The enigmatic role of ORF 6 of *Barley yellow dwarf virus*

by

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INTRODUCTION

The present study focuses on the mechanisms that regulate the life cycle of one of the most economically important viruses of cereal crops, *Barley yellow dwarf virus* (BYDV), a member of the *Luteoviridae* family (D'Arcy, 1995). By accumulating in the phloem, BYDV disrupts the transport of plant nutrients, inducing chlorosis with reddening or yellowing of the leaves, plant stunting, and poor fertilization. Ultimately, this viral infection can lead to substantial decreases in crop yield (D'Arcy, 1995).

BYDV codes for six different open reading frames (ORFs). One aspect of the BYDV infection that remains elusive is the role played by one of its ORFs. ORF6 is a small ORF located between two well-characterized cis-acting regions involved in virus replication and gene expression (Wang et al., 1997; Paul et al., 2001). It can potentially express a protein, namely P6, of about 4.3–7.2 kDa, depending on the isolate (Chalhoub et al., 1994). Importantly, ORF6 is unique to BYDV and does not share any sequence homology with any other members of the *Luteoviridae* family. Moreover, the lack of homology to any known protein, in conjunction with high sequence variability, might have also hindered the investigation of P6.

To date, no putative function has been assigned to ORF6 and, perhaps more importantly, its encoded protein has yet to be detected in plants. This constitutes a significant challenge, as evidence supports the existence of P6 or at least of the importance of the ORF (Chalhoub et al., 1994; Wang et al., 1999). ORF6 is encoded by its potential mRNA, subgenomic RNA2, which translates well in the wheat germ extract in vitro system

(Wang et al., 1999). Despite the high sequence variability among the different isolates, the first ten amino acid residues of P6 are conserved (Chalhoub et al., 1994).

Therefore, the specific aims of the present study were the following. The first aim was to (a) detect P6 in infected oat protoplasts using both well-established and creative techniques and (b) test the function of P6 in facilitating virus spread by suppressing the plant defense response against virus infection. The second aim was to investigate the function of ORF6 at the RNA level by focusing on (a) the importance of sgRNA2 with its embedded ORF6 sequence in virus-host interaction and (b) the cis-acting role that ORF6 sequence may play in viral replication and translation.

LITERATURE REVIEW

Barley yellow dwarf virus general biology

Barley yellow dwarf virus (BYDV) is the sole member of the genus *Luteovirus* in the *Luteoviridae* family (Mayo and D'Arcy, 1999). It shares some general features with the members of the *Luteoviridae* family that are grouped into the genera *Polerovirus* and *Enamovirus*. It has a monopartite, positive sense, single-stranded genomic RNA. The genome, packaged into an icosahedral particle, is about 5.7 kilobases and codes for six different open reading frames (ORFs) (Miller et al., 1997). One of the main characteristics of the *Luteoviridae* family is their tissue specificity and insect transmissibility (Harrison, 1999). *Luteoviridae* are confined in the phloem of the plant and can be transmitted only by specific aphids (Harrison, 1999). Vector specificity varies among BYDV serotypes (Power and Gray, 1995). For instance, *Cereal yellow dwarf virus*, formally BYDV-RPV, is specifically transmitted by *Rhopalosiphum padi*, BYDV-MAV by *Sitobion avenae* and BYDV-PAV serotype can be transmitted either by *Rhopalosiphum padi* or *Sitobion avenae* (Power and Gray, 1995). BYDV is transmitted in a circulative manner and does not replicate in the insects (Chay et al., 1996). Along with the plant sap, the virus reaches the hindgut of the aphids and penetrates into the haemocoel. From the haemocoel, the virus migrates to and accumulates in the accessory salivary glands. During aphid feeding, the virus particles are released into the plant phloem along with the saliva to start infection (Chay et al., 1996).

Besides this general biology, BYDV does not share other features with genus *Polerovirus* and genus *Enamovirus* of the *Luteoviridae* family (Mayo and Miller, 1999). BYDV differs not only in its genome organization but also in its gene expression and

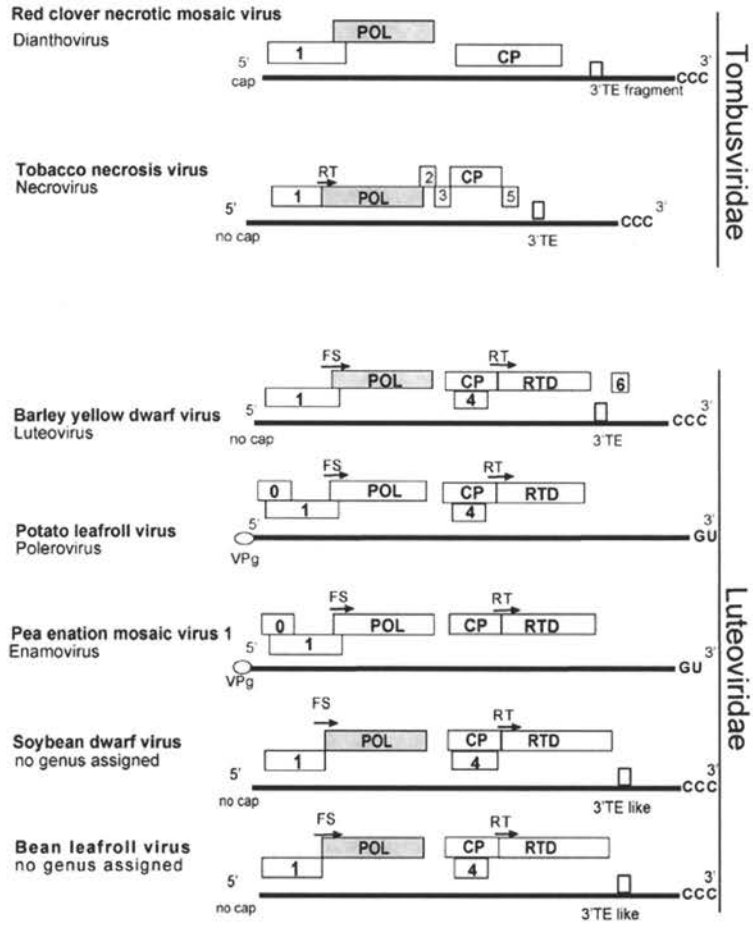

Figure 1. Genome organization of viruses related to BYDV (modified from Miller et al., 2002). VPg, genome linked viral protein; 3'TE, 3' translation element; RT, read through domain; FS, frameshifting; Pol, polymerase; CP, coat protein; RT, read through domain. Grey shading, features in common with *Tombusviridae*.

replication strategies (Mayo and Miller, 1999). In contrast to the other genera of the *Luteoviridae* family, BYDV does not have ORF0 at its 5' end (Miller et al., 1995) (Fig. 1). However, it codes for a small ORF6 at the 3' end of its genome that is unique to the genus *Luteovirus* and has no sequence homology to ORF0 (Miller et al., 1995). Moreover, BYDV does not code for a genome-linked protein, VPg (Shams-Bakhsh and Symons, 1997, Allen et al., 1999).

Different functions have been suggested for the VPg protein including initiation of RNA replication in mammalian polioviruses (Lyons et al., 2001), systemic infection of plants and translation initiation in potyviruses (Rajamaki and Valkonen, 2002, Leonard et al., 2000). In the absence of a VPg or a 5'cap structure, BYDV has evolved different and non-canonical strategies to assure its gene expression (Wang et al., 1997). A cis-acting element, that is located 5 kilobases downstream of the 5'end of the genome, controls BYDV gene expression (Wang et al., 1997). Moreover, BYDV replication is controlled by specific RNA sequences located in the 3' end that are absent in genus *Polerovirus* (Koev et al., 2002). With regard to these latter features, Miller et al. (2002) proposed a re-classification of BYDV in the *Tombusviridae* family. In fact, it appears that BYDV shares more homology with the *Tombusviridae* members rather than with the *Luteoviridae.*

Barley yellow dwarf virus gene expression

Due to the limited size of their genomes, many viruses have overlapping open reading frames (ORFs) on their single genomic strand for maximizing their gene capacity. Consequently, they have evolved a variety of translational strategies to overcome some of the host translational constraints. It is noteworthy that BYDV has evolved an array of tactics

including ribosomal frameshifting, leaky scanning, production of subgenomic RNAs and in-frame readthrough of a stop codon for regulating the expression of its polycistronic genome (Miller and Rasochova, 1997).

The genomic RNA (gRNA) codes for the first proteins necessary for the early stage of infection. P1 and P2 are the only proteins necessary for viral replication (Mohan et al., 1995). By a low rate ribosomal frameshifting mechanism, ORF2 is translated as a fusion with ORF1 and produces the putative 99 kDa RNA-dependent-RNA polymerase (Mohan et al., 1995).

Subgenomic RNAs synthesis

One of the strategies used for the expression of the downstream ORFs that are not translatable from the gRNA, is the production of subgenomic RNAs (sgRNAs). SgRNAs are truncated genomic RNAs at their 5' end but share the same 3' terminal sequences. The sgRNAs are present only during viral infection. They accumulate in infected cells but they are not packaged with the gRNA (Drugeon et al., 1999). Different models have been proposed for the production of the sgRNAs including internal initiation of the polymerase, premature termination of negative strand synthesis or discontinuous transcription of the polymerase (Miller and Koev, 2000) (Fig. 2). In an internal initiation mechanism, a full-length minus strand gRNA is produced and the polymerase complex specifically recognizes and binds to an internal site on the negative strand RNA for starting sgRNA production (Siegel et al., 1997). In a premature termination model, the polymerase prematurely terminates minus strand RNA synthesis. This truncated RNA is then used as a template for positive strand sgRNA synthesis. For *Red clover necrosis mosaic virus* (*Tombusviridae*), base pairing of a trans-activator element on RNA 2 with RNA 1 is proposed to prevent the

complete synthesis of a full-length negative strand RNA 1 (Sit et al., 1998). The termination occurs at a region adjacent to the sgRNA promoter region. In *Tomato bushy stunt virus* (*Tombusviridae*) and *Flock house virus* (Nodavirales), long distance base pairing between cis-acting regions on the gRNA proximal to the subgenomic initiation site controls sgRNA accumulation (Choi and White, 2002, Lindenbach et al., 2002). The distal base pairing of the complementary region appears to be the obstruction to polymerase progression during negative strand synthesis and contributes to the premature release of the nascent transcript from the template.

Lindenbach et al. (2002) proposed that the sequences in between the complementary region might play a role in modulating the formation of the long distance base pairing. These structures may function as a molecular switch that directs the switch from gRNA synthesis to sgRNA production (Lindenbach et al., 2002). The Nidoviridales produce their sgRNAs by a discontinuous transcription mechanism (Marle et al., 1999). The replicase starts negative strand synthesis, stalls at a specific site, disassembles from the template without releasing the nascent strand and resumes transcription in the 5' end of the gRNA. Using this strategy, each sgRNA has an identical 5' terminal sequence. In all models, the truncation of the gRNA brings the start codon of the downstream ORF close to the 5' end for easy access by the ribosomes.

BYDV produces three sgRNAs of different sizes that accumulate in infected cells in a ratio inversely proportional to their size (Kelly et al., 1994) (Fig. 3). Interestingly, three different promoters, divergent in their secondary structure and sequence, control the synthesis of the three sgRNAs (Koev and Miller, 2000).

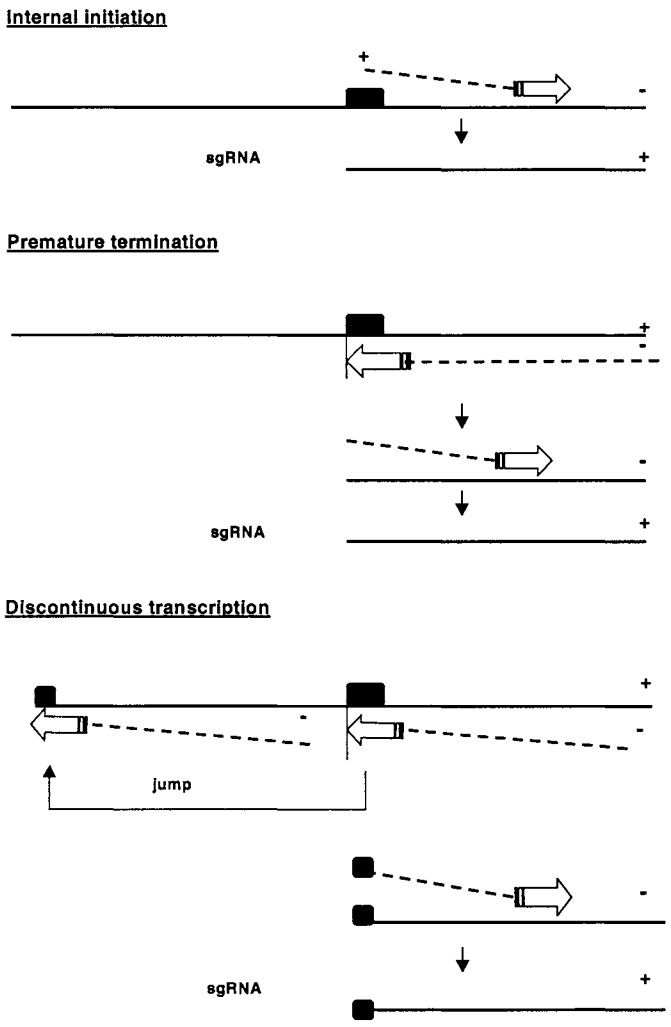

Figure 2. Different proposed models for subgenomic RNA synthesis. (modified from Miller and Koev, 2000) Box: subgenomic RNA initiation sites, thick arrow: polymerase, vertical line: termination of transcription.

This may reflect a difference in their recognition by the polymerase. The strategy used for the production of BYDV sgRNAs is being investigated. Premature termination of minus strand synthesis has been proposed for sgRNA2 synthesis (Miller et al., 2002). In this process, a stable secondary structure indispensable for gene expression, known as the translation element (3'TE), may be responsible for the RNA release (Miller et al., 2002). Koev and Miller (2000) showed that BYDV sgRNAs are critical for systemic infection of the plants but dispensable for viral replication. In fact, the sgRNAs express the proteins involved in the late stage of viral infection such as packaging, plant systemic infection and aphid transmission. In addition to their function as coding RNAs, sgRNAs play a role in regulating viral gene expression. The production of the sgRNAs regulates the proper level and timing of protein accumulation. Notably, sgRNA2 has been proposed to control the switch from early to late gene expression during infection (Wang et al., 1999).

The three BYDV sgRNAs play different roles during infection. SgRNA1 is the mRNA for ORF3, ORF4 and ORF5 (Dinesh-Kumar et al., 1992) (Fig. 3). ORF3 encodes the coat protein. ORF4, which overlaps ORF3 sequence in a different reading frame, is translated by a leaky scanning mechanism (Dinesh-Kumar and Miller, 1993) and codes for a 17kDa protein that seems to be involved in virus spread (Chay et al., 1996). ORF5 is expressed via readthrough of the ORF3 stop codon (Brown et al., 1996). The readthrough domain fused with the coat protein is required for aphid transmission (Chay et al., 1996). SgRNA2 is the messenger RNA for ORF6 that can potentially code for a 4.3-7.2 kDa protein P6 (depending on the isolate) of unknown function (Miller et al., 1995). SgRNA3, which accumulates highly during infection, does not code for any gene and its function is unknown (Kelly et al., 1994).

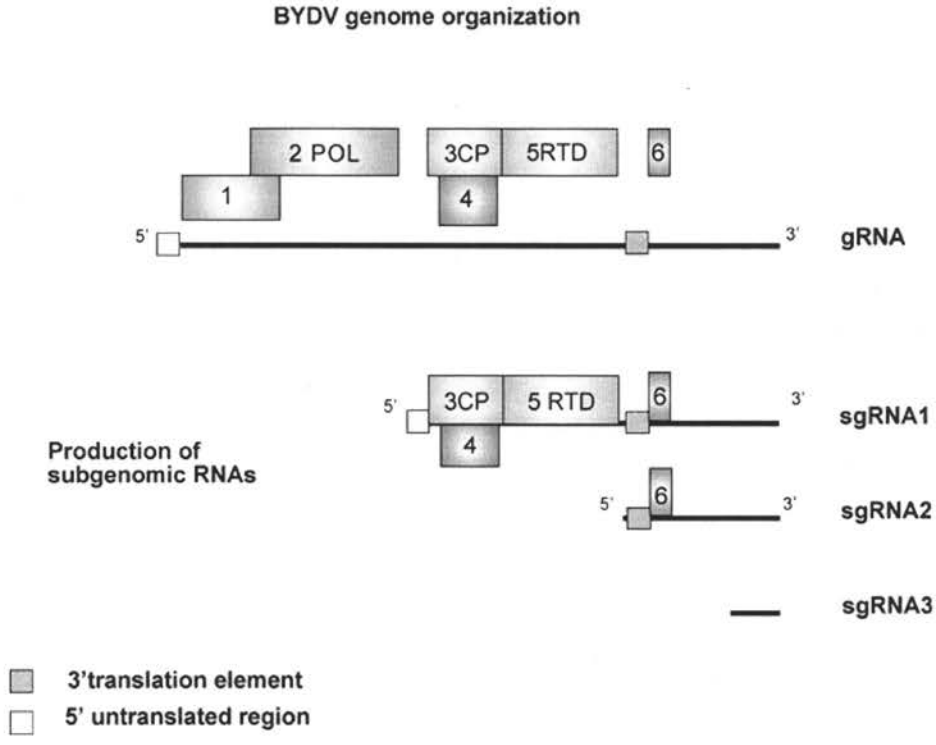

Figure 3. *Barley yellow dwarf virus* genome organization and its three subgenomic RNAs. Pol: polymerase, CP: coat protein, RTD: readthrough domain.

Translational control of Barley yellow dwarf virus

It is of great importance to understand that the ability to recruit the translation machinery determines translation efficiency. In fact, all cellular mRNAs harbor in their terminal non-coding regions two structures that are determinant for mRNA translatability: a methylated guanosine (m7GpppN) or cap structure at the 5' end of the mRNA, and a chain of adenosine bases or poly (A) tail at the 3' end (Sachs and Buratowski, 1997). The cap and the poly (A) tail, by interacting with the various translation initiation factors (eIFs), act synergistically to recruit and position the protein synthesis machinery (Sachs and Bratowski, 1997). This assures efficient RNA translatability in an environment where ribosome and translation factor supply is limited (Proweller and Butler, 1997). The recruitment of the various translation initiation elements starts with the binding of the eIF4F translation factor complex on the 5' end of the mRNA (Preiss and Hentze, 1999). The translation initiation factor eIF4G, main component of the eIF4F complex, acts as a molecular scaffold in coordinating the assembly of the various translation factors on the mRNA (Hentze, 1997). It interacts with eIF4E and PABP that bind the cap structure and the poly (A) tail, respectively (Sachs and Varani, 2000). The RNA dependent ATPase, eIF4A, binds the C-terminal domain of eIF4G and assures unwinding of RNA secondary structure that can prevent ribosome scanning (Gringas, et al., 1999). The translation factor eIF3, which binds to the ribosomal 40S subunit with eIF2-GTPase, mediates ribosomal recruitment by recognition of the positioned translation complex (Browning, 1996). Once positioned at the 5' end of the mRNA, the ribosomes start scanning for the first start codon to the 3' direction and initiate protein synthesis (Gringas et al., 1999). The interaction of the translation factors on the mRNA circularizes the capped, poly-adenylated mRNA (Wells et al., 1998). The

circularization of the mRNA may be involved in stimulating translation by recycling the translation complex for several additional rounds (Preiss and Hentze, 1999). The long distance 5'-3' communication during RNA circularization seems to be a prerequisite not only for ribosome recruitment but also for RNA stability and exclusive translation of intact mRNAs (Preiss and Hentze 1998, Wells et al., 1998). The disruption of the eIF4F/PABP-mediated circularization drastically reduces translation efficiency (Michel et al., 2000). Interaction of the various translation factors including PABP at the 3' ends of the mRNA blocks access to the deadenylating and decapping enzymes (Wilusz et al., 2001). Poly (A) tail trimming is the determining step in mRNA turnover (Ford and Wilusz, 1999, Wilusz et al., 2001). The cap structure on the deadenylated RNA is removed and the exonucleases start RNA degradation (Wilusz et al., 2001). Therefore, the two terminal structures play critical roles in coordinating the mRNA fate: template for translation or target for degradation (Wickens et al., 1997).

Like many other viruses, BYDV lacks both cap and poly (A) tail structures but still translates efficiently (Allen et al., 1999). It uses an unconventional strategy for recruiting the host translation machinery for the expression of its uncapped, non-polyadenylated genome. This novel cap-independent translation differs from the internal ribosome entry site mechanism (IRES) described in various animal viruses (Hellen and Sarnow, 2001). In IRES-mediated translation, the ribosomes are directly positioned via the specialized IRES near the initiation codon without having to scan from the 5' end of the mRNA and independently of an eIF4E-cap interaction (Hellen and Sarnow, 2001).

BYDV translation requires conserved secondary structures and RNA sequences in the 5' and 3' UTRs of the viral genome to assure RNA circularization and translation machinery

recruitment (Wang et al., 1997, Guo et al., 2000). Its cap-independent translation is mediated by a 109 nucleotide translation element (3'TE) located 5 kilobases downstream of the first start codon of the gRNA (Wang et al., 1997). The 3'TE, located at base positions 4810-4919, forms a cruciform structure that is composed of three stem-loops (SL-I, II, III) and a stem (S-IV) (Guo et al., 2000). SL-III interacts by base-pairing with a stem loop located in the 5'UTR of the gRNA (Guo et al., 2001). Any mutation on either stem-loop abolishes translation. However compensatory mutations that restored base pairing also restored translation at a comparable level to the wild type sequence (Guo et al., 2001). This underlines the importance of the complementarity between the two elements rather than their primary sequences. The TE-5'UTR long distance base pairing assures circularization of the gRNA and its translatability as efficient as a poly (A) tail/cap-mediated translation (Guo et al., 2000). TE-mediated translation requires at least one of the host translation initiation factors (Ed Allen, unpublished data). It can bind eIF4E-iso4E, one of the components of eIF4F complex that recognizes and binds the cap structure (Ed Allen, unpublished data). Guo et al., (2001) proposed that the recruitment of the translation machinery may start with the positioning of the eIF4F protein complex at the 3'end of the BYDV genome through the binding of eIF4E/eIF4G to the 3'TE. 3'TE base pairs with the 5'UTR of the gRNA. In this closed loop model, the eIF4F is transferred back to the 5' end of the genome and recruits the 43S ribosomal complex. Similar TE-mediated translation models are predicted for related *Soybean dwarf virus* (*Luteoviridae*) and *Tobacco necrosis virus* (*Tombusviridae*) (Guo et al., 2001). The 109 nucleotide cruciform structure is sufficient to direct translation in wheat germ extract in vitro system (Wang et al., 1999). However, additional sequences are needed for the competitiveness of TE-dependent RNAs for the translation machinery in protoplasts

(Wang et al., 1999). These additional sequences coincide with the 5' UTR of sgRNA2 and some downstream sequences in the 3'UTR (Guo et al., 2000). It has been proposed that the 3' UTR of BYDV genome may influence RNA translation efficiency by mimicking a poly (A) tail and assuring RNA stability (Guo et al., 2000).

The 3'TE regulates both BYDV replication and translation. The synthesis of the polymerase that is crucial for viral replication is controlled by the 3'TE. Intriguingly, the 3' TE function is not limited to a cis-acting translation control. If 3'TE enhances translation of gene in cis, it inhibits gene expression of genes in trans (Wang et al., 1999). Wang et al., (1999) showed that 3'TE or full-length sgRNA2 added in trans inhibits expression of gRNA and sgRNA1. 3'TE-trans-inhibition affects the gRNA, which codes for the early genes involved in genome replication, to greater extent than sgRNA1, which codes for late genes for virion formation and spread. There is evidence that the TE trans-inhibition is reversed with the addition of eIF4F protein complex in the reaction (Wang et al., 1999). Hypothetically, sequestration of the limited number of translation factors by sgRNA2-TE could be the basis of this trans-inhibition mechanism (Wang et al., 1999).

SgRNA2 with the TE in its 5'UTR has been proposed to serve as ribo-regulator to control the switch from early to late gene expression during infection (Wang et al., 1999) (Fig.4). During the early stage of infection, the polymerase is synthesized in a cap-independent TE-mediated way. The polymerase replicates the gRNA and produces the sgRNAs. SgRNA2 accumulates at high level. In a trans-acting regulation, the TE of sgRNA2 shuts off polymerase transcription and favors expression of the late genes of sgRNA1 needed for virus encaspidation and viral movement. BYDV virions are then able to assemble and move cell-to-cell systemically throughout the plant.

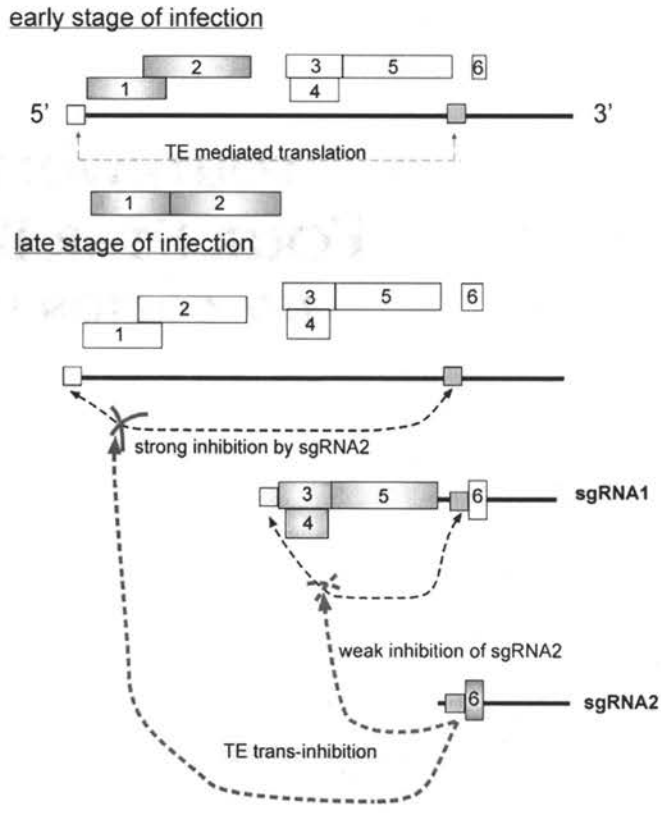

Figure 4. SgRNA2 has been proposed to serve as a ribo-regulator to control the switch from early to late gene expression. SgRNA2 with the presence of the translation element (3'TE) has the ability to trans-inhibit gene expression of gRNA to a greater extent than sgRNA1. (Modified from Wang et al., 1999).

Many animal viruses have evolved strategies to subvert host gene expression for recruiting the limited numbers of translation factors. For instance, picornaviruses shut off host gene translation by cleaving the translation factor eIF4G (Hellen and Sarnow, 2001). They require only the C-terminal of eIF4G for assembling the translation complex in an IRES-mediated manner (Hellen and Sarnow, 2001). In a similar way, NSP3 protein of rotaviruses (*Reoviridae*), by interacting with eIF4G translation factor (Piron et al., 1998), interferes with the binding of PABP to cellular mRNAs (Michel et al., 2000). NSP3-eIF4G interaction promotes rotavirus translation but shuts down host PABP-dependent translation (Michel et al., 2000).

Ability of plant viruses to shut off host gene expression has not been reported. Plant viruses have rather evolved translation strategies that increase their affinities to the host translation factors or that get around the need of some of translation elements (Futterer and Hohn, 1996). Notably, BYDV TE-mediated trans-inhibition can target both viral and non-viral genes in vitro (Wang et al., 1999). If this TE-activity is applicable in vivo, BYDV may use a unique mechanism for shutting off host gene expression via sgRNA2/TE trans-inhibition. The potential inhibition of host translation mediated by sgRNA2 accumulation during BYDV infection was investigated in this study.

Open reading frame 6 and its encoded P6 protein

Many viruses have small and conserved ORFs of unknown function in their genome. One interesting example is the pX gene of *Tomato bushy stunt virus* (*Tombusviridae*). Located at the 3' end of the genome, it can potentially encode a protein of 8 kDa (Boyko and Karasev, 1992). However, pX protein and its messenger RNA have not been detected in

infected plants (Boyko and Karasev, 1992). Mutations on pX ORF lead to a decrease of viral accumulation but in a host dependent manner (Scholthof and Jackson, 1997). pX virus mutants replicated efficiently in *Chenopodia quinoa* but poorly in cucumber or tobacco plants (Sholthof and Jackson, 1997). It has been shown that the RNA sequence of pX may play an important role as a cis acting sequence in replication rather than the encoded protein. (Sholthof and Jackson, 1997).

ORF6 of BYDV is another small ORF whose function is still a mystery. It is 128 nucleotides long in our BYDV infectious cDNA clone pPAV6 and can be expressed only from sgRNA2. ORF6 is located between two important structures required for virus replication and gene expression (Fig. 5). ORF6 sequence contains base positions 4920-5048. It is between the 3' translation element (4810-4919) and the distal frameshifting element (5050-5100) (Wang et al., 1997, Paul et al., 2001). Furthermore, a part of ORF6 overlaps with the core promoter of sgRNA2 (4810-4952) (Koev et al., 2000). Sequence analysis of ORF6 showed the presence of a conserved polypyrimidine tract of about 30 bases (Miller et al., 1988). A polypyrimidine tract is known to play an important role in some IRES-mediated translation systems by binding the cellular polypyrimidine tract-binding protein (PTB) (Hunt and Jackson, 1999). In IRES-mediated translation of picornaviruses, PTB binding is required for RNA translatability (Hunt and Jackson, 1999). Kaminski and Jackson (1998) showed that PTB is needed for restoring proper folding of mutated IRES in cardiovirus RNA and assures efficient translation. Since BYDV translation initiation depends on the proper conformation of the 3'TE structure, a similar function for the ORF6 polypyrimidine tract could have been expected.

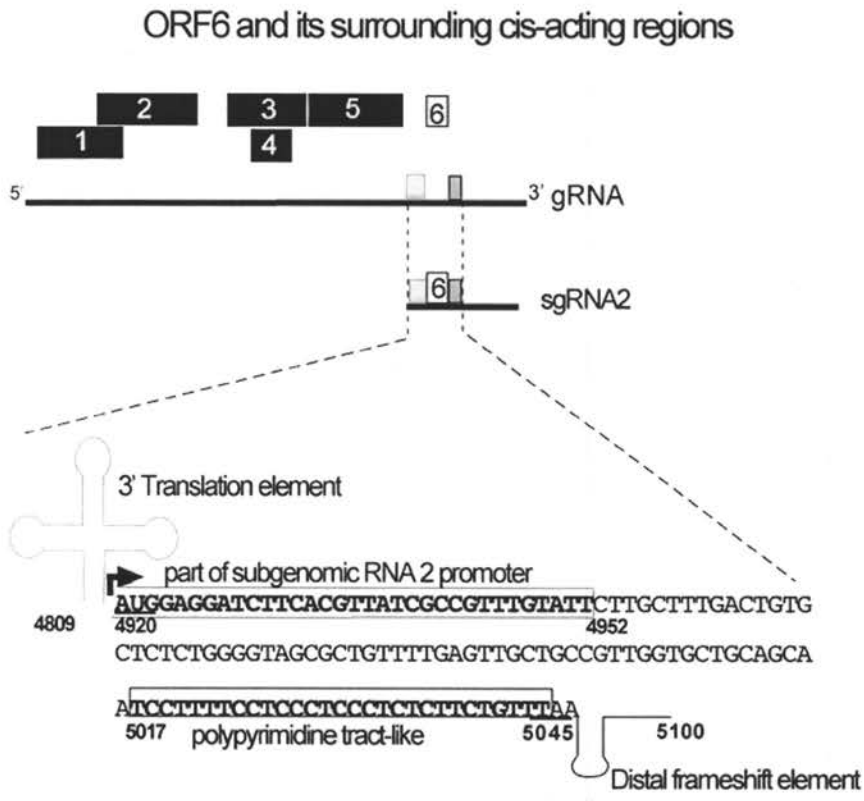

Figure 5. ORF6 sequence (nt 4920-5048) and its surrounding cis-acting regions. ORF6 is located in between two important structures involved in gene expression and viral replication: the translation element TE (nt 4809-4919) and the long distance frameshift element (nt 5050-5100). ORF6 includes in its sequence a part of the sgRNA2 promoter (nt 4920-4920) and a polypyrimidine tract at its 3' end (nt 5017-5045).

However, in recent studies Paul et al., (2001) and Elizabeth Pettit (unpublished data) revealed that the deletion of ORF6 polypyrimidine tract did not affect viral replication or TE-mediated translation efficiency.

ORF6 can potentially code for a 4.3-7.2 kDa protein depending on the isolate (Chalhoub et al., 1994). The high variability in size and in sequence of the protein, and the lack of homology to any known protein in the BLAST search database may support the irrelevance of P6. Moreover, *Soybean dwarf virus* and *Bean leafroll virus*, members of the *Luteoviridae* family with BYDV-like genome organizations, lack ORF6 or any homologous sequence to ORF6 in their 3'UTR (Rathjen et al., 1994, Domier et al., 2002) (Fig. 1).

One piece of evidence that supports the existence of ORF6 protein is the identification of its messenger RNA, sgRNA2, that is translated well in the wheat germ extract in vitro system (Wang et al., 1999). Secondly, the alignment of ORF6 sequences from different BYDV serotypes showed a change of the nucleotide sequence at the third wobble position of the codon (Chalhoub et al., 1994) (Fig. 6).

Despite the differences at the third base position, the amino acid sequence remained the same, at least for the first 10 residues (Chalhoub et al., 1994). The selection of the conserved amino acid sequence supported the idea of P6 expression (Chalhoub et al., 1994). However, P6 has not been detected in infected plants. Mastari and Lapierre (1999) reported a high hydrophobicity of P6. An activity of P6 at a membrane level could be expected. However, no putative function of P6 has been assigned. There is evidence that the deletion of ORF6 sequence or the mutation of its start codon were deleterious for BYDV replication (Mohan et al., 1995). However, Koev and Miller (2000) showed that a sgRNA2 deficient mutant that lacks in ORF6 mRNA, did not affect virus replication in protoplasts.

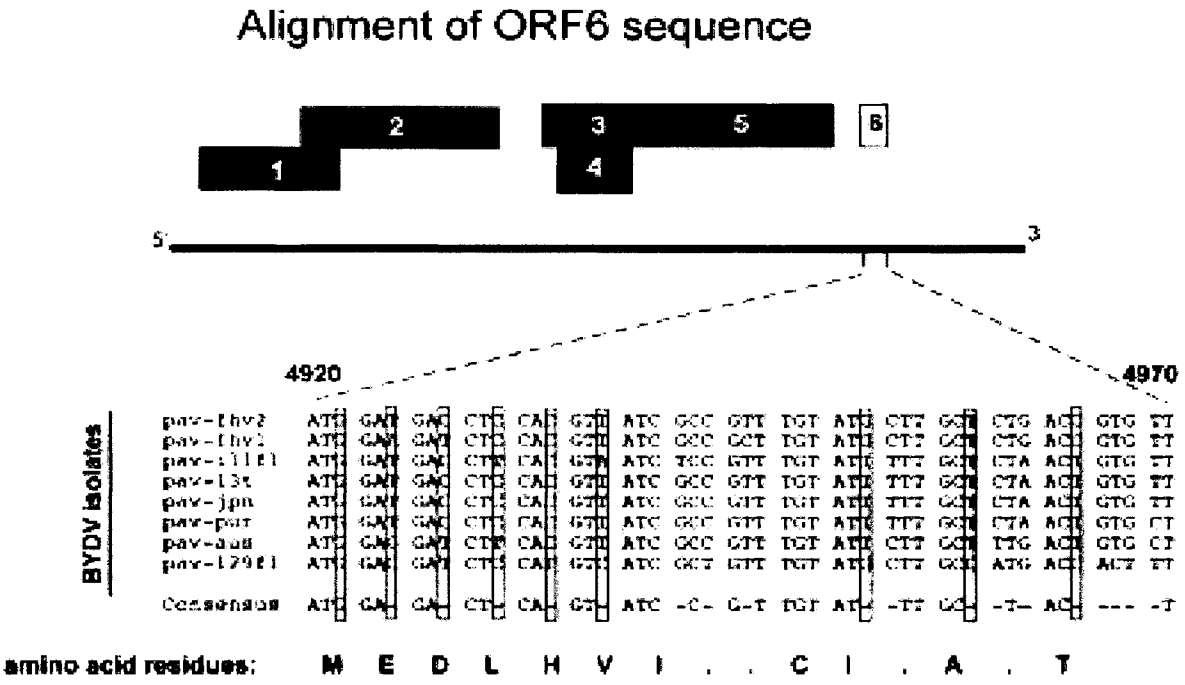

Figure 6. Alignment of ORF6 sequence of various BYDV isolates. The variation of the nucleotide at the wobble position allows conservation at least of the first ten amino acids.

Therefore, it is suggested that the small protein P6 if made, might not be directly involved in replication but rather in whole plant viral events.

MATERIALS AND METHODS

Fluorescein linked arsenic helix binder or FLAsH-EDT2 protein labeling assay. Our first approach for detecting expression of P6 protein in infected protolasts was to perform the FLAsH-labeling assay following the protocol of Griffin et al., (1998, 2000) with some modifications. The FLAsH or fluorescein linked arsenic helix ligand was provided by Aurora Bioscience Corporation in a highly toxic form. The FLAsH ligand was activated and detoxified with addition of bis-ethandithiol (EDT2) prior use. Oat protoplasts were electroporated with RNA transcripts from pPAV6, pPAV6-G4810C (sgRNA2 deficient mutant), pSG2 and pSG2 -G4922C (mutation of ORF6 start codon from AUG into AUC) cDNA clones (Wang et al., 1999). The incubation time was 48 hours for the gRNA and 4 hours for the sgRNAs, estimated time for sufficient expression of ORF6, if made. The infected protoplasts were rinsed with the washing buffer (MS media with 0.4 mM mannitol (ph 7.3) supplemented with 1mM sodium pyruvate) and incubated at room temperature in 1 ml of loading buffer (1μl of FLAsH-EDT2, 1μl EDT 2 in washing buffer). After one hour, the loading buffer was aspirated away from the protoplasts and replaced with the washing buffer containing 10μM EDT. The cells were examined under a fluorescence microscope at a maximum excitation of 508 nm and emission of 528 nm. Due to the toxicity of the product, all experiments were done under the hood.

Cloning FLAG tag at the C terminus of P6. For performing immunoblot detection of ORF6 protein in infected plants, we inserted a flag tag sequence at the 3' end of ORF6. The added 21 nucleotides code for the amino acid sequence ASP-TYR-LYS-ASP-ASP-ASP-

ASP-LYS that are specifically recognized by commercial anti-FLAG TAG antibodies from Sigma. The Flag tag sequence was inserted by a 3 steps-PCR (Koev et al., 2002) at the 3' end of ORF 6 into pPAV6 and sgRNA2 cDNA, pSG2, clones (Wang et al., 1999). PSG1 plasmid, sgRNA1 cDNA clone transcription (Wang et al., 1999), was used as template. As first step, a fragment from base positions 1402 to 2397 of pSG1 plasmid was amplified using primer, JB0586.PC: CAAGGATGCCAGCTTCTACC which annealed at base postions 1402-1421 and the downstream primer FLAG-REV 5045 which annealed at bases 2396-2358 GTCATCGTCATCCTTGTAATCAACAGAAGAGAGGGAGG. The Flag tag sequence is underlined. At the second round of PCR, the fragment from base positions 2397 to 3099 of pSG1 plasmid was amplified using the reverse primer SK020601 and primer FLAG-FOR 5044 TACAAGGATGACGATGACAAGTAAGCAAAAGACTCTCG: at bases 2397-243. The two PCR products were annealed and used as template in the third PCR. With their complementary bases, they base-paired and were elongated from their free 3' ends. A long fragment from base position 1402 to 3099 was generated and was amplified using primers SK020601 and JB0586.PC. The final PCR product was digested with Acc65I and SmaI and cloned into Acc65I-SmaI digested pPAV6. The same strategy was used to construct sgRNA2 ORF6-FLAG using pSG2 cDNA clone (Wang et al., 1999) with the universal forward primer CGCCAGGGTTTTCCCAGTCACGAC as one of the primers for the first step. The final fragment was digested with NotI and SmaI and cloned into NotI –SmaI digested pSG2.

In vitro transcription. Ambion Megascript T7 kit was used for preparing the infectious BYDV RNA transcripts. pPAV6 plasmid was linearized by cutting with SmaI. 1 μg of the

linearized DNA was incubated in the provided buffer with the various nucleotides and T7 polymerase enzyme. RNAsin was added to the reaction for inhibiting possible RNA nuclease activity. The reaction was performed at 37°C for 3 hours. The transcribed RNA was precipitated with lithium chloride. The pellet was washed with 75 % ethanol, vacuum dried and resuspended in RNAse free water. For preparing capped mRNAs, Ambion mMessage mMachine T7 kit was used. The reaction was performed for 2 hours at 37°C.

Preparation of oat protoplasts for electroporation. Oat suspension cultures were maintained in MS media at 22°C by shaking at 110 rpm in the dark. Seven days old suspension were used for preparing oat protoplasts following a modified protocol of Dinesh et al., (1993). The suspension cultures were digested for 16h, at room temperature, in the dark, at 42rpm in an enzyme solution of 0.35 % Cellulysin (Yakult Honsha Co.), 1.6 % Hemicellulase (Sigma) and 0.2 % Driselase (Sigma). The digested cells were washed twice in artificial sea water: 0.6 M mannitol (1:1) buffer and once with electroporation buffer containing 0.2 mM of spermidine. The protoplasts were resuspended in electroporation buffer. 1 ml of the resuspended protoplasts is aliquoted in the 4mm gap cuvette and 10 μg of RNA is added. The protoplasts were electroporated at a pulse of 500μF and 300V. After electroporation, the protoplasts were transferred into culture plates with 5 ml of MS media containing 0.4 mM mannitol and incubated for the designated period of time in the dark sitting on the bench. For studying BYDV replication, 48 hours is needed the accumulation of the sgRNAs. For translation assay, 4 hours is sufficient.

RNA extraction from oat protoplasts. RNA from inoculated protoplasts was extracted using the Qiagen Rneasy Plant Minikit. The inoculated oat protoplasts were pelleted by centrifugation at 700 rpm for 4 min. Lysis buffer supplied with the kit was added to the pellet prior a quick freeze and thaw in liquid nitrogen. The lysate was applied to the QIAshredder spin column and centrifuged at full speed for 2 min. The cell debris was retained in the column and the flow through fraction mixed with 95 % ethanol was applied to the Rneasy mini spin column and centrifuged for 15 sec at 8000 g. The RNA,bound to the membrane was washed twice with the washing buffer provided in the kit. The RNA was finally eluted from the membrane with 30µl of RNAse free water by centrifuging at full speed for 1 min.

Northern blot hybridization. The northern blot hybridization was performed as in Koev et al., (2000). The extracted RNAs were denatured for 15 minutes at 65 C in a 2 X sample buffer (50% formamide, 10 mM EDTA (pH 7.5), 40mM phosphate buffer (pH 6.8), 16.5% formaldehyde, 2% ethidium bromide) and put back into ice until loading in the gel. The RNA was run on a denaturing agarose gel containing 8% of formaldehyde in 10mM phosphate running buffer with 8% formaldehyde at 100V for 5 hours. The RNA was transferred to Genescreen membrane overnight. The membrane was UV cross-linked and incubated at 65 C in the pre-hybridization buffer (50% formamide, 5X SCC, 20mM potassium buffer, 1% SDS, 0.2mg/ml polyanethosulfonic acid). ^{32}P labeled probe, complementary to the 3'end of the gRNA and sgRNAs, was in vitro transcribed using Biolab's T7 polymerase enzyme. The pSp10 plasmid that was used as a template was cut with HindIII. After 3 hours in pre-hybridization buffer, the probe was added and the membrane was incubated overnight. The membrane was quickly washed with 0.1% SDS and

2X SCC and incubated for 30 minutes with 0.1% SDS and 0.1% SCC. The radioactive membrane was transferred and exposed to a Phosphoimager screen overnight before scanning on the STORM 840 Phosphoimager (Molecular Dynamics).

Wheat germ extract in vitro translation system. The in vitro translation assays were performed using the wheat germ extract kit from Promega. 0.2 pmol of transcribed RNA was incubated in the wheat germ extract supplied with 75mM of potassium acetate, the various amino-acyl-tRNAs and ^{35}S-labeled methionyl-tRNAs. For revealing the frameshift product of BYDV, the potassium acetate concentration was brought up to 100 mM. The reaction was incubated for 1 hour at room temperature. The samples were then separated in a poly-acrylamide gel that was dried and exposed on a Phosphoimager screen up to 48 hours before scanning on a STORM 840 Phosphoimager (Molecular Dynamics).

Western Immunoblot. The proteins were mixed with the SDS loading buffer and run on an poly-acrylamide gel. We followed the western blot procedure from Amersham Pharmacia Biotech. The proteins were transferred to PVDF membrane wetted with 100% methanol. The membrane was blocked overnight in PBS-tween buffer with 5% dried low-fat milk. The membrane was washed 3 times with PBS-tween buffer and then incubated with the primary antibody in a 1: 25000 dilution for 2 hours. The membrane was washed again and incubated for 1 hour with the secondary anti-body conjugated with fluorescein in a 1:600 dilution. The membrane was washed and incubated with the anti-fluorescein anti-body conjugated with alkaline phosphatase at a 1:2500 dilution for another hour. After the 3 steps rinse, the membrane was incubated with Attophos substrate with a volume of 24µl /cm2 for less than

20 minutes. The membrane was air dried and then scanned on the STORM 840 Phosphoimager (Molecular Dynamics).

SgRNA 2 Transgenic plant constructs. For testing the ability of sgRNA2 to shut off host gene expression, in collaboration with Dr David Somers (University of Minnesota) we created transgenic plants expressing sgRNA2 transcript. The first step was to construct the plasmid ScBV-sgRNA2. A PCR fragment from our full length BYDV cDNA clone, pPAV6, was amplified using primer 4809-SmaI GCACGACCCGGGAGTGAAGACAACACCA that anneals at base positions 4809-4837 and contains SmaI site (underlined) and primer 5678-EcoRI: ACTAGAGAATTCGGGTTGCCGAACTGCT which anneals at base positions 5678-5650 and with an added EcoRI site at its 5' end (underlined). The sgRNA 2 PCR product was cut with SmaI and EcoRI and cloned into plasmid pScBV 3M that was linearized with NcoI, treated with mung-bean nuclease and cut with EcoRI. The promoter of pScBV-3M plasmid is from the *Sugar cane bacilliform virus* promoter that expresses highly in monocots (Tzafrir et al.). PScBV–5320sg2 construct with truncated sgRNA 2 was generated by replacing the NcoI/ EcoRI fragment with a SmaI/ EcorI digested PCR product generated with primers 4809-SmaI and 5320-EcoRI which anneals at base position 5320-5292: GCTAGCGAATTCTCGGGTGTACATCACG. pScBV-BFTEsg2 construct with mutated TE was generated using pPAV6-BFTE plasmid as PCR template. The constructs were introduced into the plants by bombardment by the laboratory of Dr David Somers (University of Minnesota). Plants were regenerated and seeds were harvested at maturity. The second generation was screened for the presence of the constructs as described in the following sections.

Total DNA extraction from plants. The plants were placed in the dark for 3 days prior to DNA extraction to lower the carbohydrate concentration because a high level of carbohydrates may interfere with DNA isolation. Total DNA was extracted following Dellaporta's protocol (1983). 100 mg of fresh plant tissue was ground in liquid nitrogen with a mortar and pestle and incubated for 10 min at 65 C in lysis buffer (50 mM Tris pH 8, 10 mM EDTA pH 8, 100 mM NaCl, 1 % SDS and 10 mM β- mercaptoethanol). 5 M potassium acetate was added to the samples. The samples were put on ice for 20 min and centrifuged at 12,000×g for 10 min. The nucleic acids were precipitated by adding isopropanol and resuspended in 75 % ethanol with 3 M sodium acetate and put at – 20 C for at least 10 min. The samples were centrifuged for 5 min and re-washed with 75 % ethanol. The pellets were dried and resuspended in 50 µl of water.

PCR screening of the transgenic plants. PCR screening of sgRNA2 construct was performed on the total extracted genomic DNA from the transgenic plants using primers P043: CGGGATCCTGTTTCCCAGGCAGAACTTCGGTTCA which anneals at base positions 4850-4868 and primer SK020601: GGGCCCGGGTTGCCGAACTGCTCTTTCG that corresponds to the 3'end of genomic and subgenomic RNAs (nt 5677-5656). As a positive control, primers designed for annealing to the host tubulin gene were used for each sample. The PCR was performed with a denaturing temperature of 94°C for 1 min and 92°C for 40 sec, annealing temperature of 55°C for 40 sec and extension time of 1 min and 30 sec at 75°C for a total of 30 cycles. The PCR products were run on a 0.8 % agarose gel stained with ethidium bromide and visualized under UV light.

RNA extraction from plants. RNA from inoculated plants was extracted using Invitrogen TRIZOL reagent. 100 mg of fresh plant tissues were ground in liquid nitrogen and incubated for 5 min at room temperature with 1 ml of TRIZOL reagent. 0.2 ml of chloroform was added. The samples were centrifuged for 15 min at 12 000 g. 0.5 ml of isopropanol was added to the supernatant. The samples were incubated at room temperature for 10 min prior centrifugation for 10 min at 12 000 g at 9 C. The pellets were washed with 75 % ethanol, air dried and resuspended in 50μl RNAse free water.

Green fluorescence protein tagged P6. The green fluorescent gene (GFP) from jelly fish *Aequaria victoria* was cloned from pGFP-398 (that will be called pPAV6 CP-GFP), pPAV6 plasmid with the coat protein gene and a part of the read-through domain substituted with GFP sequence (plasmid provided by S. Song). The GFP gene was amplified using primer ApaI-GFP: CGGGGCCCTGGTGAGCAAGGGCGAGGAG which contains ApaI site (underlined) followed by sequence complementary to 2867-2895 and primer P020: TTGCCCCGGGACAATACGATACGGCGGCGGTAG which anneals to base position 3876-3846. GFP PCR product was digested with ApaI and cloned into ApaI-digested pPAV6 and pSG2. The ApaI restriction site was created at base position 5032 using the same strategy of 3 step PCR as for pPAV6 ORF6-FLAG construct (Koev et al., 2002). Reverse primer APAI-5032RV that annealed at base positions 5032-5005: AGAGGGCCCGAGGAAAAGGATTGCTGC was used with primer JB0586.PC for amplifying the first fragment. The second fragment was amplified using primer SK020601 with primer APAI-5032: CTCGGGCCCTCTCTTCTGTTCAAGC that anneals base position 5032-5057. The final PCR product was digested with Acc65I and SmaI and cloned into

Acc65I-SmaI digested pPAV6. The ApaI site was also created on pSG2 using the same strategy.

Construct of pPAV6 with early stop codon for ORF 6. A thymidine nucleotide was inserted at base position 4926 on pPAV6 and pSG2 by 3 step PCR using respectively pSG1 and pSG2 as a template. This insertion creates an early stop codon (UGA) 3 bases downstream of the initiation codon of ORF 6. As a first step, pSG1 was amplified using primer JB0586.PC and primer T4925 REV: GTGAAGATCACTCCATCGGCCAAACAC which anneals to base position 2262-2235. The second PCR product was amplified using primer SK020601 and primer T4926: GATGGAGTGATCTTCACGTTATCGCCG which anneals at base positions 2263-2290. The third round PCR created the final product that was digested with Acc65I and SmaI and cloned into Acc65I-SmaI cut pPAV6. The same strategy was used for cloning pSG2-T4926. Universal forward primer and T4925 REV were used for the first step PCR. The final PCR product was cut with NotI-SmaI and cloned into NotI/ SmaI cut pSG2.

pGLO18-G4920C construct for Luciferase reporter gene assay. The G into C mutation at the start codon of ORF 6 in pPAV6- G4920C was subcloned into pGL018. pGLO18 clone transcribes for a firefly luciferase gene flanked with the 5' and 3' untranslated region of BYDV (Guo et al., 2000). pPAV6-G4920C was cut with BamHI. The released fragment was cloned into BamHI cut pGLO18.

Quantification of in vivo translation using luciferase assay. This assay was done using in vitro RNA transcript of pGLO18. 1 μg of the RNA was co-electroporated into oat protoplasts with 0.2 pmol of cap-renilla luciferase-poly (A) tail RNA for normalizing electroporation efficiency. The protoplasts were harvested 4 hours post inoculation and lysed in 2.5 X passive lysis buffer (Promega) by vortexing for 15 min. The cell debris were pelleted by centrifuging for 10 min at maximum speed in a microcentrifuge. The Renilla luciferase activity was measured using the luminometer. Right before reading, 2 μl of the lysate supernatant was mixed with 50 μl of luciferase assay reagent II (Promega). Following Renilla luciferase quantification, 50 μl of STOP-and-GLO reagent (Promega) was added for firefly luciferase activity measurement.

RESULTS AND DISCUSSION

One of the specific aims of the present study was to examine whether the product of ORF6 could be detected during BYDV infection. Previous research has shown that ORF6 can be translated well from its messenger sgRNA2 in the wheat germ extract in vitro system (Wang et al., 1999). However, the P6 protein has yet to be detected in infected plants. Following a step-by-step approach, a series of currently available techniques were used to determine whether this hypothetical protein of 4.3 kDa accumulates in BYDV infected oat protoplasts.

FLAsH-EDT2 labeling detection of P6. Griffin et al. (1998) demonstrated a novel technique for labeling proteins, which uses a fluorescein arsenical helix binder or FLAsH. The ligand is membrane-permeable and can enter live cells. It recognizes and binds a rare peptide motif, namely CCXXCC (where X can be any amino acid and C a cysteine residue), located on an α helix turn (Griffin et al., 1998) (Fig. 7). The binding of the organo-arsenate compound to this specific small receptor motif causes fluorescence of the cells under an excitation wavelength of 528 nm (Griffin et al., 1998). We used this novel technique for detecting the product of ORF6, which naturally bears in its sequence the six amino acid motif CCRWCC at residues 28-33. The FLAsH ligand was provided by Aurora Bioscience Corporation and activated with the addition of bis-ethanedithiol (EDT2) solution. The oat protoplasts were inoculated with BYDV infectious transcripts (PAV6), non-replicating sgRNA2 and a P6 deficient sgRNA2 mutant, SG2-G4922C, with the ORF6 start codon mutated to AUC. As a control, non-infected cells were either treated or untreated with the

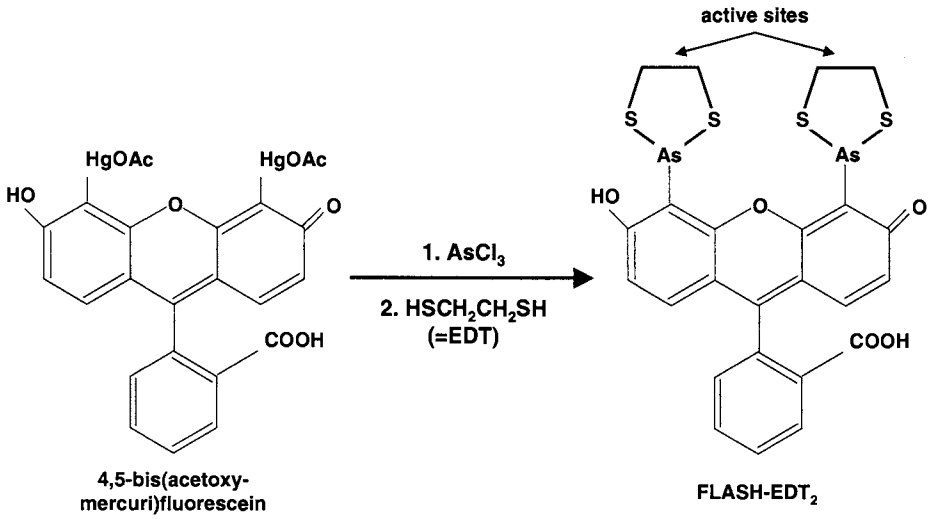

Figure 7. Fluorescein arsenical helix binder or FLAsH-EDT2 molecule.

The FLAsh ligand specifically recognizes and binds a rare peptide motif, CCXXCC (where X can be any amino acid and C a cysteine residue). The binding will induce the fluorescence of the complex. Modified from Griffin et al. (1998).

FLAsH-EDT2 solution. Forty-eight hours post inoculation (hpi), the PAV6-infected protoplasts were treated with the FLAsH-EDT2 solution. The same treatment was performed on sgRNA2 inoculated cells 5 hpi. After several washing steps to rinse off free FLAsH-EDT2 ligands, the treated cells were examined under a fluorescence microscope using an excitation wavelength of 528 nm.

The results of the assay were inconclusive. All FLAsH-treated protoplasts, both infected and non-infected, fluoresced brightly (Fig. 8). A possible explanation for this non-specific fluorescence is that the CCXXCC motif may not be rare in plant proteins. In fact, the FLAsH-labeling assay was designed for mammalian cells (Griffin et al., 1998). However we cannot rule out the possibility that the FLAsH compound fluoresces even without binding.

Fusion of Flag tag sequence to P6. A more conventional technique, namely immunoblot detection, was then attempted to reveal the product of ORF6 in infected protoplasts. A short sequence of twenty-one nucleotides coding for FLAG-tag was inserted at the 3'end of ORF6 (Fig. 9A). The fused sequence codes for an epitope site of eight amino acids that can be recognized with commercially available anti-FLAG-tag antibodies (Sigma) (Fig. 9A). If P6 accumulates during viral infection, it can be detected immunologically with the attached FLAG-tag sequence. The RNA transcripts from wild type PAV6 and PAV6 ORF6-FLAG cDNA clones, were electroporated into oat protoplasts.

It was important to ensure that the insertion of extra sequences at ORF6 base position would not disrupt the replication of BYDV and that sgRNA2, the messenger RNA of ORF6

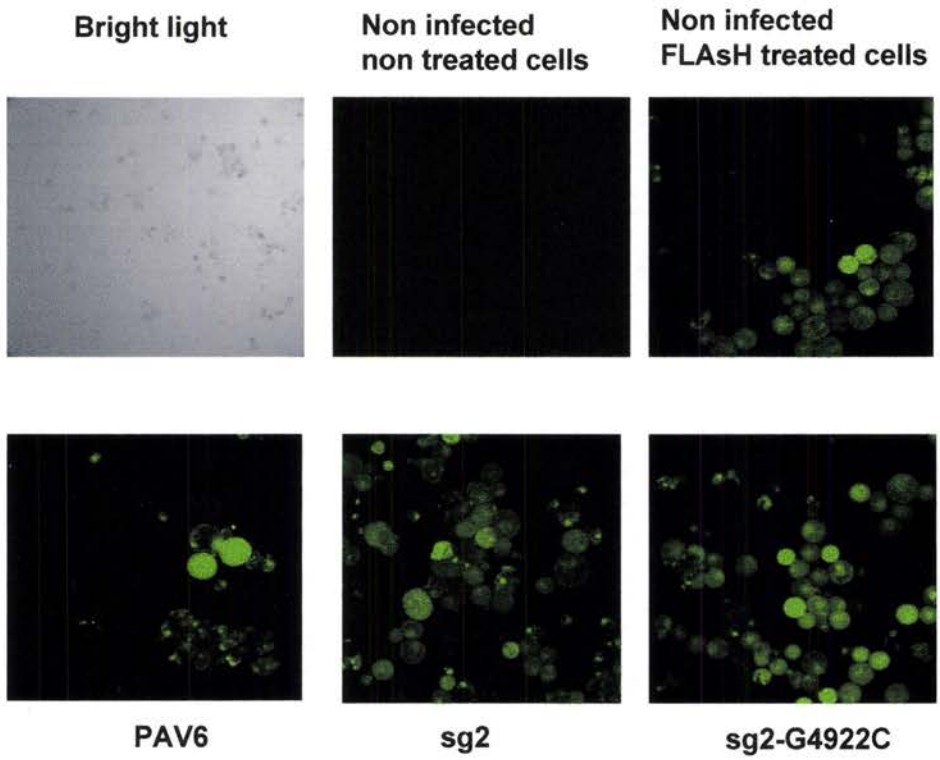

Figure 8. FLAsH labeling assay for P6 detection on infected protoplasts with infectious BYDV transcripts: PAV6, full length BYDV transcript; SG2, sgRNA2 transcript; SG2 G4922C, sgRNA2 transcript with point mutation of ORF6 start codon from AUG into AUC, sg2-G4922C. P6 naturally bears in its sequence the six amino acid motif CCRWCC that is the specific receptor of FLAsH ligand. Unspecific fluorescence was observed in the FLAsH-treated infected and uninfected protoplasts.

FLAG, would be efficiently synthesized. Total viral RNA was extracted and analyzed in a northern blot hybridization 48 hpi (Fig. 9B).

The results showed that PAV6 ORF6-FLAG was fully infectious and replicated at the same level as the wild type PAV6. On the northern blot hybridization, we noticed a higher accumulation of sgRNA3 in PAV6 ORF6-FLAG infected cells compared to the cells inoculated with wild type PAV6. This may be due to a variation in RNA quality during RNA isolation.

Total protein from PAV6, PAV6 ORF6-FLAG and non-replicating sgRNA2-FLAG inoculated protoplasts was subsequently extracted and analyzed in a western immunoblot using anti-FLAG-tag antibodies. We extracted the total protein at different time points depending on the initial inoculum: 48 hpi for the genomic RNA transcripts and 5 hpi for non-replicating sgRNA2. 5 hours were sufficient for direct translation of ORF6 from non-replicative RNA. All samples were denatured in the presence of SDS loading buffer at 70 °C for 10 minutes before running on a 12% pre-cast poly-acrylamide gel (Invitrogen). As a control, we ran spiked and non-spiked in vitro translation product of SG2-FLAG with non-infected, electroporated protoplasts in adjacent lanes with the other samples (Fig. 10A lane 1-2). Variation in ion conditions or presence of cell membranes could possibly mask the detection of the small protein or change its mobility on the gel. Such an occurrence could be detected with the spiking control.

In figure 10A (lane 1-2), we noticed a difference in mobility between spiked and non-spiked sgRNA2-FLAG in vitro products. The in vitro translation product appeared to migrate at a slower rate in the presence of cells debris. Both in vitro translation product lanes

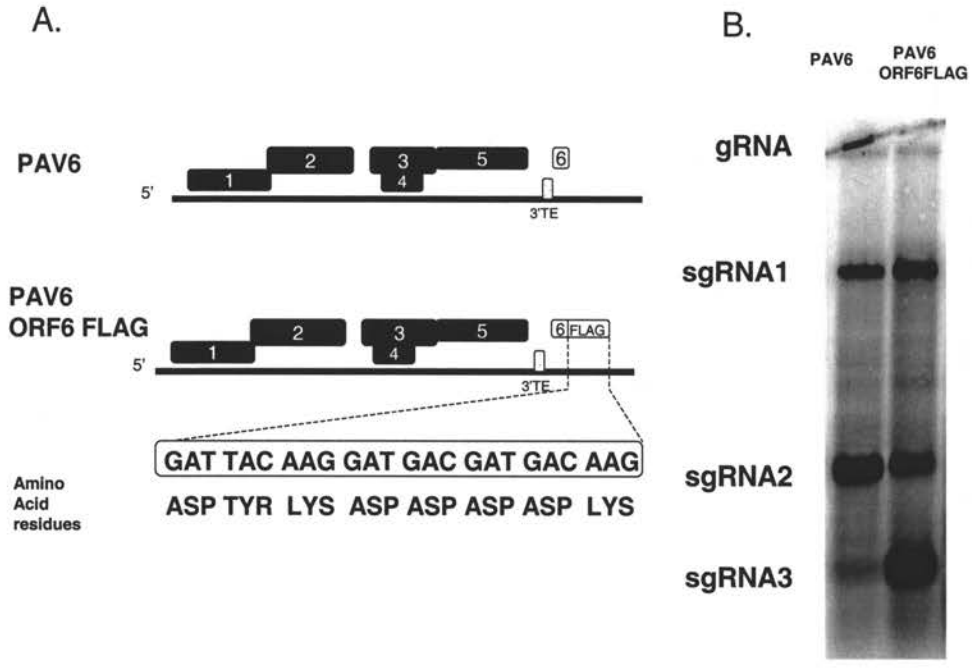

Figure 9. A. Insertion of FLAG tag sequence at the 3'end of ORF6.

The figures are not to scale. The FLAG insertion is only 21 nucleotides.

B. Northern blot analysis of total virus RNA extracted from inoculated protoplasts with both wild type PAV6 and mutant PAV6 ORF6-FLAG. The insertion of the FLAG tag sequence at ORF6 base position did not affect virus infectivity. PAV6 ORF6-FLAG replicated at the same level as the wild type PAV6.

were used as control for the identification of FLAG tagged P6 in vivo accumulation. Although sgRNA2-FLAG product translated in vitro was detected by immunoblot (lane 1-2), lane 5A revealed no detection of P6-FLAG from sgRNA2-FLAG inoculated protoplasts. A similar result was obtained from PAV6 ORF6-FLAG infected cells (lane 4). The assay was repeated several times with similar results.

A reasonable question was whether P6, even if made, was too small to be purified and detected, rapidly degraded, or expressed at a very low level. To answer this question, we extracted the total protein from amply sgRNA2-FLAG inoculated protoplasts at various time points. Specifically, the total protein was extracted at 5, 10, 24 and 48 hpi and analyzed on a western immunoblot. No FLAG-tagged P6 accumulation was detected at any time point (Fig. 10B). In summary, the well-established FLAG tagged assay was not effective in detecting the product of ORF6. The aforementioned concerns regarding a possible rapid degradation remain plausible.

Fusion of green fluorescent protein to P6. Subsequently, our focus was then shifted to the translation initiation level of ORF6 rather than the detection of its product, using a reporter gene. To visualize ORF6 translation in oat protoplasts, we used the green fluorescent reporter gene (GFP). The commonly used GFP gene from *Aequorea victoria* was inserted in the middle of ORF6, at base position 5036, in the infectious BYDV cDNA construct (Fig. 11A). During virus replication, the mRNA for the ORF6-GFP fusion should accumulate as a result of sgRNA synthesis. If GFP-tagged ORF6 is translated, the protoplasts should turn green under UV light. As a control, we used the infectious PAV6 CP-GFP cDNA clone constructed

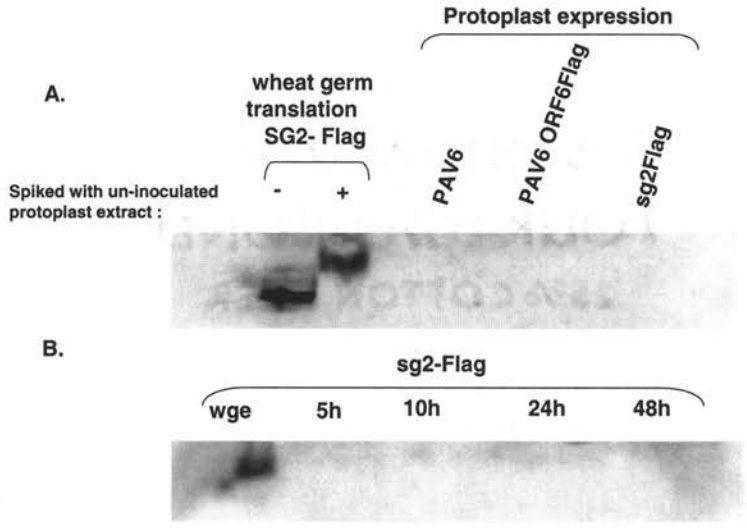

Figure 10. Western immunoblot using anti FLAG tag antibodies on total extracted protein from infected protoplasts with:

A. Infectious BYDV PAV6-ORF6–Flag and non-replicative SG2-Flag.

As a control, the wheat germ in vitro product of SG2-flag was spiked with un-inoculated, protoplasts extract (+,-). Variation in ion condition or presence of cell membrane seemed to change protein mobility on the gel (lane 2).

B. No replicating psg2-Flag RNA. The total protein was extracted at 5, 10, 24 and 48 hours post inoculation.

P6-FLAG was not detected in the infected protoplasts, nor was it translated directly form sgRNA2-FLAG. The question remained whether P6 was too expressed at a low amount or rapidly degraded.

by Sang Ik Song. In this construct, the coat protein gene of BYDV was deleted replaced with GFP gene. The length of the genomic RNA and its sgRNA1 were shorter than wild type PAV6 (Fig. 11B lane 3). The deletion of the coat protein and its substitution with GFP gene does not affect viral replication in protoplasts (S. I. Song, unpublished data).

Oat protoplasts were inoculated with PAV6 ORF6-GFP and PAV6-CP-GFP RNA transcripts. The protoplasts were examined under fluorescence microscope. No fluorescence appeared in inoculated PAV6 ORF6-GFP cells. The northern blot analysis of total extracted viral RNA from the infected protoplasts showed that the PAV6 ORF6-GFP construct replicated poorly (fig. 11B lane 2).

We performed a wheat germ in vitro translation assay to test the translation efficiency of PAV6 ORF6-GFP RNA. The in vitro assay revealed the inability of PAV6 ORF6-GFP RNA to express the frameshift product required for viral replication (Fig. 12 lane 3). Moreover, we noticed that, for the same construct, there was lower expression of the 39kDa product compared to the wild type level (Fig. 12 lane 3). ORF6 is located just between the translation element (nt 4809-4919) and the distal frameshifting element (nt 5050-5100) (Wang et al., 1997, Paul et al., 2001). The low translation efficiency and frameshift ability of PAV6 ORF6-GFP construct may be due to the disruption of the functionality of the control elements with insertion of the GFP coding sequence at that particular position.

Since the insertion of GFP into ORF6 prevented virus replication, its expression could not be tested. Alternatively, we attempted to detect GFP-tagged P6 expression using the non-replicating sgRNA2 as inoculum where ORF6-GFP shoud be directly translated. We inserted a GFP gene into sgRNA2 cDNA clone at base position 5036 and inoculated the

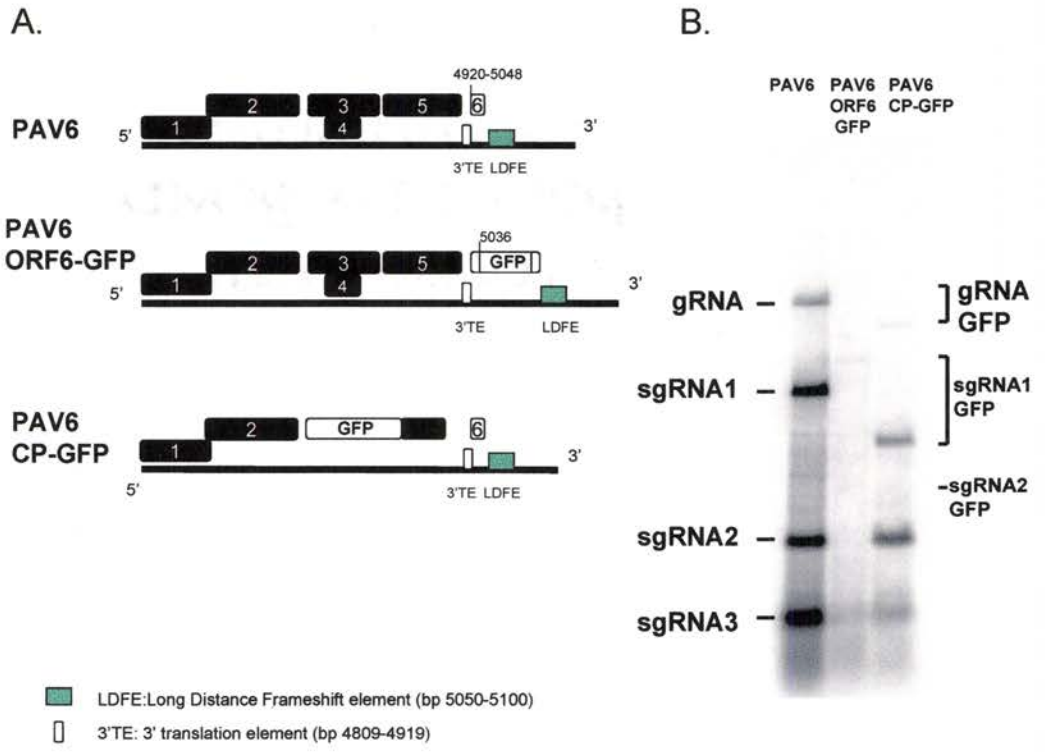

Figure 11. Fusion of green fluorescent protein sequence to ORF6

A. Insertion of green fluorescent protein sequence (GFP) at ORF6 base positioning PAV6 ORF6-GFP or in place of coat protein in PAV6. CP-GFP (constructed by Sang Ik Song).

B. Northern blot analysis of virus RNA from oat protoplasts inoculated with PAV6, PAV6 ORF6-GFP and PAV6 CP-GFP. The insertion of GFP sequence into ORF6 decreased virus infectivity (lane. 2). Thus, the expression of GFP could not be tested with this construct.

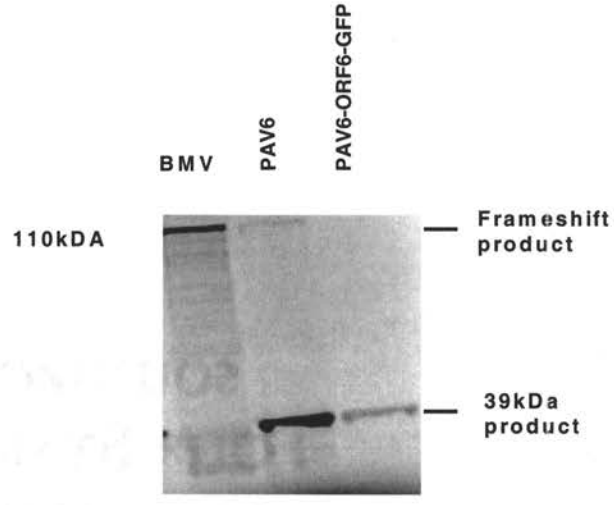

Figure 12. Wheat germ in vitro translation of wild type PAV6 and mutant PAV6 ORF6-GFP. Wheat germ in vitro translation product of BMV transcript (*Brome mosaic virus*) was used as a ladder. The frameshift product was not detected in PAV6 ORF6-GFP (lane 3). Moreover, the 39 kDa product accumulated at a lower lever thant the wild type PAV6. The insertion of GFP sequence at ORF6 base position may have disrupted the functionality of both translation and frameshift elements.

RNA transcript into the cells (Fig. 13A). The PAV6 CP-GFP construct was used as the positive control in vivo assay despite the drawback of requiring at least 24 hours for accumulating the sgRNAs and allowing their translation. The inoculated protoplasts were observed under a fluorescence microscope 5, 10 and 24 hpi. The sgRNA2-GFP inoculated protoplasts did not fluoresce (Fig. 13B) in spite of the fact that the fusion protein was expressed in the wheat germ in vitro system (Fig. 14).

It has been shown that sgRNA2 is well translated in the wheat germ extract in vitro system (Fig. 14) (Wang et al., 1999). However, the multiple approaches that were followed in the present study failed to detect the P6 protein in inoculated protoplasts. Moreover, the GFP reporter gene assay showed that translation of sgRNA2 does not seem to be initiated in protoplasts. Nevertheless, the in vitro system did show that the sgRNA2 has the ability to code for a translatable protein. The question is whether ORF6 is in a good context for translatability in vivo.

RNA circularization is a pre-requisite for ribosome recruitment in vivo but not in vitro (Wells et al., 1998). In cellular mRNAs, the circularization is mediated by the terminal cap and poly (A) tail structures (Hentze, 1997). Wang et al. (1997) showed that the 3' translation element (3'TE) of BYDV RNA, located 5 kilobases downstream of the start codon of the gRNA, plays an important role in gene expression of the uncapped, non-polyadenylated RNA. By base pairing with the 5'end of the genome, it assures circularization and translatability of the gRNA in vivo (Guo et al., 2001) (Fig. 15). It appears that the sgRNA1 uses a similar strategy to get its genes expressed (Elizabeth Pettit, unpublished). The 5'UTR of sgRNA1 presents a stem loop that potentially base pairs with 3'TE SL-III (Guo et al., 2001).

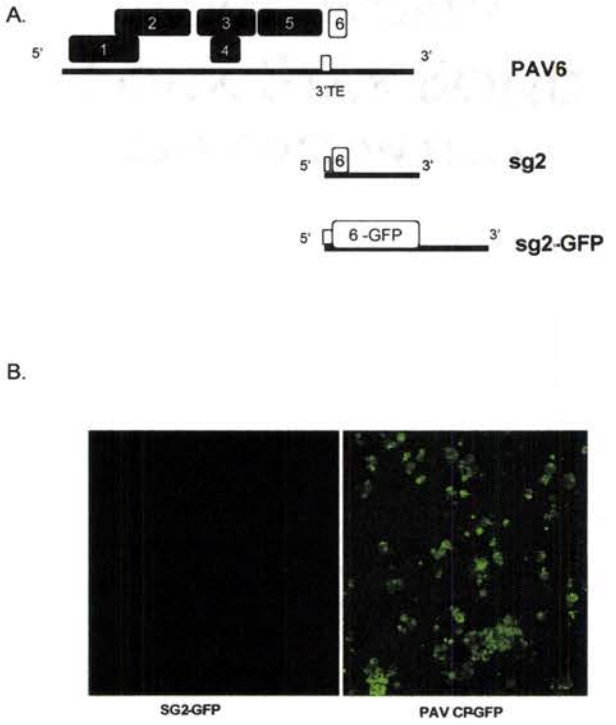

Figure 13. A. Construct showing GFP sequence insertion on sgRNA2 at ORF6 position (sg2-GFP).

B. Green fluorescent protein expression from protoplasts inoculated with sgRNA2-GFP transcript and infectious full length PAV6 CP-GFP cDNA transcript 24 hpi. No GFP expression was observed on sg2-GFP inoculated protoplasts 5, 10, 24 hpi. The picture has been taken 24 hpi.

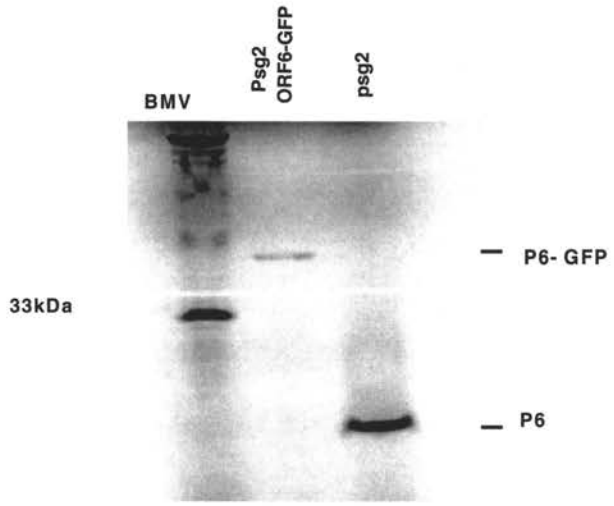

Figure 14. Wheat germ in vitro translation of pSG2 and pPSG2-GFP.

BMV (*Brome mosaic virus*) in vitro products were used as a ladder. The insertion of GFP at ORF6 position did not affect in vitro translatability of sgRNA2 (lane 2).

The 3'UTR of BYDV RNA has been reported to be indispensable for in vivo TE-mediated translation (Guo et al., 2000). The 3'UTR has been suggested to mimic the poly (A) tail function (Guo et al., 2000).

In sgRNA2, the TE is positioned in the 5'UTR of the mRNA. No potential base pairing of TE to the 3'UTR was found to support the closed loop model that is required for in vivo recruitment of the translation machinery (Fig. 15). In the absence of such base pairing, initiation of ORF6 translation appears unlikely. This is a plausible explanation of the failure to detect the product of ORF6 in infected protoplasts.

According to our results, in addition to the non-circularization of sgRNA2, the 3'UTR in sgRNA2 does not seem sufficient to fully account for the function of a poly (A) tail in the recruitment of the translation factors in vivo when the 3'TE is in the 5'UTR. Using in vivo luciferase assay, Guo et al. (2000) showed that TE located at the 5'UTR of the reporter gene could direct gene expression up to 20% only in the presence of a poly (A) tail (Guo et al., 2000).

We propose that the addition of a poly (A) tail at the 3' end of sgRNA2 would help in initiating ORF6 expression by recruiting the translation machinery via binding of PABP (poly (A) tail binding protein) (Fig. 15).

Effect of various mutations on ORF6 sequence in BYDV replication and translation.

If the P6 is not be translated, as shown earlier, this may imply that the RNA sequence of ORF6 or sgRNA2 plays a role in BYDV infection. To examine this possibility, the following two approaches were followed. First, we analyzed the importance of sgRNA2 in

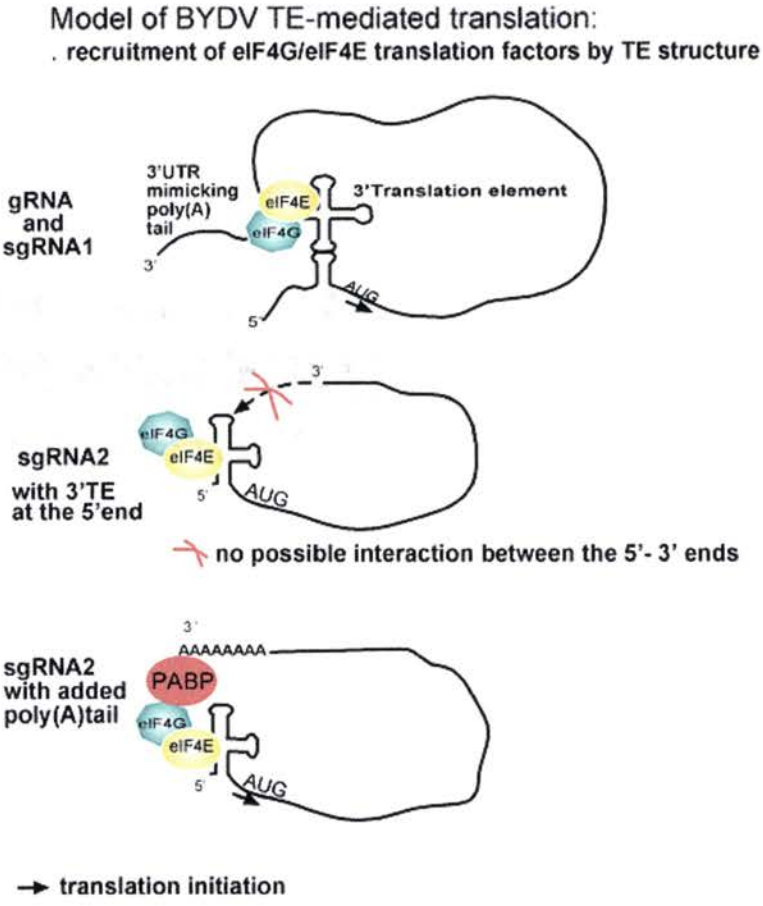

Figure 15. Closed loop model of BYDV gRNA and sgRNA1, and sgRNA2 via 3'TE-5'UTR base pairing. The translation element, 3'TE, by base pairing with the 5' end of the gRNA assures the circularization of the mRNA that seems to be a prerequisite for ribosome recruitment in vivo but not in vitro. In sgRNA2, the 3'TE is positioned in the 5'end of the mRNA. The lack of interaction of the 3'TE to the 3'UTR could explain the non-translatability of ORF6 in vivo. The addition of a poly (A) tail at the 3' end of sgRNA would help in initiating ORF6 expression via poly (A) tail binding protein (PABP).

viral-host interaction. Second, we tested the role of the ORF6 sequence as a cis-acting element involved in either viral replication or translation control.

SgRNA2 function during BYDV infection. It has been proposed that sgRNA2 serves as a ribo-regulator to control the switch from early to late virus gene expression (Wang et al., 1999). sgRNA2 with its 3'TE has the ability to trans-inhibit gene expression of the genomic and subgenomic RNAs (Wang et al., 1999). This trans-inhibition activity has been reported to inhibit viral as well as non-viral genes in wheat germ in vitro system (Wang et al., 1999). However, no experiment has been performed in vivo to study the ability of sgRNA2 to trans-inhibit host translation.

The ability of plant viruses to shut off host gene expression to favor their own translation has yet to be reported. In order to examine the potential inhibition of host gene expression by sgRNA2 during BYDV infection, we engineered transgenic oats to express sgRNA2 in collaboration with Dr. David Somers (University of Minnesota) (Fig. 16). Our main goal was to observe the phenotypes of the transgenic lines and to determine whether any abnormality in plant growth can be correlated with the expression of the sgRNA2 transgene and its trans-inhibition activity mediated by TE. As a control, additional constructs were tested in oats. Transgenic oats expressing sgRNA2 with a non-functional TE were engineered. The TE mutant is characterized by a BamHI fill-in at base position 4837 that abolishes TE function (Wang et al., 1999). If any abnormality would be observed in the transgenic sgRNA2 plants in correlation to the TE trans-inhibition effect, the sgRNA2 with TE mutant should show a normal phenotype. We analyzed the second generation of the transgenic plants obtained by self-pollination of the first generation.

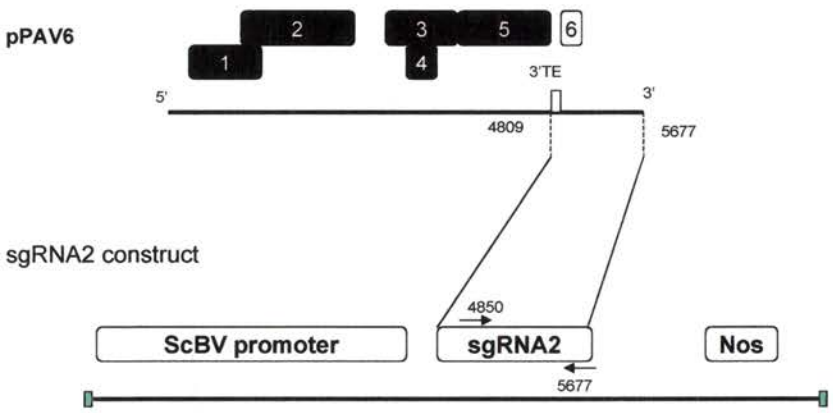

Figure 16. sgRNA2 construct inserted in oats. ScBV: *Sugar cane bacilliform virus.*

Figure 17. Second generation of the non transgenic oats (left) and transformed plants with sgRNA2 cDNA (the three plants from the right). The transformed plants showed various phenotypes. Some plants were stunted compared to the non transgenic plants. (In collaboration with Dr. David Somers, University of Minnesota).

The plants of the second generation showed various phenotypes. Specifically, some plants were stunted compared to wild type non-transgenic plants or died at a early stage of growth, whereas others were indistinguishable from the non-transgenic plants (Fig. 17).

Our main question was to determine whether the stunting phenotype of some plants was due to somaclonal variation during the cloning step or a direct effect of sgRNA2. Interestingly, the plants transformed with sgRNA with a mutated TE were indistinguishable from the non-transgenic plants. The question still remains whether, if expressed, sgRNA2 accumulation has no effect on host gene expression or, through a gene silencing response, the plant blocks sgRNA2 expression. PCR screening of the sgRNA2 gene in the whole plant genome using internal primers to sgRNA2 sequence was performed on several transgenic oats.

A fragment of about 900 nucleotides was expected. As a quality control of our extracted genomic DNA, we simultaneously performed a PCR screening of the same plants using primer annealing to host tubulin genes. Twenty out of twenty five plants tested positive with both primers (Fig. 18). A band appeared on our positive control, non-transgenic oats (lane NT). This might have been due to contamination rather than nonspecific priming.

A northern blot analysis was done on all samples that tested positive to determine whether sgRNA2 sequence accumulated. The northern blot analysis of total RNA extracted from the transgenic plants did not show the presence of sgRNA2 (Fig. 19 from lane 1-25). Lane 25 represented one of the stunted transgenic plants. Our positive controls included extracted viral RNA from infected protoplasts with infectious PAV6 (lane PAV6) and sgRNA2 transcripts (lane pSG2).

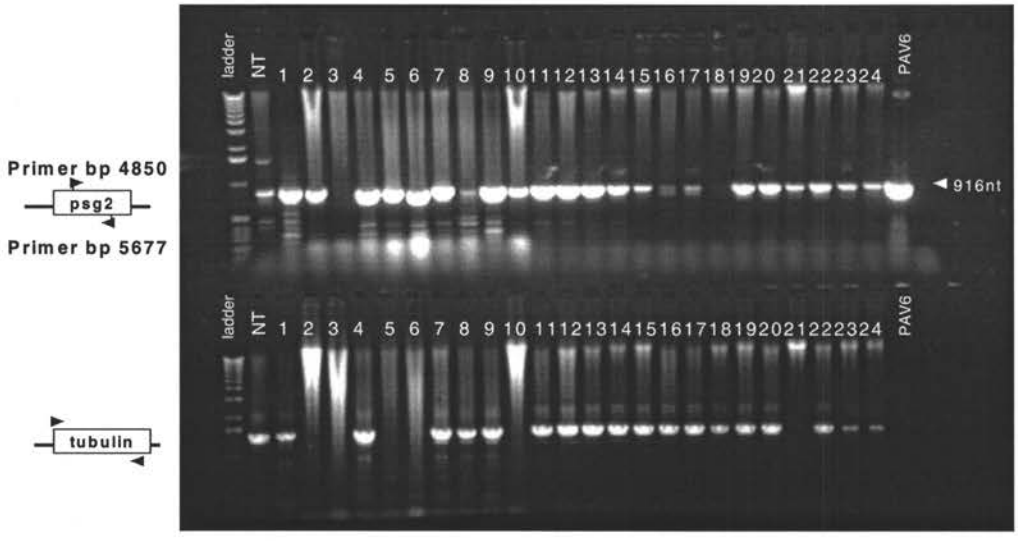

Figure 18. PCR screening of sgRNA2 transgenic plants. Primers flanking at base position 4850 and 5677 were used for detecting sgRNA2 construct inserted into host DNA genome. Primers detecting plant tubulin gene were used as control. Twenty out of twenty five plants tested positive with the accumulation of the 916 nucleotide expected fragment.

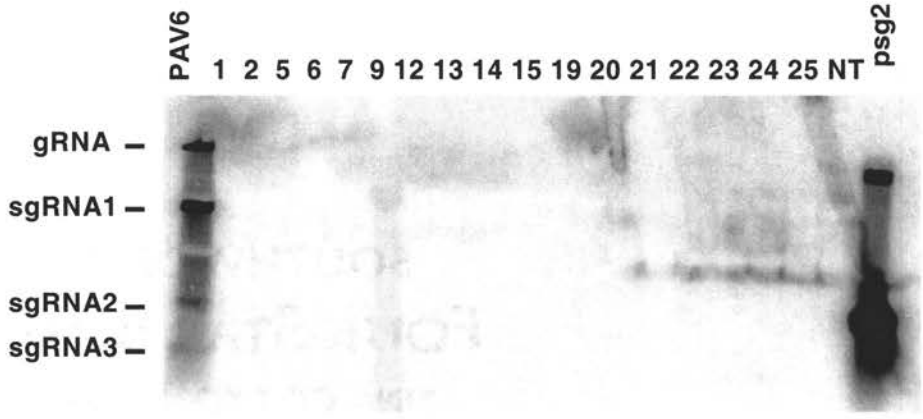

Figure 19. Northern blot analysis of sgRNA2 transcript from the transformed plants that apparently integrated sgRNA2 in their genome. Extracted viral RNA from inoculated protoplasts with PAV6 and sgRNA2 transcripts were used as control. SgRNA2 transcript was not detected in any of the plants.

The absence of sgRNA2 transcripts could be due to a plant response against foreign genes in a mechanism of gene silencing (Waterhouse et al., 2001). The detection of the small 25 nucleotides RNAs resulting from RNA degradation (Hamilton et al., 1999) and run-off transcription assays are possible directions for future research, to confirm these results. The other explanation for the failure to detect sgRNA2 transcripts is that the transgene might have been inserted in a silent region of the plant genome or the promoter got methylated, preventing the gene from being transcribed.

ORF6 as a cis-acting region controlling either replication or viral translation. Besides being a part of sgRNA2, ORF6 is positioned between, and perhaps itself includes, important regions needed for viral gene expression and its replication. The question is whether ORF6 plays a role in any viral event as a cis-acting element with its strategic position. There is evidence that the possible ORF6 involvement in replication must be at a cis level rather than in trans (Koev et al., 2000). Koev et al. (2000) showed that the sgRNA2-deficient PAV6 mutant (construct PAV6 SG2-G4809C in our study) replicated at the same level as wild type (Fig. 21 lane 1). As far as translation is concerned, additional BYDV sequences are required for efficient TE-mediated translation in vivo (Guo et al., 2000). ORF6 that is adjacent to the TE structure may be an additional region influencing viral gene expression in vivo.

To test these hypotheses, a series of mutations was constructed in ORF6 sequence on full length BYDV replicative construct (Fig. 20). Each mutant was inoculated into protoplasts. The effects on replication and translation were analyzed by a northern hybridization blot and luciferase reporter gene assay, respectively.

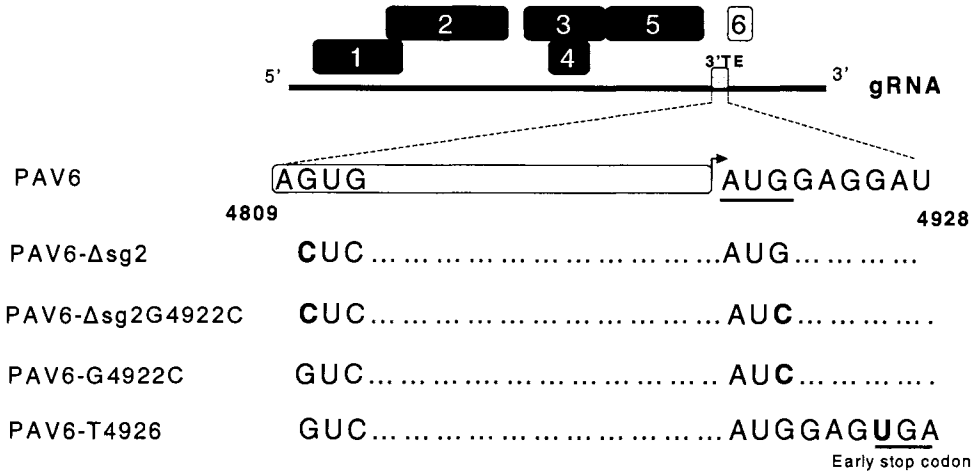

Figure 20. Mutation of various PAV6 clones in ORF6 sequence surrounding its start codon AUG.

PAV6, wild type BYDV; PAV6 Δsg2, sgRNA2 deficient mutant with a mutation of G into a C at nt 4810; PAV6 Δsg2 G4922C, PAV6 Δsg2 mutant with additional mutation of ORF6 start codon from AUG into AUC; PAV6 G4622C, mutant with the AUC point mutation at ORF6 start codon, PAV6 T4926, mutant with a U insertion three bases downstream the start codon of ORF6 creating an early stop codon.

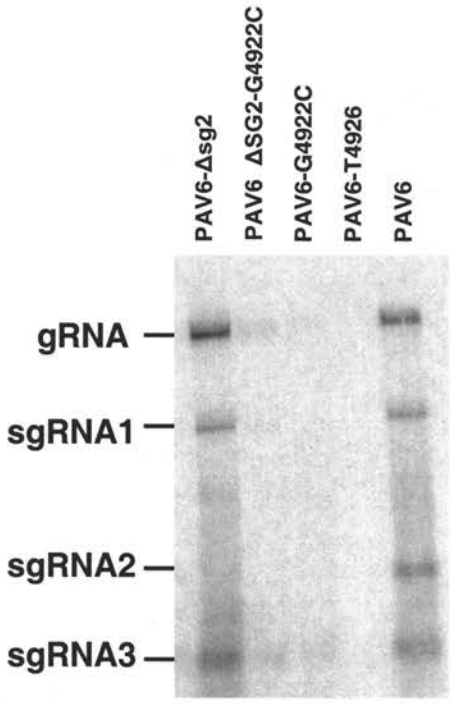

Figure 21. Northern blot of the various PAV6 mutants at ORF6 position.

PAV6-ΔsgRNA2 (lane 1) replicated the same level as the wild type PAV6 (lane 5). The mutation of ORF6 start codon from AUG to AUC (lane 2-3) and the U insertion three bases downstream of the AUG (lane 4) decreased virus replication (lane 4).

Previous results have shown that the total deletion of ORF6 sequence abolishes viral replication (Mohan et al., 1995). Partial deletion from its 3'end, corresponding to the polypyrimidine tract (nt 5017-5045) that seems important in cap-independent translation of IRES containing animal virus RNAs had no effect on either translation or viral replication (Paul et al. 2001, Elizabeth Pettit unpublished data). The mutation of ORF6 start codon in AUC (construct PAV6 G4922C) drastically decreased virus replication (Fig. 21 lane 3). However, the same point mutation did not affect TE-mediated in vitro translation (Mohan et al., 1995).

The competitive environment of the in vivo system versus in vitro may require additional sequences for TE- mediated translation efficiency. Guo et al. (2000) showed that the 105 nucleotides-TE structure is not sufficient for efficient translation in protoplasts. The ORF6 start codon is just adjacent to the TE structure. If ORF6 start codon or at least its G is unnecessary for in vitro translation, it may affect TE in vivo functionality.

To test this specific hypothesis, the translation efficiency of PAV6 G4922C mutant was analyzed in protoplasts using a luciferase reporter flanked by the 5'UTR and the 3'UTR of BYDV including 3'TE, ORF6 and the downstream sequences. Translation was determined by the luciferase activity. The luciferase assay showed that the AUG-to-AUC point mutation of the start codon of ORF6 did not affect translation in vivo (Fig. 22). The mutant translated as efficiently as the wild type. It seems that the AUG or at least the G is not playing a cis-acting role in BYDV gene expression, but does so in viral replication. A uracil base was inserted just 3 bases downstream from the start codon of ORF6, at base position 4926. This insertion creates an early stop codon. The T insertion decreased viral replication (Fig. 21 lane 4) but did not affect translatability of the RNA in vivo (data not shown).

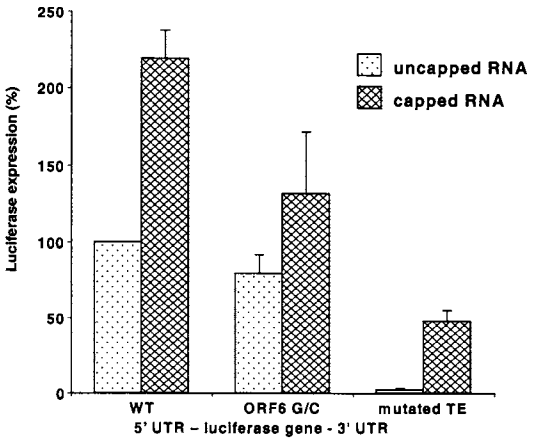

Figure 22. Luciferase activity assay. The Luciferase reporter gene was flanked with the 5' and 3' ends of BYDV (wt, wild type), with 3'end containing the point mutation of ORF6 start codon from AUG into AUC (ORF6 G/C) and with a non-functional BamHI-fill in TE (mutated TE). The ORF6 point mutation of the G into a C did not affect RNA translatability.

In conclusion, the various mutations of the ORF6 sequence revealed a cis-acting region involved in BYDV replication surrounding the start codon of ORF6 at base position 4920. Any mutation or insertion in that region drastically decreased viral replication but did not affect virus translatability (summarized in Fig. 23).

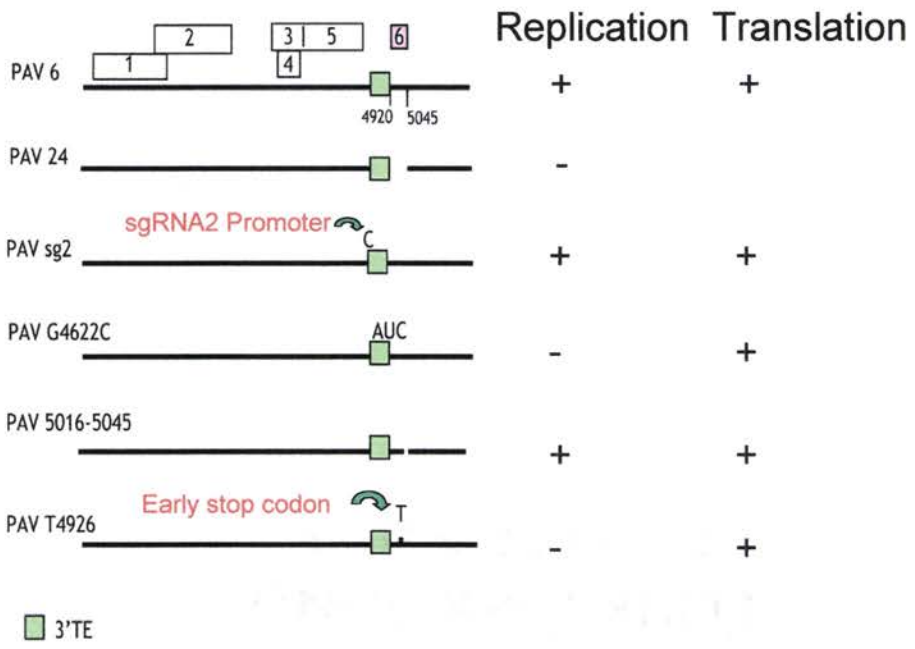

Figure 23. Mutations in ORF6 sequence that may affect BYDV replication and gene expression.

PAV 24: mutant with total deletion of ORF6 sequence.

PAV sg2: sgRNA2 deficient mutant with a point mutation at base position 4810 G into C.

PAV G4922C: mutant with a mutation of ORF6 start codon into AUC.

PAV 5015-5045: mutant with deletion of the polypyrimidine tract at the 3 end of ORF6.

PAV T4926: mutant with U insertion three bases downstream ORF6 start codon creating an early stop codon.

The various mutations of ORF6 sequence revealed a cis-,but not trans-, acting region surrounding the start codon of ORF6 invovled in BYDV replication but not in cap-independent translation of the 5'proximal ORF via 3'TE.

CONCLUSION

Based on the experiments that were conducted, the following conclusions can be drawn in reference to the specific aims of the study. Although well expressed in the wheat germ extract in vitro system, P6 was not detected in infected oat protoplasts. According to our proposed model herein, ORF6 is not in a good context for in vivo translatability. The lack of interaction between the 5'-3' ends of sgRNA2, a prerequisite for translation initiation in vivo but not in vitro, does not favor ORF6 translation in vivo. Thus, sgRNA2 itself, rather than its encoded ORF may perform a function in BYDV replication, supporting the ribo-regulator model of Wang et al. (1999).

The assay testing the possible ability of sgRNA2 to inhibit host gene expression by using transgenic oat expressing sgRNA2 was inconclusive since none of the plants appeared to transcribe sgRNA2. However, we could not rule out that the non-detection of sgRNA2 transcript may be the result of a plant defense response against foreign genes known as gene silencing (Waterhouse et al., 2000).

Notably, the ORF6 sequence, or at least the region surrounding its start codon at base position 4922, seems to play a cis-role in viral replication. Base change or insertion in that region reduced viral replication without affecting cap independent translation efficiency.

Various viral processes, including translation initiation, frameshifting, and viral replication are controlled by stable stem loops all along BYDV genome (Guo et al., 2001, Paul et al., 2001, Koev et al., 2002). RNA conformation and its stability are crucial in BYDV gene regulation. Future studies should focus on the secondary structure of ORF6. It

is plausible that the mutation at the AUG site may cause improper RNA folding, which might prove deleterious for viral replication but with no effect on translation efficiency.

APPENDIX: P6 SUPPRESSION OF GENE SILENCING ASSAY

In collaboration with Dr Ken Richards and Sebastien Pfeffer
Institut de Biologie Moleculaire des Plantes –Strasbourg, France

During viral infection, plants develop a defense mechanism called post-transcriptional gene silencing (PTGS) that specifically recognizes and targets the invading viral RNAs for degradation (Waterhouse et al., 2001). A similar mechanism has been described in different kingdoms. PTGS is known as RNA interference in nematodes and insects or quelling in fungi (Cogoni and Macino, 2000, Zamore et al 2000). PTGS in plants is analogous to the immune system of animals (Vionnet, 2001). It is directed against any invading foreign genes and their endogenous homologous genes including viruses, transposable elements or transgenes. During viral infection, the defense response relies on the recognition of the viral double stranded replicative intermediate RNA (dsRNA) that is formed during virus replication by a specific ribonuclease called dicer that directs degradation of the dsRNA in small untranslatable nucleotides of about 25 bases (Hamilton and Baulcombe, 1999, Hammond et al., 2000). A silencing signal, that is still uncharacterized, spreads systemically and initiates PTGS throughout the plant (Mlotshwa et al., 2002). PTGS attenuates viral pathogenicity and allows the plant to recover from virus infection.

Most viruses, as a survival adaptation, have evolved various strategies for overcoming this complex defense response (Brigneti et al., 1998, Anandalakshmi et al., 1998). Vionnet et al (1999) showed that most viruses encode for a suppressor of PTGS that can inhibit initiation, maintenance or spread of PTGS response. This assures virus accumulation. The activity of suppressors of gene silencing was found to be at the origin of the synergistic interaction of two unrelated viruses (Shi et al., 1997).

In a synergistic interaction, the infection of a plant with a mixture of two unrelated viruses can result in symptom severity higher than the sum of two single infections. One well-studied example is the co-infection of potyviruses, including *Pepper mottle virus* (PepMoV), *Tobacco vein mottling virus* (TVMV) and *Tobacco etch virus* (TEV), with *Potato virus X (Potexvirus)* (Vance et al., 1995). In this synergistic interaction, the level of PVX increased 5 to 10 fold and the level of the potyvirus remained unchanged (Vance et al., 1995). It was shown that the presence of the TEV helper component proteinase, HC-pro, that is in fact a suppressor of gene silencing (Brigneti et al., 1998) was responsible for the synergism (Shi et al., 1997). It enhances PVX virus accumulation and its pathogenicity (Shi et al., 1997). Similar viral synergistic interaction was described for co-infectivity of BYDV with polerovirus *Cereal yellow dwarf virus* or CYDV-RPV (Baltenberger et al., 1987, Miller et al., 1997, Wang et al., 2000). In such a dual infection, the plants were very stunted with higher accumulation of CYDV-RPV virus (Miller et al., 1997, Wang et al., 2000). This suggests the involvement of a suppressor of gene silencing by BYDV that would facilitate CYDV-RPV infection and inhibit plant defense mechanisms. In many viruses, the movement protein is involved in suppressing plant-silencing response (Vionnet et al., 1999). However, some small ORFs of previously unknown function such as gene 2b of *Cucumber mosaic virus* or gene B2 of animal F*lock house virus,* have been found to code for a suppressor of silencing (Guo and Ding 2002, Li et al 2002). For instance, Cmv2b has been shown to be able to de-activate the mobile silencing signal (Guo and Ding, 2002). On the other hand, potyviral HC-pro protein inhibits accumulation of the small RNAs by interfering at the dicer activity level (Mallory et al., 2001). In the *Luteoviridae* family, P0 of poleroviruses has been found to suppress gene silencing (Pfeffer et al 2002). In relation to

their suppression activity, Sandowy et al (2001) showed that P0 was necessary for viral accumulation.

BYDV lacks P0. However, it has the potential of expressing a small protein P6 that has not yet been detected in infected plants and the function is still unknown. P6 does not have any sequence homology to P0 or any other known suppressors. However, the deletion of ORF6 sequence or the mutation of the start codon of P6 decreases viral accumulation (Mohan et al., 1995). In addition to their similar small size, P6 seems share one more common feature with the well-studied 2b suppressor protein of *Cucumber mosaic virus.* It is encoded by its own messenger sgRNA2 that highly accumulates during infection (Miller et al., 1997). Thus, P6 seems to be a viable candidate for suppressive activity and may be responsible for the synergistic interaction between BYDV and CYDV-RPV.

In collaboration with Dr Ken Richards and Sebastien Pfeffer (Institut de Biologie Moleculaire des Plantes -Strasbourg, France), P6 activity to suppress gene silencing was tested following a prototypical assay of transient gene expression. The assay involved the use of line 16c transgenic *Nicotiana benthamiana* plants from Baulcombe's lab (Brigneti et al., 1998). This plant line constitutively expresses green fluorescent protein (GFP). Under UV light, the plants appear green fluorescent, masking the red color of the chlorophyll. Via agrobacterium-infiltration of a pBin binary vector that can transiently express GFP protein in the leaves, the gene-silencing response can be induced (Vionnet et al., 1998) (Fig. A). In such case, GFP expression is inhibited due to the induced specific degradation of all GFP messenger RNAs into small untranslatable units (Hamilton et al., 1999). Consequently, the green fluorescent patch disappears and the silenced GFP plant appears red under UV light (Fig. B A1).

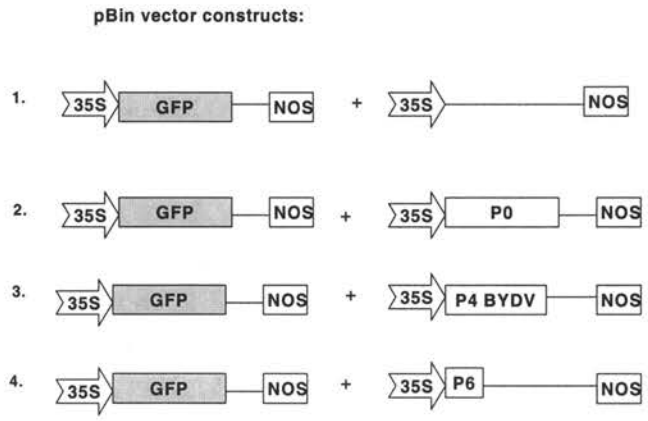

Figure A. pBin vector constructs made by Sebastien Pfeffer:

1. pBIn GFP + empty vector
2. pBin GFP + P0 of *Potato leafroll virus*
3. pBIn GFP + P4 putative movement protein of *Barley yellow dwarf virus*
4. pBin GFP + P6 of BYDV

Gene silencing can be blocked by co-inoculating pBin GFP with a suppressor of gene silencing (Johansen et al., 2001). In the presence of P0 of *Potato leafroll virus*, the GFP RNA specific degradation was halted and GFP expression restored (Pfeffer et al., 2002). The plants recovered their green fluorescent phenotype (Fig. B A2).

To test the ability of P6 to restore GFP expression in silenced plants, S. Pfeffer co-infiltrated pBin binary vector expressing GFP with P6. During simultaneous transient expression of GFP and P6, the inoculated leaves were observed under UV light: the leaves remained red indicating no suppression of the induced gene silencing response (Fig. B A3). The northern blot analysis of total plant RNA from GFP+ P6 co-inoculated leaves showed the accumulation of the small 23-25 nucleotides GFP RNAs that resulted from its degradation (Fig. B C) and the absence of the full length GFP RNA (Fig. B B). Whereas, the northern blot from GFP+ P0 co-inoculated leaves showed the disappearance of the 25 nucleotides small RNAs in correlation with the suppression of RNA specific degradation (Fig. B B).

In conclusion, this experiment showed that BYDV P6 did not show any activity of suppression of gene silencing comparable to the P0 of poleroviruses. Interestingly, the movement protein P4 of BYDV appears to show such activity (Fig. B A3). However due to the weak signal, the result is inconclusive. Further experiments should be done in a system more compatible to BYDV natural hosts. BYDV infects monocots and all gene silencing work has been performed on dicot systems. It is worth to note that Holzeberg et al. (2002) proved for the first time that gene silencing occurs also in monocots.

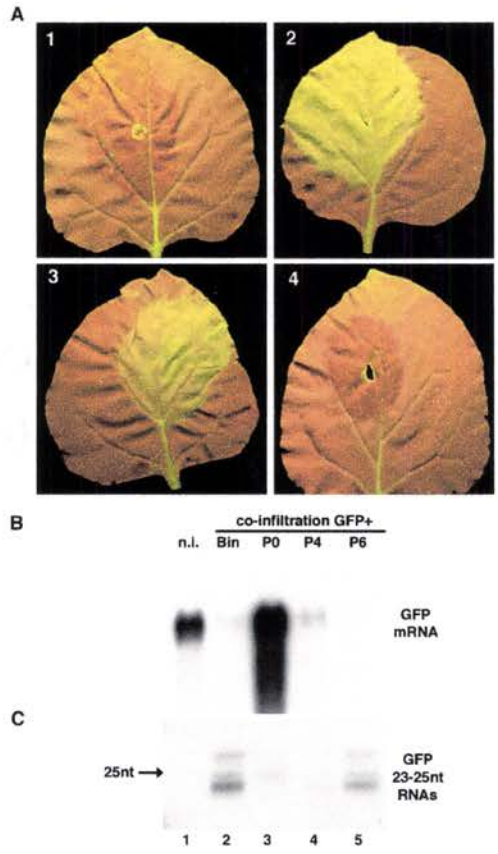

Figure. B Suppression of gene silencing assay with P6 protein of *Barley yellow dwarf virus*.

A. Agrobacterium co-infiltration of GFP transgenic *N..benthamiana* leaves with:

1. pBIn GFP + empty vector
2. pBin GFP + P0 of *Potato leafroll virus*
3. pBIn GFP + P4 putative movement protein of *Barley yellow dwarf virus*
4. pBin GFP + P6 of BYDV

B. Northern blot analysis of GFP RNA extracted from inoculated leaves from panel A

C. Northern blot analysis of small GFP RNA extracted from inoculated leaves from panel A. The small 23-25 nucleotides RNAs are absent in leaves expressing a suppressor of gene silencing.

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