Pro sequence.

twelve alanine-scanning mutations on GUS accumulation were relatively weak. The levels of GUS activity detected in protoplasts were from 63% to 128% of that found in the parental variant (Table 2.2). Statistical analysis of the data revealed that these mutants were not significantly different from the non-mutant variant ($p > 0.1$), except for the mutant A7 ($p < 0.001$). In contrast, the mutant A1 accumulated only

~1% of the GUS activity found in a non-mutant variant (Table 2.2). This result was also confirmed using the Northern hybridization analysis (Fig. 2.3, lane A1). Since A1 was the only alanine-scanning mutation located within the limits of the N-terminal, 54-residue-long peptide, this result further emphasized the particular significance of this N-terminal region in the L-Pro function. It is also possible that A1 mutation affected replication due to disturbance in the overall folding of the 5'-terminal RNA region.

2.5 Discussion

In this work, we used GUS activity as a surrogate marker of BYV genome amplification. Since the GUS activity is a final result of viral genome replication, transcription of a subgenomic RNA, and its translation, it seemed important to investigate, if the levels of GUS activity is an accurate measure of genome amplification. More specifically, we determined if the mutations in the L-Pro could selectively affect the processes of transcription or translation without affecting genomic RNA accumulation. Northern hybridization analyses demonstrated that the GUS-negative fs, DELL, and S-1 mutants failed to accumulate any detectable viral RNA indicating that each of these mutations blocked accumulation of viral RNA. Comparative analyses of the relative levels of GUS activity and RNA accumulation for the mutants A1 and N-ATG revealed similarly low levels of replication between the two types of assay. It should be noted that the sensitivity of GUS assays is much higher than that of the Northern analysis. Quantification of the RNA levels lower than 1% of the wild-type level was impractical due to the background signal. On the other hand, high signal-to-background ratio of the GUS assays allowed confident measurements of the enzymatic activity at the levels of 0.001% of the wild-type level. These results established GUS-tagged mini-BYV genome as an adequate

model with which to study amplification of BYV RNA. An additional benefit of using the mini-BYV variant is the relative ease of manipulation of the truncated genome. A similar minimal replicon was engineered recently for another closterovirus, the citrus tristeza virus (29).

The GUS-tagged mini-BYV was utilized here to reveal the roles played by each of two major domains of BYV L-Pro in genome amplification. As we demonstrated previously, the cleavage mediated by the C-terminal proteinase domain is essential for virus viability, whereas the N-terminal L-Pro domain acts as an activator of RNA amplification (26). However, it was not known if L-Pro is essential for RNA replication, and what are the specific roles played by each of the L-Pro domains.

The data presented in this work demonstrate that the mutant N-ATG-ΔALL expressing none of the L-Pro domains is capable of replicating in the tobacco protoplasts, albeit to a very low level. The results indicate that L-Pro is not necessary for a basal level replication. On the other hand, a 1000-fold decrease in RNA accumulation exhibited by the L-Pro null mutant stresses the importance of the L-Pro for efficient amplification of closterovirus genome.

The identical phenotypes of the mutants lacking the N-terminal domain only, and both N-terminal and proteinase domain suggested that the proteinase itself plays no specific role in the enhancement of genome amplification. The previous work with potyviral HC-Pro that also possesses a C-terminal papain-like proteinase domain suggested that either this domain itself, or the *cis*-cleavage mediated by this domain is indispensable for viral viability (19). Since we were able to generate a viable BYV mutant in which the need for *cis*-cleavage was abolished, we propose that the major function of the proteinase domain is to cleave between the L-Pro and bona fide replicase. However, extreme debilitation of genome amplification in the absence of the N-terminal domain of L-Pro could interfere with our ability to detect possible additional functions provided by the proteinase domain. Generation of chimeric L-Pro molecules in which proteinase domain is derived from unrelated

(e.g., animal) viruses seems to be a promising approach for addressing this important problem.

Fig. 2.3. Northern analysis of the RNA accumulation in protoplasts transfected with parental and mutant BYV variants. Lane GUS-p21, parental BYV-GUS-p21 variant; other lanes represent the corresponding mutants marked on the top and mock-transfected protoplasts (lane Mock). Arrows mark the positions of genomic RNA and subgenomic RNAs encoding GUS and p21, as well as of background bands corresponding to plant ribosomal RNAs (rRNAs; 28). The membrane was overexposed to visualize low levels of BYV RNA accumulation detected in N-ATG and A1 mutants.
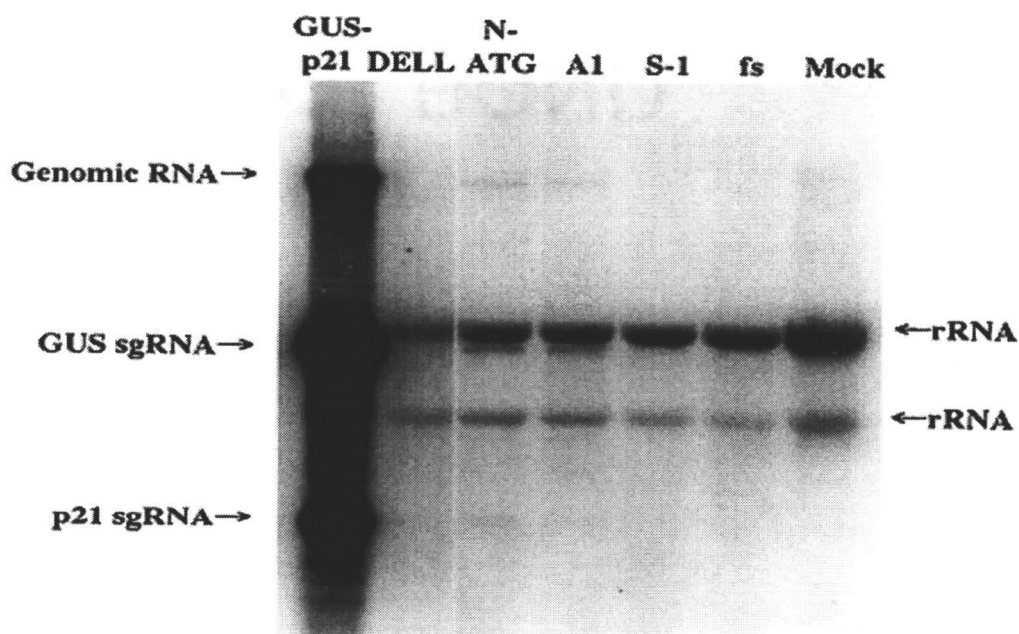
Systematic mutation analysis of the N-terminal domain revealed its unexpected structural flexibility. Indeed, 11 out of 12 alanine-scanning mutations introduced into this domain had no major effect on RNA accumulation. Computer analysis suggested that the N-terminal domain of the L-Pro possesses a non-

globular, elongated structure in contrast to the globular proteinase domain (A.R. Mushegian and V.V.D., unpublished). This type of the structure may account for the unusual tolerance of the former domain to alanine-scanning mutations. Alternatively, this domain may be required for other than genome amplification phases of the virus life cycle.

The only region in which an alanine scanning mutation was not tolerated was the very N-terminal region of the L-Pro. The A1 mutation that changed amino acids 39 to 41, resulted in a 100-fold reduction in RNA accumulation. A similar level of genome amplification was obtained with the mutant in which the ORF 1a start codon was engineered ~50 codons downstream from its natural position. In contrast, deletion of the ~50 codon-long RNA segment completely abolished genome amplification, indicating that this region of the ORF 1a functions not only as a coding sequence, but also as a (part of) *cis*-element required for RNA replication. Understanding of the multiple roles played by the L-Pro-encoding region will permit us to investigate the molecular mechanisms involved in activation of genome amplification mediated by this important part of the BYV genome.

2.6 Acknowledgements

2.7 References

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Capter 3

Functional Specialization and Evolution of the Leader Proteinases in the Family *Closteroviridae*

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3.1 Abstract

Members of the *Closteroviridae* and *Potyviridae* families of the plant positive-strand RNA viruses encode one or two papain-like leader proteinases. In addition to a C-terminal, proteolytic domain, each of these proteinases possesses a non-proteolytic, N-terminal domain. We compared functions of several leader proteinases using a gene-swapping approach. The leader proteinase (L-Pro) of the beet yellows virus (BYV, a *Closterovirus*) was replaced with L1 or L2 proteinases of the citrus tristeza virus (another *Closterovirus*), P-Pro proteinase of the lettuce infectious yellows virus (a *Crinivirus*), and HC-Pro proteinase of the tobacco etch virus (a *Potyvirus*). Each foreign proteinase efficiently processed the chimeric BYV polyprotein *in vitro*. However, only the L1 and P-Pro, but not the L2 and HC-Pro were able to rescue the amplification of the chimeric BYV variants. The combined expression of the L1 and L2 resulted in an increased RNA accumulation compared to that of the parental BYV. Remarkably, this L1-L2 chimera exhibited reduced invasiveness and inability to move from cell to cell. Similar analyses of the BYV hybrids in which only the papain-like domain of L-Pro was replaced with those derived from L1, L2, P-Pro, and HC-Pro, also revealed functional specialization of these domains. In subcellular localization experiments, distinct patterns were observed for the leader proteinases of BYV, CTV, and LIYV. Taken together, these results demonstrated that in addition to a common proteolytic activity, the leader proteinases of closteroviruses possess specialized functions in virus RNA amplification, virus invasion, and cell-to-cell movement. The phylogenetic analysis suggested that functionally distinct L1 and L2 of the citrus tristeza virus originated by a gene duplication event.

3.2 Introduction

Papain-like cysteine proteinases of the positive-strand RNA viruses are multifunctional proteins involved not only in polyprotein processing, but also in genome amplification, virus pathogenicity and spread in the infected organism, and suppression of host defenses (13-15, 26, 31, 39, 40, 43). Two major classes of the viral papain-like proteinases include 'main' proteinases that are required for the processing of nonstructural polyprotein and RNA replication (3, 13, 18, 39) and 'accessory' or 'leader' proteinases that are typically responsible for a single autocatalytic cleavage at their C-termini (1, 4, 5, 8, 9, 13, 14, 31, 43). Representatives of the each of these classes are found among diverse taxa of positive-strand RNA viruses infecting plants, animals, and fungi.

Closteroviridae and *Potyviridae* are two large families of plant viruses that share filamentous morphology of the virions, but belong to evolutionary distant lineages of the positive-strand RNA viruses, the Sindbis virus-like supergroup, and the Picornavirus-like supergroup, respectively (26). The ~10 kb genome of a typical potyvirus codes for a single polyprotein that is processed by the three proteinases (35). Among these, the helper component-proteinase (HC-Pro) is a leader proteinase that is also required for efficient genome amplification, suppression of RNA silencing, virus transport inside infected plants, and aphid transmission (6, 29). The papain-like, proteolytically active domain of HC-Pro is located in its C-terminal region (4), whereas the large N-terminal domain is implicated in all additional functions of the HC-Pro (21-23, 29).

The 15-20 kb genomes of closteroviruses are the largest and most complex among all RNA viruses infecting plants (11, 19). Two currently recognized genera of the family *Closteroviridae* are *Closterovirus* and *Crinivirus*, with the beet yellows virus (BYV) and lettuce infectious yellows virus (LIYV) as the prototype members, respectively. Although the gene content varies from one virus to another,

all closteroviruses share the strategy of gene expression. The 5'-terminal open reading frame (ORF 1; Fig. 3.1) encodes a large polyprotein that functions in genome amplification (25, 33, 36). The N-terminal part of this polyprotein encompasses the leader proteinase (1) that is traditionally abbreviated as L-Pro in BYV (33) and P-Pro in LIYV (24). Some closteroviruses, such as citrus tristeza virus (CTV), possess two tandemly organized leader proteinases that are designated L1 and L2 (20). The 3'-terminal part of a closterovirus genome harbors from 7 to 10 ORFs, which are expressed via formation of a set of 3'-coterminal subgenomic mRNAs (sgRNAs) (16, 30). In LIYV and other members of the *Crinivirus* genus, the genome is split between two RNAs; RNA 1 encodes P-Pro and replicase, whereas RNA 2 specifies most of the LIYV sgRNAs (24). It was demonstrated that the release of the BYV L-Pro from the polyprotein is mediated by the autocatalytic, papain-like domain (1, 33). This release is essential for genome replication. The N-terminal, non-proteolytic domain of the L-Pro is required for efficient accumulation of BYV RNAs; its elimination results in ~1,000-fold reduction in the RNA levels (32).

In this study we conducted the comparative analyses of the leader proteinases of plant viruses using a gene swapping approach and computer-assisted phylogenetic reconstructions. Our results indicate that the non-conserved N-terminal domains of the leader proteinases provide several distinct functions, which may vary from one proteinase to another. Moreover, conserved papain-like domains of the leader proteinases also exhibited an unexpected degree of functional specialization. In addition to autocatalytic processing, these domains were implicated in activation of genome amplification, virus invasion, and virus movement from cell to cell.

3.3 Material and methods

3.3.1 Generation of the chimeric BYV variants

To generate a cassette for replacing BYV L-Pro region with the regions encoding foreign leader proteinases, two restriction endonuclease sites were engineered into the pBYV-GUS-p21 (16) (Fig. 3.1). One of these sites, *Sac* II, was introduced as the mutation A2 described earlier (32). The second site, *Sph I*, was engineered downstream from the two glycine codons that specify a scissile dipeptide using oligonucleotide primer GG-Sph (5'-CGT.TTC.ATC.GGC-**GGC.ATG.C**AA.GAA.GAA.GCT.CCT.G; dots indicate the codons, dash separates two glycine codons, the *Sph* I site is in a boldface). This modification resulted in replacement of valine and glutamic acid codons with methionine and glutamine codons, respectively. The regions encoding TEV proteinase HC-Pro, CTV proteinases L1 and L2, combination of L1 and L2, and LIYV proteinase P-Pro were PCR-amplified using biologically-active cDNA clones of the TEV (10), CTV (36) and LIYV (25) as templates. The *Sac* II and *Sph* I sites flanking the resulting cDNA fragments were introduced concomitant with PCR and used to clone these fragments into mini-BYV (Fig. 3.1). The chimeric leader proteinases shown in Fig. 2 possessed authentic N-terminal domain of the BYV L-Pro (up to nt 1443) and papain-like proteinase domains derived from TEV (nts 1957-2436), CTV L1 (nts 1114-1563) and L2 (nts 2593-3039), and LIYV (nts 956-1336). Engineering of the corresponding chimeric variants of mini-BYV was done as described above, except that the *Sac* II site corresponded to mutation A12 rather than A2 (32). Four chimeric BYV variants shown in Table 3.3 were subcloned into the pBYV-GFP, the BYV variant tagged via insertion of the GFP gene (34). To this end, the *Nhe* I-*Eag* I

fragment of the pBYV-GFP was replaced with corresponding chimeric cDNA fragments derived from mini-BYV variants.

3.3.2 Analysis of the mutant phenotypes in vitro and in vivo

The plasmids containing cloned BYV cDNAs were linearized using *Xba* I (for *in vitro* analysis) or *Sma* I (for *in vivo* experiments) and transcribed using SP6 RNA polymerase (33). To assess the proteolytic activity of the leader proteinases, the resulting capped RNA transcripts were translated using wheat germ extracts (Promega) and ^{35}S-methionine (Amersham/Pharmacia Biotech) according to the manufacturers protocol. After 1 hr incubation at 25°C, the labeled translation products were separated by PAGE and the radioactivity in the bands corresponding to processed and unprocessed products was quantified using PhosphorImager (Molecular Dynamics) and ImageQuant, version 5 software package. This radioactivity was normalized to the number of methionine residues present in each product and used to calculate the efficiency of proteolysis as described (33). The results represent means and standard deviations from four independent reactions. The mini-BYV variants were further characterized using protoplasts isolated from the suspension culture of *Nicotiana tabacum* cells (12) or from the leaves of *N. benthamiana* (36). Protoplasts were harvested at 4 days post transfection and used to measure the GUS activity (10). Each recombinant variant was characterized in four independent transfections. The pBYV-GFP-based variants were manually inoculated to leaves of *Claytonia perfoliata* or *N. benthamiana*, and the number and diameter of the resulting fluorescent infection foci were determined at eight days post inoculation (34).

Fig. 3.1. Diagrams of the BYV genome (top) and the cDNA clone of mini-BYV variant tagged by insertion of the β-glucuronidase gene (GUS). The BYV ORFs from 1 to 8 encode leader proteinase (L-Pro), replicase that harbors methyltransferase (MET), RNA helicase (HEL), and RNA polymerase (POL) domains, 6 kDa-protein (p6), HSP70-homolog (HSP70h), 64 kDa-protein (p64), minor capsid protein (CPm), major capsid protein (CP), 20 kDa-protein (p20), and 21 kDa-protein (p21). The arrows mark the self-processing sites for the viral leader proteinases. An expanded diagram of the BYV L-Pro coding region and its chimeric variants is shown below. *Sac* II and *Sph* I, the endonuclease restriction sites engineered to facilitate generation of the replacement mutants. S1, a short N-terminal region of the L-Pro (32). NTD, N-terminal, non-proteolytic domains; Pro and pro, the papain-like, proteolytic domains of the BYV L-Pro and foreign proteinases, respectively. CTV, citrus tristeza virus (a *Closterovirus*); LIYV, lettuce infectious yellows virus (a *Crinivirus*); TEV, tobacco etch virus (a *Potyvirus*).
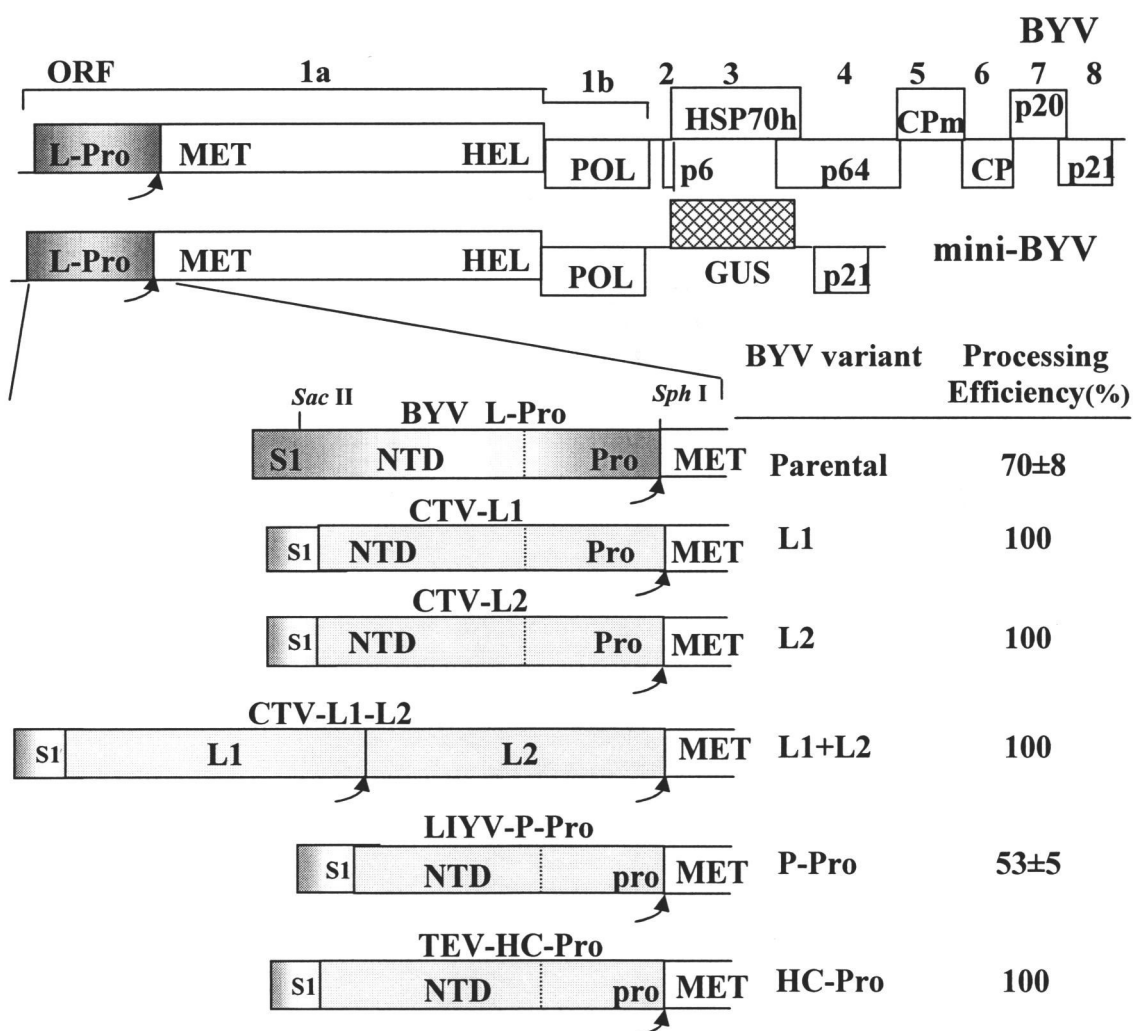
3.3.3 Subcellular localization of the leader proteinases

The expression cassette encompassing duplicated cauliflower mosaic virus 35S-promoter, tobacco etch virus leader, poly(A) signal (5), and GFP was cloned into binary vector pCB302 (42). The genes encoding GUS, L-Pro, L1 and L2, and P-Pro were PCR-amplified using the full-length cDNA clones of the TEV-GUS (10), BYV(34), CTV (36), and LIYV (25), respectively, with the concomitant addition of the *Avr* II and *Xba* I sites into their 5'- and 3'-terminal regions. The translation stop codons were introduced into amplified genes upstream from the *Xba* I sites. The modified genes were cloned downstream from the GFP gene using the *Avr* II and *Xba* I sites. The resulting plasmids were transformed into *Agrobacterium tumefaciens* strain EHA 105, and used for transient protein expression via infiltration of the bacteria to the leaves of *N. benthamiana* (28). GFP fluorescence was detected at two days post infiltration using a confocal laser scanning microscope Leica TCS 4D. For GFP imaging, a 488/568 nm excitation beam generated by krypton/argon laser was used with an RSP580 beam-splitter and BP-FITC emission filter.

3.3.4 Phylogenetic analysis

The multiple alignments of amino acid sequences were generated using the Macaw program (37). The Gibbs sampler option of Macaw was used to detect the blocks with highest sequence similarity. The Phylip package (Felsenstein, J. 1993. PHYLIP, Phylogeny Inference Package, version 3.5c; distributed by the author, Department of Genetics, University of Washington, Seattle) was used for construction of the phylogenetic trees. Hundred bootstrap replicates were obtained

using the SEQBOOT program; the trees were built using the neighbor-joining algorithm (NEIGHBOR program) or maximum likelihood algorithm (KITSCH program).

3.4 Results

To facilitate generation and characterization of interviral hybrids, we used a cDNA clone of a mini-BYV variant that was tagged by insertion of the bacterial β-glucuronidase (GUS) gene (16). This reporter gene replaced six BYV ORFs that are non-essential for the genome amplification (Fig. 3.1), and provided a convenient and sensitive marker for quantification of the amplification and expression of the viral genome. It was demonstrated that accumulation of the GUS activity strictly correlates with accumulation of the viral RNAs (32).

3.4.1 Replacement of the BYV L-Pro with heterologous leader proteinases

Each of the cDNA fragments encoding the foreign proteinase replaced almost the entire BYV L-Pro region (Fig. 3.1). The 70 codon-long, S1-part of this region was retained because it contains an RNA element that is crucial for BYV RNA amplification (32). Hence, each of the foreign leader proteinases expressed by chimeric BYV variants possessed an N-terminal extension. Two artificial restriction endonuclease sites were introduced immediately downstream from S1 region and from the L-Pro cleavage site to accommodate the foreign inserts (see Materials and Methods). The possible effect of corresponding mutations on the amplification and expression of the BYV genome was assessed using transfection of the

corresponding RNA transcript into tobacco protoplasts. It was found that the resulting double mutant amplified to the level similar to that of the parental BYV variant (data not shown).

In addition to replacing the BYV L-Pro with the CTV L1 or L2, LIYV P-Pro, or the tobacco etch potyvirus (TEV) HC-Pro, we engineered a mini-BYV variant containing both of the CTV proteinases to mimic the tandem organization of the L1- L2 region of the CTV polyprotein (Fig. 3.1). The processing of chimeric polyproteins was examined in the *in vitro* translation system. As in previous studies (1, 33), the authentic BYV L-Pro was able to process ~70% of the translation product after 1 hr incubation in the cell-free system. In contrast, processing of the chimeric translation products by the CTV L1, L2, and the TEV HC-Pro was essentially complete. In the case of LIYV P-Pro, processing efficiency was somewhat lower than that of the parental BYV variant (Fig. 3.1).

The genome amplification of the chimeric mini-BYV variants was examined using transfection of two types of protoplasts, suspension culture protoplasts derived from *N. tabacum* and *N. benthamiana* leaf protoplasts. Replacement of the BYV L-Pro with each of the CTV leader proteinases resulted in two conspicuously distinct phenotypes. The L1 was able to partially replace L-Pro function in genome amplification, whereas the L2 failed to do so (Table 3.1). However, the replacement hybrid expressing the combination of L1 and L2 reproduced more efficiently than one expressing the L1 only. In *N. benthamiana* protoplasts, this L1-L2 variant reproducibly outperformed the parental BYV variant expressing the authentic L-Pro.

The functional profile of the replacement chimera harboring LIYV P-Pro resembled that of the L1: the P-Pro supported the amplification of the chimeric genome much more efficiently in *N. benthamiana* protoplasts compared to *N. tabacum* protoplasts (Table 3.1). In contrast, the TEV HC-Pro was unable to functionally substitute the BYV L-Pro in either of the protoplast systems. These results revealed a high degree of the functional specialization of the closterovirus

leader proteinases and indicated that CTV L1, LIYV P-Pro, but not CTV L2 or TEV HC-Pro can provide functions required for the efficient amplification of the chimeric mini-BYV genome. The strikingly better performance of all three viable replacement variants in *N. benthamiana* protoplasts compared to their performance in *N. tabacum* protoplasts suggested that the function of leader proteinases is affected by species-specific host factors.

Table 3.1. GUS activity in *N. tabacum* and *N. benthamiana* protoplasts transfected with the chimeric BYV variants harboring full-size, heterologous leader proteinases (% of level in BYV-GUS-p21± SD)

BYV Variant	*N. tabacum*	*N. benthamiana*
Parental	100	100
CTV-L1	10±2	29±5
CTV-L2	<0.001	<0.001
CTV-L1-L2	19±5	185±25
LIYV-P-Pro	2±1	34±6
TEV HC-Pro	<0.001	<0.001

3.4.2 Functional specialization of the papain-like proteolytic domains

To determine if the sole function of the papain-like domains of the closteroviral and potyviral leader proteinases is autoprocessing, we engineered a series of chimeric viruses in which only the C-terminal, proteinase domain of L-Pro

was replaced with the homologous domains derived from L1, L2, P-Pro, and HC-Pro (Fig. 3.2). If this assumption were true, the papain-like domains of different viruses would be functionally equivalent to each other. *In vitro* assays revealed that the proteolytic activities of the chimeric proteinases harboring papain-like domains of the L1 and P-Pro were not significantly different from that of the parental variant (Fig. 3.2). The activity of the L2 chimera was somewhat lower, whereas the TEV proteinase domain processed ~100% of the polyprotein.

The *in vivo* experiments indicated that only the CTV proteinase domains derived from the L1 and L2 were capable of supporting limited genome amplification of the corresponding chimeric variants (Table 3.2). None of the variants that harbored LIYV or TEV proteinase domains were viable. Comparison of the data in Fig. 3.2 and Table 3.2 reveals no apparent correlation between the levels of proteolytic activity and genome amplification of the chimeric variants. It should be emphasized that unlike chimeras expressing the full-size L1, L2, P-Pro, and HC-Pro, reproduction of those expressing only the corresponding proteinase domains, did not depend on the source of protoplasts (compare Tables 3.1 and 3.2). It can be concluded that in addition to proteolytic processing, the homologous, papain-like, proteolytic domains of the closterovirus leader proteinases posses additional specialized functions required for efficient genome amplification.

Fig. 3.2. Diagrams of the chimeric variants in which authentic proteinase domain of the BYV L-Pro (Pro) was replaced with those derived from CTV L1 or L2, LIYV P-Pro, and TEV HC-Pro (each designated as pro). *Sac* II and *Sph* I, the endonuclease restriction sites engineered to facilitate generation of the chimeric variants.

BYV variant	Processing (%WT)
Wild type	100
L1-pro	105±7
L2-pro	71±5
P-pro	96±4
HC-pro	142

3.4.3 Invasiveness and cell-to-cell movement of the hybrid viruses

To examine the phenotypes of the chimeric viruses in intact plants, we employed the BYV variant tagged via insertion of the reporter gene encoding the green fluorescent protein (BYV-GFP). Several viable chimeric variants were engineered into BYV-GFP, and the corresponding RNA transcripts were mechanically inoculated to the leaves of *Claytonia perfoliata*. The multicellular infection foci formed by BYV-GFP can be easily detected and measured using the fluorescent microscope. At 8 days post inoculation, the parental virus produced on average 12 infection foci per leaf; the mean diameter of the foci was ~4 cells (Table 3.3). In contrast, numbers of the infection foci produced by the chimeric variants

were reduced by factor from 14 to 25. Moreover, all of these foci were unicellular (Table 3.3). Importantly, a similar phenotype of infection was observed when only the proteinase domain of the L-Pro was replaced with that of CTV L1.

Although *C.perfoliata* is a susceptible local lesion host of the BYV, it is not a reported host for CTV or LIYV. Because of that, this plant species could impose host-specific constraints on the functions of CTV or LIYV proteinases. To determine if the invasiveness and intercellular translocation of the chimeric viruses can be improved in a more permissive host, we inoculated leaves of *N. benthamiana* with a BYV-GFP variant expressing CTV L1 and L2. As shown above, the leaf protoplasts derived from this plant species supported very efficient amplification of the L1-L2 chimera (Table 3.1). In accord with the earlier work (34), the average number of infection foci found on *N. benthamiana* leaves was much less than that on the *C. perfoliata* leaves. The parental BYV-GFP and its L1-L2 replacement chimera produced 0.94±0.3 and 0.83±0.2 foci per leaf, respectively. The mean diameters of the infection foci were 4.3±2.1 cells for BYV-GFP and one cell for the L1-L2 chimera. Thus, the specific infectivity (invasiveness), but not the cell-to-cell movement of the L1-L2 chimera was restored in *N. benthamiana* compared to *C. perfoliata*.

Taken together, these results clearly indicated that the replacement of the authentic BYV L-Pro with proteinases derived from other members of the family *Closteroviridae* resulted in a dramatic decrease in invasiveness of the chimeric RNA transcripts. Moreover, these chimeric viruses completely lost the ability to move from cell to cell.

Table 3.2. GUS activity in *N. tabacum* and *N. benthamiana* protoplasts transfected with the chimeric BYV variants harboring heterologous proteinase domains (% of level in BYV-GUS-p21± SD)

BYV Variant	*N. tabacum*	*N. benthamiana*
Parental	100	100
CTV-L1-pro	11±3	14±2
CTV-L2-pro	2±1	3±1
LIYV-P-pro	<0.001	<0.001
TEV HC-pro	<0.001	<0.001

Table 3.3. Specific infectivity and cell-to-cell movement of the chimeric BYV variants in the leaves of *Claytonia perfoliata*

BYV-GFP Variant	Number of foci per leaf	Mean diameter (cells)
Parental	12.4±3.5	4.3±1.8
CTV-L1	0.5±0.2	1
CTV-L1-L2	0.8±0.3	1
LIYV-P-Pro	0.6±0.2	1
CTV-L1-pro	0.9±0.3	1

3.4.4 Subcellular localization of the GFP-leader proteinase fusion proteins

To determine if the closteroviral leader proteinases possess signals for targeting to specific cellular compartments, we employed *Agrobacterium*-mediated, transient expression of these proteins in the leaves of *N. benthamiana*. Each of the ORFs encoding BYV L-Pro, CTV L1 and L2, and LIYV P-Pro was fused in frame with the 3'-terminus of the GFP ORF. An analogous GFP-GUS fusion was used as a control because neither of these reporter proteins possesses specific targeting signals. Furthermore, the mol. wt of the GFP-GUS (~95 kDa) is similar to that of the GFP-L-Pro (~93 kDa) and other tested fusion products. Figure 3 shows that the green fluorescence of the GFP-GUS was uniformly distributed in the cytosol, although a fraction of the product in some cells was localized to nuclei (not shown). A similar pattern of subcellular localization was observed for the CTV L1 and L2 fusion products, whereas the GFP-L-Pro formed distinct cytoplasmic inclusion bodies (Fig. 3.3). In contrast, very little of the GFP-P-Pro was detected in the cytosol; most of the fluorescence was confined to the nuclei (Fig. 3.3).

Fig. 3.3. Subcellular localization of the GUS (control) and viral leader proteinases fused to the GFP-reporter. White color corresponds to GFP-specific fluorescence, whereas small gray bodies are the autofluorescent chloroplasts.
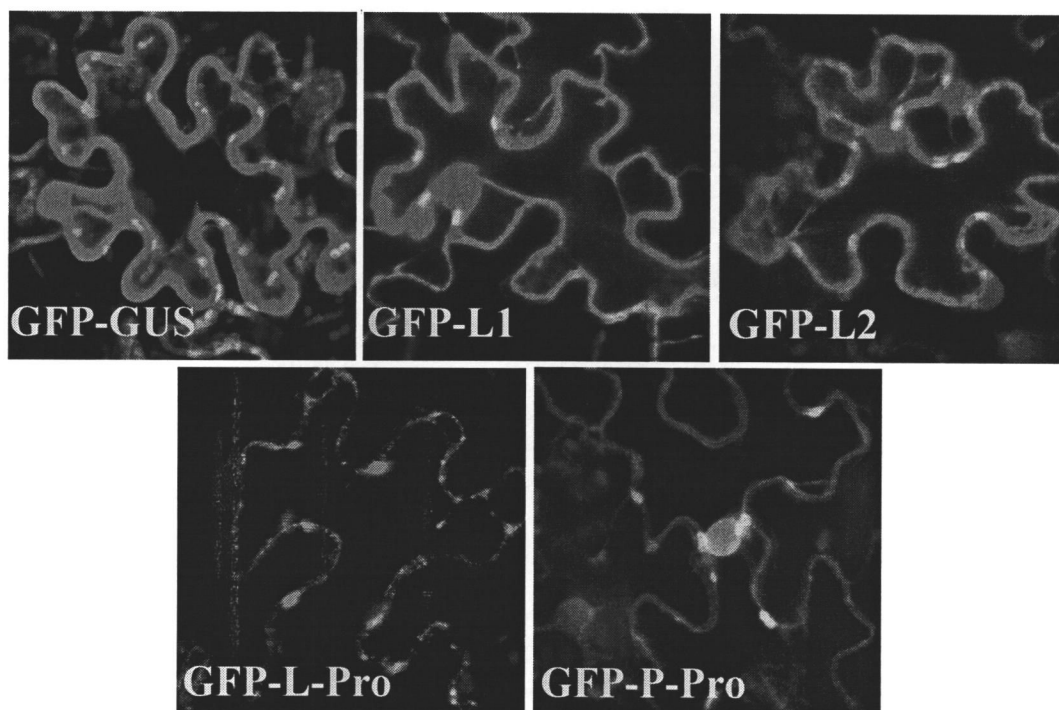
3.4.5 Phylogenetic analysis of the closterovirus papain-like proteinases

We examined the evolutionary relationships of the leader proteinases from BYV, CTV, LIYV, and two additional members of the family *Closteroviridae*, the grapevine leafroll-associated virus-2 (GLRaV-2) (44) and the little cherry virus (LChV) (17). The multiple alignment of corresponding amino acid sequences clearly revealed the conserved C-terminal domain that possessed signature motifs

common for the viral papain-like proteinases (Fig. 3.4). In contrast, extensive comparisons of the N-terminal, non-proteolytic domains revealed no amino acid motifs common for all included leader proteinases. Only a limited conservation in the regions located upstream from the proteinase domains was observed in subsets of leader proteinases (e.g., CTV L1 and L2; data not shown).

Fig. 3.4. Multiple alignment of the amino acid sequences of the papain-like proteinase domains encoded in diverse representatives of the family *Closteroviridae*. GLR, the GLRaV-2, grapevine leafroll-associated virus-2 (44); LChV, little cherry virus (17). Invariant residues are shown in bold face, blocks of conserved residues are in capital letters. The cysteine residue predicted to directly attack the scissile peptide bond, and the histidine residue also likely to participate in catalysis, are marked by asterisks. An arrow and a gap indicate a scissile bond between a glycine and another small residue (glycine, alanine, or serine).

```
                   *
BYV      EGLCYLAHAalccalqkrtfreedff-VGMYPTKFVFAKRLTEKLGPSALKHPVRgrq
GLR-L1   DGRCYLAHMrylcafycrpfresdya-LGMWPTVARLRACVEKNFGVEACGIALRgyy
GLR-L2   NGFCYLAHCryacafllrgfdpkrfd-IGAFPTAAKLRNRMVSELGERSLGLNLYgay
CTV-L1   DGQCYVRHVfdvalyfgrradlsvrraLGMYPTVGALKAYLVREYGRDSLKVPMRgty
CTV-L2   DGYCYIRHFaevslsmgriffrrdvd-LGPFPYVFEVKHRLERLYGKAALRYGVRgqy
LIYV     DGFCWLDVFada--------------NRRIPEWVKPHCLLTGSVLMSCGLWDFAkrk
LChV     NGFCWLQAFamf--------------GKIIPTFVEFIPNLNVSCLLQAGLPKSClky


                 *                      ↓
BYV      ---VSRSLFHCDVASAfsspfyslpr-FIG G
GLR-L1   ---TSRNVYHCDYDSAyvkyfrnlsg-RIG G
GLR-L2   ---TSRGVFHCDYDAKfikdlrlmsavIAG G
CTV-L1   ---TFGSVFHCLSTKSsvdlrsipnhhLVG G
CTV-L2   ---SAPRCFHCCYNDSprpmasfngyhKMG G
LIYV     mvsVSHGLLHYDRKLErssaragvrd-FVG A
LChV     mhkTGNALYHFDPNKInniyhanlnf-LVG S
```

Fig. 3.5. Dendrogram illustrating phylogenetic relations of the conserved, papain-like domains of closteroviral proteinases. The numbers indicate the results of the bootstrap analysis. The proteinase domain of the LIYV was used as an outgroup. Although the bootstrap value corresponding to grouping of the CTV L1 and L2 is rather low, affinity of these leader proteinases was further supported by the presence of conserved amino acid sequence motifs upstream from the proteinase domains (not shown).
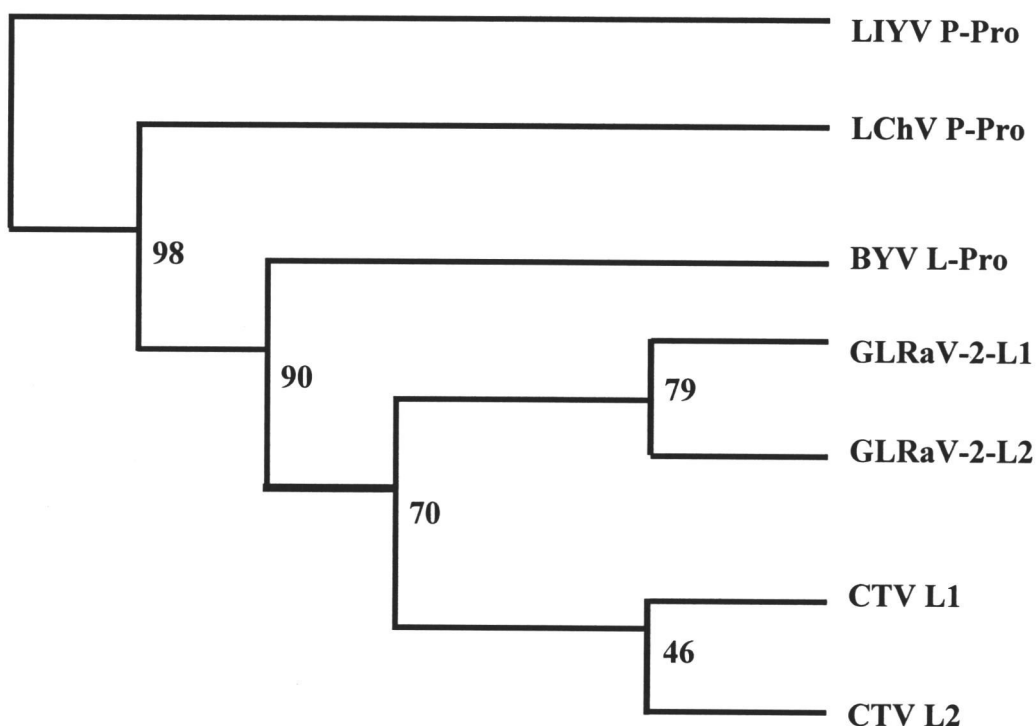
The phylogenetic trees of the papain-like domains based on the neighbor-joining algorithm (Fig. 3.5) and maximum likelihood algorithm (not shown) were very similar. The proteinase of the LIYV was selected as an outgroup in accord with the distinct genome organization and sequence relationships of the LIYV replicational proteins (19). Notably, specific affinities were observed between the L1 and L2 proteinase domains in both CTV and GLRaV-2. These pairs of the proteinases appear to be closer related to each other than to homologs present in

other family members. This tree topology suggests the intragenomic duplication as a likely scenario for the origin of the tandem genes encoding L1 and L2 of CTV and GLRaV-2.

3.5 Discussion

The leader proteinases of the positive-strand RNA viruses from the families *Potyviridae* and *Closteroviridae* share similar plans of organization possessing a variable N-terminal domain and a conserved, C-terminal, papain-like domain. In addition to polyprotein processing, these proteinases were implicated in efficient genome amplification (21, 22, 32, 33) and suggested to share the similar functional profile (12). To test this possibility, we replaced the L-Pro of BYV, a *Closterovirus*, with HC-Pro of TEV, a *Potyvirus*. Although the chimeric polyprotein was efficiently processed, the hybrid BYV was nonviable, suggesting that the functions of the L-Pro and HC-Pro in genome amplification are mechanistically different. As it was proposed recently, the HC-Pro role in genome amplification is mediated by the suppression of RNA silencing (6). The BYV L-Pro, however, lacks detectable silencing suppression activity (J. Reed, K.D. Kasschau, J.C. Carrington, and V.V.D., unpublished data).

We further asked whether the functional specialization of the L-Pro and HC-Pro is provided solely by their unrelated N-terminal domains. A chimeric protein in which the N-terminal domain of the BYV L-Pro was fused to the papain-like domain of the TEV HC-Pro failed to support genome amplification of the BYV, suggesting that despite their homology, the papain-like domains of the closteroviruses and potyviruses are functionally distinct. It should be noted that functional differences between the L-Pro and HC-Pro can not be attributed to differences in the host ranges of BYV and TEV since each of these viruses readily

infects *N. benthamiana* and several other common hosts. We can also exclude the possibility that the expression of the TEV proteinase domain or insertion of the corresponding RNA exerted an inhibitory effect on BYV amplification. Indeed, this domain was expressed from several locations within the BYV genome without affecting the viability of the resulting hybrid variants (16).

To examine functional specialization of the closteroviral leader proteinases, we swapped the corresponding genes of CTV and LIYV that belong to two distinct evolutionary lineages within the family *Closteroviridae* into BYV. Among these viruses, BYV and LIYV possess only one leader proteinase, whereas CTV possesses two, L1 and L2. Phylogenetic analysis (Fig. 3.5) suggested that the corresponding gene tandem in CTV evolved via a duplication event. It was not, however, known, if the L1 and L2 are functionally distinct, and if yes, which of them is more similar to the leader proteinases of BYV or LIYV. The ability of the CTV L1 and LIYV P-Pro to substitute for the BYV L-Pro in genome amplification indicated that these three leader proteinases belong to the same functional class. In contrast, failure of the CTV L2 to support amplification of the chimeric genome suggested that L2 function had diverged from that of L1, L-Pro, and P-Pro. This assumption was further confirmed by a phenotype of the BYV chimera that expressed both the L1 and L2. This chimera amplified almost twice as efficiently as the original BYV, providing a remarkable example of a hybrid virus that outperformed its parent.

The transient expression experiments revealed distinct patterns of subcellular localization of the leader proteinases fused with the GFP reporter. Most of the CTV L1 and L2 were uniformly distributed in cytoplasm and nucleus, whereas the LIYV P-Pro almost exclusively localized to nuclei. In contrast, the BYV L-Pro was observed predominantly in cytoplasmic inclusion bodies. It can not be excluded that the localization of the leader proteinases in a context of the virus-infected cell might be different from that observed in the transient expression experiments. Nevertheless, our results suggest that the leader proteinases of CTV,

BYV, and LIYV possess distinct intrinsic signals for interaction with the cell environment.

Duplication and functional divergence of the leader proteinase genes is not unique to closteroviruses. A tandem arrangement of the leader proteinases is found among several animal viruses from the order *Nidovirales* (9, 38, 41, 43). Although *Closteroviridae* and *Nidovirales* are phylogenetically dissimilar, they are the most complex positive-strand RNA viruses of plants and animals, respectively (26, 43). Apparently independent duplication of the leader proteinases in these viruses may be interpreted as one of the means to facilitate evolution of the larger and more complex genomes. In accord with this speculation, acquisition of the second leader proteinase in the ~20kb CTV genome is accompanied by three additional genes that have no homologs in otherwise closely related ~15 kb BYV genome.

The gene swapping experiments revealed an unexpected degree of the functional specialization of the papain-like domains of the closteroviral proteinases. Each of these domains efficiently processed the chimeric polyprotein. However, the papain-like domains of the CTV L1 and L2 supported relatively low levels of BYV genome amplification, whereas the corresponding domain of the LIYV P-Pro was completely nonfunctional (Fig. 3.2 and Table 3.2). Although the mechanistic basis for this specialization is unknown, it seems possible that the proper function of the leader proteinases requires structural compatibility between the N-terminal and C-terminal domains.

Perhaps the most important outcome of this work is a better understanding of the multifunctional nature of the closteroviral proteinases. In addition to the primary role in the autocatalytic processing, each of the studied four proteinases functions in activation of genome amplification in a host-specific manner. Moreover, at least the L-Pro is critical for the ability of BYV to establish infection in the initially inoculated cells (virus invasiveness), and to translocate from cell to cell.

The cell-to-cell movement of plant viruses proceeds through plasmodesmata and is activated by the movement proteins (27). In BYV, as many as five proteins that are encoded by a conserved gene block were implicated in virus movement. These proteins include three dedicated movement proteins (p6, HSP70h, and p64) and two capsid proteins (2, 34). Since virion assembly is a prerequisite for BYV cell-to-cell movement (2), it was possible that the debilitated movement of the chimeric BYV-GFP variants was due to the defective assembly. However, analysis of the chimeric virus progeny from the transfected protoplasts revealed normal virion assembly (data not shown). Hence, the role played in BYV movement by the replication-associated L-Pro may suggest the coordination of the genome amplification and virus translocation processes. Intriguingly, the leader proteinase of the foot-and-mouth disease virus, an *Aphtovirus*, was recently implicated in its spread within infected animals (7). Although the mechanisms of virus transport in plants and animals are different, this functional parallelism highlights evolutionary plasticity of the viral papain-like proteinases that provide a structural platform for a variety of functions.

The mechanistic basis of multifunctionality and specialization of the closteroviral leader proteinases is yet to be determined. These proteinases may act via the cleavage of or via interaction with the particular viral or host target proteins. The host-dependent mode of activation of genome amplification and its role in virus invasiveness suggest that the intracellular targets of the closteroviral leader proteinases may include host factors. In conclusion, the gene swapping approach allowed us to reveal novel functions of the viral proteinases and to generate capable interviral hybrids. Further study of these hybrids will provide an insight into molecular mechanisms underlying activities of leader proteinases in genome amplification and virus invasion, and help to design more efficient viral gene expression vectors.

3.6 Acknowledgements

3.7 References

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Chapter 4

A Replication-Competent Chimera of Plant and Animal Viruses

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4.1 Abstract

Human, animal, fungal, and plant viruses encode papain-like proteinases that function in polyprotein processing, RNA synthesis, and virus-host interactions. To compare the functional profiles of diverse papain-like proteinases, we replaced a proteinase gene of the *Beet yellows virus* (BYV) with those derived from *Equine arteritis virus* (EAV), *Foot-and-mouth disease virus* (FMDV), and the fungal virus CHV1. We found that, although each of the foreign proteinases efficiently processed the viral polyprotein, only the EAV proteinase supported vigorous replication of the chimeric BYV in plant protoplasts. This result demonstrated that the proteinases of BYV and EAV, but not FMDV or CHV1, provide a function that is critical for genome replication and that is separable from polyprotein processing. Further characterization of the BYV-EAV chimera revealed that BYV proteinase is also required for virus invasion and cell-to-cell movement. Thus, the same viral protein can combine both replication-related functions shared by plant and animal viruses and specialized functions in virus-host interactions.

4.2 Introduction

The advent of viral genomics two decades ago dramatically enhanced our understanding of the structure and evolution of viral genomes. One of the least expected outcomes of the comparative analyses of viral genomes was the discovery of similarities between RNA viruses of animals and plants (reviewed in Goldbach, 1987). Viral RNA genomes were conceptualized as mosaics of gene modules with an RNA polymerase gene being the only universally conserved module (Kamer and Argos, 1984; Koonin, 1991). The recombinational shuffling of gene modules was

proposed to be a major driving force behind the large-scale evolution of RNA viruses. Among viral gene modules, those encoding proteinases are found in all major subdivisions of viruses infecting eukaryotes (Dougherty and Semler, 1993; Babe and Craik, 1997; Guarne *et al.*, 1998). In particular, representatives of papain-like proteinases are encoded in a variety of positive strand RNA viruses (Gorbalenya *et al.*, 1991; Koonin and Dolja, 1993).

Viral papain-like proteinases may be further subdivided into main and leader proteinases. Main proteinases are responsible for the proteolytic maturation of the replicase components and are intimately involved in genome amplification (Strauss and Strauss, 1994). These proteinases are located internally in the polyprotein and possess both *cis* and *trans* cleavage activities. In contrast, leader proteinases encompass the N-terminal part of the polyprotein and normally cleave only *in cis*. Leader proteinases can be dispensable for genome replication and are implicated in the synthesis of viral mRNAs, the inhibition of cellular translation, viral pathogenicity, virus transport within the infected organism, and suppression of the host defense response (Guarne *et al.*, 1998, Kasschau and Carrington, 1998; Chinsangaram *et al.*, 1999; Suzuki *et al.*, 1999; Tijms *et al.*, 2001). To gain further insight into the remarkable functional flexibility of papain-like leader proteinases, we analyzed the ability of leader proteinases derived from the dissimilar animal and fungal viruses to function in the context of a virus-infected plant cell. In other words, we asked if a novel, chimeric viral genome could be engineered by mimicking a process of modular evolution that shaped the genomes of present-day RNA viruses (Koonin and Dolja, 1993).

Our model, the *Beet yellows virus* (BYV), belongs to *Closteroviridae* family of positive strand RNA viruses. The 15.5 kb genome of BYV encodes a broad array of proteins that have homologs in diverse RNA viruses and cellular organisms (Fig. 1) (Agranovsky *et al.*, 1994; Dolja *et al.*, 1994). For instance, the BYV replicase is similar to those of other *Sindbis virus*-like viruses, whereas the BYV Hsp70 homolog functions in virus translocation from cell to cell (Peremyslov *et al.*, 1999).

The papain-like leader proteinase (L-Pro) of BYV is somewhat atypical because of its requirement for efficient genome amplification (Agranovsky *et al.*, 1994, Peremyslov *et al.*, 1998). Although L-Pro is not essential for basal-level replication, its elimination results in a 1,000-fold reduction in the accumulation of viral RNA (Peng and Dolja, 2000). In this study, we targeted the BYV L-Pro gene for replacement with the leader proteinase genes derived from the fungal virus CHV1 (family *Hypoviridae*), *Foot-and-mouth disease virus* (FMDV, family *Picornaviridae*), and the *Equine arteritis virus* (EAV, family *Arteriviridae*). The p29 proteinase of CHV1 is dispensable for virus replication, but is a major determinant of pathogenicity (Suzuki *et al.*, 1999). The FMDV Lab proteinase is involved in inhibition of cellular mRNA translation and in virus spread in infected animals (Guarne *et al.*, 1998, Chinsangaram *et al.*, 1999). The nsp1 proteinase of EAV is essential for subgenomic mRNA synthesis, but dispensable for genome replication (Tijms *et al.*, 2001).

Characterization of BYV L-Pro replacement hybrids revealed efficient autoprocessing by each of the foreign proteinases. However, only the EAV nsp1, but not CHV1 p29 or FMDV Lab was capable of supporting the genome replication of chimeric BYV. This result indicated that BYV L-Pro and EAV nsp1 possess a common activity that is distinct from polyprotein processing and that is required for genome amplification. Efficient replication of the chimeric virus in isolated plant cells allowed us to examine the infection phenotype in whole plants. The largely reduced invasiveness and debilitated cell-to-cell movement of the BYV-EAV chimera demonstrated that EAV nsp1 failed to substitute for BYV L-Pro functions that are required for the successful development of infection in plants. This work revealed the unexpectedly complex and partially overlapping functional profiles of the leader proteinases of a plant and an animal virus. In addition, it illustrated the utility of the gene swapping approach for studies of virus evolution and host specificity.

Fig. 4.1. The genome maps of the viruses used in gene swapping experiments. CHV1, *Cryphonectria* hypovirus 1; FMDV, foot-and-mouth disease virus; EAV, equine arteritis virus; BYV, beet yellows virus; mini-BYV, a recombinant BYV variant in which six genes that are nonessential for replication were replaced with the reporter β-glucuronidase (GUS) gene. The genes coding for leader proteinases p29, Lab, nsp1, and L-Pro are shaded, whereas GUS gene is crosshatched. The arrows designate the sites for autoproteolysis by the leader proteinases. The BYV ORFs from 1 to 8 encode leader proteinase (L-Pro), replicase possessing methyltransferase (MET), RNA helicase (HEL), and RNA polymerase (POL) domains, 6 kDa-protein (p6), HSP70-homolog (HSP70h), 64 kDa-protein (p64), minor capsid protein (CPm), major capsid protein (CP), 20 kDa-protein (p20), and 21 kDa-protein (p21), respectively.
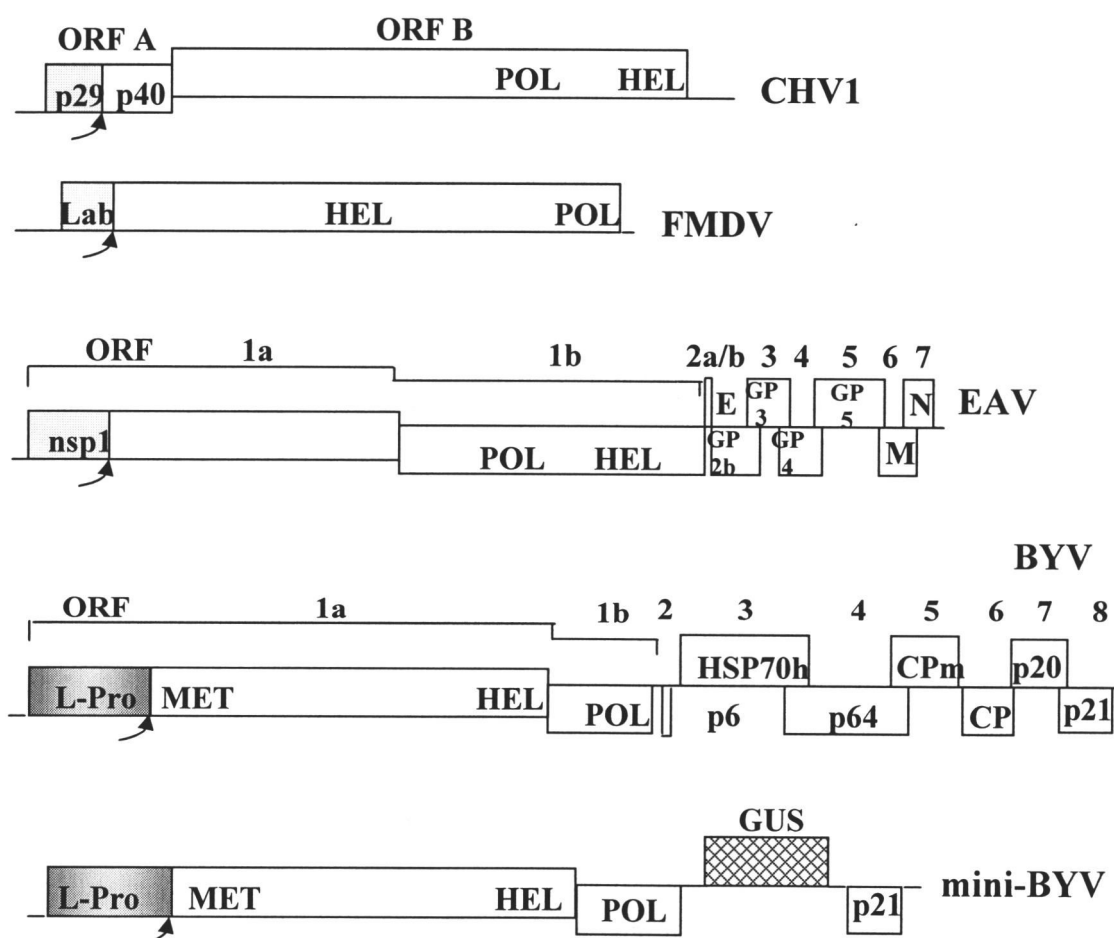
4.3 Materials and Methods

<u>4.3.1 Engineering of chimeric genomes</u>

The chimeras were generated using standard techniques of site-directed mutagenesis and genetic engineering. Restriction endonuclease sites *Sac* II and *Sph* I were introduced downstream of the S1 region and the L-Pro cleavage site, respectively (Fig. 4.2), and were used to replace the L-Pro coding region with those encoding full-size foreign proteinases. The corresponding modifications introduced into BYV ORF 1a were as follows. Three artificial Ala codons replaced codons 72-74 at the end of the S1 region to generate the *Sac* II site. This mutation is identical to the mutation A2 described previously (Peng and Dolja, 2000). The codons 590-592 located immediately downstream of the two consecutive Gly codons that specify a scissile bond were replaced with His, Ala, and Glu codons to accommodate a *Sph* I site. These modifications did not affect the accumulation of BYV RNA in transfected protoplasts (data not shown). The entire regions encoding CHV1 p29, FMDV Lab, and EAV nsp1 were PCR-amplified with the concomitant addition of the *Sac* II and *Sph* I sites at their respective 5'- and 3'-termini, digested with the corresponding restriction endonucleases, and used to replace most of the BYV L-Pro coding region in a context of the plasmid pBYV-GUS-p21 (Hagiwara *et al.*, 1999). This plasmid contained a mini-BYV-GUS genome tagged by insertion of GUS gene as shown in the Fig. 4.1.

In the CHV1-Pro, FMDV-Pro, and EAV-Pro variants shown in Fig. 4.4, the papain-like domain of the BYV L-Pro (codons 437-589 of the BYV ORF 1a) was replaced with an analogous domain derived from one of the indicated viruses. These domains encompassed codons 122-249, 29-202, and 141-261 of the CHV1, FMDV, and EAV open reading frames, respectively, and included a scissile Gly-Gly

dipeptide located at the proteinases' C-termini. To generate these replacement mutants, we used the *Sph* I site described above and a *Sac* II site that was generated by replacing ORF 1a codons 434-436 with the three Ala codons. This latter mutation is identical to the A12 mutation characterized earlier (Peng and Dolja, 2000).

The EAV nsp1 and EAV-Pro variants shown in the Fig. 4.6 were generated by replacing the *Nhe* I-*Xba* I fragment in pBYV-GFP plasmid (Peremyslov *et al.*, 1999) with corresponding fragments derived from mini-BYV-GUS variants. The pBYV-GFP contained the full-length BYV genome tagged via insertion of the green fluorescent protein (GFP) gene. The L-Pro + EAV nsp1 variant was obtained by insertion of the nsp1 coding sequence using *Sph* I site located downstream from the L-Pro region (see above). Prior to *in vitro* transcription, the plasmid DNAs were linearized by *Xba* I or *Sma* I for subsequent cell-free translation or protoplast transfection experiments, respectively.

4.3.2 Characterization of the virus phenotypes

Capped RNA transcripts derived from recombinant DNA plasmids were translated *in vitro* using wheat germ extracts or rabbit reticulocyte lysates, or transfected into isolated protoplasts as described previously (Peremyslov *et al.*, 1998). The ^{35}S-methionine-labeled translation products were separated by PAGE and quantified to assess the efficiency of proteolysis. Accumulation of RNAs and GUS activity in protoplasts were determined at four days post transfection by Northern analysis and GUS assays, respectively (Hagiwara *et al.*, 1999). In each experiment, at least four independent samples were used to obtain the mean value and standard deviation. For analysis of the specific infectivity and cell-to-cell movement, the transcripts of pBYV-GFP variants were mechanically inoculated

into *Claytonia perfoliata* plants, and visualized at 8 days post inoculation using fluorescent microscope (Peremyslov *et al.*, 1999). At least two independent inoculation experiments involving 6 leaves were done for each of the variants.

4.3.3 Subcellular localization of the GFP-proteinase fusions

The expression cassette harboring a duplicated cauliflower mosaic virus 35S promoter, the tobacco etch virus leader, a poly(A) signal (Carrington *et al.*, 1990), and the GFP gene was cloned into mini-binary vector pCB302 (Xiang *et al.*, 1999). The genes encoding GUS, BYV L-Pro and EAV nsp1 were PCR-amplified and cloned in frame downstream from the GFP gene. The resulting plasmids were transformed into *Agrobacterium tumefaciens* strain EHA 105, and the transformants were used for transient protein expression in the leaves of *Nicotiana benthamiana* as described (Llave *et al.*, 2000). Leaves were harvested at ~2 days post infiltration. For cell imaging, a confocal laser scanning microscope Leica TCS 4D equipped with krypton/argon laser (488/568 nm excitation beam) was used with an RSP580 beam-splitter and BP-FITC or LP665 filters for GFP and chlorophyll detection, respectively.

4.4 Results

4.4.1 EAV proteinase rescues the replication of BYV

To facilitate the generation and characterization of the chimeric viruses, we used a cDNA clone of a so-called "mini-BYV" genome. In this clone, six BYV genes that are superfluous for genome amplification were replaced with the reporter, β-glucuronidase (GUS) gene, under control of a BYV subgenomic mRNA promoter (Fig. 4.1). The GUS activity produced by mini-BYV in infected cells provided a sensitive combined measure of genome amplification, transcription of subgenomic mRNAs, and translation (Hagiwara *et al.*, 1999). The foreign leader proteinase genes derived from CHV1, FMDV, and EAV each replaced almost the entire BYV L-Pro open reading frame (ORF) (Fig. 4.2). The small, 5'-terminal region of this ORF designated S1 was retained because it contains an RNA element required for replication (Peng and Dolja, 2000). The RNA transcripts from the resulting cDNA clones were translated *in vitro* to test for autoproteolytic activity of the proteinases, or transfected to isolated protoplasts to examine viral replication and gene expression (Peremyslov *et al.*, 1998). The *in vitro* studies were done in wheat germ extracts and rabbit reticulocyte lysates, whereas the *in vivo* experiments were done using protoplasts from tobacco suspension cell culture and *Nicotiana benthamiana* leaves. Reproduction of the experiments in two independent model systems ensured the reliability of the results.

In vitro experiments demonstrated that each of the three foreign proteinases efficiently processed the chimeric polyprotein both in rabbit reticulocyte lysates (Fig. 4.3) and wheat germ extracts (not shown). In rabbit reticulocyte lysates, the cleavage efficiencies of the leader proteinases of CHV1, FMDV, and EAV were slightly higher than that of the authentic BYV L-Pro (Fig. 4.2 and 4.3). However,

Fig. 4.2. Engineering and characterization of chimeric BYV variants. A top diagram corresponds to the wild-type BYV L-Pro and shows its major regions. *Sac* II and *Sph* I, restriction endonuclease sites introduced to facilitate insertion of the foreign genes. Other diagrams illustrate the structure of the chimeric proteinase variants harboring CHV1 p29, FMDV Lab, and EAV nsp1. Pro, homologous, papain-like, proteinase domains in each of the foreign proteinases. ZF, putative zinc finger present in EAV nsp1. The asterisks mark mutation introduced into the nsp1 gene. C164S, mutation that replaced catalytic Cys with Ser resulting in inactivation of proteinase activity (Snijder *et al.*, 1992). C25A and C44A, replacements of the cysteines that form putative zinc finger with alanine (Tijms *et al.*, 2001). The efficiency of autoprocessing in rabbit reticulocyte lysates and the level of genome replication and expression in *N. benthamiana* protoplasts as reflected by Northern analysis and GUS activity are shown for each variant. UD, undetectable. The measurements presented in the table are means from at least four experiments expressed as percentage of the wild-type level. Representative experiments showing polyprotein processing *in vitro* and RNA accumulation in protoplasts are also presented in Figs. 4.3 and 4.4.

BYV Variant	Processing (%)	RNA level (%)	GUS activity(%)
Parental	100	100	100
CHV1-p29	107±7	UD	<0.001
FMDV-Lab	113±9	UD	<0.001
EAV-nsp1	116±6	87±12	82±7
nsp1-C164S	UD	UD	<0.001
nsp1-C25A	115±6	90±15	80±11
nsp1-C44A	113±8	82±13	77±9

quantification of GUS activity revealed that neither CHV1 p29, nor FMDV Lab was able to support a detectable level of GUS expression. Strikingly, EAV nsp1 mediated levels of chimeric virus gene expression comparable to those of the parental mini-BYV (Fig. 4.2). These results could be due to the failure of CHV1 or FMDV proteinases to support replication or transcription of the chimeric genomes. Northern hybridization analysis of the RNAs derived from transfected protoplasts did not detect any virus-specific RNAs produced by BYV-CHV1 and BYV-FMDV chimeras (Fig. 4.4, lanes p29 and Lab). In contrast, the levels of genomic and each of two subgenomic mRNAs produced by the BYV-EAV chimera were similar to those of the wild type (Fig.4.4, lane nsp1). Close correlation of the data obtained

Fig. 4.3. Processing of the wild type and chimeric polyproteins upon translation in rabbit reticulocyte lysates. M, mock-translation with no added mRNA (negative control); BMV, translation of the brome mosaic virus RNA (positive control); p29, Lab, nsp1, chimeric BYV variants CHV1-p29, FMDV-Lab, and EAV-nsp1, respectively; WT, wild type BYV RNA; (same as in Fig. 2). C164S, nsp1 mutant with inactivated catalytic center; C25A and C44A, putative zinc finger mutants of nsp1; (same as in Fig. 2). Arrows indicate the translation products of BMV RNAs 2 and 3. Gray and black arrowheads mark the unprocessed and processed products of translation of the chimeric and wild type BYV RNA.
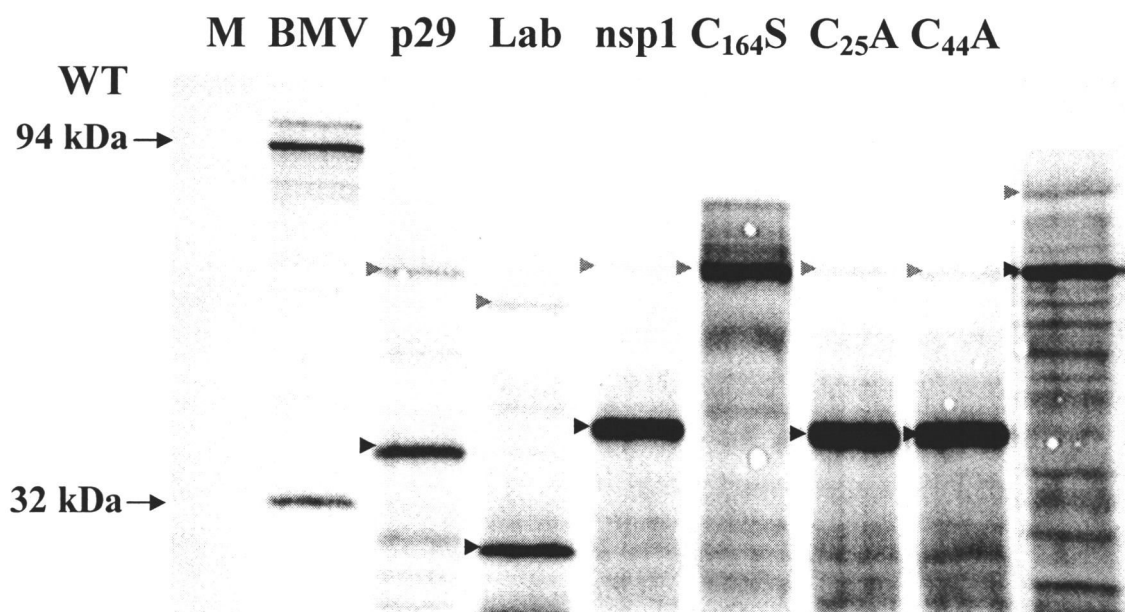
Fig. 4.4. Accumulation of the viral RNAs in transfected *N. benthamiana* protoplasts. RNAs were detected using Northern blot analysis and the probe complementary to the 3'-terminal BYV gene encoding p21. The designations are same as in Fig. 4.2.

M   WT   p29   Lab   nsp1   C25A   C44A C164S

genomic RNA➡

sg RNA (GUS)➡

sg RNA (p21)➡
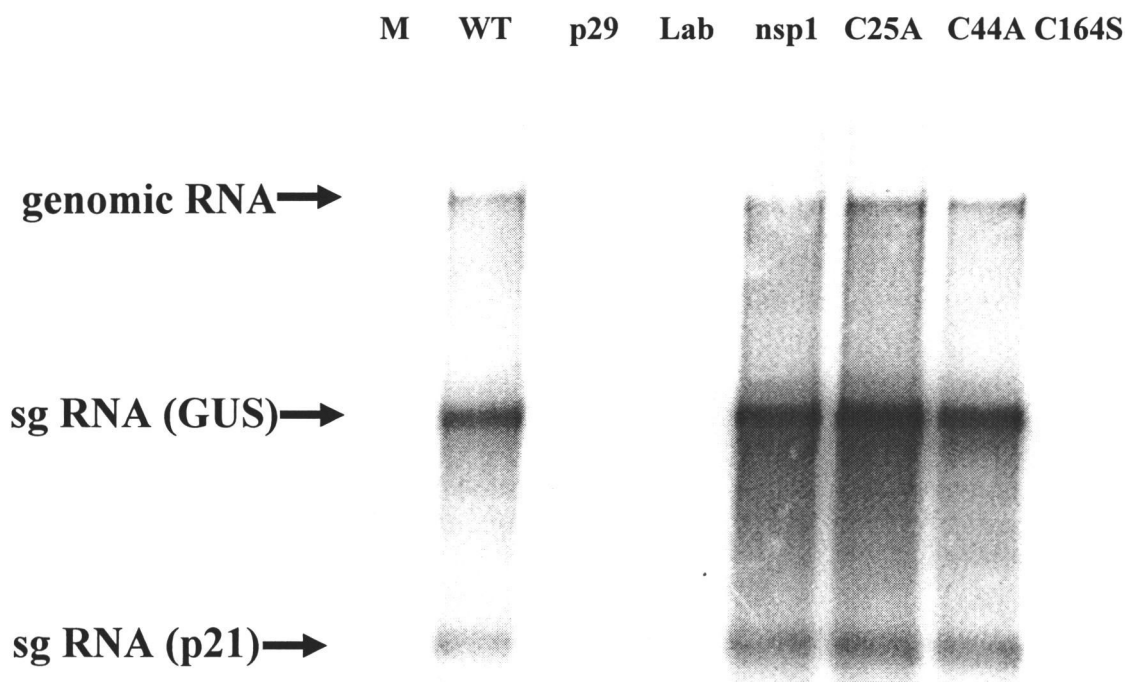
using GUS assays and Northern analysis (Figs. 4.2 and 4.4) indicated that genome amplification, as well as the synthesis and translation of the GUS mRNA were affected in a similar way in all variants.

To determine if processing of the chimeric polyprotein in the BYV-EAV hybrid relied on the proteolytic activity of EAV nsp1, rather than on incidental processing by a cellular proteinase, we tested nsp1 mutant C164S that contains an inactivated catalytic center (Snijder *et al.*, 1992). This mutant variant failed to process the polyprotein (Fig. 4.3, lane C164S) and to replicate (Fig. 4.4, lane C164S), confirming the requirement for nsp1 proteinase activity.

It was demonstrated recently, that the nsp1 activity in transcription of the EAV subgenomic RNAs is mediated by a putative zinc finger localized outside of

the proteinase domain, close to the protein's N-terminus. Substitution of Ala for zinc finger-forming Cys-25 or Cys-44 resulted in debilitation of EAV transcription (Tijms *et al.*, 2001). To test if the zinc finger is also important for the nsp1 function in a context of plant cell infection, we introduced C25A and C44A mutations into BYV-EAV chimera. As expected, these mutations located outside of the C-terminal, proteinase domain did not affect the processing activity (Fig. 4.3). Likewise, the accumulation of GUS activity and viral RNAs in C25A and C44A mutants were indistinguishable from that of the parental BYV-EAV chimera (Figs. 4.2, 4.4). Because of that, we concluded that zinc finger is not required for nsp1-mediated replication of BYV.

Taken together, these data demonstrated that the efficient replication of the BYV-EAV chimera in isolated plant cells was dependent on the functions provided by the foreign leader proteinase. One of these functions is the autoprocessing required for the release from the replicase polyprotein encoded in ORF 1a (Peremyslov *et al.*, 1998). However, this function alone was insufficient to rescue replication of the BYV-CHV1 and BYV-FMDV chimeras. These results revealed that the BYV L-Pro and EAV nsp1 share an additional activity that is critical for efficient replication of BYV genome.

4.4.2 Dual function of the papain-like proteinase domains

Each of the four proteinases employed in this study possesses an N-terminal, non-proteolytic domain, and a C-terminal, papain-like domain (Fig. 4.2). It seemed reasonable to assume that functional specialization of these two-domain proteins is provided by their dissimilar N-terminal domains, whereas the homologous proteinase domains serve the sole purpose of polyprotein processing. If that were the case, the papain-like domains should be functionally interchangeable.

To test this assumption, we designed chimeric leader proteinases in which the authentic N-terminal domain of the BYV L-Pro was fused to the papain-like proteinase domains derived from CHV1, FMDV, or EAV (Fig. 4.5). Although each of the resulting variants was competent in processing, only the variant that possessed the EAV proteinase domain supported the efficient amplification and expression of the BYV genome (Fig. 4.5). In contrast, chimeric BYV variants

Fig. 4.5. Functional specialization of papain-like domains in viral leader proteinases. Note that only the proteinase domain of the L-Pro was replaced with homologous foreign domain in chimeric variants. These domains are designated 'Pro' for CHV1 p29, 'Lb' for FMDV Lab, and 'β-Pro' for EAV nsp1. The efficiency of processing and the level of genome replication and expression as reflected by GUS activity are shown for each variant. Other designations are the same as in the legend to Fig. 4.2.

| BYV L-Pro	BYV variant	Processing (%)	GUS activity (%)
S1 N-terminal domain Pro MET	Parental	100	100
CHV1-Pro / S1 N-terminal domain Pro MET	CHV1-Pro	88±7	<0.001
FMDV-Pro / S1 N-terminal domain Pro MET	FMDV-Pro	77±7	<0.001
EAV-Pro / S1 N-terminal domain Pro MET	EAV-Pro	59±2	56±5

harboring papain-like proteinase domains of CHV1 or FMDV were nonviable. Comparison of the results presented in Figs. 4.2 and 4.5 suggests that the proteinase domains of BYV and EAV, but not those of CHV1 and FMDV, share a specialized

function required for BYV reproduction. It should be emphasized that this function is distinct from autoproteolytic activity, since all tested viral proteinases were competent in polyprotein processing.

4.4.3 Subcellular targeting of the leader proteinases

To determine if the viral leader proteinases harbor autonomous signals for targeting to particular cellular compartments, we analyzed their localization in plant cells. Each viral proteinase was fused to the GFP reporter and transiently expressed in the *N. benthamiana* leaves. A fusion of GFP and GUS was used as a control because neither GFP nor GUS possesses subcellular targeting signals. As expected, GFP-GUS fluorescence was distributed uniformly throughout the cytoplasm that, in plant cells, is confined largely to the cortical region and to transvacuolar cytoplasmic strands (Fig. 4.6, panel GFP-GUS). A similar distribution was observed for the GFP-p29 and GFP-Lab fusion proteins. As described recently, the GFP-L-Pro localized to discrete bodies, which were most abundant in the cortical cytoplasm (Fig. 4.6) (Peng *et al.*, 2001). Very similar distribution was observed for the GFP-nsp1 fusion (Fig. 4.6, panel GFP-nsp1). It is yet to be determined, what the relation is between the formation of the cytoplasmic bodies and the mechanism underlying the activation of genome replication by the leader proteinases of BYV and EAV. However, the correlation between the ability of L-Pro and nsp1 to activate BYV replication and the distinct pattern of their subcellular localization suggests that the formation of cytoplasmic bodies is a functionally important property of these proteinases.

Fig. 4.6. Subcellular localization of the viral leader proteinases fused to green fluorescent protein (GFP). The green color corresponds to the GFP fluorescence; the red spots represent the autofluorescent chloroplasts. The GFP-GUS was used as a control fusion product that is distributed uniformly throughout the cortical cytoplasm and cytoplasmic strands. The GFP-p29 and GFP-Lab are distributed in cytoplasm similar to GFP-GUS. The GFP-L-Pro and GFP-nsp1 localize to cytoplasmic inclusion bodies.
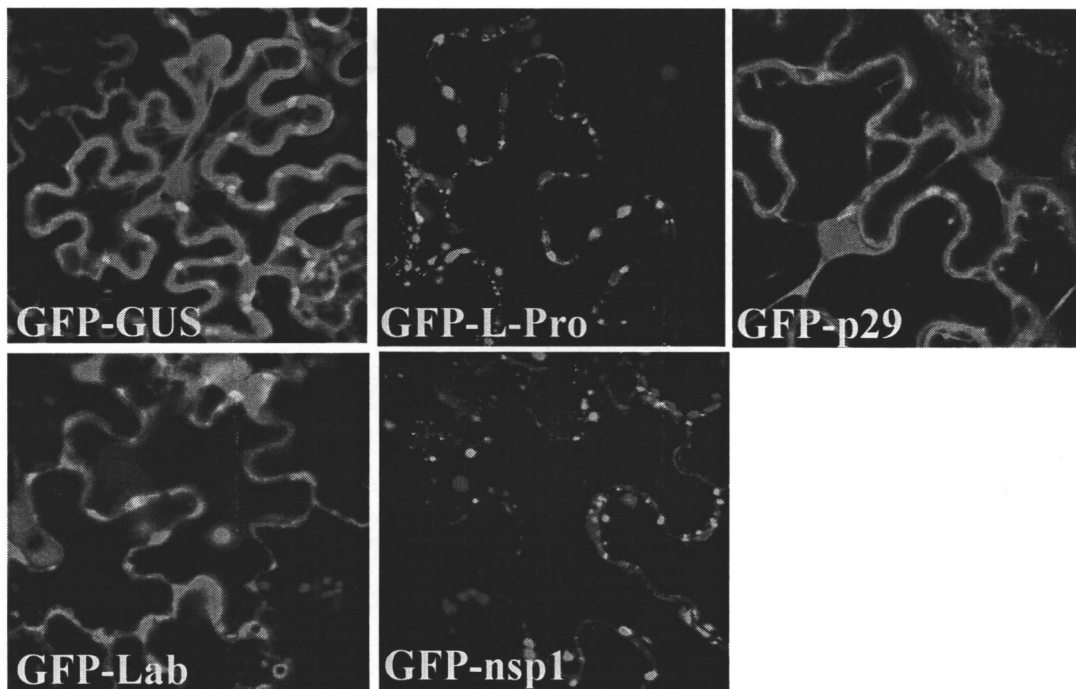
4.4.4 The EAV leader proteinase fails to rescue invasion and spread of the chimera

We further investigated whether EAV nsp1 or its proteinase domain could complement the L-Pro functions throughout the BYV life cycle in infected plants. In a normal infection, virus replication in the initially inoculated cells is followed

Fig. 4.7. Cell-to-cell movement and specific infectivity of the parental BYV-GFP and its chimeric derivatives in plants. A diagram of the BYV variant tagged via insertion of the reporter GFP gene is shown at the top; the diagrams of the examined chimeric variants and representative images of the green fluorescent infection foci are shown below. Dark-red areas surrounding green cells correspond to uninfected, autofluorescent, cells. L-Pro + EAV-nsp1, chimeric BYV-GFP variant that harbors both the BYV L-Pro and EAV nsp1. Other designations as in Figs. 4.1, 4.3 and 4.4.
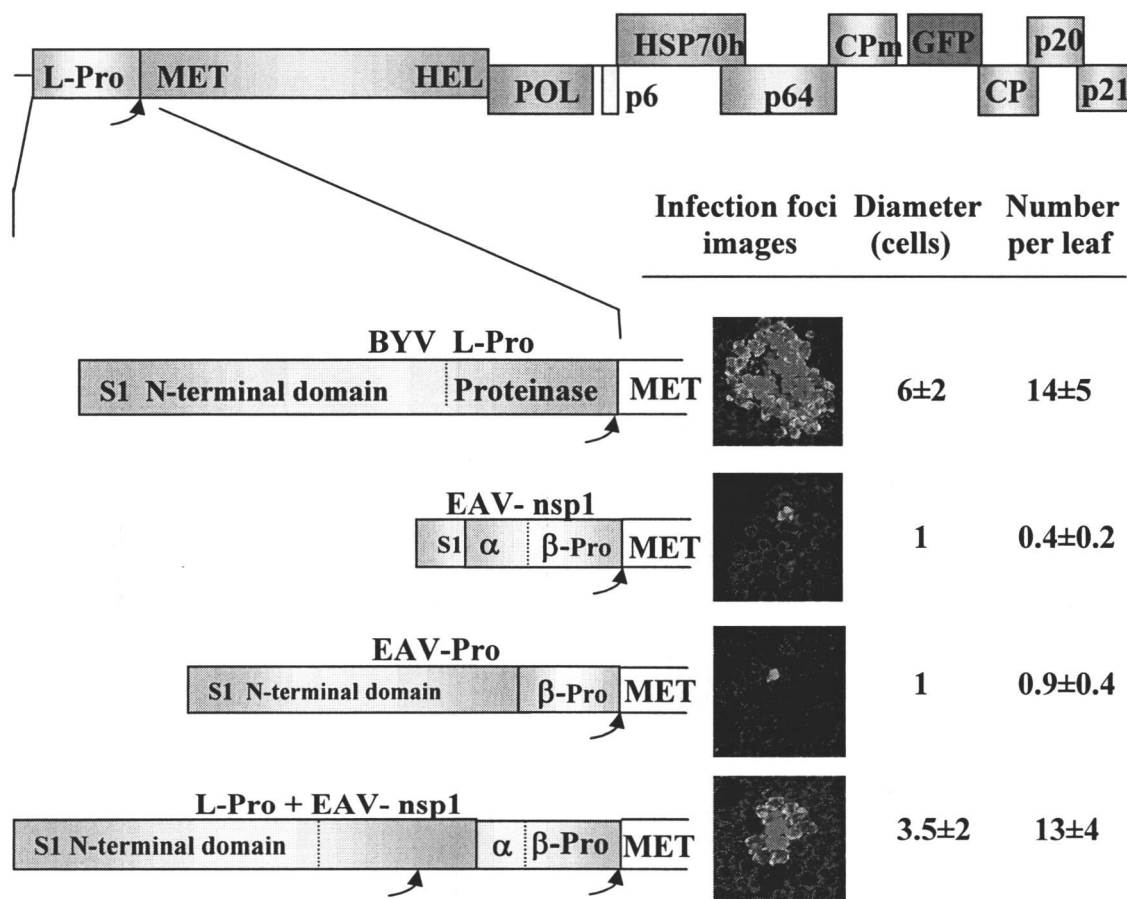
by a symplastic virus spread from cell to cell. To test the phenotypes of BYV-EAV chimeras during plant infection, we employed a previously characterized BYV-GFP variant (Peremyslov *et al.*, 1999). This variant possesses the entire complement of viral genes required for virus replication, assembly, and transport within infected plants. In addition, it is tagged by insertion of the GFP gene that is expressed from an autonomous BYV subgenomic mRNA promoter (Fig. 4.7). The GFP expression provides a convenient means for quantifying specific infectivity and intercellular translocation of the BYV-GFP (Peremyslov *et al.*, 1999).

As shown in Fig. 4.7, plant leaves inoculated with BYV-GFP developed multicellular fluorescent infection foci. However, the BYV-EAV chimera in which most of the L-Pro was replaced by the EAV nsp1 was unable to move from cell to cell. The same phenotype was observed when plants were inoculated by the chimera in which only the papain-like domain of the L-Pro was replaced with that of EAV nsp1 (Fig. 4.7). This result suggested that the authentic BYV L-Pro and its proteinase domain play an essential role in the cell-to-cell transport of the virus. Alternatively, the RNA region encoding the proteinase domain rather than protein itself may be required for virus transport.

We have also observed that the number of infection foci per leaf (specific infectivity or invasiveness) produced by each of the two BYV-EAV chimeras was at least an order of magnitude lower than that in the parental BYV-GFP variant (Fig.4.7). Since these same chimeras exhibited efficient replication in isolated protoplasts (Figs. 4.2, 4.4, and 4.5), these data indicate that the ability of the virus to replicate in individual cultured cells must be distinguished from its ability to establish infection in the host tissue. It can be concluded that although EAV nsp1 activates BYV replication in isolated protoplasts, it cannot support efficient virus invasion and local spread in whole plants.

It is not known if the mechanisms by which BYV L-Pro and EAV nsp1 activate amplification of the viral RNA in plant cells are similar or distinct. However, it could be anticipated that if these mechanisms do not overlap, the

combined expression of the L-Pro and nsp1 might produce a synergistic phenotype. To test this assumption, we engineered and analyzed the mini-BYV and BYV-GFP chimeras that expressed both L-Pro and nsp1 from the same polyprotein (Fig. 4.7). The corresponding mini-BYV chimera replicated in protoplasts to 82±11% of the level found for the parental variant. This apparent lack of synergy suggests that the mechanisms by which L-Pro and nsp1 activate BYV RNA amplification may be similar.

In plants, the BYV-GFP chimera established multicellular infection foci that were comparable in abundance to those produced by the parental BYV-GFP (Fig. 4.7). The somewhat smaller size of these foci compared to those formed by the original BYV-GFP could be due to interference of nsp1 with the L-Pro function in BYV translocation from cell to cell. Alternatively, the increased size of the viral genome may hamper the movement machinery of BYV. Thus, the expression of the L-Pro restored virus invasiveness and its ability to move symplastically. However, combined production of the authentic L-Pro and EAV nsp1 did not result in a more aggressive phenotype. This lack of synergy is compatible with the overlapping mechanisms of L-Pro and nsp1 action in infected plant cells.

4.5 Discussion

The concurrent progress in understanding the modular nature of viral RNA genomes and in generating cDNA clones of RNA viruses allows the examination of new combinations of gene modules. Such experimental evolution via engineering chimeric viruses has proven to be a powerful approach for studying gene functions and developing viruses into biotechnological tools (Conzelmann and Meyers, 1996; Scholthof *et al.*, 1996; Lu and Wimmer, 1996). Most of this previous work involved the swapping of genetic elements between viruses that infect similar host

organisms. An interesting exception was provided by Siegel *et al.* (1997) who showed that the RNA polymerase of a plant virus is capable of accurate, albeit inefficient, RNA synthesis of an animal virus. In this work, we succeeded in generating a vigorously replicating interviral hybrid and demonstrated functional compatibility between the replication machinery of a plant virus, BYV, and a proteinase activator of RNA synthesis derived from an animal virus, EAV.

BYV and EAV do not seem to share a common ancestor more recent than that of all eukaryotic positive-strand RNA viruses (Dolja *et al.*, 1994; Snijder and Meulenberg, 1998; Ziebur *et al.*, 2000). However, the genomes of these viruses do share the distinction of being among the most complex RNA genomes. Both BYV and EAV possess unusually large replicases, generate multiple subgenomic mRNAs, and encode papain-like, leader proteinases, L-Pro and nsp1, respectively. The expression of the EAV nsp1 by the chimeric BYV resulted in the efficient rescue of L-Pro function indicating that BYV and EAV have evolved a common mechanism that is mediated by a papain-like proteinase. The virtual absence of sequence similarity between BYV and EAV suggested that this mechanism might be aimed not at the viral RNA or protein, but rather at a conserved host factor. Moreover, we found that this mechanism involves two distinct functions of the leader proteinases. One of these functions, the proteolytic processing of the viral polyprotein can be rescued by any of the three tested heterologous leader proteinases. In contrast, the other function, which is critical for replication of the chimeric BYV genome, was provided only by EAV nsp1, and not by the CHV1 p29 or FMDV Lab proteinases.

It is important to stress that, although EAV nsp1 supported the efficient amplification of BYV in cultured plant cells, the BYV-EAV chimera had a grossly reduced ability to establish an infection in plants. This result implies that the abilities of the virus to replicate in cultured cells and to invade the host tissue are genetically separable. Moreover, it indicates that there are tissue-specific virus-host

interactions in the initially inoculated cells that can not be reproduced in cell cultures.

The striking contrast between the efficient replication and reduced invasiveness of the BYV-EAV chimera suggests that the authentic BYV L-Pro is involved in at least two facets of the virus-host interaction: one is conserved among animal and plant systems and can be rescued by the EAV nsp1, while the other is plant-specific and can not be provided by an animal virus proteinase. The latter type of interaction, however, may involve an RNA signal located within the L-Pro coding region.

A conspicuous example of a multifaceted plant defense system that grossly affects virus infectivity is provided by the RNA silencing response that targets viral RNA for degradation (Vance and Vaucheret, 2001). Some components of this system are common in plants, animals, and fungi, while others are unique to plants (Sharp, 2001). To cope with the host defense, viruses have evolved a counterdefensive response mediated by the suppressors of RNA silencing (Voinnet *et al.*, 1999). Interestingly, the best studied suppressor of RNA silencing, a potyviral protein HC-Pro is a papain-like, leader proteinase (Kasschau and Carrington, 1998; 2001). This protein is not only able to prevent or reverse RNA silencing, but is also critical for efficient virus replication and systemic invasion. Similar to BYV L-Pro, these functions of HC-Pro are separable from its proteolytic activity (Kasschau and Carrington, 2001). However, two lines of evidence indicate that the mechanisms underlying HC-Pro and L-Pro functions are not identical. First, HC-Pro is incapable of functionally replacing L-Pro; the resulting chimera is nonviable (Peng *et al.*, 2001). Second, unlike HC-Pro, L-Pro is unable to revert RNA silencing induced by a dsRNA (J.E. Reed, K. Kasschau, J.C. Carrington and V.V.D., unpublished results).

To establish systemic infection, plant viruses multiply in the initially inoculated cells, and actively move from cell to cell through plasmodesmata (Maule, 1994; Carrington *et al.*, 1996; Lazarowitz and Beachy, 1999; Citovsky and

Zambryski, 2000; Oparka and Roberts, 2001). Previous work demonstrated that BYV cell-to-cell movement requires virion formation and three movement proteins (Peremyslov *et al.*, 1999; Alzhanova *et al.*, 2000; 2001). One of these movement proteins, the Hsp70 homolog, is found in tight association with the virions and in plasmodesmata (Medina *et al.*, 1999; Napuli *et al.*, 2000). The complete restriction of the BYV-EAV chimera to single, inoculated cells indicated that the authentic L-Pro is also required for successful translocation of BYV from cell to cell. However, L-Pro is not associated with plasmodesmata (Fig. 4.5) or virions (unpublished data), indicating that the role of the L-Pro in virus movement is indirect. It is also possible that the RNA region that encodes L-Pro plays an additional role in virus assembly that is intimately involved in BYV movement (Alzhanova *et al.*, 2001). Furthermore, the requirement of L-Pro for both efficient replication and spread may suggest coordination of these processes in BYV-infected plants. Previously, replication-associated proteins were implicated in the cell-to-cell movement of a bromovirus (Traynor *et al.*, 1991) and a potyvirus (Carrington *et al.*, 1998). Although the mechanisms of virus transport in animals and plants are fundamentally different, it seems interesting that certain EAV nsp1 mutants are spread-defective in animal cell culture (M.A. Tijms and E.J.S., unpublished data).

In a concurrent study, we compared the leader proteinases encoded in diverse members of a *Closteroviridae* family using very similar gene swapping approach (Peng *et al.*, 2001). The obtained results revealed high degree of functional specialization among closteroviral leader proteinases and domains thereof. Moreover, these results confirmed the pivotal role played by the BYV L-Pro in virus invasion and spread, and reinforced the suggestion of host-specific mode of the L-Pro action throughout BYV life cycle (Peng *et al.*, 2001).

There are three aspects of this work that are related to biotechnology. First, this study expands the capabilities of plant viruses as gene vectors that offer a facile approach for superexpression or silencing of genes in plants (Baulcombe, 1999). The large capacity of BYV vectors is highlighted by the expression of two fully

functional foreign proteins, GFP and the EAV leader proteinase. Second, functional hybrids of plant and animal viruses are potentially useful for the development of vaccines or antiviral drugs in relatively inexpensive plant-derived systems. Third, the demonstrated uncoupling of virus replication in cell culture and the ability to establish infection in host tissue indicates that virus invasiveness provides an additional target for antiviral therapies.

In conclusion, we generated a hybrid of plant and animal viruses that replicates efficiently in isolated plant cells. Characterization of this hybrid allowed us to reveal functions of a leader proteinase in virus genome amplification, invasion of a host tissue, and cell-to-cell translocation, and propose that the mechanism underlying these functions is aimed at virus-host interactions. This work can also be viewed as a step toward the ultimate goal of making designer viruses producing useful proteins or even desired phenotypes of infection.

4.6 Acknowledgements

4.7 References

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Chapter 5

Conclusion

Leader proteinase of the beet yellows virus functions in long-distance transport

Chih-Wen Peng

5.1 Abstract

The 66-kDa leader proteinase (L-Pro) of the *Beet yellows virus* (BYV) possesses non-conserved N-terminal domain and conserved, papain-like, C-terminal domain. Previous work revealed that the N-terminal domain functions in RNA amplification and virus invasion. Here we apply alanine-scanning mutagenesis to complete functional analysis of the entire L-Pro molecule throughout the virus life cycle. This analysis indicated that the C-terminal domain of the L-Pro possesses genetically separable functions in proteolysis and RNA amplification. Examination of the L-Pro role in BYV cell-to-cell movement revealed that none of the 20 examined replication-competent mutants was movement-defective. These results implied that the L-Pro is not a dedicated movement protein. In contrast, seven of the L-Pro mutations affected the long-distance transport of BYV to various degrees, whereas three mutations completely abolished the transport. Since these mutations were introduced throughout the protein molecule, both principal domains of the L-Pro function in virus transport. We conclude that in addition to previously identified functions of the L-Pro, it also serves as the BYV long-distance transport factor.

5.2 Introduction

The papain-like, leader proteinases provide a particulary illuminating example of the multifunctional nature of the viral proteins. These proteinases were found in a number of evolutionary diverse lineages of the positive-strand RNA viruses infecting animals, fungi, and plants (5, 7, 8, 14, 23). In addition to the immediate role in a processing of the viral polyproteins, these proteinases were

implicated in genome replication, synthesis of the subgenomic mRNAs, virus spread, and various aspects of virus-host interactions (3, 18, 21, 22). Among the positive-strand RNA viruses of plants, the papain-like, leader proteinases were characterized in the members of two viral families, *Potyviridae* and *Closteroviridae* (9, 20). The potyviral helper component-proteinase (HC-Pro) and the closteroviral leader proteinase (L-Pro) share two-domain structure with the N-terminal, non-proteolytic domain and the C-terminal, papain-like domain. The HC-Pro functions in self-processing, efficient genome amplification, long-distance transport, and aphid transmission (2, 4, 13, 19). At least some of these activities are due to the ability of HC-Pro to suppress RNA silencing, a host defense response that targets viral RNA for sequence-specific degradation (11, 12).

The L-Pro of the *Beeet yellows virus* (BYV), a prototype *Closterovirus*, is encoded in a 5'-proximal part of the BYV ORF 1a (Fig. 1). The early work revealed involvement of the BYV L-Pro in self-processing and efficient genome amplification, and suggested that the functional profile of the L-Pro overlaps that of the potyviral HC-Pro (1, 6, 18). More recent study demonstrated that although the L-Pro is not essential for basal-level RNA replication, deletion of the N-terminal domain results in 1,000-fold reduction of the RNA accumulation (15).In addition, a short RNA element indispensable for genome amplification was identified within the 5'-terminal region of the L-Pro ORF.

Interestingly, some of the closteroviruses, such as *Citrus tristeza virus* (CTV), encode two tandemly organized leader proteinases, L1 and L2 (10). Comparative analysis of the closteroviral and potyviral proteinases using gene swapping approach indicated that BYV L-Pro and CTV L1 belong to the same functional class, whereas CTV L2 belongs to another (16). This analysis also demonstrated that potyviral HC-Pro can not functionally replace BYV L-Pro, suggesting that these proteinases are mechanistically distinct. Moreover, it was found that the homologous, papain like domains of the L-Pro, L1, L2, and HC-Pro are functionally specialized. Although each of these domains efficiently processed

chimeric BYV polyprotein, only that of the L1 was capable of partial rescue of the proteinase function in RNA amplification (16).

A similar approach was used to compare leader proteinases from a broader range of plant, fungal, and animal viruses (17). Surprizingly, it was found that the replacement of BYV L-Pro with the leader proteinase of the *Equine arteritis virus* (EAV) resulted in a replication-competent BYV-EAV chimera. Furter analysis of this chimera revealed its defective invasiveness and inability to move from cell to cell. These results suggested that the authentic BYV L-Pro is required for virus ability to establish infection in the initially-inoculated plant tissue (17).

Here we employ reverse genetics to complete functional analysis of the BYV L-Pro. We confirm that the papain-like domain of the L-Pro plays important role in RNA amplification that is separable from its primary role in polyprotein processing. We also reveal a novel function of the L-Pro in virus long-distance transport in a host plant. The unique and complex functional profile of the BYV L-Pro highlights evolutionary plasticity of the viral leader proteinases that provide structural platform for diverse biological activities.

5.3 Results

5.3.1 Mutation analysis of the proteinase domain

Our previous gene-swapping experiments indicated that in addition to primary function in polyprotein processing, the proteinase domain of the L-Pro plays an additional role in RNA amplification. To further substantiate this conclusion, we employed alanine-scanning mutagenesis of the proteinase domain

(Fig. 5.1). Fifteen mutants were generated and numbered from A13 to A27 in continuation of the

Fig. 5.1. Mutagenic analysis of the proteinase domain of L-Pro. Each of the selected residues within the proteinase domain was replaced with an alanine residue. Among these residues, C^{509} (in bold face) is one of the enzyme catalytic residues. The site of the autocatalytic processing is located between two consecutive glycine residues, Gly^{588} and Gly^{589}. Other designations are as in Fig. 2.1.
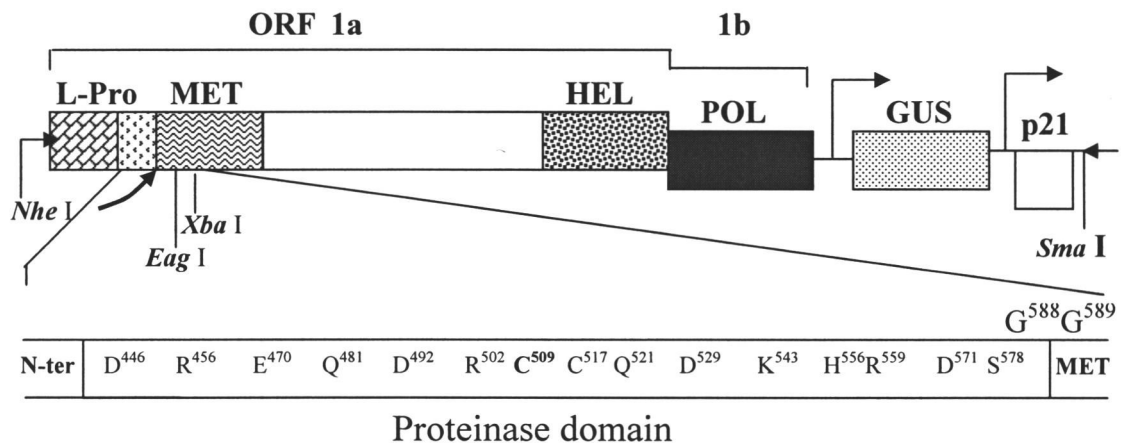
reviously described twelve mutants that targeted the N-terminal domain. The processing activity of each mutant was tested in a cell-free translation system (Fig. 5.2). The mutants were arbitrarily classified into three classes in accord with their ability to process viral polyprotein. Class I contained eleven mutants with the processing efficiency comparable to that of the wild type (Table 5.1). Three class II mutants exhibited a processing rate that was reduced to 50-60%, whereas the only class III mutant A19 was processing-incompetent due to a replacement of the catalytic cisteine residue (Table 5.1).

Table 5.1. Mutation analysis of the proteinase domain of BYV L-Pro[a].

BYV variant	Mutation	Processing efficiency[b]	GUS activity[b]
A13	$D_{446}A$	97±7	<0.001
A14	$R_{456}A$	100±8	100±13
A15	$E_{470}A$	67±3	87±10
A16	$Q_{481}A$	95±5	95±12
A17	$D_{492}A$	93±4	125±17
A18	$R_{503}A$	92±7	43±3
A21	$Q_{521}A$	99±8	102±15
A23	$K_{543}A$	95±6	114±13
A24	$H_{556}A$	72±5	95±10
A25	$R_{559}A$	81±6	86±10
A27	$S_{578}A$	104±5	92±11
A20	$C_{517}A$	54±4	27±6
A22	$D_{529}A$	58±4	2±1
A26	$D_{571}A$	59±3	3±1
A19	$C_{509}A$	UD[c]	<0.001

[a]The mutant variants are divided into classes I to III (from top to bottom) according to efficiency of processing. These efficiencies are ≥67% for class I, ≤54% for class II, and

[b]Expressed as per cent of the levels found for the wild-type. Means and standard deviations are shown.

[c]Undetectable.

These efficiencies are ≥61% for class I, ≤50% for class II, and zero for class III.

[b]Expressed as per cent of the infected vs inoculated plants.

[c]Not applicable.

Fig. 5.2. Processing of the wild type and mutant L-Pro variants upon translation in wheat germ extract. M, mock-translation with no added mRNA (negative control); BMV, translation of the brome mosaic virus RNA (positive control); A13-A18, A21-23, A25-27, translation of the first set of mutant variants (panel A); A19, A20 and A24, translation the variants with mutations in or close to the proteinase active site (Panel B). P1, nonprocessed product; P2, processed product.

(A)
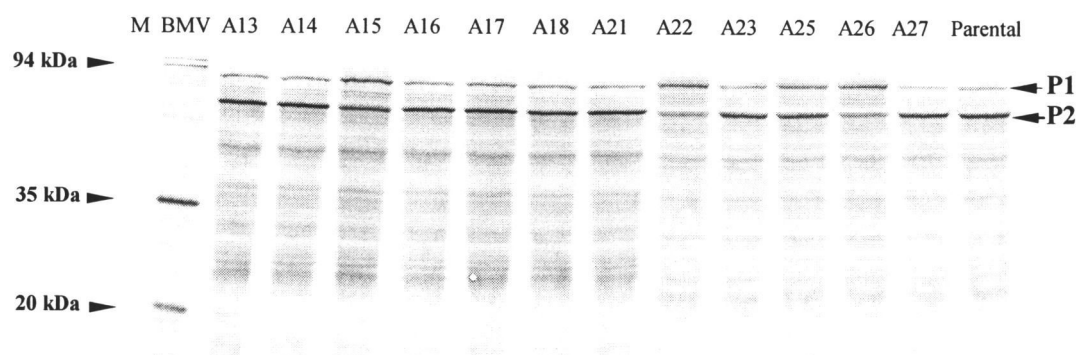
(B)
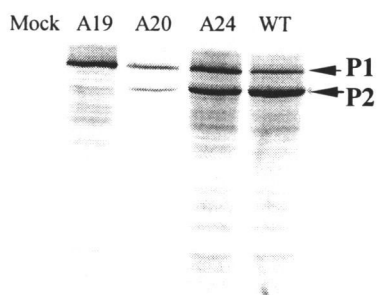
The effects of mutations on the level of RNA accumulation were tested in protoplast transfection experiments using mini-BYV variant tagged by insertion of the reporter β-glucuronidase (GUS) gene. As it was demonstrated previously (see

chapters 2 and 4), GUS assays provide a sensitive and accurate measure of the accumulation of viral RNA. As expected, processing-deficient class III mutant failed to replicate to detectable level. In contrast, all of the class II and class III mutants were replication-competent (Table 5.1). Among the class I mutants, A18 exhibited significantly reduced GUS activity, whereas A13 was replication-incompetent. Furthermore, despite the very similar processing efficiencies, the levels of GUS activity varied from 2 to 27% among the tree class II mutants (Table 5.1).

Two major conclusions can be drawn from the presented genetic analysis. First, these data reconfirmed that the autocataclytic processing by the L-Pro is a prerequisite for genome replication. Second, several mutations had differential effects on polyprotein processing and RNA amplification. This lack of strict correlation between the two processes provides a genetic evidence for a role of proteinase domain in RNA amplification that is separable from its primary function in processing.

5.3.2 Replication-competent L-Pro mutants are functional in cell-to-cell movement

As it was suggested by previous analyses of the chimeric BYV variants (chapters 3 and 4), L-Pro could be indirectly involved in potentiating virus movement from cell to cell. To further investigate this possibility, we studied 20 alanine-scanning mutants that replicated efficiently in the transfected protoplasts. Each of the mutations was introduced into genetic background of the BYV variant tagged via insertion of the reporter gene encoding green fluorescent protein (BYV-GFP). The parental BYV-GFP and its mutant variants were inoculated onto leaves of the *Claytonia perfoliata*, and virus ability to move from cell to cell was assessed

Table 5.2. Systemic infectivity of the BYV-GFP and its mutant variants [a].

BYV variant	Number of inoculated/ infected plants	Systemic infectivity[b]	Time of symptom appearance (weeks)
BYV-GFP	16/18	89	3
A3	13/18	72	6
A4	11/18	61	3
A6	14/18	78	3
A8	13/18	72	3
A10	14/18	78	3
A11	16/18	89	3
A14	12/18	67	3
A16	14/18	78	3
A18	11/18	61	3
A23	11/18	61	3
A27	15/18	83	3
A2	1/18	6	3
A9	8/18	44	3
A12	9/18	50	3
A15	4/18	22	5
A21	9/18	50	4
A25	7/18	39	4
A5	0/18	0	N/A[c]
A7	0/18	0	N/A
A17	0/18	0	N/A

[a]The BYV-GFP variants are arbitrarily divided into classes I to III (from top to bottom) according to efficiency of systemic infection. TABLE 1. Mutation analysis of the proteinase domain of BYV L-Pro[a].

eight days post inoculation via measuring the resulting infection foci under epifluorescence microscope.

As shown in Table 5.2, each of the tested mutants was capable of forming green fluorescent infection foci. The number of these foci per leaf (specific infectivity) was similar for BYV-GFP and its mutant variants. Likewise, none of the mutants exhibited significant reduction in the size of infection foci: the mean diameters of these foci were more than 4 cells for all variants (Table 5.2).

Since the characterized mutations were located throughout the L-Pro molecule, these results suggested that neither the N-terminal, nor proteinase domain of the L-Pro plays a primary role in BYV cell-to-cell movement. Thus, the cell-to-cell movement defects of the L-Pro replacement mutants described previously may be attributed to their reduced invasiveness. Alternatively, it can not be excluded that none of the tested point mutations resulted in major changes in L-Pro structure required to affect its function in cell-to-cell movement.

5.3.3 L-Pro is required for the long-distance transport of BYV

Three principal phases in the life cycle of a plant virus include cell-autonomous replication, cell-to-cell movement, and long-distance transport through the plant vascular system. In order to complete functional profiling of the L-Pro, we examined its possible involvement into the long-distance transport of BYV. To this end, each of the 20 alanine-scanning mutants that were competent in replication and intercellular spread were inoculated onto BYV systemic host *Nicotiana benthamiana* using agroinfection. Three independent experiments each involving 6 plants were conducted for parental BYV-GFP and each of the mutant variants. The competence of the virus in long-distance transport that is synonymous with systemic infectivity was assessed by appearance of the symptoms and GFP expression in the upper, non-inoculated leaves.

Fig 5.3. Systemic transport of the BYV-GFP and its mutant variants. The systemic symptoms seen as green fluorescent areas were photographed at four weeks and eight weeks postinoculation. (Rows A and B, respectively). The BYV-GFP variants are marked between the rows.
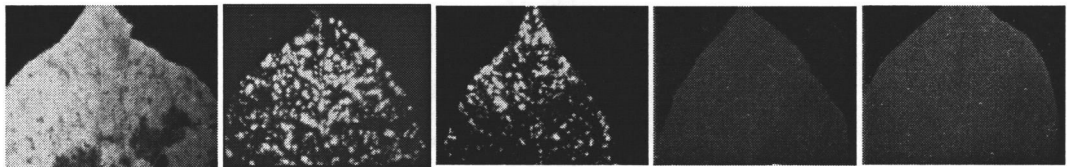
(A)
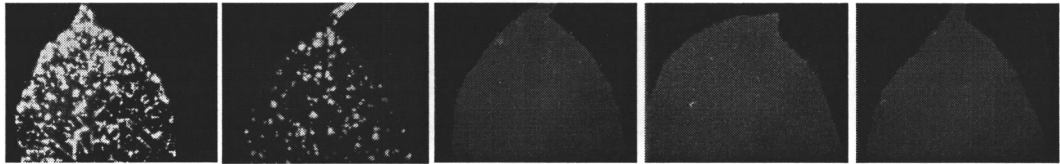
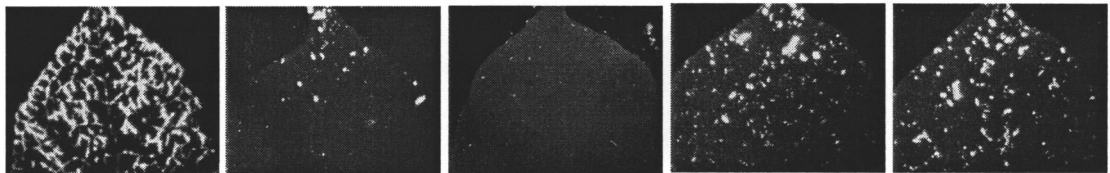
(B) BYVGFP   A2   A3   A5   A7
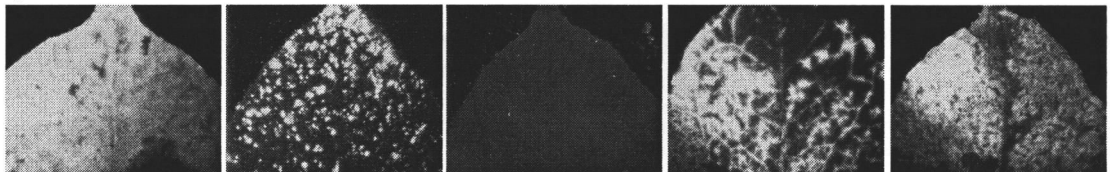

(A)

BYVGFP   A15   A17   A21   A25

(B)

As seen from the Table 5.3, ~90% of the plants inoculated with parental BYV-GFP exhibited symptoms of the systemic infection and expressed GFP in upper leaves by 3-4 weeks post inoculation. Among the mutant variants, 10 were not significantly different from BYV-GFP in that by the week four they systemically infected more than 50% of the inoculated plants. Mutant A3 exhibited delayed systemic spread: it was detected in the upper leaves only after 6 weeks post inoculation. Systemic transport of the six mutants (A2, A9, A12, A15, A21, and A25) was affected to varying degrees; they were able to systemically infect from only 6% to 50% of the inoculated plants. Strikingly, the remaining three mutants, A5, A7, and A17, failed to establish systemic infection in any of the inoculated plants (Table 5.3). These results are further illustrated in Fig. 5.3, which shows the reduced spread or complete absence of the green fluorescent areas in the upper leaves of plants inoculated with the affected mutants.

Taken together, these results demonstrate that the L-Pro is required for systemic transport of BYV. Since transport-defective mutations were identified both in the N-terminal and proteinase domains, this additional function involves each of these principal domains.

5.4 Conclusions

The major objective of this study was to define a functional profile of the BYV leader proteinase, or L-Pro. L-Pro is a 66-kDa protein expressed from the virion RNA as an N-terminal part of the replicase polyprotein. Computer analysis revealed that L-Pro possesses two principal domains, a non-conserved N-terminal domain and a conserved, C-terminal, papain-like domain. To assess the functional significance of these putative domains, we conducted extensive genetic study using alanine-scanning mutagenesis and gene swapping. This study revealed that the L-

Pro provides an illuminating example of a multifunctional viral protein that enables progression of virus infection through all of its major phases.

Our results confirmed that the computer-predicted domains of the L-Pro correspond to its functional domains with the C-terminal domain being both essential and sufficient for polyprotein processing. This autocatalytic processing was demonstrated to be a prerequisite for virus genome amplification. It was found, however, that the function of papain-like domain is not limited to proteolysis. Our analyses revealed that this domain plays additional role in genome amplification that is genetically separable from its role in proteolysis.

Functional analysis of the N-terminal domain revealed that although it is not essential for basal level replication, its inactivation results in dramatic reduction in the genome amplification and expression. It is yet to be determined if the function of the L-Pro domains in genome amplifications are independent or interdependent. It seems likely that relative orientation of these domains is important for their individual or integral activities. Our discovery of the role played by L-Pro in BYV long-distance transport provides an example of activity that requires an entire L-Pro molecule. Indeed, the mutations that affected virus transport were found in each of the L-Pro domains.

As powerful as it is, extensive alanine-scanning mutagenesis did not allow us to identify all of the L-Pro functions. None of the introduced mutations affected virus invasiveness or cell-to-cell movement. In contrast, the alternative approach of replacing L-Pro ORF with the ORFs that encode leader proteinases of diverse viruses revealed these additional functions. We found that such replacement can rescue L-Pro functions in polyprotein processing and genome amplification. However, the replication-competent chimeric viruses exhibited grossly reduced invasiveness and complete failure to move from cell to cell. In summary, we demonstrated that BYV L-Pro functions include polyprotein processing, genome amplification, virus invasion into plant tissues, and virus long-distance transport via

vascular system. Thus, L-Pro provides activities that are essential through the entire life cycle of the BYV.

In addition, this study has a strong evolutionary aspect. We conducted comparative analysis of a broad range of leader proteinases derived from plant, fungal, and animal viruses. We found that these proteinases belong to several distinct, but overlapping functional classes that provided examples of both divergent and convergent evolution of the conserved papain-like domain. Finally, we succeeded in generating a replication-competent chimera of plant and animal viruses and thus demonstrated feasibility of intelligent design of novel virus genomes. Such feasibility has potential applications in experimental virus evolution and biotechnology.

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