Gene regulation of RNA viruses with uncapped and non-polyadenylated genomic RNA

by

Ruizhong Shen

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Program of Study Committee:
W. Allen Miller, Major Professor
Jeffrey K. Beetham
Bryony C. Bonning
Gloria M. Culver
David J. Oliver

Iowa State University

Ames, Iowa

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ABSTRACT

New roles of RNAs as regulators of gene expression have emerged and expanded in recent years. However, gene regulation by viral RNA *in trans* is less noted and not well understood. *Barley yellow dwarf virus* (BYDV) is a positive sense RNA virus with a single genomic RNA (gRNA). As it replicates it generates three subgenomic RNAs (sgRNA). Data in this dissertation show that BYDV sgRNA2 serves as a regulatory RNA to control viral gene expression. *In vitro*, BYDV sgRNA2 preferentially inhibits translation of genomic RNA (gRNA) compared to sgRNA1. *In vivo*, BYDV sgRNA2 inhibits translation of gRNA, but has little effect on translation of sgRNA1. These data support and modify a trans-regulation model proposed previously.

I also report that the 3' cap-independent translation element (3'TE) of BYDV functions differently *in cis* and *in trans* in plant cells. *In cis*, the 3'TE confers cap-independent translation and increases translation of capped RNAs as well. *In trans*, the 3'TE or the 3'TE-containing sgRNA2 serves as a riboregulator to negatively regulate viral replication, most likely via inhibition of translation. Thus a viral subgenomic RNA can perform important regulatory functions instead of acting as a messenger RNA.

RNAs of many important plant and human viruses are translated efficiently in the absence of a 5' cap structure and/or a poly(A) tail that are normally required for translation. The translation mechanism of the uncapped and non-polyadenylated RNA of *Tobacco necrosis virus* (TNV) has not been well investigated. Here, I identify a cap-independent translation element (TE) in the 3' UTR of TNV strain D (TNV-D) that shares many features with BYDV 3'TE, even though it is in a different family. TNV-D and other members of

genus *Necrovirus* may initiate translation by a BYDV-like TE-mediated cap-independent translation mechanism.

Finally, I show that sequence at the 3' end of TNV-D RNA functionally mimics a poly(A) tail. A phylogenetically conserved double-stem-loop structure is replaceable by, but cannot substitute for a poly(A) tail. The full-length 3' UTR of TNV-D is sufficient to functionally replace a poly(A) tail. Data suggest the poly(A) mimic facilitates translation efficiency. Thus, translation of TNV RNA in plant cells requires both cap-mimic and poly(A) mimic elements. This research provides new insight into our understanding of gene regulation, especially that of RNA viruses with uncapped and non-polyadenylated genomic RNA.

CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

In my dissertation research, I investigated gene regulation of RNA viruses with uncapped and non-polyadenylated genomic RNA, by using *Barley yellow dwarf virus* (BYDV) and *Tobacco necrosis virus* (TNV) as models. Specifically, I showed 1) BYDV subgenomic RNA2 (sgRNA2) functions as a trans-regulator to control viral gene expression; 2) Translation of uncapped and non-polyadenylated RNA of TNV in plant cells needs both cap mimic and poly(A) mimic elements.

My dissertation contains seven chapters. Chapters 2-5 contain four manuscripts co-authored by my major professor, Dr. W. Allen Miller. Chapter 2, "Translational control of *Barley yellow dwarf virus* gene expression by its subgenomic RNA 2 *in trans*" is written and formatted for submission to the *EMBO Journal*. Chapter 3, "Subgenomic RNA as a *trans*-regulator: a viral subgenomic RNA negatively regulates viral replication" is prepared for submission to the *Journal of Virology*. Chapter 4, "The 3' untranslated region of *Tobacco necrosis virus* RNA contains a BYDV-like cap-independent translation element" is accepted by the *Journal of Virology*. Dr. Miller made constructive revision on the discussion section of this Chapter and also made editorial corrections throughout the dissertation. Chapter 5, "A poly(A) tail mimic at the 3' end of an uncapped, nonpolyadenylated viral RNA" is prepared for submission to the *Journal of Virology*. Chapter 6 contains research on "the effect of BYDV subgenomic RNA2 on host gene expression". In Chapter 7 are general conclusions of my dissertation research. This chapter (Chapter 1) introduces the background of my research.

Translation control of viral gene expression

Translation control is a major step of gene regulation, especially for viruses that rely on the host cell for propagation. Although most viruses have their own polymerase for genomic replication and/or transcription, viruses do not encode the translation machinery but completely depend on the host translation machinery to fulfill viral protein synthesis. Because of this dependency, viruses have adopted many regulatory mechanisms from their hosts and evolved many new strategies to regulate their gene expression and to usurp host machinery (Fig 1).

The majority of translation control occurs during the whole process of translation initiation, including formation of 43S pre-initiation complex, assembly of eIF4F complex on the mRNA cap structure, ribosome scanning, and AUG selection. Viruses exploit and take advantage of these checkpoints to control gene expression and to redirect host translation machinery in favor of viral protein synthesis.

In the stage of formation of 43S pre-initiation complex, phosphorylation of eIF2α is a major point of translation initiation control (30). Viruses may not have direct translation control mechanisms on this stage. However, many viruses develop mechanisms to disrupt the phosphorylation of eIF2α via inhibition of serine/threonine protein kinase (PKR) (20, 28-30, 107). The disruption of eIF2α phosphorylation maintains the translation competency of the host cell, which is required for virus to complete its replicative cycle. It may also prevent the cellular antiviral response apoptosis.

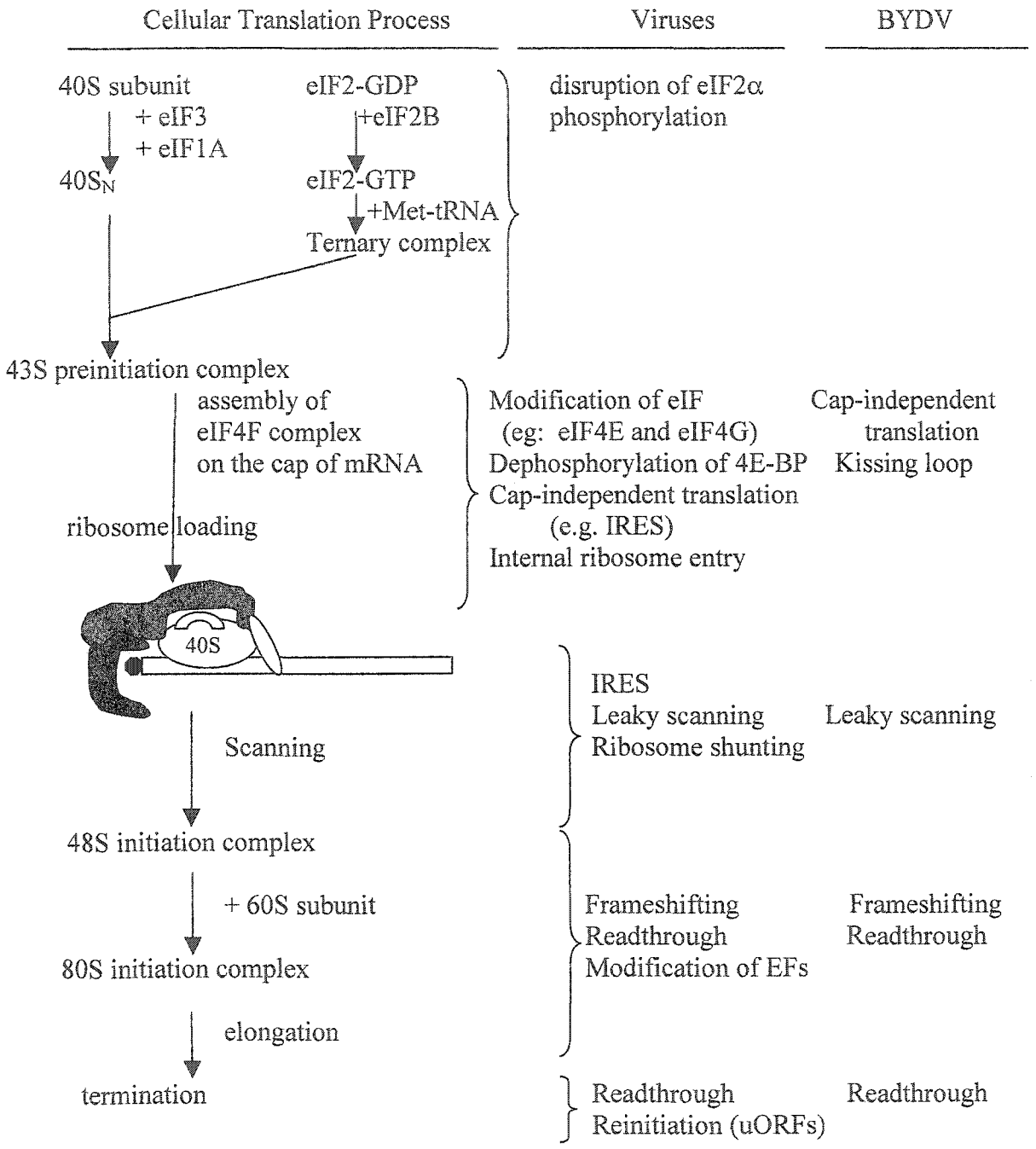


Fig 1: The process of eukaryotic mRNA translation and translation control of viruses. The left panel is the scheme of eukaryotic mRNA translation; the middle panel is viral translation control mechanisms and regulation sites; the right panel is translation control mechanisms evolved in barley yellow dwarf virus (BYDV).

In the stage of assembly of eIF4F complex on the mRNA cap structure, the affinity of eIF4E for the cap structure confers a second major point of translation initiation control (30, 45, 104). Many viruses evolved cap-independent translation mechanisms to control gene expression (43, 44, 52, 82, 112, 116, 118). One of the well-known examples is the internal ribosome entry site (IRES). Viruses also modify translation initiation factors to shutoff host gene expression and to redirect host machinery for viral protein synthesis. The modification of translation initiation factors at this stage includes dephosphorylation of eIF4E (53, 104), cleavage of eIF4G (41, 46), and dephosphorylation of the 4E-Binding protein (4E-BP) that results in an inactive eIF4E/4E-BP complex (40).

In the stage of ribosome scanning and AUG selection, the viral translational control mechanisms include the IRESs (52, 57, 82, 105), ribosome shunt (25, 48, 91), and leaky scanning (51, 101, 106). IRESs overcome cap-dependence, inefficiency of the 5'-UTR, and host antiviral response. Ribosome shunt combines the cap-mediated ribosome entry, 5'-scanning, and internal initiation together (30). Leaky scanning associates with non-optimal AUG context (51) and overcome the restriction of limited viral genome size.

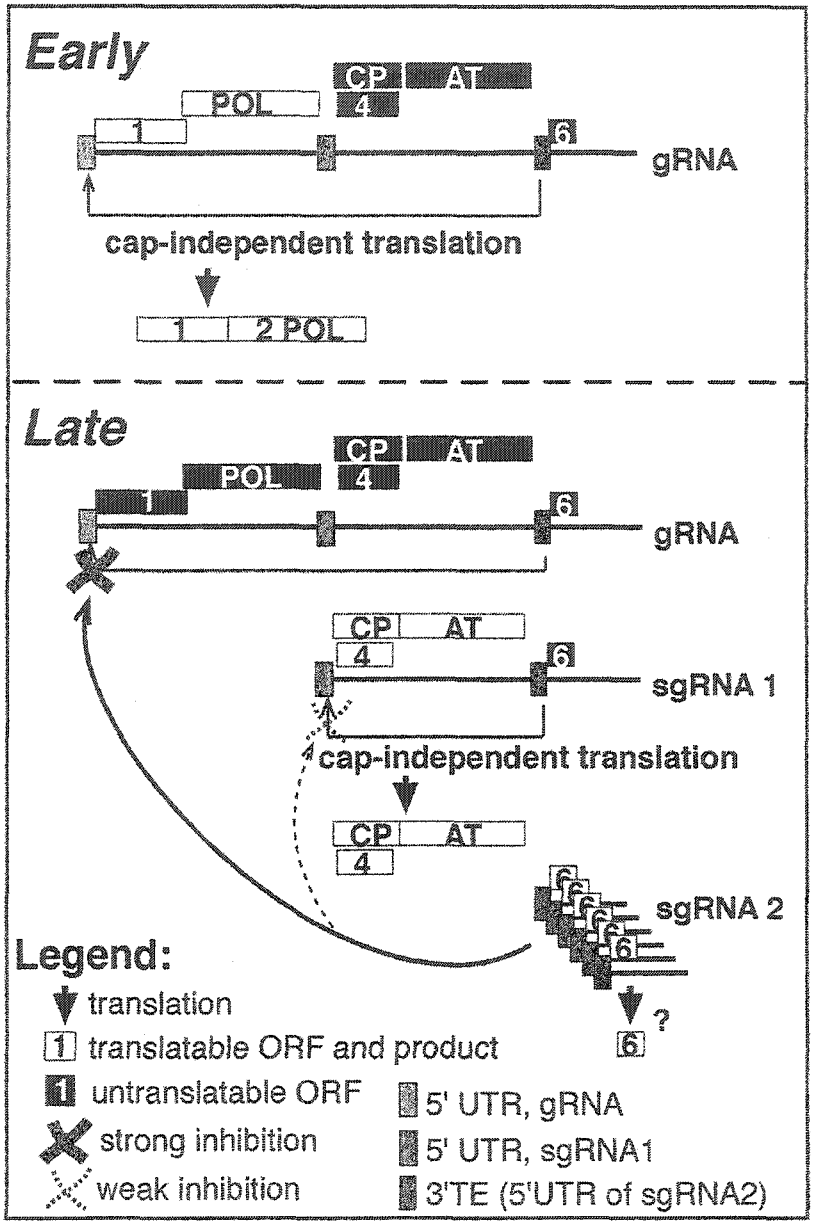
Viral translational control also takes place during elongation and termination. The mechanisms during these two stages include frameshifting, readthrough, reinitiation, and elongation factor modification. In frameshifting, the translating ribosome shifts position by +1 or −1 and the reading frame changes (38). Readthrough proceeds through an in-frame termination codon, for example, by encoding glutamine at UAG or tryptophan/ selenocysteine at UGA (38, 110). In reinitiation mechanisms, a short upstream ORF affects the translation of the major downstream ORF (37). Modification of elongation factor

facilitates the viral mRNA translation efficiency (54, 55) or reduces the efficiency of cellular but not viral mRNA translation (126).

Barley yellow dwarf virus (BYDV) is the type member of genus *Luteovirus* in the family *Luteoviridae* (67). It has a positive strand RNA genome with a size of 5677 nt. BYDV has evolved a diverse set of translational control strategies (Fig. 1), such as cap-independent translation, ribosomal frameshifting, leaky scanning, and stop codon readthrough, to regulate viral gene expression (review in 73-75). Significantly, many of these translation events are regulated *in cis* by sequences hundreds to thousands of bases downstream. Cap-independent translation and frameshifting are regulated by long distance base-pairing (7, 43).

Another translational control mechanism of BYDV was proposed from previous in vitro translation experiments (117). In the proposed model, BYDV subgenomic RNA2 (sgRNA2), via the translation element (TE), functions as a riboregulator to control viral gene expression (Fig. 2 and detail in section *Barley yellow dwarf virus* gene expression). In chapter 2, I demonstrate and modify the novel translational control mechanism of BYDV. In chapter 3, I show another mechanism of gene regulation used by BYDV: sgRNA2 acts as a *trans*-regulator to negatively regulate viral replication.

BYDV may also shut off host genes and selectively facilitate viral gene expression (116). In wheat germ extract, the TE of BYDV trans-inhibited translation of capped, polyadenylated mRNA lacking any BYDV sequence, whereas the defective TE, which differs only by having a GAUC duplication in the BamH I site (TEBF), has no such effect. Therefore, the high level accumulation of BYDV subgenomic RNA2 (sgRNA2),



(Wang, S., et al., 1999, RNA 5:728-738)

Fig 2: Trans-regulation model of BYDV gene expression (Wang et al, 1999). Early in BYDV infection, only ORF1 and ORF2 are translated via TE-mediated cap-independent translation. Viral RdRp are produced, virus replicate, genomic (g) RNA and sgRNAs accumulate. The high level accumulation of sgRNA2, via its TE, selectively inhibited translation of gRNA relative to sgRNA1 in trans. The BYDV viral life cycle switches from the early stage to the late stage.

which contains the TE in the 5' end, may shut off host genes in favor of viral gene expression. The effect of BYDV sgRNA 2 on host gene expression is reported in Chapter 6.

Trans-regulatory RNA in virus

It is a well-established concept that RNA structures are widely involved in gene regulation. Most of them function in cis. Trans-acting RNAs are also reported to control translation of their target mRNAs by binding UTRs or by other mechanisms. In recent years, diverse classes of non-coding RNAs have been discovered (3, 5, 27, 61, 97). These non-coding RNAs function at the RNA level in trans. As riboregulators, their main function is posttranscriptional regulation of gene expression (27). They have varied functions, such as DNA markers *Xist* and *H19* involving dosage compensation and imprinting (11, 56), development timing RNAs *lin*-4 and *let*-7 in *Caenorhdabditis elegans* (4), abiotic stress signals OxyS RNA and DsrA RNA (3, 62), and biotic stress signals *his-1* RNA (6). In recent years, more attention has been drawn to small non-coding RNAs. These small non-coding RNAs can control transcription or translation. They include microRNAs (miRNA), small interfering RNAs (siRNA), small nucleolar RNAs (snoRNA), and small nuclear RNAs (snRNAs) (5, 15, 50, 58, 119).

Trans-acting regulatory RNAs from viruses have also been reported. *Red clover necrotic mosaic virus* (RCNMV) RNA2 has a 34nt trans-activator sequence, which is required for transcription of sgRNA from RNA1 (102). The trans-activator fulfills its function via base pairing between RNA1 and RNA2. This is an example of transcriptional regulation by trans-regulatory RNA in virus. *Flock house virus* (FHV) sgRNA transactivates the replication of RNA2 (1, 26). The trans-activation is mediated by sgRNA rather than by

its translation product. FHV RNA2, not its translation product, down-regulates synthesis of sgRNA from RNA1 (127). *Adenovirus* virus-associated (VA) RNAs are required for efficient expression of late viral genes (65, 111). The non-coding, 160 nts VA RNAs are common to all adenoviruses and transcribed by RNA polymerase III (65, 120). VA RNAs protect against dsRNA-activated inhibitor (DAI)-mediated phosphorylation of eIF-2α by binding DAI (100). *Epstein-Barr virus* (EBV) EBER RNAs are also transcribed by RNA polymerase III and may have similar function as VA RNAs (9, 19). Among these viral regulatory RNAs, only VA RNAs, and EBER RNAs are non-coding RNA. Others function as both a coding RNA and a non-coding regulatory RNA. Such a phenomenon occurs with tmRNA, which serves as both a message RNA and a function–specific tRNA (39).

BYDV sgRNA2 is a potential example of trans-regulatory RNA that functions at the translational level. In chapter 2 and 3, I show that *Barley yellow dwarf virus* (BYDV) sgRNA2 does act as a riboregulator to temporally control translation and negatively regulate viral replication.

Luteoviridae

Luteoviridae is a family of plant viruses that include three genera: *Luteovirus, Polerovirus,* and *Enamovirus.* It also includes more than ten unassigned species. Luteoviridae are phloem-limited, aphid transmitted viruses with a positive single strand RNA genome. The virions contain 28% RNA and are icosahedral with a diameter of 24-30nm (72). The three genera have distinctly different genome organizations (Fig 3), cytopathological effects, and serological properties (67).

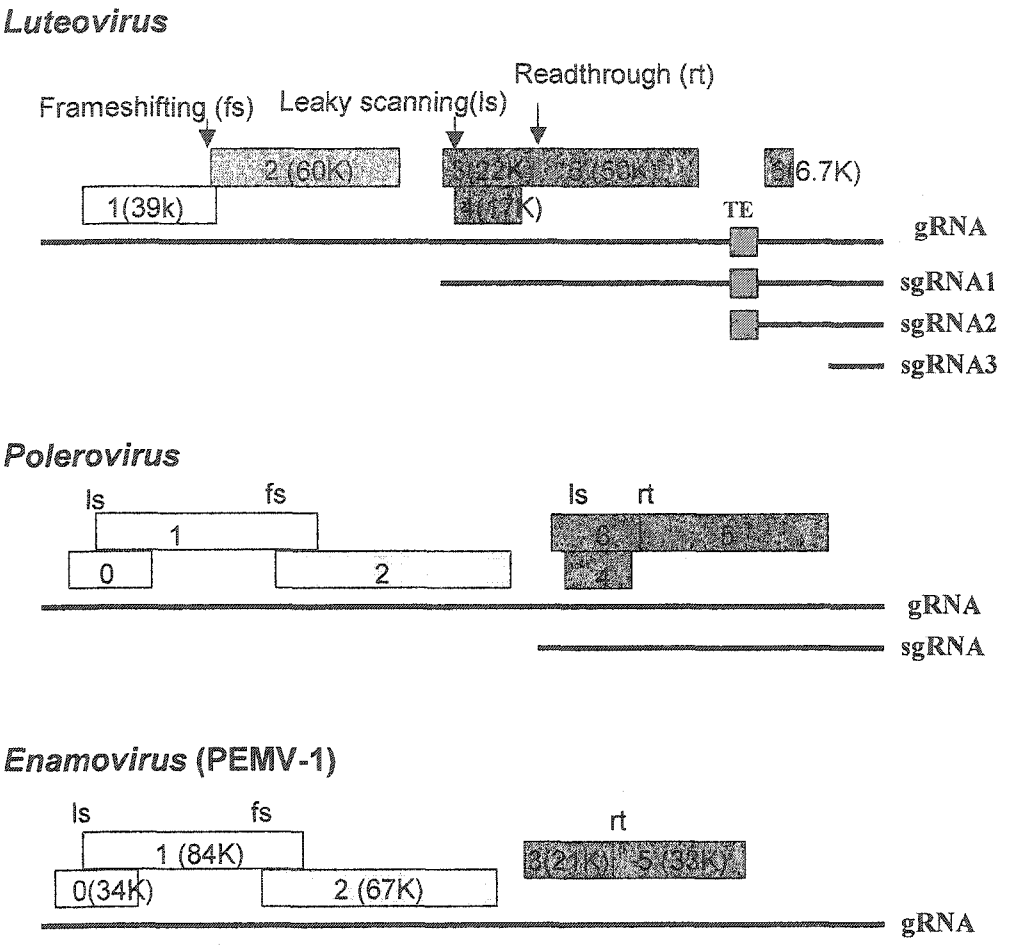


Fig 3: Genome organization of luteoviruses. Open reading frames are numbered and the molecular weight of the encoded protein is indicated in brackets. Bold lines indicate gRNA and sgRNAs. Unshaded ORFs have little similarity with other viruses. ORFs with the same fill schemes share high similarity. Sites of frameshifting (fs), leaky scanning (ls), and readthrough (rt) are shown. Hatched boxed indicate TE.

The genome of viruses in genus *Luteovirus* lack open read frame (ORF) 0. The RNA-dependent RNA polymerase (RdRp) of genus *Luteovirus* belongs to the carmovirus-like supergroup. It is translated via –1 ribosomal frameshifting from ORF1 into ORF2 at the ORF1 stop codon (12, 23). *Luteovirus* has three subgenomic RNAs (sgRNA). Genomic RNA (gRNA) and sgRNAs lack a genome-linked protein (VPg) or any other known modification (2, 67). Viral proteins are translated via a cap-independent mechanism (116, 118). The intergenic region between ORF2 and ORF3 is about 100nt. The type species is BYDV PAV (67).

The genome of *Polerovirus* has ORF0 but lacks ORF6 that present in *Luteovirus* (67). The RdRp of *Polerovirus* belongs to sobemovirus-like supergroup. It is also translated via –1 ribosome frameshifting from ORF1 into ORF2, but at a considerable distance upstream of the ORF1 stop codon (68). Polerovirus has only one sgRNA and its RNAs have VPg at the 5' end (66, 77). The intergenic region between ORF2 and ORF3 is about 200nt. The type member is *Potato leafroll virus* (67).

The genus Enamovirus has only one member, *Pea enation mosaic virus*-1 (PEMV-1) (67). The genome structure of PEMV-1 resembles those of poleroviruses. It has a VPg and ORF0. PEMV-1 lacks ORF4 and its ORF5 is relatively short (Demler and de Zoeten, 1991). PEMV-1 can replicate in protoplasts, but in nature, it is found only in association with PEMV-2, which is an umbravirus (66, 67).

Barley yellow dwarf virus gene expression

BYDV is the type member of genus *Luteovirus*. BYDV RNA encodes six ORFs (Fig 3 and (73)). ORF 1 and 2 are translated from genomic RNA (gRNA). ORF 2 encodes the

activity domain of RNA-dependent RNA polymerase (RdRp) and is translated as a fusion of ORF 1 via –1 ribosomal frameshifting (7, 12, 23, 80). The function of ORF 1 product is not known. ORF 3, 4, and 5 are translated from subgenomic RNA 1 (sgRNA1) (24). ORF 3 encodes the coat protein. ORF 4 encodes the movement protein and is translated via leaky scanning (18). ORF 5 encodes the readthrough domain of 72k protein (ORF 3 + 5) via functional recoding (14) and is required for aphid transmission (13, 17). ORF 6 is located within sgRNA2 and its function is unknown. So far, the ORF 6 product has not been detected in vivo. An interesting feature is that most of these translation events are controlled by sequences hundreds to thousands of bases downstream and long distance communication. Here, I will focus on what we know about cap-independent translation and how the data lead to the trans-regulation model of BYDV (Fig. 2).

BYDV gRNA and sgRNAs lack both 5'-cap and 3'-poly (A) tail; yet they are translated efficiently. A 3'- translation enhancer element (TE), which confers cap-independent translation in both the 3'-UTR and 5'-UTR, was identified in the viral 3'-UTR (116, 118). The BYDV 5'-UTR is required for the TE to fulfill its cap-independent translation function from the 3'-UTR, whereas the TE can function alone in the 5'-UTR (116). Thus the viral 5'-UTR only confers communication with 3'-TE (43). RNA structure prediction by MFOLD, nuclease probing, and mutagenesis revealed that the TE folds in a cruciform structure with three stem-loops (SL) and one stem (stem-IV) (Fig. 5) (44). TE mutants with deletion of any of the stems lost its cap-independent translation function. Stem-IV is necessary for TE function in either UTR. SL-1 resides in a 17nt tract that is completely conserved in *Luteovirus* members, unassigned luteovirus *Soybean dwarf virus* (SDV), and unrelated *Tobacco necrosis virus* (TNV). The loop sequence GGAAA of SL-1 is important

for TE function. The secondary structure but not the sequence of SL-II is necessary for TE function. The loop sequence of SL-III is required only in the 3'-UTR context and base pairs with the loop of SL-IV of BYDV 5'-UTR (44). Co-variation mutageneses showed that the long distance base-pairing (kissing loop) between the SL-III of TE and the SL-IV of BYDV 5'-UTR is essential for cap-independent translation mediated by TE in 3'-UTR (43). As the 18nt conserved tract, the kissing loop is also phylogenetically conserved in Luteovirus member SDV and in TNV (43). To function as a cap-independent translation element, the TE recruits translation factors and delivers them to the 5'-UTR via long distance base-pairing (E. Allen and W.A. Miller, personal communication; 43).

Another significant feature is that the TE trans-inhibits translation of gRNA much more that sgRNA1 does (117). 100-fold molar excess of TE inhibits translation of gRNA by 50%, whereas the same level of translation inhibition of sgRNA1 needs 400-fold excess of TE. BFTE has little inhibitory effect. When equal amounts of gRNA and sgRNA1 were present in the same reaction, a 300-fold excess of TE reduced translation of gRNA by 11-fold, whereas it reduced that of sgRNA1 by only 20%. Wild-type sgRNA2 inhibited gRNA translation more efficient than TE did. The inhibition did not require the expression of ORF 6, but required a functional TE. Based on these data, a trans-regulation model of gene expression was proposed (Fig 2): Early in BYDV infection, only ORF1 and ORF2 are translated via TE-mediated cap-independent translation. Viral RNA-dependent RNA polymerase (RdRp) is produced; virus replicate, gRNA and sgRNAs accumulate. The high level accumulation of sgRNA2, via its TE, selectively inhibited translation of gRNA relative to sgRNA1 *in trans*. The BYDV viral life cycle switches from the early to the late stage (117). In chapter 2, I demonstrate and modify this novel translational control mechanism of

BYDV. We also proposed that the accumulation of sgRNA2 inhibits viral replication, via the inhibition of translation of RdRp from gRNA. In chapter 3, I show sgRNA2 does inhibit viral replication.

Cap-dependent translation

With few exceptions, all nuclear-encoded mRNAs in eukaryotes have a 5' m^7GpppN cap structure and a poly(A) tail. The 5' cap structure and poly(A) tail function synergistically to facilitate mRNA translation efficiency in animal, plant, yeast cells (31, 94, 95, 109). The mechanism underlying the synergy is the formation of a closed loop mRNA (47, 121). Eukaryotic initiation factor (eIF) 4E binds the 5' cap, poly(A) binding protein (PABP) binds the poly(A) tail, both eIF4E and PAPB bind to eIF4G, therefore a closed loop is formed. This closed loop greatly facilitates the translation initiation via efficiently recruiting the 43S initiation complex to the 5' untranslated region (UTR) of mRNA.

Many viral mRNAs and some cellular mRNAs have a cap structure but lack a poly(A) tail, yet they translate effectively. These RNAs use cap-dependent, poly(A)-independent translation. Generally, a specific sequence within 3' UTR replaces the function of a poly(A) tail (detail in section Translational control by 3' untranslated region and poly(A) tail). The capped, nonpolyadenylated RNAs of *Tobacco mosaic virus* (TMV) (32, 33, 36), *Rotavirus* (89), and *Brome mosaic virus* (BMV) (33) have functional alternatives for the poly(A) tail in the 3'-UTR. The metazoan histone mRNA also lacks a poly(A) tail and has a stem-loop structure that is a functional mimic of poly(A) tail (79, 124).

Cap-independent translation

Many viral mRNAs lack a cap structure and/or a poly(A) tail but translate efficiently. The important strategy that they have evolved to overcome the lack is cap-independent translation. *Tobacco etch virus* (TEV) mRNA is naturally polyadenylated but lacks a cap; its 5'-UTR is a functional alternative for a cap (16, 35). The TEV 5'-UTR confers cap-independent translation on reporter mRNAs. The 5' leader and poly(A) tail interact to synergistically enhance the translation efficiency.

The animal picornavirus RNAs have a VPg and a poly(A) tail. The VPg is cleaved off soon after entering the host cell, thus, the RNAs translate as uncapped mRNAs (8). Cis-acting RNA elements IRESes have evolved to confer cap-independent translation and allow direct internal ribosome entry. IRESes were first discovered in poliovirus (PV), an *Enterovirus* in the *Picornaviridae*, and in encephalomyocarditis virus (EMCV), a *Cardiovirus* in the *Picornaviridae* (52, 82). When the 5'-UTR of PV or EMCV was inserted between the two cistrons of reporter dicistronic mRNA, it efficiently enhanced expression of the downstream cistron. Picornavirus IRESes are typically about 450nt long. They are highly structured and contain numerous AUG triplets; at the 3' end they have a 22-25nt segment starting with a 10 nt oligopyrimidine tract and ending with an AUG triplet (51). Salt-washed 40S ribosome subunits cannot bind picornavirus IRESes in the absence of initiation factors (86). Binding of 40S ribosome subunit to picornavirus IRESes requires the same set of initiation factors as normal mRNA except for no requirement for eIF4E, and the central domain of eIF4G is sufficient to fulfill the eIF4G requirement (81, 86, 88). An exception is hepatitis A virus IRES, which requires eIF4E and intact eIF4G (10, 51).

IRESes are also found in hepatitis C virus (HCV) and members of genus *Pestivirus*, both of which belong to *Flaviviridae* family and have an uncapped and non-polyadenylated

RNA (92, 113, 115). HCV and pestivirus IRESes are located in the 300-310 nt immediately preceding the authentic initiation codon or include the beginning of the first ORF. They have very similar secondary structures (49). Compared to picornavirus IRESes, HCV and pestivirus IRESes have a significant feature: salt-washed 40S ribosome subunits can bind directly to these IRESes at/near the correct initiation site in the absence of all initiation factors except eIF3 (85, 87). UV-crosslinking and mutation analyses revealed that the binding occurs between the ribosomal protein S9 and the IRES domain II (87). HCV IRES is bound to the solvent side of the 40S subunit in the proposed path of the mRNA through the subunit (105). The HCV IRES induces structural change in the 40S ribosome subunit, which may activate or promote initiation of translation without the help of certain canonical initiation factors (105). The initiation efficiency of HCV and pestivirus IRESes is very sensitive to local secondary structure at and around the initiation codon (51). These features of HCV and pestivirus IRESes resemble prokaryotic translation initiation (51).

Another group of naturally uncapped and non-polyadenylated RNAs has evolved a different cap-independent translation mechanism from IRES. They confer cap-independent translation via translation enhancement sequences in their 3'-UTR and do not confer internal ribosome entry. This group includes RNAs of viruses in *Luteovirus* BYDV (43, 44, 116, 118) and in the diverse *Tombusviridae* family: *Satellite tobacco necrosis virus* (STNV) (21, 70, 112), *Turnip crinkle carmovirus* (TCV), (96), *Hibiscus chlorotic ringspot carmovirus* (HCRV) (59), *Tomato bushy stunt tombusvirus* (TBSV) (125), and *Red clover necrotic mosaic dianthovirus* (RCNMV) (76). The BYDV TE has been discussed in detail in the section on *Barley yellow dwarf virus* gene expression.

The uncapped and non-polyadenylated *Satellite tobacco necrosis virus* (STNV) RNA is a monocistronic messenger. A 120nt translational enhancer domain (TED) in the 3'-UTR confers the cap-independent translation initiation (21, 70, 112). The TED binds eIF4E and may act primarily by recruiting the translational machinery to the RNA (70). It is unclear how the translational machinery is transferred to the 5'-UTR. The STNV 5'-UTR and TED have potential base-pairing to fulfill this bridge function, however, mutagenesis analyses does not support it (70).

The 3' UTR of RCNMV RNA1 has a cap-independent translation element with many properties similar to BYDV TE (76). RCNMV RNA1 and BYDV RNA contain a 18 nt conserved sequence, with one or two base differences, in their 3' UTRs (116). In the 18 nt conserved sequence, mutations known to knock out the BYDV TE function also eliminated function of the RCNMV TE (76). Cap-independent translation mediated by the TBSV translation enhancer was detectable only *in vivo* (125). This sequence overlaps cis-acting replication elements and is more 3'-proximal than the BYDV-like TEs (125). A 180 nt sequence including an essential hexanucleotide, GGGCAG, in the 3' UTR of HCRV confers cap-independent translation (59). This sequence functions with the IRES of encephalomyocarditis virus (59). The TCV translation enhancer located at the 5'-end of the 255 nt 3' UTR, is 150 nts long, and requires the 5' UTR to achieve optimal translation efficiency (96).

Tobacco necrosis virus (TNV) is the type member of genus *Necrovirus* in the *Tombusviridae* family. TNV RNA has no 5' cap (63) and no 3'poly(A) tail (69). Phylogenetic and secondary structure analyses predict the presence of a BYDV TE-like structure in all members in the genus *Necrovirus*. However, there has been no experimental

evidence to support the existence of a TE in the necroviruses. In chapter 4, I report that such a TE indeed exists in the 3' UTR of TNV strain D (TNV-D).

Translational control by the 3' untranslated region and poly(A) tail

Translational control is a major step of gene regulation for RNA viruses, oocytes, and other systems with little or no transcriptional control. Most of translational control elements and features in mRNAs exist in the 5' and 3' untranslated regions (Fig. 4) (71, 83, 84, 103, 123). The 5' m7GpppN cap, Kozak consensus sequence, upstream AUG(s), upstream open reading frame (uORF), internal ribosome entry sites (IRES), and iron-responsive element (IRE) are well-known examples of these control elements and features in 5' UTRs. 3'UTRs also contain many translational control elements, such as cytoplasmic polyadenylation elements (CPE), AU-rich elements (AREs), and an array of diverse binding sites for regulatory proteins (60, 98, 122, 123). On average, 3' UTRs are substantially longer that 5' UTRs (84). Average 5' UTR length is roughly constant for all taxa, but average 3' UTR length varies significantly (84). Consequently, the 3' UTR is a region with great regulatory potential. For example, translational controls by 3' UTR elements are essential in both male and female gametogenesis, early embryonic development, stem-cell proliferation, sex determination, neurogenesis, and erythropoiesis (60, 98, 122).

The 3' poly(A) tail is an important and well-studied element in determining translational efficiency. The poly(A) tail regulates both stability and translational efficiency of mRNAs. The 5' cap and poly(A) tail function synergistically to facilitate efficient translation initiation via circularization of mRNA (31, 47, 93, 94, 109, 121). eIF4E binds the 5' cap and is associated with eIF4G. eIF4G also binds poly(A) binding protein (PAPB),

which binds to the poly(A) tail. Thus mRNA is circularized (31, 47, 93, 94, 109, 121). The mRNA circularization provides a framework to understand how elements within 3' UTRs can control translation. However, some mRNAs lack a cap and/or poly(A) tail. How these mRNAs translate efficiently is an interesting and not well-understood question.

Many viral mRNAs and some cellular mRNAs have a cap structure but lack a poly(A) tail. Generally, a specific sequence within the 3' UTR replaces the function of the poly(A) tail. The 3' UTR pseudoknot domain of TMV RNA can functionally substitute for a poly(A) tail in plant and animal cells (34, 36). BMV 3' UTR has a similar effect as TMV 3' UTR in regulating translational efficiency of non-polyadenylated mRNAs in carrot protoplasts (33). Both BMV and TMV 3' UTRs are dependent on a 5' cap to function (34, 36). The 3' end consensus sequence of rotavirus is a functional alternative for the poly(A) tail, and its function depends on rotavirus protein NSP3 (89). The metazoan histone mRNAs also lack a poly(A) tail and have a stem-loop structure functionally mimicking poly(A) tail (34, 79, 124). The histone mRNA 3' terminal stem-loop is necessary and sufficient to support translation of non-polyadenylated mRNA and functionally depends on a 5' cap (34) and stem-loop binding protein (SLBP) (79, 99, 124).

Proteins binding to poly(A)-mimic sequences also have been found. These proteins functionally mimic PAPB. Rotavirus protein NSP3, a functional analogue of PAPB, binds to rotavirus poly(A) mimic sequence and eIF4G (22, 42, 89, 90, 114). The simultaneous interaction of NSP3 with the poly(A) mimic and eIF4G is necessary for efficient translation of rotavirus mRNA (114). NSP3 binding evicts PAPB from eIF4G (89). Host protein p102 binds both the 5'-leader and 3'- upstream pseudoknot domain of TMV (108). SLBP binds the poly(A)-mimic sequence of metazoan histone mRNA and is required for efficient

translation of histone mRNA both in vivo and in vitro (79, 99, 124). SLBP functions by interaction with eIF4G and eIF3 (64). The coat protein of *Alfalfa mosaic virus* (*Bromoviridae*) is proposed to act as a functional equivalent of PAPB (78), but the 3' UTR of AMV can not be replaced by a poly(A) tail (33).

TNV RNA has no 5' cap (63) and no 3'poly(A) tail (69). Whether a specific sequence within 3' UTR replaces the function of poly(A) tail is unclear. In chapter 5, I discovered that sequence at the 3' UTR of TNV strain D functionally mimics a poly(A) tail.

Tobacco necrosis virus gene expression

TNV-D has a positive single-stranded RNA genome with a size of 3762 nt. It encodes six open reading frames (Fig. 4). Viral proteins p22, p82, and p7 are translated from genomic RNA. p82 contains the active site of the viral RNA-dependent RNA polymerase and is probably translated via readthrough of the p22 ORF stop codon. Both p22 and p82 are required for virus replication. The downstream ORFs are translated from subgenomic mRNAs (69). p7a and p7b are translated from subgenomic RNA1. p7, p7a, and p7b are required for infection of plants. Coat protein p29 is translated from sgRNA2 and required for systemic infection and vector specificity (69). p22, p7a, and p29 are presumably translated via a cap-independent translation mechanism. The translation mechanisms of p7 and p7b are unclear. TNV RNA has no 5'cap (63) and no 3'poly(A) tail (69), yet it translates efficiently. Previously, we proposed the presence of a 3' TE structure in TNV strain A, based on conserved sequence and predicted secondary structure (44). Further phylogenetic and secondary structure analyses predict the presence of a similar TE structure in all members in genus *Necrovirus* (Fig. 5). In the *Necrovirus* TE-like structure, the loop at the end of a stable

stem-loop also has potential to base pair to a loop in the 5' UTR of *Necrovirus* RNAs (Fig. 5, bold). However, there has been no experimental evidence to support the existence of a TE in necroviruses. In chapter 4, I showed that the 3' UTR of TNV-D RNA contains a BYDV-like cap-independent translation element. In chapter 5, I demonstrated that the 3' UTR of TNV strain D RNA has a poly(A) mimic.

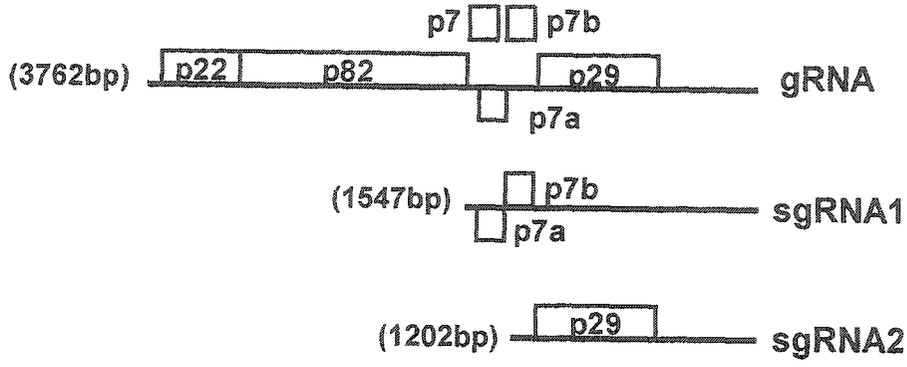


Fig. 4: Schematic of *Tobacco Necrosis Virus* strain D genome organization. Boxes represent open reading frames. Black lines represent genomic RNA (gRNA) and subgenomic RNAs (sgRNA). The sizes of gRNA and sgRNAs are indicated in brackets.

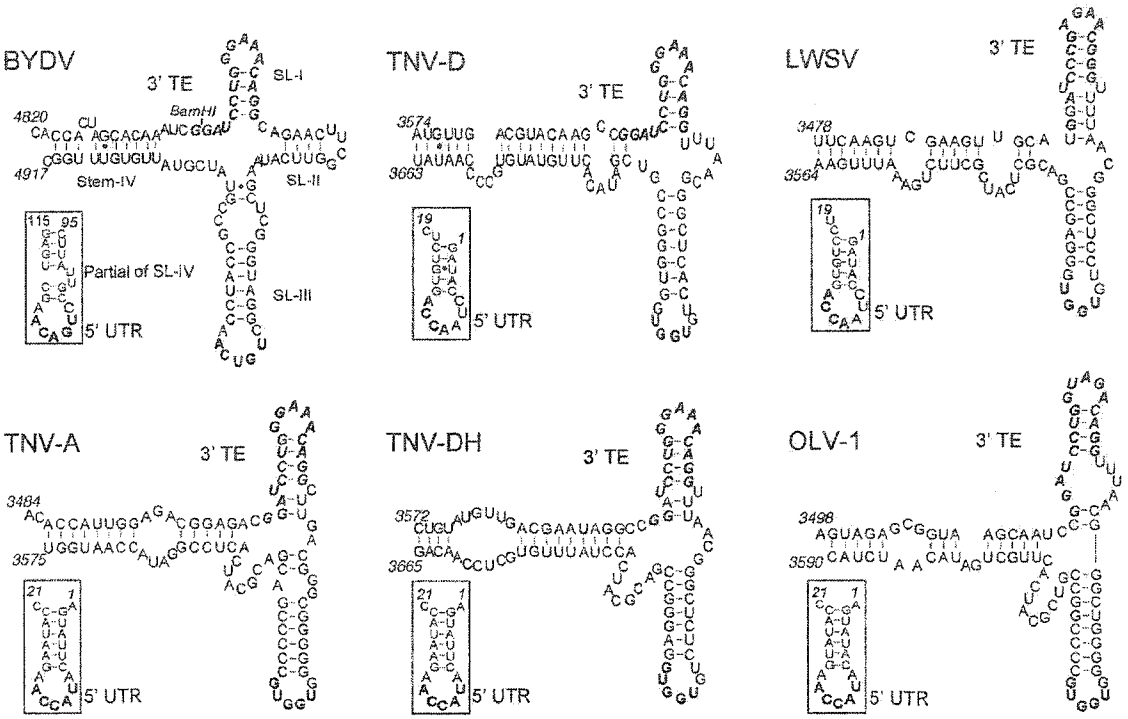


Fig. 5. Secondary structures of BYDV TE and putative *Necrovirus* TEs predicted by MFOLD (128). The structure of BYDV TE has been confirmed by structure probing (44). Bold italic: 18 nt conserved tract. Bold: potential base pairing between TEs and corresponding 5' UTR. Relevant portions of 5' UTRs are shown in rectangles. TNV-D: TNV strain D UK isolate (Genebank accession #: D_00942). TNV-DH: TNV strain D Hungary isolate (NC_003487). TNV-A: TNV strain A (NC_001777). OLV-1: *Olive latent virus 1* (NC_001721). LWSV: *Leek white stripe virus* (NC_001822).

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CHAPTER 2. TRANSLATIONAL CONTROL OF *BARLEY YELLOW DWARF VIRUS* GENE EXPRESSION BY ITS SUBGENOMIC RNA 2 *IN TRANS*

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Ruizhong Shen and W. Allen Miller

Abstract

It has been well established that proteins function as gene regulatory factors. The roles of RNAs as trans regulatory molecules are emerging and have expanded in recent years. However, gene regulation by regulatory viral RNA *in trans* is less noted and not yet well understood. Uncapped and nonpolyadenylated *Barley yellow dwarf virus* (BYDV) RNA has a translation element (3' TE) in its 3'untranslated region that confers cap-independent translation *in cis*. *In vitro* experiments led us to propose a trans-regulation model for BYDV translation. In this model, BYDV subgenomic RNA2 (sgRNA2), via its TE, functions as a riboregulator to selectively inhibit translation of genomic RNA relative to subgenomic RNA1 *in trans*. Therefore, the viral life cycle switches from the early to the late stage. Here we used reporter constructs to test the model both *in vitro* and *in vivo*. *In vitro*, BYDV sgRNA2 preferentially inhibited translation of gRNA versus sgRNA1. *In vivo*, BYDV sgRNA2 inhibited translation of gRNA, but has little effect on translation of sgRNA1. The 5' UTRs of gRNA and sgRNA1 determine the differential inhibition of translation of gRNA and sgRNA1 by sgRNA2 *in trans*. Both the *in vitro* and *in vivo* data prove the trans-regulation model. Our data show that sgRNA2 functions as a regulatory RNA to temporally control

viral gene expression. It reveals a new function for a viral subgenomic RNA and a novel translational control mechanism by a regulatory viral RNA.

Key words: BYDV /regulatory RNA/ subgenomic RNA /translational control

Introduction

Translational control is a widespread and important means of gene regulation. It can occur temporally and spatially at global or mRNA-specific levels. Messenger RNA-specific translation control usually results from the interaction of RNA-binding protein(s) with regulatory elements within 5' and/or 3' untranslated regions (UTRs) (Mazumder et al., 2003; Wickens et al., 2002; Wilkie et al., 2003). For example, iron regulatory proteins (IRPs) repress translation of iron-responsive element (IRE)-containing mRNAs by binding IRE within the 5' UTRs (Theil and Eisenstein, 2000; Thomson et al., 1999). PUF family proteins repress translation or enhance decay of target mRNAs by binding 3' UTRs elements (Wickens et al., 2002). Poly(A)-binding protein (PABP) plays a crucial role in poly(A)-mediated translational control by binding the poly(A) tail and other translation factors (Wilkie et al., 2003; Jacobson, 1996). More and more trans-acting RNAs are also reported to control translation of their target mRNAs by binding UTRs or by other mechanisms.

In addition to ribosomal RNAs and transfer RNAs, other classes of diverse non-coding RNAs have been discovered (Erdmann et al., 2001; Altuvia and Wagner, 2000; Ambros, 2003; Lease and Belfort, 2000a; Reinhart et al., 2000). These non-coding RNAs lack protein-coding capacity and function at the RNA level *in trans*. As riboregulators, their main function is posttranscriptional regulation of gene expression (Erdmann et al., 2001). They have varied functions, such as DNA markers *Xist* and *H19* involving dosage

compensation and imprinting (Brannan and Bartolomei, 1999; Kelley and Kuroda, 2000), development timing RNAs *lin*-4 and *let*-7 in *Caenorhdabditis elegans* (Ambros, 2000), abiotic stress signals OxyS RNA and DsrA RNA (Altuvia and Wagner, 2000; Lease and Belfort, 2000b), and biotic stress signals *his-1* RNA (Askew et al., 1994). In recent years, more attention has been drawn to small non-coding RNAs. These small non-coding RNAs can control transcription and translation. They include microRNAs (miRNA), small interfering RNAs (siRNA), small nucleolar RNAs (snoRNA), and small nuclear RNAs (snRNAs) (Ambros, 2003; Carrington and Ambros, 2003; Huttenhofer et al., 2002; Kiss, 2002; Weiner, 2003). Trans-acting regulatory RNAs from viruses have also been reported, such as *Red clover necrotic mosaic virus* (RCNMV) RNA2 (Sit et al., 1998), *Flock house virus* (FHV) subgenomic RNA (sgRNA) (Albarino et al., 2003; Eckerle and Ball, 2002), FHV RNA2 (Zhong and Rueckert, 1993), *Adenovirus* virus-associated (VA) RNAs (Mathews and Shenk, 1991), and Epstein-Barr virus (EBV) EBER RNAs (Bhat and Thimmappaya, 1983; Clarke et al., 1990). In this report we show that *Barley yellow dwarf virus* (BYDV) sgRNA2 acts as a riboregulator to control viral gene expression. BYDV sgRNA2 potentially encodes a small ORF that varies from 4.3 to 7.2 kDa. However, a protein product of the small ORF has not been detected *in vivo* (Rakotondrafara and Miller, personal communication).

Barley yellow dwarf virus (BYDV) has a positive single-stranded RNA genome of 5677 nts and encodes six open reading frames (ORF) (Fig. 1) (Miller et al., 1997; Miller et al., 2002). BYDV genomic RNA (gRNA) and subgenomic RNAs (sgRNAs) have no 5'-cap and no 3'-poly(A) tail (Allen et al., 1999). Cap-independent translation of BYDV RNAs is conferred by a 105 nt cap-independent translation element (3'TE) in the 3' untranslated

region (UTR). (Guo et al., 2000; Guo et al., 2001; Wang et al., 1997). The 3'TE fulfills the cap-independent translation function by recruiting the translational machinery and conveying the recruited machinery to the 5'UTR (E. Allen and W.A. Miller, personal communication; (Guo et al., 2001)). The 3'TE also inhibits translation *in trans* (Wang et al., 1997; Wang et al., 1999). In wheat germ extract, both the 3' TE and sgRNA2, which harbors the TE at its 5' end, trans-inhibit the translation of gRNA much more than that of sgRNA1 (Wang et al., 1999). Subgenomic RNA2 inhibits gRNA translation about ten times more efficiently than does the 3'TE alone. The inhibition does not require expression of ORF 6, but it requires a functional TE. When gRNA, sgRNA1, and sgRNA2 are all present at ratios approximating that in infected cells, translation of gRNA is almost totally inhibited while sgRNA1 remains as an efficient messenger (Wang et al., 1999). Based on these *in vitro* data, a trans-regulation model of gene expression is proposed (Wang et al., 1999): early in BYDV infection, only ORF1 and ORF2 (replicase genes) are translated via TE-mediated cap-independent translation. The viral RNA-dependent RNA polymerase is produced. Then, viral RNA is replicated, and gRNA and sgRNAs accumulate. The high level accumulation of sgRNA2, via the 3'TE, selectively inhibits translation of gRNA relative to sgRNA1 *in trans*. Structural proteins are then preferentially synthesized from sgRNA1. The BYDV viral life cycle switches from the early to late stage (Fig. 1) (Wang et al., 1999).

Here we tested the trans-regulation model both *in vitro* and *in vivo* by using reporter constructs. *In vitro*, BYDV sgRNA2 preferentially inhibits translation of gRNA versus sgRNA1. *In vivo*, BYDV sgRNA2 inhibits translation of gRNA, but has little effect on translation of sgRNA1. Our data show that BYDV sgRNA2 functions as a riboregulator to

temporally control viral translation. It reveals a new function for a viral subgenomic RNA and a novel translational control mechanism by a trans-regulatory viral RNA.

Results

BYDV sgRNA2 differently inhibits translation of gRNA and sgRNA1 *in vitro*

To test the trans-regulation model, we developed two reporter constructs, GfLuc and SG1rLuc (Fig. 2A). GfLuc is the reporter construct for gRNA, which encodes firefly luciferase ORF flanked by the UTRs of BYDV (Guo et al., 2000). SG1rLuc is the reporter construct for sgRNA1, which encodes renilla luciferase ORF flanked by the 5' UTR (nts 2670-2842) and 3' UTR (nts 4565-5677) of BYDV coat protein. To examine the validity of these two reporter constructs to represent gRNA and sgRNA1, we tested whether GfLuc and SG1rLuc behave the same as gRNA and sgRNA1 did in *in vitro* translation experiments.

In wheat germ extract, sgRNA2 trans-inhibits the translation of gRNA much more than that of sgRNA1 (Wang et al., 1999). When gRNA, sgRNA1, and sgRNA2 are all present at ratios approximating that in infected cells, translation of gRNA is almost totally inhibited while sgRNA1 remains as an efficient message (Wang et al., 1999). Here, we first tested the ability of sgRNA2 to inhibit translation of GfLuc or SG1rLuc *in trans* in wheat germ extract. A 5-fold molar excess of sgRNA2 inhibited translation of GfLuc to 52%, whereas the same amount of excess sgRNA2 inhibited translation of SG1rLuc to 78% and more than 10-fold molar excess of sgRNA2 was required to achieve 50% inhibition of SG1rLuc translation (Fig. 2B). When the molar excess of sgRNA2 to GfLuc or SG1rLuc was increased, the difference of sgRNA2 inhibition effects on GfLuc and SG1rLuc were dramatically increased (Fig. 2B). A 10-fold molar excess of sgRNA2 reduced translation of

GfLuc by 10-fold, but only reduced translation of SG1rLuc by less than two-fold (Fig. 2B). A 20-fold excess of sgRNA2 caused 50-fold drop in translation of GfLuc, but only 3-fold drop in translation of SG1rLuc (Fig. 2B). BFsgRNA2, which contains GAUC duplication in a Bam HI_{4837} site, had little or no inhibitory effects on the translation of GfLuc or SG1rLuc (Fig. 2B).

To more closely mimic the natural infection, gRNA reporter GfLuc, sgRNA1 reporter SG1rLuc, and sgRNA2 were presented in the same wheat germ extract translation reaction. Like the results with only GfLuc (or SG1rLuc) and sgRNA2 presented in the same reaction (Fig. 2B), sgRNA2 inhibited translation of GfLuc much more than that of SG1rLuc (Fig. 3A). More interestingly, the differential inhibition effects of sgRNA2 on translation of gRNA and sgRNA1 reporters were amplified when GfLuc, SG1rLuc, and sgRNA2 added together in wheat germ extract (compared Fig. 3A, Fig. 2B). BFsgRNA2 had little or no effects on the translation of GfLuc or SG1rLuc (Fig. 3A). Because sgRAN2 trans-inhibits translation of GfLuc much more than that of SG1rLuc (Fig. 2B, 3A), we predicted that the ratio of GfLuc product/sg1rLuc product would decrease in the same reaction when the excess sgRNA2 was increased. Indeed, the ratio decreased 3, 10, and 33-fold with 5, 10, and 20-fold excess sgRNA2, respectively (Fig. 3B).

Taken together, our data show that BYDV sgRNA2 differentially inhibits translation of gRNA and sgRNA1 reporters in wheat germ extract. Reporter constructs for gRNA and sgRNA1, GfLuc and SG1rLuc, behave in a similar way to gRNA and sgRNA1 themselves.

Coding region does not contribute to the differential inhibition effects

A possible cause of differential inhibition of GfLuc and SG1rLuc by sgRNA2 is due to the coding region. To examine this, we constructed another sgRNA1 reporter construct, SG1fLuc, and tested the effect of sgRNA2 on its translation *in trans* in wheat germ extract. SG1fLuc encodes firefly luciferase ORF flanked by BYDV coat protein 5' UTR (nts 2670-2842) and a shortened 3' UTR (nts 4809-5677) (Fig. 2A). We found SG1fLuc and SG1rLuc behave very similar (Fig. 4). Thus, as expected, coding regions do not account for the differential effects of sgRNA2 on translation of GfLuc and SG1rLuc.

BYDV PAV6, but not PAV6ΔSG2, differentially inhibits translation of GfLuc and SG1rLuc *in vivo*

Having validated that reporter constructs GfLuc and SG1rLuc truly represent gRNA and sgRNA1, we further tested the trans-regulation model in oat protoplasts by using the two constructs. We developed a 2-step electroporation method. First oat protoplasts were inoculated with infectious BYDV PAV6 or PAV6ΔGS2 RNA by electroporation. PAV6ΔSG2 has one point mutation at position 4810 (G to C) of PAV6, which prevents sgRNA2 synthesis (Koev and Miller, 2000). After 24-hour incubation, protoplasts were inoculated again with GfLuc or SG1rLuc. Then firefly luciferase and renilla luciferase were analyzed after another 4-hour incubation. Inoculation of 1 and 2pmol of PAV6 RNA in the first step caused 80% and 52% drop in translation of gRNA reporter GfLuc, but only caused 20% and 9% drop in translation of sgRNA1 reporter SG1rLuc, respectively (Fig. 5A). Inoculation of 1 and 2pmol of PAV6ΔSG2 RNA in first step also caused 42% and 28% drop in translation of gRNA reporter GfLuc, respectively, and both caused less than 10% drop in

translation of sgRNA1 reporter SG1rLuc (Fig. 5A). Thus, PAV6ΔSG2 has much less inhibitory effects on translation of Gfluc.

When GfLuc and SG1rLuc were inoculated together in the second step electroporation, inoculation of 1pmol of PAV6 RNA in first step caused 88% drop in translation of GfLuc, but did not inhibit translation of SG1rLuc (Fig. 5C). Thus, as observed *in vitro*, the differential inhibition effects of PAV6 on translation of GfLuc and SG1rLuc were amplified when both reporters were presented together. The ratio of GfLuc product/SG1rLuc product was decreased 17-fold (Fig. 5D). PAV6ΔSG2 also had much less inhibitory effects on the ratio and translation of Gfluc and SG1rLuc (Fig. 5C, 5D). PAV6 and PAV6ΔSG2 had similar replication level except the later did not produce sgRNA2 (Fig. 5B, (Koev and Miller, 2000)). Thus, sgRNA2 is responsible for the differential inhibition of GfLuc and SG1rLuc. sgRNA2 and TE sequence within gRNA and sgRNA1 probably contributed to the inhibition by PAV6ΔSG2.

BYDV sgRNA2 expressed in transgenic *Arabidopsis* differentially inhibits translation of gRNA and sgRNA1 *in vivo*

We showed that sgRNA2 is responsible for the differential inhibition of GfLuc and SG1rLuc *in vivo* in the previous section. But we were not clear whether sgRNA2 alone is sufficient for the inhibition function *in vivo*. To examine the trans-inhibition effects of sgRNA2 out of other potential regulatory elements in BYDV RNA, we constructed transgenic *Arabidopsis* lines expressing sgRNA2 under the control of estradiol inducible promoter. Fresh protoplasts were then prepared from leaves and transcription of sgRNA2 and BFsgRNA2 was induced by β-17-estradiol (Fig. 6B). Four hours after PEG transfection

of protoplasts with GfLuc and SG1rLuc, luciferase activities were measured. BYDV sgRNA2 transcribed from transgenic Arabidopsis caused the ratio of GfLuc product/SG1rLuc product drop 60%, while BFsgRNA2 caused 36% drop (Fig. 6A). The sgRNA2 and BFsgRNA2 transcribed from transgenic *Arabidopsis* is capped and polyadenylated. Thus, capped and polyadenylated sgRNA2 without involvement of other BYDV elements is sufficient to differentially inhibit translation of gRNA and sgRNA1.

Discussion

Subgenomic RNA2 preferentially trans-inhibits translation of gRNA versus sgRNA1 both *in vitro and in vivo*

BYDV sgRNA2 has been showed previously to preferentially trans-inhibit translation of gRNA versus sgRNA1 in wheat germ extract (Wang et al., 1999). Here we reported that BYDV sgRNA2 trans-inhibits translation of gRNA, but has little or no effect on translation of sgRNA1 *in vivo* (Fig. 5A, 5C). The specificity of trans-inhibition by sgRNA2 is illustrated by the inability of BFsgRNA2 to trans-inhibit (Fig. 2 and 3). BFsgRNA2 is the *Bam* HI fill-in mutant of sgRNA2 and has a GAUC duplication in *Bam* HI_{4837} site (Wang et al., 1999). These data prove the trans-regulation model. Interestingly, although sgRNA2 preferentially inhibits translation of gRNA and gRNA reporter *in vitro* (Fig. 2, Fig. 3, and (Wang et al., 1999)), it does also trans-inhibit translation of sgRNA1 (Wang et al., 1999) and sgRNA1 reporter constructs SG1rLuc and SG1fLuc (Fig. 2-4). The difference of inhibition of sgRNA1 by sgRNA2 between *in vitro* and *in vivo* conditions suggested that the trans-regulation model needs modification. In the modified trans-regulation model, we propose: early in BYDV infection, only ORF1 and ORF2 (replicase genes) are translated via TE-

mediated cap-independent translation. The viral RNA-dependent RNA polymerase is produced. Then, viral RNA is replicated, and gRNA and sgRNAs accumulate. The high level accumulation of sgRNA2 trans-inhibits translation of gRNA. Replication is inhibited and structural proteins are translated from sgRNA1. The viral life cycle enters its late stage.

Another interesting observation is that the differential inhibition effects of sgRNA2 on translation of gRNA and sgRNA1 reporters were amplified when GfLuc, SG1rLuc, and sgRNA2 were added together to mimic natural infection both *in vitro* and *in vivo* (compare Fig. 2B and 3A, Fig. 5A and 5C). This suggests another level of gene regulation, in which BYDV RNAs are well coordinated with each other.

5'UTRs of gRNA and sgRNA1 determine the differential inhibition effects

The differential inhibition effects of sgRNA2 on translation of GfLuc and SG1rLuc reported here recapitulated the results of Wang et al. (Wang et al., 1999). They showed that the differential inhibition effects of sgRNA2 on translation of gRNA and sgRNA1. Thus, the coding regions are not responsible for the differential effects. This is supported by the observation that another sgRNA1 reporter (SG1fLuc) with a different coding region behaved in a manner similar to SG1rLuc did (Fig. 4). The differential inhibition of translation by sgRNA2 was previously proposed due to the different 5' UTR sequences of gRNA and sgRNA1 and the proximity of 3'TE to the start codon (Wang et al., 1999). Our data disprove the second reason. The distances between the start codon and the 3'TE in GfLuc and SG1rLuc are 1652 and 1193 nts, respectively (Fig. 2A). There is only a 459 nt difference between the two distances, much smaller than the difference of 2712 nts between that of gRNA and sgRNA1. Secondly, two sgRNA1 reporters, SG1rLuc and SG1fLuc, behaved

similarly, but the distances between the start codon and the 3'TE in SG1fLuc and SG1rLuc are 1652 and 1193 nts, which are the same as that in GfLuc and SG1rLuc. Thus, we conclude that the 5'UTRs of gRNA and sgRNA1 determine the differential inhibition of translation of gRNA and sgRNA1 by sgRNA2 *in trans*. We do not exclude the possibility that host factor(s) may be involved.

***Barley yellow dwarf virus* sgRNA2 is a riboregulator and functions at the translation level**

Our data provide evidence that BYDV is a riboregulator. BYDV sgRNA2 is a sense RNA and functions *in trans* to inhibit the translation of gRNA. Wang et al. (Wang et al., 1999) have shown *in vitro* that the inhibition of translation does not require expression of ORF6. On the other hand, protein product of the potential small ORF6 within sgRNA2 has not been detected *in vivo* despite much effort (Rakotondrafara and Miller, person communication). Thus, sgRNA2 functions as a regulatory RNA, not a messenger RNA, at the level of translation.

Other trans-regulatory RNAs from viruses have also been reported. RCNMV RNA2 has a 34nt trans-activator sequence, which is required for transcription of sgRNA from RNA1 (Sit et al., 1998). The trans-activator fulfills its function via base pairing between RNA1 and RNA2. This is an example of transcriptional regulation by trans-reglatory RNA in virus. FHV sgRNA transactivates the replication of RNA2 (Albarino et al., 2003; Eckerle and Ball, 2002). The trans-activation is mediated by sgRNA rather than by its translation product. FHV RNA2, not its translation product, down-regulates synthesis of sgRNA from RNA1 (Zhong and Rueckert, 1993). *Adenovirus* virus-associated (VA) RNAs are required

for efficient expression of late viral genes (Mathews and Shenk, 1991; Thimmappaya et al., 1982). The non-coding, 160 nts VA RNAs are common to all adenoviruses and transcribed by RNA polymerase III (Mathews and Shenk, 1991; Weinmann et al., 1974). VA RNAs protect against dsRNA-activated inhibitor (DAI)-mediated phosphorylation of eIF-2α by binding DAI (Schneider et al., 1985). EBV EBER RNAs are also transcribed by RNA polymerase III and may have similar function as VA RNAs (Bhat and Thimmappaya, 1983; Clarke et al., 1990). Among these viral regulatory RNAs, only BYDV sgRNA2, VA RNAs, and EBER RNAs are non-coding RNAs. Others function as both a coding RNA and a non-coding regulatory RNA. Such a phenomenon occurs with tmRNA, which serves as both a message RNA and a function –specific tRNA (Gillet and Felden, 2001).

Potential mechanism(s) of trans-inhibition of translation of gRNA by sgRNA2

Considering the nature of RNA, regulatory RNAs of gene expression could use two mechanisms. One mechanism is the RNA-RNA interaction, i.e. regulatory RNA base pairs to target RNA(s). The base pairing could change the secondary structure of target RNA(s), block protein(s) binding the target RNA(s), or recruits protein(s) to the target RNA(s). For example, the miRNAs and siRNAs function via RNA-RNA interaction (Ambros, 2003; Carrington and Ambros, 2003; Nelson et al., 2003). The *E. coli* oxidative stress signals *OxyS* RNA acts as a global regulator to activate or repress the expression of 40 genes, including the transcriptional activator gene *fhlA* and sigma(s) subunit of RNA polymerase gene *rpoS (Altuvia et al., 1997; Altuvia et al., 1998; Zhang et al., 1998).* The *OxyS* RNA represses translation of *fhlA* and *rpoS* by pairing with a complementary sequence overlapping the ribosome-binding site of the *fhlA* and *rpoS* mRNA, thus blocking ribosome binding. E. coli

DsrA RNA also represses or enhances translation of different transcription factors by sequence-specific RNA-RNA interaction (Review in Lease and Belfort, 2000a). Whether BYDV sgRNA2 adopts such a mechanism needs further investigation.

The second mechanism is that regulatory RNAs function as molecular decoys to compete with protein(s) binding to target RNAs or protein(s). VA RNAs are one example of regulatory RNAs using such a mechanism. VA RNAs compete with dsRNA for binding DAI and therefore, block autophosphorylation of DAI activated by dsRNA. Thus, phosphorylation of eIF-2α mediated by phosphorylated DAI is blocked (Mathews and Shenk, 1991; Schneider et al., 1985). We previously proposed TE/sgRNA2 trans-inhibits gene expression by titering out the necessary and/or limiting translation initiation factor(s) ((Wang et al., 1997), Shen and Miller manuscript in preparation). But it is hard to explain why sgRNA2 trans-inhibits only translation of gRNA but not that of sgRNA1. Other elements, such as host factor(s) and/or the 5' UTRs of gRNA and sgRNA1, may also be involved in the mechanism. Although further investigations are needed to elucidate the mechanism, the selective translation inhibition by a viral sgRNA reported here shows a novel translational control strategy by a trans-regulatory RNA in viruses.

A novel function of viral sugenomic RNA

Subgenomic RNAs are 5'-truncated, 3' co-terminal versions of viral genomic RNA, and are synthesized during viral replication from gRNA. SgRNA is a strategy used by many viruses to express their 3' proximal genes. In this report, we revealed a new function for a viral subgenomic RNA: BYDV sgRNA2 functions as a riboreguilator to temporally control viral gene expression. BYDV sgRNA2 preferential inhibits translation of ORF 1 and 2

(nonstructural proteins) from gRNA, but have little effect on translation of ORF 3, 4 and 5 (structural proteins) from sgRNA1 (Fig. 5A, 5C). The shutoff of BYDV early genes (nonstructural proteins) expression by accumulation of sgRNA2 allows an additional level of temporal control. The first level of temporal control for BYDV is the subgenomic RNAs synthesis, which ensures that non-structural proteins are expressed after structural proteins are expressed and RNAs are replicated.

We previously reported that BYDV sgRNA2, also as a RNA regulator, negatively regulates the replication of BYDV RNA (Shen and Miller, Manuscript in preparation). The phenomenon that a viral sgRNA functions as a regulator RNA is also reported for insect nodavirus *Flock house virus* (FHV). In FHV, the replication of RNA2 is dependent on the synthesis of RNA3 from RNA1 (Albarino et al., 2003; Eckerle and Ball, 2002). RNA3 is a subgenomic RNA synthesized from RNA1. The trans-activation of RNA2 replication by RNA3 does not require the translation products from RNA3. And the RNA3 sequence in the context of RNA1 cannot transactivate the replication of RNA2 (Eckerle and Ball, 2002). As a riboregulator, FHV sgRNA functions at the level of replication. BYDV sgRNA2 reported here functions at the level of translation. BYDV sgRNA2 trans-inhibits replication (Shen and Miller, manuscript in preparation). Our data (including those reported here) suggest that the mechanism is inhibition of translation of BYDV genomic RNA. The selective translation inhibition by a viral sgRNA reported here shows a novel function for a viral subgenomic RNA, which acts as a riboregulator to temporally control translation.

Materials and Methods

Plasmids and RNA constructs

The full-length infectious clone of BYDV-PAV, pPAV6, was used for transcribing infectious BYDV genomic RNA (Di et al., 1993). The sgRNA2 knockout mutant clone of BYDV-PAV, pPAV6ΔSG2, was previously referred to as SG2G/C (Koev and Miller, 2000). It has one point mutation at position 4810 (G to C), which prevents sgRNA2 synthesis. pSG2 and pSG2BF allow T7 transcription of sgRNA2 and its mutant BFsgRNA2, respectively (Wang et al., 1999). BFsgRNA2 contains a GAUC duplication at the *BamH* I site (BF) of sgRNA2 that destroys the *in vitro* trans-inhibition function of sgRNA2 (Wang et al., 1999).

Clone pGfLuc was constructed in (Guo et al., 2000), where it was called p5'UTR-LUC-TE869-$(A)_{60}$. GfLuc is a gRNA reporter and encodes the firefly luciferase ORF flanked by the UTRs of BYDV. pRenilla-CP393 was cloned by replacing nts 2843-4565 of BYDV with renilla ORF. pSG1rLuc was cloned by ligating the *Bst1107* I-*Bsm* I fragment of pRenilla-CP393 into *Bst1107* I/*Bsm* I-cut pSG1 (Koev et al., 1999). SG1rLuc is a sgRNA1 reporter and encodes renilla luciferase flanking by BYDV coat protein UTRs (Fig. 2A). pSG1fLuc is cloned by replacing 5' UTR of pGfLuc with the 5'UTR of coat protein. SG1fLuc is another sgRNA1 reporter and encodes firefly luciferase ORF flanking by UTRs of coat proteins with a shorten 3' UTR (Fig. 2A).

All constructs were verified by automatic sequencing at the Nucleic Acid Facility of Iowa State University on an ABI377 sequencer (Applied Biosystems, Foster City, CA).

In vitro transcription and translation

All RNAs are uncapped and were synthesized by *in vitro* transcription by using the T7 MegaScript kits (Ambion, Austin, TX) as per manufacturer's instructions. All constructs were linearized with *Sma* I before transcription. *In vitro* translation in wheat germ extract (Promega) and luciferase assay were performed as in Shen and Miller (Manuscripts accepted). All luciferase assays were performed in at least three independent experiments, each of which was conducted in duplicate or triplicate.

2-step electroporation

In the first step, oat protoplasts were inoculated with infectious BYDV PAV6 or PAV6ΔGS2 RNA by electroporation and incubated for 24 hours at room temperature. In second step, protoplasts were inoculated again with GfLuc, SG1rLuc, or both. Then firefly luciferase and renilla luciferase were analyzed after another 4-hour inoculation. Oat (*Avena sativa* cv. Stout) protoplasts were prepared and electroporated with RNA as described in Dinesh-Kumar and Miller (Dinesh-Kumar and Miller, 1993). Luciferase assays were performed as in Shen and Miller (manuscript accepted), and the Promega Stop-N-Glo™ (Madison, WI) system was used to assay both luciferase activities.

Northern blot analysis

Total RNAs were extracted from oat protoplasts 24-hour post-inoculation or *Arabidposis* 8-hour after induction by using the Trizol reagent (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. RNAs were then analyzed by Northern blot as described in (Koev et al., 1999). A ^{32}P-labeled probe complementary to the 1.5 kb 3' end of BYDV-PAV genome RNA was used to detect BYDV gRNA and sgRNAs (Koev et al., 1999).

Construction of transgenic *Arabidopsis* lines, and preparation and transfection of protoplasts

Binary vectors pERSG2 and pERSG2BF were constructed by insertion of PCR-amplified BYDV sgRNA2 and BFsgRNA2 into *Xho* I /*Spe* I-cut pER8 (Zuo et al., 2000), respectively. Transformation of *Agrobacterium tumefaciens strain* GV3101::pMP90 was done as in (Shen and Forde, 1989) by using MicroPulser (Bio-Rad). Transformation of *Arabidopsis thaliana* Col-0 ecotype was carried out by floral dip as in (Clough and Bent, 1998). T4 seeds were used for experiments. Fresh protoplasts were prepared from 4-6 week-old leaves as in (Sheen, 2002). Expression of sgRNA2 and BFsgRNA2 was induced for 4 hours by adding 15μm of β-17-estradiol into the media. β-17-estradiol was prepared in dimethyl sulfoxide (DMSO). Then protoplasts were transfected with GfLuc and SG1fLuc by using PEG as in (Sheen, 2002) and cultured in media containing 15μm of β-17-estradiol. Luciferase activities were analyzed 4-hour after transfection.

Acknowledgements

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Figure Legends

Fig. 1. Trans-regulation model of BYDV gene expression. In the early stage of BYDV infection, viral RNA-dependent RNA polymerase is produced via TE-mediated cap-independent translation of gRNA (1). Viral replication and transcription occur (2). Viral RNAs accumulate and viral proteins are produced (1, 2, and 3). The accumulation of sgRNA2 trans-inhibits translation of BYDV RNA-dependent RNA polymerase (RdRp) from gRNA (4), hence inhibits the replication of BYDV RNAs (5). However, the synthesis of structural proteins is less affected (4). Genomic RNAs are used for package (6). Thus, virus enters the late stage of its life cycle in which structural proteins are synthesized and viruses are packaged (3 and 6).

Fig. 2. A. Schematic of gRNA reporter GfLuc and sgRNA1 reporters SG1rLuc and SG1fLuc. B. Differential effects of sgRNA2 and BFsgRNA2 *in trans* on translation of GfLuc and SG1rLuc. 0.2pmol GfLuc (or SG1rLuc) and indicated molar excess fold of sgRNA2 or BFsgRNA2 transcripts were added to 25μl wheat germ translation system and translated for 1 hour at 25^0C. The activity of GfLuc (or SG1rLuc) without sgRNA2 added was defined as 100%.

Fig. 3. Differential effects of sgRNA2 or BFsgRNA2 *in trans* on translation of GfLuc and SG1rLuc in the same reaction. 0.2pmol GfLuc, 0.2pmol SG1rLuc, and indicated molar excess fold of sgRNA2 or BFsgRNA2 transcripts were added together to 25μl wheat germ translation system and translated for 1 hour at 25^0C. A. The activities of GfLuc and SG1rLuc

were plotted individually against sgRNA2 and BFsgRNA2 fold excess. The activity of GfLuc (or SG1rLuc) without sgRNA2 added was defined as 100%. B. Ratio changes of GfLuc/SG1rLuc with increased molar excess fold of sgRNA2 or BFsgRNA2.

Fig. 4. Effects of coding region on trans-inhibition of translation of sgRNA1 reporters SG1rLuc and SG1fLuc by sgRNA2. 0.2pmol SG1rLuc (or SG1fLuc) and indicated molar excess fold of sgRNA2 or BFsgRNA2 transcripts were added to 25μl wheat germ translation system and translated for 1 hour at 25^0C. The activity of SG1rLuc (or SG1fLuc) without sgRNA2 added was defined as 100%.

Fig. 5. Differential effects of PAV6 and PAV6ΔSG2 replication on translation of GfLuc and SG1rLuc in oat protoplasts. 24 hours after inoculation of PAV6 or PAV6ΔSG2 RNA, oat protoplasts were electroporated again with 1pmol GfLuc, 1pmol SG1rLuc, or both. Luciferase activities were analyzed 4 hours later. A. 1pmol GfLuc (or SG1rLuc) was inoculated into infected oat protoplasts. B. Northern blot analysis of replication of PAV6 and PAV6ΔSG2. C. The activities of GfLuc and SG1rLuc were plotted individually against amount of viral RNA inoculums. 1pmol GfLuc and 1pmol SG1rLuc were inoculated together into infected oat protoplasts. D. Ratio changes of GfLuc/SG1rLuc with increased viral RNA inoculums.

Fig. 6. A. Effects of sgRNA2 and BFsgRNA2 transcribed from fresh transgenic *Arabidopsis* leaf protoplasts induced by β-17-estradiol on the ratio of GfLuc/SG1rLuc. β-17-estradiol was prepared in dimethyl sulfoxide (DMSO). B. Northern blot analysis of expression level

of sgRNA2 and BFsgRNA2 from fresh transgenic *Arabidopsis* leaf protoplasts induced by β-17-estradiol. 1, sgRNA2 *in vitro* transcripts. 2, Vector line mock induced with DMSO. 3, BFsgRNA2 line mock induced with DMSO. 4, sgRNA2 line mock induced with DMSO. 5, Vector line induced with β-17-estradiol. 6, BFsgRNA2 line induced with β-17-estradiol. 7, sgRNA2 line induced with β-17-estradiol.

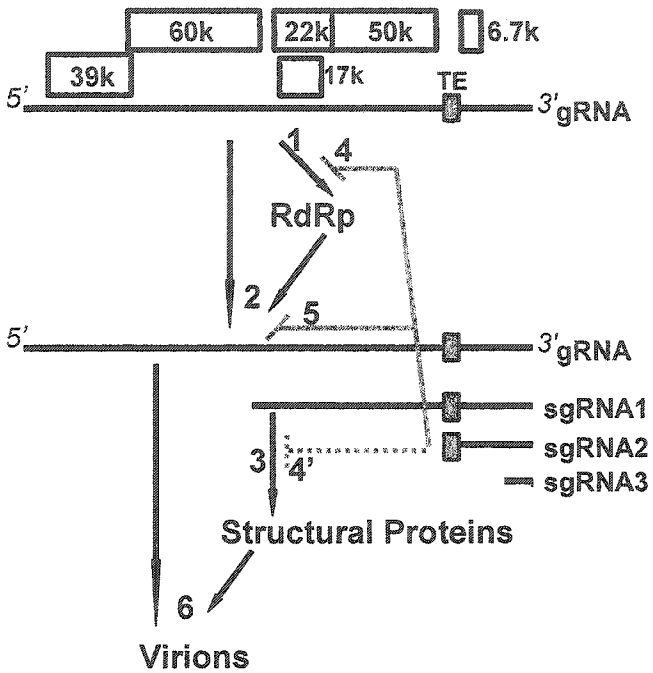
60k
22k
50k
6.7k
39k
17k
TE
5'
3'gRNA
1
4
RdRp
2
5
5'
3'gRNA
sgRNA1
sgRNA2
sgRNA3
3
4'
Structural Proteins
6
Virions

Fig. 1

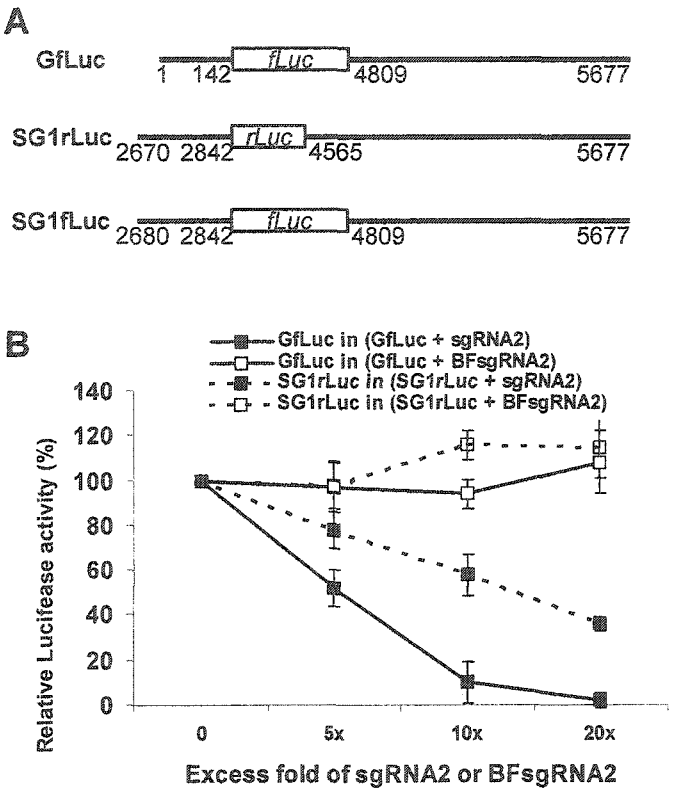
A
GfLuc
1 142
fLuc
4809
5677
SG1rLuc
2670 2842
rLuc
4565
5677
SG1fLuc
2680 2842
fLuc
4809
5677
B
GfLuc in (GfLuc + sgRNA2)
GfLuc in (GfLuc + BFsgRNA2)
SG1rLuc in (SG1rLuc + sgRNA2)
SG1rLuc in (SG1rLuc + BFsgRNA2)
Relative Lucifease activity (%)
140
120
100
80
60
40
20
0
0
5x
10x
20x
Excess fold of sgRNA2 or BFsgRNA2

Fig. 2

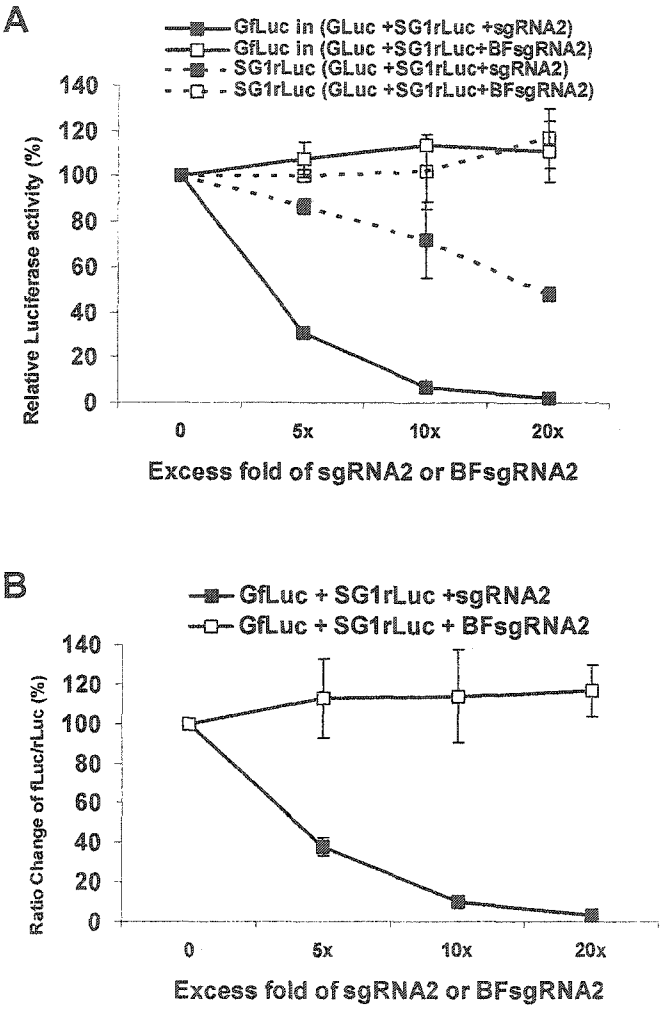
A
GfLuc in (GLuc +SG1rLuc +sgRNA2)
GfLuc in (GLuc +SG1rLuc+BFsgRNA2)
SG1rLuc (GLuc +SG1rLuc+sgRNA2)
SG1rLuc (GLuc +SG1rLuc+BFsgRNA2)
Relative Luciferase activity (%)
140
120
100
80
60
40
20
0
0
5x
10x
20x
Excess fold of sgRNA2 or BFsgRNA2
B
GfLuc + SG1rLuc +sgRNA2
GfLuc + SG1rLuc + BFsgRNA2
Ratio Change of fLuc/rLuc (%)
140
120
100
80
60
40
20
0
0
5x
10x
20x
Excess fold of sgRNA2 or BFsgRNA2

Fig. 3

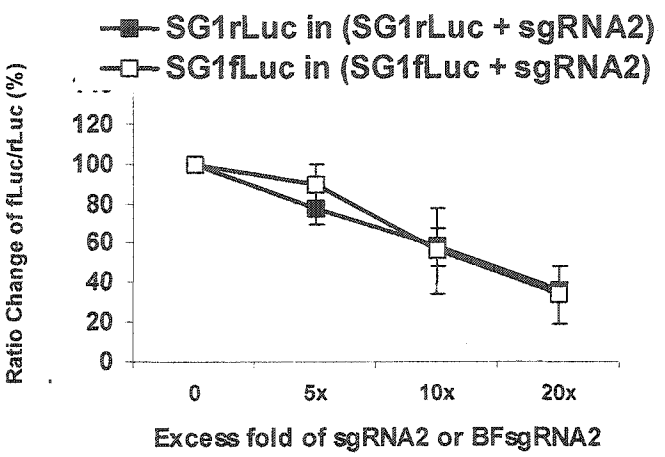
SG1rLuc in (SG1rLuc + sgRNA2)
SG1fLuc in (SG1fLuc + sgRNA2)
Ratio Change of fLuc/rLuc (%)
120
100
80
60
40
20
0
0
5x
10x
20x
Excess fold of sgRNA2 or BFsgRNA2

Fig. 4

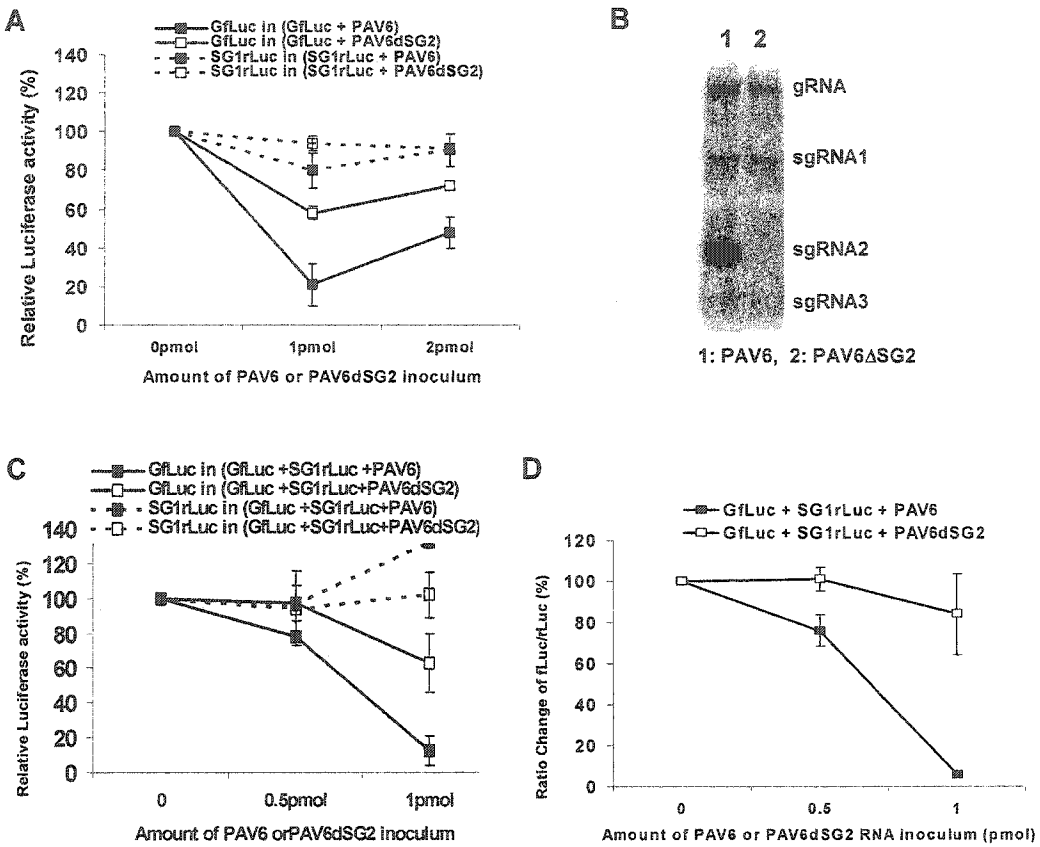
A
GfLuc in (GfLuc + PAV6)
GfLuc in (GfLuc + PAV6dSG2)
SG1rLuc in (SG1rLuc + PAV6)
SG1rLuc in (SG1rLuc + PAV6dSG2)
Relative Luciferase activity (%)
140
120
100
80
60
40
20
0
0pmol
1pmol
2pmol
Amount of PAV6 or PAV6dSG2 inoculum
B
1 2
gRNA
sgRNA1
sgRNA2
sgRNA3
1: PAV6, 2: PAV6ΔSG2
C
GfLuc in (GfLuc +SG1rLuc +PAV6)
GfLuc in (GfLuc +SG1rLuc+PAV6dSG2)
SG1rLuc in (GfLuc +SG1rLuc+PAV6)
SG1rLuc in (GfLuc +SG1rLuc+PAV6dSG2)
Relative Luciferase activity (%)
140
120
100
80
60
40
20
0
0
0.5pmol
1pmol
Amount of PAV6 orPAV6dSG2 inoculum
D
GfLuc + SG1rLuc + PAV6
GfLuc + SG1rLuc + PAV6dSG2
Ratio Change of fLuc/rLuc (%)
120
100
80
60
40
20
0
0
0.5
1
Amount of PAV6 or PAV6dSG2 RNA inoculum (pmol)

Fig. 5

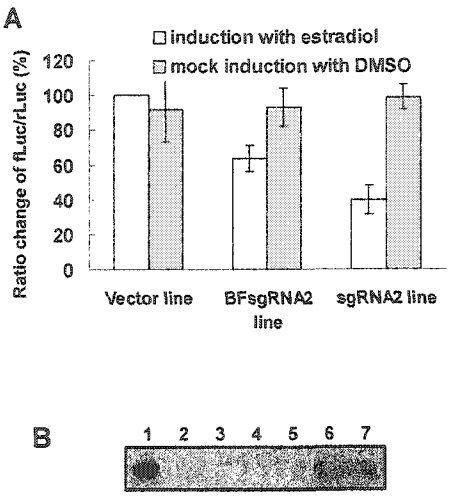
A
induction with estradiol
mock induction with DMSO
Ratio change of fLuc/rLuc (%)
120
100
80
60
40
20
0
Vector line
BFsgRNA2 line
sgRNA2 line
B
1 2 3 4 5 6 7

Fig. 6

CHAPTER 3. SUBGENOMIC RNA AS A *TRANS*-REGULATOR: A VIRAL SUBGENOMIC RNA NEGATIVELY REGULATES VIRAL REPLICATION

A paper to be submitted to the *Journal of Virology*

Ruizhong Shen and W. Allen Miller

Abstract

Gene expression of RNA viruses is often controlled by primary and secondary structures of viral RNA *in cis*. However, gene regulation conferred by viral RNA *in trans* is less noted and not yet well understood. *Barley yellow dwarf virus* (BYDV) RNA has a cap-independent translation element (3'TE) in its 3' untranslated region that confers cap-independent translation *in cis*. *In vitro* experiments led us to propose that in natural infection, accumulation of sgRNA2, which contains TE in its 5'UTR, would trans-inhibit BYDV replication by inhibiting translation of the viral polymerase from genomic RNA. Here we tested the hypothesis. We showed that: (1) nonreplicating TE or sgRNA2 RNA *in trans* inhibits BYDV replication; (2) Replicating TE RNA introduced into *Brome mosaic virus* (BMV) trans-inhibits BYDV replication; (3) sgRNA2 from natural infection of BYDV trans-inhibits GFP expression from BMV RNA ; (4) sgRNA2 from natural infection of BYDV trans-inhibits translation of reporter mRNA; 5) BYDV 3'TE *in cis* enhances GFP expression from BMV RNA. We conclude that the BYDV TE serves different roles *in cis* and *in trans*. *In cis*, it confers cap-independent translation and increases translation of capped and uncapped RNA. *In trans*, it functions as a riboregulator to negatively regulate viral replication, via inhibition of translation, and switches gRNA from translation to

encapsidation and replication by the existing viral polymerase. Our data reveal that viral subgenomic RNA do not always serve as message RNAs, and instead can perform important regulatory functions.

Introduction

The dependence of viruses on host cellular machinery for propagation has led viruses to adopt many strategies to orchestrate viral and host gene expression in favor of maximum viral reproduction. Because most RNA viruses replicate in the cytoplasm of their host cells, translation rather than transcription is often the major step at which viral gene expression is regulated. Many viral translational control strategies are conferred by RNA structures in *cis* (Gale et al., 2000; Macdonald, 2001; Mazumder et al., 2003). In recent years, diverse regulatory RNAs, known as riboregulators, have been discovered in both prokaryotes and eukaryotes (Altuvia and Wagner, 2000; Lease and Belfort, 2000; Reinhart et al., 2000). Riboregulators function in *trans* and mainly post-transcriptionally. Only a few *trans*-regulatory RNAs from, or related to, viruses have been reported (Albarino et al., 2003; Das et al., 1998; Eckerle and Ball, 2002; Sit et al., 1998). Here we show that subgenomic RNA 2 of *Barley yellow dwarf virus* (BYDV) acts as a riboregulator to negatively regulate viral replication *in trans*.

BYDV is the type member of genus *Luteovirus* in the family *Luteoviridae*. BYDV RNA has a complex set of regulatory primary and secondary structures that confer many non-canonical translational control mechanisms (Miller et al., 2002). Such mechanisms include cap-independent translation (Guo et al., 2001; Wang et al., 1997; Wang et al., 1999),

–1 ribosomal frameshifting (Barry and Miller, 2002; Paul et al., 2001), leaky scanning (Chay et al., 1996), and stop codon readthrough (Brown et al., 1996). BYDV has a positive sense RNA genome of 5677 nts and encodes six open reading frames (ORFs) (Fig. 1; Miller et al., 1997; Miller et al., 2002). Its genomic RNA (gRNA) and subgenomic RNAs (sgRNAs) have no 5'-cap and no 3'-poly(A) tail (Allen et al., 1999), yet they are translated efficiently. A 105 nt cap-independent translation element (3'TE) in the 3' untranslated region (UTR) of BYDV RNA facilitates efficient translation (Guo et al., 2000). The 3'TE binds translation factors (E. Allen and W.A. Miller, personal communication) and these presumably recruit the ribosome. The TE is brought into proximity with the 5' end where translation initiates by direct base pairing to the 5' UTR (Guo et al., 2001). The 3'TE functions both in the 5'UTR and in the 3'UTR (Guo et al., 2000). Most of the 869 nt 3'UTR of BYDV gRNA is required for full cap-independent and poly(A) tail-independent translation in oat protoplasts (Guo et al., 2000; Wang et al., 1999). SgRNA2 corresponds to the 869 nt 3'UTR of BYDV RNA and the TE is at the 5' end of sgRNA2 (Fig. 1). SgRNA2 encodes a small ORF (ORF 6) that varies from 4.3 to 7.2 kDa and is poorly conserved between isolates. Despite much effort, we have no evidence that ORF 6 is translated *in vivo* (Rakotondrafara and Miller, person communication).

In addition to conferring cap-independent translation in *cis*, the 3'TE inhibits translation *in trans*, *in vitro* (Wang et al., 1997; Wang et al., 1999). In wheat germ extract, both the 3' TE and sgRNA2, which harbors the TE at its 5' end, trans-inhibit the translation of BYDV genomic RNA (gRNA) and (to a lesser extent) sgRNA1 (Wang et al., 1999). The inhibition does not require translation of ORF 6, but it requires a functional TE. Based on these *in vitro* data, we proposed that in natural infections, accumulation of sgRNA2 inhibits

translation of RNA-dependent RNA polymerase from gRNA, thus viral replication would be inhibited. Here we tested this hypothesis. We found that both replicating and nonreplicating TE inhibit BYDV replication *in trans*. sgRNA2 from natural infection of BYDV trans-inhibits translation of reporter gene and gene expression from BMV RNA with or without TE, which suggests that BYDV sgRNA2 inhibits viral replication via inhibition of translation. We also showed that TE *in cis* increases translation of capped and uncapped RNA. Our data strongly suggest that BYDV 3'TE/sgRNA2 functions as a riboregulator to negatively control viral replication.

Results

Nonreplicating TE and sgRNA2 RNAs trans-inhibit accumulation of BYDV RNA

In wheat germ extract, both BYDV 3' TE and sgRNA2, which harbors the TE at its 5' end, trans-inhibit the translation of BYDV genomic RNA (gRNA) and sgRNA1 (Wang et al., 1999). In natural infection, the molar ratio of sgRNA2 to sgRNA1 or gRNA is similar to the level that inhibits translation of gRNA and sgRNA1 *in vitro*. Thus, we predict that addition of excess 3'TE or sgRNA2 during inoculation with BYDV RNA would inhibit BYDV replication via premature inhibition of translation of the RNA dependent RNA polymerase (RdRp) from genomic RNA. To test this prediction, we co-inoculated oat protoplasts with the 105 nt TE or 109 nt nonfunctional mutant TE (TEBF) transcripts and wild-type BYDV RNA PAV6. TEBF contains GAUC duplication in the *Bam*H I_{3591} site of TE (Wang and Miller, 1995). The accumulated BYDV gRNA and sgRNAs levels at 24 hours post-inoculation (hpi) were detected by northern blot hybridization. When co-inoculated with PAV6 RNA into oat protoplasts, a 10-fold excess of nonreplicating TE105 RNA trans-

inhibited BYDV replication including sgRNA accumulation (Fig. 2A, lane 1 and 3). The defective mutant TEBF did not inhibit PAV6 replication (Fig. 2A, lane 4).

The trans-inhibitory effects of the TE were dose-dependent (Fig. 2B, lane 3-7). As low as 2.5-fold excess TE RNA inhibited BYDV RNA accumulation (Fig. 2B, lane 3). Ten-fold excess TE almost abolished BYDV replication (Fig. 2A, lane 1 and 3; Fig. 2B, lane 5). Surprisingly, when we increased the molar ratio of TE:PAV6 to 20:1, replication of BYDV was recovered (Fig 2B, lane 6 and 7). Nonreplicating full-length sgRNA2 and its counterpart containing the TEBF mutation, sgRNA2BF, had similar effects as those of TE and TEBF RNAs (data not shown). Thus, nonreplicating TE and sgRNA2 does trans-inhibit the accumulation of BYDV RNA as predicted.

Replicating TE trans-inhibits the accumulation of BYDV RNA

To examine the effects of TE, *in trans*, in the replicating context but still isolated from other potential regulatory element in BYDV RNA, we developed an expression system from an unrelated virus, BMV. In a different family from BYDV, BMV is a tripartite virus and has three genomic RNAs. RNA 1 and 2 are required for virus replication, RNA3 are not. All BMV RNAs are capped (Dasgupta et al., 1975), so it has no apparent need for a cap-independent translation element. To monitor gene expression and to avoid complications caused by encapsidation, the BMV coat protein ORF was replaced with that of GFP (Fig. 3). The TE or TEBF of BYDV was inserted into the intergenic region between the 3a and coat protein genes of BMV RNA3 (Fig. 3). This places the TE in the 3' UTR of the 3a gene on RNA 3 and in the 5' UTR of the GFP-encoding subgenomic RNA 4 that is generated from

RNA 3. The resulting viruses were designated as BMV.TEGFP and BMV.TEBFGFP (Fig. 3).

We co-inoculated oat protoplasts with BYDV PAV6 and various BMV constructs (Fig. 3) that presented replicating TE or its nonfunctional counterpart, TEBF. To ensure that the effects were conferred specifically by the TE, we also included tRNA and wild-type BMV.GFP as controls. When co-inoculated with BYDV PAV6, BMV.TEGFP inhibited accumulation of BYDV RNA (Fig. 4A, lane 3), whereas BMV.TEBFGFP (Fig. 4A, lane 4), tRNA (lane 5), and BMV.GFP (lane 6) did not inhibit BYDV RNA accumulation. The inhibitory effects conferred by the TE from replicating BMV.TEGFP were dose-dependent (Fig. 4B). When the co-inoculated BMV.TEGFP was increased from 1 to 4 μg, the amounts of BYDV gRNA and sgRNAs decreased (Fig. 4B, lane 2, 3, and 4). However, when co-inoculated with 8 μg of BMV.TEGFP, BYDV RNA accumulation was inhibited less (Fig. 4B, lane 5).

Subgenomic RNA2 from natural BYDV infection trans-inhibits gene expression of BMV RNA with and without TE

Having established that the TE and sgRNA2 trans-inhibit BYDV RNA replication *in vivo*, we set out to test the mechanism of the inhibition. Based on our *in vitro* data (Wang et al., 1999), we proposed that TE and sgRNA2 inhibit BYDV RNA replication via premature inhibition of translation of the RdRp from genomic RNA. Because of the difficulty of detecting RdRp, we used BMV.GFP and BMV.TEGFP as sensors to test whether that sgRNA2 could trans-inhibit translation *in vivo*. We co-inoculated BMV.GFP with wild-type BYDV infectious transcript PAV6 RNA, or with a mutant, PAV6ΔSG2 RNA. PAV6ΔSG2

contains a point mutation (G4810C) that knocks out sgRNA2 synthesis but has little effect on BYDV RNA accumulation (Fig. 6B) (Koev and Miller, 2000). When co-inoculated with BMV.GFP, the wild-type BYDV transcript PAV6 reduced GFP expression from BMV.GFP by 2 to 6 fold. PAV6ΔSG2 had much less inhibitory effect (Fig. 5A, 5C). GFP expression levels in protoplasts co-inoculated with BMV.GFP and PAV6ΔSG2 were similar to those inoculated with BMV.GFP only ((Fig. 5A, 5C). The degrees of inhibition of GFP expression by PAV6 were similar at different time points (Fig. 5A). BMV.GFP has no effect on the accumulation of BYDV RNAs (Fig. 5B).

The above experiment shows that sgRNA2 inhibits translation of RNA lacking the TE. To examine whether sgRNA2 could inhibit translation of TE-containing RNA *in vivo*, which mimics the natural BYDV infection, we co-inoculated oat protoplasts with PAV6 or PAV6ΔSG2 RNA and BMV.TEGFP RNA. PAV6 reduced GFP expression from BMV.TEGFP, whereas PAV6ΔSG2 only slightly reduced the expression level of GFP (Fig. 5C). Northern blot analysis showed that PAV6 and PAV6ΔSG2 also inhibited the replication of RNA 3 and 4 of BMV.GFP and BMV.TEGFP (Fig. 5D). Thus, accumulated sgRNA2 trans inhibits expression of mRNAs whether or not they harbor the TE. However, we cannot differentiate whether the inhibition is due to reduced translation, replication/transcription, or a combination of these.

Subgenomic RNA2 from natural BYDV infection inhibits translation of reporter mRNA

To test whether the inhibition of gene expression by BYDV sgRNA2 is at the level of translation, we tested the effect of BYDV infection on translation of non-replicating reporter mRNA construct in oat protoplasts. A 2-step electroporation method was developed. First oat protoplasts were inoculated with infectious BYDV PAV6 or PAV6ΔGS2 RNA by electroporation. After 24-hour incubation to allow genomic replication and accumulation of sgRNAs, protoplasts were inoculated again with reporter cap-fLuc-$A_{(60)}$, which is capped and polyadenylated firefly luciferase gene. Then firefly luciferase activities were analyzed after another 4-hour incubation. Inoculation of 1pmol PAV6 RNA in the first step caused 60% drop in translation of cap-fLuc--$A_{(60)}$, whereas inoculation of PAV6ΔSG2 RNA in first step had little effect on translation (Fig. 6A). Again, PAV6 and PAV6ΔSG2 had similar levels of RNA accumulation (Fig. 6B) (Koev and Miller, 2000). Thus, the inhibition of gene expression by BYDV sgRNA2 functions at the level of translation.

TE *in cis* enhances gene expression from BMV RNA

We also examined TE's effect on BMV gene expression *in cis*. In oat protoplasts infected with recombinant BMV RNAs, the insertions of TE and TEBF had no effect on BMV.GFP RNA replication and synthesis of BMV RNA4 (Fig. 7A). This agreed with previous reports that insertion of a foreign gene within 17 bases downstream of the RNA4 start site did not affect subgenomic RNA synthesis (French et al., 1986). By using flow cytometry and UV-microscopy, we found that GFP expression levels from BMV.TEGFP were 2 to 5 fold higher than those from BMV.GFP (Fig. 7B and 5C). In contrast, the TEBF leader, which differs from TE by only four bases, reduced GFP expression to near background levels (Fig. 7B and 5C). This may be caused by the secondary structure of

TEBF, which could hinder the ribosome scanning to the start codon. BMV.TEGFP-infected cells fluoresced more brightly than BMV.GFP-infected cells (Fig. 5C), and the TE increased the number of cells expressing detectable levels of GFP. The percentage of oat protoplasts with green fluorescence was 6.5% (3.3, standard deviation) in BMV.TEGFP-inoculated cells, 2.2% (0.7) in the BMV.GFP-inoculated group, and 0.25% (0.12) in the BMV.TEBFGFP-inoculated group. Because insertion of TE had little effect on replication (Fig. 7A), these data suggest that the insertion of TE in 5' UTR of non-BYDV mRNA increased translation. This was unexpected because all four BMV RNAs are reported to have a 5' cap. Thus, the TE also increases the translation of capped mRNA.

Discussion

Subgenomic RNA2 trans-inhibits the accumulation of BYDV RNA

Positive sense RNA in the *Nidoviridae*, *Togaviridae*, and many plant virus families synthesizes subgenomic RNAs to express 3' proximal genes. In this report, we showed that the 3'TE in three different contexts (replicating BMV, nonreplicating 3'TE, and nonreplicating sgRNA2) inhibited BYDV RNA accumulation *in trans* (Fig. 2 and 4). The nonfunctional TEBF sequence that differs from 3'TE by only a four base duplication did not inhibit *in trans*. In natural infection, the trans-function of TE is fulfilled in the context of sgRNA2. Thus, BYDV sgRNA2 trans-inhibits accumulation of BYDV RNAs via its TE. This reveals viral subgenomic RNA do not always serve as message RNAs, and instead can perform important regulatory functions.

The inhibitory effects of the TE and sgRNA2 on BYDV replication and transcription were dose-dependent (Fig. 2B, Fig. 4B). This dose dependency supported our hypothesis that 3'TE and sgRNA2 trans-inhibited translation of capped and uncapped mRNAs by competing for translation initiation factor(s) (Wang et al., 1997). Surprisingly, the replication of BYDV was restored when the molar ratio of TE:PAV6 was increased to 20:1 and 40:1. This pattern of inhibition and restoration was highly reproducible. One possible explanation is that at 10-fold or lower molar excess, TE binds the rate-limiting factors, eIF4E/eIFiso4E, sequestering it from participating in translation of viral genes. At higher concentrations, the TE may be abundant enough to increase the probability of base pairing to the viral 5'UTR by the kissing stem-loop interaction *in trans* instead of *in cis*. Thus the trans-added TE may stimulate translation by delivering translation factors to the 5'UTR *in trans*. A second possibility is that 3'TE could not fold into the functional secondary structure when the concentration of 3'TE reached a threshold in our experimental conditions, preventing trans-inhibition that occurs at lower concentration.

Subgenomic RNA2 trans-inhibits gene expression from RNA with or without the TE

Wild type PAV6 trans-inhibited GFP expression from an unrelated virus, BMV with or without the TE, whereas PAV6ΔSG2 did not (Fig. 5A and 5C). Thus, the decreased expression levels of GFP were caused by BYDV sgRNA2. There are at least two explanations for the differential effect of PAV6 and PAV6ΔSG2 on the translation of GFP from BMV. The first is that specific TE secondary structure presents only in the sgRNA2 context, but not full-length viral RNA, and is required for the trans-inhibition function. The TE sequence in the context of gRNA and sgRNA1 could not inhibit GFP expression from

BMV. When present in gRNA and sgRNA1, the sgRNA2 sequence (3'UTR) may fold in an alternative structure to confer its cap-independent translation function. A similar observation was found in *Flock house virus* (FHV) (Albarino et al., 2003; Eckerle and Ball, 2002). In FHV, the subgenomic RNA from RNA1 trans-activates replication of RNA2. However, the subgenomic RNA sequence embedded in the context of RNA1 cannot support RNA2 replication. Another possible cause is that the lower level accumulation of TE sequence in PAV6ΔSG2 infected protoplasts, compared with PAV6-infected protoplasts, was insufficient for the inhibition effect. Our *in vivo* data here did not support the second possibility. Because even the molecular ratio of TEBF/sg2BF: PAV6 was increased up to 40-fold, we still did not observe inhibition of BYDV replication (data not shown). Increasing the co-inoculated BMV.TEBFGFP up to 8μg also did not confer an inhibitory effect on BYDV replication (Fig. 4B, lane 5).

Feedback regulation of BYDV gene expression by its sgRNA2

The correlation between functions of TE *in cis* and ability to inhibit virus replication *in trans* provides strong evidence that the trans-inhibition uses the same factors as for cis-stimulation. Competition studies showed both sgRNA2 and 3'TE trans-inhibited translation of gRNA *in vitro* (Wang et al., 1999). Here, we showed that sgRNA2 trans-inhibited translation of reporter mRNA (Fig. 6) and GFP expression from BMV *in vivo* (Fig. 5). These data suggest that TE and sgRNA2 trans-inhibited BYDV replication and transcription most likely by inhibiting translation, thus preventing the production of the RNA-dependent RNA polymerase (RdRp). But we could not rule out that it could also function at the level of replication/transcription. Combined with previous reported results, we propose a feedback

regulation mechanism (Fig. 8): In the early stage of BYDV infection, viral RdRp is produced via TE-mediated cap-independent translation of gRNA (Wang et al., 1997). The RdRp then carries out viral RNA replication and sgRNA synthesis. Viral RNAs accumulate with sgRNA2 particularly abundant. Accumulated sgRNA2, via its TE, trans-inhibits translation of BYDV RdRp from gRNA. The synthesis of viral structural proteins fromsgRNA1 is not affected. Genomic RNA is switched from translation to replication and encapsidation, i.e. gRNA is available for replication by the existing RdRp and for encapsidation.

We also propose TE/sgRNA2 trans-inhibits host translation by titering out the necessary and/or limiting translation initiation factor(s). Our data, trans-inhibition assays (Wang et al., 1997), and *in vitro* binding assays (E. Allen and W.A. Miller, personal communication) support this hypothesis. *In vitro* binding assay revealed that TE specifically binds eIF4E and other wheat germ extract proteins *in vitro* (E. Allen and W.A. Miller, personal communication). Moreover, exogenous added eIF4F reverses the trans-inhibition effect caused by TE *in vitro* (Wang et al., 1997).

Barley yellow dwarf virus sgRNA2 functions as a regulatory RNA, not a messenger RNA

We show here that RNA harboring the BYDV 3'TE trans-inhibits translation of other TE-containing RNAs *in vivo*, as well as translation of RNA without TE. Thus, the TE serves as a riboregulator as proposed from previous *in vitro* translation experiments (Wang et al., 1999). The TE is a sense RNA and functions *in trans*, probably, at the level of translation. Subgenomic RNA2 from viral natural infection conferred the observed trans-inhibition of

BMV RNA4 translation via the 3'TE (Fig. 5A, 5C). These *in vivo* results confirmed *in vitro* observation that TE trans-inhibits translation of capped mRNA lacking any BYDV sequence (Wang et al., 1997). Our results also support the notion that 3'TE out-competes mRNA for translational machinery (Wang et al., 1999) (E. Allen and W.A. Miller, personal communication). In viral natural infection, TE functions in the context of sgRNA2. Evidence showed that the potential small ORF6 within sgRNA2 couldn't be translated (Rakotondrafara and Miller, person communication). *In vitro* data also showed that inhibition of translation by sgRNA2 does not require expression of ORF6 (Wang et al., 1999). Thus, sgRNA2 functions as a regulatory RNA, not a messenger RNA.

Other trans-regulatory RNAs from or related to viruses also have been reported (Albarino et al., 2003; Das et al., 1998; Eckerle and Ball, 2002; Sit et al., 1998). A 34nt trans-activator sequence in RNA2 of *Red clover necrotic mosaic virus* is required for transcription of sgRNA from RNA1 (Sit et al., 1998). The trans-activator fulfills its function via base pairing between RNA1 and RNA2. This is an example of *in trans* RNA-mediated transcriptional regulation in virus. The replication of *Flock house virus* RNA2 is dependent on the synthesis of subgenomic RNA from RNA1 (Albarino et al., 2003; Eckerle and Ball, 2002). Dasgupta group reported that a 60nt small inhibitor RNA (IRNA) from yeast *Saccharomyces cerevisiae* specifically inhibited internal ribosome entry site (IRES)-mediated translation of viral RNAs and did not interfere with cap-independent translation of cellular mRNAs *in vivo* and *in vitro* (Das et al., 1994; Das et al., 1998). *Adenovirus* virus-associated (VA) RNAs are required for efficient expression of late viral genes (Mathews and Shenk, 1991; Thimmappaya et al., 1982). VA RNAs protect against dsRNA-activated inhibitor (DAI)-mediated phosphorylation of eIF-2α by binding DAI (Schneider et al., 1985).

TE functions differently *in cis* and *in trans*

Our data showed that a viral translation element, BYDV 3'TE, functions differently *in cis* and *in trans*. First, 3'TE *in cis* enhanced GFP expression from BMV. The 3'TE enhanced GFP expression most likely by pulling down translation initiation factor(s) close to the cap structure, which increased efficiency of translation initiation. It is unlikely that 3'TE stimulated GFP expression through internal initiation. Wang et al. (Wang et al., 1997) didn't detect stimulation of translation of the downstream gene when the TE was located at the intergenic region of a bicistronic mRNA. Secondly, sgRNA2 from natural BYDV infection inhibited GFP expression from BMV *in trans*. Thirdly, both replicating and nonreplicating TE, or sgRNA2 trnascripts *in trans* inhibited BYDV replication, most likely by inhibiting translation of BYDV genomic RNA. Thus, 3'TE functions differently *in cis* and *in trans*. *In cis*, 3'TE confers cap-independent translation (Wang et al., 1997) and increases translation of capped RNA (Fig. 5, 7). *In trans*, 3'TE or 3'TE-bearing RNA (e.g. sgRNA2) serves as a riboregulator to trans-inhibit mRNA translation and BYDV replication. These data demonstrate that BYDV 3'TE/sgRNA2 functions as a riboregulator to control viral gene expression via different *in cis* and *in trans* functions. Our data reveal a new function for a viral subgenomic RNA and a novel mechanism of gene regulation by a trans-regulatory viral RNA.

Materials and Methods

Plasmids

Infectious BYDV-PAV genomic RNA was transcribed from the full-length clone, pPAV6 (Di et al., 1993). The sgRNA2 knockout mutant clone of BYDV-PAV, pPAV6ΔSG2, was referred to previously as SG2G/C (Koev and Miller, 2000) and differs from pPAV6 by a point mutation at position 4810 (G to C), which prevents sgRNA2 synthesis. pTE and pTEBF are clones for T7 transcription of the 105 nt TE RNA and its nonfunctional mutant TEBF (Wang et al., 1997). pSG2 and pSG2BF allow T7 transcription of the 869 nt sgRNA2 and its nonfunctional mutant sgRNA2BF, respectively (Wang et al., 1999). Both pTEBF and pSG2BF contain a GATC duplication in the *Bam*H I site (BF) in the 3'TE. The duplication destroys the cap-independent translation function of the 3'TE (Wang et al., 1997; Wang et al., 1999).

Brome mosaic virus (BMV) RNA clones were kindly provided by A. L. N. Rao (University of California, Riverside). pT7B1, pT7B2, and pT7B3 are clones for T7 transcription of BMV RNA1, RNA2, and RNA3, respectively (Dreher et al., 1989). pT7B3EGFP is a clone of BMV RNA3 with the coat protein gene replaced by enhanced green fluorescent protein (GFP) gene (Rao, 1997). To construct pT7B3TEGFP for T7 transcription of BMV.TEGFP RNA3, the 109nt fragment corresponding to the 3'TE (4809-4918) was amplified from pPAV6 by PCR using the upstream primer, 5'-GG*AGATCT*ATGTCCTAATTCAGCGTATTAATAGTGAAGACAACACCA-3', and the downstream primer, 5'-CCTGAA*GTCGAC*ATTCGGCCAAACACAATACGATA-3'. The PCR products were cut with *Bgl* II and *Sal* I (in italics), then ligated with pT7B3EGFP that had also been digested with *Bgl* II and *Sal* I. The same strategy was used to clone

pT7B3TEBFGFP except the template for PCR was pSG2BF. The pT7B3TEGFP and pT7B3TEBFGFP constructs were verified by sequencing at the DNA Sequencing and Synthesis Nucleic Acid Facility of Iowa State University on an ABI377 sequencer (Applied Biosystems, Foster City, CA).

RNA preparation and infection of protoplasts

The capped and uncapped RNAs were synthesized by *in vitro* transcription by using the T7 mMESSAGE mMACHINE or MegaScript kits (Ambion, Austin, TX) as per manufacturer's instructions. For transcription of infectious RNAs, BYDV constructs were linearized with *Sma* I to give a perfect genomic 3' end. pT7B1, pT7B2, pT7B3GFP, pT7B3TEGFP were linearized with *Bam*H I. pT7B3TEGFP was linearized with *Tth*111 I. Oat (*Avena sativa* cv. Stout) protoplasts were prepared and inoculated with RNA as described in Dinesh-Kumar and Miller (Dinesh-Kumar and Miller, 1993). Except when explicitly stated otherwise, 10 μg of RNA transcript was used for BYDV inoculation and 4 μg of BMV RNAs 1, 2, and 3 in a molar ratio of 1:1:2 were used for BMV inoculation.

2-step electroporation

In the first step, oat protoplasts were inoculated with infectious BYDV PAV6 or PAV6ΔGS2 RNA by electroporation and incubated for 24 hours at room temperature. In the second step, protoplasts were inoculated again with cap-fLuc-$A_{(60)}$. Then firefly luciferase activities were analyzed after another 4-hour incubation.

Protein Analysis

Oat protoplasts were analyzed for GFP expression 24, 48, 72, and 96 hr after inoculation by flow cytometry by using an ELITE ESP fluorescence-activated cell sorter (Beckman-Coulter, Anaheim, CA) at the Cell and Hybridoma Facility of Iowa State University. All data presented in this report were obtained from at least three independent experiments.

Northern blot hybridization

Total RNA was extracted from protoplasts by using the RNeasy plant RNA isolation kit (QIAGEN, Los Angeles, CA) as per manufacturer's instructions. For the time course of sgRNA2 accumulation, protoplasts were collected at 24, 48, and 72 hpi. RNA was then extracted from these cells and analyzed by Northern blot as described previously (Koev et al., 1999). A ^{32}P-labeled probe complementary to the 1.5 kb 3'-terminal sequence of BYDV-PAV RNA was used to detect BYDV gRNA and sgRNAs (Koev et al., 1999). Because of the low replication level of BMV RNAs 3 and 4 in oat protoplasts, we could hardly detect these two RNAs by using BMV tRNA-like structure probe. Instead, we used a ^{32}P-labeled probe complementary to the full-length GFP gene sequence RNA to detect recombinant BMV RNAs 3 and 4.

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Figure Legends

Fig. 1. Schematic of *Barley yellow dwarf virus* genome organization. Boxes represent open reading frames (ORFs) with the sizes of encoded proteins indicated in kilodaltons (K). Black lines represent genomic RNA (gRNA) and subgenomic RNAs (sgRNAs). The *in vivo* translation element (TE), which includes the entire sgRNA2, is located between two dashed lines. Gray boxes present the *in vitro* TE.

Fig. 2. Effects of nonreplicating TE on BYDV replication. BYDV RNA were co-inoculated with a 10-fold excess of *in vitro* transcript TE or TEBF (A), or with increasing excess molar ratios of TE (B), into oat protoplasts. After 24hrs incubation, protoplasts were collected, and total RNA were extracted and analyzed by northern blot. gRNA and sgRNAs are indicated. A. effects of a 10-fold excess of *in vitro* transcript TE or TEBF on PAV6 replication. lane 1 and 3: PAV6 + 10-fold excess TE; lane 2: PAV6; and lane 4: PAV6 + 10-fold TEBF. The bottom panel shows the RNA loading. B. effects of increasing excess molar ratios of TE on PAV6 replication. lane 1: mock; lane 2: PAV6; and lanes 3 to 7: PAV6 + 2.5, 5, 10, 20, and 40-fold excess TE, respectively.

Fig. 3. Schematic of *Brome mosaic virus* genome organization. Boxes represent ORFs with the gene names indicated above. CP: coat protein. GFP: green fluorescent protein. Black ovals indicate 5'cap. Cloverleaves stand for 3' tRNA-like structure. Arrows show synthesis of the subgenomic RNA (RNA 4) of *Brome mosaic virus* (BMV). Gray boxes are the *in vitro* TE of *Barley yellow dwarf virus* (BYDV). The sequence of BMV subgenomic core

promoter, the secondary structure of BYDV TE, and start codon of GFP gene (underlined) are showed in the dotted box.

Fig. 4. Effects of replicating TE from BMV on BYDV replication. Northern blot analyses were done as in Figure 2. *Barley yellow dwarf virus* gRNA and sgRNAs are indicated. *Brome mosaic virus* RNA3 and RNA4 were also detected because they contain BYDV TE. A. lane 1: mock; lane 2: PAV6; lane 3: PAV6 + 4 μg of BMV.TEGFP; lane 4: PAV6 + 4 μg of BMV.TEBFGFP; lane 5: PAV6 + 4 μg of tRNA; and lane 6: PAV6 + 4 μg of BMV.GFP. The bottom panel shows RNA loading. B. effects of increasing BMV RNA inoculums on BYDV replication. lane 1: PAV6; lanes 2 to 5: PAV6 + 1, 2, 4, and 8 μg of BMV.TEGFP, respectively. The bottom panel shows RNA loading.

Fig. 5. Effects of subgenomic RNA2 (sgRNA2) of BYDV, *in trans*, on the expression of GFP from BMV. A. Effects of sgRNA2 on the expression of GFP from BMV. BYDV RNA was co-inoculated with BMV.GFP into oat protoplasts. At different time points post inoculation, a portion of cells was collected and GFP fluorescence intensities were measured by using flow cytometry. PAV6: wild-type BYDV. PAV6ΔSG2: one base mutation (G4810C) of PAV6 that knocks out sgRNA2 synthesis. hpi: hours post-inoculation. Vertical bars indicate standard deviation. Each point is the mean of at least 3 replicates. B. RNA accumulation of PAV6 and PAV6ΔSG2 in oat protoplasts. At different time points post inoculation, another portion of cells was collected. Total RNA were extracted and used for northern blotting analysis. The bottom panel shows the RNA loading. gRNA and sgRNAs are indicated. C. Oat protoplasts infected with BMV.GFP, BMV.TEGFP alone, or with

PAV6 or PAV6ΔSG2. Pictures were taken under microscope with UV-light after 24hpi. D. RNA accumulation of BMV.GFP and BMV.TEGFP in oat protoplasts. Protoplasts from C were collected after 24hpi. Total RNA were then extracted and used for northern blotting analysis. A ^{32}P-labled probe complementary to full-length GFP gene sequence were used to detect recombinant BMV RNA 3 and 4. The bottom panel shows RNA loading control. RNA 3 and 4 are indicated.

Fig. 6. A. Differential effects of PAV6 and PAV6ΔSG2 replication on translation of reporter construct cap-fLuc-$A_{(60)}$. 24 hours after inoculation of PAV6 or PAV6ΔSG2 RNA, oat protoplasts are electroporated again with 1pmol cap-fLuc-$A_{(60)}$. Luciferase activities are analyzed 4 hours later. B. Northern blot analysis of replication of PAV6 and PAV6ΔSG2. Line 1, PAV6. Line 2, PAV6ΔSG2.

Fig. 7. A: RNA 3 and 4 accumulation of recombinant BMV. Oat protoplasts infected with BMV.GFP, BMV.TEGFP, or BMV.TEBFGFP are collected 24hrs after inoculation. Total RNA was extracted and used for northern blotting analysis. A ^{32}P-labled probe complementary to full-length GFP gene sequence were used to detect recombinant BMV RNA 3 and 4. The bottom panel shows RNA loading control. RNA 3 and 4 are indicated. B: Effects of translation element (TE) of BYDV on the expression of GFP from BMV *in cis*. GFP fluorescence intensities were measured as Fig. 5. Mock: oat protoplasts were electroplated without RNA. hpi: hours post inoculation. Vertical bars indicate standard deviation. Each value is a mean of at least 3 replicates.

Fig. 8. Feedback regulation of BYDV gene expression. In the early stage of BYDV infection, viral RNA-dependent RNA polymerase (RdRp) is produced via TE-mediated cap-independent translation of gRNA (1). The RdRp then carries out vial RNA replication and sgRNA synthesis (2). Viral RNAs accumulate with sgRNA2 particularly abundant (2). Accumulated sgRNA2, via its TE, trans-inhibits translation of RdRp from gRNA (3). The synthesis of viral structural proteins is not affected. Genomic RNA switches from translation to replication and encapsidation, i.e. gRNA is available for replication by the existing RdRp and for encapsidation.

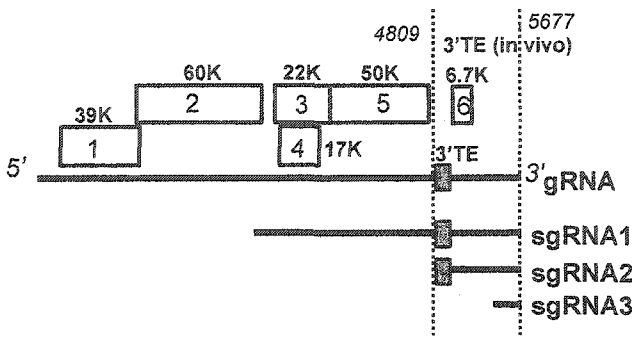
4809
3'TE (in vivo)
5677
60K
22K
50K
6.7K
39K
2
3
5
6
1
4
17K
3'TE
5'
3'gRNA
sgRNA1
sgRNA2
sgRNA3

Fig. 1

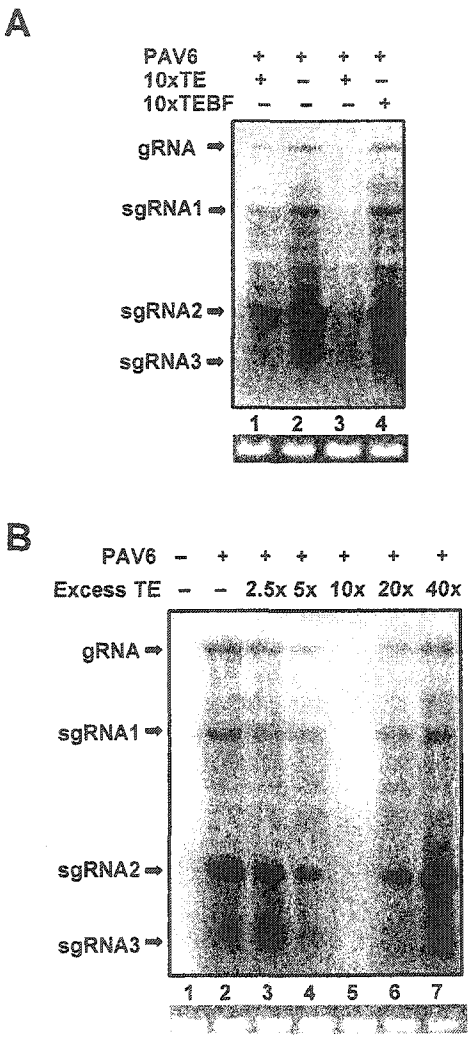
A
PAV6 + + + +
10xTE + – + –
10xTEBF – – – +
gRNA
sgRNA1
sgRNA2
sgRNA3
1 2 3 4
B
PAV6 – + + + + + +
Excess TE – – 2.5x 5x 10x 20x 40x
gRNA
sgRNA1
sgRNA2
sgRNA3
1 2 3 4 5 6 7

Fig. 2

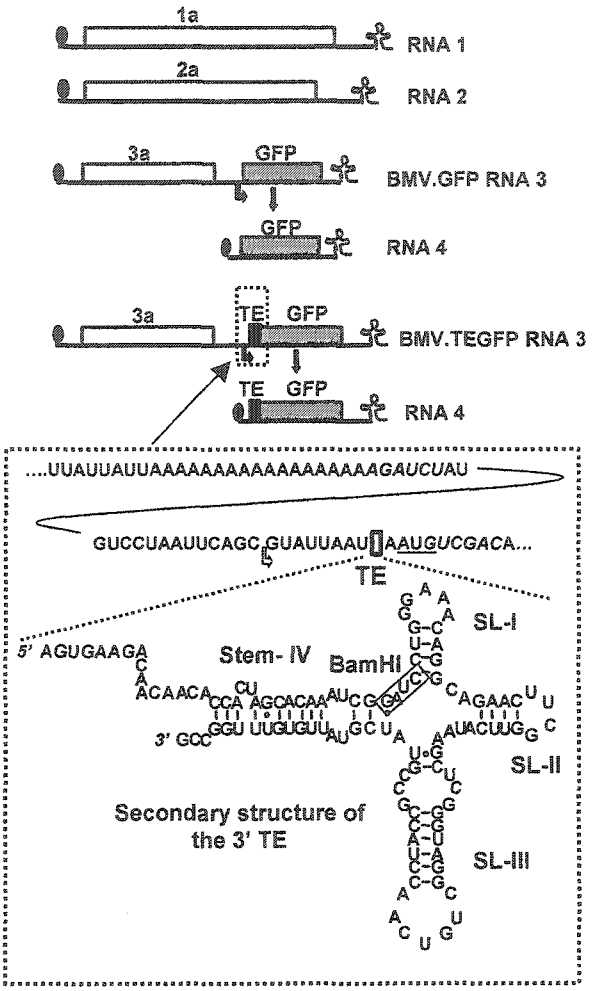
1a
RNA 1
2a
RNA 2
3a
GFP
BMV.GFP RNA 3
GFP
RNA 4
3a
TE
GFP
BMV.TEGFP RNA 3
TE
GFP
RNA 4
....UUAUUAUUAAAAAAAAAAAAAAAAAAAGAUCUAU
GUCCUAAUUCAGCGUAUUAAUAAUGUCGACA...
TE
5' AGUGAAGA
Stem- IV
BamHI
SL-I
SL-II
SL-III
Secondary structure of
the 3' TE

Fig. 3

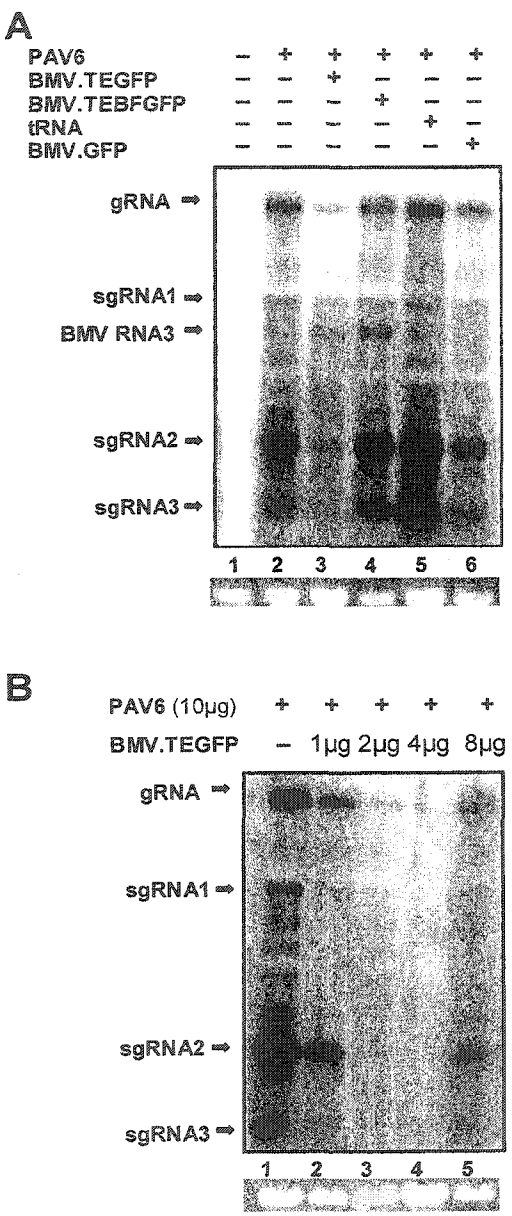
A
PAV6 – + + + + +
BMV.TEGFP – – + – – –
BMV.TEBFGFP – – – + – –
tRNA – – – – + –
BMV.GFP – – – – – +
gRNA
sgRNA1
BMV RNA3
sgRNA2
sgRNA3
1 2 3 4 5 6
B
PAV6 (10µg) + + + + +
BMV.TEGFP – 1µg 2µg 4µg 8µg
gRNA
sgRNA1
sgRNA2
sgRNA3
1 2 3 4 5

Fig. 4

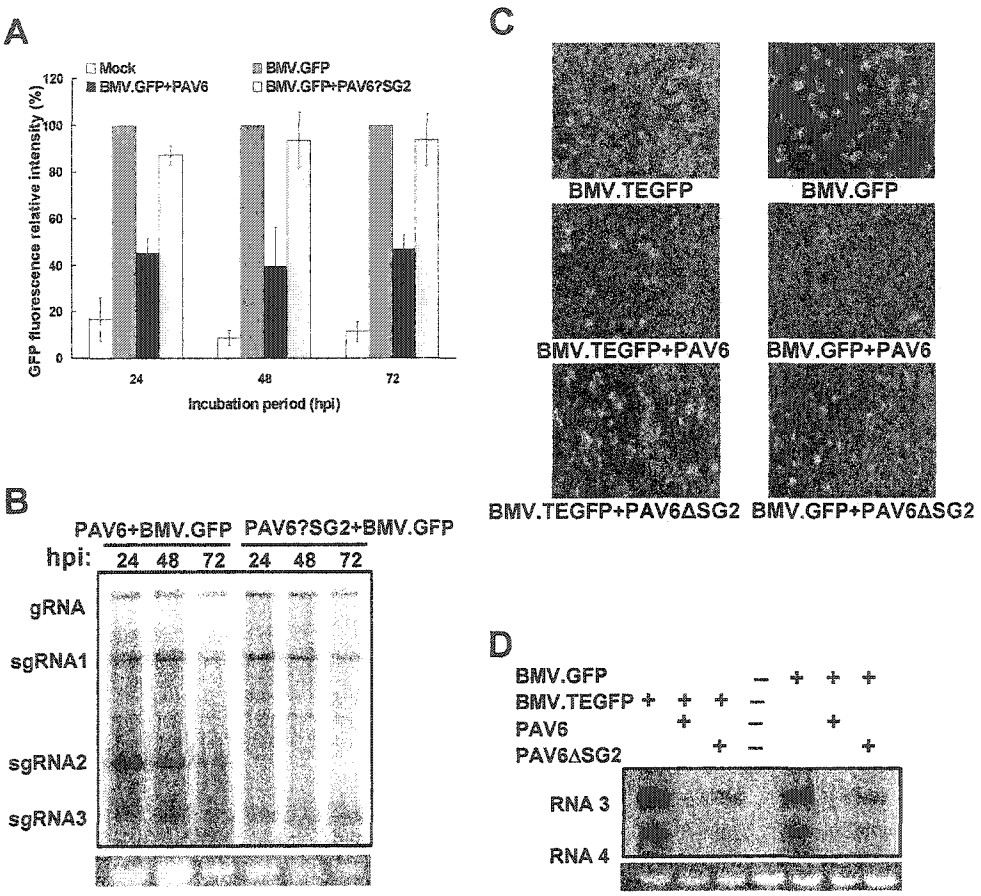
A
Mock
BMV.GFP
BMV.GFP+PAV6
BMV.GFP+PAV6?SG2
GFP fluorescence relative intensity (%)
120
100
80
60
40
20
0
24
48
72
Incubation period (hpi)
B
PAV6+BMV.GFP
PAV6?SG2+BMV.GFP
hpi:
24 48 72 24 48 72
gRNA
sgRNA1
sgRNA2
sgRNA3
C
BMV.TEGFP
BMV.GFP
BMV.TEGFP+PAV6
BMV.GFP+PAV6
BMV.TEGFP+PAV6ΔSG2
BMV.GFP+PAV6ΔSG2
D
BMV.GFP
BMV.TEGFP
PAV6
PAV6ΔSG2
RNA 3
RNA 4

Fig. 5

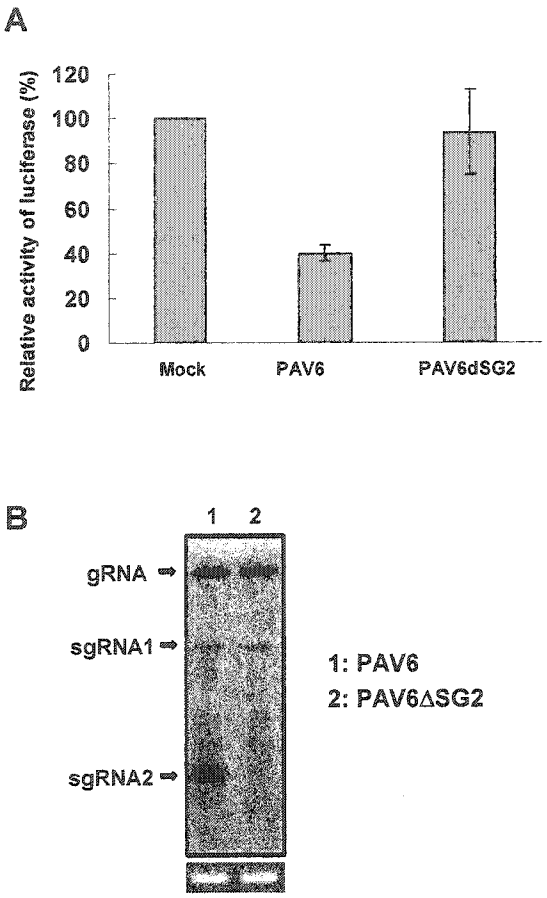
A
Relative activity of luciferase (%)
120
100
80
60
40
20
0
Mock
PAV6
PAV6dSG2
B
1
2
gRNA
sgRNA1
sgRNA2
1: PAV6
2: PAV6ΔSG2

Fig. 6

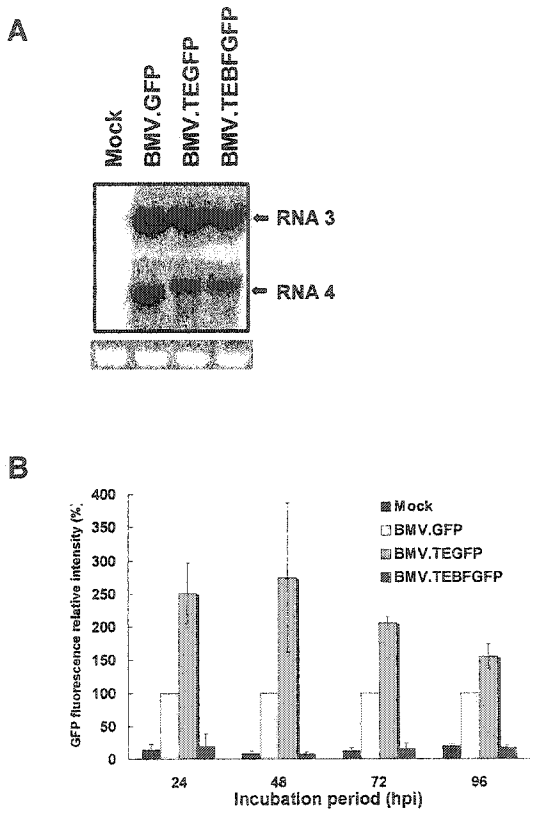
A
Mock
BMV.GFP
BMV.TEGFP
BMV.TEBFGFP
RNA 3
RNA 4
B
GFP fluorescence relative intensity (%)
400
350
300
250
200
150
100
50
0
Mock
BMV.GFP
BMV.TEGFP
BMV.TEBFGFP
24
48
72
96
Incubation period (hpi)

Fig. 7

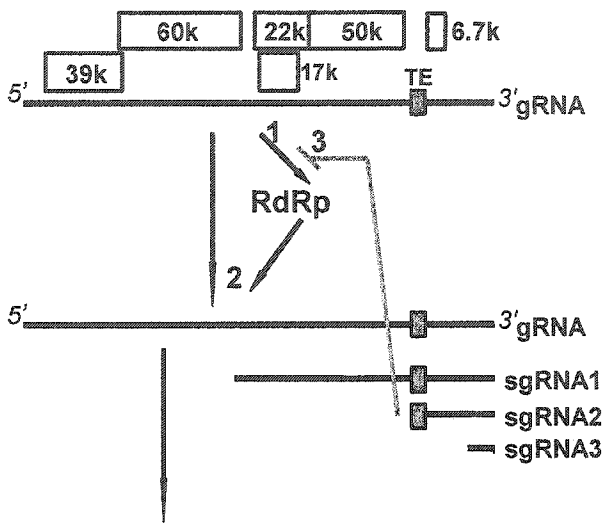
60k
22k
50k
6.7k
39k
17k
TE
5'
3'gRNA
1
3
RdRp
2
5'
3'gRNA
sgRNA1
sgRNA2
sgRNA3
Genomic RNA is available for replication by the existing RdRp and for encapsidation

Fig. 8

CHAPTER 4. THE 3' UNTRANSLATED REGION OF *TOBACCO NECROSIS VIRUS* RNA CONTAINS A BYDV-LIKE CAP-INDEPENDENT TRANSLATION ELEMENT

A paper accepted by *the Journal of Virology*

Ruizhong Shen and W. Allen Miller

Abstract

RNAs of many viruses are translated efficiently in the absence of a 5' cap structure. The *Tobacco necrosis necrovirus* (TNV) genome is an uncapped, non-polyadenylated RNA whose translation mechanism has not been well investigated. Computational analysis predicted a cap-independent translation element (TE), within the 3' untranslated region (UTR) of TNV RNA, that resembles the TE of *Barley yellow dwarf luteovirus* (BYDV). Here we report that such a TE indeed exists in the 3' UTR of TNV strain D. Like the BYDV TE, the TNV TE (i) functions both in vitro and in vivo, (ii) requires additional sequence for cap-independent translation in vivo, (iii) has similar secondary structure and the conserved sequence: CGGAUCCUGGGAAACAGG, (iv) is inactivated by a four base duplication in this conserved sequence, (v) can function in the 5' UTR, and (vi) when located in its natural 3' location, likely form long-distance base pairing with the viral 5' UTR that is conserved and probably required. The TNV TE differs from the BYDV TE by having only three helical domains instead of four. Similar structures were found in all members of genus *Necrovirus* of the *Tombusviridae* family, except *Satellite tobacco necrosis virus* (STNV), which harbors a different 3' cap-independent translation domain. The presence of the BYDV-like TE in

select genera of different families indicates that phylogenetic distribution of TEs does not follow standard viral taxonomic relationships. We propose a new class of cap-independent translation element called the BYDV-like TE, or BTE.

Introduction

The 5' m^7GpppN cap structure and 3' poly(A) tail on eukaryotic mRNAs function synergistically to facilitate efficient translation initiation (10, 32, 33, 37). Eukaryotic initiation factor (eIF) 4E binds the 5' cap, poly(A) binding protein (PABP) binds the poly(A) tail, and both eIF4E and PAPB bind to eIF4G, forming a closed loop (18, 34, 43). This closed loop is a prerequisite for efficient translation initiation of most mRNAs, as it seems to enhance recruitment of the 43S ribosomal initiation complex to the 5' untranslated region (UTR) of the message (12, 36).

Many viral mRNAs lack a cap structure and/or a poly(A) tail, yet translate efficiently. Sequences have evolved that functionally replace the 5' cap and/or poly(A) tail. For example, the uncapped RNAs of picornaviruses, *Hepatitis C virus* (HCV), and the *Discistroviridae* harbor internal ribosome entry sites (IRES) (9, 17, 39, 44). IRESes, which are located upstream of the translated open reading frame (ORF), recruit the ribosome to the mRNA via a variety of mechanisms (7, 31). Like picornaviral RNAs, *Tobacco etch potyvirus* (TEV) mRNA is polyadenylated and uncapped. Its 5' UTR is a functional alternative for a cap and has modest IRES activity (4, 11, 13).

A group of naturally uncapped and non-polyadenylated plant viral RNAs has evolved a different cap-independent translation mechanism. They carry out cap-independent translation via elements in their 3' UTRs and do not utilize internal ribosome entry. This

group includes RNAs of viruses in the diverse *Tombusviridae* family: *Satellite tobacco necrosis virus* (STNV) (6), *Turnip crinkle carmovirus* (TCV), (35), *Hibiscus chlorotic ringspot carmovirus* (HCRV) (20), *Tomato bushy stunt tombusvirus* (TBSV) (45), and *Red clover necrotic mosaic dianthovirus* (RCNMV) (29). A well-studied example from a virus in a different family is the cap-independent translation element (TE) in the 3' UTR of *Barley yellow dwarf luteovirus* (BYDV) (15, 16, 40, 41). The BYDV TE confers cap-independent translation by recruiting translation factors (E. Allen, personal communication) and interacting with the 5' UTR via long-distance base pairing (15).

In contrast to internal ribosome entry, the 3' TE-5' UTR interaction appears to facilitate ribosome scanning from the 5' end (15), like normal capped mRNA (21). An 18 nt sequence, the TE secondary structure, and base pairing between 5' and 3' UTRs are conserved in the 3' UTRs of members of *Luteovirus* and *Necrovirus* genera in the *Luteoviridae* and *Tombusviridae* families, respectively (Fig. 1) (15). However, there has been no experimental evidence to support the existence of a TE in the necroviruses.

In this report, we investigate an isolate of the D strain of *Tobacco necrosis necrovirus* (TNV-D) from the United Kingdom. TNV-D has a positive sense, single-stranded RNA genome of 3762 nt (5). It encodes six open reading frames (ORFs) (Fig. 2A). Viral proteins p22, p82, and p7 are translated from genomic RNA. p82 contains motifs of the viral RNA-dependent RNA polymerase and is probably translated via readthrough of the p22 ORF stop codon. The downstream ORFs are translated from subgenomic mRNAs (24, 30). p7a and p7b are translated from subgenomic RNA1. p7, p7a, and p7b are required for infection of plants. Coat protein, p29 is translated from sgRNA2 and required for systemic infection and

vector specificity (24, 30). p22, p7a, and p29 are translated presumably via a cap-independent translation mechanism. The translation mechanisms of p7 and p7a are unclear.

TNV RNA has no 5' cap (23) and no 3'poly(A) tail or tRNA-like structure (24), yet it translates efficiently. Here we report that there is a BYDV-like TE in the 3' UTR of TNV-D RNA that confers efficient cap-independent translation. Our data suggest that RNAs of all *Necroviruses*, but not the satellite virus of TNV (STNV), initiate proteins synthesis by highly similar TE-mediated mechanism as BYDV RNA. Similar structures are present in the *Dianthovirus* genus (29) of the *Tombusviridae* and the *Luteovirus* genus of the *Luteoviridae*, but absent in other genera of these families. This suggests recent recombination between viruses of these two families. Because the BYDV-like TE is not limited to BYDV, we propose that it represents a new class of cap-independent translation element called the BYDV-like TE, or BTE.

Materials and methods

Plasmids

All clones were verified by automated sequencing at the Iowa State University DNA Sequencing and Synthesis Facility. Plasmid pTNV-D is a full-length infectious clone of TNV-D, kindly provided by R. H. A. Coutts, Imperial College, London (5). We used two steps to construct plasmid pTLucT, the template for transcription of TLucT RNA, which consists of a luciferase ORF flanked by the 5' and 3' UTRs of TNV-D. First, the full-length 5' UTR of TNV-D was PCR-amplified from pTNV-D by using primers: TCC*CCGCGG*TAATACGACTCACTATAGATACCTAACCAGTGTCTC (T7 promoter is

underlined) and TTG*GCGCGC*AGCTGATTACTTAATCACTGAGACACTGGTTAGG. The PCR product was cut with *Sac* II and *Bss*H II (italics) and ligated into *Sac* II/*Bss*H II-digested pBLucB. pBLucB, constructed as described in ref (16) where it was called p5'UTR-LUC-TE869-$(A)_{60}$, encodes the luciferase ORF flanked by the UTRs of BYDV. The resulting clone was named pTLucB. In the second step, the full-length 3' UTR of TNV-D was PCR-amplified from pTNV-D by using primers: CGG*GGTACC*TTGCTTTCATAGATCCG and TCC*CCCGGG*TTCCTAGAGAGATCT. The PCR product was cut with *Acc*65 I and *Sma* I (italics) and ligated into *Acc*65 I/*Sma* I-digested pTLucB to obtain pTLucT, or ligated into *Acc*65 I/*Sma* I-cut pBLucB to obtain pBLucT. Internal deletions d3462-3510 and d3462-3554 were cloned by ligating PCR-amplified small fragments of 3' UTR into *Acc*65 I/*Sma* I-digested pTLucT. Plamid pTLucTBF with GATC duplication at the *Bam*H I site were constructed from pTLucT by cutting, Klenow-filling and re-ligating the *Bam*H I site.

Standard PCR-mediated, site-directed mutagenesis was used to construct pTNVD3*, pTLucT*, pT*LucT, pT*LucT*, pB*LucT, pBLucT*, and pT*LucT*, as in (15, 16). To clone the TNV-D TE into the 5' UTR, the 107 nt TNV-D TE (nt 3566-3672) was PCR-amplified from pTNV-D by using primers: TTG*GCGCGC*TACAATATATGTTGACGTACAAG and GAC*GCGCGC*CGACAACCAATATTGGGGCACAT. The PCR product was cut with *Bss*H II and ligated into *Bss*H II-digested pTE105-LUC (16). The resulting plasmid was named pTELucAn. To mutate the two AUG codons of the TNV-D TE, the 107 nt TE was PCR-amplified from pTNV-D by using primers: TTG*GCGCGC*TACAATATAAGTTGACTTACAAG and

GAC*GCGCGC*CGACAACCAAAATTGGGGCACCTACAAGT. The PCR product was cut with *Bss*H II (italics) and ligated into *Bss*H II-digested pGL051A. The resulting clone was pTE2LucAn. The same strategy was used to clone pTE2BFLucAn and pTE2*LucAn, but instead of using pTNV-D as template, pTLucTBF and pTLucT* were used respectively.

In vitro transcription and translation

Capped and uncapped RNAs were synthesized by in vitro transcription by using the T7 mMESSAGE mMACHINE or MegaScript kits (Ambion, Austin, TX) as per manufacturer's instructions. The plasmids were digested with *Sma* I or at other sites as indicated in figures. For TNVD, TNVD3*, TNVD5*, and TNVD5*3* transcripts used in Fig. 5B, in vitro transcription templates were PCR-amplified. In vitro translation in wheat germ extract (Promega), SDS-PAGE, phosphorimager analysis, and luciferase assay were performed as described by Wang and Miller (42) and Guo et al. (16). All luciferase assays were performed in at least three independent experiments, each of which was done in triplicate. Luciferase activities are normalized to TLucT, whose luciferase activity is defined as 100%.

In vivo translation

Oat (*Avena sativa* cv. Stout) protoplasts were prepared and electroporated with RNA as described by Dinesh-Kumar and Miller (8). Luciferase assays were performed as in Guo et al. (16), except we included a renilla luciferase reporter as an internal control. The Promega (Madison, WI) Stop-N-Glo™ system was used to assay both luciferase activities. The internal control has a renilla luciferase ORF flanked by the 5' UTR and 3' UTR of the

firefly luciferase gene from pGEMLUC (Promega, Madison, WI), and is capped and polyadenylated. Firefly luciferase activities were normalized with renilla luciferase activity to minimize variation between samples.

Results

The putative TNV TE is phylogenetically conserved

Previously, we proposed the presence of a 3' TE structure in TNV strain A, based on conserved sequence and predicted secondary structure (16). Further phylogenetic and secondary structure analyses predict the presence of a similar TE structure in all members in genus *Necrovirus* (Fig. 1). As in the BYDV TE, all *Necrovirus* TEs have a conserved 18 nt tract and a stem-loop structure (Fig. 1, bold italic and SL-I). The 18 nt tract includes the essential sequence, GGAUCC, which comprises a *Bam* HI site in the cDNA clone. For convenience, we refer to it as the *Bam* HI site even though it is in RNA. A structural homolog of stem-loop II (SL-II) in the BYDV TE is missing in all of the *Necrovirus* structures. The loop (L-III) of SL-III of the BYDV TE base pairs to a loop in the 5'UTR (15). In the *Necrovirus* TE-like structure, the loop at the end of a stable stem-loop has a conserved sequence, GUGGUG that differs from BYDV (Fig. 1), but it also has potential to base pair to a loop in the 5' UTR of *Necrovirus* RNAs (Fig. 1, bold). We refer to this stem-loop in the necrovirus TE-like structures as SL-III because it resembles that of SL-III of BYDV.

Sequence in the TNV-D 3'UTR confers cap-independent translation in vitro and in vivo.

To determine if the TE-like structure in TNV-D RNA functions as a cap-independent

translation element, truncated TNV-D RNAs, containing or lacking this structure, were transcribed from full-length clone pTNVD (Fig. 2A) and translated in wheat germ extract. The amount of transcript added in all cases (0.2 pmol) was well below saturating (41, 42), so the levels of translation product were proportional to the translation efficiency of the mRNA. *In vitro* transcription of *Xho* I-linearized pTNVD yields the full-length, infectious genomic RNA transcripts (3). Significant amounts of the main translation product, p22, were translated from uncapped full-length TNV-D RNA (Fig. 2B, lane 2). The faint band migrating at approximately 29 kDa is probably coat protein (p29) that was shown previously to be translated at low levels from TNV-A genomic RNA in wheat germ extract but not *in vivo* (25).

We define a cap-independent translation element as a sequence essential for translation of uncapped mRNA that can be replaced functionally by addition of a 5'cap and not by addition of a poly(A) tail. Therefore, we compared translation of capped and uncapped transcripts of all constructs. Presence of a 5' m^7GpppG cap on the *Xho* I-linearized TNVD transcript increased translation by less than two-fold (Fig. 2B, lanes 2 and 3). Very similar amounts of p22 were obtained from all capped transcripts regardless of the 3' truncations (Fig. 2B, Lanes 3, 5, 7, 9, and 11). However, uncapped transcripts with 3' truncations yielded one-sixth to one-twentieth as much p22 as uncapped full-length TNV-D RNA (Fig. 2B, even-numbered lanes) and one-seventh to one-twentieth of p22 compared to their capped counterparts. Thus, translation of TNV-D RNA is cap-independent and this requires sequence downstream of the *Bsm*B I_{3482} site.

To test whether the TNV-D 3' UTR can confer cap-independent translation on a heterologous gene, we replaced the coding region of TNV-D RNA with the firefly luciferase

coding region (fLuc, Fig. 3A) and translated the resulting RNA, TLucT, in wheat germ extract and in oat protoplasts. As seen with genomic RNA, capped TLucT containing the full-length viral UTRs yielded about 40% more translation product than uncapped TLucT (Fig. 3B, *Sma* I) in wheat germ extract. Thus, replacement of coding regions with the luciferase ORF did not affect the ability of TNV-D UTRs to support cap-independent translation.

Cap-independent translation elements function differently *in vitro* and *in vivo*, thus, we performed all experiments both *in vitro* and *in vivo* to better understand the cap-independent translation of TNV-D. We found that TLucT also translated cap-independently *in vivo* (Fig. 3C, *Sma* I). Luciferase activity in oat protoplasts transfected with uncapped TLucT was at least 3000-times greater than background. Presence of a cap on TLucT RNA increased translation by only 50% (Fig. 3C, *Sma* I), similar to the stimulation seen *in vitro*. Thus, TNV-D UTRs confer cap-independent translation of a heterologous gene both *in vitro* and *in vivo*.

We next set out to map the 5' and 3' boundaries of the 3' UTR sequence required for cap-independent translation. To this end, a series of truncations and internal deletions of TNV-D 3' UTR was made from reporter construct TLucT (Fig. 3A). In wheat germ extract, deletion of the 3'-terminal 104 nucleotides (nt) of the 3' UTR decreased translation of uncapped RNA by less than two-fold (Fig. 3B, *Ssp* I and *Bgl* II). However, truncation up to the *Bam*H I_{3591} site caused a ten-fold decrease in translation of uncapped transcripts (Fig. 3B, *Bam*H I). Addition of a 5' cap restored translation of all these RNAs to wild-type levels (Fig. 3B). Truncation to the *Acc*65 I_{3457} site, located just 3 nt downstream of the Luc stop codon, abolished the cap-independent translation. Addition of a 5' cap increased the translation

more than 25-fold, but still to only 25% of uncapped TLucT (Fig. 3B, *Acc*65 I). Deletions of nts 3462-3510 and 3462-3554 caused only a small decrease in translation of uncapped TLucT (Fig. 3D). Therefore, sequence upstream of nt 3555 and downstream of 3659 is not necessary to obtain at least 50% cap-independent translation in vitro. We hereafter defined the region spanning nts 3555-3659 as the *in vitro* cap-independent translation element (*in vitro* TE).

We examined the boundaries of the 3' UTR required for cap-independent translation *in vivo* by introducing the above set of mutant transcripts into protoplasts (Fig. 3A, 3C, and 3E). Truncations to the *Bgl* II_{3754} or *Ssp* I_{3659} sites reduced luciferase expression from uncapped RNAs by about 7-fold. Addition of a 5' cap increased translation of these truncations and full-length TLucT about two-fold, so expression of the truncated transcripts remained about six to eight-fold below that from capped full-length TLucT RNA (Fig. 3C, *Bgl* II and *Ssp* I). Truncation to the *Bam*H I_{3591} site abolished cap-independent translation activity. Addition of a 5' cap gave measurable translation but luciferase activity remained far below the wild type level (Fig. 3C, *Bam*H I). These data show that sequence downstream of the *Ssp* I_{3659} site is required for efficient gene expression, but it has only a slight, if any, effect on cap-independence of the expression. This is because stimulation by addition of a cap is similar (about 2-fold) in the full-length RNA and RNAs truncated at *Bgl* II_{3754} or *Ssp* I_{3659} sites. Thus the 3' border of the *in vivo*-defined cap-independent translation element is nt 3659.

Deletion of bases 3462-3510 reduced luciferase expression of uncapped RNA by 50% (Fig. 3E, d3462-3510). Deletion of 3462-3554 virtually abolished the cap-independent translation activity (Fig. 3E, d3462-3554). Addition of a 5' cap had little, if any, effect on

translation (Fig. 3E). These data showed that the 5' border of the *in vivo* cap-independent translation element is located downstream of nt 3510 and that sequence between 3511- 3555 is necessary for translation of capped or uncapped RNA. Thus, we conclude that the sequence between bases 3511 and 3762 is required for efficient (≥50% of wild type) *in vivo* cap-independent translation.

Taken together, our data show that the boundaries of the sequence required for cap-independent translation are similar *in vitro* and *in vivo*, but that additional sequences at the very 3' end (downstream of nt 3745) and between nts 3511-3554 are needed for full expression of capped and uncapped RNAs *in vivo* only. Thus, (portions of) another type of translation element(s) and/or a stability element(s) required only *in vivo*, exist outside of the in *vitro*-defined TE.

To test whether the *Bam*H I_{3591} site in the conserved 18 nt tract (bases 3589-3606) is necessary for cap-independent translation as it is in the BYDV TE (42), we constructed TLucTBF, a TLucT mutant with a four-base duplication (GAUC) in the *Bam*H I_{3591} site, and tested its translatability. In wheat germ extract, the translation efficiency of TLucTBF is one-fifth of that of TLucT (Fig. 3F). Addition of a 5' cap restored translation to the wild-type level (TlucT). In oat protoplasts, TLucTBF lost all translatability (Fig. 3G). Addition of a 5' cap increased translation more than 80-fold. Thus, the GAUC duplication in the *Bam* HI site has strong negative effects in the TNV-D TE, as it does in the TE of BYDV.

TNV-D TE functions in the 5' UTR

The BYDV TE can function in the 5' UTR, in place of the natural viral 5' UTR (16). To test whether the TNV-D TE shares this property, we constructed TELucAn (Fig. 4A). In

TELucAn, the firefly luciferase ORF is flanked by the TNV-D TE (nts 3566-3672) as the 5'UTR and a 67 nt vector sequence followed by a 60 base poly(A) tail as the 3' UTR. There are two AUGs in the TNV-D TE, which, being out of frame and upstream of the LUC start codon, would be expected to inhibit translation initiation at the luciferase start codon. Thus, we mutated these two AUGs to AAG and we altered the predicted complementary bases to maintain the predicted secondary structure in TE2LucAn (Fig. 4A).

In wheat germ extract, uncapped TE2LucAn had similar translation efficiency as uncapped TLucT (Fig. 4B). Addition of a 5' cap had no effect on translation of TE2LucAn. Uncapped TELucAn had a translation efficiency similar to that of negative control TLucTBF. Unlike TLucTBF, addition of a 5'cap did not restore translation of TELucAn to the TLucT level. This result is consistent with ribosome entry at the 5' end followed by scanning to the first AUG codon. The first AUG in TELucAn is upstream of, and out-of-frame of, the luciferase start codon, so initiation at this AUG would greatly reduce translation of luciferase. As expected, a negative control, TE2BFLucAn, which contains the GAUC duplication at the *Bam* HI site, translated as poorly as TLucTBF RNA (Fig. 4B). Addition of a 5'cap to TE2BFLucAn restored translation to near the translation level of TLucT and TE2LucAn. Thus, TNV-D TE functions in the 5' UTR to confer cap-independent translation *in vitro*.

In oat protoplasts, uncapped TE2LucAn did not translate as efficiently as uncapped TLucT. However, it is important to note that uncapped TE2LucAn gave luciferase activity that was 1000 to 4000-fold above background, 22-fold above TLucTBF, and six-fold above uncapped TE2BFLucAn (Fig. 4C). Similar translation of constructs with the BYDV TE in the 5' UTR was observed previously (16). The relatively low translation of TE2LucAn in

vivo may result from the secondary structure of the TE in the 5' UTR impeding scanning 40S subunits, and/or the ectopically located TE may not interact efficiently with the artificial poly(A) tail to form a closed loop that facilitates translation in vivo. Also, the sequence necessary for full TE activity in vivo may be absent in this construct.

Loop sequences in the 3' TE and 5' UTR participate in cap-independent translation.

In BYDV, we found that the TE required the presence of the viral 5' UTR only when the TE was located in the 3' UTR. The BYDV TE recruits the translational machinery (E. Allen, personal communication), and that the viral 5' UTR is needed only to communicate with the 3' TE via long-distance base pairing (15). Like BYDV, the TNV-D TE has a stem-loop that with potential to base pair to a stem loop in the 5' UTR (Fig. 5A). This potential long-distance base pairing exists in all *Necrovirus* RNAs (Fig. 1). To test the base pairing hypothesis, we introduced mutations expected to disrupt and restore the potential base pairing, and examined their effects on cap-independent translation both in viral genomic RNA and in reporter gene contexts. Point mutations were introduced into the 5' UTR loop (T*LucT) and the loop of 3' TE SL-III (TLucT*). Each mutation reduced translation five-fold in wheat germ extract, and about 50-fold *in vivo* (Fig. 5 C,D). Thus, the loop sequence in the 5' UTR is crucial for activity of the TE in the 3' UTR context. However, combining the 5' and 3' UTR mutations, which should restore base pairing, did not restore cap-independent translation (T*LucT*, Fig. 5 C,D). Thus, either the double mutant did not fold as predicted to restore the long distance base pairing, or sequence of at least one of the altered loops is important.

We next determined whether the mutations in loop III of the TE inhibited the TE's ability to recruit ribosomes, in addition to the predicted disruption of long distance base pairing. To test this, we measured the ability of the loop III-mutant TE to confer cap-independent translation in the 5' UTR context. In wheat germ extract, no long distance base pairing between UTRs is necessary with the TE in the 5' UTR, but the TE must retain the ability to recruit ribosomes in the absence of a cap. The mutant TE2*LucAn failed to support cap-independent translation at 5' UTR both *in vitro* and *in vivo* (Fig. 4B and 4C, TE2*LucAn). Thus, the point mutations in TE loop III knocked out TE function altogether and we are unable to conclude whether long-distance base pairing is required, because it is not possible to restore TE function in the compensatory double mutant (T*LucT*), even if long-distance base pairing is restored.

The *BYDV 5' UTR allows cap-independent translation by the TNV-D 3' TE.*

To further test the role of 5' UTR-3' UTR interaction in TE-mediated cap-independent translation, we tested luciferase constructs containing all four possible combinations of TNV and BYDV UTRs. Because loop III of the BYDV TE is different from loop III of the TNV-D TE, we expected that the 5' UTR of TNV would not support translation when combined with the 3' UTR of BYDV and vice versa. Indeed, cap-independent translation of the construct with the TNV 5' UTR and BYDV 3' TE (TLucB) was very low in wheat germ extract (Fig. 6C) and undetectable in protoplasts (Fig. 6D). Surprisingly, the reciprocal construct with the BYDV 5' UTR paired with the TNV 3' TE (BLucT) gave significant luciferase activity (about 30% of the all-BYDV UTR construct,

BLucB) *in vitro* (Fig. 6C) and even in the more competitive *in vivo* conditions (Fig. 6D), where BLucT translates at least 30-fold more efficiently than TLucB.

The above result can be explained by the complex structure of the BYDV 5' UTR. Normally, loop III of the BYDV TE base pairs to stem-loop IV (SL-IV) of the BYDV 5' UTR to mediate the 3'-5' communication (Fig. 6A) (15). In contrast, phylogenetic analysis supports base pairing of loop III of the TNV TE to the 5' proximal stem-loop, SL-I, of the TNV 5' UTR (which is much shorter than that of BYDV and has no structural homolog to SL-IV of the BYDV 5' UTR). The BYDV 5' UTR has a 5'-proximal stem-loop (SL-I) that resembles that of TNV. Thus, we propose that the TNV 3' TE stem-loop III can base pair to SL-I of the BYDV 5' UTR (Fig. 6A). This explains why the hybrid construct BLucT facilitates cap-independent translation. To further investigate this, point mutations were introduced into the loop of BYDV SL-I in BLucT (construct B*LucT), loop III of the TNV TE (BLucT*), and in both positions (B*LucT*). As predicted, the point mutations destroyed cap-independent translation (Fig. 6 E,F). However, the double mutants did not restore translation because the TNV TE does not tolerate changes to loop III (Fig. 4 B,C, TE2*LucAn; and Fig. 5). Importantly, point mutations in SL-I of the 142 nt BYDV 5' UTR destroyed cap-independent translation on a construct with the TNV 3' TE (B*LucT). In contrast, SL-I of the BYDV 5' UTR is unnecessary for function of the BYDV 3' TE (15). Thus, the BYDV 3' TE and the TNV 3' TE appear to interact with different loops in the BYDV 5' UTR to facilitate cap-independent translation.

Discussion

TNV-D RNA has a BYDV-like cap-independent translation element in its 3'UTR.

Here we identified a cap-independent translation element in the 3' UTR of TNV, which shares the following properties with the TE of BYDV. (*i*) The TNV-D TE is at most 105 nt long and allows translation of uncapped viral and nonviral mRNAs as efficiently as corresponding capped mRNAs *in vitro* (Fig. 2, and Fig. 3B, 3D). (*ii*) Deletion of the sequence causes a many-fold decrease in translation of uncapped mRNAs. Addition of a 5' cap to these mRNAs restores translation to the wild type level (Fig. 2, and Fig. 3B). (*iii*) The predicted secondary structure of the TNV-D and other necrovirus TEs has features in common with the known BYDV TE structure (Fig. 1). (*iv*) The TEs of TNV-D and BYDV share an 18 nt sequence: CGGAUCCUGGGAAACAGG that is well conserved among members of *Luteovirus* and *Necrovirus* genera (Fig. 1 and refs. (15, 16)). (*v*) A four-base duplication (GAUC) in the *Bam*H I site in the conserved sequence abolishes the TE function. (*vi*) When located in the 3' UTR (its natural location), the TE depends on the viral 5' UTR to function. (*vii*) When located in the 5' UTR (with AUG triplets altered), the TNV-D TE allows similar *in vitro* translation efficiency as the combination of TNV-D 5' and 3' UTRs (Fig. 4B). Thus, the viral 5' UTR serves only for the long-distance 5'-3' communication. (*viii*) When tested in protoplasts, a longer sequence is required for efficient translation, and deletion or mutation of the TE had much more drastic negative effects on activity than in wheat germ extract (Fig. 3C,E). (*ix*) The extra sequence needed only *in vivo* is needed for translation of capped and uncapped mRNAs. (*x*) Our data strongly support but do not prove that long-distance base pairing between the TNV 3' TE and the 5' UTR is required for cap-independent translation, as is known for BYDV UTRs (15).

A cap-independent translation element from genus *Dianthovirus* (*Tombusviridae* family) also fits in this class of BYDV-like TEs. Previously we showed that the dianthoviruses contain the 18 nt conserved sequence, with one or two base differences, in their 3' UTRs (41). More recently, the 3' UTR of a dianthovirus RNA (RCNMV RNA 1) was shown to have a cap-independent translation element with many of the properties listed in the previous paragraph (29). In the 18 nt conserved sequence, mutations known to knock out the BYDV TE function also eliminated function of the RCNMV TE (29). The RCNMV TE has predicted secondary structural homologs to stem-IV and SL-I but differs in other ways (below). In summary, we now define a class of cap-independent translation elements, called BTEs (BYDV-like TEs) present in at least three plant virus genera, that are defined by (*i*) the ability to powerfully stimulate translation of uncapped mRNA, (*ii*) location in the 3' UTR, (*iii*) presence of a highly conserved 18 nt sequence, and (*iv*) similar secondary structures.

Differences between the Necrovirus, Dianthovirus and Luteovirus TEs.

There are notable differences that distinguish the TEs of each genus discussed above. The predicted structures of the TE of all necroviruses lack a structural homologue to stem-loop II of the BYDV TE. Previous deletion analysis revealed that deletion of SL-II knocked out BYDV TE function, while mutations that disrupted the BYDV SL-II merely reduced TE activity, and double mutations that restored SL-II, restored BYDV TE function (16). We speculate that SL-II does not participate directly in factor or ribosome recruitment, but that the alterations to SL-II had deleterious effects on the overall structure of the BYDV TE. Thus, the function of SL-II is unclear. It may participate in a function other than translation,

such as a subgenomic RNA promoter (19), which is unique to luteoviruses. While the RCNMV RNA1 TE contains predicted structural homologous to stem IV and SL-I, it contains two *more* predicted stem-loops between SL-I and stem IV, than the BYDV TE and thus three more than predicted in the TNV TE. It is not obvious which is the functional homolog to SL-III. In fact, Mizumoto et al. (26) showed that the RCNMV RNA1 TE could function in the presence of a nonviral 5' UTR. Thus, although complementarity between the RCNMV RNA1 3' and 5' UTRs can be predicted (WAM, unpublished), its role, if any, is unclear.

While base pairing between 3' and 5' UTRs appears to be necessary for luteovirus and necrovirus TEs, the loop of SL-III that is complementary to a loop in the 5' UTR has a different sequence in each genus (12). Deleterious point mutations in the 5' UTR loop of TNV indicated its importance in allowing the 3' TE to function, but compensating mutations could not restore activity. Thus the sequence of loop III is very important as well as the probable long-distance base pairing. We also found the BYDV loop III to be very sensitive to base changes. Only a U to A point mutation was allowed to compensate for a point mutation in the 5' UTR, and even this mutation reduced translation efficiency (12). Other covarying mutations in loop III did not restore BYDV TE activity (L. Guo, A. Rakotondrafara, personal communications). Thus, the long-distance base pairing may be sensitive to non-Watson-Crick structural changes and/or the sequence of loop III is required for interactions with a protein(s) necessary for cap-independent translation.

Comparison to other classes of 3' cap-independent translation elements: taxonomic implications

Non-BYDV-like 3' cap-independent translation elements have been detected in other viruses in the large, diverse *Tombusviridae* family. These include TCV and HCRV in genus *Carmovirus*, TBSV in genus *Tombusvirus*, and the satellite virus of TNV (STNV). None of these RNAs harbors a 3' UTR that bears sequence or structural similarity to a BTE. The 3' element of STNV RNA stimulates cap-independent translation as efficiently as BTEs in vitro and in vivo, is about the same size, and is located at the 5' end of a long 3' UTR (6), but its sequence and structure are entirely different from those of BTEs (6, 26, 38, 40, 41). How the different TNV and STNV cap-independent translation elements compete for the host translational machinery is an interesting unanswered question.

Cap-independent translation mediated by the TBSV translation enhancer was detectable only *in vivo* (45). This sequence overlaps cis-acting replication elements and is more 3'-proximal than the BTEs (45). A 180 nt sequence including an essential hexanucleotide, GGGCAG, in the 3' UTR of HCRV confers cap-independent translation (20). This sequence functions with the IRES of encephalomyocarditis virus (20). The TCV translation enhancer located at the 5'-end of the 255 nt 3' UTR, is 150 nts long, and requires the 5' UTR to achieve optimal translation efficiency (35).

The fact that BTEs are in all known or probable members of the *Luteovirus* genus, but not the two other genera of the *Luteoviridae* family, and in only two of several genera of the *Tombusviridae* has significant evolutionary implications. Either the BTE evolved independently in each family or, more likely, recombination took place between ancestral members of *Luteoviridae* and *Tombusviridae* (27). Additional homology between the

replicase genes of genus *Luteovirus* and the *Tombusviridae*, especially the dianthoviruses, suggests that genus *Luteovirus* may be more appropriately assigned to the *Tombusviridae* (28).

Additional sequence required for translation in vivo.

The additional portions of the 3' UTR required only for in vivo translation may facilitate binding of translation initiation factor(s) and/or other trans-acting factor(s) to the TNV-D TE, enhance the interaction between UTRs, increase the stability of RNA, or all of the above. We found that a double stem-loop structure at the extreme 3' end of TNV-D RNA functionally mimics a poly(A) tail (Chapter 5), i.e. the additional sequence needed for translation in vivo can be replaced by a poly(A) tail, but not by a 5' cap, to obtain an efficient mRNA. BYDV RNA also contains a "poly(A) mimic" function downstream of the 3' TE (16). These elements are not needed in vitro probably because the excess ribosomes present in wheat germ extract provide far less competitive translation conditions for an mRNA than in vivo, in which many host mRNAs compete for limiting ribosomes. How these various functional domains in the viral 3' UTR interact with each other and with host factors to recruit ribosomes remains to be investigated.

The cap-independent translation mechanism of TNV-D TE

Based on phylogenetic comparisons (Fig. 1) and experimental data with BYDV, we speculate that the highly conserved sequence that includes the *Bam*HI site and stem-loop I plays a key role in recruiting translation factors, and that the long, G,C-rich stem-loop III, serves to project loop III outward to be accessible to the 5' UTR to which it must base pair and to any proteins that facilitate this long-distance interaction. Stem IV may also project the

entire TE and isolate the TE from intramolecular base pairing with flanking sequences in the RNA.

There are some revealing variations in the 18 nt conserved sequence among the necroviruses. In all but one case, loop I fits the pentaloop consensus, GNRNA. A stem-loop involved in anti-termination of bacteriophage lambda transcription also fits this motif (22). The fourth base of the GNRNA loop protrudes outward, allowing the remaining four bases to form the same stabilizing interactions as in a GNRA tetraloop (22). Interestingly, *Leek white stripe necrovirus* (LWSV) has only a four base loop I, and it does not fit the GNRA consensus. The stem also has unique base changes, but covariations maintain the SL-I helix (Fig. 1). While these exact mutations were not tested, alteration of BYDV loop I to contain only four bases, destroyed BYDV TE activity (15). Thus either the LWSV TE tolerates differences that other TEs do not, or it may be cloned from a nonviable mutant in the LWSV quasispecies population that was used for sequencing.

Why translational control via the 3' UTR?

We speculated previously that having the 3' UTR facilitate translation initiation at the 5' end serves as a switch to prevent collisions of ribosomes and replicase on BYDV RNA (1). This is inspired by studies that showed that synthesis of poliovirus negative strand RNA is completely blocked by translating ribosomes (2, 14). Thus RNA synthesis requires prior removal of ribosomes from the viral genome (2). Now we suggest that the following mechanism we proposed for BYDV also applies to all viruses in the *Tombusviridae.* After translation of the viral replicase (p82 in TNV) facilitated by the 3' cap-independent translation element, the replicase would begin copying the viral RNA from the 3' end. As it

proceeds in the 5' direction on the viral template RNA, the replicase would disrupt base pairing (or other form of interaction) of the 3' cap-independent translation element with the 5' UTR. This would shut off translation initiation at the 5' end while the replicase is still in the 3' UTR, and clear the upstream ORFs of ribosomes by the time the replicase reaches them (see details in Fig. 5, ref. 1). This would allow efficient replication of viral RNA, unimpeded by ribosomes. Subsequently, when enough RNA accumulates, some molecules will be free of replicase and able to form the long-distance interactions that facilitate translation, and the cycle would begin again. This model provides an elegant means by which positive strand virus RNA may achieve the potentially conflicting roles of both genome and messenger RNA.

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Figure Legends

Fig. 1. Secondary structures of BYDV TE and putative *Necrovirus* TEs predicted by MFOLD (46). The structure of BYDV TE has been confirmed by structure probing (16). Bold italic: 18 nt conserved tract. Bold: potential base pairing between TEs and corresponding 5' UTR. Relevant portions of 5' UTRs are shown in rectangles. TNV-D: TNV strain D UK isolate (Genebank accession #: D_00942). TNV-DH: TNV strain D Hungary isolate (NC_003487). TNV-A: TNV strain A (NC_001777). OLV-1: *Olive latent virus 1* (NC_001721). LWSV: *Leek white stripe virus* (NC_001822).

Fig. 2. Effect of 3' truncations on translation of TNV-D RNA *in vitro*. (A) Genome organization of TNV-D RNA. Restriction enzyme sites used for truncation are shown with base number in parentheses. (B) Translation products of capped (C) or uncapped (U) TNV-D RNA truncated at the indicated restriction enzyme sites. The prominent band is p22. The predicted 104 kDa readthrough product (p22 + p82) was not detected under these translation conditions. Lane 1: translation products of BMV RNAs with mobilities in kilodaltons (kDa) shown at left. Translations were performed in wheat germ extract (Promega) with 0.2 pmol of RNA and [^{35}S]-methionine in a 25 µl reaction at 25^{0}C for 1 h. Products were separated on an SDS, 10% polyacrylamide gel and detected with a STORM 840 Phosporimager and quantified by ImageQuant 5.2 (Amersham) software.

Fig. 3. Deletion mapping of TNV-D 3' UTR sequences required for cap-independent translation. (A) Map of TLucT and its mutants. Truncation transcripts are named after the

restriction enzyme used for truncation. Restriction sites are numbered according to their position in the TNV-D genome. Deletion transcripts are named by the deleted bases. fLuc: firefly luciferase ORF. TNV-D 5' and 3' UTRs are indicated by bold lines with blank areas indicating deleted portions. *In vitro* translations (B,D,F) were performed as Fig. 2. Relative luciferase activity in oat protoplasts (C,E,G) was determined following cell lysis 4 h after electroporation with 1 pmol of the indicated transcript, and assayed as in Materials and Methods. Luciferase assays were performed in at least three independent experiments, each of which was in triplicate. Luciferase activities are normalized to that yielded by uncapped TLucT (defined as 100%). Standard deviations are indicated. (B,C) Effect of 3' truncations on translation of the TLucT transcript. (D,E) Effect of deletions near the 5' end of the TLucT 3' UTR on luciferase expression. (F,G) Effect of a four-base duplication (GAUC) in the *Bam*H I_{3591} site on cap-independent translation of TLucT. TLucTBF differs from TLucT only by a GAUC duplication at the conserved *Bam*H I_{3591} site.

Fig. 4. Function of the 105 nt TNV-D TE in the 5' UTR. (A) Map of transcripts showing the 105 nt portion of the TNV-D 3' UTR that was placed in the 5' UTR of TELucAn, MFOLD-predicted secondary structure of TNV-D TE, and mutated regions (boxed). Mutated bases are in bold. Names of mutants are shown outside the rectangles. TE2LucAn is the parent construct for TE2BFLucAn and TE2*LucAn mutants. (B) Relative luciferase activity of RNAs with 105 nt TNV-D TE or its mutants as 5' UTR *in vitro*. (C) Relative luciferase activity of RNAs with 105 nt TNV-D TE or mutants as 5' UTR *in vivo*.

Fig. 5. Effect of mutations in the potential base pairing between TNV-D 3' TE and 5' UTR on cap-independent translation. (A) Secondary structures of the TNV-D 3' TE and a conserved stem-loop at the 5' end of the 5' UTR. Dashed lines: potential base pairing. Bold italic: the conserved 18 nt tract. Mutated bases are in bold. (B) Translation of TNV-D gRNA with wild-type or mutant UTRs in wheat germ extract. 5* indicates mutation at 5' UTR. 3* indicates mutation at 3' UTR. 5*3* indicates mutations at both UTRs, which restores the potential base pairing. The main translation product of gRNA, p22, is indicated. Assays were done as in Fig. 2. (C) and (D): Relative luciferase activity of TLucT with wild-type or mutant UTRs in wheat germ extract (C) and in oat protoplats (D). * denotes mutation shown in (A). Assays are performed as Fig. 3B and 3C.

Fig. 6. Translation of reporter constructs with all combinations of TNV-D and BYDV UTRs. (A) Secondary structures of BYDV 5' UTR, BYDV 3' TE, TNV-D TE, showing potential base pairing (bold bases) of selected portions of BYDV 5' UTR with the 3' TEs. Mutated bases are in circles. (B) Maps of reporter constructs. Genomic position of UTRs are indicated by numbers. T indicates TNV-D UTR (black) and B indicates BYDV UTR (gray). (C) and (D) Relative luciferase activities of RNA transcripts with wild type UTRs in wheat germ extract and oat protoplasts, respectively. (E) and (F) Relative luciferase activities yielded by wild type or mutant BLucT transcripts in wheat germ extract (E) and oat protoplasts (F), respectively. * denotes mutations shown in panel A.

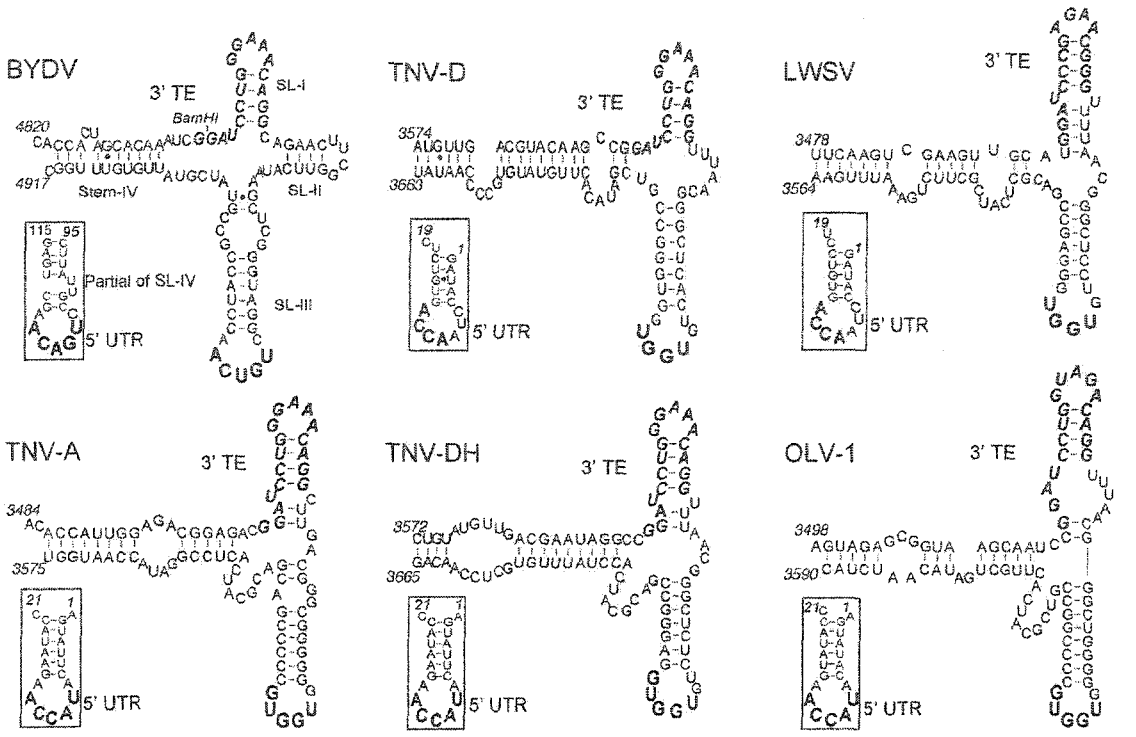
BYDV
3' TE
SL-I
BamHI
Stem-IV
SL-II
Partial of SL-IV
SL-III
5' UTR
TNV-D
3' TE
5' UTR
LWSV
3' TE
5' UTR
TNV-A
3' TE
5' UTR
TNV-DH
3' TE
5' UTR
OLV-1
3' TE
5' UTR

Fig. 1

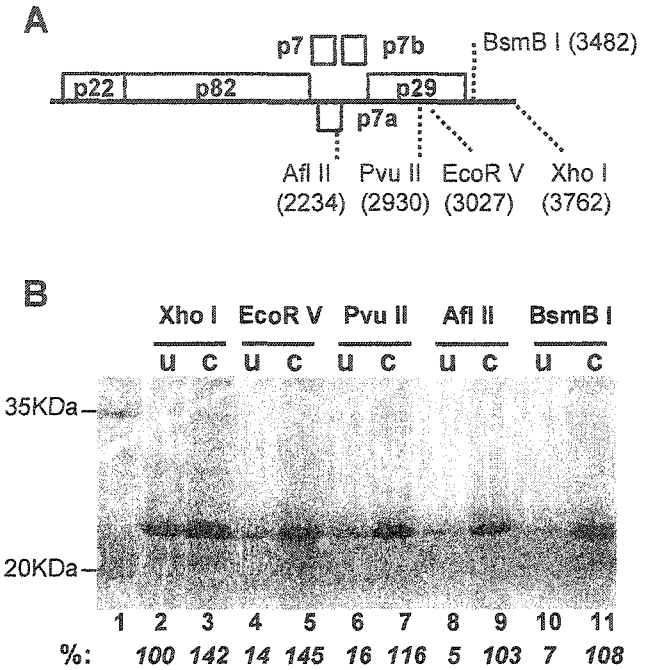
A
p7
p7b
BsmB I (3482)
p22
p82
p29
p7a
Afl II
(2234)
Pvu II
(2930)
EcoR V
(3027)
Xho I
(3762)
B
Xho I
EcoR V
Pvu II
Afl II
BsmB I
u c u c u c u c u c
35KDa
20KDa
1 2 3 4 5 6 7 8 9 10 11
%: 100 142 14 145 16 116 5 103 7 108

Fig. 2

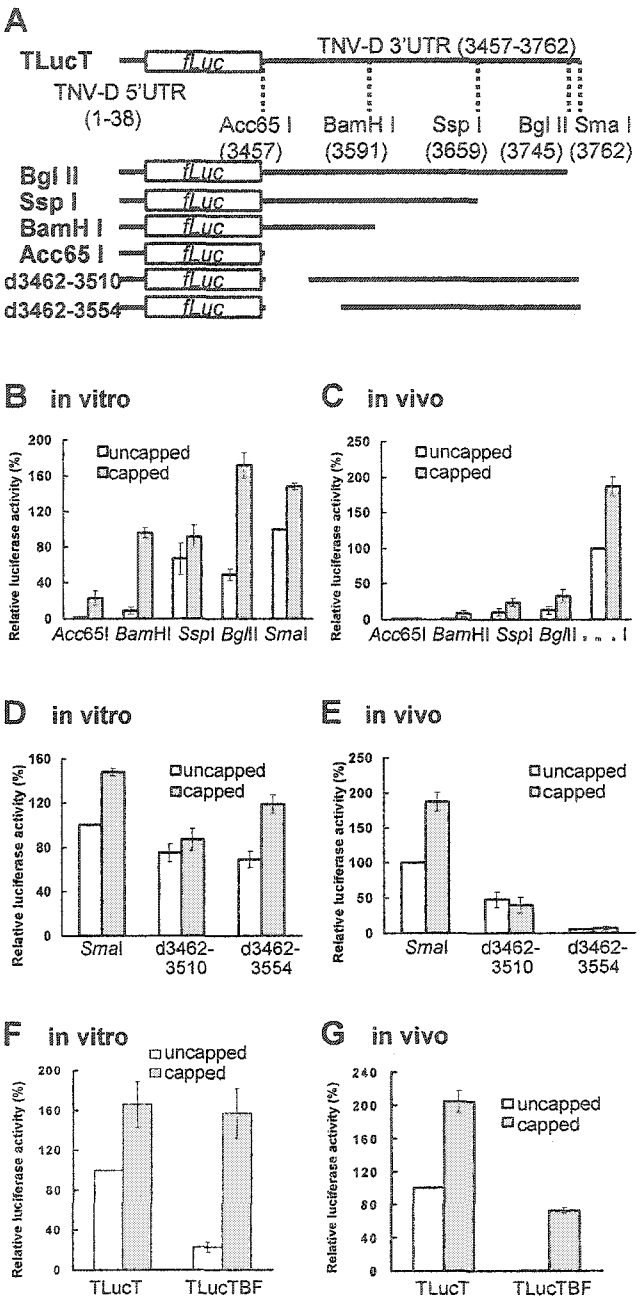
A
TLucT
fLuc
TNV-D 3'UTR (3457-3762)
TNV-D 5'UTR
(1-38)
Acc65 I
(3457)
BamH I
(3591)
Ssp I
(3659)
Bgl II
(3745)
Sma I
(3762)
Bgl II
Ssp I
BamH I
Acc65 I
d3462-3510
d3462-3554
B in vitro
C in vivo
Relative luciferase activity (%)
uncapped
capped
Acc65I BamHI SspI BglII SmaI
D in vitro
E in vivo
SmaI
d3462-3510
d3462-3554
F in vitro
G in vivo
TLucT
TLucTBF

Fig. 3

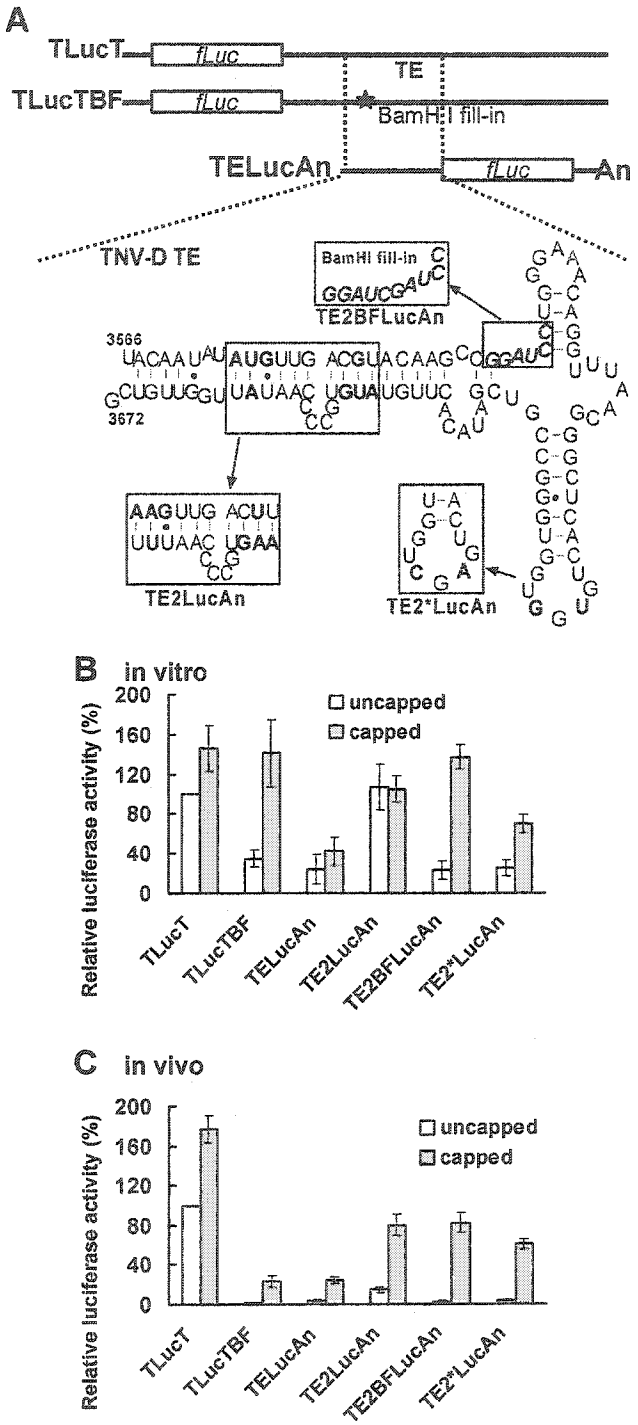
A
TLucT
fLuc
TE
TLucTBF
fLuc
BamHI fill-in
TELucAn
fLuc
An
TNV-D TE
BamHI fill-in
GGAUCGAUC
TE2BFLucAn
3566
3672
AUGUUG ACGUACAAG
UGUAUGUUC
AAGUUG ACUU
UUUAAC UGAA
TE2LucAn
TE2*LucAn
B in vitro
Relative luciferase activity (%)
200
160
120
80
40
0
uncapped
capped
TLucT
TLucTBF
TELucAn
TE2LucAn
TE2BFLucAn
TE2*LucAn
C in vivo
Relative luciferase activity (%)
200
160
120
80
40
0
uncapped
capped
TLucT
TLucTBF
TELucAn
TE2LucAn
TE2BFLucAn
TE2*LucAn

Fig. 4

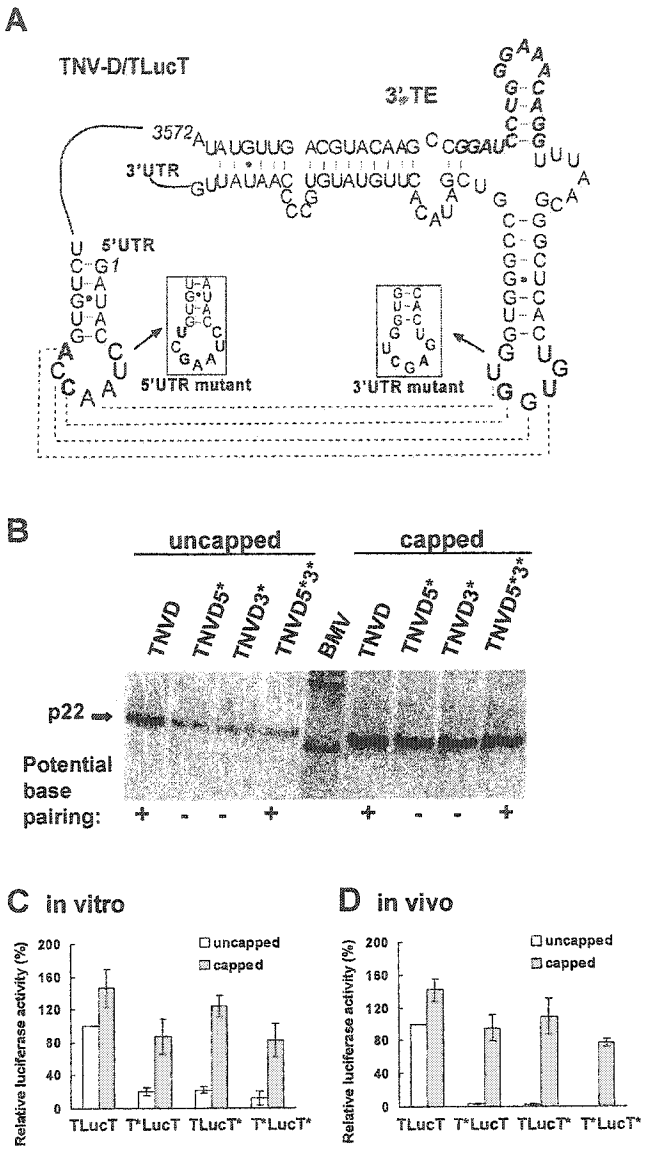
A
TNV-D/TLucT
3'TE
3572
3'UTR
5'UTR
5'UTR mutant
3'UTR mutant
B
uncapped
capped
TNVD
TNVD5*
TNVD3*
TNVD5*3*
BMV
p22
Potential base pairing:
C in vitro
D in vivo
Relative luciferase activity (%)
uncapped
capped
TLucT
T*LucT
TLucT*
T*LucT*

Fig. 5

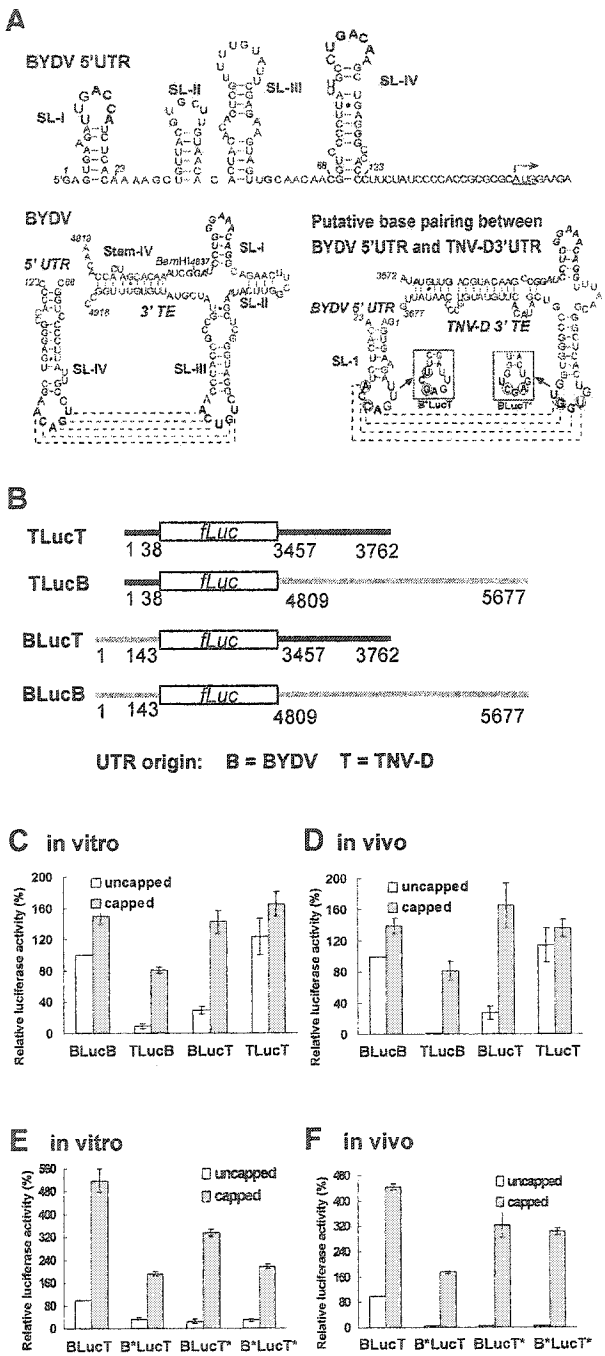
A
BYDV 5'UTR
SL-I
SL-II
SL-III
SL-IV
BYDV
5' UTR
Stem-IV
SL-I
SL-II
3' TE
SL-IV
SL-III
Putative base pairing between
BYDV 5'UTR and TNV-D3'UTR
TNV-D 3' TE
B
TLucT
TLucB
BLucT
BLucB
fLuc
1 38
3457
3762
4809
5677
1 143
UTR origin: B = BYDV T = TNV-D
C in vitro
D in vivo
uncapped
capped
Relative luciferase activity (%)
BLucB TLucB BLucT TLucT
E in vitro
F in vivo
BLucT B*LucT BLucT* B*LucT*

Fig. 6

CHAPTER 5. A POLY(A) TAIL MIMIC AT THE 3' END OF AN UNCAPPED, NONPOLYADENYLATED VIRAL RNA

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Ruizhong Shen and W. Allen Miller

Abstract

The 3762 nt genomic RNA of *Tobacco necrosis virus* (TNV) (*Tombusviridae* family) is naturally uncapped and nonpolyadenylated, but is translated efficiently. Our previous data showed that the 3' untranslated region (UTR) of TNV RNA harbors a functional cap-independent translation element (TE) that resembles that of *Barley yellow dwarf virus* (BYDV, *Luteoviridae*). As with the BYDV TE, additional, unmapped sequences in the TNV 3' UTR are needed for efficient cap-independent translation *in vivo*, but not *in vitro*. The role(s) of the extra sequence is not clear. Here, we determined that this extra sequence functionally mimics a poly(A) tail and not a 5' cap in stimulating translation. Truncations and deletions downstream of nt 3662 caused loss of translation, which was restored by adding a 60 nt poly(A) tail, but not by the presence of a 5' cap. The same effects were caused by point mutations tested in this region. Thus, the sequence between nt 3662-3762 is a poly(A) mimic sequence (PAM). Secondary structure prediction revealed a double stem-loop structure, which is phylogenetically conserved among all necroviruses. Mutation analyses established that the double stem-loop structure is important for the PAM function, as well as for viral replication. Physical and functional stability assays suggested the PAM facilitated translation initiation. The double stem-loop structure is functionally replaceable

by, but cannot functionally substitute for a poly(A) tail. However, the full-length 3' UTR of TNV-D is sufficient to functionally replace a poly(A) tail.

Introduction

Translational control is a major step of gene regulation for RNA viruses, oocytes, and other systems with little or no transcriptional control. Most translational control elements and features in mRNAs exist in the 5' and 3' untranslated regions (22, 25, 26, 36, 43). On average, 3' UTRs are substantially longer that 5' UTRs (26). The average 5' UTR length is roughly constant for all taxa, but the average 3' UTR length varies significantly (26). Consequently, the 3' UTR is a region with great regulatory potential. 3'UTRs contain many translational control elements, such as cytoplasmic polyadenylation elements (CPE), AU-rich elements (AREs), and an array of diverse binding sites for regulatory proteins (17, 34, 42, 43). For example, translational controls by 3' UTR elements are essential to both male and female gametogenesis, early embryonic development, stem-cell proliferation, sex determination, neurogenesis, and erythropoiesis (17, 34, 42).

The 3' poly(A) tail is an important and well-studied element in determining translational efficiency. The poly(A) tail regulates both stability and translational efficiency of mRNAs (14). The 5' cap and poly(A) tail function synergistically to facilitate efficient translation initiation via circularization of mRNA (6, 13, 30, 31, 38, 41). Eukaryotic initiation factor (eIF) 4E binds the 5' cap and is associated with eIF4G. eIF4G also binds poly(A) binding protein (PAPB), which binds to the poly(A) tail. Thus mRNA is circularized (6, 13, 30, 31, 38, 41, 15, 32, 38). The mRNA circulation provides a framework to understand how elements within 3' UTRs can control translation. However, some

mRNAs lack a cap and/or a poly(A) tail. How these mRNAs translate efficiently is an interesting and not well-understood question.

Many viral mRNAs and some cellular mRNAs have a cap structure but lack a poly(A) tail. Generally, specific sequences within 3' UTR replace the function of a poly(A) tail. The RNAs of *Tobacco mosaic virus* (TMV) (8, 9), *Rotavirus* (27), and *Brome mosaic virus* (7) have functional alternatives for the poly(A) tail in the 3' UTR. Metazoan, but not plant, histone mRNA also lacks a poly(A) tail and has a stem-loop structure functionally mimicking a poly(A) tail (24, 44). Corresponding binding proteins are also found for some of these poly(A) functional alternatives. Examples include host protein p102, which binds both the 5'- leader and 3'- upstream pseudoknot domain of TMV (37); rotavirus protein NSP3, which binds rotavirus poly(A) mimic sequence and eIF4G (4, 10, 27, 29, 39); and SLBP (stem-loop binding protein) for metazoan histone mRNA (24, 35, 44). The coat protein of *Alfalfa mosaic virus* (BMV, *Bromoviridae*) is proposed to act as a functional equivalent of PAPB (23). However, the 3' UTR of AMV cannot be replaced by a poly(A) tail (7).

Tobacco necrosis virus (TNV) is the type member of genus *Necrovirus* in the *Tombusviridae* family. TNV RNA has no 5' cap (19) and no 3'poly(A) tail (21). In this report, we used an isolate of TNV strain D from the United Kingdom (TNV-D) as model. TNV-D has a positive sense single-stranded RNA genome of 3762 nt. It encodes six open reading frames (Fig. 1). We reported previously that TNV-D RNA has a translation element (TE) within the 3' UTR that functionally mimics a 5' cap (Fig. 1, Chapter 4). The TNV TE confers cap-independent translation both *in vitro* and *in vivo*. When located in the 3' UTR (its natural location), the TE depends on the viral 5' UTR to function. It also functions in the

5' UTR when the AUG triplets within the TE are altered. Longer sequence is required for efficient cap-independent translation *in vivo* than *in vitro*. A 105 nt sequence (3555-3659) is sufficient for cap-independent translation in wheat germ extract. Full length 3'UTR is required for efficient cap-independent translation *in vivo* (Chapter 4). However, the role of the extra sequence is not clear. Here, we report that this extra sequence required for translation *in vivo* can be replaced by a 60nt poly(A) tail, but not a 5' cap. This suggests that the extra sequence has a poly(A)-mimic function. The extra sequence is also predicted to form a double stem-loop structure. Mutation analyses showed that this double stem-loop structure is important for the poly(A)-mimic function, as well as for viral replication.

Materials and Methods

Plasmids and RNA constructs

All clones were verified by automated sequencing at the Iowa State University DNA Sequencing and Synthesis Facility. Plasmid pTNV-D is a full-length infectious clone of TNV-D, kindly provided by R. H. A. Coutts, Imperial College, London (2). pTLucT is the template for TLucT, which has a firefly luciferase ORF as a reporter flanked by the 5' and 3' UTRs of TNV-D (Chapter 4). D3720, D3700, D3680, and d3726-3738 were constructed by replacing the 3' UTR of TLucT with the respective shortened 3' UTR of TNV-D generated by PCR. In D3720, nts 3721-3744 were deleted. In D3700, nts 3701-3759 were deleted. Nts 3681-3759 were deleted in D3680. D3748 and D3661 were truncations of TLucT at *Bgl* II_{3745} and *Ssp* I_{3659}, respectively.

Mutants within the stem-loops were constructed by using standard PCR-mediated, site-directed mutagenesis as in (11, 12). VLucAn was described in Guo et al. (12), in which

a 60 base poly(A) tail was inserted into the *Stu I/Sal I* site of pGEMLUC (Promega, Madison, WI) (12). VLucT122 was constructed by replacing the 3' UTR of VLucAn with a 122 nt sequence from TNV-D 3' UTR (nts 3641-3762), which includes the PAM sequence. V indicates sequence from vector, and T indicates sequence from TNV-D. The template for *in vitro* transcription of VLucV294 was pGEMLUC linearized with *Ssp I*. TLucT122 and TLucV294 were constructed by replacing the 5' UTR of VLucT122 and VLucV294 with the 5' UTR of TNV-D. TLucT171 was constructed by replacing the 3' UTR of TLucT122 with the 171 nt (nts 3592-3762) sequence from the 3' UTR of TNV-D. TLucTBF has the full length 3' UTR of TNV-D with a GUAC duplication in a *BamH I* site. The cap-independent translation function of TE is destroyed by this duplication (Chapter 4).

In vitro transcription

Capped and uncapped RNAs were synthesized by *in vitro* transcription using the T7 mMESSAGE mMACHINE and MegaScript kits (Ambion, Austin, TX) as per manufacturer's instructions, respectively. Templates for RNAs with a poly(A) tail were linerized with *Vsp I*. Template for VLucV294 was pGEMLUC linearized with *Ssp I*. Templates for D3661 and D3748 were pTLucT linearized with *Ssp I* and *Bgl II*, repectively. All other templates were digested with *Sma I*.

In vivo translation

Oat (*Avena sativa* cv. Stout) protoplasts were prepared and electroporated with RNA as described in (5) . Luciferase assays were done as in Shen and Miller (Chapter 4). We included a capped and polyadenylated renilla luciferase reporter as an internal control, and

the Promega Stop-N-Glo™ (Madison, WI) system was used to assay both luciferase activities. All luciferase assays were performed in triplicates in at least three independent experiments. Firefly luciferase activities were first normalized with renilla luciferase activity to minimize variation between samples. The luciferase activities of all constructs were then compared to TLucT, whose luciferase activity is defined as 100%.

Northern blot hybridization

Total RNA was extracted from protoplasts by using the Trizol reagent (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. For TNV-D replication assays, NT-1 protoplasts were used and incubated for 24 hr after electroporation. For physical stability assays, oat protoplasts were used and incubated for 0 to 9 hr after electroporation. Total RNAs were extracted from these cells and analyzed by Northern blot analysis as described previously (16). A ^{32}P-labled probe, complementary to the 107 nt TE of TNV-D, was used to detect TNV-D gRNA and sgRNAs.

Stability assay

Physical stability assays were done as described in (40). Functional stability assays were done as described in (3). Protein accumulation (A) as a function of time (t) was analyzed by using the first order kinetics equation: $A(t) = A_0e^{-kt}$. A function $y = a\cdot\ln(t) + b$ was achieved from the logarithmic trend line of curve protein accumulation (A) vs. time (t) by using Microsoft Excel. Constant k was calculated by giving an arbitrary time t = 60 min and the function. We then calculated the functional half time $t_{1/2} = \ln(1/2)^* (1/k)$

Results

A sequence within the TNV-D 3' UTR functionally mimics a poly(A) tail.

We showed previously that the *in vivo* TE and *in vitro* TE have the same 3' boundary, but additional sequence downstream of nt 3659 is needed for full expression of capped and uncapped RNAs *in vivo* (Chapter 4). These observations suggest that another type of translation element and/or stability element exists downstream of nt 3659 and is required *in vivo* only. Because poly(A) tails are necessary for translation of typical mRNAs *in vivo* but not in wheat germ extract (WGE), we proposed that the additional sequence could be replaced by a poly(A) tail. To test the function of this extra 103 nt sequence in translation in vivo, we made a series of deletions and truncations in this region on reporter construct TLucT, then examined the translatability of these mutated RNAs with cap and poly(A), with cap only, with poly(A) only, or with no cap and no poly(A). TLucT encodes the firefly luciferase ORF flanked by TNV-D 5' UTR and 3' UTR (Chapter 4).

Based on the effect of deletions on translation, the 103 nt sequence could be separated into approximately three regions. Region I includes nts 3721-3762. Deletion of this region caused translation to drop about 2.5 fold from TLucT (Fig. 2, TLucT, D3748, and D3720). Addition of a 5' cap has little, if any, effect on translation. However, addition of a 60 nt poly(A) tail increased translation 5 to 6-fold and restored it to a similar level as TLucT with a poly(A) tail. Addition of a 5' cap and a poly(A) tail had a similar effect on translation as did poly(A) only. No synergistic effect between cap and poly(A) was observed (Fig. 2, TLucT, D3748, and D3720). Region II includes nts 3681-3720. The translation level of RNAs with region II deletions dropped 14 to 20-fold from TLucT. Again, addition of a 5' cap has little, if any, effect on translation. Addition of a 60 nt poly(A) tail increased translation about 8-

fold, but could not restore translation to the level of TLucT. Cap and poly(A) also had no synergistic effect (TLucT, D3700, D3680). Region III includes 3662-3680. Deletion of region III caused a 17-fold reduction in translation. Addition of a 60 nt poly(A) tail increased translation 4.5-fold. Interestingly, addition of a 5' cap increased translation 2.5-fold. Cap and poly(A) showed synergistic effect. A 37-fold increase was observed in D3661 RNA by adding both a 5' cap and a poly(A) tail. The translation level of capped, polyadenylated D3661 RNA was similar to capped, polyadenylated TLucT RNA (Fig. 2, TLucT, D3661).

Taken together, our data showed that the extra sequence required for efficient translation of TNV-D *in vivo* can be replaced by a poly(A) tail to restore translation. Thus, we defined this sequence as a poly(A)-mimic sequence (PAM), even though its mechanism of translation stimulation is unknown. The core sequence of the PAM is located downstream of nt 3661.

The poly(A) mimic sequence has a phylogenetically conserved double stem-loop structure

Next we used the MFOLD program (46) to predict the secondary structure of this poly(A) mimic sequence. Nucleotides 3680-3762 are predicted to form a double stem-loop structure (Fig. 3). Nts 3661-3679 base pair to sequence upstream of the TE to form an extended TE stem-loop IV (SL-IV, Fig. 3B). A 15 nt single-stranded tract (nts 3725-3739) separates these two stem-loops and is named bridge thereafter. Within the bridge, there is a type I AU-rich instability element (ARE) (45). Stem-loop II has two internal bulges. The upper bulge can potentially base pair to the 3' terminal four bases "ACCC" (potential base

pairing bases are in rectangles). A similar interaction is required for *Tomato bushy stunt tombusvirus* (TBSV) replication silencer to down-regulate complementary RNA synthesis (28).

The double stem-loop structure is phylogeneticlly conserved among all members of the genus *Necrovirus* (Fig. 3). Double stem-loop structures of all necroviruses can be grouped into two. Group I contains those from TNV Strain D UK isolate (TNV-D), TNV strain D Hungary (TNV-DH), *Leek white stripe virus* (LWSV), and *Beet black scorch virus* (BBSV). Group II contains TNV strain A (TNV-A) and *Olive latent virus 1* (OLV-1). Group I double stem-loop structure has a similar secondary structure as that of TNV-D, but LWSV and BBSV have no type I ARE within the bridge (Fig. 3). Group II differs from group I by having only one internal bulge and no type I ARE (Fig. 3). Except for the BBSV Loop-II (L-II), all loops in the predicted double stem-loop structures of necroviruses are tetraloops.

Stem-loop I is important for the poly(A) mimic function

To determine the primary and secondary structures required for the poly(A) mimic function, we made mutations in the stems and loops, and deletion of the bridge in the double stem-loop structure (Fig. 4 and 5). Based on secondary structure predictions by MFLOD (46), single mutations were made to disrupt the stem-loom structure while compensatory mutations restored the structure (Fig. 4A and 5A). Deletion of the bridge (d3726-3738) decreased luciferase activity more than 3-fold (Fig. 4B). The Loop I mutation (LI-mut) had little effect on translation. SL-I mutations (C3753G and G3744C) disrupted the SL-I structure. C3753G and G3744C caused a 2.5- and 4-fold decrease in translation,

respectively. Interestingly, compensatory mutation (SI-re) restored translation to wild-type level. Addition of a poly(A) tail restored translation of all mutants to the level of polyadenylated TLucT (Fig. 4B). Thus, the bridge and the secondary structure of SL-I are important to the poly(A)-mimic function.

To examine effects of the bridge and SL-I mutations on TNV-D replication, we subcloned these mutations into full-length TNV-D genomic RNA and analyzed their replication in tobacco NT-1 protoplasts (Fig. 4C). Because translation is necessary for production of viral replicase, mutations that knock out translation should prevent replication. Bridge deletion (d3762-3738) decreased the replication of TNV-D to an undetectable level. Stem I mutations (C3753G and G3744C) and Loop I mutation (LI-mut) dramatically decreased the replication of TNV-D. Remarkably, compensatory mutation (SI-re) increased the replication of TNV-D at least to 10-fold higher than the wild-type level. With the exception of L-I mutation, the effects of other mutations on translation are correlated with their effects on replication. However, whether this correlation is solely responsible for reduced replication is not clear and needs further investigation.

Stem-loop II is important for the poly(A) mimic function

We did similar analysis on SL-II as in SL-I (Fig. 5). Mutations in Loop II (LII-mut) and disrupting Stem II (G3697C and C3704G) caused a 2 to 3-fold decrease in translation (Fig. 5B). Addition of a poly(A) tail restored translation of all mutants to the level of polyadenylated TLucT. The double mutant containing G3697C and C3704G is predicted to restore the stem. Indeed this construct (SII-re) restored translation to wild-type levels. These observations corresponded to replication assays (Fig. 5C). LII-mut and Stem II mutation

G3697C decreased the replication of TNV-D to undetectable levels. Stem II mutation C3704G significantly decreased the replication of TNV-D. SII-re restored the accumulation of TNV-D to a level higher than wild type. Thus, the secondary structure of SL-II and the primary sequence of L-II are important for the function of the TNV-D poly(A)-mimic, as well as for TNV-D replication.

Mutations have no effect on physical and functional stabilities of TLucT

The expression differences of mutants could be caused by changes in 1) translation efficiency, 2) RNA physical stability, and 3) RNA functional stability. To distinguish these possibilities, we performed both physical stability and functional stability assays. Northern blot assays showed that all mutants tested had similar degradation rates, which means that mutations did not affect physical stability (*Fig. 6C*). Functional half-life is the time in which the protein accumulation rate halves. Time-course analysis of protein accumulation showed all mutants had a similar functional half-life, which shows that mutations have no effect on functional stability as well (Fig. 6A and 6B). Thus, mutations did not significantly affect the physical and function stabilities of TLucT. These data suggest that the poly(A)-mimic sequence reported here increased the translational efficiency of the RNA.

Full length TNV-D 3' UTR, but not the double stem-loop structure, is sufficient to replace a poly(A) function

Having established that the 3'-terminal double stem-loop structure can be replaced by a poly(A) tail, we further tested whether it is sufficient to replace a poly(A) tail (Fig. 7). All constructs in this poly(A)-replacement experiment were capped. To create transcript

VLucT122, we replaced the 3' UTR of the firefly luciferase gene in the VLucAn construct (12) with a 122 nt sequence from the TNV-D 3' UTR (nts 3641-3762), which includes the double stem-loop sequence (V indicates vector sequence, T indicates TNV-D sequence, Fig. 7). We compared the translation level of VLucT122 with and without a poly(A) tail to VLucAn and VLucV294 in oat protoplasts. VLucAn has a 67 nt vector sequence followed by a 60 nt poly(A) as its 3' UTR, and VLucV294 has a 294 nt vector-derived sequence as its 3'UTR. We found that VLucT122 and VLucV294 had similar translation level, only 7% of that of VLucAn. Addition of a poly(A) tail increased the translation of VLucT122 to the level of VLucAn (Fig. 7, VLucAn, VLucV294, and VLucT122). Thus, the TNV-D 122 nt sequence containing the PAM is not sufficient to replace a poly(A) tail.

The inability of the 122 nt sequence to replace a poly(A) tail in the above construct may be caused by 1) 5' UTR of TNV-D is required for the PAM function, 2) additional upstream 3' UTR sequence is needed, or 3) the definition of PAM is over-simplified. To test these possibilities, we first replaced the 5' UTR of VLucT122 and VLucV294 with the 5' UTR of TNV-D to construct TLucT122 and TLucV294 (Fig. 7) and examined their translatability. Our data showed no statistically significant difference between luciferase activity from constructs containing the vector 5' UTR or the TNV-D 5' UTR (Fig. 7). Thus, the 5' UTR of TNV-D is not able to restore the PAM function of the construct with the 122 3'-terminal TNV-D bases. Secondly, we replaced the 122 nt 3' UTR of TLucT122 with longer TNV-D 3' UTR sequence to construct TLucT171 and TLucTBF. TLucT171 has a 171 nt sequence from the 3' UTR of TNV-D (nts 3592-3762). TLucTBF has the full length 3' UTR of TNV-D with a GAUC duplication in a *Bam* HI site that destroys the cap-independent translation function of the TE (Chapter 4). The translation of TLucT171 was

4.2-fold higher than that of TLucT122, 3.2-fold higher than that of TLucV294, and was about 40% as efficiently translated as VLucAn. The translation of TLucTBF was even higher than that of VLucAn, with a 70% increase (Fig. 7). Thus, a longer sequence is needed for full PAM function in these replacement experiments. The full-length 3' UTR of TNV-D can functionally replace a poly(A) tail (Fig. 7).

Discussion

A double stem-loop at the 3' end of TNV-D RNA functionally mimics a poly(A) tail

mRNAs lacking a poly(A) tail have been reported to use a poly(A)-mimic sequence within the 3' UTR to fulfill the function of poly(A) tail (8, 9). Here we reported that a double stem-loop structure located at the 3' end of TNV-D RNA functionally mimics a poly(A) tail. Deletions in this region caused significant drops in translation that was able to be restored by addition of a 60 base poly(A) tail, but not a cap (Fig. 2). Similar results are observed with point mutations (Fig. 4 and 5).

Based on the effect of deletions on translation, the TNV-D PAM could be divided into three regions. Coincidently, deletion-defined Region I (nts 3721-3762) approximately corresponds to SL-I and the bridge, and Region II (nts 3681-3729) corresponds to SL-II (Fig. 2 and 4A). Region III (nts 3661-3679) is upstream of the double stem-loop structure. Instead, it base pairs to sequence upstream of TE to form an extended TE SL-IV (Fig. 3B). Deletions of Regions I and II have additive deleterious effects on translation (Fig. 2). Region II probably has another type of translation and/or stability element because addition of both a cap and a poly(A) tail can not restore translation to the level of TLucT (Fig. 2). Another possibility is that sequence in Region II could interact with another region and that

interaction could be required for efficient translation. Region III contains both poly(A) mimic sequence and cap-mimic sequence. Either cap or poly(A) alone can partially restore translation of RNAs with Region III deletions, and cap and poly(A) have synergistic effects on the deletion RNA (Fig. 2, D3661). The 3' boundary of the cap-mimic sequence, TE, is defined previously at nt 3659 (Chapter 4). Data shown here suggest that the 3' boundary of *in vivo*-defined TNV TE is between nts 3662-3680.

Comparison of TNV-D PAM and other poly(A)-mimic sequences

Other poly(A)-mimic sequences have been found located within the 3' UTRs of non-ployadenylated viral and cellular mRNAs. The 3' UTR pseudoknot domain of TMV RNA can functionally substitute for a poly(A) tail in plant and animal cells (8, 9). BMV 3' UTR has a similar effect as TMV 3' UTR in regulating translational efficiency of non-polyadenylated mRNAs in carrot protoplasts (7). Both BMV and TMV 3' UTRs are dependent on a 5' cap to function (8, 9). The 3' end consensus sequence of rotavirus is a functional alternative for the poly(A) tail, and its function depends on rotavirus NSP3 (27). The metazoan histone mRNAs also lacks a poly(A) tail and has a stem-loop structure functionally mimicking a poly(A) tail (8, 24, 44). The histone mRNA 3' terminal stem-loop is necessary and sufficient to support translation of non-polyadenylated mRNA and functionally depends on a 5' cap (8) and SLBP (24, 35, 44).

The TNV-D poly(A)-mimic sequence reported here functions in the absence of a 5' cap (Fig. 2). This is the most striking difference from other known poly(A)-mimic sequences. It includes a double stem-loop structure conserved among all necroviruses (Fig. 3). The deletion-defined poly(A)-mimic sequence is not sufficient to replace a poly(A) tail.

However, a longer sequence (171 nt) can partially replace a poly(A) tail (Fig. 7). Full-length 3' UTR of TNV-D is sufficient to replace a poly(A) tail, allows 70% higher translation than a 60 base poly(A) tail does, and increases translation more than 27-fold compared to a 294 nt vector 3' UTR sequence (Fig. 7). Hence, full-length 3' UTR contains an efficient poly(A)-mimic sequence, as well as a cap-mimic region (Chapter 4). At this point we have not determined if the two functions can be completely separated, but clearly the terminal double stem-loop is not necessary for cap-independent translation. The ability of TNV-D 3' UTR to functionally replace a poly(A) tail is compatible to other poly(A)-mimic sequences. The histone mRNA stem-loop allows similar or lower translation compared to a 50 base poly(A) tail and increases translation 12.5 to 22-fold compared to a 44 nt vector sequence (8). TMV and BMV 3' UTRs confer 63- and 57-fold higher translation efficiency of the GUS gene, and 48- and 40-fold higher translation efficiency of luciferase than an unspecific length of vector 3' UTR (8, 9).

The TNV-D PAM contains a class I ARE, UUUAUUUA, within the bridge (Fig. 3 and 4A). Deletion of the ARE-containing bridge decreases translation about 3-fold (Fig. 4B, d3726-3738) and has a similar physical stability and functional stability as wild-type (Fig. 6, d3762-3738). Thus, the class I ARE in TNV-D PAM doesn't induce mRNA instability. This observation agrees with others: Class I AREs do not necessarily cause mRNA instability in their natural context (1, 18). The function of the ARE within the TNV-D PAM remains to be investigated. However, lack of conservation of the ARE motif in other necroviruses (Fig. 2) sheds doubt on the biological significance of the ARE-like sequence in TNV-D.

Advantage of viral RNA with a poly(A)-mimic sequence instead of a poly(A) tail

The dependence of viruses on host cellular machinery for propagation has led viruses to evolve many strategies to orchestrate viral and host gene expression in favor of maximum viral reproduction. Some viral and cellular mRNAs use a poly(A)-mimic sequence instead of a poly(A) tail. Why viruses have evolved such a sequence is an interesting question. Research with *Rotavirus* poly(A)-mimic sequence offers a hint for this yet to be answered question (27). *Rotavirus* NSP3 interacts with eIF4GI and evicts PABP from binding to eIF4F, while eIF4A and eIF4E remain bound on eIF4GI (27). Thus, *Rotavirus* NSP3 would compete with PABP for binding eIF4F, and translation of cellular polyadenylated mRNAs would be shut off in favor of viral translation (27). Other advantages are also possible. A poly(A) mimic sequence could obviate the need for PAPB, thus avoiding the need to complete with cellular mRNAs for PAPB. RNA with a poly(A) mimic sequence also could have a reduced requirement for limiting translation initiation factor(s), thus having a competitive advantage over cellular mRNAs.

Mechanism of how TNV-D PAM functions

Many studies suggest that a poly(A) tail is required only when a mRNA competes with other capped and polyadenylated mRNAs for limiting translation initiation factors and ribosomes (6, 31, 33). Translation stimulation mediated by the poly(A) tail involves recruitment of the 40S ribosomal subunit to the mRNA by the PABP-poly(A) tail complex (38). In wheat germ extract, the translation conditions are far less competitive compared to that *in vivo*. This probably accounts for the fact that the TNV-D PAM is only required for efficient translation *in vivo*, but not in wheat germ extract (Chapter 4). The TNV-D PAM, required only for *in vivo* translation, may facilitate binding of translation initiation factor(s)

to the TNV-D TE, increase recruitment of the ribosomal 40S subunit to the mRNA by a similar mechanism as a poly(A) tail (38), enhance the circularization of mRNA, or all of the above. These possibilities also could explain why the PAM is still needed while TNV-D RNA is circularized by long-distance base pairing between the 5' and 3' UTRs. Further investigation is needed to examine these possibilities.

Proteins binding to other poly(A)-mimic sequences have been found. Rotavirus protein NSP3, a functional analogue of PAPB, binds to rotavirus poly(A) mimic sequence and eIF4G (4, 10, 27, 29, 39). The simultaneous interaction of NSP3 with the PAM and eIF4G is necessary for efficient translation of rotavirus mRNA (39) . NSP3 binding evicts PAPB from eIF4G (27). Host protein p102 binds both the 5'-leader and 3'- upstream pseudoknot domain of TMV (37). SLBP binds the poly(A)-mimic sequence of metazoan histone mRNA and is required for efficient translation of histone mRNA both *in vivo* and *in vitro* (24, 35, 44). SLBP functions by interaction with eIF4G and eIF3 (20). These proteins functionally mimic PAPB. We speculate TNV-D PAM also needs such a PAPB analogue(s) to function. Whether such a protein(s) exists and how TNV-D poly(A)-mimic functions remains to be investigated.

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Figure Legends

Fig. 1. Schematic of *Tobacco necrosis virus* genome organization. Open boxes represent open reading frames (ORFs) with protein names in or beside boxes. Shadowed boxes are the *in vitro*-defined translation element (TE). Black lines represent genomic RNA (gRNA) and subgenomic RNAs (sgRNAs).

Fig. 2. Deletion mapping of TLucT 3' terminal sequences required for sufficient cap-independent translation in oat protoplasts. Left panel: maps of TLucT and its mutants with names on left of each contruct. Numbers show the position in the TNV-D genome. fLuc: firefly luciferase ORF. TLucT 5' and 3' UTRs are indicated by black lines with blank areas indicating deleted portions. Right panel: the relative luciferase activity of TLucT and its deletion mutants. Luciferase assays were performed in at least three independent experiments, each of which was in triplicate. Standard deviations are indicated.

Fig. 3. Secondary structures of 3' terminal sequence of TNV-D and other members of *Necrovirus*. Rectangles: bases that potentially base pair between the upper bulge of Stem-loop II and corresponding 3' terminal bases. SL-I: Stem-loop I. SL-II: Stem-loop II. TNV-D: TNV strain D UK isolate (Genebank accession #: D_00942). TNV-DH: TNV strain D Hungary isolate (NC_003487). TNV-A: TNV strain A (NC_001777). OLV-1: *Olive latent virus 1* (NC_001721). LWSV: *Leek white stripe virus* (NC_001822). BBSV: *Beet black scorch virus* (NC_004452).

Fig. 4. Effect of bridge deletion and SL-I mutations on TLucT translation and TNV-D replication. A: map of deletions and mutations. Mutated bases are shown in bold. Name of constructs are shown above rectangles. B: Relative luciferase activity of TLucT, bridge deletion (d3726/3738), and SL-I mutations. C: Northern blot result of TNV-D with indicated SL-I mutations. The bottom panel shows the RNA loading.

Fig. 5. Effect of SL-II mutations on TLucT translation and TNV-D replication. A: map of SI-II mutation. Mutated bases are shown in bold. Name of constructs are shown above rectangles. B: Relative luciferase activity of TLucT and SL-II mutations. C: Northern blot result of TNV-D with indicated SL-II mutations. The bottom panel shows the RNA loading.

Fig. 6. A: Time course of Luciferase activity accumulation. B: mRNA functional half-life calculated from (A). C: Northern blot results of TLucT and its mutations in oat protoplasts.

Fig. 7. The ability of sequence from TNV-D 3'UTR to replace a poly(A) tail. Left panel: maps of constructs with names on the left. The size and origin of 3' sequence are indicated. Right panel: relative luciferase activity of each construct. Luciferase assays were performed in at least three independent experiments, each of which was in triplicate. Standard deviations are indicated.

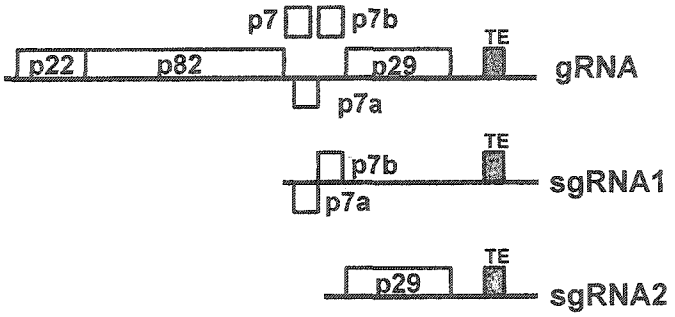
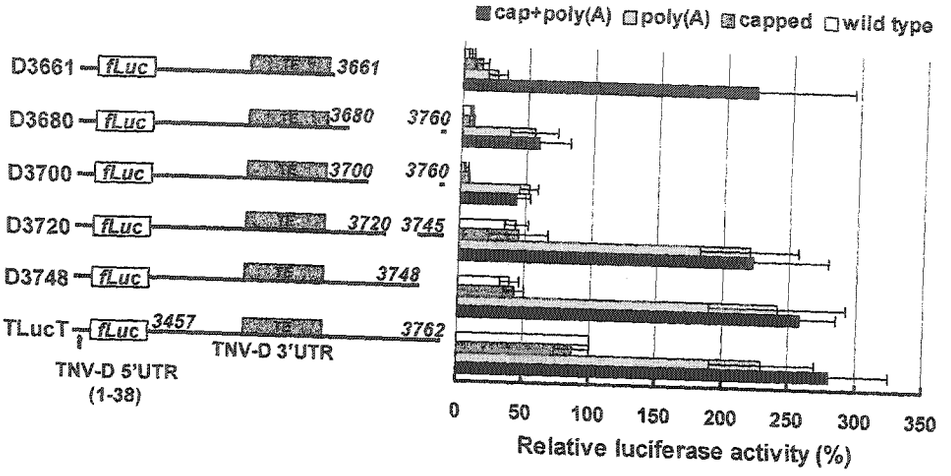


Fig. 1


cap+poly(A)
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TNV-D 5'UTR
(1-38)
0
50
100
150
200
250
300
350
Relative luciferase activity (%)

Fig. 2

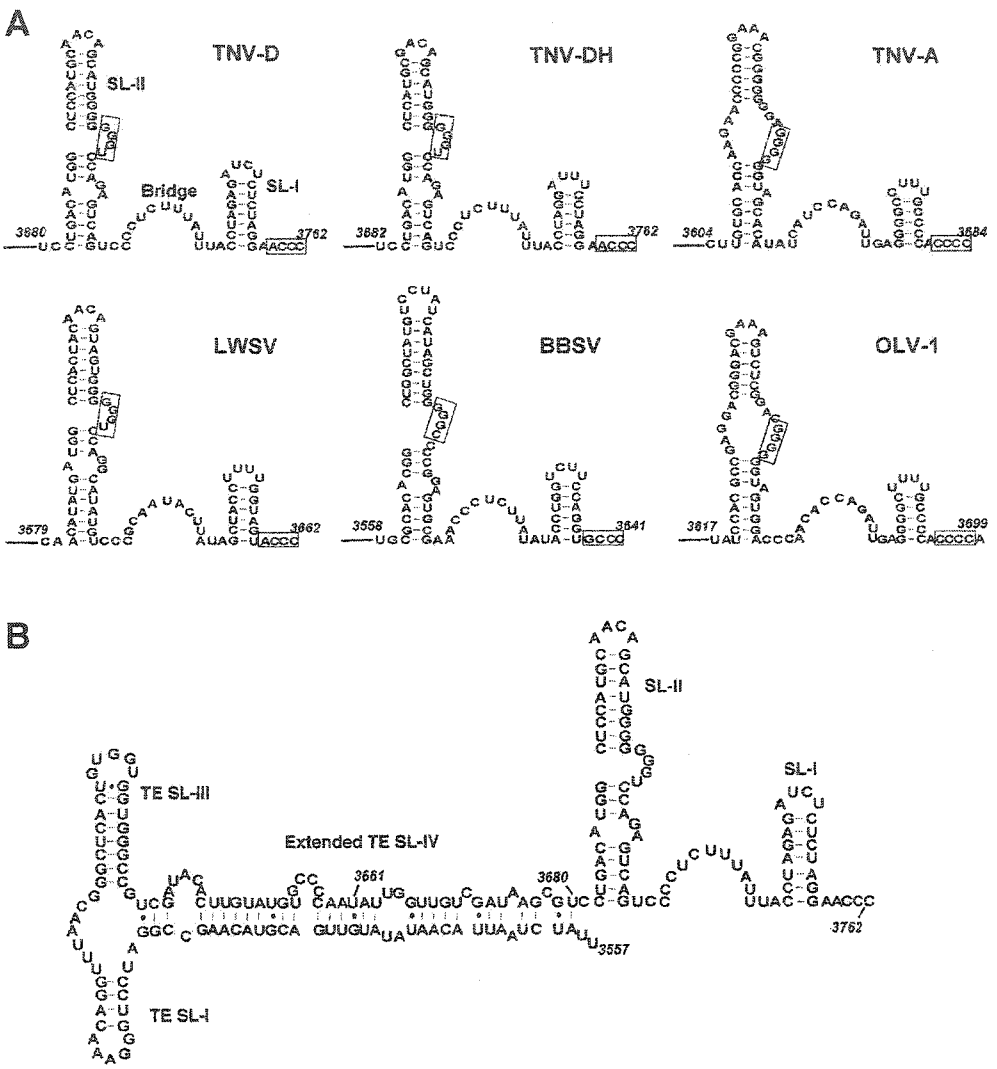
A
TNV-D
SL-II
Bridge
SL-I
TNV-DH
TNV-A
LWSV
BBSV
OLV-1
B
SL-II
SL-I
TE SL-III
Extended TE SL-IV
TE SL-I

Fig. 3

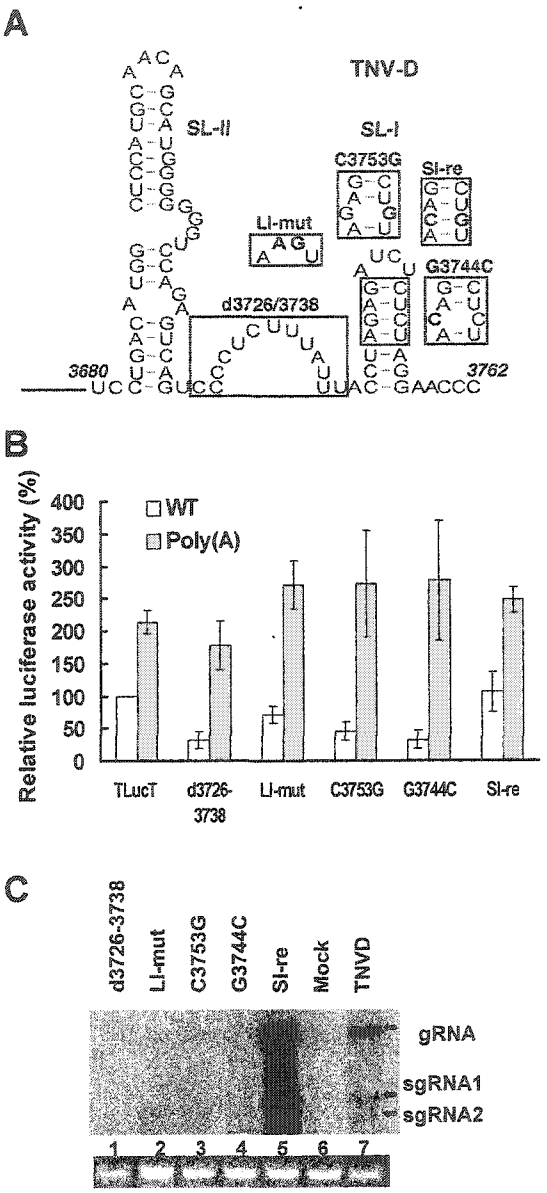
A
TNV-D
SL-II
SL-I
C3753G
SI-re
LI-mut
G3744C
d3726/3738
3680
3762
B
Relative luciferase activity (%)
WT
Poly(A)
400
350
300
250
200
150
100
50
0
TLucT
d3726-3738
LI-mut
C3753G
G3744C
SI-re
C
d3726-3738
LI-mut
C3753G
G3744C
SI-re
Mock
TNVD
gRNA
sgRNA1
sgRNA2
1 2 3 4 5 6 7

Fig. 4

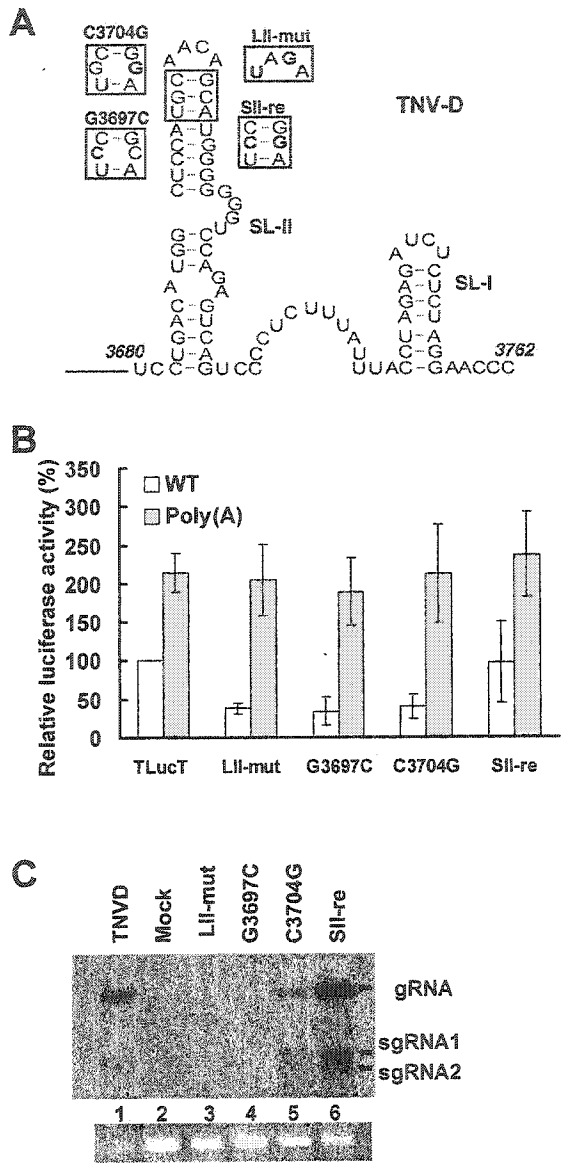
A
C3704G
LII-mut
G3697C
SII-re
TNV-D
SL-II
SL-I
3680
3762
B
Relative luciferase activity (%)
350
300
250
200
150
100
50
0
WT
Poly(A)
TLucT
LII-mut
G3697C
C3704G
SII-re
C
TNVD
Mock
LII-mut
G3697C
C3704G
SII-re
gRNA
sgRNA1
sgRNA2
1 2 3 4 5 6

Fig. 5

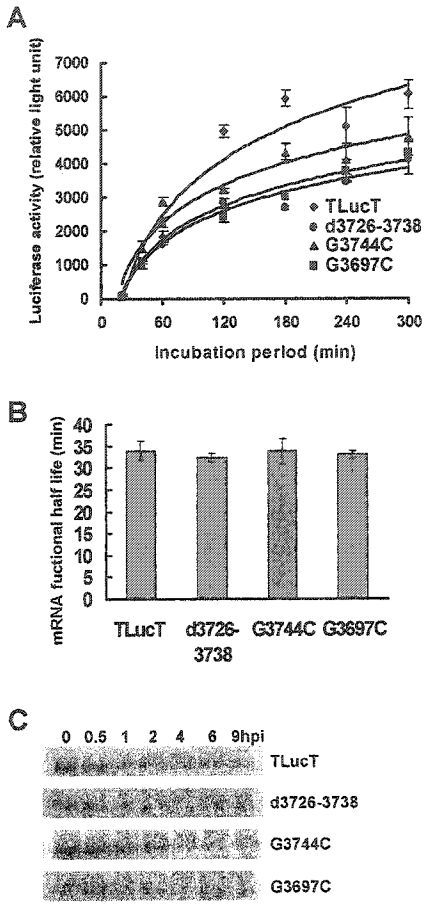
A
Luciferase activity (relative light unit)
7000
6000
5000
4000
3000
2000
1000
0
0
60
120
180
240
300
incubation period (min)
TLucT
d3726-3738
G3744C
G3697C
B
mRNA fuctional half life (min)
40
35
30
25
20
15
10
5
0
TLucT
d3726-3738
G3744C
G3697C
C
0 0.5 1 2 4 6 9hpi
TLucT
d3726-3738
G3744C
G3697C

Fig. 6

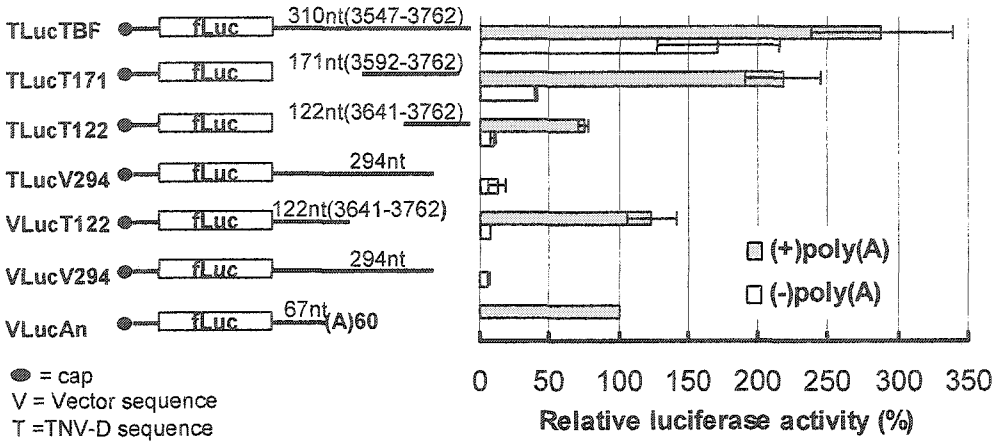
TLucTBF
fLuc
310nt(3547-3762)
TLucT171
fLuc
171nt(3592-3762)
TLucT122
fLuc
122nt(3641-3762)
TLucV294
fLuc
294nt
VLucT122
fLuc
122nt(3641-3762)
VLucV294
fLuc
294nt
VLucAn
fLuc
67nt
(A)60
= cap
V = Vector sequence
T =TNV-D sequence
(+)poly(A)
(-)poly(A)
0 50 100 150 200 250 300 350
Relative luciferase activity (%)

Fig. 7

CHAPTER 6. THE EFFECT OF *BARLEY YELLOW DWARF VIRUS* SUBGENOMIC RNA 2 ON HOST GENE EXPRESSION

Ruizhong Shen and W. Allen Miller

Abstract

Many animal viruses shut off host gene expression in favor of viral gene expression. In plant virus infection, only transient inhibition has been reported. *In vitro* and *in vivo* data lead us to propose *Barley yellow dwarf virus* (BYDV) may shut off host gene expression via its sgRNA2. Here we report that sgRNA2 from natural BYDV infection inhibits translation of capped and polyadenylated reporter gene lacking any BYDV sequence. However, host gene shutoff induced by BYDV sgRNA2 was not observed under our experimental conditions. Further investigation is needed.

Introduction

Viruses rely on host cell machinery to carry out the synthesis of viral proteins and nucleic acids. They must regulate host cell metabolism in favor of their reproduction. Many animal viruses shut off host gene expression in favor of viral gene expression (Aranda and Maule, 1998; Lyles, 2000). Host gene shutoff can be achieved via different mechanisms, such as inhibition of host transcription (Yuan et al., 2001; Yuan et al., 1998; Zimmerman et al., 1963), inhibition of host RNA processing (Fresco et al., 1987; Qiu et al., 1995), disruption of host RNA transport (Alonso-Caplen et al., 1992; Flint and Gonzalez, 2003; Qiu and Krug, 1994), degradation of host mRNA (Everly et al., 2002), and inhibition of host

translation (Padilla-Noriega et al., 2002; Porter, 1993; Zhang et al., 1994). However, host gene shutoff induced by plant viruses has not been well studied. Transient inhibition of host gene expression has been observed in pea tissues infected with *Pea seed-borne mosaic virus*, *Pea early browning virus*, *White clover mosaic virus*, and *Beet curly top virus* (Aranda et al., 1996; Escaler et al., 2000; Wang and Maule, 1995) and in *Cucurbita pepo cotypledons* infected with *Cucumber mosaic virus* (Havelda and Maule, 2000).

Barley yellow dwarf virus (BYDV) is the type member of genus *Luteovirus* in the family *Luteoviridae* (Mayo and D'Arcy, 1999). It has a positive strand RNA genome with a size of 5677 nt. BYDV RNAs lack a cap and a poly(A) tail. BYDV has evolved a diverse set of translational control strategies, such as cap-independent translation, ribosomal frameshifting, leaky scanning, and stop codon readthrough, to regulate viral gene expression (reviewed in (Miller et al., 1997; Miller et al., 1995; Miller et al., 2002). Whether BYDV also regulates host genes has not been studied.

In wheat germ extract, the 3' cap-independent translation element (3'TE) of BYDV, but not its nonfunctional *Bam*H I-fill-in (3'TEBF) mutant, trans-inhibits translation of capped, polyadenylated mRNA lacking any BYDV sequence (Wang et al., 1997). sgRNA2 from natural BYDV infection *trans*-inhibits GFP expression from *Brome mosaic virus* (BMV) RNA with or without the 3'TE in oat protoplasts (Chapter 3). Therefore, we proposed that high level accumulation of sgRNA2 in BYDV infected cell shuts off host translation and selectively facilitates viral gene expression. In this report, we used flow cytometry cell sorting, 2-dimensional electrophoresis, transgenic *Arabidopsis* expressing sgRNA2, and other techniques to examine the effect of 3'TE or sgRNA2 on host gene expression. Host gene shutoff by BYDV sgRNA2 or 3'TE was not observed under our

experimental conditions. But we observed that sgRNA2 *trans*-inhibited the expression of reporter gene without any viral sequence. This suggests that sgRNA2 may still shut off host gene expression in natural infections of BYDV.

Results and Discussion

BYDV sgRNA2 trans-inhibits the expression of a reporter gene lacking BYDV sequence in oat protoplasts

To test the hypothesis that high level accumulation of sgRNA2 in BYDV infected cells shuts off host gene expression, we first examined the ability of sgRNA2 to inhibit translation of capped, polyadenylated nonviral mRNA in oat protoplasts. A two-step electroporation method was developed (Chapter 2). First, oat protoplasts were inoculated with infectious BYDV PAV6 or PAV6ΔSG2 RNA by electroporation. PAV6ΔSG2 has one point mutation at position 4810 (G to C) of PAV6, which prevents sgRNA2 synthesis (Koev and Miller, 2000). PAV6 and PAV6ΔSG2 have similar replication levels except that the latter does not produce sgRNA2 (Fig. 1B) (Koev and Miller, 2000). After a 24-hour incubation, protoplasts were inoculated again with the reporter gene cap-fLuc-$A_{(60)}$. Then firefly luciferase activities were analyzed after another 4-hour incubation. Inoculation of PAV6 RNA in the first step caused a 55-75% drop in translation of cap-fLuc--$A_{(60)}$, whereas inoculation of PAV6ΔSG2 RNA in the first step only caused a 6-25% drop in translation of the reporter (Fig. 1). Thus, sgRNA2 does inhibit translation of nonviral, capped and polyadenylated mRNA in BYDV natural infection. These data strongly support our hypothesis.

No conclusive results were achieved by cell sorting of infected protoplasts

Having established that sgRNA2 from BYDV natural infection inhibits translation of reporter gene, we tested the effects of BYDV infection on host gene expression. Because of the low percentage of protoplasts infected by BYDV (~10%), it was not feasible to directly analyze the effect of PAV6 infection on host gene expression. The majority of uninfected cells would mask the effects of BYDV infection on host gene expression. Thus, we used flow cytometry to sort out infected oat protoplasts, by tagging BYDV with GFP in construct PAV6-GFP (Fig. 2B). The open reading frames (ORFs) 3, 4, and most of ORF 5 (nts 2858-4593) of BYDV RNA were replaced with a GFP ORF. After cell sorting, the cells were pulse-labeled with [^{35}S]-methionine for 2-3 hours, lysed, and subjected to SDS-PAGE and phosphorimage analysis. We observed that BYDV infection appeared to have both inhibitory and enhancement effects on host gene expression, even at the same time point (Fig. 2). The inconsistency is most likely due to the low yield and viability of sorted cells, the inaccuracy of sorted cell counting, and the unequal loading of SDS-PAGE gel. During the process of cell sorting, the fragile oat protoplasts could lyse. Flow cytometry counts each object (including cell debris and intact cells) passing through the channel equally. So the cell counting is not accurate, which caused unequal loading during SDS-PAGE analysis. The lysis of oat protoplasts during the processes of cell collection from the sorting output and methionine labeling could cause more unequal loading.

Measures were taken to improve the infection percentage by serial viral passage and by using BMV as expression vector, and to select infected cells by engineering BYDV with

Bar gene and hygromycin phosphotransferase (HPT) gene. Except for the BMV system that improved the infection percentage, the other approaches failed.

We developed an expression system from an unrelated virus, BMV and improved the infection percentage from at most 10% to 20-30%. The TE or TEBF of BYDV was inserted into the intergenic region between the 3a gene and GFP gene of BMV RNA3 (Fig. 3A). The coat protein ORF of BMV RNA3 was replaced by the GFP ORF. This places the TE in the 3' UTR of the 3a gene on RNA 3 and in the 5' UTR of the GFP-encoding RNA 4 that is generated from RNA 3 (Fig. 3A). The resulting viruses were designated as BMV.TEGFP and BMV.TEBFGFP (Chapter 3). By using BMV, we also could test the effects of TE independent of the context of other potential regulatory elements in BYDV RNA. Cell sorting of oat protoplasts infected by BMV constructs showed similar results as that by PAV6-GFP (Fig. 3B). We also did not observe host gene shutoff by the 3'TE expressing from BMV.TEGFP in 2-dimension gels (Fig. 3C). One possible explanation is that the 3'TE of BYDV may need to be in the context of sgRNA2 to shut off host gene expression. A second possibility is that the 20-30% infection level was not high enough to allow detection of the inhibition effects even in 2-dimension gel.

BYDV sgRNA2 expressed in transgenic *Arabidopsis* does not inhibit host gene expression

Next, we constructed transgenic *Arabidopsis* plants expressing the sgRNA2/TE of BYDV to further test whether sgRNA2 inhibits host gene expression. By using transgenic plants, all cells will have the 3'TE or sgRNA2 expressed. If the 3'TE and sgRNA2 shuts off host protein synthesis as proposed, transgenic plants expressing 3'TE or sgRNA2 under the

control of a constitutive promoter would be sick or dead. We would not be able to obtain transgenic *Arabidopsis* lines and could not distinguish the deleterious effects of the TE or sgRNA2 from the effect of insertion position. So an inducible estrogen receptor-based XVE system was used (Zuo et al., 2000) to control the expression of the TE and sgRNA2. The XVE system is highly inducible (up to eight fold higher compared to a constitutive 35S promoter), tightly controlled, and yields no detectable gene expression without induction. No toxic and adverse physiological effects have been observed in transgenic *Arabidopsis* (Zuo and Chua, 2000; Zuo et al., 2000).

Fresh protoplasts were then prepared from transgenic leaves and transcription of sgRNA2 and BFsgRNA2 was induced by β-17-estradiol for 4-7 hours (Fig. 4A). The expression of host genes was analyzed by [^{35}S]-methionine labeling (2-3 hours), SDS-PAGE, and phosphorimage analyses. We did not observe shutoff of host protein accumulation (Fig. 4B). There are several possible explanations: 1) The sgRNA2 transcribed from transgenic *Arabidopsis* is capped and polyadenylated, and made in the nucleus. Addition of a cap and a poly(A) tail may destroy the ability of sgRNA2 to shutoff host gene expression. 2) The sgRNA2 transcribed from transgenic *Arabidopsis* is made in the nucleus. It is unclear whether sgRNA2 is transported to cytoplasm. 3) The accumulation level of sgRNA2 is lower than that in natural BYDV infection, resulting in not enough to shutoff host gene expression. 4) BYDV viral protein(s) is required for the function. 5) A host protein(s) is required for the function. But *Arabidopsis* is not the host of BYDV and does not have the protein(s) required. 6) We did not find the right timing for sgRNA2 to shutoff host genes.

Conclusions

We did not observe global host gene shutoff induced by BYDV sgRNA2 under our experimental conditions. However, sgRNA2 from natural BYDV infection does inhibit translation of nonviral, capped and polyadenylated reporter mRNA. This observation suggests that sgRNA2 of BYDV may shut off host gene expression in natural viral infection. Further investigations are needed to test the hypothesis of host gene shutoff.

Materials and Methods

Plasmids

Infectious BYDV-PAV genomic RNA was transcribed from the full-length clone, pPAV6 (Di et al., 1993). The sgRNA2 knockout mutant clone of BYDV-PAV, PAV6ΔSG2, was previously referred to as SG2G/C (Koev and Miller, 2000). It has one point mutation at position 4810 (G to C), which prevents sgRNA2 synthesis. *Brome mosaic virus* (BMV) RNA clones, pT7B1, pT7B2, and pT7B3, were kindly provided by A.L.N. Rao and used for T7 transcription of BMV RNA1, RNA2, and RNA3, respectively (Dreher et al., 1989). pT7B3EGFP is a clone of BMV RNA3 with the coat protein ORF replaced by enhanced green fluorescent protein (GFP) ORF (Rao, 1997). pT7B3TEGFP and pT7B3TEBFGFP were described in Chapter 3. pPAV6-GFP was constructed by replacing the open reading frames (ORF) 3, 4, and most of ORF 5 (nts 2858-4593) of BYDV RNA with GFP gene. pVLucAn was used for T7 transcription of cap-fLuc-$A_{(60)}$ and described in (Guo et al., 2000), in which a 60 base poly(A) tail was inserted into the *Stu I/Sal I* site of pGEMLUC (Promega, Madison, WI).

In vitro transcription

Capped and uncapped RNAs were synthesized by *in vitro* transcription by using the T7 mMESSAGE mMACHINE or MegaScript kits (Ambion, Austin, TX) as per manufacturer's instructions. All BYDV constructs were linearized with *Sma* I before transcription to give a perfect genomic 3' end. pT7B1, pT7B2, and pT7B3GFP were linearized with *Bam*H I. pT7B3TEGFP and pT7B3TEBFGFP were linearized with *Tth*111 I. pVLucAn was linearized with *Vsp I*.

Two-step electroporation

At the first step, oat protoplasts were inoculated with infectious BYDV PAV6 or PAV6ΔSG2 RNA by electroporation and incubated for 24 hours in MS-media at room temperature. At the second step, protoplasts were collected, resuspended in electroporation buffer, and inoculated again with 1 pmol cap-fLuc-$A_{(60)}$. Then firefly luciferase activities were analyzed after another 4-hour inoculation. Oat (*Avena sativa* cv. Stout) protoplasts were prepared and electroporated with RNA as described in Dinesh-Kumar and Miller (1993). Except when explicitly stated, 10 μg of RNA transcript was used for BYDV inoculation and 4 μg of BMV RNAs 1, 2, and 3 in a molar ratio of 1:1:2 were used for BMV inoculation.

Northern blot analysis

Total RNAs were extracted from oat protoplasts 24-hour post-inoculation or *Arabidposis* protoplasts 8-hour after induction by using the Trizol reagent (Invitrogen,

Carlsbad, CA) as per manufacturer's instructions. RNAs were then analyzed by Northern blot as described in (Koev et al., 1999). A ^{32}P-labeled probe complementary to the 1.5 kb 3' end of BYDV-PAV genome RNA was used to detect BYDV gRNA and sgRNAs (Koev et al., 1999).

Construction of transgenic *Arabidopsis* lines and preparation of protoplasts

Binary vectors pERSG2 and pERSG2BF were constructed by inserting PCR-amplified BYDV sgRNA2 and BFsgRNA2 into *Xho* I /*Spe* I-cut pER8 (Zuo et al., 2000), respectively. Transformation of *Agrobacterium tumefaciens strain* GV3101::pMP90 was done as in (Shen and Forde, 1989) by using MicroPulser (Bio-Rad). Transformation of *Arabidopsis thaliana* Col-0 ecotype was carried out by floral dip as in (Clough and Bent, 1998). T3 or T4 seeds were used for experiments. Fresh protoplasts were prepared from 4-6 week-old leaves as in (Sheen, 2002). Expression of sgRNA2 and BFsgRNA2 was induced for 4-7 hours by adding 15μM of β-17-estradiol (final concentration) into media. β-17-estradiol (10mM) was prepared in dimethyl sulfoxide (DMSO). Then, protoplasts were pulse-labeled with [^{35}S]-methionine for 2-3 hours and subjected to SDS-PAGE and phosphorimagery analysis as in Wang and Miller (Wang and Miller, 1995).

Analyses of protein synthesis

Luciferase assays were performed as in Chapter 4. 2-dimensional electrophoresis was performed as Amersham's manual (Amersham, Piscataway, NJ). Sorted cells or induced protoplasts were pulse-labeled with [^{35}S]-methionine for 2-3 hours. Labeled cells were and subjected to SDS-PAGE and phosphorimagery analysis as in Wang and Miller (Wang and

Miller, 1995). Oat protoplasts infected with GFP-tagged BYDV or BMV were sorted by flow cytometry at the Cell Facility of Iowa State University, Ames, Iowa.

Acknowledgements

The authors thank S. Song for construction of pPAV6-GFP, Ming Lin from David Oliver's Lab for the competent *Agrobacterium tumefaciens strain* GV3101::pMP90 cell and for technical advice on the construction of transgenic *Arabidopsis*. We also thank A. L. N. Rao for providing pT7B3EGFP and infectious clones of BMV. This research was funded by grants from USDA/NRI (2001-35319-10011) and NIH (RO1 GM067104-01A1).

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Figure Legends

Fig. 1. A. Differential effects of PAV6 and PAV6ΔSG2 replication on translation of cap-fLuc-$A_{(60)}$. 24 hours after inoculation of PAV6 or PAV6ΔSG2 RNA, oat protoplasts were electroporated again with 1 pmol cap-fLuc-$A_{(60)}$. Luciferase activities were analyzed 4 hours later. B. Northern blot analysis of replication of PAV6 and PAV6ΔSG2.

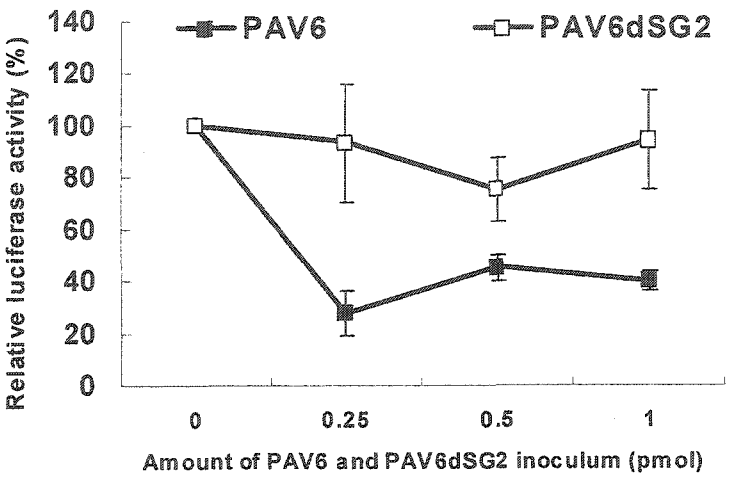
Fig. 2. A. Effects of PAV6GFP infection on host gene expression. Oat protoplasts infected by PAV6GFP were sorted by flow cytometry. After pulse-labeling with [^{35}S]-methionine for 2-3 hours, oat protoplasts were subjected to SDS-PAGE and phosphorimagery analysis. PAV6GFP lanes indicate the sorted cells with GFP fluorescence, i.e. cells infected by viruses. (-) lanes indicate the sorted cells without fluorescence, i.e. not infected by viruses. Mock lanes indicate cells mock infected and passed through the same flow cytometry procedure. Time points indicated are hours post-inoculation (hpi). B. Output of cell sorting by flow cytometry. Population D is cells with GFP fluorescence, i.e. cells infected by viruses. Population E is cells without fluorescence and was used as negative control in (-) lanes.

Fig. 3. Effects of BMV.TEGFP and BMV.GFP infection on host gene expression. A. Schematic of *Brome mosaic virus* genome organizations. Boxes represent ORFs with the

genes indicated above. CP stands for coat protein. Black ovals indicate 5'cap. Cloverleaves indicate 3' tRNA-like structure. Arrows show synthesis of the subgenomic RNA (RNA 4) of *Brome mosaic virus* (BMV). Black boxes are the in vitro TE from *Barley yellow dwarf virus* (BYDV). B. Effects of BMV.TEGFP and BMV.GFP infection on host gene expression. Assays were performed as Fig. 2. C. 2-D electrophoresis analysis of total proteins from oat protoplasts infected by BMV.TEGFP or BMV.GFP without cell sorting.

Fig. 4. A. Northern blot analysis of expression level of sgRNA2 and BFsgRNA2 from fresh transgenic *Arabidopsis* leaf protoplasts induced by β-17-estradiol. V: Vector lines. SG2: sgRNA2 lines. BFSG2: BFsgRNA2 lines. Mocked induced: mock induced with DMSO. Estradiol induced: induced with 15μM β-17-estradiol. B. Effects of BYDV sgRNA2 expressed form transgenic *Arabidopsis* on host gene expression. U: mocked induced by DMSO. I: induced by 15μM β-17-estradiol.

A


PAV6
PAV6dSG2
Relative luciferase activity (%)
140
120
100
80
60
40
20
0
0
0.25
0.5
1
Amount of PAV6 and PAV6dSG2 inoculum (pmol)

B

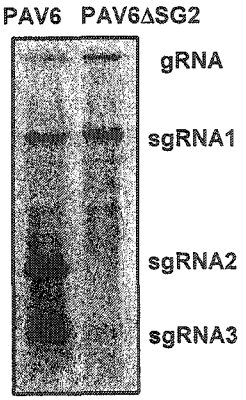
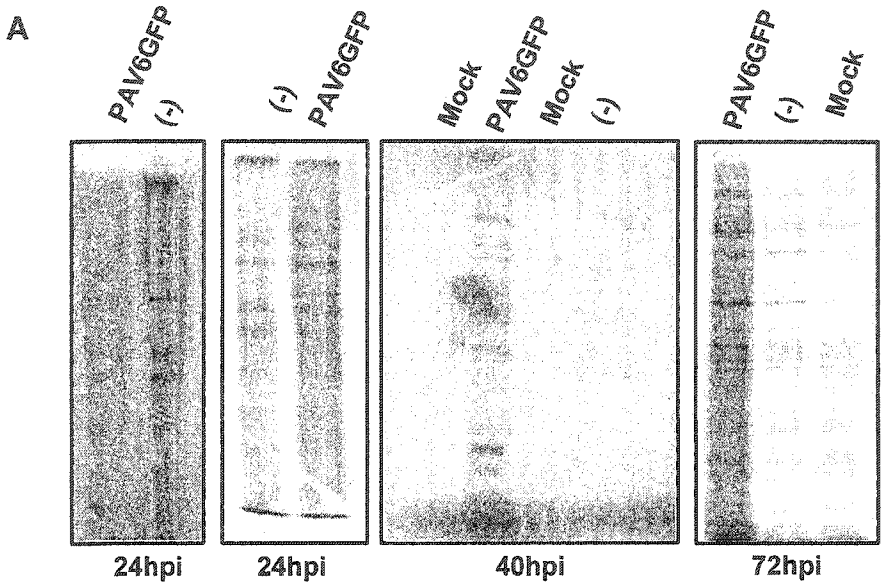
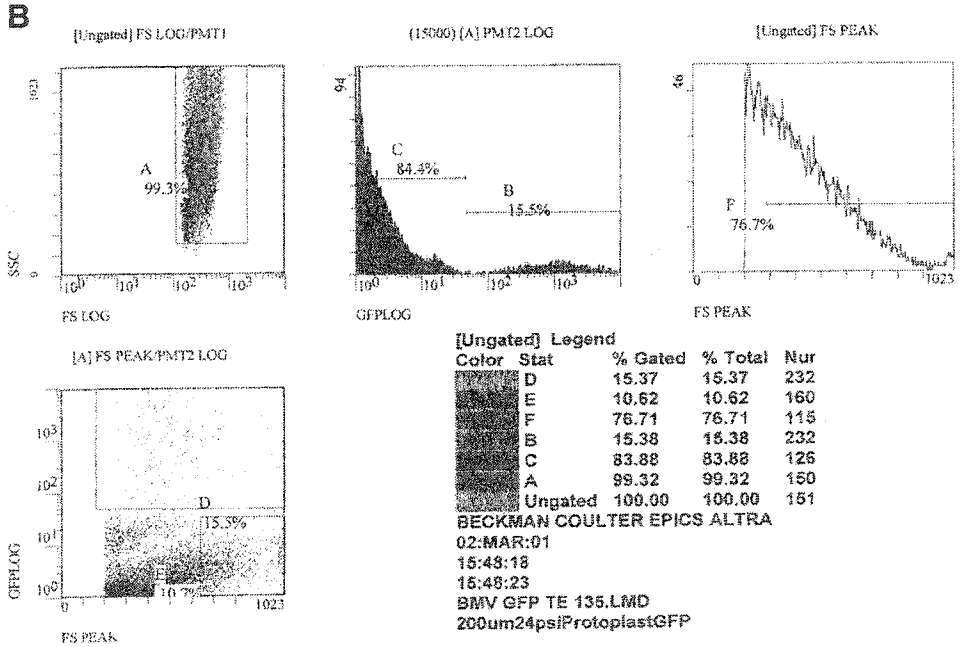

PAV6
PAV6ΔSG2
gRNA
sgRNA1
sgRNA2
sgRNA3

Fig. 1



B



[Ungated] Legend

Color	Stat	% Gated	% Total	Nur
	D	15.37	15.37	232
	E	10.62	10.62	160
	F	76.71	76.71	115
	B	15.38	15.38	232
	C	83.88	83.88	126
	A	99.32	99.32	150
	Ungated	100.00	100.00	151

BECKMAN COULTER EPICS ALTRA
02:MAR:01
15:48:18
15:48:23
BMV GFP TE 135.LMD
200um24psiProtoplastGFP

[Ungated] FS LOG/PMT1

Region	Number	%Total	%Gated	X-Median 50.0	X-Mean	Y-Median 50.0	Y-Mean
A	15000	99.32	99.32	336.8	384.8	640.0	670.4

(15000) [A] PMT2 LOG

Region	Number	%Total	%Gated	X-Median 50.0	X-Mean	Y-Median 50.0	Y-Mean
B	2322	15.38	15.48	982.2	1892.4	###	###
C	12667	83.88	84.45	1.6	3.5	###	###

[A] FS PEAK/PMT2 LOG

Region	Number	%Total	%Gated	X-Median 50.0	X-Mean	Y-Median 50.0	Y-Mean
D	2321	15.37	15.47	526.0	550.1	982.2	1893.2
E	1604	10.62	10.69	748.0	779.3	4.1	5.9

Fig. 2

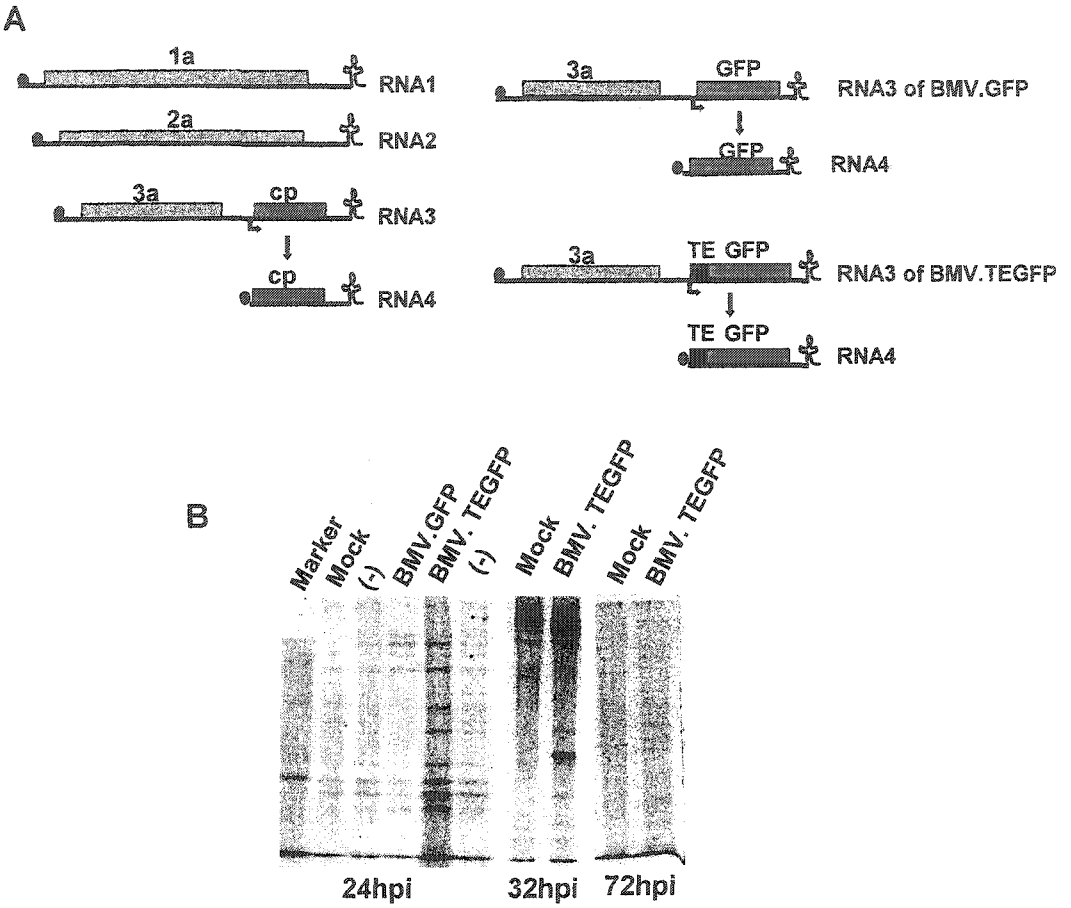

A
1a
RNA1
2a
RNA2
3a
cp
RNA3
cp
RNA4
3a
GFP
RNA3 of BMV.GFP
GFP
RNA4
3a
TE GFP
RNA3 of BMV.TEGFP
TE GFP
RNA4
B
Marker
Mock
(-)
BMV.GFP
BMV. TEGFP
(-)
Mock
BMV. TEGFP
Mock
BMV. TEGFP
24hpi
32hpi
72hpi
C
BMV.GFP
BMV.TEGFP

Fig. 3

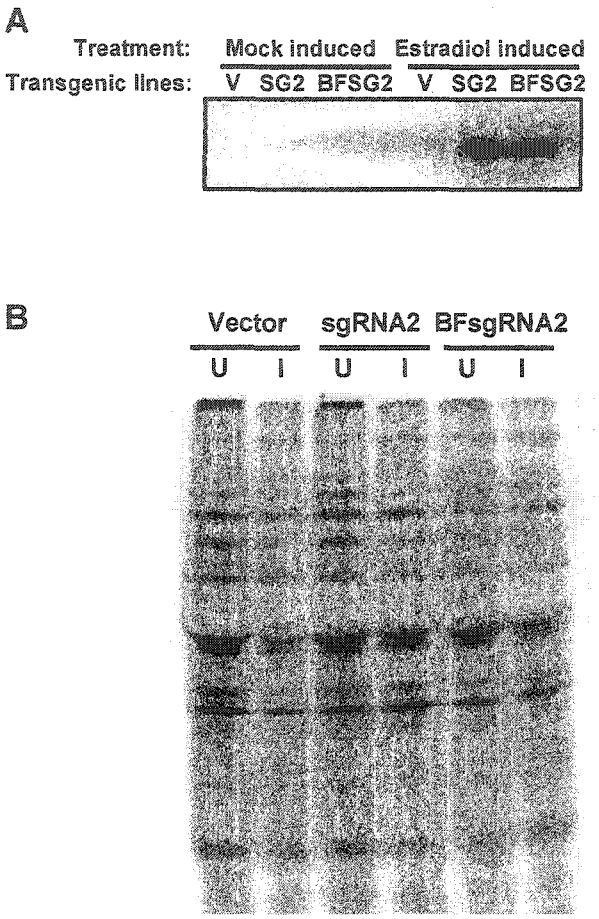

A
Treatment:
Mock induced
Estradiol induced
Transgenic lines:
V
SG2
BFSG2
V
SG2
BFSG2
B
Vector
sgRNA2
BFsgRNA2
U
I
U
I
U
I

Fig. 4

CHAPTER 7. GENERAL CONCLUSIONS

The dependence of viruses on host cellular machinery for propagation has led viruses to adopt many strategies to orchestrate viral and host gene expression in favor of maximum viral reproduction. In my dissertation research, I investigated the mechanisms of gene regulation of *Barley yellow dwarf virus* (BYDV) and *Tobacco necrosis virus* (TNV). Both BYDV and TNV have an uncapped and non-polyadenylated genomic RNA. I showed that BYDV sgRNA2 functions as a trans-regulatory RNA to temporally control viral gene expression and to inhibit viral replication. My research reveals that viral subgenomic RNA can perform important regulatory functions instead of acting as a messenger RNA. It extends the functions of viral subgenomic RNAs and shows that trans-regulatory RNA also regulates viral gene expression. I also demonstrated that the 3' untranslated region (UTR) of TNV RNA contains a BYDV-like cap-independent translation element and a poly(A) mimic sequence. Translation of TNV RNA in plant cells requires both elements. My research enriches our knowledge of gene regulation, especially that of RNA viruses with uncapped and non-polyadenylated genomic RNA.

Trans-regulation of BYDV gene expression by its subgenomic RNA 2

It has been well established that proteins function as gene regulatory factors. The roles of RNAs as trans regulatory molecules are emerging and expanding in recent years. However, gene regulation by regulatory viral RNA *in trans* is less noted and not yet well understood. In my dissertation research, I showed that sgRNA2 functions as a regulatory RNA to temporally control vial gene expression. *In vitro*, BYDV sgRNA2 preferentially

inhibits translation of gRNA versus sgRNA1. *In vivo*, BYDV sgRNA2 inhibits translation of gRNA, but has little, if any, effect on translation of sgRNA1. The 5' UTRs of gRNA and sgRNA1 determine the differential inhibition of translation of gRNA and sgRNA1 by sgRNA2 *in trans*.

These data prove and modify the trans-regulation model proposed previously based on *in vitro* data. In the modified trans-regulation model, I propose: early in BYDV infection, only ORF1 and ORF2 (replicase genes) are translated via TE-mediated cap-independent translation. Viral RNA-dependent RNA polymerase (RdRp) is produced. The RdRp then carries out viral RNA replication and sgRNA synthesis. Viral RNAs accumulate with sgRNA2 particularly abundant. Accumulated sgRNA2, via its TE, trans-inhibits translation of RdRp from gRNA. The synthesis of structural proteins is not affected. Genomic RNA is available for replication by the existing RdRp and for encapsidation.

Results from this project reveal a function for a viral subgenomic RNA and a translational control mechanism by a trans-regulatory viral RNA. However, the underlying mechanism(s) is unclear. To regulate gene expression, regulatory RNAs could use two mechanisms: 1) RNA-RNA interaction, i.e. regulatory RNA base pairs with target RNA(s). Whether BYDV sgRNA2 adopts such a mechanism needs further investigation. 2) RNA-protein(s) interaction, i.e. regulatory RNAs function as molecular decoy to compete protein(s) binding to target RNAs or protein(s). We previously proposed TE/sgRNA2 trans-inhibits gene expression by titering out the necessary and/or limiting translation initiation factor(s). But this could not explain why sgRNA2 trans-inhibits only translation of gRNA but not that of sgRNA1. Other elements, such as host factor(s) and/or the 5' UTRs of gRNA

and sgRNA1, may also be involved in the mechanism. Further investigation is needed to elucidate the mechanism(s).

Subgenomic RNA as a *trans*-regulator: A viral subgenomic RNA negatively regulates viral replication

BYDV RNA has a translation element (3'TE) in its 3' untranslated region that confers cap-independent translation *in cis*. In my dissertation research, I showed that the 3'TE functions differently *in cis* and *in trans* in plant cells. *In cis*, the 3'TE confers cap-independent translation and increases translation of capped and uncapped RNA. *In trans*, the 3'TE or the 3'TE-containing sgRNA2 serves as a riboregulator to inhibit viral replication, most likely via inhibition of translation. Specifically, I demonstrated: 1) Nonreplicating TE and sgRNA2 *in trans* inhibits BYDV replication. 2) TE from replicating *Brome mosaic virus* (BMV) trans-inhibits BYDV replication. 3) sgRNA2 from BYDV natural infection inhibits GFP expression from BMV RNA *in trans*. 4) The BYDV 3'TE *in cis* enhances GFP expression from BMV RNA. 5) sgRNA2 from natural infection of BYDV trans-inhibits translation of reporter mRNA. These data reveal another function for a viral subgenomic RNA and another mechanism of gene regulation by a trans-regulatory viral RNA.

The effect of BYDV sgRNA2 on host gene expression

Many animal viruses shut off host gene expression in favor of viral gene expression. In plant virus infection, only transient inhibition has been reported. *In vitro* and *in vivo* data lead us to propose BYDV, via its sgRNA2, shuts off host gene expression. In my dissertation research, I found that sgRNA2 from natural BYDV infection inhibits translation

of capped and polyadenylated reporter gene without any BYDV sequence. This observation supports the hypothesis of host gene shutoff by BYDV sgRNA2. However, host gene shutoff induced by BYDV sgRNA2 was not observed under our experiment conditions. Further investigation is needed to test whether natural infection of BYDV shuts off host gene expression.

The 3' UTR of TNV RNA contains a BYDV-like cap-independent translation element

RNAs of many viruses are translated efficiently in the absence of a 5' cap structure. The translation mechanism of uncapped and non-polyadenylated RNAs of TNV has not been well investigated. Computational analysis predicted a BYDV-like cap-independent translation element (TE) within the 3' UTR of TNV RNA. In my dissertation research, I identified such a TE in the 3' UTR of TNV strain D (TNV-D). The TNV-D TE shares many features with the TE of BYDV: 1) the TNV TE functions both *in vitro* and *in vivo*, 2) longer sequence is required for cap-independent translation *in vivo*, 3) a four-base duplication in a conserved *Bam*H I site abolishes TE function, 4) the TNV TE functions in the 5' UTR, 5) long-distance base pairing between the 5' UTR and the 3' TE is conserved and likely required, and 6) TNV-D and other members of *Necrovirus* may initiate proteins synthesis by the same TE-mediated cap-independent translation mechanism as BYDV RNA.

The additional portions of the 3' UTR required only for *in vivo* translation may facilitate binding of translation initiation factor(s) and/or other trans-acting factor(s) to the TNV-D TE, enhance the interaction between UTRs, increase the stability of RNA, or all of the above. Further research is needed to elucidate many unanswered questions, such as what is the mechanism of TNV-D TE? What protein(s) binds to the TE and where? What is the

minimum sequence requirement of TE *in vitro* and *in vivo*? Do the TE/sgRNAs of TNV-D function *in trans* as BYDV TE/sgRNA2 does?

A poly(A) tail mimic at the 3' end of an uncapped, nonpolyadenylated viral RNA

Messenger RNAs lacking a poly(A) tail have been reported to use a poly(A)-mimic sequence within the 3' UTR to fulfill the function of poly(A) tail. In my dissertation research, I discovered that a double stem-loop structure located at the 3' end of TNV-D RNA functionally mimics a poly(A) tail. Deletions in this region cause a significant drop in translation that is able to be restored by addition of a 60 base poly(A) tail, but not by a cap. Similar results are observed with point mutations within this region. The double stem-loop structure is phylogenetically conserved among all necroviruses. Mutation analyses established that the double stem-loop structure is important for the poly(A) mimic function, as well as for viral replication. Physical and functional stability assays suggested that the poly(A) mimic facilitated translation initiation, not stability of the RNA. The double stem-loop structure is functionally replaceable by, but cannot functionally substitute for a poly(A) tail. However, the full-length 3' UTR of TNV-D is sufficient to functionally replace a poly(A) tail.

Why a poly(A)-mimic sequence, instead of a poly(A) tail, evolved in some viral and cellular RNAs is an interesting question yet to be answered. A poly(A) mimic sequence could obviate the need for PABP, thus could avoid to compete with cellular mRNAs for PAPB and could offer a means to shut off translation of cellular polyadenylated mRNAs in favor of viral translation. RNA with a poly(A) mimic sequence also could have a reduced requirement for limiting translation initiation factor(s), providing a competitive advantage

over cellular mRNAs. Future research should be done to answer these potential advantages of a poly(A) mimic sequence.

The poly(A) mimic of TNV-D is required only for *in vivo* translation. It may facilitate binding of translation initiation factor(s) to the TNV-D TE, increase recruitment of the ribosomal 40S subunit to the mRNA by a similar mechanism as a poly(A) tail, enhance the circularization of mRNA, or all of the above. These possibilities also could explain why the poly(A) mimic is still needed while TNV-D RNA is presumably circularized by long-distance base pairing between 5' and 3' UTRs. Further investigation is needed to examine these possibilities and the mechanisms of how the poly(A) mimic of TNV-D functions.

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