

Organization and expression of single-stranded positive-sense RNA plant viruses

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Abstract

A majority of plant viruses have positive-sense RNA as their genome. These viruses are classified as mono-, bi- or tripartite based on the presence of 1, 2, or 3 RNA segments as their genomes. The constraints imposed by eukaryotic protein synthesizing machinery for translation of only the first 5' ORF as well as the need for optimal utilization of the limited genetic material necessitate these viruses to evolve different strategies to express their genes. These strategies include termination at a non-conventional stop signal, read through of leaky termination codon, polyprotein processing, frame shifting, generation of subgenomic mRNAs and internal initiation. Despite wide variation in particle morphology, host plant, vector transmission, etc., an analysis of the genomic sequence and strategies of expression in these viruses has led to their classification into a limited number of supergroups. Many interviral relationships not envisaged earlier have emerged and this has helped in understanding the molecular mechanisms of viral replication, translocation, etc. Determination of genomic sequences of a large number of viruses will help in further strengthening the established evolutionary relationships among various groups of plant viruses.

Key words: Eukaryotic RNA viruses, genomes, positive-sense RNA plant viruses.

1. Introduction

Eukaryotic RNA viruses show a wide variation in the structure and organization of their genomes. In the plant kingdom, viruses whose genomes are made up of RNA are far more abundant than the DNA viruses¹. This is in contrast to the situation in the animal kingdom, where both RNA and DNA viruses are equally abundant. The reasons, if any, for such an asymmetry in the distribution of RNA and DNA viruses in the two kingdoms is not clear. The plant viruses are classified into different taxonomic groups, based on their particle morphology, structure and genomic organization (Fig. 1). A vast majority (>75%) of plant RNA viruses possess single-stranded RNA (ssRNA) of positive polarity as their genome^{1,2}. Their genomes, in turn, are formed of either a single (monopartite) or two (bipartite) or three molecules (tripartite) of RNA. In the case of bi- and tripartite viruses, the RNA is encapsidated in separate particles. Further, the genomic RNA contains various terminal structures such as genome-linked proteins (VPg) or cap structures at their 5' terminus and a poly (A) tail or a tRNA-like structure at the 3' end.

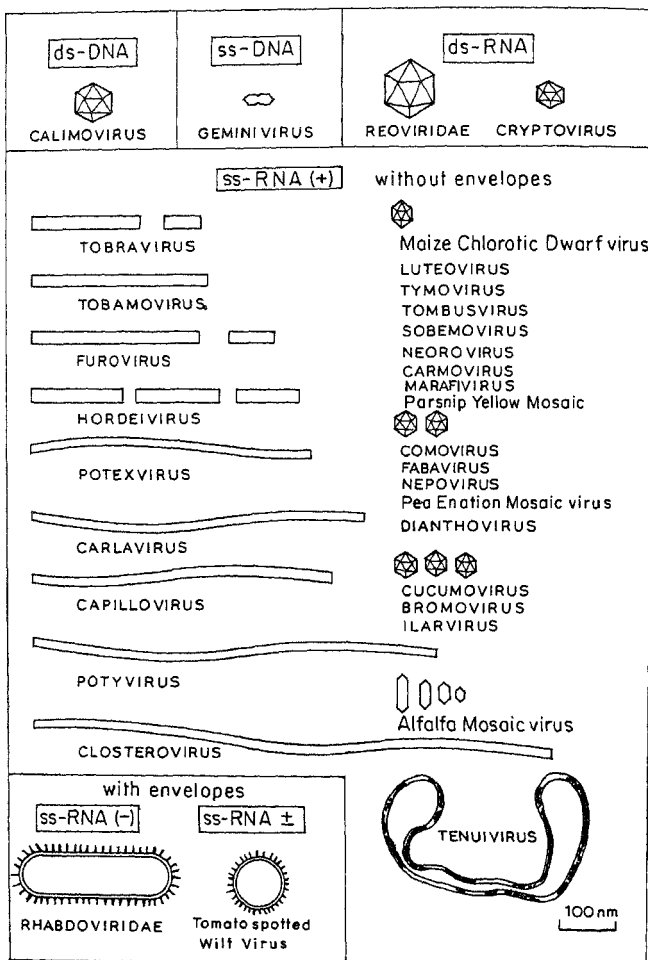


FIG. 1. Particle morphology and nature of genomes in plant viruses²⁴ (reproduced with permission).

Despite a wide variability in particle morphology, structure and organization of their genomes, the nucleotide sequence of several of the positive-sense RNA viruses revealed many interviral relationships and offered the opportunity for a more systematic study of these viruses on the basis of their phylogenetic relationships.

This article presents a brief review of the organization and expression of ss, positive-sense RNA plant viruses. Elucidation of the genomic organization and expression of *Physalis mottle virus* (PhMV) carried out in our laboratory is also presented in some detail.

2. Strategies of expression of the genomic RNA

The number of RNA molecules found in plant viruses range from 1 for the satellite tobacco necrosis virus to 12 for some reoviruses. Many of the positive-sense RNA viral genomes code for about 4 to 7 proteins. The limited size of the genome and its large information content necessitates the development of a variety of strategies for the expression of the genomic RNA. The eukaryotic ribosomes are adapted to translating only the ORF immediately downstream from the 5' end of an mRNA. Any other downstream ORF normally remains untranslated. The RNA viruses have evolved a variety of mechanisms to ensure that all their genes are accessible to the eukaryotic protein-synthesizing system. These alternative strategies are discussed by Joshi and Haenni³ and by Morch and Haenni². Some of the important strategies are listed below with examples.

2.1. Termination at a non-conventional stop signal

The termination of translation at a non-conventional stop signal results in the synthesis of two proteins with the same N-terminus whose sequences were identical over the length of the shorter protein (Fig. 2). The mechanism(s) involved in such premature chain terminations are not known. When alfalfa mosaic virus total RNA was translated in a reticulocyte lysate system, in addition to the full-length translation product, two shorter polypeptides were also synthesized. This premature termination was ascribed to the lack of charged gln-tRNA which could be overcome by an exogenous addition of glutamine⁴. It was not clear whether premature termination is actually a strategy for expression of the genome or an artifact of the *in vitro* translation system.

2.2. Read-through of a leaky termination codon

Here again, two proteins which are co-N-terminal are produced from the same RNA by the suppression of a stop codon (usually UAG) by suppressor tRNAs (Fig. 2). This mechanism is exhibited by several viral RNAs, for example, in carnation mottle virus (CarMV) *in vitro* translation of the RNA in reticulocyte lysate resulted in the synthesis of four virus-specific polypeptides of apparent molecular weight (M_r) 100, 77, 38 and 30 kDa. A comparison of partial peptide maps and translation in the presence of calf liver amber suppressor tRNA, demonstrated that P30, P77 and P100 were a series of overlapping polypeptides generated by a double read-through mechanism⁵.

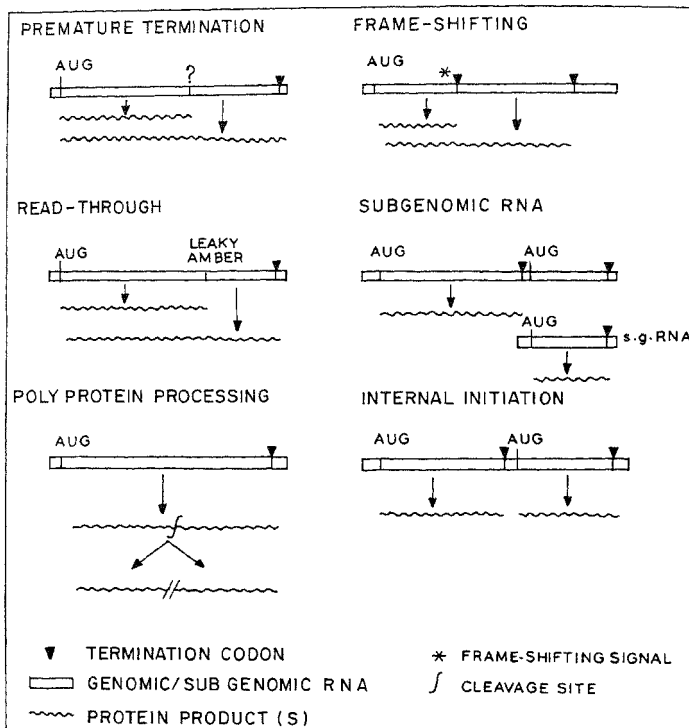


FIG. 2. The different strategies of expression of positive-sense RNA plant viral genomes (reproduced with permission).

2.3. Polyprotein processing

In this mechanism, the genomic RNA is translated into a long polypeptide which is proteolytically processed to yield the mature functional proteins (Fig. 2). Several families of positive-(+ve) sense RNA viruses are known to utilize this strategy which involves, either a virally coded protease or a proteolytic domain in the polyprotein or a specific host protease, for example, in potyviruses the genomic RNA was translated to give a polyprotein which was post-translationally cleaved into as many as seven functional proteins⁶.

2.4. *Frame shifting*

This mechanism results in two proteins coded in different reading frames, the 5' region of the distal ORF overlapping with the 3' sequence of the proximal ORF (Fig. 2). The synthesis of multiple products is achieved *via* ribosomal frame shifting during the process of translation⁷. In order to account for a 99 kDa product observed in *in vitro* translation studies of Barley yellow dwarf virus, it was suggested that the ORF1 which was in -1 reading frame with respect to ORF2 was translated in such a way that a ribosomal frame shifting could allow the termination codon of ORF1 to be read through leading to the transframe product of size 99 kDa⁷.

2.5. *Subgenomic RNAs*

Many viruses contain, besides the genomic RNA, smaller RNA species corresponding to their internal or 3' genes (Fig. 2). These smaller RNAs are usually encapsidated along with the genomic RNA in viral nucleocapsids. They serve as mRNAs for the synthesis of viral proteins. These subgenomic RNAs (sgRNAs) are generated from the genomic RNA by internal initiation on the negative strand during replication⁸. The use of subgenomic RNAs is one of the mechanisms by which the synthesis of viral proteins could be regulated during the replication cycle. Subgenomic RNAs are involved in the expression of internal genes of several groups of +ve-sense RNA viruses such as the tymo, tobamo, bromo and cucumo viruses.

2.6. *Internal initiation*

A direct entry of the ribosome to an internal AUG was suggested as a possibility in the case of several positive-sense RNA viruses of plants and animals, *e.g.*, in the translation of poliovirus RNA, ribosomes directly bind to an internal sequence in the 5' untranslated region, and are then translocated to the initiator AUG^{9,10}.

3. Features of organization and expression of the plant viral genomes

Table I lists the several different strategies adopted by RNA plant viruses for the expression of their genome. Some common features of organization and expression in these viruses have emerged through *in vitro* translation of the viral RNAs and determination of their genomic sequences. Analysis of these sequences has revealed certain striking sequence homologies among non-structural proteins of plant and animal viruses. This has led to the idea that many plus-strand RNA virus groups may be classified into two major superfamilies and that viruses within these superfamilies may have a common evolutionary origin¹¹⁻¹⁴. There is also some sequence similarity between the members of the two superfamilies. A third supergroup comprising the luteo and related viruses was proposed by Habili and Symons¹⁵. The organization and expression of the genomes in each of these classes are briefly described in the following section.

Table I
Strategies of expression employed by different groups of plant viruses

<i>Virus group</i>	<i>Multi-partite</i>	<i>Read-through</i>	<i>Trans-frame</i>	<i>Poly-protein</i>	<i>s.g.RNA</i>
Nepo, Como	+			+	
Bromo, Cucumo, Alfalfa, Ilar, Hordei	+				+
Tobra, Furo, Diantho	+	+			+
Sobemo				+	+
Tobamo		+			+
Luteo			+		+
Carmo		+			+
Potex					+
Tombus		+			+
Poty				+	
Tymo		?		+	+

3.1. *The picorna-like plant viruses*

The como, nepo and poty ssRNA plant virus groups have several features common to polio virus, a picorna virus in their genomic organization and expression. The genomic organization of cowpea mosaic virus (CPMV), a comovirus, tomato black ring virus (TBRV), a nepovirus and tobacco vein mottling virus (TVMV), a potyvirus are presented in Fig. 3 along with poliovirus (a picorna virus) to illustrate these similarities. Polio, como and nepo viruses are spherical while potyviruses are flexuous rods. The como and nepo group of plant viruses consist of two RNAs as their genome. Simultaneous presence of both these RNAs is essential for disease manifestation. In spite of these differences, the organization of the genomes is similar as illustrated in Fig. 3.

The viruses belonging to this supergroup have a small protein, VPg, covalently linked to the 5' end of the genome and a poly A tail at the 3' terminus. These modifications are believed to protect the RNA from degradation. Viruses of this group follow polyprotein processing as the major strategy of expression of proteins. The polyproteins synthesized from long ORFs are processed by viral-coded proteases. These proteases have unusual specificities such as cleavage at gln-ser, gln-met or gln-gly bonds. Overexpression of such viral-coded proteases could be of use in protein sequencing although no such attempt has been made to exploit the unusual specificity of these proteases so far.

As evident from Fig. 3, the order of arrangement of the sequence which leads to functional proteins after processing is similar in this supergroup. The region 3' to that corresponding to the viral coat proteins VP1, VP2 and VP3 in polio virus is followed by the non-structural proteins in the order, protease 2A, polypeptide 2C,

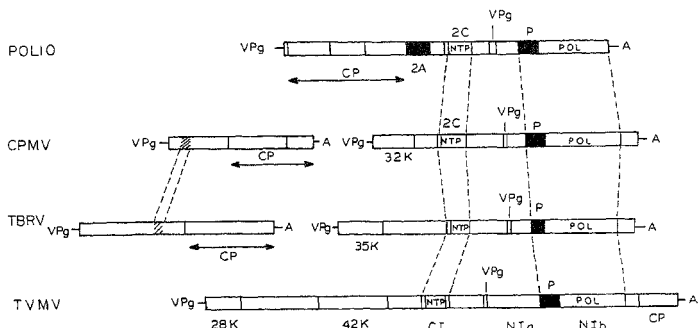


FIG. 3. Picorna-like superfamily of plant viruses¹³. Comparison of the genomic organization in oliovirus (Picorna virus) and CPMV (comovirus), TBRV (nepovirus) and TVMV (potyvirus) = ORFS, ---- : indicates homologous regions; : homologous domain of protein involved in transport, VPg : viral protein genome linked, CP : coat protein, P: Cysteine protease domain, 2A: Protease 2A, 2C : Polypeptide 2C with NTP binding motif, CI : Cytoplasmic inclusion protein, NIA, NIB : Nuclear inclusion protein a and b, Pol. : RNA-dependent RNA polymerase (replicase) (reproduced with permission).

VPg, cysteine-protease and polymerase. This arrangement of coding sequences corresponding to non-structural proteins is similar in all the members of this group. However, coat protein gene is present at the 3' terminus of the RNA of como, nepo and poty viruses. These coat proteins are generated by processing the respective polyproteins. The three-dimensional structure of the coat protein and their organization is similar in CPMV and poliovirus. The asymmetric unit of the viral coat consists of three 8-stranded β -barrel domains. A large and small protein subunit constitutes the asymmetric unit in CPMV. The large subunit folds into two β -barrel domains while the small subunit folds into a similar third barrel domain¹⁶. In poliovirus, the three β -barrel domains originate from three proteins VP1, VP2 and VP3¹⁷. In both the cases, the protein subunits are formed from precursor protein of size 60 kDa. In view of similarity in the three-dimensional structure of these two viruses, it is anticipated that in nepoviruses also, the asymmetric unit might consist of three β -barrel domains which could be present on a large protein (60 kDa) that does not undergo processing to smaller proteins.

The function of several proteins generated by the polyprotein processing has been established in recent years by an analysis of the expression of the mutants of full-length cDNA transcripts. For example, in CPMV the 24-kDa viral-coded protease of the B-RNA (the cysteine-protease domain region, Fig. 3) acts *cis* on the 200 kDa polyprotein of B-RNA (the larger size RNA) and *trans* on the polyprotein of mRNA (smaller size RNA)^{18,19}. In poty viruses the genome codes for a poly protein of size 340 kDa which is processed to give various products. Site-directed mutagenesis of the

49 kDa protease (corresponding to cysteine protease region in Fig. 3) showed the catalytic triad His 234, Asp 269, and Cys 339 to be involved in catalysis²⁰. This protease is cleaved autocatalytically from the polyprotein and subsequently it catalyzes the other cleavage reactions⁶. In this respect, it is similar in function to the 24 kDa viral-coded protease of CPMV.

The polypeptide 2C region contains a nucleotide triphosphate (NTP)-binding motif similar to the NTP-binding site of several GTP- or ATP-using enzymes. This region is conserved in all these viruses. Unlike in other supergroups, the polymerase domain is located towards the 3' end of the genome and is found to bear extensive homology with each other. The GDD motif typical of polymerases is conserved in all of them. Thus despite differences in size and shape as well as the genomes being divided in *com*o and *nepo* viruses, the genomic organization and expression of these viruses are similar.

3.2. *Sindbis-like plant viruses*

The tobamo, tobra, hordei, bromo, cucumo, alfalfa, furo, potex and tymo group of plant viruses show similarity in the amino acid sequence of the non-structural proteins and gene arrangements with the sindbis virus, an alfa virus. Hence, these plant viruses have been classified under this supergroup (Fig. 4). All these viruses have a 5' cap. However, their 3' termini are different. The members of this group show a wider variation in their gene structure and translation strategy than the picorna virus-like supergroup of viruses. Highest degree of similarity is observed, however, between alfa, bromo and cucumo viruses. They all have a tripartite genome structure and their three non-structural proteins exhibit sequence homology²¹⁻²³. Hordei viruses which also have tripartite genome have similar genomic organization. Although tobamo viruses have non-divided genome (*monopartite*) they have similar genome organization. The three distinct conserved domains in the tripartite viruses corresponding to nsp₁, nsp₂, and nsp₄ of sindbis virus are also found in TMV except that it is present on one polypeptide, while in tripartite viruses the domains are spread over two polypeptide chains. Similarly in tobra, furo, potex and tymo viruses they are present on the same polypeptide chain. In Sindbis virus these are present on a single polyprotein which gets subsequently processed to three proteins (nsp₁, nsp₂ and nsp₄). This suggests that despite variation in the genome structure and translation strategy employed, all sindbis-related viruses end up with functionally equivalent proteins.

3.3 *Luteovirus-like superfamily*

The tombus, carmo, luteo, sobemo, and diantho group of plant viruses belong to this supergroup (Fig. 5). Members of this group have a small genome size. They all share extensive homology in the putative replicases^{15,24}. Sequence motifs of nucleic acid helicases and RNA polymerases previously considered to be specific for one or the other of the two superfamilies are found to occur together within the new luteo virus-like superfamily. Thus, this new superfamily has provided an evolutionary link between the other two supergroups.

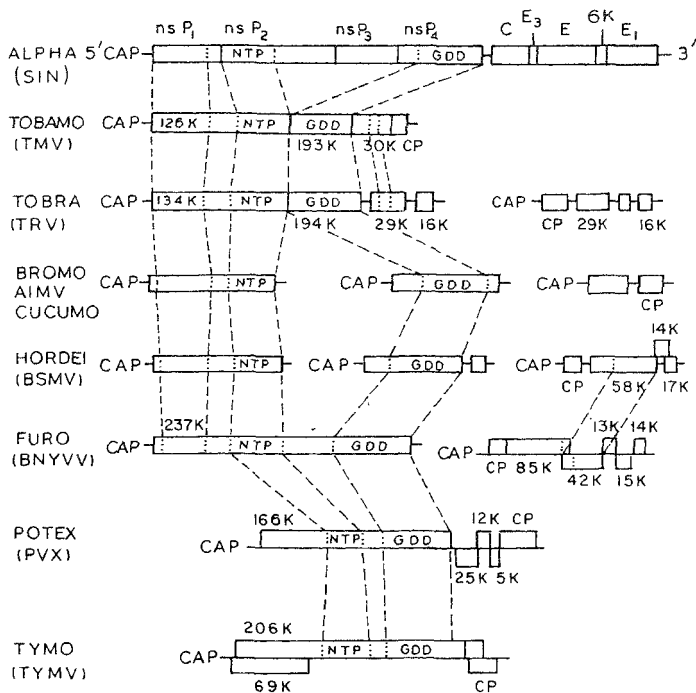


FIG. 4. A comparison of genomic organization in sindbis-like superfamily of plant viruses¹³. = : ORFS; --- : indicates homologous regions; CP : coat protein; nsP₁, nsP₂, nsP₃ and nsP₄ : non-structural protein p1, p2, p3 and p4; NTP: nucleotide binding motif; GDD: conserved polymerase binding motif (reproduced with permission).

Determination of complete genomic sequence of members of each group of viruses and their comparison with the known sequence will strengthen the established evolutionary relationships among the various groups of plant viruses.

3.4. Tymoviruses

We have used PhMV, a member of the tymovirus group of plant viruses, as a model

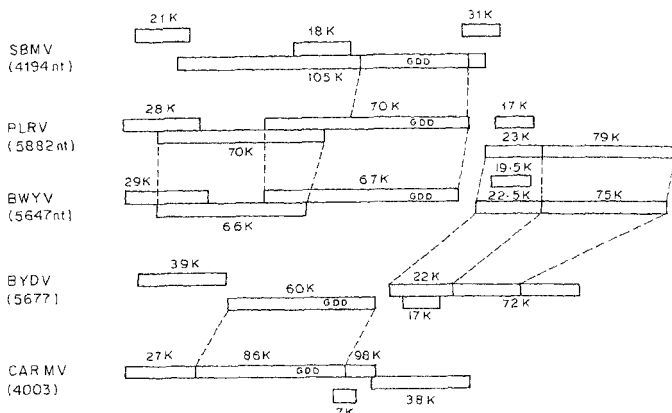


Fig. 5. Genomic organization in SBMV, PLRV, BWYV, BYDV and CarMV, members of the luteovirus-like superfamily of plant viruses^{1, 15}. = : ORFs, --- : indicates homologous regions, GDD : conserved polymerase binding motif (reproduced with permission).

to understand the architecture, genomic organization and expression in these viruses. The following section summarizes our findings along with those of other tymoviruses. Tymoviruses comprise a large group of positive-sense ssRNA viruses with a monopartite genome encapsidated within an icosahedral protein capsid of 180 identical subunits²⁵. The genomic RNA is approximately 6.3 kb with a 5' CAP and tRNA-like structure at 3' terminus. The capsid protein has a molecular weight of 20 kDa. The capsid structure is maintained essentially by protein-protein interactions and hence the presence of empty capsids in purified preparations. A characteristic feature of tymoviruses is the release of RNA from intact particles by a variety of chemical and physical treatments without disruption of capsid structure²⁶⁻³⁰. However, reconstitution of the virus from isolated coat protein has not been possible in any tymovirus studied so far.

PhMV has features similar to those of other tymoviruses. However, in PhMV, the intrinsic polyamines are easily exchanged with monovalent cations leading to the release of RNA and formation of empty shells at alkaline pH³⁰. The amino acid sequence of the coat protein was determined by manual micro sequencing methods and the sequence compared with other plant viruses³¹. An analysis of the 3' terminal 1225 genomic sequence of PhMV determined from corresponding cDNA clones has shown that the genomic organization of this virus is similar to other tymoviruses³².

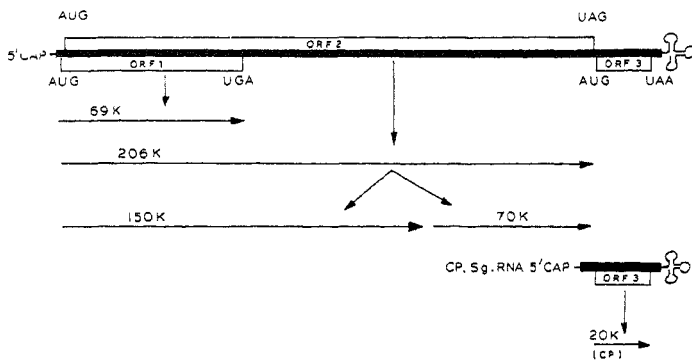


FIG. 6. Genomic organization and expression in tymoviruses. ORF1: overlapping protein ORF (OPORF), ORF2: replicase protein ORF (RPORF), ORF3: coat protein ORF (CPORF), □: genomic/sub-genomic RNA, —: protein products, CP: coat protein.

The deduced coat protein sequence corresponded with that determined earlier by protein sequencing except for the absence of a dipeptide lys-leu at position 110–111. A comparison of the coat protein sequences of tymoviruses revealed that they all lack the basic amino-terminal arm usually found in viruses stabilized by RNA-protein interactions. It is possible that polyamines in tymoviruses replace the role of the basic amino terminal arm in stabilizing the virus^{33,34}. A phylogenetic tree constructed after aligning the coat protein sequences of tymoviruses showed that PhMV originally named Belladonna mottle virus (I) is a separate tymovirus and not a strain of Belladonna mottle virus (E)³².

Tymoviruses have a compact genomic organization. In turnip yellow mosaic virus (TYMV), only 192 out of 6318 nucleotides are non-coding (3%)³⁵. The genomic sequence of five tymoviruses, TYMV (E)³⁵, TYMV (CL)³⁶, eggplant mosaic virus³⁷, ononis yellow mosaic virus³⁸ and kennedy yellow mosaic virus³⁹ have been determined. All of them have similar genomic organization. The largest ORF, RPORF, codes for a protein of size 206 kDa, called the replicase protein. It contains the characteristic GXXGXGKTS and GDD sequence motifs that resemble other members of the sindbis supergroup. The genomic organization of tymoviruses is presented in Fig. 6. In addition to RPORF, the genome codes for a protein of size 69 kDa that overlaps extensively with the RPORF at 5' terminus. The coat protein gene is located at the 3' end which is followed by a non-coding sequence of 100–150 nucleotides. The terminal 80 nucleotides at 3' end can be folded into a tRNA-like structure which can be aminoacylated with valine in most of the tymoviruses studied^{32,40–44}. The functional significance of the aminoacylatability *in vivo* is not clear. However,

competition experiments with short transcripts corresponding to the tRNA-like structure has implicated this region to be essential for binding the viral replicase⁴⁵.

In vitro translation studies on TYMV⁴⁶, PhMV⁴⁷ and other tymoviruses has shown that tymoviruses follow several strategies for the expression of their genes such as proteolytic processing, overlapping reading frame, and generation of subgenomic RNA for the expression of the coat protein gene⁴⁸. Recently, Bozarth *et al*⁴⁹ suggested that the ORF1-encoded 69 kDa protein could be a protein involved in cell-to-cell movement of the virus. Mutations in this ORF as well as in the ORF for the coat protein would throw light on the involvement of specific domains in viral assembly and movement within the host. Site-directed mutagenesis in certain regions of the 206 kDa replicase ORF, on the other hand, has helped in the identification of the protease domain within this ORF which is involved in the processing of the 206 kDa protein⁵⁰. The 206 kDa replicase protein undergoes proteolytic processing to yield 150 and 70 kDa protein products. The NTP- and polymerase-binding motifs are present in the C-terminal portion of 206 kDa protein. The functionally active replicase protein *in vivo* has a size of 110 kDa⁵¹. Thus, it is not clear which of the processed proteins is the true replicase. It would be interesting to mutagenize specific regions corresponding to polymerase binding or NTP binding in the replicase gene to establish their functional significance. A comparison of the 206k ORF of tymoviruses with those of other viruses has shown that the replicase protein of tymoviruses is closely related to those of potex and carla viruses suggesting possible evolutionary relationship among these viruses⁵².

In vitro translation studies on tymoviruses has shown that the coat protein is not translated from the intact genome but is translated instead *via* a subgenomic messenger RNA^{43,47,53,54}. It has been demonstrated by Gargouri *et al*⁶ that the mechanism of synthesis of the subgenomic messenger RNA for the coat protein in TYMV occurs by internal initiation on the full-length negative-sense RNA generated during replication. The conserved region upstream of the CPORF (Tymobox) might play a regulatory role in the generation of the subgenomic RNA⁵². As evident, tymoviruses in general have an organization similar to members of the sindbis supergroup but the strategies of expression of viral proteins are different.

4. Conclusions

It is evident from the foregoing discussion that some common features underlie the genomic organization of positive-sense RNA plant viruses. The genome sizes range from 4kb in small icosahedral viruses to about 9kb in rod-shaped potyviruses. The genomes, being compact, exploit in full their coding potential, with overlapping genes and the use of all the three reading frames. In many cases, the reading frames for the different ORFs alternate along the length of genomic RNA, *i.e.*, two adjacent genes are present in different reading frames. This could be a mechanism to ensure the separate identity of the 3' gene product from the plausible read-through product of the 5' gene. The use of alternating reading frames by adjacent genes becomes essential, when there is a junctional overlap between the genes. The order of arrangement of genes along the length of the genomic RNA has some common features in

the different groups of positive-sense RNA viruses. In general, the RP gene is located 5' proximal, while the CP gene is 3' proximal in the sindbis and luteo superfamilies. This arrangement ensures the expression of the replicase protein in the early stages of infection, when it is required for the synthesis of the negative-strand RNA. The positive-strand RNA viral nucleocapsids do not contain the polymerase enzyme which consequently is made *de novo* after the virus enters the host cell. The CP, on the other hand, is required only in the later stages of the infection and is therefore synthesized *via* a subgenomic RNA in some of the positive-sense RNA viruses. The synthesis of the CP subgenomic RNA is facilitated by the polymerase (resulting from early gene expression) initiating synthesis at the 'promoter' sites on the negative-strand RNA. Thus, the 5'→3' tandem arrangement of the RP gene with the other non-structural/structural protein genes would ensure synthesis of the template negative strand upon which the subgenomic RNAs required for the expression of the 3' and internal genes are made. In picorna-like viruses the RP gene is 3' proximal. However, in this group the major strategy for expression is polyprotein processing.

The 5' terminus of the genomic RNAs of these viruses in general possesses either a 7mGpppG structure similar to the eukaryotic mRNAs or covalently linked to a small protein, VPg. Both these structures play a primary role in protecting the genomic RNA from exonucleolytic degradation. In picorna viruses, the VPg plays the role of a primer for replication of the minus- and plus-strand RNA. Among the plant viruses with a 5' VPg (como, nepo, and potyviruses), the protein is essential for infectivity of some, but not all RNAs. The 5' terminal structure is followed by a stretch of nucleotides forming a leader sequence, upstream of the first ORF. This noncoding region is shown to contain regulatory signals such as the ribosome-binding sequences and the plus-strand promoter sequences.

The 3' terminal region of most of the positive-sense RNA viruses either has a poly(A) tail or a tRNA-like structure. The role of different regions of genome in viral replication, translocation and vector transmission is being investigated through site-directed mutagenesis of full-length cDNA transcripts. Such studies are expected to throw light on the mechanism of virus infection at the molecular level.

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Abbreviations

Physalis mottle virus	PhMV
Carnation mottle virus	CarMV
Cowpea mosaic virus	CPMV
Tomato black ring virus	TBRV
Tobacco vein mottling virus	TVMV

Southern bean mosaic virus	SBMV
Potato leaf roll luteovirus	PLRV
Beet western yellow mosaic virus	BWYMV
Barley yellow dwarf virus	BYDV
Turnip yellow mosaic virus	TYMV
Tobacco mosaic virus	TMV
Tobacco rattle virus	TRV
Alfalfa mosaic virus	ALMV
Barley stripe mosaic virus	BSMV
Potato virus-X	Potex
Beet necrotic yellow vein virus	BNYVV
Replicase protein	RP
Coat protein	CP

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