

ジャガイモウイルスゲノムの5'末端領域
の機能に関する研究

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Studies on the functions of the 5'terminal region
of potato virus Y genome

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Chapter 1 **Introduction**

1-1. The Economic Importance of Plant Viruses

Losses Caused by Viruses

Plant viruses cause significant losses to most, if not all, major crops around the world and they can also limit the production of specific crops to certain areas. There are no fully collated figures for world-wide losses due to viruses, but some estimates illustrating the magnitude of the problem are given in Table 1-1 (Hull & Davies, 1992). It is likely that in tropical countries losses will be much greater, not only economically but also practically as they limit the availability of basic subsistence food crops. On top of these losses are the costs of insecticides and other chemicals used for virus vectors control, and losses caused by other pathogens in plants weakened by virus disease.

Control of Viruses by Conventional Protection

There are three basic ways by which virus diseases are currently controlled. One, which is applied primarily to annual crops, is the use of healthy planting material and/or the eradication of infected plants. For instance, in many temperate climate countries, there are schemes by which certified virus-free potatoes are produced in areas where vectors are few and are subsequently grown in more productive regions. The second, also the major approach for the control of viruses in annual crops, involves various cultural practices. These include the timing of planting so as not to coincide

with an influx of virus vectors, or the use of chemicals to control virus vectors (Sama *et al.*, 1990). The third approach, breeding resistance to the virus or its vector into the crop, is generally regarded as being the best in the long term. In host plants with resistance to a virus the response of the plant to infection is reduced or eliminated (Yeh *et al.*, 1988). All three of these conventional protections have not been sufficiently successful to prevent great losses in crops due to virus infections (see Table 1-1).

Control of Viruses by Nonconventional Protection

With increasing knowledge on genome organizations and gene functions of many plant viruses, new promising concepts of nonconventional resistance appear. These concepts involve transformation of plants with viral nucleic acid sequences (antisense nucleic acids, satellite sequences, defective interfering molecules, structural and non-structural genes) that interfere with the viral infection cycles. For instance, it was shown that the expression of coat protein genes of many plant viruses in transgenic plants delayed the development of viral diseases (reviewed by Beachy, 1990; Beachy *et al.*, 1990). Tobacco plants transformed with a cistron of potato virus Y (PVY) Nla protease are resistant to PVY (Vardi *et al.*, 1993).

Recently, an attempt to screen natural inhibitor(s) of PVY protease from products of soil microorganisms showed that 3-amino-3-deoxyglucose (3AG) specifically inhibited Nla protease activity of PVY-O *in vitro*, and 3AG sprayed to PVY-O infected tobacco leaves reduced the titer of PVY-O in infected plants dramatically to one sixtieth (Hidaka, 1996).

The concept of nonconventional resistance or protection is opening up a whole range of new possibilities for virus control. However, the

aforementioned approaches not only depend on the development and improvement of plant biotechnology, but also greatly rely on the further understanding of the viral genome organization and gene functions.

Table 1-1 Some Examples of Crop Losses Due to Viruses

Crop	Virus	Countries	Loss/year
Rice	Rice tungro	Southeast Asia	\$1.5 x 10 ⁹
	Rice ragged stunt	Southeast Asia	\$1.4 x 10 ⁸
	Rice hoja blanca	South America	\$9.0 x 10 ⁶
Barley	Barley yellow dwarf	UK	£6 x 10 ⁶
Wheat	Barley yellow dwarf	UK	£5 x 10 ⁶
Potato	Potato leafroll	UK	£3.5 x 10 ⁷
	Potato virus Y		
	Potato virus X		
Beet	Beet yellows	UK	£5-50 x 10 ⁶
	Beet mild yellows		
Citrus	Citrus tristeza	worldwide	£9-24 x 10 ⁶
Cassava	African cassava mosaic	Africa	\$2 x 10 ⁹

(Hull & Davies, 1992, with modification)

1-2. The Genome Structure and Function of Potyviruses

The Organization and Evolution of Plant Viral RNA Genome

Approximately 75% of the plant viruses studied so far, and many of the economically important ones, have a single-stranded RNA genome of positive polarity (Table 1-2). These viruses are classified in distinct taxonomic groups and show a wide variation in capsid morphology. Their genomes are either segmented or unsegmented, and have various terminal structures such as genome-linked proteins or cap-structures at the 5'-end, and a poly(A)-tail or tRNA-like structure at the 3'-end. Nevertheless, sequence comparisons of the non-structural proteins they encode have demonstrated that most of them are somehow genetically interrelated. Moreover, most plant RNA viruses appear to have relatives among the animal RNA viruses. Hence, it has been proposed (Goldbach, 1986; 1987) that the como-, nepo- and potyviruses may be arranged in a "super group" of picornavirus-related plant viruses (Fig. 1-1). What all "picorna-like" plant viruses share, and what indicates that they are all genetically related to the animal picornaviruses, are the following properties:

1. their RNAs have a protein (VPg: viral protein genome-linked) covalently linked to the 5'-end and a poly(A) tail at the 3'-end;
2. their RNAs are expressed by the production of so-called "polyproteins" from which the mature functional proteins are derived by proteolytic processing;
3. they encode a number of non-structural proteins exhibiting significant amino acid sequence homology and
4. these conserved proteins, which have all been shown or suggested to be

involved in the viral RNA replication process, are encoded by similarly ordered gene sets.

The Organization and Expression of Potyviral Genes

The genus *Potyvirus* [named after its type species potato virus Y (PVY)] is the most rapidly growing and the largest of the 47 plant virus genera currently recognized (Murphy *et al.*, 1995). It contains at least 180 species (or 30 % of all known plant viruses) which cause significant losses in agricultural, pasture, horticultural and ornamental crops (Ward & Shukla, 1991). These viruses are unique in the diversity of inclusion bodies that are formed during the infection cycle (Lesemann, 1988). A feature shared by all potyviruses is the induction of characteristic pinwheel or scroll-shaped inclusion bodies in the cytoplasm of the infected cells. Although some of these viruses are transmitted by mites, and possibly by whiteflies, the predominant transmission of potyviruses is by aphids. Many potyviruses also induce cytoplasmic amorphous inclusion bodies and some form nuclear inclusions (Knuhtsen *et al.*, 1974). Virions are flexuous and rod-shaped, 680 to 900 nm long and 11 to 15 nm wide, made up of about 2,000 units of a single structural protein surrounding one molecule of ssRNA of approximately 10 kb and messenger polarity. It contains one long open reading frame which is translated into a large polyprotein. This polyprotein is subsequently cleaved into smaller functional proteins (Fig. 1-2).

The different gene products into which the potyviral polyprotein is cleaved by *cis* and/or *trans* are, from the N to the C terminus of the polyprotein, the first protein (P1), the helper component/protease protein (HC-Pro), the third protein (P3), the first putative 6K protein (6K1), the cylindrical inclusion

protein (CI), the second 6K peptide (6K2), the nuclear inclusion 'a' protein (NIa: VPg and protease), the nuclear inclusion 'b' protein (NIb) and the capsid protein (CP).

P1: P1 protein catalyzes autoproteolytic cleavage between itself and adjacent HC-Pro (Verchot *et al.*, 1991). The protease domain is located at the C-terminus half of this protein. Although P1 protein has been proposed extensively in the literature to function as a movement protein involved in cell-to-cell transport of virus in plants (Domier *et al.*, 1987; Atabekov & Taliinsky, 1990), recent reports indicate that P1 protein plays little role in virus movement (Verchot & Carrington, 1995a, 1995b). P1 protein shows single-stranded RNA binding activity *in vitro* (Brantley & Hunt, 1993; Soumounou & Laliberte, 1994) and functions in *trans* as an accessory factor for genome amplification (Verchot & Carrington, 1995b).

HC-Pro: HC-Pro is a helper factor for acquisition and transmission of virus by aphids (Huet *et al.*, 1994; Atreya & Pirone, 1993). The N-terminal and central regions are involved in genome amplification and pathogenicity (Atreya *et al.*, 1992; Dolja *et al.*, 1993; Klein *et al.*, 1994; Kasschau & Carrington, 1995). The C-terminal region of HC-Pro comprises a proteinase that catalyzes autoproteolytic cleavage between itself and the neighboring P3 protein (Carrington *et al.*, 1989). HC-Pro plays a specific role in long-distance transport as a *trans*-acting factor (Cronin *et al.*, 1995).

P3: P3 is a protein whose function is least understood. The protein could be one of the proteolytic cofactors in the regulation of polyprotein processing. The observation that P3 is intimately associated with CI at early stage of infection may indicate that this protein is involved in viral life cycle (Rodriguez-Cerezo *et al.*, 1993). An insertion mutant in P3 encoding region

fails to produce viral RNA in plants which indicates that P3 is involved in RNA replication (Klein *et al.*, 1994).

6K1: The function of this polypeptide is not yet known, it may play a role in RNA replication. The presence of this protein in polyprotein is actually not quite clear.

CI: It has been proposed that this protein may be involved in cell-to-cell movement of the virus, in conclusion to the observations that CI was associated with plasma membrane, plasmodesmata and cell wall at an early stage of infection (Lawson & Hearon, 1971; Baunoch *et al.*, 1991). CI may play a role in RNA replication, based on the presence of a large helicase-like sequence (Gorbalenya *et al.*, 1989) and the fact that such an enzyme activity has been reported for plum pox virus CI (Lain *et al.*, 1991).

6K2: An insertion mutant in this peptide fails to produce detectable amounts of progeny viral RNA in plants, suggesting this protein has a function in RNA replication (Klein *et al.*, 1994).

N1a: N1a protein contains two functional domains: the N-terminal domain being the VPg and the C-terminal domain being the protease (Dougherty & Parks, 1991; Gorbalenya *et al.*, 1989; Yoshida *et al.*, 1993). The protease recognizes possibly eight cleavage sites on the polyprotein (Riechmann *et al.*, 1992; Kim *et al.*, 1995). The VPg may be involved in RNA replication.

N1b: This protein has been postulated as the potyviral RNA-dependent RNA polymerase in the synthesis of progeny viral RNA, based on the presence of conserved sequence motifs characteristic for these enzymes (Domier *et al.*, 1987; Poch *et al.*, 1989).

CP: The major role of CP is to encapsidate viral RNA (Shukla & Ward, 1989). CP could also affect host range, symptom induction (Saito *et al.*,

1987), and aphid transmission (Atreya *et al.*, 1991). Recent reports show that CP plays specific roles in cell-to-cell and long-distance transport of virus (Dolja *et al.*, 1994; 1995)

Table 1-2 Genomes of Plant Viruses

Genome Type	Number of Viruses	% of Total
single-stranded DNA	26	4
double-stranded DNA	13	2
single-stranded RNA(+) sense	470	76
single-stranded RNA(-) sense	85	14
double-stranded RNA	26	4

(From Goldbach *et al.*, 1989)

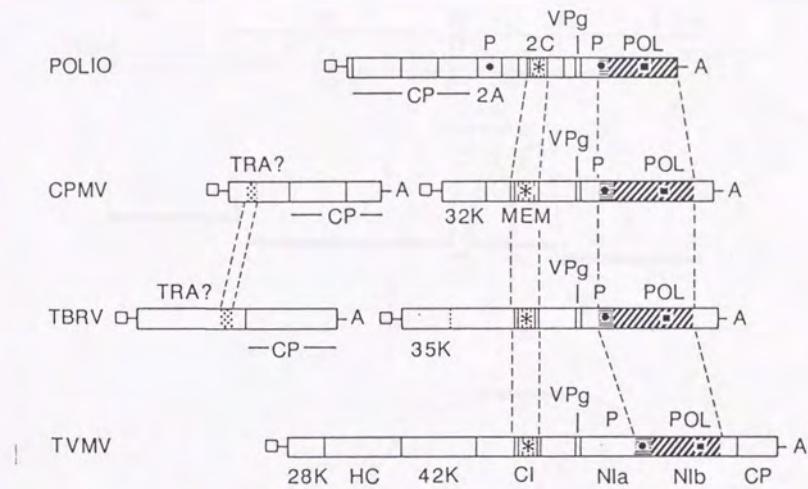


Fig. 1-1 Comparison of the genomes of picornaviruses (polio), comoviruses (CPMV), nepoviruses (TBRV) and potyviruses (TVMV). Coding regions are indicated as open bars, VPg as open squares and polyadenylate sequences as A. Regions of amino acid sequence homology in the gene products are indicated by similar shading. Other symbols: CP, capsid protein(s); TRA, putative transport function; P, protease; MEM, membrane-binding; POL, core RNA-dependent RNA polymerase; HC, helper component; *, nucleotide binding domain; ●, cysteine protease domain; ■, conserved polymerase domain.

(From Goldbach *et al.*, 1989)

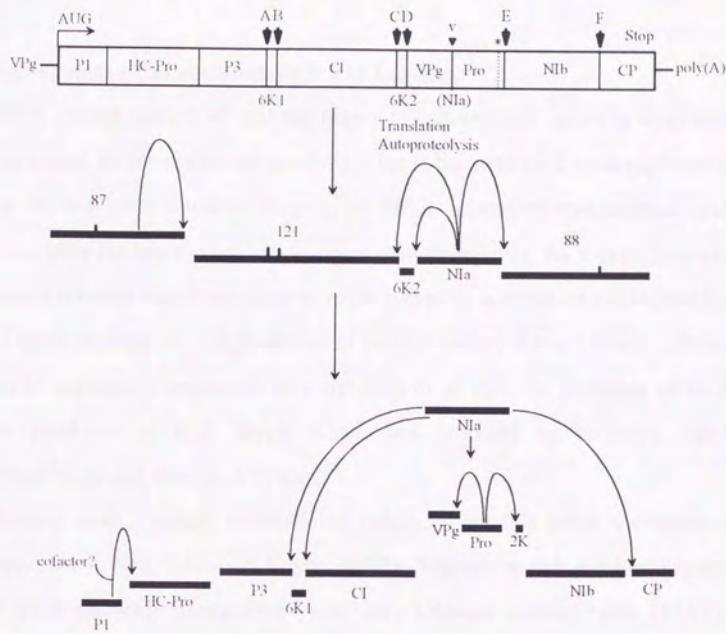


Fig. 1-2 Schematic representation of the potyviral polyprotein processing based on the results of polyprotein cleavage analysis obtained *in vitro* and in *E. coli*, and on amino acid sequence features of several proteins. (Riechmann *et al.*, 1992; Kim *et al.*, 1995, with modifications)

1-3. Enhancement of Translation by Viral Leaders and Their Potential Application in Biotechnology

Enhancement of Translation by Viral Leaders

RNA viruses cause host cell machinery to express their genes in ways that are not used for host genes themselves. This is because viral gene expression must be regulated without reliance on DNA-dependent transcription and viruses have far more compact genomes than their hosts. As a consequence, translation rather than transcription, often serves as a major step in regulating viral gene expression. Single-stranded positive sense RNA viruses contain specific nucleotide sequences that are used to elevate the synthesis of viral gene products to high levels which are required to facilitate rapid multiplication and spread of viruses.

Among plant viruses, translational enhancement has been documented widely (see review, Turner & Foster, 1995). Reports include the best known and most intensely researched virus, i.e., tobacco mosaic virus (TMV), members of the potyvirus group, and even a small satellite RNA of tobacco necrosis virus. These enhancers consist of the 5' untranslated nucleotide regions (5'UTR) of genomic or subgenomic RNA molecules (Table 1-3). The presence of these sequences at 5' termini of mRNA molecules results in an increase in translation of a downstream open reading frame. Enhancement values range from 2 to 100 fold with different viruses, different reporter genes and in different systems. This indicates, possibly, that more than one mechanism may be exploited to attain this enhancement property. This enhanced expression of viral gene products at the translational level may, therefore, be different from the universally accepted scanning model for

translational initiation, and it yet is another example of how these versatile organisms have adapted to maximize gene expression and subsequent multiplication and spread.

The potential of plant viral translational enhancers in biotechnology for increased gene expression

The biotechnological applications of these leaders could be quite dramatic. Most analyses of these plant viral leaders have shown that these viral translational enhancers operate efficiently and independently of their related coding sequence and they could easily be exploited to enhance the expression of specific targeted genes in a transgenic system. Viral translational enhancers should be useful to elevate the expression of foreign genes in transgenic plants in addition to the control at the level of transcription, and that may be exploited to maximize protein production in transgenic plants.

Table 1-3 Characteristics of Translational Enhancers

Translational leader	Rabbit reticulocyte	Wheat germ	<i>E. coli</i> t ₇ P promoter	Protoplasts or suspension culture	Xenopus oocytes	Transgenic plants	Insensitivity to cap analog	Internal initiation, bicistronics
AMV RNA4 5' UTR	+	+	+	+	tobacco	N	N	N
TMV ω 5' UTR	+	+	+	+	tobacco, carrot	+	+	N
PVX 5' UTR	+	+	+	+	barley, tobacco	N	N	N
BMV RNA3 5' UTR	N	N	N	+	tobacco	-	N	N
TYMV 5' UTR	N	N	N	+	N	N	N	N
TEV 5' UTR	+	N	N	N	tobacco	+	+	N
PsbMV 5' UTR	N	N	N	N	tobacco, pea	N	N	N
PPV 5' UTR	+	+	N	N	N	N	+	N
PVY 5' UTR	+	+	N	N	N	N	+	N
TuMV 5' UTR	N	N	N	N	tobacco, suspension culture	N	+	N

+ indicates that translational enhancement occurs in the system. - indicates that the leader does not show translational enhancement properties in the system. N (not determined) indicates that the leader has not been tested for the property or in the system in question.

(From Turner & Foster, 1995)

1-4. Objectives of This Study

Potato virus Y (PVY), an aphid-transmitted potyvirus, with many strains and variations, is a worldwide-spread and serious pathogen of major solanaceous crops, causing various symptoms of mottle, necrosis and distortion of leaves, reduced growth, and fruit malformation in potato, tomato, pepper, chilli, eggplant and tobacco (de Bokx & Huttinga, 1981). PVY ranks number one in top ten viruses in Mediterranean and Africa, and number two in Southeast Asia and Europe (Milne, 1988). The yield losses caused by PVY in potato may go up to 90%. Since the potato is the most important non-cereal world food crop and is next only to rice, wheat and corn as a major crop in terms of total food production (Bajaj, 1987), PVY is a very important plant virus to be studied.

Therefore, this study was undertaken to understand the functions of the 5' terminal region of potato virus Y ordinary strain (PVY-O) genome. The research was carried out on the following lines.

1. To clone a 5 kb cDNA corresponding to the 5'-half of PVY-O genome and to determine its nucleotide sequence;
2. to analyze the function of the 5'UTR of PVY-O by a strategy using GUS as a fused reporter;
3. to detect the P1 protein in infected tobacco plants by Western blot analysis; and to determine the C-terminal position of P1 protein on polyprotein.

Chapter 2

Cloning and Sequencing of 5 kb cDNA Corresponding to 5'-Half of PVY-O Genomic RNA

The analysis of the complete genomic RNA sequence for individual potyviruses has advanced the understanding of potyviral genome structure, as well as suggested functions for several mature viral proteins. A number of potyviral genomes have been sequenced completely: tobacco etch virus strains HAT and NW (TEV-HAT and TEV-NW; Allison *et al.*, 1986, Chu *et al.*, 1995), tobacco vein mottling virus (TVMV; Domier *et al.*, 1986), plum pox virus strains NAT and D (PPV-NAT and PPV-D; Maise *et al.*, 1989, Teycheney *et al.*, 1989), potato virus Y strains N and H (PVY-N and PVY-H; Robaglia *et al.*, 1989, Thole *et al.*, 1993), zucchini yellow mosaic virus (ZYMV; Balint *et al.*, 1990), pea seed-borne mosaic virus (PSbMV; Johansen *et al.*, 1991), papaya ringspot virus (PRSV; Yeh *et al.*, 1992), soybean mosaic virus (SMV; Jayaram *et al.*, 1992), turnip mosaic virus (TuMV; Nicolas & Labiberte, 1992), pepper mottle virus (PepMoV; Vance *et al.*, 1992), potato virus A (PVA; Puurand *et al.*, 1994), peanut stripe virus (PStV; Gunasinghe *et al.*, 1994) and brome streak mosaic virus (BrSMV; Gotz & Maiss, 1995).

This chapter reports the molecular cloning and sequencing of PVY-O genomic RNA, which is essential to study the molecular basis for its genome structure and function.

Materials and Methods

Virus Purification and RNA Isolation

PVY-O was propagated in tobacco (*Nicotiana tabacum* Samsun NN). Tobacco leaf tissue (300 g), 10 weeks after inoculation, was homogenized in a homogenizer (Nissei, Japan) for 5 min at 4°C in 1L of solution containing 0.5M potassium phosphate buffer pH 7.5, 0.01 M EDTA and 0.1% 2-mercaptoethanol. The homogenate was filtrated through cheesecloth and the filtrate was centrifuged at 4,000 g for 10 min. The supernatant was clarified by the addition of Triton X-100 (1% final concentration) and polyethylene glycol (PEG 6,000) (4 % final concentration) and 0.1 M NaCl while stirring for 5 h at room temperature. The precipitated virus was collected by centrifugation (7,000 g for 15 min) and resuspended in 0.5 M potassium phosphate buffer, pH 7.5, 0.01 M MgCl₂. The virus was further purified through a 20% sucrose cushion (190,000 g for 1.5 h) prior to centrifugation in a 10-40% linear sucrose gradient (80,000 g for 2 h). The virus zone was removed and diluted with the same volume of 10 mM Tris-HCl buffer, pH 7.5 and collected by centrifugation at 260,000 g for 2 h.

RNA was isolated from purified virions by proteinase K (2mg/ml) and SDS (2.5%) treatment followed by phenol-chloroform extraction and ethanol precipitation.

cDNA Synthesis and Cloning

4 µg viral RNA was reverse transcribed using a synthesized 17 mer primer (3'-ACTGTGGAGACACGCTA-5', complementary to nt 5017 to 5033 of PVY genomic RNA) and a cDNA synthesis system kit (Amersham, U.K.) according to the manufacturer's instruction. The ds cDNAs were methylated and ligated with *Eco*RI linkers at both ends. After digestion with *Eco*RI, the ds cDNAs were inserted into the *Eco*RI site of the plasmid pBR322, and transformed into *E.coli* strain HB101. All subsequent DNA manipulations were performed according to the standard procedures (Ausubel *et al.*, 1987).

Southern Hybridization

Four clones (termed pVY3, pVY4, pVY118 and pVY263) among the 150 clones containing cDNA inserts, were digested with *Sal* I and *Pst* I, separated on a 1% agarose gel and blotted to a nylon transfer membrane (HybondTM-N+, Amersham, UK). The 0.77 kb *Eco*RI fragment (containing nucleotide sequence corresponding to genomic RNA from 4704 to 5470 bases) of pVY87 (Yoshida, 1994) was used as a probe, which was labelled with [α -³²P]-dCTP using a random primer DNA labelling kit (BcaBest labelling kit, Takara, Japan). The filter was prehybridized in 6 x SSC, 50 % (v/v) formamide, 5 x Denhardt's reagent, 0.1 % SDS and 250 μ g/ml denatured salmon sperm DNA at 45°C for 4 h, and hybridized with the labelled probe for 16 h. The filter was washed at 65°C in 2 x SSC and subsequently in 1 x SSC twice for 10 min, and finally in 0.1 x SSC for 30 min.

Nucleotide Sequencing

The cDNA clone pVY263 was used for DNA sequence determination. Appropriate restriction fragments were subcloned into pUC118 and sequenced by the method of Taq Dye Terminator Cycle Sequencing on a 373A DNA Sequencer using a PRISMTM DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems Inc, USA). The 5'-terminal genomic sequence was determined by primer extension method using reverse transcriptase (Amersham, UK) with the 5' end-labeled synthetic oligonucleotide primer (3'-GTTGCGTCTTGATAT-5', complementary to nt 34 to 52 of PVY-O genomic RNA). The RNA sequencing reactions were essentially identical to those given by Ausubel *et al.* (1987) with modifications of the dNTP and ddNTP concentrations (Table 2-1).

Results and Discussion

Virus Purification and RNA Isolation

PVY-O was purified from infected tobacco leaves and the yield was about 800 µg/100 g leaf tissues, based on the O.D.₂₆₀ determined with a spectrophotometer (Beckman DU-65 Spectrophotometer, USA). The isolated viral RNA was approximately 10 kb in size (Fig. 2-1) and this RNA sample was used for cDNA synthesis.

Synthesis and Screening of cDNA

The ss- and ds- cDNAs were synthesized, and the result of electrophoresis showed that the most predominant bands were around 5 kb (Fig. 2-2). Initial screening of the cDNA clones by restriction enzyme mapping and southern hybridization analysis (Fig. 2-3) identified one of the longest clones termed pVY263. This clone covered the coding region of polyprotein precursor for P1, HC-Pro, P3 and part of CI, and 167 bp of 5' UTR. The relationship of the pVY263 clone to PVY-O genome and the sequencing strategy are shown in Fig. 2-4. The sequence of the 5' extreme end which was not included in any cDNA clones analyzed was determined by direct RNA sequencing using reverse transcriptase and a synthetic primer (Fig. 2-5).

Nucleotide Sequence Analysis of 5'-half genomic RNA of PVY-O

The nucleotide sequence of pVY263 and RNA direct sequence revealed that this part of genome contained 5016 bases (Fig. 2-6). Computer analysis of this sequence showed a long unique ORF encoding a polyprotein of 1608 amino acids, starting at nucleotide position 186. The sequence surrounding the ATG codon at position 186, CCTCAAATG is in reasonable agreement with the consensus

ribosomal recognition sequence of eukaryotic mRNAs, CCRCATG noted by Kozak (1987). The first three nucleotides were not determined (Fig. 2-5) due to the presence of covalently linked amino acid residues of VPg, that might have resulted in premature termination of the reverse transcriptase reactions. However, comparison of PVY-O 5'UTR to those of other PVY strains PVY-N, PVY-H and PVY-Th showed that the extreme 5' termini of the RNAs shared a similar structure and were well conserved (Fig. 2-7). Presumably, the first three nucleotide residues of PVY-O could be adenosines as well.

Genome Organization of PVY-O RNA

When the 5'-half nucleotide sequence of this work was combined with that of the 3'-half of PVY-O reported by Hidaka *et al.* (1992), a total of 9699 bases was revealed. A 5'UTR of 185 bases precedes the unique long ORF which can encode a polyprotein of 3061 amino acids with a calculated M_r of 350 kDa, followed by a 3' UTR of 331 bases. Proceeding from the N-terminus to the C-terminus of the polyprotein, at least 9 functional proteins can be predicted. They are P1, HC-Pro, P3, CI, 5K, VPg, NIa-Pro, NIb and CP (Fig. 2-4, see upper part). Comparison of the complete amino acid sequence of PVY-O polyprotein to those of PVY-N (Robaglia *et al.* 1989) and PVY-H strains (Thole *et al.* 1993) showed 96.4 % and 92.4 % similarities, respectively (Fig. 2-8).

Conserved Motifs and Structures in PVY-O 5'UTR

A 185 bp 5'UTR of PVY-O is rich in A and T residues, and has remarkably few G residues. Like in other potyviruses, within the first 25 nucleotides of each 5' UTR of PVY strain there is a highly conserved sequence motif, "Box a" (ACAACAU) (Turpen, 1989). A second conserved region "Box b" (UCAAGCA) (Turpen, 1989) is present in PVY-O, PVY-N and PVY-Th, but absent in PVY-H

(Fig. 2-7). Furthermore, the 5'UTR of PVY-O contains a threefold repeat of UUUCA. This UUUCA penta-nucleotide block occurs in the 5'UTR of PVY-N and PVY-Th four times, and in PVY-H five times. The penta-nucleotide UUUCA is also present in 5'UTR of most of other potyviruses, but not well conserved in the 5'UTR of the potyvirus-related virus group (comoviruses, nepoviruses and picornaviruses). Therefore, this consensus nucleotide may play a role in potyviral life cycle. In addition, duplicate or triplicate CAA triplets with one or more nucleotides inserted between this motif are present throughout the 5'UTR of PVY strains (Fig. 2-7). It has been shown (Gallie & Walbot, 1992) that the core regulatory element of translational enhancement conferred by the TMV 5'UTR consists of the combination of one copy of the 8 base direct repeat and a 25 base (CAA)_n region.

Conserved Amino Acid Motifs in P1 Protein

Verchot *et al.* (1991) have reported that the TEV P1 protein functions as a third protease cleaving autocatalytically at its C terminus. The amino acid residues of His²¹⁴, Asp²²³, Ser²⁵⁶ and Asp²⁸⁸ in P1 protein of TEV are required for optimal autoproteolytic activity (Verchot *et al.*, 1992). Those residues are well conserved in PVY-O P1 protein. Another consensus sequence FI(V)VRG (Mavankal & Rhoads, 1991) is also found in the P1 protein of PVY-O (Fig. 2-9). Those conserved residues in P1 protein of PVY-O could also play a key role in cleavage of its own C terminus. Three predicted cleavage sites (A), (B) or (C) between P1 and HC-Pro polyprotein by Domier *et al.* (1986), Mavankal and Rhoads (1991) and Hellman *et al.* (1988), respectively, are shown in Fig. 2-9.

Table 2-1 Concentrations of dNTPs and ddNTPs in A, C, G and T mixes (in μ M) for direct sequencing of PVY RNA

	A	C	G	T
dATP	200	200	200	50
dCTP	200	200	50	200
dGTP	200	50	200	200
dTTP	50	200	200	200
ddATP	500	-	-	-
ddCTP	-	500	-	-
ddGTP	-	-	500	-
ddTTP	-	-	-	500

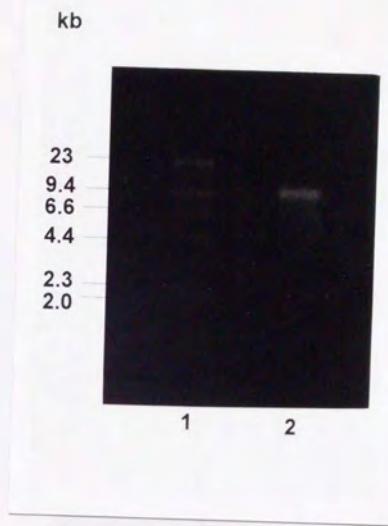


Fig. 2-1 Electrophoresis of purified PVY-O RNA on 0.8% agarose gel in MOPS buffer (Ausubel *et al.*, 1987). Lane 1 λ -Hind III markers, Lane 2 PVY-O genomic RNA. The size of DNA molecular markers in kb are indicated at the left

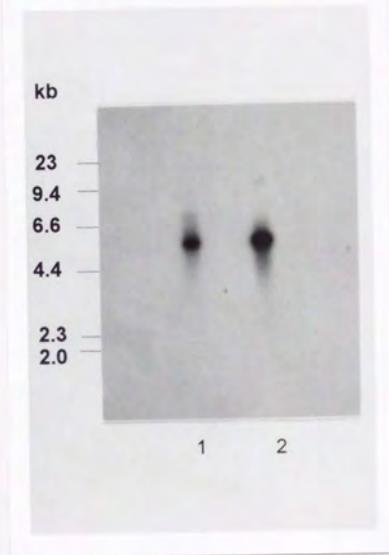


Fig. 2-2 An autoradiograph of a dried agarose gel containing ^{32}P -labelled single-strand cDNA (lane 1) and double-strand cDNA (lane 2).

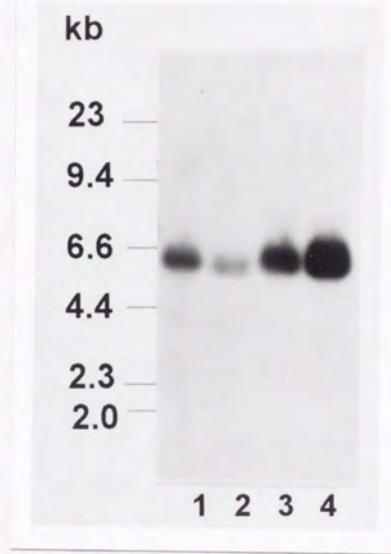


Fig. 2-3 Southern hybridization analysis of clones which contain PVY-O genomic inserts. Lane 1 to 4 represent pVY3, pVY4, pVY118 and pVY263, respectively (for details, see Materials and Methods).

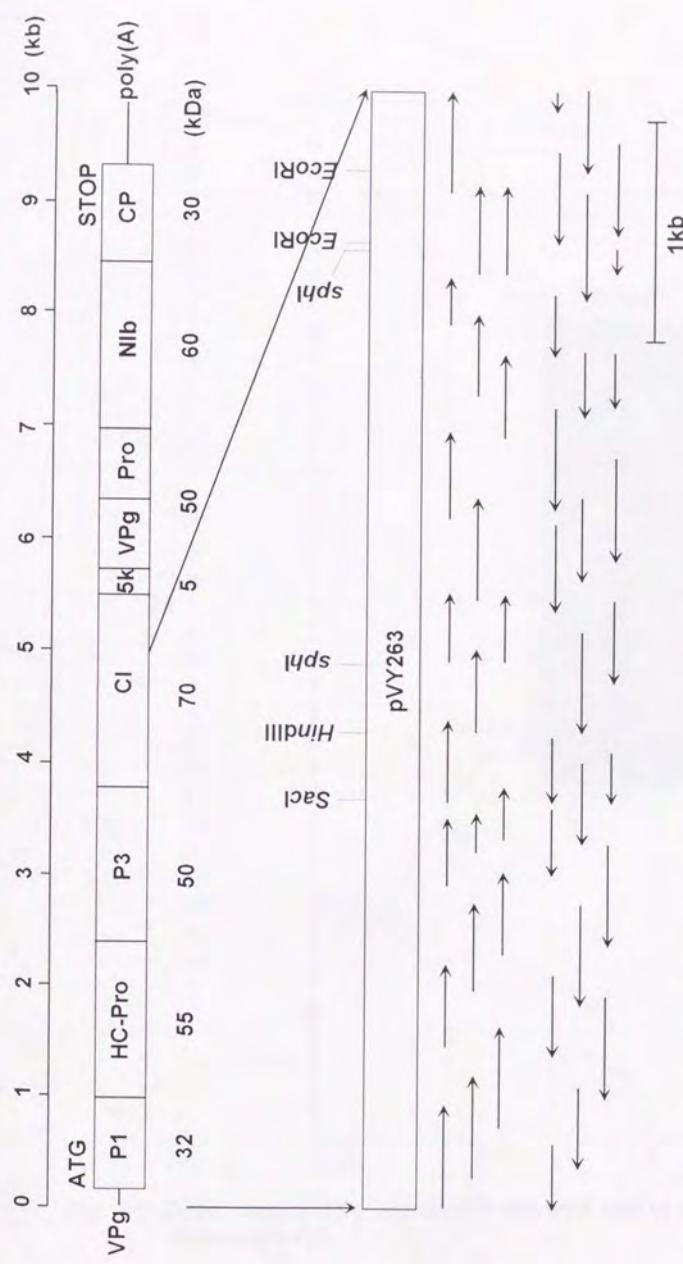


Fig. 2-4 Restriction map and sequence strategy of PVY-O clone pVY263. The PVY-O genomic RNA and its translated products are shown above the clone pVY263. The horizontal arrows below the clone indicate the length and direction of the DNA strands sequenced.

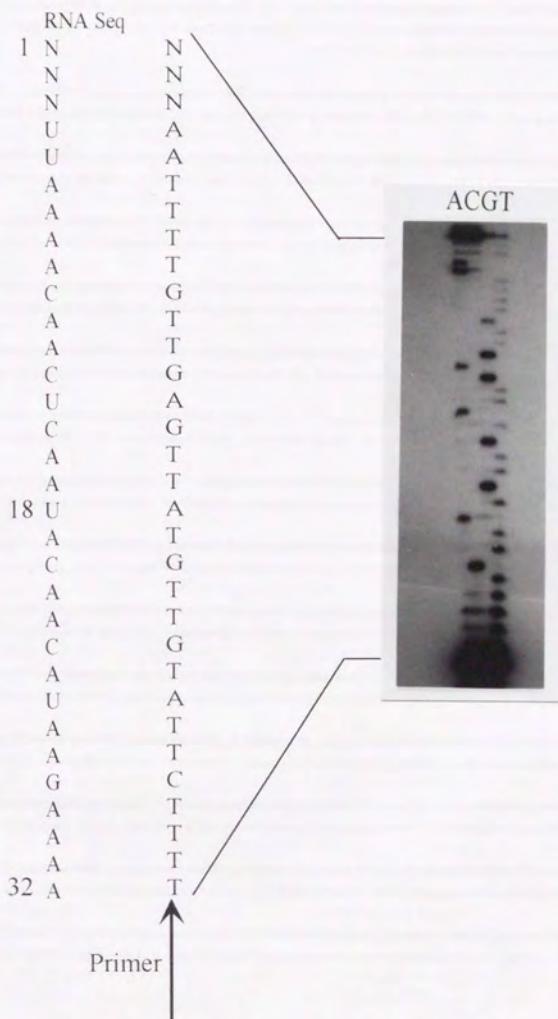


Fig. 2-5 Direct sequence analysis of 5'-extreme end of viral RNA of PVY-O

Fig. 2-6 The nucleotide sequence of the clone pVY263 and its deduced amino acid sequence. The lower-case letters represent the nucleotides which were determined by direct RNA sequencing. The primers used for cDNA cloning and RNA sequencing are underlined.

Fig. 2-6 continued

CTGAGATCTGA-TTTAATTGAACCAACGGACCACTAGCGGAAACGTTTAAACACGTATCTAGTGACCCATTCTCAAGAACGCCAACATGCTATGCTGAAATAATGATATTG 4080
 GlySerValLeuLeuIleGluProThrArgProLeuAlaGlnAsnValPheLysGlnLeuSerSerAspProPhePheLysLysProThrLeuArgMetArgGlyAsnSerIlePheGly

 GCTCTTCGAACTCTCGCTCATGACTAGC99GTTGGCTGCACTATTTO90CAAATACTGCTCACTAGCTCAGITCAAAITTTGAATATTGATGAGTGCCATGTTCTGAGATCTT 4200
 SerSerProIleSerValMetThrSerGlySerAlaLeuAsnAsnArgSerGlnLeuIleGlnPheAsnSerValLeuIlePheAspGlyCysHisValLeuAspProSer

 CGGCAATGGCGTTCCGCACTGCTGAGCTGTTATCATCAACATGCAAAAGTAAAGGTGTCAGCTACTCCAGTGGGAAGGAGGTGAATTCACAACACAGCAGCAGTCAAACTAA 4320
 AlaMetAlaPheArgSerLeuSerValTyrosineGlnIleCysLysValLeuIlyValSerAlaThrArgProValGlyArgGluValGluPheThrThrGlnGlnProVallysLeuIle

 TAGTGAGGACACACTGTTTCCAACTTGTGATGACAAAGGTTTAAACAAATGCTGATGTTGTTCAAGCTGACTTGTAAGTGTGAGATACAATGAAATG 4440
 ValGluAspThrLeuSerGlnSerAspValAsnGlnGlySerIleAsnAsnValIleGlnPheGlySerAsnValLeuValTyrosineSerSerTyrAsnGlnValAsp

 ATA CCTTGCTTAAGCTCTAACGGCACAGAAATATGATGGTCAACAAAGGTTGATGGCAAACTGAAACATGAAACGCGTTGCTAGAAATGTCACAAAAGGAAACUAGTGCGGACACATTG 4560
 ThrLeuAlaIleLeuThrAspIleAsnMetMetValThrIleAspValAspGlyArgValGluMetIleHisGlyCysLeuGluIleValThrLysGlyThrSerAlaArgProHisPheVal

 TGTAGACAAACATAATTGAAATGAGATGTTGGACATAGACGGTGTGGATTTGGTTGAAAGTCTCACCGTTCTTGGACATGACAATAAGGCGATGCTTACAATAAGG 4680
 ValAlaIleThrAsnIleIleGluAsnGlyValThrLeuAspIleAspValValAspPheGlyLeuIlyValSerProIleLeuAspAsnArgSerIleAlaTyrAsnLysVal

 TGAATGTTAGCTATGGTGAAGAAATTCAAAAGGCTGGGTCTGTTGGACGCTTCAAGAAAGGAGTTGCATTGGCATTGGACACACTGAGAAAGGAAATTATTGAAATTCAGCAGCATGGCG 4800
 SerValSerTyrGlyGluArgIleGlnArgLeuGlyArgValGlyArgPheLysLysGlyValAlaIleLeuArgIleGlyHisThrGluLysGlyIleIleGluIleProSerMetValAla

 CTACTGAGGCAAGCTCTGCTGTTGGCTATAATAATTGGCAAGTGATGACAGGAGGCGCTCTCAACTAGCTGATGGCAATTGTACTGCTGAGCCAGGTTAAAAACATGCAAGCAATTGAAAT 4920
 ThrGluAlaIleLeuAlaIleGlyAsnLeuProValMetThrGlyGlyValSerThrSerLeuIleGlyAsnCysThrValArgGlnValLysThrMetGlnGlnPheGluLeu

 TGAATGCTCTTCTTATCCAGAATTGTTGGCTATGATGATGATCAATGATGATCTATCATACATGACATTCTAAGAAAGTATAAAACTGAGATTGATGAAACCTCTGIGGAGATGGAAATTG 5040
 SerProPhePheIleGlnAsnAsnPheValAlaIleAspGlySerMetHisProIleIleHisAspIleLeuLysLysTyrLysLeuArgAspCysMetThrProLeuCysAspGlyIle

Fig. 2-6 continued

	Box a				Box b			
	PVY-O	PVY-N	PVY-Th	PVY-H	PVY-O	PVY-N	PVY-Th	PVY-H
NNNUUAAAAC	AACCUAUAUC	AACUADAGAA	AAACAAACGCA	GAACACCUUA	UAAACGCTU.	..AUTUCUAC		
.AAUTUAAAAC	AACCUAUAUC	AACUADAGAA	AAACAAACGCA	AAACACCUUA	UAAACGCU.	..AUTUCUAC		
.AAUTUAAAAC	AACCUAUAUC	AACUADAGAA	AAACAAACGCA	AAACACCUUG	UAAACGCTU.	..AUTUCUAC		
.AAATUAAAAC	AACCUAUAUC	AACUADAGAA	AAACAAACGCA	AAACACCUUG	CAAAG <u>CGUU</u> CAACUCUAAU			
UCAAGCAACU	UGCUUAAGUU	CAGUUUAAU	CAUUCUUG	CAAUCUCUUA	AAACAAAUAU	GGAAACCAAUU		
UCAAGCAACU	UGCUUA <u>GUUU</u>	CAGUUUAAU	CAUUCUUG	CAUUCUCUUA	GACCAAAUAU	GGAAACCAAUU		
UCAAGCAACU	UGCUUA <u>GUUU</u>	CAGUUUAAU	CAUUCUUG	CAUUCUAAU	AAACAAAUAU	GGAAACCAAUU		
UCAAAACAAU	UGGUUA <u>GUUU</u>	C <u>AA</u> UUCGUA	CUTUCAUCAA	CAAAC <u>GUUU</u> C <u>AA</u> DUC <u>GU</u>	GUAAAGGAUUC			
UCA <u>AC</u> UCAAC	AAGU <u>AA</u> UUC	A. UCACUCC	AACCCAAUC	GGAUCCUCUAUG				
UCA <u>AC</u> UCAAC	AAGCA <u>AA</u> UUC	A. UCACUCC	AACCA <u>AA</u> UUC	AGAUCCUCUAUG				
UCA <u>AC</u> UCAAC	AAGCA <u>AA</u> UUC	A. UCACUCC	AACCA <u>AA</u> UUC	AGAUCCUCUAUG				
GUAAUUCAGU	A <u>GUU</u> UUC	A <u>GUU</u> UUC	AAAU <u>GCAGA</u>	AGAUCAUCUAUG				

Fig. 2-7 Multiple sequence alignment of PVY-O 5' NTR with those been published previously: PVY-N (Robaglia *et al.* 1989), PVY-H (Thole *et al.* 1993) and PVY-Th (Thornbury *et al.* 1990). The dots indicate gaps inserted into the sequences to improve sequence alignment. UUUCA motifs are boxed. Conserved "Box a" and "Box b" are indicated by shadowed boxes. CAA motifs are in dash-lined boxes. The viral translation initiation codons are underlined.

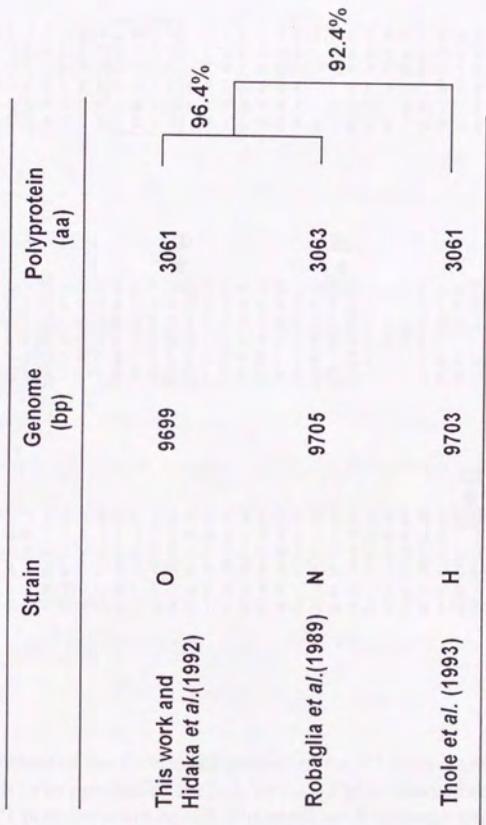


Fig. 2-8 Comparison of the amino acid sequence of polyprotein of PVY-O to those of PVY-N and PVY-H

PVY-O	188 V R T A [H] M M G L R R R V [D]	F R C D T W T V G L L Q R L A R T D K W S N Q
PRSV	452 V Q L R I [H] M N G I R A R Q [D]	V S S S P D M E L L F T Q F C K F L V G H K P
TEV	210 A S V R I [H] M Y G E R K R V D [L]	R I D N W Q Q E T I L L D L A K R F K N E R V
TVMV	161 I D V A [H] A K G H R R R I [D]	C R M H R R E Q R T M H M F M R K T T K T E
PPV	212 T H V R I [H] L D G S K P R Y [D]	L V I D E A T K K I L Q L F A N T S G F H H V
PVV-N	188 V R T A [H] M M G L R R R V [D]	F R C D M W T V G L L Q R L A R T D K W S N Q

PVY-O	225 V R T I N I R R [G D S G]	V I L D T K S L K G H F G R S S G D L [F I V R G S H E]
PRSV	489 I K S K N I T F G S S G	I L F K P K F A D N V G R Y F G D Y [F V V R G R L G]
TEV	247 D Q S K L T F G S S G	L V L R Q G S Y G P A H W Y R H G M [F I V R G R S D]
TVMV	197 V R S K H I R R K G D S G I	V I L L T Q K I K G H L S G V R D E F F I V R G T C D
PPV	249 H K K G E V T P G M S G F V V N P M N	L S D P M Q V Y D T D L F I V R G K H N
PVV-N	225 V R T I N I R R G D S G	V I L N T K S L K G H F G R S S G G L [F I V R G S H E]

(A)	(B)	
↓	↓	
PVY-O	264 G K L Y [H] A R S K V T [Q S] V L N S M I Q [F S]	N A D N F W K G L D G N W A
PRSV	527 G K L F [H] G R S K I L A R S V Y A K M D Q Y N D	V A E K F W L G F N R A F L
TEV	284 G M L V [H] A R A K Y T F A V C H S M T H Y [S] D K S I S E A F F I P Y S K K F L E	
TVMV	236 D S L L [H] A R A R F S Q S I T L R A T H F S T G D I	F W K G F N A S F Q E
PPV	288 S I L V [H] S R C K V S K K Q S N E I I H Y [S] D	P G K Q F S D G F T N S F M Q
PVV-N	264 G K L Y [H] A R S R V T [Q S] I L N S M I Q [F S]	N A D N F W K G L D G N W A

(C)		
↓		
PVY-O	300 R M R Y P S D H T C V A G I L P V E D C G K V A A L M A [H S] I L	
PRSV	565 R H R K P T D H T C T S D M D V T M C G E V A A L A T I I L F	
TEV	324 L R P D G I S H E C T R G V S V E R C G E V A A I L T Q A L S	
TVMV	273 Q K A I G L D H T C T S D L P V E A C G H V A A L M C Q [S] L F	
PPV	326 C K L R E T D H Q C T S D L D V K E C G G Y V A A L V C Q A I I	
PVV-N	300 R M R Y P S D H T C V A G I L P V E D C G R V A A L M A [H S] I L	

Fig. 2-9 Alignment of the C-terminal portion of the P1 proteins and the N-terminal portion of the HC-Pro proteins of six potyviruses. The conserved amino acids essential for the P1 proteinase are boxed. The predicated cleavage sites (A), (B) and (C) by Domier *et al.* (1986), Mavankal & Rhoads (1991) and Hellman *et al.* (1988), respectively, for liberating the P1 and HC-Pro proteins are indicated by arrows. The most conserved amino acids of the dipeptides are indicated by shadowed boxes.

Chapter 3 Characterization of 5' Untranslated Region of PVY-O in Tobacco Protoplasts

Introduction

An ever growing number of plant viral translational enhancers has been documented in recent years. The first reported example of a plant viral leader acting as a translational enhancer was that of the 37 nucleotides 5'UTR from the RNA 4 molecule of alfalfa mosaic virus (AIMV) (Jobling & Gehrke, 1987). In *in vitro* system, the presence of this leader results in as much as a 35-fold enhancement of the expression of the foreign barley α -amylase messenger and a 6- to 7-fold enhancement of the human interleukin 1 β gene. The most widely documented enhancer is the 5'UTR (68 bases) of the TMV genomic RNA molecule. Reports of translational enhancement properties have been derived from both *m vivo* and *m vitro* studies (Gallie *et al.*, 1987a; Gallie *et al.*, 1987b; Sleat *et al.*, 1987; Sleat *et al.*, 1988). Extensive mutation and deletion of the leader have identified one direct repeat sequence (ACAAUUAC) and the (CAA)_n sequence as the functional motif region responsible for translational enhancement (Gallie *et al.*, 1988; Gallie & Walbot, 1992).

In case of potyviral 5'UTRs, fusion of the 144 nucleotide leader of TEV to a reporter gene encoding GUS enhanced protein expression 8- to 21-fold *m vitro* and *m planta* (Carrington & Freed, 1990). Their data showed that the functional region of the leader is present within the 3'-terminal 63 nucleotides. Another characterized potyvirus translational enhancer is the 143 nucleotide 5'UTR of PSbMV. This enhancer has been shown to be effective *m vivo* in both pea and tobacco protoplasts with enhancement levels of about 20-fold (Nicolaisen *et al.*, 1992). A deletion of this leader that leaves only the first 83 nucleotides of the

authentic enhancer doubles the level of translational enhancement. Nicolaisen and coworkers (1992) have suggested that a deletion which causes disruption of secondary structures may be responsible for the stimulation of translational efficiency. Some preliminary data also implicated that 5'UTR of TuMV as translation enhancer *in vivo* (Basso *et al.*, 1994).

Recently, the 5'UTR (185 bases) of PVY has been associated with an enhancement at the translational level (Levis *et al.*, 1992; Levis & Astier-Manifacier, 1993). The presence of this leader results in a stimulation of translation in *in vitro* system. In an attempt to identify the region(s) involved in translational enhancement, a hybrid arrest translation method has been employed. The results have indicated that the central functional region of the leader is present within the first 16 residues of the genomic RNA molecule, as the addition of an oligonucleotide complementary to this region totally inhibits translational enhancement properties.

The present study was carried out to analyze the properties of the PVY-O 5'UTR, i.e., to investigate whether the 5'UTR functions as a translational enhancer in tobacco protoplasts, as well as whether specific nucleotide sequence motifs in the 5'UTR are required. Various engineered derivatives of the PVY-O 5'UTR were fused to the GUS reporter gene and their effects on translation of downstream GUS in tobacco protoplasts were examined.

3-1. 5'UTR of PVY-O Increases the Translation of Downstream GUS Encoding Region in Tobacco Protoplasts

Materials and Methods

Plant Materials

6-8 weeks-old tobacco (*Nicotiana tabacum* cv Samsun NN) plants grown in a controlled greenhouse were used for protoplast isolation.

PCR Amplification of PVY-O 5' UTR

The PVY-O 5'UTR including viral AUG was amplified by polymerase chain reaction (PCR) using the plasmid pVH9 (containing 1~313 nucleotide residues of PVY-O genome) (Sonoda, 1996) as a template and two synthetic oligonucleotide primers [5'GGTCTAGAAATTAAAACAACTC3' (primer 1) and 5'AAGGATCC CATTGAGGATCTG3' (primer 2)]. The conditions for PCR were as follows: denaturing at 94°C for 8 sec, annealing at 60°C for 15 sec, and extension at 72°C for 20 sec, amplification for 30 cycles with an Air Thermo-Cycler (Idaho Technology Corp. USA). The underlined nucleotides denote the restriction sites used for cloning. The amplified DNA was then inserted into the *Xba*I/*Bam*H-digested pUC119 vector, leading to pVH185 (Fig. 3-1). The fidelity of the PCR was confirmed by sequencing in both strands as described in chapter 2.

Construction of the Transient Expression Vectors

Plasmids pBI121 (Jefferson *et al.*, 1987), pUC119 and pVH185 were used for the construction of the transient expression vector pGUS and pENGUS (Fig. 3-2). Briefly, the *Eco*RI-*Hmd*III restriction fragment containing the CaMV35S promoter - *gus* - NOS terminator cassette from pBI121 was cloned at *Eco*RI/*Hmd*III sites of vector pUC119 to generate plasmid pGUS. An ATG codon was

introduced in *Xba*I/*Bam*HI sites of pGUS as follows: First, the *Xba*I-digested pGUS was ligated with two kinased linkers (5'CTAGAAATGG3' and 5'GATCCCATT3'), after recovery from an agarose gel, it was digested with *Bam*HI and then self-ligated. The plasmid pENGUS was generated by cloning the *Xba*I-*Bam*HI fragment which contains PVY-O 5'UTR from pVH185 into the *Xba*I/*Bam*HI sites of pGUS (Fig. 3-2). The plasmid pENGUS has PVY-O 5'UTR preceding the GUS encoding region. Both plasmids were amplified in *E. coli* HB101.

Isolation and Purification of the Transient Expression Vectors

The transient expression vectors were isolated and purified essentially as described by Ausubel *et al.* (1987). The plasmids isolated by the alkali-method were purified by two cycles of CsCl-ethidium bromide density gradient centrifugation (100,000 rpm, 3h), with a TL-100 ultracentrifuge (Beckman, USA). The plasmid yields were determined in triplicates using a spectrophotometer and the purity of the plasmids was checked on an agarose gel.

Protoplast Isolation

Young leaves were excised from 6-8 weeks old tobacco plants and surface-disinfected with 0.5% (v/v) sodium hypochlorite solution for 10 min, then thoroughly rinsed three times in sterile distilled water. After disinfection, the undersurfaces of leaves were removed with a forceps and then floated in 15 ml of CPW solution (Frearson *et al.*, 1973) with 9% mannitol (CPW-9M), containing 1% cellulase Onozuka R-10 and 0.2% macerozyme (Table 3-1). Incubation was done for 3 h in the dark at 25°C. Protoplasts were purified by filtration through a nylon mesh (100μ) and washed three times with CPW-9M and once with solution T (30 mM CaCl₂, 13 % mannitol w/v) by gentle centrifugation (90 x g, 5 min).

PEG-Mediated Transformation of Protoplasts

Transfer of the plasmid DNA molecules into tobacco protoplasts was performed essentially as described by Ballas *et al.* (1988). Briefly, samples of 2 x 10⁶ protoplasts were suspended in 1 ml of solution T, 25 µg of plasmid DNA and 85 µg of calf thymus DNA (carrier DNA) each in a volume of 25 µl and 0.4 ml of PEG 6000 (40 % in solution T) were added to protoplasts sequentially. After mixing gently, the resulting suspension was incubated for 30 min at 26°C with gentle shaking (50 rpm). After dilution with 30 ml of cold mannitol (13 %), and 30 min incubation on ice, the protoplasts were collected by centrifugation (90 x g for 5 min). The protoplasts obtained were then resuspended in 18 ml of culture medium [MS salts and vitamines (Murashige and Skoog, 1962), 1 % sucrose, 7 % mannitol and 0.5 mg/l of 2,4-D] and cultured in two 9 cm petri dishes for 24 h in the dark at 25°C. In control experiment, the protoplasts were treated with 25 µl of water.

Fluorometric Analysis of GUS Activity

GUS activity in protoplasts was assayed essentially as described by Jefferson (1987). 24h after transformation, the protoplasts were collected by centrifugation and suspended in 200 µl of GUS extraction buffer (50 mM sodium phosphate buffer pH 7.0, 10 mM EDTA, 0.1 % Triton X-100, 0.1 % sarkosyl and 10 mM β-mercaptoethanol). To facilitate lysis, the cell suspension was passed immediately 10 times through a 23-gauge needle attached to a syringe. The mixture was centrifuged 10,000 x g for 10 min. 500µl of MUG substrate buffer [1 mM 4-methylumbelliferyl β-D-glucuronide (MUG) in GUS extraction buffer] was added to 100 µl of supernatant. The final mixture was vortexed and then incubated at 37°C. Samples (100 µl) were removed after 0, 5, 15 and 30 min of incubation and added to 1.9 ml of 0.2 M Na₂CO₃. Conversion of MUG to methylumbelliferone

(MU) was measured with a spectrofluorometer (FP-777, JASCO Corporation, Japan). The concentration of MU that accumulated in the reactions was calculated from standard curves obtained with known concentrations of MU. One unit of GUS activity was defined as 1 pimole of MU released/min/mg of extracted protein. Total protein concentration in each extract was measured using a protein assay kit (Bio-Rad Laboratories, USA).

In situ Localization of GUS Activity in SDS-PAGE

In situ localization of GUS activity in SDS-PAGE was done according to Gallie *et al.* (1987). Mainly, samples of protoplast extracts containing equivalent amounts of protein were incubated with an equal volume of gel loading buffer (125 mM Tris-HCl pH 6.8, 20 % glycerol, 10 % β -mercaptoethanol, 4.6 % SDS and 0.004 % BPB) at room temperature for 15 min, followed by SDS-polyacryamide gel electrophoresis in a 7.5 % gel (Laemmli, 1970) at 40 V for 16 h. The gel was rinsed 4 times in 100 ml GUS extraction buffer for a total of 2 h, incubated on ice in MUG substrate buffer for 30 min, and transferred to a glass plate at 37°C for 30 min. The gel was then sprayed with 0.2 M Na₂CO₃ and photographed under long-wavelength ultraviolet light.

Results and Discussion

To study the role of the 5'UTR in translational initiation and enhancement, the PVY-O 5'UTR was fused to a GUS encoding sequence (Fig. 3-2). The start codon in pGUS was situated within the local sequence context, AGAAUGG (initiator codon underlined) (Fig. 3-6), the initiator codon of pENGUS resided within the sequence UCAAUGG (Fig. 3-5). The chimeric genes were expressed transiently in tobacco protoplasts following PEG-mediated transformation with plasmid pGUS and pENGUS. Assaying protoplasts extracts by GUS-activity gel (Fig. 3-3)

showed that the plasmid pENGUS enhanced expression considerably (Fig. 3-3, lane 3). Accurate fluorometric quantitation of the GUS activity revealed that fusion of the PVY-O 5'UTR to the GUS gene (pENGUS) provided approximately 3900 units of GUS activity which is a fifteen-fold increase compared to the control plasmid pGUS (approximately 250 units) (Fig. 3-4).

Levis & Astier-Manifacier (1993) showed that PVY 5'UTR stimulates the GUS translation *in vitro*. The results of the present study showed that PVY-O 5'UTR also enhances translation of reporter GUS in tobacco protoplasts. The enhancement of GUS expression caused by the viral 5'UTRs could theoretically be due to an increase in transcription efficiency, as well as an increase in translation efficiency. However, previous investigations using mRNA transcripts, with or without viral 5'UTRs, introduced directly into protoplasts have demonstrated that the enhancement effect was the direct consequence of the 5'UTRs at the level of translation (Carrington & Freed, 1990; Gallie *et al.*, 1987; Gallie *et al.*, 1988).

Table 3-1 Enzyme Solution Composition for Tobacco Protoplast Isolation

Enzyme	Cellulase Onozuka R-10 Macerozyme R-10 (Yakult Honsha, Japan)	1 % 0.2 %
Osmotic agent	Mannitol	9 %
CPW salt	KH ₂ PO ₄ KNO ₃ CaCl ₂ 2H ₂ O MgSO ₄ 7H ₂ O KI CuSO ₄ 5H ₂ O	mg/l 27.2 101.0 1480.0 246.0 0.16 0.025
pH		5.7

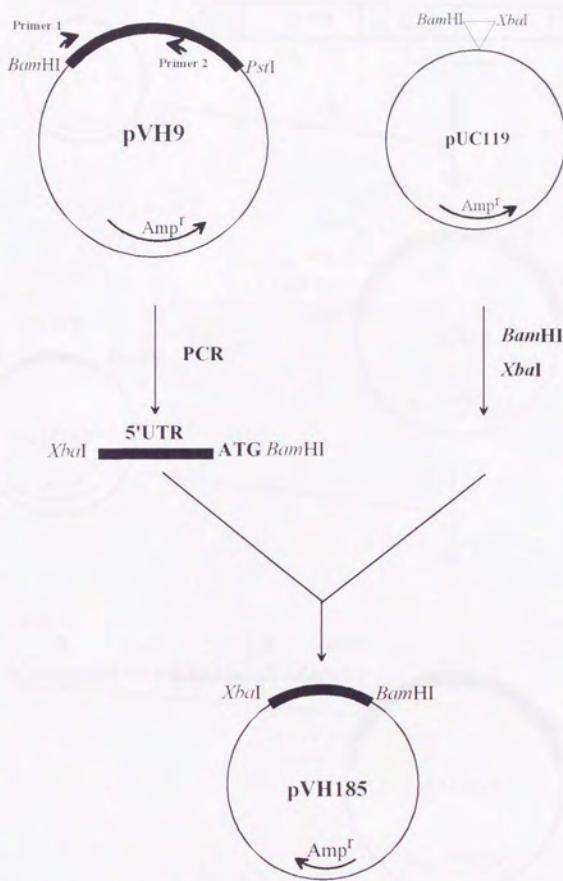


Fig. 3-1 Construction of the intermediate plasmid pVH185

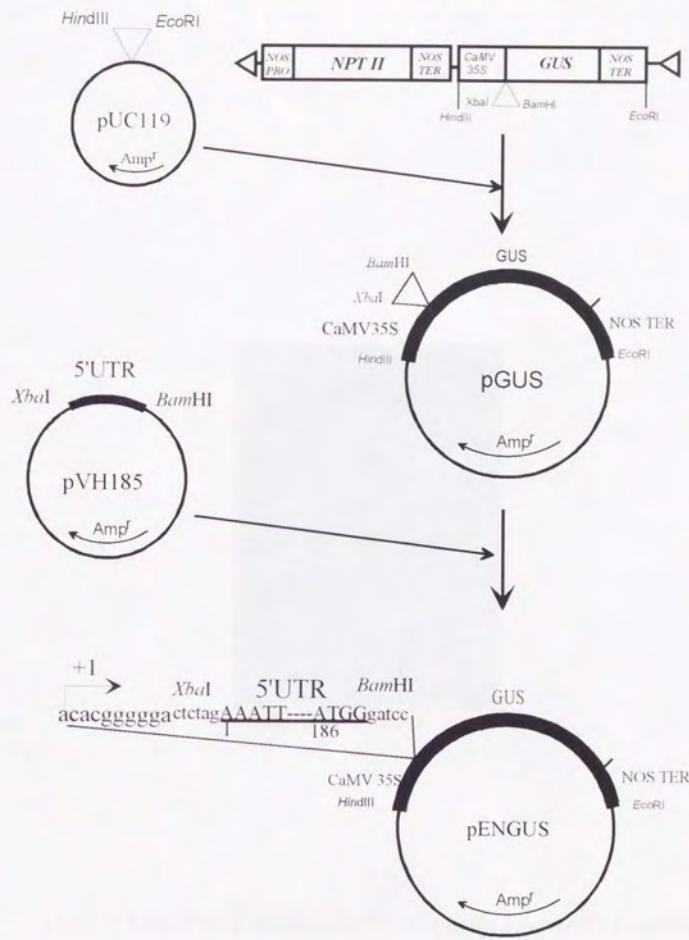


Fig. 3-2 Construction of the transient expression vector pGUS and pENGUS



1 2 3

Fig. 3-3 SDS-PAGE analysis of GUS activity from PEG transformed tobacco protoplasts.
track 1, no plasmid (mock); track 2, plasmid pGUS;
track 3, plasmid pENGUS

GUS activity (4-MU pmole/min/mg protein)

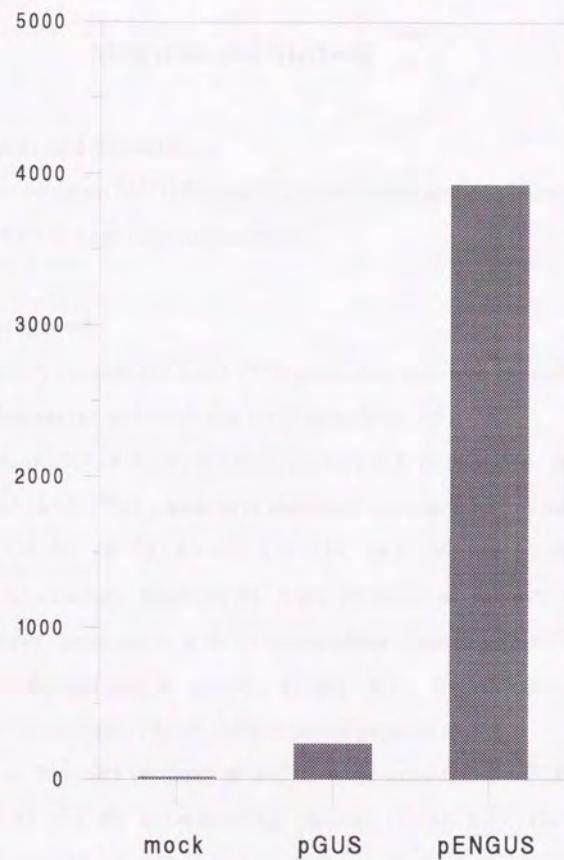


Fig.3-4 Comparison of GUS activity from pGUS and pENGUS transformed tobacco protoplasts. The average values of six independent experiments are shown.

3-2. Mutational Analysis of 5'UTR of PVY-O for Altered Ability to Enhance Translation

Materials and Methods

Bacterial Strains and Plasmids

Escherichia coli strain MV1184 and CJ236, the intermediate plasmid pVH185, pGUS and pENGUS were used in this study.

Plasmid Construction

All the plasmids contain the CaMV35S promoter, followed by either PVY-O 5'UTR or its derivatives and a reporter GUS gene (Fig. 3-5).

The plasmids pENGUS-1, pENGUS-2, pENGUS-3, pENGUS-4, pENGUS-5, pENGUS-6 and pENGUS-7 which have deletions corresponding to the 5'UTR at 1~16, 19~25, 34~46, 68~75, 85~89, 136~158, and 176~188 (including viral initiation codon) residues, respectively, were obtained as follows: After site-directed deletional mutagenesis with an intermediate plasmid pVH185 (Fig. 3-1) and synthetic oligonucleotide primers (Table 3-2), the deletion-containing fragments were cloned into *Xba*I/*Bam*HI-digested plasmid pGUS.

The 1~130 or 131~185 residues of 5'UTR were amplified by PCR using the plasmid pVH185 and the corresponding primers (Table 3-2). The amplified products were cloned into *Xba*I/*Bam*HI-digested plasmid pGUS to generate pENGUS-8 and pENGUS-9, which have 3'-terminal 55 residues or first 130 residues of the 5'UTR respectively, to precede the GUS encoding region.

The nucleotide sequences of deleted regions and PCR products were verified on both strands as described in chapter 2.

All the plasmids were purified by two cycles of CsCl-ethidium bromide density

gradient centrifugation as described in chapter 3-1.

Secondary Structure Analysis of PVY-O 5'UTR

The PVY-O 5'UTR (185 bases) was analyzed for possible secondary structure using Zuker's program.

Electroporation

Tobacco protoplasts were prepared as described in chapter 3-1, and transfer of the plasmid DNA molecules into tobacco protoplasts was performed essentially as described by Fromm *et al.* (1985) with some modifications. Briefly, each sample of 2×10^6 protoplasts was resuspended in 1 ml of Hepes-buffered saline (10 mM Hepes, pH 7.2, 140 mM NaCl, 4.5 mM CaCl₂, 330 mM mannitol) with or without plasmid DNA (25 µg/ml). The electrical pulse was supplied by a Bio-Rad gene pulser™ power supply set (USA) at 200V, 960 µF. The solution containing the protoplasts was held at 0°C for 10 min after the electric pulse. The protoplasts were pelleted by gentle centrifugation (90 x g, 5 min), and resuspended in MS medium. The culture condition was the same as described in chapter 3-1.

Results

Secondary Structure of PVY-O 5'UTR

The PVY-O 5'UTR has been examined for possible secondary structures. The results are shown in Fig. 3-5. There are two stem loop structures within the 5'UTR, one is formed from 25~118 residues and the other from 126~168 residues.

The Rationale behind the Construction of 5'UTR Based Mutants to Test for Alteration in Translation Enhancement.

Potyviral 5'UTRs are multifunctional regions involved in translational enhancement (Turner & Foster, 1995) and virus replication (Klein *et al.*, 1994). Inspection of the PVY 5'UTR revealed several conserved motifs and structures (Fig. 2-7). The "Box a" and "Box b" are well conserved among potyviral strains (Turpen, 1989). Although the 5'UTRs from different potyviruses vary in length, they all contain the UUUCA penta-nucleotide domain, ranging in 1~5 fold repeats. Besides, the (CAA)_n element which was identified as a functional motif for translational enhancement (Gallie & Walbot, 1992) is also present in PVY strains (Fig. 2-7). In *in vitro* system, Levis *et al.* (1992) showed that the first 16 nucleotides of PVY 5'UTR was absolutely needed for translational enhancement activity. Furthermore, each of the predicted stem-loop structures of PVY-O may play different roles in translational initiation and enhancement. In an attempt towards delineating those sequences necessary for translational enhancement, the PVY-O 5'UTR based deletions were thus designed (Fig. 3-6, Table 3-3), and their functions were investigated in tobacco protoplasts.

Mutational Analysis of 5'UTR in Tobacco Protoplasts

The plasmids containing 5'UTR or its derivatives prior to the GUS encoding gene, were electroporated into tobacco protoplasts. 24 h after transformation, the GUS activities were assayed (Table 3-4). The GUS activity of an intact 5'UTR containing pENGUS was taken as 100%.

① pENGUS-1

Deletion of the first 16 residues results in about 43% stimulation of the translation of GUS. In other words, this result shows that the first 16 residues can be regarded as a dispensable part of the 5'UTR for translational enhancer *in planta*.

② pENGUS-2 and pENGUS-4

Deletion of the conserved "Box a" (pENGUS-2) showed no obvious changes on translation of GUS, and deletion of "Box b" (pENGUS-4) showed a 28% higher GUS activity than that of pENGUS.

③ pENGUS-3

About 36% increased GUS activity was detected upon the deletion of the CAA containing region.

④ pENGUS-5 and pENGUS-6

Deletion of the first UUUCA penta-nucleotide repeat (pENGUS-5) showed 56% of GUS acitivity increase as compared to control pENGUS. Interestingly, that deletion of the second and third penta-nucleotide repeats (pENGUS-6) resulted in reduction of GUS activity. The pENGUS-5 caused a doubling in translation efficiency in comparison to pENGUS-6.

⑤ pENGUS-7

When viral AUG and its upstream region were deleted, the GUS activity was decreased considerably (21% GUS activity of control pENGUS). In this case, the wild type GUS initiation codon has been used for translation (Fig. 3-6), and initiator codon of pENGUS-7 resided within the sequence CUUAUGU.

⑥ pENGUS-8 and pENGUS-9

The pENGUS-8 which contains only the 3'-terminal 55 bases of the PVY-O 5'UTR resulted in 28% increasing of GUS activity compared with pENGUS. On the contrary, pENGUS-9 (having first 130 bases of 5'UTR) reduced the translation of GUS to 58% of control pENGUS. In this case, the pENGUS-8 doubled GUS activity as compared to pENGUS-9.

Discussion

The mechanisms responsible for the translation enhancing activity of the non-

translated sequences preceding the AUG codon are not well defined. Recent experiments suggested that different mechanisms might be involved in translation enhancing activity of different viral 5'UTRs (Sleat *et al.*, 1988; Jackson *et al.*, 1990; Carrington & Freed, 1990; Levis & Astier-Manifacier, 1993; Basso *et al.*, 1994).

In an attempt to assess the conserved motifs and other proposed functional structures of PVY-O 5'UTR involved in translation enhancement, a series of deletional mutations were introduced into the PVY-O 5'UTR. Those deleted sequences were fused to the reporter GUS gene and their effects on translation of downstream GUS gene in tobacco protoplasts were examined.

The first 16 nucleotides of 5'UTR have been suggested by Levis *et al.* (1992) to have an essential function on initiation of translation *in vitro*. They, therefore, suggested that this region might be an attachment site for ribosomes. If this observation holds also for *in planta* system, then deletion of this region may result in the loss of translational initiation and enhancement. However, the results in the present study showed that the first 16 bases were completely dispensable for translational initiation and enhancement (Table 3-4). The translational activity of GUS was even stimulated upon the deletion of this region. The reasons for these contradictory results were unclear. One possible explanation is that differences exist between *in planta* and *in vitro* systems with respect to factors influencing translation efficiency. Even between eukaryotic systems those observations are not uncommon (Gallie *et al.*, 1988).

Nicolaisen *et al.* (1992) showed that the shortened 5'UTR of PSbMV caused an enhancement of translation compared to the authentic PSbMV 5'UTR. Therefore, the absence of a stable secondary structure has been suggested to play a major role in determining the ability of 5'UTRs to enhance translation. The stimulation of GUS translation caused by pENGUS-2, pENGUS-3, pENGUS-4 and pENGUS-5 could be explained by the disruption of the predicted secondary

structure by the deletions in 5'UTR. Truncating first 130 bases of 5'UTR that leaves only the rest 55 residues of 5'UTR (pENGUS-8) does show higher GUS activity.

However, the absence of a stable secondary structure alone may not solely account for this enhancement phenomenon, because the partial or complete deletions of the 3'-terminal 55 residues of 5'UTR, pENGUS-6 and pENGUS-9, respectively, result in the reduction of GUS activity in comparison with an intact 5'UTR (pENGUS). The pENGUS-9 has shown only half of the GUS activity compared to that of pENGUS-8. This result indicates that the 3' terminal 55 bases of PVY-O 5'UTR function differently from the rest part of 5'UTR. In other words, this region could not be regarded as a dispensable part for translational enhancement.

It seems that three conserved UUUCA motifs within 5'UTR, function differently in the translational enhancement process. Since deletion of the first UUUCA leads to stimulation of GUS translation, while the deletion of the second and third UUUCA motifs caused the reduction of GUS activity.

Although the mechanisms of translational enhancement are not well understood, specific motifs, or binding domains for plant host translation factors or ribosomes, as well as the secondary configuration of 5'UTR may be involved in this process. Further research is required to understand the functions of PVY-O 5'UTR in translational enhancement.

Table 3-2 Synthetic primers used for construction of the 5'UTR derivatives containing vectors

Plasmid	Primer
<u>For Site-Directed Deletion</u>	
pENGUS-1	5'TTCTTATGTTGTATTCTAGAGTCGACC3'
pENGUS-2	5'TCTGCGTTGTTTCTTATTGAGTTGTTTAAT3'
pENGUS-3	5'AATAAGCGTTATAAGTTTCTTATGTTGTA3'
pENGUS-4	5'CTGAAACTTAGCAAGGTGAGAATAAGCGTT3'
pENGUS-5	5'AGGAAATGAATTAAACCTTAGCAAGTTGCTT3'
pENGUS-6	5'ATTGGTTGGAAGTGTGATGGTTCCAATATTG3'
pENGUS-7	5'GTACCCGGGGATCCAGATTGGTTGGAAG3'
<u>For PCR</u>	
pENGUS-8	5'GGTCTAGAAACCATTCAAC3' 5'AAGGATCCCATTGAGGATCTG3'
pENGUS-9	5'GGTCTAGAAATTAAAACAACTC3' 5'AAGGATCCCATTCAATATTG3'

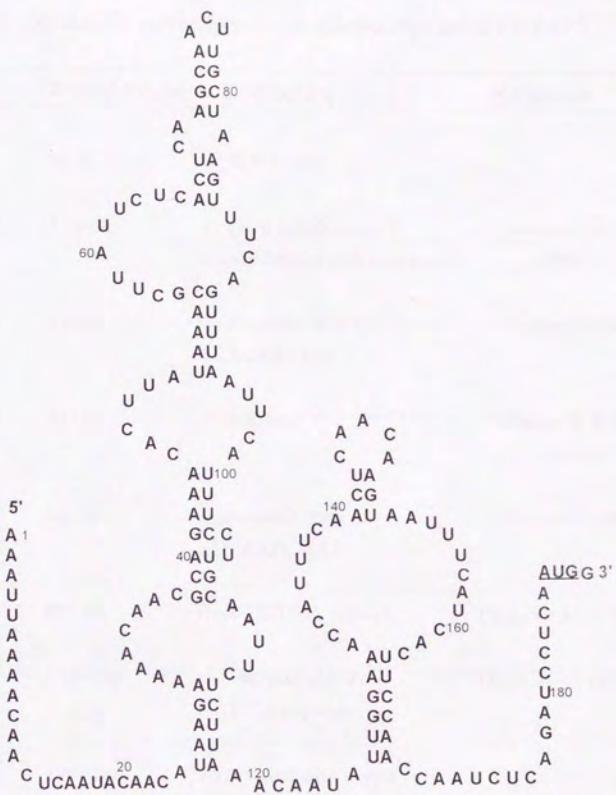


Fig. 3-5 Predicted secondary structure of the PVY-O 5'-NTR for the plus strand. The viral initiation codon is underlined.

Table 3-3 Structures and properties of deleted regions of PVY-O 5'UTR

Plasmid	Deleted Region	Structure	Reference
pENGUS	no deletion	full-length	
pENGUS-1	1~16	<i>in vitro</i> inhibition of translational enhancement	Levis <i>et al.</i> , (1992)
pENGUS-2	19~25	conserved 'Box a' (CACAAACAU)	Turpen (1989)
pENGUS-3	34~46	(CAA) motif	Gallie & Walbot (1992)
pENGUS-4	68~75	conserved 'Box b' (UCAAGCAA)	Turpen (1989)
pENGUS-5	85~89	first UUUCA repeat	Thole <i>et al.</i> (1993)
pENGUS-6	136~158	second and third UUUCA repeats	Thole <i>et al.</i> (1993)
pENGUS-7	176~188	viral initiation codon and its upstream region	
pENGUS-8	1~130	first predicted stem-loop structure	This work
pENGUS-9	131~185	second predicted stem-loop structure	This work

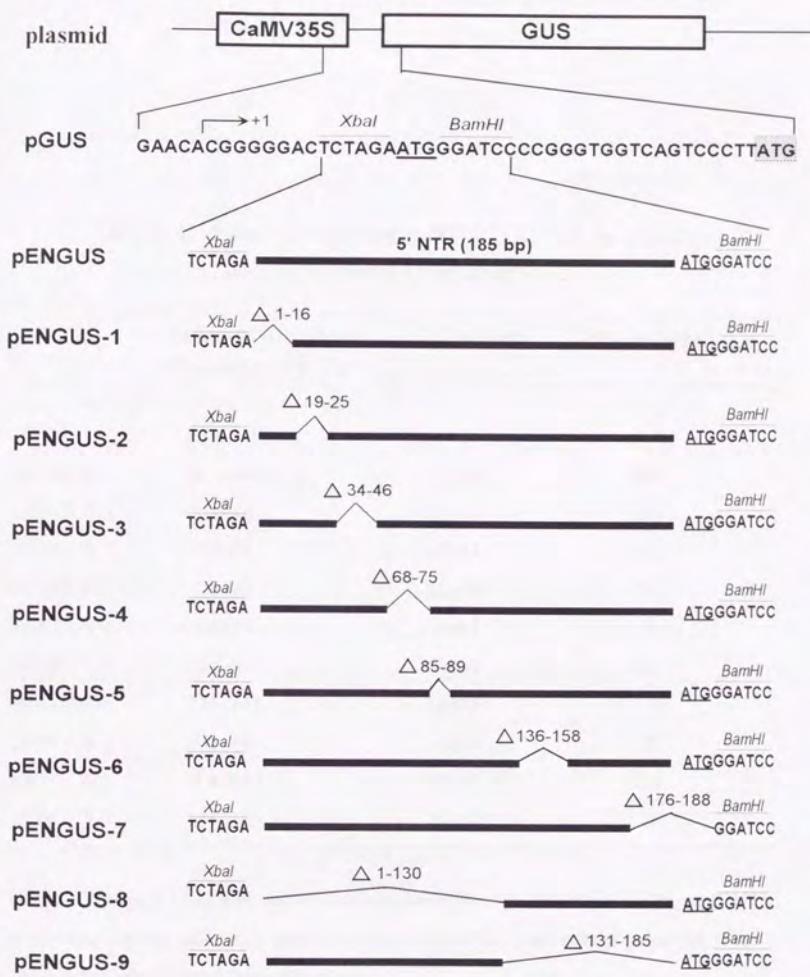


Fig. 3-6 A schematic representation of PVY-O 5'UTR and its derivatives examined for alterations in translation enhancement *in planta*. The boxed ATG codon corresponds to codon 1 in wild-type GUS. The ATG initiation codon is underlined. The arrow indicates the transcriptional start site.

Table 3-4 Effect of Deletions in PVY-O 5'UTR on Translation of GUS in Tobacco Protoplasts

Plasmid	Deleted Nucleotide Position in 5'UTR	GUS activity (4-MU pmole min mg protein)	Percentage (%)
pGUS	no 5'UTR	805	4
pENGUS	no deletion	19118	100
pENGUS-1	1~16	27301	143
pENGUS-2	19~25	20285	106
pENGUS-3	34~46	25969	136
pENGUS-4	68~75	24484	128
pENGUS-5	85~89	29838	156
pENGUS-6	136~158	14872	78
pENGUS-7	176~188	4016	21
pENGUS-8	1~130	24497	128
pENGUS-9	131~185	11120	58

The percentage of GUS activity was calculated compared to that of the full-length 5'UTR containing plasmid pENGUS which was taken as 100%. The average values of at least three independent experiments are shown.

Chapter 4 Characterization of PVY-O P1 Protein

The P1 protein is derived from the N-terminal region of the potyviral polyprotein. This protein catalyzes autoproteolytic cleavage between itself and HC-Pro protein. Its catalytic activity *in vitro* requires a cellular factor present in extracts from plant (Verchot *et al.*, 1991). The C-terminal proteolytic domain resembles chymotrypsin-like serine proteinases (Verchot *et al.*, 1992), while the N-terminal half was shown to be dispensable for proteolysis as well as genome amplification, and systemic infection (Verchot & Carrington, 1995b). The P1 protein exhibits single-strand RNA binding activity *in vitro* (Brantley & Hunt, 1993; Soumounou & Laliberte, 1994), and is the least conserved protein among potyviruses (Domier *et al.*, 1987; Vance *et al.*, 1992; Marie-Jeanne Tordo *et al.*, 1995), ranging in size from 30 kDa to 63 kDa.

The P1 protein has only recently been detected from the extracts of plants infected with tobacco vein mottling potyvirus (Rodriguez-Cerezo & Shaw, 1991), pea seedborne mosaic potyvirus (Albrechtsen & Borkhardt, 1994), and zucchini yellow mosaic potyviruses (Wisler *et al.*, 1995). Besides, there were only two reports on the determination of cleavage sites between P1 and HC-Pro polyprotein *in vitro*. The cleavage between the P1 protein and HC-Pro was shown to occur between Phe²⁵⁶/Ser²⁵⁷ in TVMV (Mavankal & Rhoads, 1991) and Tyr³⁰⁴/Ser³⁰⁵ in TEV polyprotein (Verchot *et al.*, 1992).

To further elucidate the roles of P1 protein in the potyviral life cycle, polyclonal antiserum was raised against the PVY-O P1 protein in the present study, and its presence in infected plants has been examined. By employment of the GUS fusion strategy, substitution and deletion mutagenesis, and transient assay, the cleavage site between P1 and HC-Pro polyprotein has been determined in tobacco protoplasts.

4-1. Detection of PVY-O P1 Protein in Infected Tobacco Plants

Materials and Methods

Tobacco (*Nicotiana tabacum* cv. Samsun NN) was used for PVY-O infection. *Escherichia coli* strain HB101 was used for the production of fusion protein.

Construction and Expression of P1 Protein as a Fusion Protein

The cDNA clone pVY263 and bacterial expression vector pMal-c (Guan *et al.*, 1987) were used for the construction of plasmid pMal-P1 (Fig. 4-1). pVY263 was cut with the restriction enzyme *Bgl* I and blunt-ended with T4 DNA polymerase. After ligation with a Smurft linker (5'TTAAGTTAACTTAA3'), the plasmid was digested with *Hpa* I and *Bgl* II, and *Bgl* II site was filled in with T4 DNA polymerase. The 607bp fragment covering nucleotides from 297 to 905 of PVY-O genome was cloned into plasmid pMal-c that was cut with *Sac* I and blunt-ended with T4 DNA polymerase. The resulting plasmid pMal-P1 was transferred into *E. coli* HB101. Clones containing the insert in the proper orientation were grown in L-broth containing 50 μ g/ml ampicillin at 37°C until O.D.₆₆₀=0.5 was reached. The expression of the fusion protein was then induced for 3h by addition of isopropyl β -D-thiogalacto-pyranoside (IPTG) at a final concentration of 0.3 mM. The bacteria were harvested and lysed by ultrasonic treatment (Branson Sonifier Model 200) in a lysis buffer (10mM sodium phosphate buffer pH 7.0, 30mM NaCl, 0.25% Tween-20, 2mM DTT). Lysates were centrifugated at 15,000 rpm for 10 min and supernatant was obtained. MalE-P1 fusion protein was purified by an amylose resin affinity column according to the instruction of the manufacturer (BioLabs, New England). The eluted samples were dialysed against distilled water overnight at 4°C. Protein concentration was estimated with a Bradford reagent (Bio-Rad, USA).

Preparation of Antiserum against MaLE-P1 fusion Protein in Rabbit

The antiserum to MaLE-P1 fusion protein was prepared in a rabbit (New Zealand White) by an initial immunization with 1mg of antigen emulsified in Freund's incomplete adjuvant (Difco Laboratories USA). Subsequent injection was carried out at 3 weeks after first immunization. The collected serum was purified by a protein A column (AmpureTM PA Kit, Amersham Japan) and preadsorbed with acetone-precipitated healthy tobacco proteins for 2 h at room temperature or overnight at 4°C before use.

Ouchterlony Diffusion Test

Ouchterlony diffusion test was carried out with the sera before- and after-immunization to the MaLE-P1 fusion protein, in 9 cm petri dishes containing 20 ml of 1.2% agarose dissolved in 20 mM sodium phosphate buffer (pH 7.0) and 3% PEG 4000, as described by Oshima (1993).

Western Blotting Analysis

Leaves from healthy or PVY-O infected tobacco plants were harvested at 20 days post-inoculation. Extracts were prepared by grinding in a mortar with 3ml/g tissue of either extraction buffer (ES buffer: 7.5 mM Tris-Cl pH 6.8, 9 M urea, 7.5% 2-ME, 4.5% SDS) (Rodriguez-Cerezo & Shaw, 1991) or a buffer as used by Albrechtsen & Borkhardt (1994) (100 mM Tris-Cl pH 8.0, 2% SDS, 10% glycerol, 5% 2-ME, here referred to as AB buffer). Homogenates were squeezed through a single layer of moistened cheese cloth, boiled and then centrifuged at 5,000g for 5 min. Supernatant was submitted to 10% SDS-PAGE using 15µl (equivalent to 45 mg of fresh tissue) per lane, and followed by electroblotting onto polyvinylidene difluoride membrane (Immobilon-P, millipore, USA) overnight at 50V (Towbin *et al.* 1979). The membranes were blocked for 1 h with a solution of 5% nonfat dry milk in TBS-T (20 mM Tris-HCl, pH7.5, 150 mM NaCl, 0.1%

Tween-20) and then incubated for 2 h with the antiserum diluted 1000-fold in TBS-T solution. After three washes with TBS-T, the membranes were incubated with goat anti rabbit IgG coupled to horseradish peroxidase secondary antibody (Bio Rad, USA) for 1.5 h. The membranes were washed three times with TBS-T and the immunocomplexes were detected using the enzyme chemiluminescence (ECL, Amersham, USA) as described by the manufacturer.

Results

Preparation of P1 Antibody in Rabbit

The production of antibody against MalE-P1 fusion protein was undertaken in order to investigate the presence of P1 protein in PVY-O infected tobacco cells. To obtain the antigen, a cDNA fragment (from nt 297 to 905) of PVY-O corresponding to a 22 kDa fragment of the polyprotein (from amino acid position 42 to 244) was cloned into the bacterial expression vector pMal-c to produce the recombinant plasmid pMal-P1 (Fig. 4-1). The insert was cloned into downstream of the *malE* gene, which encodes a maltose binding protein (Guan *et al.*, 1987).

pMal-P1, as well as pMal-c were used to transform the *E.coli* HB101. After induction in culture by IPTG, bacteria transformed with pMal-P1 produced a soluble protein with a molecular weight of approx. 67kD as determined by SDS-PAGE (Fig. 4-2, lane 3). This is in a good agreement with the predicted molecular weight of 45kDa from MalE plus 22 kDa from the insert. Bacteria transformed with the vector pMal-c produced a 45kDa MalE protein (Fig. 4-2, lane 1).

The fusion protein was purified from bacterial lysates by an amylose resin affinity column. Analysis of the products eluted with elution buffer (10 mM maltose, 20 mM Tris-HCl pH7.4, 200 mM NaCl, 5 mM DTT) by SDS-PAGE revealed several proteins, among which the predominant protein had a molecular

weight of approximately 67kDa (Fig. 4-2 lane 5). The partially purified MaLE-P1 fusion protein was used to immunize a rabbit.

Ouchterlony diffusion test was carried out to detect serological reaction of the antiserum to MaLE-P1 fusion protein (Fig.4-3, A). 8 µg of MaLE-P1 showed a co-precipitation line with the immunized serum, even 2 µg of the protein showed a co-precipitation line with 10-fold diluted serum. These results indicated that the immunized serum reacted strongly in diffusion tests with MaLE-P1 protein. In contrast to this, there was no reaction with the serum obtained before immunization (Fig. 4-3, A).

Detection of P1 Protein in Plants Infected with PVY-O

To investigate the presence of P1 protein in PVY-O infected tobacco plants, the leaf tissue was extracted with AB or ES buffer and subjected to 10% SDS-PAGE. The proteins were electroblotted to a membrane and probed with the fusion protein antiserum (Fig. 4-4). The antiserum reacted specifically to a protein with molecular weight of 32 kDa that was present only in plant tissue infected with PVY-O (Fig. 4-4, lanes 2 and 3), but not in extracts from healthy plant tissues (Fig. 4-4, lane 1). Although extracts of PVY-O infected tissues with both AB and ES buffer showed 32 kDa P1 protein band, the latter gave a much more satisfactory result (Fig.4-4, lane 2).

Discussion

The protein encoded by the N-terminal region of PVY-O genome was detected in infected tobacco plants in this study. This coding region has been presumed to be present in the PVY-O genome but its product had not previously been detected in plants. A cDNA cloning was employed and expressed in bacteria to obtain sufficient amounts of the fusion protein to raise polyclonal antibodies, and these

have been used for detection of the protein in infected plants. The detected PI protein of PVY-O is about 32kDa.

Two buffer systems were used for the protein extraction. It clearly showed that the ES buffer gave much better extraction than buffer AB. Since ES buffer contains a very high concentration of urea (9 M), the extraction condition is much more stringent than AB buffer. The results indicate that the PI protein might be located at or associated with the cell membrane or cell wall fractions.

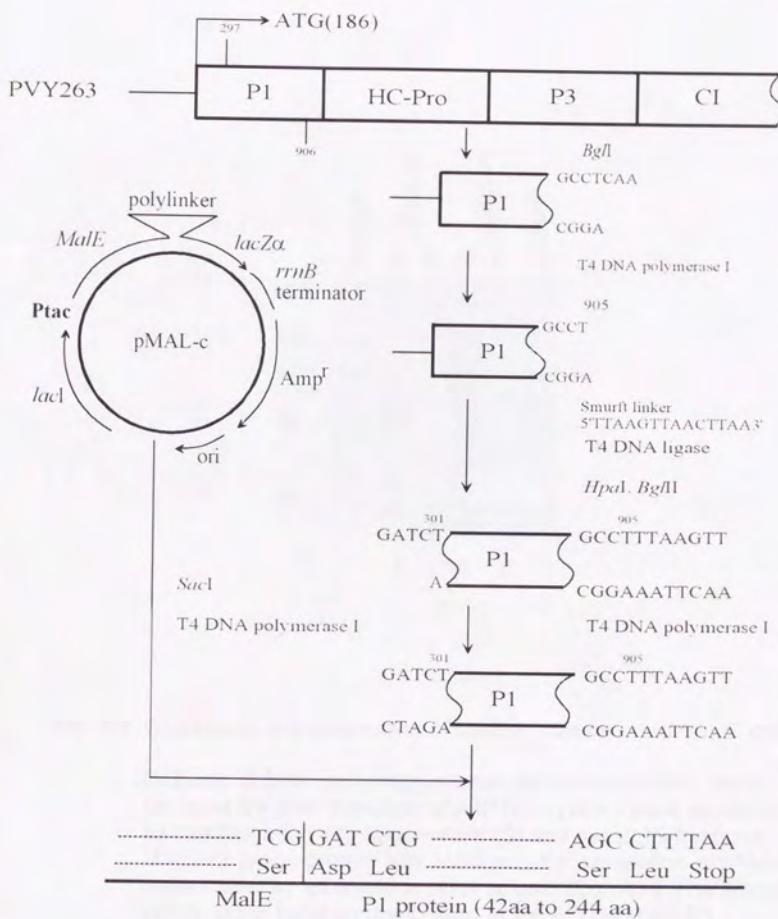


Fig. 4-1 Scheme of construction of recombinant plasmid pMal-P1

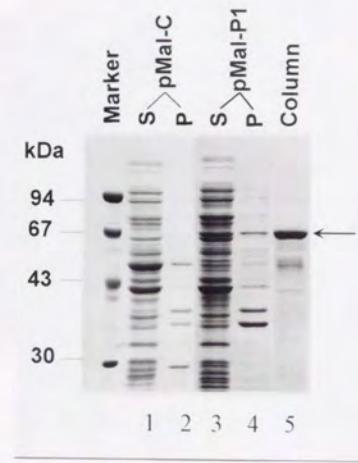


Fig. 4-2 Expression and purification of MalE-P1 fusion protein in *E. coli*

Cultures of cells containing plasmid pMal-c or pMal-P1 were sampled 3 h after induction with IPTG. Lysates were separated by centrifugation into supernatant (S) and pellet (P) fractions. MalE-P1 fusion protein was obtained after separation of pMal-P1 supernatant by an amylose resin column (Column). The arrow points to the band corresponding to the 67kDa MalE-P1 fusion protein.

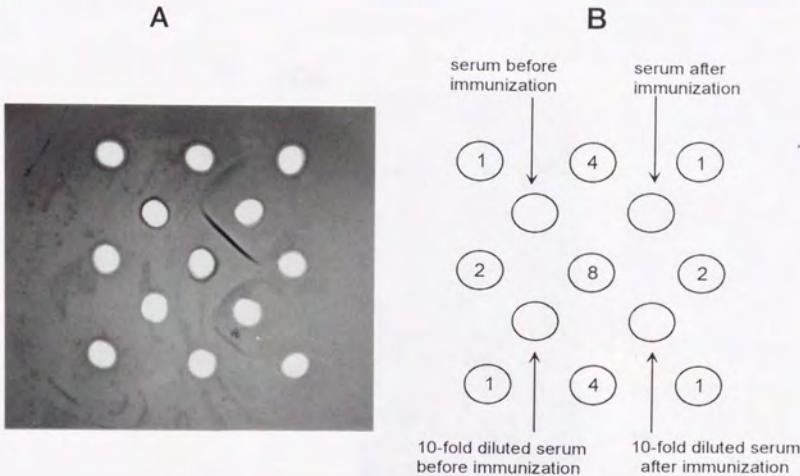


Fig. 4-3 Serological analysis of antiserum to MalE-P1 fusion protein by Ouchterlony diffusion test.

Numbers 1,2,4 and 8 indicate the concentration of MalE-P1 fusion protein at μg , respectively.

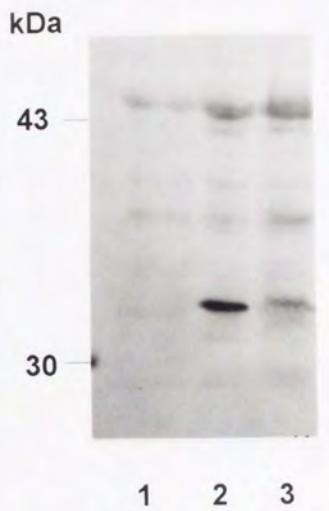


Fig. 4-4 Western blot analysis of PVY-O P1 protein in extracts from healthy and PVY-O infected tobacco leaves, using antiserum against the MaIE-P1 fusion protein

Healthy tobacco leaves (Lane 1), PVY-O infected tobacco leaves extracted by ES buffer (Lane 2) or AB buffer (Lane 3) (see Materials and Methods for details)

The sizes of protein standards, in kDa, are indicated at the left.

4-2. Identification of Cleavage Site of P1 Protein of PVY-O in Tobacco Protoplasts

Materials and Methods

Strains, Plasmids, Plant Materials

Most recombinant and mutagenized plasmids were cloned in *Escherichia coli* MV1184. Single-stranded plasmid DNA was prepared from *E.coli* CJ236 by infection with the defective phage M13K07. The numbering of all nucleotides and amino acid residues, starting from the PVY-O genome 5' end and N terminus of the polyprotein, respectively, was based on the sequence described in chapter 2.

Two PVY-O cDNA fragments with different length were subcloned into the *Bam*H-I-*Sph*I digested pUC119 vector to yield two intermediate plasmids p369 and p291. The plasmid p369 contains a cDNA fragment representing PVY-O nucleotides 1~1292, which encodes a total of 369 amino acids of the P1 protein and a part of adjacent HC-Pro protein, while p291 contains a cDNA fragment having nucleotides 1~1058, encoding a polypeptide with 291 amino acids.

Site-Directed Mutagenesis

Double amino acid replacements of Gln²⁷⁵/Ser²⁷⁶, Phe²⁸⁴/Ser²⁸⁵, and His³²⁷/Ser³²⁸ with Gly/Ala, respectively, in plasmid p369 were carried out by site-directed mutagenesis using uracil-containing, single-stranded DNA as a template. The oligonucleotides (5'TGAGTTCAAAACGCGCCCAGTAAC~~T~~TAGAA3' for Gln²⁷⁵/Ser²⁷⁶ to Gly/Ala, 5'ATTATCAGCATTGGCGCCCTGGATCATGA3' for Phe²⁸⁴/Ser²⁸⁵ to Gly/Ala, 5'GCACGGGAGGATGGCGCCTGCCATCAA TGCA3' for His³²⁷/Ser³²⁸ to Gly/Ala) specifying the underlined base changes were synthesized and used as primers for each mutagenesis. All the three mutants were selected by digestion with *Bba*I which were newly introduced by the mutation.

The resulting plasmids were named pA, pB and pC, respectively. The letters A, B and C stand for the three predicted cleavage sites between PI and HC-Pro polyprotein.

The PVY-O polypeptide encoding sequences for the amino acid residues of 284~291, 285~291, 286~291 and 287~291 were deleted individually from plasmid p291 by site-directed deletional mutagenesis. The resulting plasmids were termed p283, p284, p285 and p286, respectively. The 283, 284, 285 and 286 represent the numbers of amino acid residues of viral protein encoded by corresponding plasmids.

The nucleotide sequence around each mutation site was verified by sequencing as described in chapter 2.

Construction of polypeptide/GUS Fusion Transient Expression Vectors

The plasmid p369-GUS (Fig. 4-5) encoding polypeptide/GUS fusion protein was obtained from plasmid pGUS (Fig. 3-1) and p369.

Three plasmids encoding polypeptide/GUS-substitution variants pA-GUS, pB-GUS and pC-GUS were generated from plasmid pGUS, pA, pB, and pC (Fig. 4-7)

Four additional plasmids encoding polypeptide/GUS-truncate variants p283-GUS, p284-GUS, p285-GUS and p286-GUS were generated from plasmids pGUS, p283, p284, p285 and p286 (Fig. 4-9), respectively.

All the above transient expression vectors were constructed by the following strategies. The cDNA containing plasmids p369, pA, pB, pC, p284, p285 and p286 were cut with *Sph*I and blunt-ended with Klenow fragment, and then digested with *Bam*HI. After recovery from agarose gel, the cDNA fragments were fused in frame with the N-terminus of GUS gene in the *Bam*HI-*Sma*I digested plasmid pGUS (See Fig. 4-5, as an example). The resulting plasmids were electroporated into tobacco protoplasts and used for GUS transient expression

assay. Fluorometric and *in situ* SDS-PAGE analysis of GUS activity was done as described previously.

Western Blotting Analysis of Tobacco Protoplasts

48 h after transformation, the protoplasts were collected by gentle centrifugation and suspended in GUS extraction buffer or ES buffer and lysed completely. The mixture was centrifuged (5,000 rpm, 10 min) and the supernatant was boiled for 5 min, then submitted to 9% and 12% SDS-PAGE for GUS and P1 protein analysis, respectively. The subsequent steps were done as described previously. For GUS detection, 4000-fold diluted anti-GUS serum (Clontech Laboratories Inc. USA) was used.

Results

The Fusion Protein Encoded by Plasmid p369-GUS Is Processed in Tobacco Protoplast

Cleavage between the P1 and HC-Pro polyprotein of PVY was proposed to occur between (A) Gln²⁷⁵/Ser²⁷⁶, (B) Phe²⁸⁴/Ser²⁸⁵ or (C) His³²⁷/Ser³²⁸ by Domier *et al.* (1986), Mavankal & Rhoads (1991) and Hellmann *et al.* (1988) based on the amino acid sequence alignment with other potyviral polyproteins. A transient expression plasmid p369-GUS has 369 amino acid residues (all three predicted cleavage sites are included) from the N-terminus of PVY-O polyprotein fused in frame to N-terminus of GUS. The p369-GUS fusion protein has a molecular weight of approx. 108 kDa. This fusion plasmid was electroporated into tobacco protoplasts to examine whether the translated fusion protein precursor could be cleaved. The result of Western analysis using a GUS antiserum showed a specific band around 76kDa, a little larger than GUS standard (Fig. 4-6, lane 3), while in

control, this band was not present (Fig. 4-6, lane 2). This data indicated that the fusion protein precursor produced from p369-GUS underwent cleavage in tobacco protoplasts. The cleavage site of this precursor could not be known based on the size of resulting product.

Substitution of Phe²⁸⁴/Ser²⁸⁵ Failed to Detect GUS Activity in Tobacco Protoplasts

To determine the authentic cleavage site between PI and HC-Pro polyprotein, three substitution plasmids pA-GUS, pB-GUS and pC-GUS, i.e., replacement of Gln²⁷⁵/Ser²⁷⁶, Phe²⁸⁴/Ser²⁸⁵ and His³²⁷/Ser³²⁸ to Gly/Ala, respectively, were constructed (Fig. 4-7). After electroporation into tobacco protoplasts, the extracts of transformed protoplasts were assayed for GUS activity fluorometrically. As shown in Fig. 4-8 pA-GUS and pC-GUS showed similar GUS activity with that of p369-GUS (having more than 2000 units), while pB-GUS had only very low GUS activity, about the same as in control. These data indicate that after replacement of Phe²⁸⁴/Ser²⁸⁵, the fusion protein precursor is not cleaved. Surely, GUS activity could not be detected due to the state of fusion in which 369 amino acid residues fused to its N-terminus. This result of amino acid substitution suggested that Phe²⁸⁴/Ser²⁸⁵ is a cleavage site between PI and HC-Pro polyprotein.

The Phe²⁸⁴ is the C-terminus of PI protein of PVY-O

Although the Phe²⁸⁴/Ser²⁸⁵ is a possible cleavage site between PI and HC-Pro, it is interesting to address whether those two amino acid residues are absolutely required for proteolysis in tobacco protoplasts. Single amino acid truncates around Phe²⁸⁴/Ser²⁸⁵ were prepared. Four plasmids encoding fusion proteins containing GUS fused to the C-terminus of the proteolytic domain terminating at Gln²⁸³, Phe²⁸⁴, Ser²⁸⁵ or Asn²⁸⁶ were constructed (Fig. 4-9). Fusion proteins containing a functional cleavage site were expected to undergo proteolysis, generating a 32kDa

P1 protein and a 68 kDa GUS protein. Whereas fusion proteins with disrupted cleavage sites were predicted to accumulate a non-processed 102 kDa precursor. Both GUS active-gel *in situ* staining and GUS western analysis showed that p283-GUS yielded no GUS activity (Fig. 4-10 A, lane 4) or a GUS band (Fig. 4-10 B, lane 4). Processed GUS product and its activity were observed from p284-GUS, p285-GUS and p286-GUS (Fig. 4-10, lanes 5, 6 and 7). These results indicate that amino acid residues beyond Phe²⁸⁴ are dispensable and that Phe²⁸⁴ is necessary for proteolysis. Phe²⁸⁴ is most probably the C-terminus of P1 protein.

Discussion

Although the antiserum specific reaction to P1 has led to the detection of this viral protein from infected plants, extremely small amount of P1 was present in infected plants (Rodriguez-Cerezo & Shaw, 1991; Albrechtsen & Borkhardt, 1994; Wisler *et al.*, 1995). Besides, unlike the other viral proteins (for example CP and CI) which accumulate in infected plants with amounts to be isolated for amino acid sequence analysis and other characterizations, the P1 protein is normally only detected in very young plant materials (Albrechtsen & Borkhardt, 1994). Therefore, it could be very difficult to determine the P1 cleavage site or its C-terminal in infected plants.

Mavankal & Rhoads (1991) and Verchot *et al.* (1992) have determined the P1 cleavage sites of TVMV and TEV potyviruses by the techniques of site-directed mutagenesis and *in vitro* translation systems. Their results showed that the cleavage requires a cofactor which was only born in plants.

The present study was carried out to elucidate the cleavage site between P1 and HC-Pro polyprotein of PVY-O in tobacco protoplasts. The results showed that processing occurred in protoplast system at Phe²⁸⁴/Ser²⁸⁵ of PVY-O polyprotein

and Phe²⁸⁴ is indispensable for proteolysis (Fig. 4-10). The results also showed that although Ser²⁸⁵ was not absolutely required for proteolysis, it was needed for optimal cleavage activity since replacement of Ser²⁸⁵ with Gly (p284-GUS) reduced GUS activity considerably (Fig. 4-10, lane 5).

Failure to detect the unprocessed fusion protein (p283-GUS) which could theoretically react to GUS antibody may be due to the degradation of this protein in protoplasts. This suggestion is supported by the observation that in p284-GUS, the GUS activity is always much less than those of p285-GUS and p286-GUS (Fig. 4-10, lane 5, 6 and 7). The translation efficiencies should be quite similar with the same batch of protoplast preparation, the same length and construction of 5'UTR (for details see chapter 3) prior to fusion protein encoding region, identical plasmid amounts, and the same electroporation and culture conditions. However, the unprocessed p284-GUS precursor has never been detected by anti-GUS antibody (Fig. 4-10, lane 5). The result supports the assumption that a fusion protein with less efficiency of processing is very liable to degradation. Similarly, the results of NIa cistron (containing VPg and protease domains) *in vitro* translation and processing have shown that only the VPg could be detected (Laliberte *et al.*, 1992), suggesting the protease had undergone degradation.

To address the possibility that unprocessed fusion protein precursor may be associated with cell membrane fraction based on observation that P1 protein seems to locate at cell membrane fraction (see previous section), the transformed protoplasts were extracted with ES buffer. Nevertheless, the precursors could not be detected by both anti P1 and anti GUS antibodies (data not shown).

Another possible explanation for no detection of fusion protein precursor is that GUS antibody may be not specifically good for the detection of fusion protein precursor, since the state of fusion could cause modification of epitopes of GUS which are important for GUS IgG recognition and reaction.

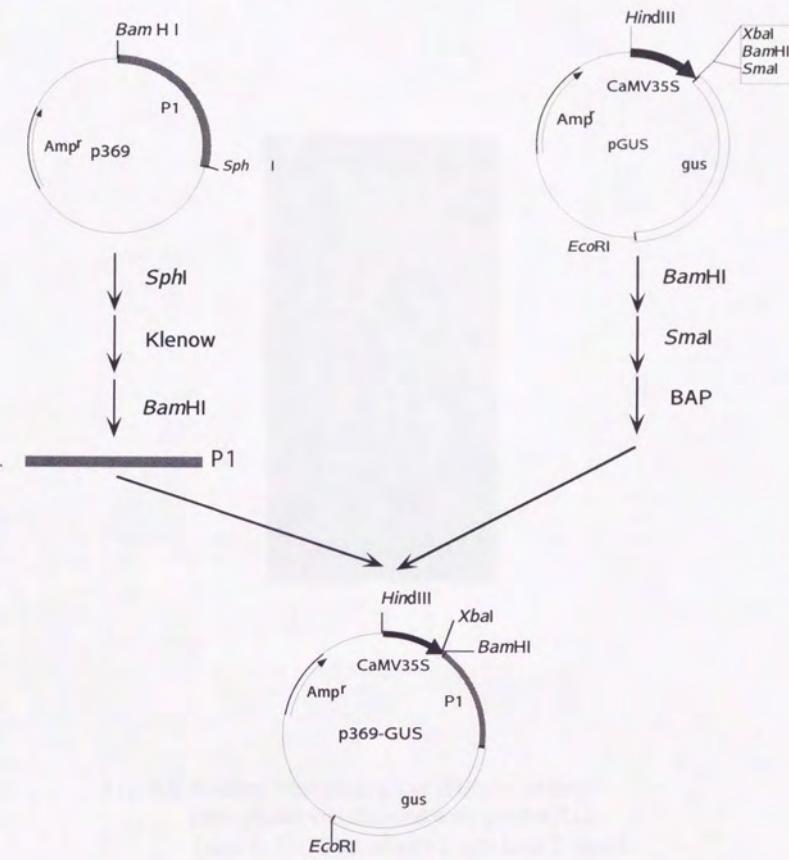


Fig.4-5 Construction of the p369-GUS plasmid

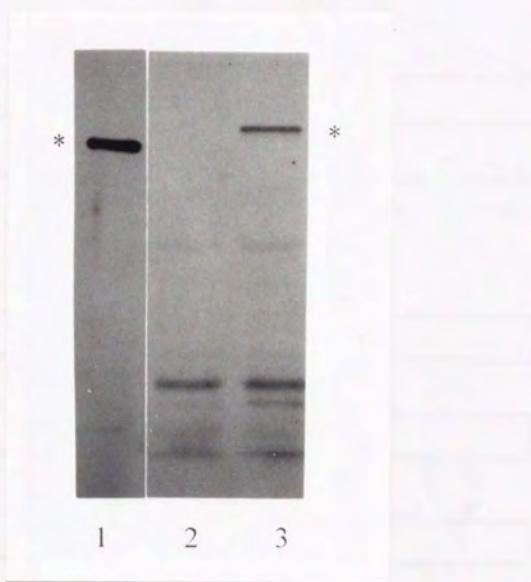


Fig. 4-6 Western blot analysis of GUS in tobacco protoplasts transformed with p369-GUS.
Lane 1, GUS standard (1 μ g); lane 2, mock
lane 3, p369-GUS. The asterisks represent standard GUS and processed GUS.

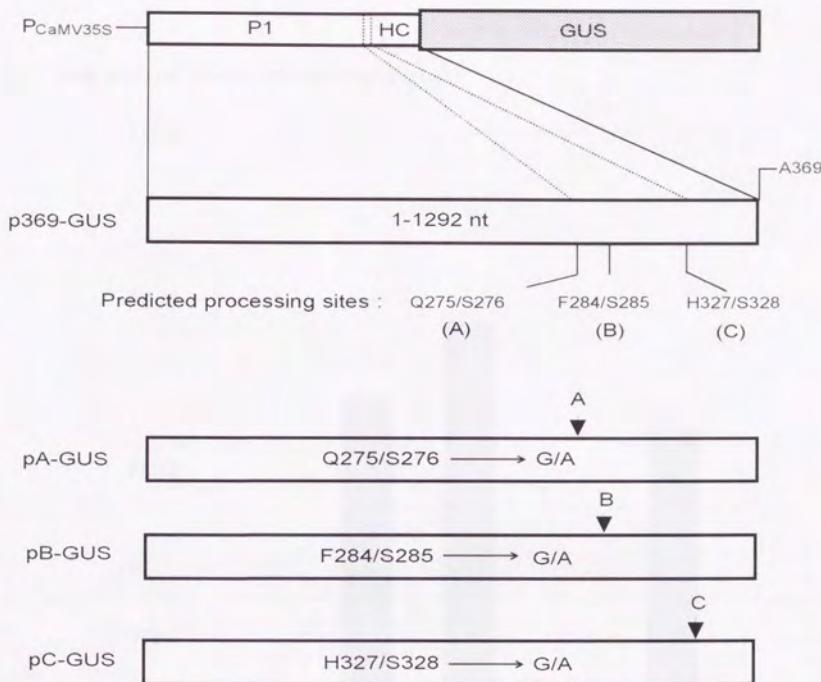


Fig. 4-7 Diagrammatic representation of relevant portion of p369-GUS recombinant plasmid and three mutagenized derivatives pA-GUS, pB-GUS and pC-GUS.
 The solid arrows above the diagram indicate the positions of mutation resulting in amino acid substitution.

GUS activity (4-MU pmol/min/mg protein)

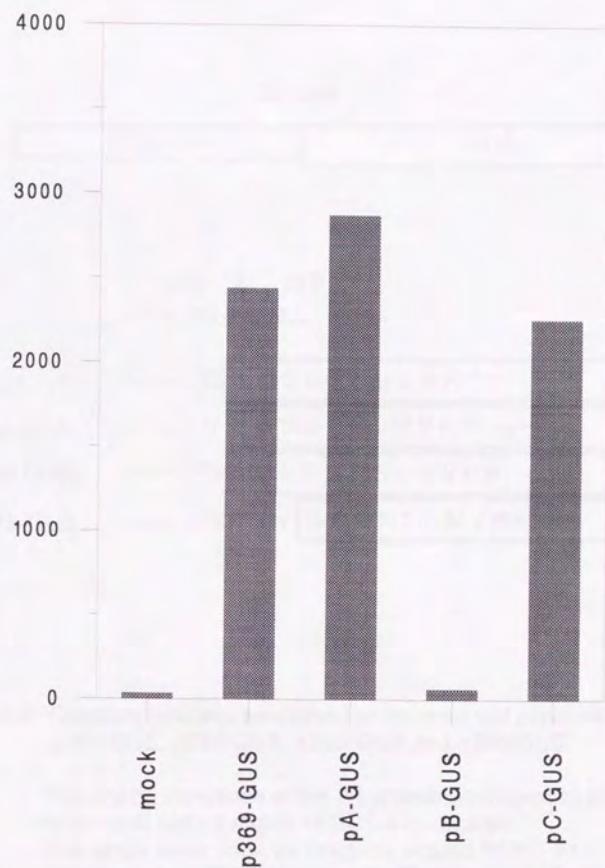


Fig. 4-8 GUS activity analysis from tobacco protoplasts transformed with p369-GUS, pA-GUS, pB-GUS and pC-GUS

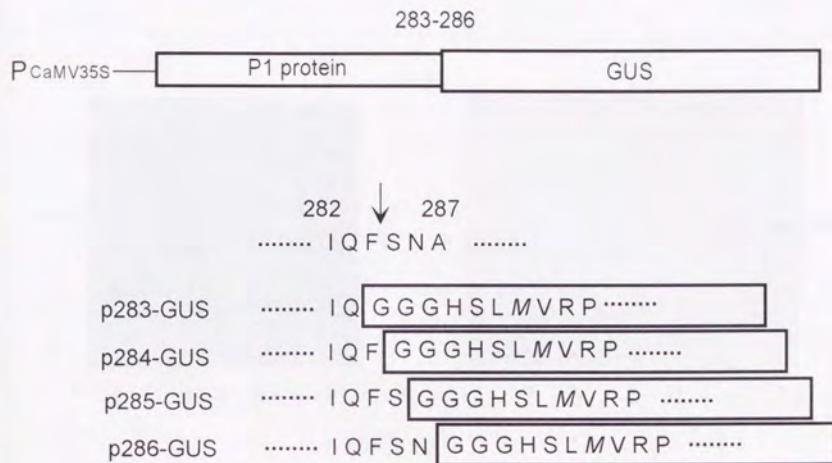


Fig.4-9 Diagrammatic representation of recombinant plasmids p283-GUS, p284-GUS, p285-GUS and p286-GUS

The coding sequence of the P1 protein was fused to the N-terminal coding region of GUS as indicated.

The single-letter code for residues around P1/HC-Pro cleavage site (IQFSNA) is shown. The cleavage site is indicated by an arrow. The GUS sequence was fused adjacent to codons for Gln283, Phe284, Ser285 and Asn286 in four constructs listed. Italic *M* represents GUS original initiation codon.

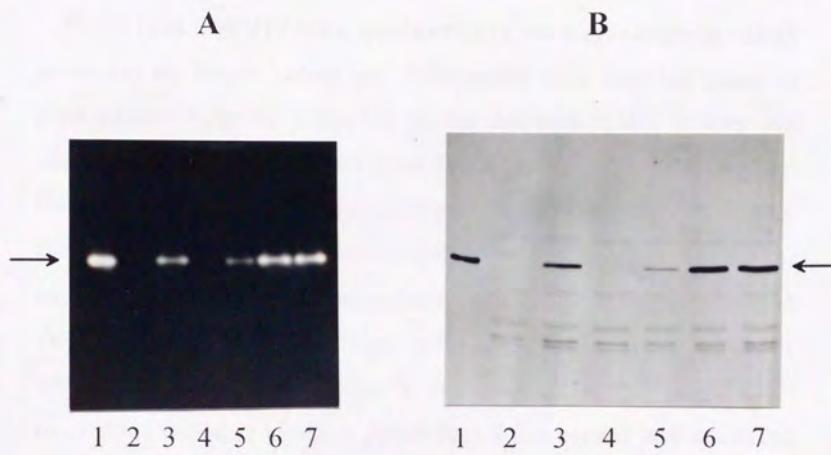


Fig. 4-10 Translation and processing of fusion proteins encoded by pENGUS, p283-GUS, p284-GUS, p285-GUS and p286-GUS in tobacco protoplasts

A: *In situ* analysis of GUS by 7.5% SDS-PAGE;
 B: Western blot analysis of GUS by anti GUS antiserum
 Lanes 1, GUS standard 1 µg; 2, mock; 3, pENGUS;
 4, p283-GUS; 5, p284-GUS; 6, p285-GUS; 7, p286-GUS
 Arrows indicate the GUS products

Chapter 5

Summary

Potato virus Y (PVY) is the type species of the *Potyvirus* genus, which constitutes the largest known and economically most important genus of plant viruses. Intensive researches on the potyvirus cDNA cloning and sequencing have led to a greater understanding of its genome structure and life cycle. The monopartite ~10 kb RNA genome contains a genetically encoded polyadenylate tail at the 3'-end, a single open reading frame for translation of a large polyprotein, and a protein termed VPg covalently linked to the 5'-terminus. The large polyprotein subsequently undergoes autoproteolysis to yield at least 9 functional viral proteins. The 5'-untranslated region (5'UTR) of potyviruses shares several well conserved motifs or structures. The special genome organization of potyvirus has resulted in its specific way on gene regulation, which may be different from those of other organisms. With the increasing knowledge on genome organization and function of potyviruses, it becomes possible to improve economically important plants by transformation with viral genes for increased viral resistance. Therefore, to study potyvirus genetically has both theoretical and practical significance.

The present study was carried out to understand the genome structure and function of PVY ordinary strain (PVY-O). A cDNA clone corresponding to 5'-half of the genome was prepared and its nucleotide sequence was determined. The function of the 5'UTR was analysed in tobacco protoplast system, and finally the P1 protein was detected in infected plants by Western

blot analysis and its C- terminus on polyprotein was determined.

Genetic Analysis of 5'-half Genome of PVY-O

PVY-O was purified from infected tobacco leaves and viral RNA was isolated. A 5 kb cDNA corresponding to the 5'-half of the genome was cloned from viral RNA with a 17 mer synthetic oligonucleotide primer. Nucleotide sequence determination of this clone, together with the 3'-half nucleotide sequence reported by our group showed that the PVY-O genome has a total of 9699 bases. A 5'UTR of 185 bases precedes the unique long ORF which encode a polyprotein of 3061 amino acids with a calculated M_r of 350 kDa, followed by a 3'UTR of 331 bases. Proceeding from the N- to C-terminus of the polyprotein, at least 9 functional proteins can be predicted: P1, HC-Pro, P3₁, CI, 5K, VPg, NIa-Pro, NIb and CP. Comparison of the complete amino acid sequence of PVY-O polyprotein to those of PVY-N and PVY-H strains showed 96.4% and 92.4% similarities, respectively.

Characterization of 5'UTR in Tobacco Protoplasts

It has been documented widely that the plant viral 5'UTRs are acting as translational enhancers. Like other potyviruses, PVY-O 5'UTR is rich in A and T residues and has remarkably few G residues. Some well conserved motifs and structures are also present in this region. To investigate whether the PVY-O 5'UTR functions as a translational enhancer, as well as whether specific nucleotide sequences in the 5'UTR are needed, various mutational derivatives of the PVY-O 5'UTR were fused to a GUS reporter gene and their function on translation of downstream GUS in tobacco protoplasts were

examined.

Two transient expression plasmids pGUS and pENGUS were constructed. The plasmid pGUS was generated by placing CaMV35S-gus-Nos terminator cassette of pBI121 into the *Eco*RI/*Hmd*III sites of pUC119. The plasmid pENGUS was essentially the same as pGUS but harboring 5'UTR prior to GUS encoding region. Analysis of protoplasts transformed with the plasmids revealed that pENGUS stimulated translation of GUS considerably. Accurate fluorometric quantitation of GUS showed that fusion of the 5'UTR to GUS gene (pENGUS) increased GUS activity 15 times compared to the pGUS which contained no 5'UTR.

Based on computer-assisted secondary structure prediction of 5'UTR, two stem-loop structures were observed within 1~130 and 131~185 nucleotide regions. Accordingly, 1~130 or 131~185 nucleotide residues of 5'UTR were amplified by PCR and cloned into *Xba*I/*Bam*HI-digested pGUS. Upon the GUS activity analysis of transformed protoplasts, plasmid pENGUS-9 containing the 1~130 residues provided only 58% GUS activity of pENGUS which contains an intact 5'UTR. On the other hand, plasmid pENGUS-8 having 131~185 residues exhibited higher GUS activity. Namely, pENGUS-8 doubled GUS acitivity compared to that of pENGUS-9. Deletions were made of seven conserved motifs and structures within 5'UTR (1~16, 19~25, 34~46, 68~75, 85~89, 136~158 and 176~188) respectively, to test for altered translational efficiency on downstream GUS. All the deletions within 1~130 bp region showed higher GUS activity. In contrast to this, the deletions in 131~185 bp region declined GUS activity. The results indicate that the deletions which cause the disruption of secondary structure of 5'UTR alone may not account for translational enhancement, and 3'-terminal

55 nucleotide residues of PVY-O 5'UTR likely play a much important role in translational enhancement.

Characteriztion of P1 Protein of PVY-O

The multifunctional P1 protein is derived from the N-terminal region of the potyviral polyprotein. To study P1 protein of PVY-O, a cDNA covering 70% of P1 encoding region was fused to the downstream of *malE* of plasmid pMal-c. The MalE-P1 fusion protein was produced in *E. coli* and an antiserum against this fusion protein was raised in a rabbit. The collected antiserum specifically reacted to a protein with molecular weight of approx. 32 kDa in infected tobacco leaves, but only after extracted with a buffer containing 9 M urea. P1 protein, therefore, is probably located at or associated with cell membrane or cell wall fractions.

Based on the amino acid sequence alignment with other potyviruses, and reports of *in vitro* polyprotein cleavage analysis with TEV and TVMV, three possible cleavage sites Gln²⁷⁵/Ser²⁷⁶, Phe²⁸⁴/Ser²⁸⁵, and His³²⁷/Ser³²⁸ for liberating P1 protein from polyprotein could be predicted for PVY-O. Therefore, a cDNA fragment encoding 369 amino acids of the polypeptide which contains all three predicted cleavage sites was fused to the N-terminus of the GUS gene, and the fusion plasmid p369-GUS was constructed for producing a 108 kDa fusion protein precursor. Analysis of extracts from the p369-GUS transformed protoplasts with anti GUS antibody revealed a band (~76 kDa) specifically reacting to GUS antibody, which is a little greater than the GUS standard. The result indicates that the fusion protein precursor was cleaved in tobacco protoplasts. Replacement of Gln²⁷⁵/Ser²⁷⁶, Phe²⁸⁴/Ser²⁸⁵, and His³²⁷/Ser³²⁸ with Gly/Ala, respectively, in p369-GUS

showed that when Phe²⁸⁴/Ser²⁸⁵ was substituted, no GUS activity could be detected in transformed tobacco protoplasts. Furthermore, an additional four plasmids were constructed by single amino acid truncating around Phe²⁸⁴/Ser²⁸⁵. The results of GUS activity assays by both *in situ* SDS-PAGE and Western blotting showed that Phe²⁸⁴ is indispensable for the cleavage activity and Phe²⁸⁴ is most probably the C-terminus of P1 protein.

Chapter 6

論文の内容の要旨

応用生命工学専攻

平成5年度博士課程進学

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論文題目

ジャガイモYウイルスゲノムの5'末端領域の機能に関する研究

Studies on the functions of the 5' terminal region
of potato virus Y genome

*Potyvirus*はジャガイモYウイルス(potato virus Y; PVY)をタイプ種とする植物ウイルスの一属で、全植物ウイルスの約30%を含み、また多数の農作物に被害を及ぼしている。このウイルスのゲノムはmRNA活性を持つ約10 kbの一本鎖RNAであり、5'末端には自身がコードする蛋白質(VPg)が結合し、3'末端にはpoly(A)鎖が存在する。ゲノムRNAからは約350 kDの巨大な单一のポリプロテイン(PP)しか翻訳されず、翻訳後PP自身が持つ三種類のプロテアーゼ活性により少なくとも9個の機能性蛋白質へプロセシングされるという遺伝子発現の特徴を持つ。そして、これらの機能性蛋白質によりウイルスの複製がなされ、ウイルスは増殖拡散していく。一方、上述のようにそのゲノムの5'末端には真核生物のmRNAの翻訳開始に必要とされるcap構造が無く、代わりにVPgが結合しているため、その翻訳開始機構は一般的なscanning modelとは異なっていると考えられる。本研究では、potyvirusの増殖過程の機構を解明することを目的に、PVY普通系統(PVY-O)を研究材料として、まず、ゲノムRNAの5'側約5 kbをクローン化し、塩基配列を決定することによりPVY-Oのゲノムの構造を明らかにした。さらに、ゲノムの5'末端非翻訳領域(5'UTR)及びPPのN末端に位置するPI蛋白質の機能の解析を行った。

1. PVYゲノムRNAのcDNAのクローン化と構造解析

PVY-O を感染させたタバコの葉からウイルスを分離し、ゲノム RNA を調製した。まず、その中央部（ゲノムの 5017-5033 ネクレオチド部位）に相補的な 17mer の合成 DNA をプライマーとして 5' 側 5014 bp の cDNA をクローニングし、その塩基配列を決定した。また、逆転写酵素を用いてウイルス RNA を直接 sequence することにより、ゲノム RNA の 5' 末端部 18 塩基の配列を決定した。今回決定した 5' 側の塩基配列と本研究室で既に決定されていた 3' 側の約 5 kb の塩基配列を合わせることにより、PVY-O ゲノムの全塩基配列が明らかになった。ゲノムは 9699 塩基より成り、3061 アミノ酸からなるポリプロテインをコードする ORF が唯一確認された。Potyvirus に属する他のウイルスで既に報告のあるポリプロテインのアミノ酸配列との相同性及び切断部位のコンセンサス配列から、PVY-O ポリプロテインは N 末端より P1, HC, P3, C1, 5K, VPg, NIa-Pro, NIb, CP の少なくとも 9 個の機能性蛋白質が結合したものであると推定できた。また、PVY-O のポリプロテインの推定アミノ酸配列は、PVY の necrotic 株及びハンガリー株のものに対しそれぞれ 96.4% と 92.4% の相同性を示した。一方、ゲノムの 5' UTR は 185 bp であり、この中には他の potyvirus でよく保存されている 7 種の配列があった。また、この領域は AT-rich であり、G が非常に少ないという特徴があった。3' 末端には 331 bp の非翻訳領域が存在していた。

2. PVY-O ゲノムの 5' UTR の翻訳段階における役割の解析

(1) 5' UTR の GUS 遺伝子の翻訳に対する作用

幾つかの potyvirus で 5' UTR が蛋白質合成のエンハンサーとして機能することを示唆する報告がある。PVY-O ゲノムの 185 bp の 5' UTR の蛋白質合成に対する作用を解析するために、植物細胞中での転写活性の高い CaMV35S プロモーターの下流に β -glucuronidase (GUS) 遺伝子をつないだプラスマド pGUS、並びにプロモーターと GUS 遺伝子の間にウイルスの翻訳開始コドンを含む 5' UTR を挿入したプラスマド pENGUS を構築し、タバコプロトプラストにPEG 法によって導入した。24 時間培養したプロトプラストの示す GUS 活性を蛍光法により測定した結果、どちらのプラスマドを導入した場合も GUS 活性が見られたが、GUS の上流に 5' UTR を挿入した pENGUS では 5' UTR を持たない pGUS に比べ 16 倍以上の GUS 活性を示した。この結果から PVY-O の 5' UTR は蛋白質合成に対するエンハンサーとして機能することが認められた。

② 5'UTR の機能部位の解析

5'UTR がどの様なメカニズムで蛋白質合成のエンハンサーとして働いているのかを調べるために以下の実験を行なった。コンピューターによる 5'UTR の塩基配列の解析により、5'UTR には 1~130 ヌクレオチド部位及び 131~185 ヌクレオチド部位の領域で 2 つのステムループ構造を形成していることが予想された。そこで、それぞれの領域を GUS 遺伝子の上流に連結した 2 種類のプラスミドを構築した。それらをエレクトロポレーション法によってタバコプロトプラストに導入したところ、1~130 の領域を持つプラスミドでは 5'UTR の全長を含むプラスミドに比べ GUS 活性が約 50 % に低下した。それに対し、131~185 の領域を持つプラスミドでは全長を含むプラスミドと同等以上の GUS 活性を示した。PVY-O の 5'UTR 中にも potyvirus でよく保存されている 7 個の配列が 1~16, 19~25, 34~46, 68~75, 85~89, 136~158 及び 176~188(ATG を含む)の位置に存在する。そこで、5'UTR 全長からそれを欠失した 7 種類の断片を構築し、これらを GUS 遺伝子の上流に連結したプラスミドを構築しタバコプロトプラストに導入した。それらが示す GUS 活性を解析した結果、1~130 の領域に存在する 5 ケ所を欠失させた場合には、5'UTR 全長を含むプラスミドの場合よりもむしろ高い GUS 活性を示した。一方、130~185 の領域の 2 ケ所に欠失を導入すると、GUS 活性の減少がみられた。以上の結果から、PVY-O の 5'UTR の 131~185 ヌクレオチド部位中にあるエレメントの方がより強く蛋白質合成の増強に関っていると考えられる。

3、PVY-O の P1 蛋白質の検出とその C 末端の切断部位の決定

P1 蛋白質はポリプロテインの N 末端部に存在し、他のウイルス蛋白質と比べて potyvirus 間でのアミノ酸配列の相同意が最も低い蛋白質である。今までに P1 が一本鎖 RNA 分子或いは一本鎖 DNA へ特異的に結合すること、そして植物体の何らかの因子の存在下で自身の C 末端を切断することなどがわかっている。tobacco vein mottling virus (TVMV) と tobacco etch virus (TEV) の P1 蛋白質については、*in vitro* の実験でその切断部位がわかっているが、それ以外の potyvirus の P1 蛋白質の切断部位についての報告は無く、また P1 蛋白質を感染植物体内で検出したという報告も少ない。そこで、PVY-O の P1 蛋白質を感染植物体中で検出すると共に、タバコプロトプラストを用いた *in vivo* の解析によりその C 末端の切断部位の決定を行った。

(1) P1 蛋白質の抗血清の作製と P1 蛋白質の検出

プラスミド pMal-c を用いて、*maIE* 遺伝子の下流に PVY-O の P1 蛋白質の ORF の中央部約 70% の領域をインフレームに連結した。このプラスミドを大腸菌に導入し、產生されるマルトース結合蛋白質と P1 蛋白質との融合蛋白質を精製し、これに対する抗血清をウサギで調製した。これを用いて PVY-O を感染させたタバコの葉の抽出液に対しウエスタンプロッティングを行った結果、感染葉を 9 M urea を含む buffer で抽出した場合のみ約 32 kDa の P1 蛋白質が検出された。したがって、P1 蛋白質は植物細胞の膜成分に結合しているのではないかと予想される。

(2) P1 蛋白質の C 末端切断部位の決定

TVMV と TEV における P1 蛋白質の切断部位をもとに、Gln²⁷⁵/Ser²⁷⁶, Phe²⁸⁴/Ser²⁸⁵, His²⁷⁷/Ser²⁷⁸ の 3ヶ所を PVY の P1 蛋白質切断部位の候補として推定した。そこで、PVY-O の PP の N 末端部 369 アミノまでの領域の下流に GUS を連結した融合蛋白質を生産する発現プラスミドを構築し、これよりタバコプロトプラスト中で產生される蛋白質の形態を抗 GUS 抗体を用いたウエスタンプロッティングにより解析した。検出された蛋白質の分子量が本来の GUS のものよりわずかにしか大きくなかったことから、產生された融合蛋白質は何らかの切断をうけていると結論できた。さらに、この 3ヶ所のそれぞれに部位特異的変異を導入した 3種類の変異体を構築し同様に解析したところ、Gln²⁷⁵/Ser²⁷⁶ 及び His²⁷⁷/Ser²⁷⁸ を Gly/Ala に置換した場合には融合蛋白質の切断が GUS 活性の発現により確認されたが、Phe²⁸⁴/Ser²⁸⁵ を Gly/Ala に置換した場合には切断により生じる GUS 活性は全く検出されなかった。さらに、Phe²⁸⁴/Ser²⁸⁵ 部位周辺の 283 番から 286 番までのアミノ酸を 1つずつ欠失した融合蛋白質をタバコのプロトプラストで生産したところ、Phe²⁸⁴ を欠失した場合にのみ融合蛋白質の切断により生じる GUS 活性は検出できなくなった。以上のことより、P1 蛋白質の C 末端はポリプロテインの 284 番のフェニルアラニンであると決定した。

以上、本研究において、PVY-O の全ゲノム構造を明らかにし、P1 蛋白質の C 末端の切断部位を初めて決定した。さらに、PVY-O の 5'UTR が蛋白質合成におけるエンハンサーとしての役割を持つことを明らかにした。これにより新しい蛋白質合成の調節機構の存在を示すことができた。

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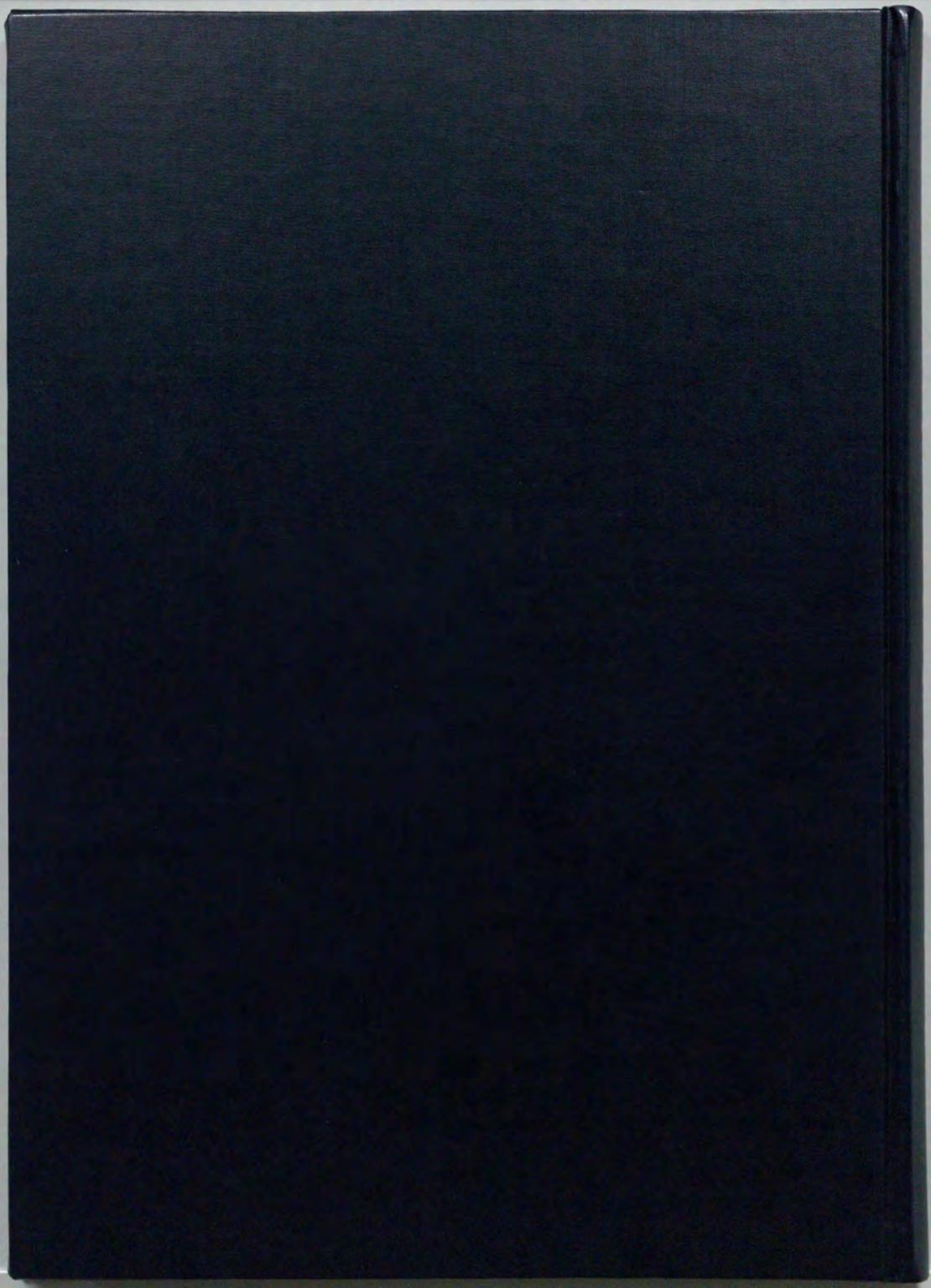
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inches
cm

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

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