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Characterization of barley yellow dwarf virus subgenomic RNAs

by

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ABSTRACT

Transcription of subgenomic RNAs (sgRNAs) is a common strategy used by many positive strand RNA viruses of plants and animals to regulate viral gene expression. Barley yellow dwarf virus (BYDV) produces three nested 3’-coterminal subgenomic RNAs in infected cells. SgRNA1 serves as the messenger for the structural and movement proteins. sgRNA2 encodes a small open reading frame (ORF6), that appears not to be translated. The role of sgRNA3 is unknown, as it encodes no ORFs. Neither sgRNA2 nor sgRNA3 is needed for BYDV RNA replication in oat protoplasts. However, sgRNA2 does function as a riboregulator of viral translation in virus-infected protoplasts. This dissertation focuses on the control of synthesis of sgRNA2, and the biological roles of sgRNAs 2 and 3 in plants.

The first aim is to determine the primary and secondary structures in BYDV RNA required for synthesis of subgenomic RNA2. The minimal promoter for sgRNA2 was previously mapped to a 143 nt region (nt 4810-4952) just downstream of its putative transcription start site at nt 4809. This region encompasses the 3’ BYDV cap-independent translation element (BTE, nts 4814-4918) that is essential for virus replication. Deletion of the entire 3’ BTE from within a duplicated copy of the sgRNA2 promoter did not affect sgRNA2 synthesis from this promoter, confirming the functional independence of the sgRNA2 promoter from the 3’ BTE. I also found that a small stem-loop containing the conserved hexanucleotide sequence GUGAAG at its 5’ end supports basal levels of sgRNA2 synthesis. All functional sgRNA2 promoter constructs retained potential base pairing between sequences flanking the BTE. This reveals that the sgRNA2 promoter is split by the embedded 3’ BTE and that both the primary and secondary structures are required for
sgRNA synthesis. Such an overlapping arrangement of translational and transcriptional control signals has not been observed in other viruses.

The second aim is to examine the roles of sgRNA2 and sgRNA3 in whole plant infections. Infectivity of viral RNA containing mutations that knock out synthesis of one or both of these RNAs was tested. All sgRNA knockout mutants infected oat plants and usually showed normal levels of viral RNA accumulation, disease onset, and symptoms. ELISA revealed that coat protein levels in plants infected with the mutants deficient in sgRNA2 alone, or sgRNA2 and sgRNA3 were about double that observed in plants infected with wild-type virus. SgRNA3 knockout mutant virus gave wild-type levels of coat protein. SgRNA2 was found to modestly inhibit virion accumulation, while the absence of sgRNA3 in virus infections had little effect on virus accumulation and disease development in infected oats. These surprising results are the first example of subgenomic RNAs that are apparently dispensable for virus infection. They indicate that sgRNA2 must play only a minor role as a riboregulator of viral gene expression. However, absence of sgRNAs 2 and/or 3 may reduce virus fitness in subtle ways that were not detected in my experiments.
CHAPTER 1. GENERAL INTRODUCTION

Luteoviridae

The Luteoviridae is a family of plant viruses that includes the genera Luteovirus, Polerovirus, and Enamovirus (figure 1). The three genera are distinguished by biological characteristics such as serological properties and genome organization. During the late 18th century, potato crops in Europe were affected by a disease known as “potato curl”, which is believed to be one of the earliest known reports of disease caused by a luteovirus (44). Typically, plants infected by luteoviruses exhibit stunted growth, yellowing or reddening of leaves and leaf rolling. However, it was not until the 1950s that scientists reported the discovery of luteoviruses in crops showing symptoms involving leaf yellowing that were transmitted by aphids (85, 86). In infected plants luteoviruses are confined to the phloem tissue. Barley yellow dwarf virus (BYDV), type member of the genus Luteovirus, can infect all members of the Graminae (97), while Potato leafroll virus (PLRV), the type member of the genus Polerovirus, infects only members of the Solanaceae. However, Beet western yellows virus (BWYV) can infect 150 species in 23 dicotyledonous families (96). The only member of the genus Enamovirus is Pea enation mosaic virus-1 (PEMV-1). Luteoviridae are distributed worldwide, cause significant yield losses in important crops and are considered to be one of the most economically important groups of plant viruses (12, 67).

Luteoviruses have isometric icosahedral (T=3) virus particles that are made up of 180 subunits of a single 21-23 kDa coat protein and are 25-30nm in diameter. Each virus particle contains one positive-sense, single-stranded RNA molecule of about 5.6-5.8 kb and accounts for
28-37% of the virion composition (79). In nature, luteoviruses cannot be mechanically transmitted and rely solely on aphid vectors for their transmission, a feature unique to luteoviruses. They are transmitted in a circulative non-propagative manner (36). Thus, the virus does not replicate within the aphid vector but circulates throughout the haemolymph and can remain there for weeks until the aphid transmits the virus during feeding. Luteoviruses share complex and intimate relationships with their aphid vectors in a highly specific manner, such that each luteovirus can be efficiently transmitted only by one or a few aphid species (36, 52, 95).

**Barley Yellow Dwarf Virus**

*Barley yellow dwarf virus* belongs to the genus *Luteovirus* of the *Luteoviridae* family. The genome of BYDV encodes six open reading frames (ORFs) on a single (+) sense RNA that is 5677-nts in length (Figure 2). The genomic RNA (gRNA) serves as the mRNA for translation of the viral replication proteins encoded by ORFs 1 and 2. ORF 2, which encodes the RNA-dependent RNA polymerase (RdRp), is translated via -1 ribosomal frameshifting and is expressed as a fusion to the protein encoded by ORF 1 (8, 21, 89). BYDV generates two sgRNAs that serve as mRNA for translation of the remaining ORFs. ORFs 3, 4 and 5, whose protein products are needed during intermediate and late stages in viral infection, are translated from sgRNA1. ORF 3 encodes the 22-kDa coat protein. ORF 4, translated by leaky scanning, encodes a 17-kDa protein that is required for systemic infection in plants (15). The protein encoded by ORF 5 is needed for aphid transmission and is an extension of the coat protein. It is expressed via read-through of the ORF 3 stop codon (10). SgRNA2 may serve as the mRNA for ORF 6, a small and highly variable 4.3-6.7-kDa protein of unknown function. This protein, which has been expressed *in vitro*, has not been detected in plants. SgRNA2 may also serve as a riboregulator of viral translation by mediating the switch from early to late gene expression in
BYDV (103). More detail about sgRNA2 and its promoter will be discussed in chapters 2 and 3 of this dissertation. SgRNA3 is a mystery because it accumulates to high levels in plants and does not code for any ORF (54).

BYDV’s genomic RNA and sgRNAs have no 5’ cap or 3’ poly(A) tail, but are able to translate efficiently. The 3’ cap-independent translation element (BTE), a 105 nt (pos. 4814-4918) region located in the 3’ untranslated region (UTR) of the BYDV genome, functionally mimics a 5’ cap (38, 127). Sequences located downstream of the 3’ BTE have been found to functionally substitute for a poly(A) tail (128). This 3’ BTE is able to circularize the viral RNA by base-pairing with additional sequences located in the 5’ UTR (39). This interaction facilitates delivery of ribosomes to the first AUG at the 5’ end of the viral genomic RNA (gRNA). The 3’ BTE is also positioned at the 5’ end of subgenomic RNA2 (sgRNA2), which overlaps a region that has been identified as the core promoter of sgRNA2 (59). Other findings suggest that sgRNA2 plays a regulatory role in viral translation because it inhibits translation of the gRNA in trans, thus potentially mediating a switch to late gene expression of proteins encoded by sgRNA1 (103, 104).

**Size limitations of RNA viruses**

Viruses are remarkable entities that must encode all of the genes needed to elicit a successful infection within its host using only a limited amount of coding sequence. In fact, viruses often lack intergenic regions and overlap many of their coding sequences to maximize use of their genomes. Even the intergenic space is functionally important, further demonstrating the efficient use of their genomes. Genome sizes of viruses vary between species, but those with RNA genomes are generally smaller. This size limitation is primarily due to the high error rates of viral RdRps, which lack a proofreading and repair mechanism, during viral replication (25,
26). However, genus *Coronavirus* of the *Coronaviridae* family of animal viruses in the order *Nidovirales*, is an exception to this general size rule and have the largest known RNA genomes; they often exceed 30 kb. Also, the *Closteroviridae* family of plant viruses has RNA genomes larger than 15 kb. The fact that RNA viruses as large as these can remain viable or even exist is a remarkable feat in itself and is a testament to the resiliency of these tiny infectious agents. However, coronaviruses are known to have high mutation rates and high frequencies of recombination during viral replication (20, 100). Many plant RNA viruses overcome this problem by having segmented genomes, a feature of plant viruses that is rarely observed in positive sense animal viruses. Dividing the genome into smaller sizes helps reduce the potential for errors that can occur during replication (92). Segmented genomes are also a strategy used by plant RNA viruses for gene expression (27, 137). In the face of these inherent genome limitations, viruses have evolved a number of clever strategies to overcome these restrictions.

**Subgenomic RNAs**

Most viruses that infect plants have positive-sense (+) single-stranded (ss) RNA genomes and encode more than one open reading frame (ORF) (78). These messenger sense genomes are readily expressed once inside the host, but are functionally monocistronic like eukaryotic cellular mRNAs. Thus, the virus is forced to utilize a variety of ingenious strategies to express all of its genes and does so at the level of transcription and translation (30). Like normal eukaryotic mRNAs, the first ORF of the viral genome is translated while downstream ORFs usually remain translationally silent. These downstream genes generally encode proteins that are needed during intermediate and late stages of viral infection (78). These include structural proteins such as coat protein and those needed for movement of the virus within the host. While some viruses make use of novel translation events such as frameshifting, read through of stop codon and leaky
scanning to overcome this problem, many (+) sense RNA viruses transcribe viral subgenomic (sg) mRNAs to express their downstream genes (30, 59, 69). This is the most common strategy used by plant viruses of either positive or negative polarity for regulation of gene expression at the transcriptional level.

Subgenomic RNAs (sgRNAs) were first discovered in *brome mosaic virus* (BMV) and later in *tobacco mosaic virus* (TMV), where the coat protein (CP) was shown to be expressed from a sgRNA (48, 106). Since that time, subgenomic RNA production has been reported in several other RNA viruses, both plant and animal (70). Analysis of many non-structural protein genes such as the RNA-dependent RNA polymerase (RdRp) and helicase has revealed striking similarities between the genome features of different groups of plant and animal viruses.

Positive-stranded RNA plant viruses are divided into four distinct supergroups, based on sequence motifs in the RdRp and other genome related features. They are the plant picorna-, alpha-, carmo- and sobemo-like supergroups (Table 1). Of these four supergroups, only the alpha-, carmo-, and sobemo-like viruses produce sgRNAs. The plant “carmo-like” viruses of the *Luteoviridae* (75, 78) and *Tombusviridae* (129, 130) families utilize this strategy of transcribing sgRNAs for gene expression, as do the “alpha-like” animal viruses families *Coronaviridae* (49, 62, 99, 113), *Arteriviridae* (87, 120, 121), *Togaviridae* (64), *Caliciviridae* (81), *Nodaviridae* (93) and *Astroviridae* (80). Plant “alpha-like” viruses that produce sgRNAs include members of the plant virus families *Bromoviridae* (2, 29, 77), *Closteroviridae* (3), along with the *Tobravirus* (68), *Carlavirus* (139), *Tymovirus* (58), *Potexvirus* (6, 63), * Hordeivirus* (51, 114, 141), *Tobamovirus* (47) and *Furovirus* (5) genera. The “sobemo-like” supergroup produces sgRNAs and includes the genera *Sobemovirus* (115) and the Luteoviridae, *Potato leaf roll virus* (PLRV) and *Pea enation mosaic virus*-1 (PEMV-1) (78). However, the plant “picorna-like” supergroup of
viruses, which include the sequiviruses and members of the Comoviridae and Potyviridae virus families, do not need to produce subgenomic RNAs because their genes are expressed as a single polyprotein (137).

Subgenomic RNAs are usually 5’-truncated versions of the genomic RNA, with the same 3’ end as the viral genomic RNA (gRNA) (78). This means that they are direct copies of the 3’ end of the viral genome, sharing identical nucleotide sequence from internal regions of the genomic RNA at its 5’ end and being co-terminal with sequences to the 3’ end of the genomic RNA. These truncated versions of the genomic RNA position ORFs that were once located downstream of the 5’ proximal ORF in the viral genomic RNA to a 5’ proximal position in the subgenomic mRNA, thus allowing them to be translated efficiently. These downstream ORFs encode proteins required during intermediate or late stages of infection and include the coat and movement proteins. One way of envisioning this clever strategy would be to view the virus as converting the 3’ half of its genome into one or more mini-genomes that serve as the mRNA for each inaccessible downstream ORF.

During infection, these RNAs usually accumulate to higher levels than genomic RNA, with shorter sgRNAs being the most abundant. This is true in BYDV where sgRNA3 accumulates to very high levels in plants (54). The promoters of smaller sgRNAs are located closer to the 3’ end of the genome and are expressed at much higher levels than sgRNAs expressed from upstream promoter sequences. This position dependent expression has been observed in other viruses such as BMV, CMV and TMV (71).

3’ co-terminal sgRNAs are the most common type of sgRNA reported among plant viruses, however, 5’-coterminal sgRNAs have been reported for viruses of the Closteroviridae and Flexiviridae virus families. Citrus tristeza virus (CTV), with the largest known plant virus
genome and a member of the *Closteroviridae* family, produces several 5’ co-terminal sgRNAs of various sizes that are produced by termination during genomic RNA synthesis at 3’ controller elements (16, 37). One of these unusual RNA species, identified as a low molecular weight tristeza (LMT RNA), was shown to be around 800 nts long and accumulates to rather high levels in infected plants (16). A second and slightly smaller LMT RNA 740 nts in length was also present during infections. Larger 5’ co-terminal sgRNAs, called large molecular weight tristeza (LaMT), have also been identified (16). These ~10 kb long RNA species accumulate to lower levels in plants. All evidence points to these single-stranded RNA species being of positive polarity with no analogous complementary negative strand sgRNA.

*Grapevine virus A*, of the genus *Vitivirus*, produces a set of three 5’ co-terminal sgRNAs that range in size from 5 to 6 kb (31). Such novel RNAs have also been observed in *Sindbis virus* (SIN) (131), *Apple chlorotic leaf spot virus* (ACLSV) (33) and *Citrus leaf blotch virus* (CLBV) (123) infections. The function of these unusual sgRNAs is presently unknown, although there is some evidence that the two LMT RNA species in CTV are regulated. For the remainder of this review, all references to subgenomic RNAs refer to 3’ co-terminal sgRNAs.

The 5’ ends of many viral sgRNAs have been determined experimentally. These studies have revealed that the 5’ and 3’ ends of many sgRNAs share similarities with the genomic RNAs that they are derived from (69). This has been observed in viruses such as BYDV, BMV, AlMV, CMV and RCNMV, where the gRNA and sgRNA 5’ ends share the same nucleotide and is typical of viruses that produce sgRNAs (7, 69, 73, 118, 138). One clear example is *Beet black scorch virus* (BBSV), in which the primary and secondary structures of the 5’ ends of the sgRNAs resemble those of viral genomic RNA (figure 3)(132). Some sgRNA 5’ ends are localized in intergenic regions of the genome or in coding regions of upstream ORFs. In some
cases, the promoter itself may overlap a coding region, even though the 5’ end of the sgRNA maps to an intergenic location. This is why many subgenomic promoters (SGP) are mapped in ectopic locations.

In RCNMV, 13 nts of the sgRNA 5’ termini are identical to 14 terminal nucleotides in the 5’ termini of gRNA1 (138). Because sgRNAs are 3’ co-terminal with the gRNA, they would by default share the same 3’ end. The 3’ end structures among viruses can vary considerably. Plant viral 3’ ends may contain tRNA-like structures, poly (A) tails or an OH. However, this is not the case for the sgRNA derived from RNAγ of Barley stripe mosaic virus (BSMV). While all three genomic RNA segments possess tRNA-like structures at their 3’ ends, including a poly(A) stretch about 200 nts upstream, sgRNAγ possesses the poly(A) stretch at its 3’ end (114). Such features exhibited by sgRNAs may contribute to their recognition by the viral replicase (91).

The similarities of these primary and secondary structures in the 5’ termini of genomic and sgRNAs may suggest a role in synthesis of these RNAs, including being part of the promoter itself (35). However, details concerning viral replicase-template recognition are not fully understood. How the viral replicase distinguishes between the various primary and secondary structures of the viral genome to initiate minus-strand, plus-strand and/or sgRNA synthesis must still be explored.

Most subgenomic RNAs contain only one ORF and usually encode the viral coat protein. This is the most common type of sgRNA, as is the case for BMV, where the coat protein is translated from RNA4, a sgRNA derived from genomic RNA3 of this tripartite virus (105). In TBSV, the coat protein is expressed from sgRNA1, which is one of two sgRNAs produced by this virus (94). Turnip yellow mosaic tymovirus (TYMV) also uses a sgRNA to express its coat protein gene (101). However, some viral subgenomic RNAs serve as the mRNA for more than
one viral gene, as has been observed in luteoviruses and tombusviruses. In BYDV, sgRNA1 is the mRNA for the coat protein (CP), its read-through extension product, and the movement protein (60). The second subgenomic RNA in TBSV serves as the mRNA for the two nested genes, p19 and p22, which are involved in cell-to-cell movement and suppression of gene silencing, respectively (46, 102, 124). This is another remarkable example of how viruses combine strategies to express their genes. In the above examples, the virus does not produce a sgRNA for each downstream ORF. Instead, it maximizes the use of its coding sequence by overlapping ORFs and compensates at the level of translation by using novel strategies to express multiple genes, nested or not, from the same mRNA.

**Role and Function of sgRNAs**

In many ways, subgenomic RNAs can be viewed as delayed messengers that are deployed by the virus at specific times during the infection cycle. This precise timing of the appearance and differences in levels of synthesis of sgRNAs allows for the temporal regulation of gene expression during late stages of infection (43, 56, 126). Therefore, when products encoded by these RNAs are needed, the virus regulates their expression at both the transcriptional and translational level. This has been observed in viruses that produce multiple sgRNAs, such as BYV, CTV, and TMV, where the timing of synthesis differs between sgRNAs (19, 43, 82). In TMV, the CP and MP are differentially expressed from their sgRNAs during later stages of infection (19). Both the timing and accumulation of these sgRNAs differ from one another and point to the complex regulation that is involved. Differences in accumulation of sgRNAs in BYDV point to regulation of these RNAs by possibly different mechanisms also (59). In the necrovirus, *Tobacco necrosis virus strain A* (TNV-A), synthesis of the smaller 1.3 kb sgRNA is delayed by 1 hour compared to the larger 1.6 kb sgRNA (74). *Beet yellows*
Closterovirus (BYV) differentially regulates the transcription of its sgRNAs, in which the messenger for the small P6 protein, encoded by ORF 2, is synthesized at a much lower yield than that of the abundantly produced P22 messenger, encoded by ORF 6 (45, 90).

Subgenomic RNAs and their protein products have been confirmed experimentally and their contribution to the infection process is certainly significant. For many viruses, in particular those where the CP is expressed via a sgRNA, the cycle of infection would be incomplete without them. In Alfalfa mosaic virus (AMV), infectivity is lost in the absence of RNA4, which is the mRNA for the CP (40, 116). Binding of CP to the 3’ termini of AMV genomic RNAs was absolutely critical for virus replication and has been shown to be functionally analogous to poly(A) binding protein (PABP) binding poly(A) tails of mRNAs (83). However, the roles of some highly expressed sgRNAs are not entirely clear, as is the case for sgRNA3 in BYDV and RNA 1A in Cherry leaf roll virus (CLRV) (9, 59). It is likely that these RNAs have regulatory roles in viral processes.

**Subgenomic Promoters**

Over the years, subgenomic promoters have been studied and mapped in many viruses (5, 7, 29, 37, 51, 53, 59, 60, 63-65, 72, 84, 101, 117, 119, 122, 125, 126, 138). These cis-acting elements are defined as all sequences that lead to the production of subgenomic RNAs (59). Generally these sequences refer to the promoter sequence in the plus-sense but are recognized by the polymerase in the minus-strand. In promoter mapping studies, these sequences can be placed elsewhere within the genome for further characterization and give rise to new subgenomic RNAs. The boundaries of many promoters have been discovered through deletion mutagenesis of sequences overlapping the sgRNA transcription start sites by a few hundred bases. The promoters of many sgRNAs may serve as sites for recombination during replication (24, 76, 98).
This process increases the genetic diversity among virus populations and potentially rescues viral genomes that have acquired mutations in critical regions during replication (13). The luteovirus genome is thought to have resulted from a recombination event between a dianthovirus and a polerovirus (34).

Promoters can function differently when analyzed in vitro or in vivo and usually require additional sequence for full promoter function in vivo. Promoters in Bromoviruses have been shown to consist of a core promoter region, which is able to support basal levels of transcription, and one or more properly spaced enhancer regions. These regions boost overall synthesis of sgRNAs in viruses and are usually located upstream or downstream of the promoter transcription start site, sometimes overlapping it. In BMV they are located between nts +1 to +16, a poly(A) stretch upstream at nts -20 to -37, and (3X) UUA repeats between nts -38 to -48 (72). Practically all viral subgenomic promoters share a few common features. In many viruses, the first nucleotide is identical in both the gRNA and sgRNA. Also, this nucleotide appears to be conserved in some viruses since mutations either abolish or greatly reduce synthesis of the sgRNA (119). Finally, sequence homologies, such as AU- or U-rich regions, usually are found upstream of the transcription start site and can extend up to 20 nt from the 5’ ends of these RNAs (72).

In the majority of viruses, the promoter regions are located largely upstream of the sgRNA transcription site (+1) and can be as small as 24 nts or larger than 100 nts (78). However, the promoters of BNYVV and BYDV differ compared to other viruses and have sgRNA promoters that are located mostly downstream of their transcription start sites. In BNYVV, the sgRNA promoter has been mapped to a position from -16 to between +108 and +208 of the transcription start site (5). In a truly remarkable case, the promoter for RCNMV is
split between its two genomic RNAs (figure 4). The promoter is formed through an intermolecular interaction between 8 nts of a loop sequence of a stem-loop predicted to form in a 34 nt region of RNA2 and a complementary sequence located upstream of the sgRNA transcription start site on RNA1 (110). This interaction is required for trans-activation of sgRNA synthesis.

Promoter sequences responsible for the synthesis of RNA4 in BMV, a subgenomic RNA derived from genomic RNA3, have been characterized more extensively than any other known promoter. It has been mapped to a region that extends 74 to 95 nt upstream and 16 nt downstream of the transcription (+1) start site in minus strand RNA 3. However, the minimal core promoter for BMV was mapped to a 20 nt region 3’ of the sgRNA transcription start. Nucleotides at positions -11, -13, -14 and -17, relative to the sgRNA start site (+1), were shown to be required for efficient synthesis (108). These highly conserved nucleotides occur in the subgenomic promoters of other plant alpha-like viruses. In the closely related Cowpea chlorotic mottle virus (CCMV), also a bromovirus, only three of these nucleotides (-17, -14, and -13) are conserved, but the same four bases are required for sgRNA synthesis, including additional bases at positions -20, -16, -15, and -10 (1). The promoter for AMV was mapped to a position between -8 and -55 nts upstream of the transcription start site (118). A conserved hairpin structure within the core promoter, which incorporates many of the conserved bases in its loop, was also shown to be essential for sgRNA synthesis in AMV and BMV and is a feature that is conserved in other Bromoviridae (50) (figure 5). This triloop hairpin is equivalent in function to the stem loop C hairpin structure in the minus strand promoter, which is recognized by the viral replicase (14, 55). The AUA loop motifs of these hairpins are able to recruit the RdRp for minus strand and
sgRNA synthesis and were shown to have similar structures to iron-responsive elements, which are involved in protein binding (42).

In the *tombusvirus* CNV, the sgRNA2 promoter was mapped to a region 20 nts upstream and 6 nt downstream of the transcription start site (53). Zhang *et al* showed that synthesis of this sgRNA was regulated by a distal RNA element (DE) located 1000 nts upstream of the transcription start site in *Tomato bushy stunt virus* (TBSV). This *cis* RNA-RNA interaction involves the base pairing of a 12 nt long sequence with a core element (CE) located just 11 nts upstream of the sgRNA transcription start site (figure 6). Subgenomic RNA synthesis was efficiently supported by this long distance interaction (140). A similar long distance interaction has been observed in the *potexvirus*, PVX. Base pairing between a conserved octanucleotide sequence motif (AACUAAAC) in the 5’ non-translated region (NTR) and sequences (GUUAAGUU) in the sgRNA1 and sgRNA3 promoters, affected genomic and sgRNA synthesis when complementary between these sequences are reduced (6, 56, 57, 63). A core promoter-like sequence for *Bamboo mosaic potexvirus* (BaMV) was mapped to nt -30 to +16 (63). The full promoter contained two upstream enhancer sequences at positions -59 to -31 and -90 to -60, respectively. Additional enhancer sequence was located downstream of the transcription start site at nt +17 through +52 (63).

The hordeivirus *Barley stripe mosaic virus* (BSMV) is a tripartite virus that produces three subgenomic RNAs. Two of the sgRNAs produced, sgRNAβ1 and sgRNAβ2, are derived from genomic RNAβ. The boundaries of sgRNAβ1 promoter was mapped to position -29 to -2 relative of the transcription start site (51). This promoter region is located near an important *cis*-acting element required for RNAβ replication. It is located upstream of the transcription start site at a position that spans from -107 to -74 (51). The promoter for sgRNAβ2 was mapped to
position -32 to -17 relative to its transcription start site (51). The promoter of a third sgRNA, derived from RNAγ, was mapped to position -21 to +2, relative to its transcription start site (51).

*Citrus tristeza virus* (CTV) produces 9 to 10 sgRNAs that are regulated in a temporal and quantitative manner. The minimal core promoter for the highly expressed major CP sgRNA was mapped to a region -47 to -5 nts upstream of its transcription start site (37). Interestingly, the promoter of this sgRNA is located entirely within a non-coding region, while the promoter for the lower expressed minor CP (CPm) sgRNA is located within an upstream coding region (37). The promoters for the two sgRNAs produced in the carmovirus TCV mapped to a region encompassing nts -90 to +6 for the smaller 1.45 kb sgRNA and nts -90 to +4 for the larger 1.7 kb sgRNA (126). Hairpin structures located immediately upstream of their transcription start sites have been predicted to form based on MFOLD analysis (126). In another carmovirus, *Hibiscus chlorotic ringspot virus* (HCRSV), the promoter for its two sgRNAs have been mapped to positions -112 to +6 for sgRNA1 and to positions -126 to +6 for sgRNA2 (65).

Promoters can vary among related viruses or differ greatly within the same virus. This is especially true for BYDV (59, 78). The minimum core promoters for all three BYDV sgRNAs have been previously mapped (59, 60) and a further characterization of the sgRNA2 promoter is discussed in chapter 2 of this dissertation. A unique feature of BYDV is that each of its three promoters is different from the other, structurally and in primary sequence. Although the three promoters differ, sgRNA1 and 2 do share a common hexanucleotide sequence (*GUGAAG*) at their 5’ ends that is also found at the 5’ termini of the gRNA. Unlike sgRNA1, the promoter of which is located primarily upstream of its start site, promoters for sgRNA2 and 3 are located largely downstream of their sgRNA 5’ ends. The 5’ end of sgRNA2 has been mapped to nt 4809 and the 5’ end of sgRNA3 has been mapped to nt 5348 (54). The minimum sequence required
for sgRNA2 synthesis was mapped to a 143 nt region located between nt 4810 and 4952. The minimum core promoter for sgRNA3 was mapped to a 44 nt region located between nt 5345 and 5388, with most of the promoter sequence located downstream of its start site (nt 5348). Interestingly, synthesis of both sgRNAs was not affected by point mutations to their published 5’ terminal base (59). However, point mutations to G residues (nts 4810 and 5351) close to their published 5’ ends completely abolished sgRNA synthesis of both RNAs in their natural location (59). Neither mutation had an effect on virus replication at 24 hpi (59).

The surprising divergent features exhibited by each of the promoters in BYDV may be due to the compact nature of the virus genome itself. The locations of these promoters and their tendency to overlap coding regions and other important cis-acting elements could give rise to very different promoters. Such differences, in both primary and secondary structure, could then allow for the temporal regulation of gene expression from these late messengers at the level of transcription and translation (59).

**Mechanisms of sgRNA Synthesis**

The mechanisms of sgRNA synthesis are still unclear for many viruses. Three models explaining the mechanisms used to generate subgenomic RNAs have been proposed (figure 7). They are: (i) internal initiation (11, 32, 41, 77, 78, 109, 126), (ii) premature termination (110, 129), and (iii) discontinuous transcription (4).

In internal initiation, (+) strand sgRNA transcription is initiated internally by the replicase at a promoter located on a genomic length (-) strand RNA (77). This is the only mechanism that has been demonstrated unequivocally in plant viruses and alphaviruses (108, 118, 126). It was first demonstrated in vitro for BMV in which sgRNA4 was generated from minus strand RNA3 templates using an RdRp isolated from infected plant cells (77). Evidence
supports the idea that the primary sequence of the promoter alone is sufficient for recognition by the viral RdRp (1). The core promoter of BMV is also recognized by the RdRp of the related cowpea chlorotic mottle virus (CCMV). The nucleotide at position -20 was essential in supporting sgRNA synthesis in CCMV but was not required for BMV (1). Other viruses that use internal initiation as a mechanism for generating sgRNAs include: AMV, BNYVV, CMV, TCV and TYMV. In AMV, binding of the viral replicase to the sgRNA promoter and a second cryptic promoter at the 3’ end of the genome potentially induces a conformational change in the RNA and results in initiation of sgRNA synthesis (119).

In the premature termination model, subgenomic length (-) strand is generated via termination during (-) strand synthesis (110). It is believed that the viral replicase stalls at some sequence and/or structure during minus strand synthesis and eventually dissociates from the viral (+) strand template. This could be caused by the secondary structure itself, binding of a protein(s) to the secondary structure or long-distance interactions with other RNA elements combined with protein binding (78). Such a scenario could act as a barrier to the viral replicase and cause its disassociation. This subgenomic length (-) strand then would serve as template for (+) strand synthesis of the sgRNA. There are questions concerning whether sgRNAs can serve as templates for their own amplification because some evidence shows a lack of sgRNA replication in experiments where the sgRNA was co-electroporated with its genomic RNA (unpublished data). Studies conducted on the dsRNA versions of PVX and BSMV sgRNAs revealed that the 3’ ends of the (-) strand RNAs lacked an extra unpaired G nucleotide that was shown to be present on the genomic RNA and is necessary for (+) strand synthesis (22, 23). Some have even suggested that sgRNAs may not be available for replication because of translation. However, a sgRNA (RNA3) in Flock house virus (FHV) was shown to be replicated
by the viral replicase in the absence of genomic RNA1 and it produced (+) stranded mRNAs from (-) strand RNA3 transcripts (28).

In addition to FHV, studies in both RCNMV and TBSV have yielded evidence that supports this model (17, 18, 110, 140). In RCMV, sgRNA transcription is activated \textit{in trans} through an interaction between its two genomic RNA segments. This interaction could potentially form a structure that stalls the replicase and prevent it from forming full-length (-) strand genomic templates (110). In TBSV, the \textit{in cis} interactions between the activator sequence (AS1) and receptor sequence (RS1) for sgRNA1 synthesis serves a similar function (18). This is also the case with sgRNA2 where both the distal element A (DE-A)/core element A (CE-A) and the AS2/RS2 distal elements are involved in a long distance interaction just upstream of the sgRNA transcription start site, ~1,100 and ~2,100, respectively (17, 66, 140). This mechanism is also thought to function in \textit{Closteroviruses} and \textit{Toroviruses} (37, 111). The third mechanism, called discontinuous transcription, has been observed only in \textit{Coronaviridae} and \textit{Arteriviridae}, where the 5’ ends of the subgenomic RNAs are acquired from the 5’ end of the viral genome by polymerase hopping during (-) strand synthesis (88, 99, 112, 121).

\textbf{Conclusions and dissertation organization}

Regulation of sgRNA synthesis by specific promoter elements is a major step in the control of viral gene expression. Understanding the mechanisms of how viruses synthesize sgRNAs will greatly facilitate our understanding of how viruses replicate. Determining how the virus decides between sgRNA synthesis and gRNA synthesis will aid with disrupting viral replication and gene expression. Because virus hosts are not known to use the process, inhibition of sgRNA synthesis is a potential target for antivirals.
Studying transcription and gene expression strategies using plant virus models offers several advantages over animal virus models, mainly because of their genome size, which is usually smaller, and their ease of use in *in vitro* and *in vivo* systems. Plant viruses are also easy to propagate in whole plants and are more amenable to replication studies. Since some plant viruses have been found to be very similar to animal viruses (61), discoveries of molecular mechanisms within plant virus models can potentially contribute greatly to similar mechanisms used by animal viruses that are considered medically important. (+) sense RNA viruses are also being used to express foreign genes from subgenomic RNAs (133, 134, 136). Understanding how sgRNAs are synthesized can improve these expression systems, which have already lead to exciting medical applications like expressing protein-based pharmaceuticals (107, 133-135).

Given that BYDV is unique in that it has three subgenomic RNA promoters that are different in both primary and secondary structure and uses novel transcriptional and translational events to control its gene expression, any knowledge generated from this study will contribute significantly to what is currently known about promoter function in both plant and animal virus studies.

The research conducted in this dissertation is an effort to further establish the role of primary and secondary structures in the sgRNA2 promoter of BYDV that support sgRNA synthesis. I also investigate the roles of sgRNA2 and sgRNA3 in whole plant infections. My dissertation contains 4 chapters and 4 appendices. Chapter 2 contains the manuscript entitled “Functional analysis of a plant viral subgenomic RNA promoter that contains an embedded translation element” and is co-authored by my major professor Dr. W. Allen Miller. It will be prepared for submission to the Journal of Virology. Chapter 3, which is also co-authored with my major professor, contains the manuscript “Barley yellow dwarf virus subgenomic RNA
knockout mutants replicate and cause disease in oats plants” and will be submitted to Molecular Plant Microbe Interactions. Chapter 4 contains the general summary of the dissertation. Appendix 1 contains my contribution as second author to a protocol published in Current Protocols in Microbiology. It is entitled “Preparation and electroporation of oat protoplasts from cell suspension culture”. Appendix 2 contains modifications that I made to improve our aphid transmission protocol. Appendix 3 details the cloning process involving the construction of two gateway destination vectors derived from our sgRNA2 and sgRNA3 knockout mutant clones. Appendix 4 contains a Northern blot analysis that provides evidence for PAV6-129 having a sgRNA3 and possibly a fourth sgRNA. Finally, appendix 5 contains additional photos of virus symptoms from oats infected with wild-type virus and knockout mutants discussed in chapter 3.

Dr. Miller contributed to all aspects of the editing process of this dissertation. They include minor corrections to grammar and revisions to the discussion sections of chapters 2 and 3. Chapter 1 discusses the background of my research and includes a thorough discussion on sgRNAs and the cis-acting elements that produce them.

References


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Table 1. (+) sense Plant Viruses that Produce sgRNAs (ssRNA)

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+= number of sgRNAs vary within genus and/or sgRNAs not fully characterized.
Figure 1. Genome organizations of the three genera of the *Luteoviridae*. Bold lines represent genomic RNA. Numbered boxes indicate open reading frames (ORFs) and in parenthesis, the molecular weight of each encoded protein represented in kilodaltons. The grey box represents the 3’ BTE. Abbreviations followed by black arrows represent the following translational strategies used by luteoviruses: leaky scanning (ls), frameshifting (fs) and readthrough (rt).
**Figure 2.** Genome organization of *Barley yellow dwarf virus*. Genomic RNA and sgRNAs are represented by bold lines. Numbered boxes indicate open reading frames (ORFs) and parenthesis indicate the molecular weight of each encoded protein represented in kilodaltons. The grey box represents the 3’ BTE. A northern blot analysis of BYDV RNAs in infected cells is shown on the right. Total RNA isolated from oat protoplasts was used in the analysis and details of the procedure are described in the materials and methods section of chapter 2.
Figure 3. *Black beet scorch virus* (BBSV) 5’ termini comparison (modified from [Yuan et al., 2006]). Predicted stem-loop structures formed at the 5’ end of the BBSV genomic and sgRNAs. Conserved bases are highlighted. Secondary structures were predicted by the author using CLC workbench.
Figure 4. Genome organization of *Red clover necrotic mosaic virus* (RCNMV). Bold lines represent genomic RNAs. Boxes indicate open reading frames (ORFs) and their corresponding protein products labeled inside (CP, coat protein; MP, movement protein). Arrow indicates the subgenomic promoter +1 transcription start site and its corresponding sgRNA is represented as a dashed line. The green box represents sequences involved in the *in trans* basepairing interaction that regulates sgRNA transcription (110). TABS, *trans*-activating binding sequence; TA, *trans*-activator sequence.
Figure 5. Predicted secondary structures of the AMV and BMV minus-strand core promoters that sgRNA synthesis (Haasnoot et al., 2000 and Kao, 2002). Arrows indicate sgRNA transcription start sites. Tri-loop nucleotides are highlighted in green.
Figure 6. Long distance base pairing that controls *Tomato bushy stunt genome* (TBSV) sgRNA synthesis (Lin *et al.*, 2004). Genomic RNAs and sgRNAs are shown as bold lines. Boxes indicate open reading frames (ORFs) and protein products labeled. Arrows indicate subgenomic promoter transcription start sites. Green arrows represent the AS1/RS1 interaction essential for sgRNA1 synthesis. Red and blue arrows represent AS2/RS2 and DE-A/CE-A sequences involved in sgRNA2 transcription. AS, activator sequence; RS, receptor sequence; DE, distal element; CE, core element.
Figure 7. Models for subgenomic RNA synthesis (modified from [Miller & Koev, 2000]). (+) and (-) sense genomic RNAs represented as red and blue lines, respectively. Green circles, viral replicases. Blue boxes, subgenomic RNA transcription start sites. Orange boxes, subgenomic RNA termination sequences. Short thick lines, 5’ genomic RNA leader (magenta) or complement leader sequence (blue). Vertical bars depict termination of transcription by viral replicase.
CHAPTER 2. FUNCTIONAL ANALYSIS OF A PLANT VIRAL SUBGENOMIC RNA PROMOTER THAT CONTAINS AN EMBEDDED TRANSLATION ENHANCER ELEMENT

A paper to be submitted to the Journal of Virology

Jacquelyn Jackson and W. Allen Miller

Abstract

Transcription of subgenomic mRNAs serves as a common strategy used by positive strand RNA viruses for regulating gene expression. During infection, Barley yellow dwarf virus (BYDV) transcribes three subgenomic RNAs. These RNAs are not required for BYDV RNA replication in oat protoplasts and their promoters have been previously mapped. The minimal promoter for sgRNA2 was mapped to a 143 nt region (nt 4810-4952) just downstream of its putative transcription start site at nt 4809. This region overlaps with the 3’ BTE (4810-4920), a cap-independent translation element, and is essential for virus replication. Here we show that the embedded 3’ BTE is not required for sgRNA2 synthesis. The remaining sequences are predicted to form a small 6 base-pair stem-loop structure, which contains the conserved hexanucleotide sequence GUGAAG and promoter start site at position 4810. This small stem-loop was able to support basal levels of sgRNA2 synthesis. This conserved GUGAAG sequence, which is important for sgRNA2 synthesis, is also present at the 5’ end of the gRNA and SL2 of the sgRNA1 promoter. Promoter sequences immediately flanking the remaining 6-base-pair stem-loop were also not needed for sgRNA
synthesis. These results show that the sgRNA2 promoter may be split by the embedded 3’BTE and that both the primary and secondary structures are required for sgRNA synthesis.

**Introduction**

The genomic RNAs (gRNAs) of many (+) sense RNA viruses encode more than one open reading frame (ORF). Like normal eukaryotic mRNAs, the first ORF of the viral genome is translated while downstream ORFs usually remain translationally silent. While some viruses make use of novel internal translation initiation events to overcome this problem, many (+) sense RNA viruses instead transcribe viral subgenomic (sg) mRNAs to express their downstream genes (16). These genes generally encode proteins that are needed during intermediate and late stages of viral infection. Subgenomic RNAs are usually 5’-truncated versions of the genomic RNA, but have the same 3’ end as the viral gRNA. This truncation positions ORFs that are located downstream of the 5’ proximal ORF in the viral gRNA to 5’ proximal positions in the subgenomic mRNA, thus allowing them to be translated efficiently (12). The mechanisms and control of sgRNA synthesis are unclear for many viruses and need to be investigated.

*Barley yellow dwarf virus* is a plus-stranded RNA virus that belongs to the genus *Luteovirus* of the *Luteoviridae* family. The genome of BYDV encodes six open reading frames (ORFs) on a single (+) sense RNA that is 5677 nts long (figure 1). The gRNA serves as the mRNA for translation of ORFs 1 and 2. ORF 2, which encodes the RNA-dependent RNA polymerase (RdRp), is translated via -1 ribosomal frameshifting and is expressed as a fusion to the protein encoded by ORF 1 (2, 5, 18). BYDV generates three sgRNAs in infected cells. ORFs 3, 4 and 5, whose protein products are needed during intermediate and
late stages in viral infection, are translated from sgRNA1. ORF 3 encodes the 22-kDa coat protein. ORF 4, translated by leaky scanning, encodes a 17-kDa protein that is required for systemic infection in plants (4). ORF 5 is a C-terminal extension of the coat protein needed for aphid transmission (4). It is expressed via read-through of the ORF 3 stop codon (3). SgRNA2 may serve as the mRNA for ORF 6, a small and highly variable 4.3-6.7-kDa protein of unknown function. However, this protein, which has been translated from sgRNA2 in vitro, has not been detected in plants or in infected protoplasts (22). SgRNA3 is a mystery because it accumulates to high levels in plants and does not code for any ORF (11).

Subgenomic promoters have been studied and mapped in many viruses and are defined as all sequences that lead to the production of subgenomic RNAs (16). Previous work in our lab has determined the primary and secondary structures required for function of the sgRNA1 promoter and the approximate boundaries of the sgRNA2 and sgRNA3 promoters have been mapped (12, 13). The minimal promoter for sgRNA2 is 143 nt long (nt 4810 to 4952) and overlaps the 3’ cap-independent translation element (BTE), which is also contained at the 5’ end of the subgenomic RNA (12). A unique feature of BYDV is that each promoter is different from the other, structurally and in primary sequence. However, sgRNA1 and 2 do share a common hexanucleotide sequence (GUGAAG) at their 5’ ends. Unlike sgRNA1, whose promoter sequence is located primarily upstream of its start site, promoter sequences for sgRNA2 and 3 are located largely downstream of their sgRNA 5’ ends.

SgRNA2 has also been shown to function as a riboregulator of viral translation. It plays a key role in a trans-inhibition of translation model proposed by our lab (21). In this
model, sgRNA2 is expected to affect the viral life cycle during late stages of infection. Based on *in vitro* and *in vivo* data, sgRNA2 selectively inhibits translation of the gRNA and allows translation from sgRNA1, thus mediating the switch from early to late gene expression (28). Therefore, it would be of great interest to determine how sgRNA2 is synthesized and regulated.

Three models explaining the mechanisms used to generate subgenomic RNAs have been proposed. They are: (i) internal initiation, (ii) premature termination, and (iii) discontinuous transcription. In internal initiation, (+) strand transcription of sgRNA is initiated internally by the replicase at a promoter located on a genomic length (-) strand RNA (15). This has been demonstrated in plant viruses and *alphaviruses* (1, 15, 26). In the premature termination model, subgenomic length (-) strand is generated via termination during (-) strand synthesis (23). This subgenomic length (-) strand serves as template for (+) strand synthesis of the sgRNA (23). Viruses such as RCNMV and FHV show evidence in support of this mechanism (7, 23). The third mechanism, called discontinuous transcription, has been observed only in *Coronaviridae* and *Arteriviridae*, where the 5’ ends of the subgenomic RNAs are acquired from the 5’ end of the viral genome by hopping during (-) strand synthesis (24). Although the mechanisms used by most viruses to generate subgenomic RNAs are still largely unknown, we propose that BYDV, depending on the sgRNA, uses both the internal initiation and premature termination mechanisms to generate its sgRNAs.

In this study, we aim to precisely map the 143 nt long minimal promoter region (nt 4810-4952) and determine the primary and putative secondary structures required for sgRNA2 synthesis. We show that the promoter is functionally independent from the 3’ BTE
and may be split between the 3’ BTE. We demonstrate that the sequences which form the base of the promoter’s predicted secondary structure, is able to support promoter activity.

Materials and Methods

Plasmids. All mutant constructs used in this study were based on the construct pPAV6, our full-length infectious clone of BYDV, serotype PAV (5). Constructs were confirmed by sequencing on an Applied Biosystems 3730xl DNA Analyzer at the Iowa State University DNA Sequencing Facility. MFOLD (Zuker, 1989) was used to predict the secondary structure of all mutants to ensure that predicted or altered structures were maintained (29). Constructs used in this study were made from the following plasmids: the BYDV infectious clone, pPAV6; the sgRNA2 knockout mutant SG2G/C (ΔSG2), which contains a G to C point mutation at nt 4810 and does not synthesize a sgRNA2 and ΔSG2-gway, which is the gateway destination vector for the sgRNA2 knockout mutant. A Gateway Conversion Cassette™ (frame A) was inserted into the HpaI<sub>3785</sub> and KpnI<sub>4154</sub> sites of ΔSG2 to create a destination vector for use with the Gateway cloning system. The frame A cassette sequence was PCR amplified with the following primers: 5’frameA, which contained half the HpaI restriction site sequence as a 5’ extension and 3’FrameA, which has a KpnI restriction site 5’ extension (Table 1). The conversion cassette was PCR amplified, digested with KpnI and ligated into ΔSG2, which was cut with the same enzymes.

Constructs for sgRNA2 promoter mapping studies were based on the subgenomic RNA2 knockout mutant, ΔSG2 (SG2G/C) and/or the gateway version of this knockout
mutant (12). For promoter mapping studies, a duplicate copy of the promoter region was placed upstream in ORF 5 between the \textit{HpaI}_{3785} and \textit{KpnI}_{4154} restriction sites. Promoter regions, either wild-type or mutant, were PCR amplified with primers that contained flanking \textit{HpaI} and \textit{KpnI} restriction sites (Table 1). Mutant promoter regions were PCR amplified from the following constructs: pGL009, pGL036, pGL037, pGL039, pGL040, pGL042a, pGL043, pGL088, and pGL118 (9, 10). Products were digested with \textit{HpaI} and \textit{KpnI}, gel purified on and 0.8% agarose gel and subcloned into either pPAV6 (for use as an additional control) and/or ΔSG2, which were also digested with the same enzymes (12, 13). Promoter mutants were also developed using the gene tailor system. The mutations, contained in the 5’ mutagenic primer (Table 1), were introduced through PCR amplification of the entire ΔSG2 genome. Genome length PCR products were transformed into competent DH5α-T1R cells and plasmid DNA isolated. Clones were confirmed through DNA sequencing.

For creating promoter mutants using the gateway cloning system, promoters were PCR amplified with primers containing a 4-base CACC 5’ TOPO extension (Table 1). Resulting PCR products were used to make entry clones using the TOPO cloning system (Invitrogen), in which PCR products containing the 5’ TOPO extension (CACC) were cloned into a TOPO-D vector. Short oligo duplexes containing the 16-base promoter stem-loop and 5’ TOPO extension were also used to create entry clones (Table 1). Entry clones were used in a recombination reaction with the ΔSG2-gway destination vector to obtain the final expression clone.

**Electroporation of oat protoplasts and Northern blot analysis.** Oat protoplasts were prepared from oat cell suspension cultures and electroporated with wild-type or mutant
RNA transcripts as previously described (6). RNA transcripts were generated with the Megascript T7 RNA in vitro transcription kit (Ambion, Austin, Texas). Protoplasts were electroporated with 10 μg of RNA and allowed to incubate for 24-48 hours post-inoculation (hpi). Total RNA (15 to 20 μg) from infected protoplasts was extracted using Trizol and analyzed by Northern blot analysis as described (20). Positive-stranded RNA was detected using a $^{32}\text{P}$-labeled riboprobe that is complimentary to the 3’ terminus of PAV6 RNA.

The probe was generated from the plasmid pSP10 (6), which was linearized with HindIII and transcribed with T7 RNA polymerase. RNA was blotted onto GeneScreen nylon membranes (Dupont), hybridized with the probe and exposed to a PhosphorImager (Molecular Dynamics, Sunnyvale, California) for 1 to 24 hours. Bands of RNA were quantified by using the ImageQuant analysis software (Molecular Dynamics) and/or the Quantity One analysis software (Biorad).

**RNA sequence and structure analysis.** Sequence alignments of BYDV and related viruses were performed using Clone Manager 7 software. RNA secondary structure was determined using version 3.2 of the MFOLD program at the website (http://www.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi) and CLC combined workbench3 software.

**Results**

**The 3’ BTE and the sgRNA2 promoter are functionally independent.**

In previous studies, the sgRNA2 core promoter was shown to be approximately 143 nt long (nts 4810-4952) and overlaps with the 3’ BTE (nts 4810-4920). To further characterize the primary and secondary structural elements of the sgRNA2 promoter,
mutations were made to the 3’ BTE domain in a duplicate copy of the promoter which was tested in an ectopic location. This was necessary because the BTE is essential for virus translation and thus replication. The BTE in the natural location was left intact. Previously, Koev et al mapped the core promoter in an ectopic location at the KpnI4154 site in ORF 5 of ΔSG2, in which the wild-type sgRNA2 promoter was knocked out by a G to C point mutation at nt 4810 (12). However, the artificial sgRNA2 migrated near the abundant 18S ribosomal RNA on gels. This interfered with detection of the artificial sgRNA2 by Northern blot hybridization because ribosomal shadowing sometimes produced a faint band during northern analysis, making it difficult to observe artificial sgRNA (sgRNA2A) accumulation. To avoid this potential problem, the duplicate promoter was inserted between the HpaI3785 and KpnI4154 restriction sites to create a deletion large enough for the artificially generated sgRNA to migrate ahead of the ribosomal front, thus allowing the band to be clearly seen (figure 2). A gateway conversion cassette was also inserted in this region to convert the sgRNA2 knockout infectious clone, ΔSG2, into a gateway destination vector. This allowed for easy insertion of mutant promoters at this upstream location.

The role of the BTE, if any, in synthesis of sgRNA2 from the ectopic promoter was examined because the 3’ BTE is contained within the sgRNA2 promoter near its 5’end and it has a critical function in translation. Mutations to the 3’BTE that were known to have a negative effect on translation (9, 10) were introduced into ΔSG2 at the ectopic location. These mutants also contained an extra 35 bases of upstream sequence because previous studies demonstrated that sequences upstream of the transcription start site enhanced promoter activity (12).
We first tested the effect of mutations in the highly conserved stem-loop I (SL-I) of the 3’ BTE on sgRNA2A synthesis (figure 3A). Mutant RNA transcripts were electroporated into oat protoplasts, incubated for 48 hrs and total RNA isolated for Northern blot analysis. Lane 3 in Fig. 3B shows the viral RNAs generated by the ectopic promoter control, which has a wild-type copy of the sgRNA2 promoter, including 35 bases of upstream sequence (4775-4809). When SL-I was deleted (figure 3B, lane 4), the sgRNA2A/gRNA ratios revealed that synthesis was reduced to 40% when compared to control levels in lane 3. Mutations made to the loop of SL-I also reduced sgRNA2A synthesis in mutants LI-m2 and LI-m1 (lanes 5 and 6) to 40% and 30%, respectively. Although mutations to SL-I of the ectopic 3’ BTE reduced synthesis of sgRNA2A significantly compared to wild-type, they did not abolish synthesis completely.

With the exception of the SL-II deletion mutant, mutations to SL-II of the ectopic 3’ BTE were less dramatic than those to SL-I (figure 4A). Deletion of SL-II (figure 4B lane 4) reduced synthesis by 70% and disrupting and restoring the stem had little effect (figure 4B, lanes 5 and 6). However, a slight increase in synthesis was observed in the SL-II restore mutant compared to the control in lane 3. Given that SL-I is a highly conserved structure in luteoviruses, it is not surprising that the effects on sgRNA2A synthesis were more dramatic when compared to the effects of mutations to SL-II.

Previous studies have shown that SL-III of the 3’ BTE plays a critical role in BYDV cap-independent translation through a base pairing interaction with SL-D in the 5’UTR of the viral genomic RNA (10). Therefore, mutations to SL-III were of particular interest. Five SL-III mutants were constructed that harbored mutations shown previously to abolish cap-independent translation (figure 5A) (9, 10, 19). Mutations to the loop sequence of SL-III had
little effect on sgRNA2A synthesis (figure 5B, lane 5). However, an interesting mutant containing a U to A point mutation in L-III of the 3’ BTE (ΔSG2XP2-LIII m2), had a marked effect on overall viral RNA replication, despite not having any adverse effects on sgRNA2A synthesis (figure 5B, lane 6). Subgenomic RNA ratios actually reveal a slight increase in sgRNA2A accumulation compared to the control and the LIII mutant ΔSG2XP2-LIII m1. However, translation of this mutant in wheat germ extract was only 15% as efficient as wild-type virus (figure 5C, lane 8). We propose that this is due to base pairing of the mutant L-III in the ectopic BTE base-pairing to the wild-type L-III in the 3’ BTE in its native location. This base pairing (kissing stem-loop interaction) between SL-III s of the two copies of the BTE in the viral genome would prevent the wild-type 3’BTE from base pairing to SL-D in the 5’ UTR, which has the same loop sequence (UGACA) as the mutant SL-III in the ectopic sgRNA2A promoter (Fig. 5A). This result supports previous work done by Guo et al., which showed that long distance base-pairing between L-III of the 3’ BTE and L-D in the 5’ UTR is necessary for cap-independent translation and thus replication (9). An identical mutation in L-III of the 3’ BTE in its natural context reduced replication to undetectable amounts. Most likely, in the experiment in Fig. 5, the mutant L-III in the ectopic BTE serves as a decoy to cause the wild-type BTE to base pair to it, instead of to SL-D in the 5’ UTR.

Mutations that disrupted the stem of the ectopic SL-III had unexpected effects. Mutants SIII-m1, SIII-m2 and SIII-r (figure 5B, lanes 7-9), which harbored a disrupted and restored version of SL-III of the 3’ BTE, drastically reduced overall viral accumulation compared to wt virus and the ectopic promoter control (figure. 5B, lanes 1 and 4, respectively). However, the amount of sgRNA2A relative to other viral RNAs was not
greatly reduced in these mutants, indicating that the mutations did not affect sgRNA2 promoter activity, but by some other mechanism reduced viral replication overall.

To test whether the 3’ BTE could function from the ectopic location and restore the non-functional mutant BTEBF, we tested the replication of mutant BF-SG2XP2wt, which is essentially the BTEBF mutant with a wild-type copy of the sgRNA2 promoter (including the BTE) in the ectopic location. The mutation, BTEBF, contains a four base GAUC duplication at the \textit{Bam}HI\textsubscript{4837} site in the 3’ BTE. This mutation was shown previously to abolish cap-independent translation and thus virus replication (9, 27). In \textit{in vitro} translation studies, BTEBF translates to 5% of wt. The presence of the ectopic promoter with the wild-type BTE restored replication of the BTEBF mutant virus (figure 5B, lane 2) and translation was restored to 74% of wild-type (figure 5D, lane 6), which explains the ability of the virus to recover replication. This shows that the 3’ BTE can function outside of its natural location and confer cap-independent translation and permit virus replication.

These results demonstrate that the sgRNA2 promoter and the 3’ BTE are functionally independent, despite their overlapping sequences. Although some of the mutations within the 3’ BTE affected overall viral RNA accumulation, they had relatively minor affects on sgRNA2A synthesis, relative to the other viral RNAs.

\textbf{3’ BTE is not necessary for sgRNA synthesis.}

Because none of the mutations abolished sgRNA2A synthesis in the above studies, several mutants were made to test the sgRNA2 promoter function in the absence of the 3’ BTE. Two mutants were constructed in which all stem-loops SL-I, SL-II and SL-III (nts 4841-4899), or the entire 3’ BTE (nts 4818-4926) were completely deleted (Fig. 6A). Surprisingly, Northern blot hybridization revealed that the 59 base deletion of all three stem-
loops did not significantly affect sgRNA synthesis (Fig. 6B, ∆SG2XP2-SL1-3 del, lane 5).

Even more surprising was the observation that deleting the entire 108 base 3’ BTE (Fig. 6B, ∆SG2XP2-∆BTE, lane 6) resulted in sgRNA2A accumulation to a level similar to that of the control virus. The levels of sgRNA1 and gRNA also seemed to have increased slightly in this mutant. These results show that none of the 3’ BTE is needed by the promoter for sgRNA2 synthesis. Therefore we conclude that the sgRNA2 promoter contains an embedded translation control element that is not necessary for sgRNA2 synthesis. The deletion also included the start codon for ORF 6 and show that this AUG is not needed for sgRNA2 synthesis. Also, the wt promoter was tested in reverse orientation to see if it would generate a sgRNA2A. Surprisingly, this mutation was lethal to the virus (figure 6B, ∆SG2XP2-revp, lane 4).

Deletion of the BTE narrows the sequences necessary for sgRNA2 synthesis to the first 8 bases of the minimal promoter (nts 4810-4817), not including the extra 35 bases of upstream sequence (nts 4775-4809), and the last 26 bases of the core promoter (nts 4927-4952). In the absence of the 3’ BTE, the remaining promoter sequences spanned 69 bases, including the 35 bases upstream of the transcription start site. Mfold analysis of the remaining promoter sequence predicts a 16-base stem-loop structure formed by the lower base of the wild-type sgRNA2 promoter, with the flanking sequences not forming part of the stem (see the ∆BTE mutant in fig. 6A).
sgRNA2 promoter may be split by the 3’ BTE.

To test whether the stem-loop, which is what remains in the absence of the BTE sequence, could support sgRNA2 synthesis, all sequences flanking the predicted stem-loop were deleted. This included the extra 35 bases upstream of the transcription start site sequence and sequences flanking the 3’ side of the predicted stem-loop (bases 4935-4952). The progeny RNA of this mutant was analyzed via Northern blot hybridization and it was unexpectedly able to support synthesis of an artificial sgRNA2 of the expected size, albeit at a much lower level than ΔSG2XP2-ΔBTE (fig. 6, lane 7). These results show that the promoter was only 20% active with just the stem-loop structure and 60% more active with the flanking sequences present, thus revealing the importance of sequences flanking the remaining stem-loop structure and their contribution to promoter activity.

Many of the constructs tested in this study were made using the TOPO and Gateway cloning systems. Our sgRNA2 knockout mutant, ΔSG2, was converted into a gateway destination vector and used to make many of our ectopic promoter mutants (figures 6-7). The TOPO and Gateway cloning processes left an extra 45-50 bases that flanked the ectopic promoter and predicted secondary structural analysis revealed that the Gateway sequences could potentially form a rather significant stem-loop (figure 7A). Given that the relatively small virus-derived stem-loop, which remained after deletion of the BTE, supported sgRNA2A synthesis in replication studies, we decided to examine the influence of the flanking Gateway sequences to rule out any possible contribution to promoter activity.

To determine whether the flanking sequence and/or secondary structure introduced by the Gateway sequence was influencing promoter activity, we tested a construct in which all of the flanking gateway sequence was deleted, leaving only the stem-loop sequence flanked
by the HpaI and KpnI restriction sites (Fig. 7B, ΔSG2XP2-pSL). This mutant was still able to generate an artificial sgRNA2A of the expected size at levels similar to the mutant ΔSG2XP2-pSLg, which contains flanking gateway sequences (figure 7C, lanes 3 and 4). This result shows that the flanking gateway sequences did not influence promoter activity and that the stem-loop itself is able to support promoter activity, albeit at very low levels. As a control, a mutant that was flanked by the gateway sequence but did not contain any viral sequence seemed to produce an artificial sgRNA at very low levels (figure 7C, lane 10). However, sequences just upstream of the flanking A’s could potentially act as an alternate transcription start site and support low level synthesis. A G to C point mutation was also introduced into the remaining stem and results showed that it did appear to produce very low levels of sgRNA2A similar to mutants in lanes 7 and 10 (figure 7C, lane 6). Interestingly, this mutation in the wt context of the promoter abolished sgRNA2 synthesis. This result points to the importance of the primary structure, i.e. the sequence itself. We tested another mutant that contained a disruption of the stem from the 5’ side. It also appeared to produce a sgRNA2A at very low levels (Fig. 7C, lane 7). This result points to the importance of the secondary structure. However, a mutation to the 3’ side of the predicted helix and the combination of mutations in both strands predicted to restore the stem-loop, did produce a sgRNA2A (figure 7C, lanes 8 and 9). Again, suggesting the importance of both the primary sequence and secondary structure.

Disruption of the of the stem in the mutant ΔSG2XP2-3’dis, produced a similar effect seen in promoter mapping studies of sgRNA1, in which disruption of the structure at the start site (SL2) increased sgRNA1A synthesis (13). This could explain the observed increase in synthesis of this mutant compared to the controls in lanes 3 and 4. Also, MFold analysis of
the 3’ disrupt mutant revealed the formation of a potential 4-base stem-loop structure that resembles the first three bases of the wild-type stem-loop, thus allowing it to support synthesis. The restore mutant is essentially the base of the sgRNA2 promoter in reverse orientation. Interestingly, this mutation is lethal to the virus when the 3’ BTE is present. Although both of these mutants appear to synthesize sgRNA2A at higher levels than the other mutants, the ratios reveal that the actual relative synthesis is similar. It should be noted that the “shadow” from the ribosomal 18S RNA on the blot, can create the appearance of a faint band just below the shadow. This may explain the apparent faint band and/or low level synthesis in some lanes (lanes 1, 6, 7 and 10). Interestingly, the apparent bands observed for mutants in lanes 6, 7 and 10 all seem to be at the same level and do not appear to be distinct bands (compare with lanes 3 and 4). Therefore, we believe that the apparent bands observed in these lanes are actually background (compare with lane 1). The results reveal the importance of the conserved hexanucleotide sequence at the 5’ end of sgRNA2. It is also present at the 5’ ends of the gRNA and sgRNA1 promoter, where it is required (13).

A deletion mutant reported by Koev et al 2000 from previous border mapping experiments completely abolished sgRNA2A transcription when all bases downstream of base 4900 to the end of the promoter were removed. Interestingly, these bases include those that form the 3’ side of the 16-base stem-loop, thus possibly explaining its attenuated function. The attenuated function of this mutant confirms the importance of sequences that comprise the 3’ side of the stem and supports the notion that the sgRNA2 promoter may be split by the 3’ BTE.
Discussion

We were extremely surprised that a small region of deleted promoter sequence, consisting of a 16 base stem-loop and lacking over 100 bases between the two strands of the helix, was able to generate any sgRNA. However, there is some precedent for this. In *Hibiscus chlorotic ringspot virus* (HCRSV), Li *et al* 2006 reported sgRNA synthesis from minimal promoter sequences 30 bases in length that formed stable stem loops after most of the original promoter was deleted (14). HCRSV transcribes two sgRNAs and their corresponding promoters share a similar two-stem-loop (SL1 + SL2) structure located immediately upstream of the transcription start site. Both SL1 and the upper portion of SL2 enhance transcription, while the basal portion of SL2, which contains the transcription start site and the conserved hexanucleotide (CCCUUU) sequence, was found to be essential for promoter activity. They demonstrated that the remaining 30 bases, which contained the basal region of SL2, were sufficient for supporting transcription levels of 16% and 9% for the mini sg1 and sg2 promoters, respectively (14). Similarly, the remaining 16 bases of BYDV’s sgRNA2 promoter supports comparable levels of sgRNA synthesis with approximately half the bases reported for HCRSV. Interestingly, the 30 bases comprising each of the HCRSV mini-promoters formed the same helical structures found at the base of their respective wild-type promoter structures. This was also the case with BYDV, where the helical structures of the remaining stem-loop and the base of the wild-type promoter are identical. The base of the wild-type sgRNA2 promoter in BYDV also contains the sgRNA transcription start site and a conserved hexanucleotide sequence similar to HCRSV. However, unlike HCRSV, the sgRNA2 promoter of BYDV overlaps (or flanks) a translation enhancer element that coincides with the 5’ UTR of sgRNA2.
The 16 bases form a single six base stem loop that contains the conserved hexanucleotide sequence GUGAAG, which includes the start site at position 4810. This conserved GUGAAG sequence is also present at the 5’ end of the gRNA and SL2 of the sgRNA1 promoter. But GUGAAG is not sufficient for sgRNA synthesis because it is also present at position 655 within the genome and no sgRNA is associated with it. Mfold analysis of the latter region shows that the secondary structure is different than that of the promoter regions, which may imply that the secondary structure in which this conserved sequence occurs may be important. However, while the GUGAAG motif is also required for sgRNA1 synthesis, the stem-loop that contains it attenuates, and is unnecessary for sgRNA1 synthesis (12). Unlike sgRNA2, the key promoter elements are upstream of the sgRNA1 start site. The GUGAAG motif is conserved in other luteoviruses and most, but not all, occurrences of this sequence are associated with the start sites of sgRNA promoters.

The conserved hexanucleotide (CCCUUU) sequence in HCRSV is located within the stem structure formed by the mini-promoter and in the base of the native promoter structure. The initiation nucleotides of its two sgRNAs are at G residues (nt 2178 and 2438) and mutations to this nucleotide abolish transcription of the sgRNA. This was also observed in BYDV and in other viruses such as BMV and TCV (8, 12, 26). In BYDV, synthesis of sgRNA2 was also abolished in a mutant reported by Mohan et al. (1995), which included a partial deletion of ORF 5 (nts 3788-4515) and a G4922 to C4922 base change that knocked out the start codon of ORF6 (17). Replication was not abolished in this mutant, suggesting a role for ORF6 in sgRNA2 stability (17). A second mutant, PAV13, which contained the same deletion mutation but a normal start codon, also replicated and produced a sgRNA2. The results presented here show that the G at position 4922 is not necessary for promoter
function. However, this does not explain the role of ORF 6 in promoter function, since the start codon for ORF6 would still be present at the wt location and potentially compensate for its absence at the ectopic location.

The secondary structures formed by two overlapping elements, a subgenomic RNA promoter and a translation enhancer, and the importance for function of these elements may point to a type of structural cooperation. In such a mutual relationship, the secondary structure of one element is necessary for the structural integrity of the other. In the case of the BTE, computer analysis of the sgRNA2 promoter suggests a possibility for disruption of the 3’ BTE if this 16 base sequence is removed. If the BTE does not form, then the virus cannot replicate. For the promoter, proper formation of the 3’ BTE could potentially be important if the sgRNA is generated by premature termination. The BTE has been shown to bind translation initiation factor eIF4G (25) and possibly other host factors and this RNA-protein interaction could act as a barrier to the viral replicase during minus strand synthesis. However, results presented here show that the promoter functions well in the absence of the BTE. Thus, the reason for the overlap may be simply to conserve sequence space rather than to serve as a co-regulatory mechanism of gene expression. In conclusion, the results of these experiments indicate that the sgRNA2 promoter is split with two key domains separated by the unnecessary 3’BTE in the middle. We know of no other virus with this type of sgRNA promoter. Perhaps this allows regulation of translation and sgRNA synthesis.

Acknowledgements

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References


**Figure Legends**

Figure 1. Genome organization of *barley yellow dwarf virus*. Genomic RNA and sgRNAs are represented by bold lines. Numbered boxes indicate open reading frames (ORFs) and parentheses indicate the molecular weight of each encoded protein in kilodaltons. The grey boxes represent the 3’ BTE. POL: RNA dependent RNA polymerase; CP, coat protein; AT, required for aphid transmission.

Figure 2. Expression construct used to map the sgRNA2 promoter. Bold lines represent the gRNA and sgRNAs. The bold dashed line represents the artificial sgRNA produced by the ectopic promoter. Small grey boxes represent the 3’ BTE and small grey horizontal boxes with arrows represent the sgRNA2 promoter region. At right is an image of a Northern blot hybridization of total cellular RNA from oat protoplasts 48 h after inoculation with a construct containing the ectopic sgRNA promoter in a ΔSG2 background (the natural promoter is knocked out). Mobility of the ribosomal front is indicated.

Figure 3. Northern blot hybridization RNA from cells inoculated with BTE SL-I mutants. (A) Secondary structure of the 3’ BTE stem loops showing introduced mutations. A portion of the 3’ half of the genome is shown for reference and the location of the mutated BTE at the ectopic location are indicated. Red bases represent wt loop sequence and gray bases represent wt stem sequence. Boxed red and gray bases show the entire stem-loop I deletion. Blue bases indicate the mutations made to loop I. (B) Northern blot analysis of total RNA extracted from oat protoplast (~48 hpi) that were infected with wild-type and indicated mutant transcripts. Control lane was inoculated with no RNA. Corresponding genomic and
sgRNAs are indicated by labels on left side of blot. Bands were detected on a Phosphorimager and quantified using ImageQuant 5.2. Relative synthesis of mutants vs. wild-type synthesis (shown below each lane) was calculated as $\frac{[\text{sgRNA2A}]}{[\text{gRNA}]}$ relative to $\Delta$SG2XP2wt.

Figure 4. Northern blot analysis of protoplasts inoculated with BTE SL-II mutants. (A) Secondary structure of 3’ BTE and SL-II mutations. A portion of the 3’ half of the genome is shown for reference and the location of the mutated BTE at the ectopic location are indicated. Red and gray bases indicate the wild-type sequence. Blue bases indicate mutations made to SL-II. Boxed red and gray bases show the SL-II deletion mutation. (B) Northern Blot Analysis of total RNA from oat protoplasts infected with wild-type and ectopically expressed sgRNA2 promoter mutant transcripts (~48 hpi). Control lane was inoculated with no RNA. Bands were detected on a Phosphorimager and quantified using ImageQuant 5.2. Relative synthesis of mutants vs. wild-type synthesis (shown below each lane) was calculated as $\frac{[\text{sgRNA2A}]}{[\text{gRNA}]}$ relative to $\Delta$SG2XP2wt.

Figure 5. Northern analysis of protoplasts inoculated with BTE SL-III mutants. (A) Secondary structure of 3’ BTE and SL-III mutations. Red bases indicate wild-type sequence and blue bases indicate mutated sequence. Boxes indicate region where mutations were made. (B) Image of northern blot showing RNAs from oat protoplasts inoculated with the indicated mutant transcripts. Control lane was inoculated with no RNA. Bands were detected on a Phosphorimager and quantified using ImageQuant 5.2. Relative synthesis of mutants vs. wild-type synthesis (shown below each lane) was calculated as
[sgRNA2A]/[gRNA] relative to ΔSG2XP2wt.  (C) *In vitro* translation of mutant (ΔSG2XP2-LIII-m2) containing the U to A point mutation in SL-III of the 3’ BTE (lane 8). Other lanes are controls. Molecular masses in kilodaltons for BMV (left) and the BYDV ORF 1 (39K) and frameshift product (99K) are shown at right. (D) *In vitro* translation of BF-SG2XP2wt control (lane 6), which performed poorly in replication studies. The control is in lane 4. Molecular masses in kilodaltons for BMV (left) and the BYDV ORF 1 (39K) and frameshift product (99K) are shown at right.

Figure 6. Northern blot analysis of progeny of BYDV transcripts containing large deletions in the ectopic sgRNA2 promoter. (A) Predicted secondary structure of the sgRNA2 promoter depicting the deletions in the 3’ BTE. (B) Northern blot hybridization of RNA from protoplasts inoculated with the indicated ΔSL1-3 and ΔBTE deletion mutants. The ΔSG2XP2-pSLg mutant harbors additional deletions of the 35 upstream bases and the bases 3’ of the remaining stem in the ectopic sgRNA2 promoter. Predicted secondary structures of the ectopic sgRNA2 promoter regions are shown above each lane with BTE-derived sequence in black and adjacent non-BTE portion of the promoter region in magenta. Control lane was inoculated with no RNA. Ribosomal RNA indicated in panel under Northern blot. Bands were detected on a Pharos Phosphorimager (Bio-Rad Laboratories Inc., Hercules, CA, USA) and quantified using Quantity One analysis software (Biorad). Relative synthesis of mutants vs. wild-type synthesis (shown below each lane) was calculated as [sgRNA2A]/[gRNA] relative to ΔSG2XP2wt.
Figure 7. Accumulation of sgRNAs in BYDV mutants containing small stem-loops in the ectopic sgRNA2 promoter region. (A) Predicted secondary structure of the remaining stem-loop of the ectopic promoter (highlighted) flanked by the gateway sequence. Extra A’s were added to separate viral stem-loop from the gateway sequence. Restriction sites in ORF 5 (HpaI and KpnI) where the gateway conversion cassette was inserted, is indicated. Non-viral sequence derived from the cloning process is indicated by brackets. (B) Secondary structure of the remaining stem with flanking gateway sequence, without flanking gateway sequence, with the G to C point mutation, disrupt and restore mutations, and non-viral sequence. (C) Northern analysis of RNA from protoplasts inoculated with BYDV transcripts containing the indicated mutations (and predicted secondary structures) in the ectopic promoter region. ΔSG2XP2-pSLg contains the 16 nt viral stem-loop flanked by gateway sequence as in panel A. ΔSG2XP2-pSL lacks the flanking gateway sequence. ΔSG2XP2-GtoC contains a G to C point mutation in the ectopic 16 nt stem-loop that obliterates sgRNA2 synthesis in its natural context (ΔSG2). ΔSG2XP2-5’dis, ΔSG2XP2-3’dis, ΔSG2XP2-5’3’res, contain mutations that disrupt (dis) and restore (res) the six base pair helix. ΔSG2XP2-non viral replaces the stem-loop sequence with 15 bases of non-viral sequence shown in panel B. In the control lane, no viral transcript was added during electroporation. Ribosomal RNA indicated in panel under Northern blot. Bands were detected on a Pharos Phosphorimager (Bio-Rad Laboratories Inc., Hercules, CA, USA) and quantified using Quantity One analysis software (Biorad). Relative synthesis of mutants vs. wild-type synthesis (shown below each lane) was calculated as [sgRNA2A]/[gRNA] relative to ΔSG2XP2wt.
## TABLE 1. Primers used for mapping the sgRNA2 promoter

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### Construction of Gateway destination vector

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*Altered bases are indicated. TOP15' extensions and PvuII and KpnI recognition sequences are underlined; and deletions shown as underlined spaces. (*) primers used in Genetool cloning.*
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.
CHAPTER 3. BARLEY YELLOW DWARF VIRUS SUBGENOMIC RNA KNOCKOUT MUTANTS REPLICATE AND CAUSE DISEASE IN OAT PLANTS

A paper to be submitted to Molecular Plant-Microbe Interactions

Jacquelyn R. Jackson, Sijun Liu* and W. Allen Miller

*Constructed pPAV6-129 and first showed it to be infectious. All other work including writing of the chapter was performed by Jacquelyn Jackson.

Abstract

Barley Yellow Dwarf Virus produces three subgenomic RNAs (sgRNAs) during its infection cycle. sgRNA1 serves as the messenger for translation of the coat protein, the coat protein C-terminal extension (read-through domain), and a protein required for systemic infection in plants (P4). In contrast, sgRNA2 and sgRNA3 are not believed to be mRNAs. Instead, sgRNA2 appears to function as a riboregulator of viral translation in virus-infected protoplasts (26). SgRNA3 does not encode an ORF and its role is unknown. To understand the roles of sgRNA2 and sgRNA3 in BYDV infections, infectivity of viral RNA containing knockout mutants of these RNAs were tested in whole plant infections. We report that these sgRNA knockout mutants elicit infection in oats and induce similar symptoms and disease onset as the wild-type virus. An ELISA revealed that coat protein levels in the sgRNA2 and double knockout mutants were significantly higher than those induced by the control virus. There was no significant difference between the levels of coat protein in the control and sgRNA3 knockout mutant. These results reveal an unexpected modest inhibitory effect of
sgRNA2 on virion accumulation, and a lack of effect of sgRNA3 on virus accumulation and disease symptoms.

**Introduction**

*Barley Yellow Dwarf Virus* (BYDV) is a luteovirus with a single-stranded plus-sense RNA genome and causes serious losses worldwide in many small grain crops (19). It produces three subgenomic RNAs during infection, two of which serve as the mRNAs for its 3’ proximal ORFs (16). Subgenomic RNA1 serves as the messenger for the coat protein, the aphid transmission readthrough product, and the movement protein (7, 17). Subgenomic RNAs usually serve as messengers for viral proteins required late in infection. However, in BYDV, two of its sgRNAs appear not to be messengers for any viral protein, but are both produced abundantly in infections. Subgenomic RNA2 harbors ORF 6, which theoretically encodes a small protein (P6) of unknown function, but sgRNA2 probably does not serve as an mRNA. Its protein product has been detected only by *in vitro* translation. Translatability of sgRNA2 and presence of P6 has not been detected *in vivo*, despite much effort (27).

Subgenomic RNA3 does not code for any ORF and accumulates to high levels during infection (14, 16). Both RNAs are thought to have some type of regulatory role during infections, but this has not been established for sgRNA3. SgRNA2 has been shown to mediate a switch from early to late gene expression by selectively inhibiting translation of the gRNA and allowing translation from sgRNA1, thus functioning as a riboregulator of viral translation (26, 29). Other viruses, such as *Cherry leaf roll virus* (CLRv), also produce sgRNAs that accumulate to high levels yet have questionable function (2, 16). Thus, viral sgRNAs may have regulatory roles instead of and/or in addition to serving as mRNAs.
The 5’ end of sgRNA2 contains the 3’ BTE, which is a translation enhancer element that confers cap-independent translation to the viral genomic RNA and sgRNA1 (13). It is required for sgRNA2 to mediate the switch between early and late gene expression. As sgRNA2 accumulates during infection, it selectively *trans* inhibits translation of replication products from the genomic RNA and favors the translation of sgRNA1 (27). Therefore, sgRNA2 functions as a riboregulator of viral gene expression (26).

While sgRNA2 controls translation of gRNA and sgRNA1 in protoplasts, its’ role in whole plants has not been examined. Here, we investigate the roles of sgRNA2 and sgRNA3 in whole plant infections. Infectivity in oat plants of BYDV transcripts containing mutations that knock out synthesis of these sgRNAs was observed. Surprisingly, knockouts were equally as infectious as wild-type virus and the absence of the sgRNAs appeared to have no effect on disease symptoms. We also examine the hybrid infectious clone, PAV-129. This hybrid virus, which is based on the isolate BYDV-PAS, is more severe than BYDV-RPS and has no recognizable sgRNA 3 promoter sequence (3). The PAV-129 hybrid virus possesses BYDV-IL sequence at its 5’ end and PAV-129 sequence comprising the 3’ half of the genome (15).

**Materials and Methods**

**Virus isolates, plant material and aphid vector.** Five constructs were used in this study. The wild-type BYDV-PAV cDNA clones used were pPAV6 (6) and pPAV-129 (15). Subgenomic RNA knockout constructs were derived from the infectious clone pPAV6. The sgRNA2 and sgRNA3 knockout mutants, pSG2G/C and pSG3G/C2, were constructed previously by (16). The double sgRNA knockout mutant pSGDK was derived from
pSG2G/C and pSG3G/C2. The mutations from these two constructs were combined by digesting the pSG2G/C and pSG3G/C2 cDNA clones with BamHI and ligating the G/C point mutation containing fragment from pSG2G/C into the pSG3G/C2 vector. pPAV6-129, a chimeric clone containing bases 1-2854 of pPAV6 and bases 2855-5677 of BYDV-PAS isolate PAV129, was constructed as described previously (15). The desired mutant was confirmed by sequencing. These constructs were used for inoculation of oat protoplasts and aphid feeding for passage into healthy oat plants. Rhopalosiphum padi, the bird cherry-oat aphid, was used for passage of the virus into healthy oats. Non-viruliferous aphids were maintained in a growth chamber on healthy Avena sativa L. cv. Clintland 64 oat seedlings at 22-24°C under a 12 hr night and 12 hr day cycle.

**In vitro translation of viral RNA transcripts and electroporation of oat protoplasts.** Oat protoplasts were prepared from oat cell suspension cultures and electroporated with wild-type or mutant RNA transcripts as described previously (7). RNA transcripts were generated with the Megascript T7 RNA in vitro transcription kit (Ambion, Austin, Texas). Protoplasts were electroporated with 200-400 μg of RNA and allowed to incubate for 24-48 hours post-inoculation (hpi).

**Northern blot hybridization.** Total RNA (15 to 30 μg) from infected protoplasts and infected oat leaves were extracted using Trizol and analyzed by Northern blot analysis as described (25). Positive-stranded RNA was detected using a 32-P-labeled riboprobe that was complementary to the 3’ terminus of BYDV. The probe was generated from the plasmid pSP10 (7), which was linearized with HindIII and transcribed with T7 RNA polymerase. RNA was blotted onto GeneScreen nylon membranes (Dupont), hybridized with the probe and exposed to a PhosphorImager (Molecular Dynamics, Sunnyvale, California) for 5 to 24
hours. Bands of RNA were quantified by using the ImageQuant analysis software (Molecular Dynamics).

**Virus purification and aphid transmission of BYDV and BYDV mutants.** Methods used to isolate a crude extract of virus from infected oat protoplast were based on Chay et al. (1996). Protoplasts infected with wild-type and mutant viral RNA transcripts were harvested at 700 x g for 5 min in 50 ml conical tubes. Total RNA was isolated from 1 ml of combined cells for northern blot analysis. Cells were either kept at -80°C until further use or used immediately by adding 300 μl of 10 mM of sodium phosphate buffer pH 7.0. Cells were sonicated on ice at 2-3 second intervals for 6 to 8 times and centrifuged at 8000 x g for 5 min. Supernatant was then centrifuged at 75,000 rpm for 30 min (Sorval Discovery M150 centrifuge, S150AT rotor) and pellets resuspended in 100 μl of 10 mM sodium phosphate buffer pH 7.0. Samples were diluted 1:1 with 100 μl of 50% sucrose and loaded onto Parafilm™ membranes for membrane feeding of virus-free *R. padi*. Aphids were given an acquisition access period of 16-18 hr and placed onto five 5 day old oat seedlings (*Avena sativa* L. cv. Clintland 64) contained in a GA-7 vessel. Aphids were given an inoculation access period of 24 to 24 hrs and seedlings were soaked with Orthenex insecticide solution before transfer to soil and placement in growth chamber. Virus was passaged into healthy oats 4– 6 weeks post inoculation using virus-free *R. padi* that were fed on infected tissue for 24-48 hrs. Oats were maintained in a growth chamber at 18°C with 12 hr days and 12 hr nights.

**Standard reverse transcription-polymerase chain reaction (RT PCR) and primers.** Reverse transcription of viral RNA carried out with Superscript III™ RT (Invitrogen). Total RNA was isolated from 0.1 g of infected tissue. For 1st strand synthesis,
1 μl of 25 pmol viral specific 3’ end primer, pSG2 (5’ GGGTTGCGA ACTGCTTTTCGAGTG 3’), was combined with 3 μl (5-7 μg) of total RNA, 2 μl of 10 mM dNTP mixture and 7 μl of nuclease-free water. Sample was mixed and incubated at 65°C for 5 minutes and quickly placed on ice. 1 μl of RNasin (Promega), 4 μl of 5X cDNA buffer, 1 μl of 0.1M DTT and 1μl of Superscript III™ reverse transcriptase (Invitrogen) was added to each sample. Each sample was incubated for 12 minutes at 37°C, 12 minutes at 42°C, 12 minutes at 47°C, 12 minutes at 51°C and 12 minutes at 55°C. The cDNA was stored at -80°C for later use or used immediately in a 50 μl PCR reaction. Viral genome fragments were amplified in a reaction that contained 5 μl of 10X Pfx buffer, 1 μl of 50 mM MgSO4, 6 μl of 2.5 mM dNTPs, 1 μl of 25 pmole 3’ pSG2 reverse primer (5’ GGGTTGCGA ACTGCTTTTCGAGTG 3’), 1 μl of 25 pmole 5’ border forward primer (5’ CCCTCCAAGGAACAGTTGTC 3’), 1 μl of 1st strand cDNA template and 1 μl of Pfx DNA polymerase (Invitrogen). The PCR reaction was cycled on an MJ thermocycler with a 1 minute hot start at 94°C, 29 cycles of 94°C for 1 minute, 52°C for 1 minute, 72°C for 6 minutes and a final elongation step at 72°C for 10 minutes. PCR products (2 μl) were analyzed on a 0.8% agarose gel stained with ethidium bromide and observed under UV light. PCR products were sequenced and aligned using the BOXSHADE 3.21 server web tool (www.ch.embnet.org/software/BOX_form.html).

**Enzyme linked immunoabsorbant assay (ELISA).** The ELISA procedure was carried out using a compound ELISA kit for *Barley yellow dwarf virus*-PAV (Agdia). Protein was extracted from 1 ml of infected oat protoplasts and diluted in 2 ml (1:20) of the kit supplied general extraction buffer. Infected oat tissue (0.1 g) was ground in liquid
nitrogen and resuspended in 1 ml (1:10) of general extraction buffer. 100 μl of each sample in replicates of four was loaded onto pre-coated plates and incubated overnight (14 to 16 hrs) at 4°C. Plates were washed 4X (shaken on third wash) with 1X PBST (Phosphate Buffered Saline Tween-20) and 100 μl of enzyme conjugate was added to each well. Plates were incubated for 2 hr at room temperature and washed 4X (shaken on third wash) with 1X PBST. 100 μl of PNP (p-nitro phenyl phosphate) solution was added to each well and incubated for 1 hr. Optical densities of test wells were measured on a BIO-TEK plate reader at 405 nm for 3-4 times at half hour intervals using synergy software.

Results

Virus isolation from oat protoplasts.

We investigated the ability of point mutation mutants ΔSG2 (knockout of sgRNA2), ΔSG3 (knockout of sgRNA3) and DK (double knockout of sgRNAs 2 and 3) to replicate in whole plants. We also tested a chimeric transcript, PAV6-129. The 5’ half (including 5’ UTR and ORFs 1 and 2) of this transcript are identical to transcript PAV6, while the 3’ half (including ORFs 3-6 and the 3’ UTR) are derived from a severe isolate of BYDV-PAS known as PAV-129 (3, 15). PAV-129 diverges so much from PAV6 that no homology to the sgRNA3 promoter region of PAV6 is apparent. Viral RNA transcripts were electroporated into oat protoplasts and allowed to incubate at room temperature for 48 hpi. Total RNA was isolated from a 1 ml aliquot of cells for Northern blot analysis to confirm virus replication (figure 1A). Virus was detected in all samples and produced sharp bands that clearly showed the viral gRNA and sgRNAs. It should be noted that the PAV6 probe, pSP10, does not detect PAV6-129 efficiently due to sequence differences, hence the weaker signal produced.
in PAV-129-infected cells does not reflect a lower virus titer, as evident when the blot is probed with a PAV-129-specific probe (Fig. 1B).

There appeared to be a reduction in gRNA and sgRNA1 relative to sgRNA2 in the sgRNA3 knockout mutant, yet the double knockout mutant (DK) gRNA accumulated wild-type levels of gRNA and sgRNA1 (figure 1A). In cells inoculated with the DK mutant, a new RNA band that migrated slightly slower than sgRNA3 arose. Analysis of the progeny from the DK mutant showed that the presence of this band can vary in different infections (See figures 1A and 5).

**Aphid transmission of BYDV and BYDV mutants.**

To observe virus replication in plants, virus mini-preps were performed on the remaining protoplasts infected with each construct and the crude extract was fed to virus-free aphids through parafilm membranes overnight. Aphids were transferred to healthy 5 day old oat seedlings for a 24-48 hr transmission period. Young plants were subsequently monitored for symptoms.

The PAV6-129 hybrid RNA was infectious in oats. Symptoms appeared as early as 7 dpi on oats infected with the PAV6-129 infectious clone, which was earlier than seen with PAV6 which showed an average disease onset time of two to four weeks. Like the uncloned PAV-129 (3), the PAV6-129 hybrid induced more severe symptoms than the PAV6 infectious clone. These symptoms included severe stunting, reddening and curling of leaves (figure 2). Yellowing and reddening of leaves was observed on all infected plants, with much less stunting of plants infected with PAV6 and it mutants, compared to PAV6-129 infected plants. Surprisingly, all sgRNA knockout mutants were infectious, and plants
infected with sgRNA knockouts looked phenotypically similar to PAV6 infected plants (figure 3). Notching of leaves was also observed in some infected plants (figure 4A, C and F). Interestingly, these results show that the sgRNA 2 and 3 knockout viruses are able to cause disease in oats similarly to the wild-type PAV6 infectious clone.

**Northern blot analysis of infected plants.**

To confirm the presence of virus in plants exhibiting BYDV-like symptoms, Northern blot analysis was performed. Viral RNA was detected in all plants at ~7 wpi, except for one of the plants that was inoculated with the sgRNA3 knockout virus (figure 5). The RNA from the PAV6-129 infected plant is barely detectable due to the use of a PAV6-derived 3’ probe which differs significantly from the PAV-129 sequence. It is clear that RNA from sgRNA knockout virus-infected plants accumulated to similar levels as that of wild-type (PAV6)-infected plants. Also, the knocked out sgRNAs did not accumulate in plants infected with the knockout mutant viruses, indicating that the viruses did not revert to wild-type and that infections were not caused by contaminating wild-type virus. An exception was the presence of an RNA of unknown origin in the double knockout-infected plant #4 (P11) (figure 5). This resembles the new sgRNA that appeared sporadically in DK-infected protoplasts shown in figure 1A. These results reveal that neither sgRNA2 nor sgRNA3 is necessary for infection of plants, and corroborates the results observed in protoplast replication assays (see figure 1A).

**RT-PCR and sequence analysis of progeny BYDV RNA in infected oats.**

Because of the unexpected replication of the sgRNA knock-out viruses, we sequenced the progeny to confirm that the mutant viruses had not reverted to wild-type. RT-PCR analysis was performed on 5-7 μg of total RNA isolated from infected plants and resulting
PCR fragments were sequenced. Amplification products were observed in all plants, except the uninfected plant (C) and the nonviruliferous aphid-infected plant (HA). Because PAV6-specific primers were used to amplify the targeted region, the PAV-129 infected plant (P3) gave very little product of the expected size due to lack of sequence homology in the 3’ half of the genome. (figure 6B). Alignments of the region surrounding the sgRNA2 promoter revealed that the G4810C point mutation was still present in the sgRNA2 and double knockout mutants (figure 6C). Interestingly, the sgRNA3 knockout plant P9 (ΔSG3 infected plant #5) has an apparent G to C point mutation at the sgRNA2 transcription start site and still produces a sgRNA2. This result could be an artifact of sequencing, especially since it has been established that such a mutation abolishes sgRNA synthesis. Alignment of sgRNA3 promoter sequences showed that the point mutation is maintained in both sgRNA3 knockout and double knockout mutants. These results verify that the mutation is carried within the viral genomic RNA among virus populations infecting and accumulating in the plant.

**ELISA analysis of structural proteins.**

Based on the role of sgRNA2 as a riboregulator that selectively inhibits gRNA but not sgRNA1 translation, we speculated that the absence of sgRNA2 might affect coat protein (CP) levels translated from sgRNA1. Thus, we analyzed the levels of coat protein in infected plants and protoplasts to look for other possible effects that the absence of these sgRNAs might have. Examining viral protein products could potentially reveal details concerning their roles that may not have been obvious by observing only symptoms and RNA accumulation. Oat protoplasts were inoculated with 10 μg of wild-type or knock out mutant transcripts. After 48 hrs, triplicate samples for each construct were analyzed by ELISA plate with 4 replicates per sample. Absorbance readings were positive for each sample and were
greater than 3-fold higher than the mock inoculated and blank controls. The samples from protoplasts inoculated with sgRNA2 and double knockout mutant viruses had 1.5 to 2-fold higher levels of coat protein compared to wild-type (PAV6) virus (figure 7). There was no difference between the sgRNA3 knockout mutant and wild-type virus. Similar results were obtained in repeated experiments. These results indicate that in wild-type virus, sgRNA2 has a slight negative effect on virion accumulation at 48 hpi. This is unexpected, given the role for sgRNA2 in acting as a riboregulator (26, 27). The role of sgRNA3, if any, remains an enigma.

We next quantitated virion (or coat protein) levels in infected whole plants. Protein was extracted from infected plant tissue (~7 wpi) and resuspended in general extraction buffer. There were at least two plant replicates per construct, and several leaves were pooled from each plant for ELISA in which 4 replicates were used per pooled leaf sample. The same general trend was observed as in protoplasts but with more extreme effects of deleting sgRNA2 (figure 8). The absence of sgRNA2 caused coat protein levels in the sgRNA2 and double knockout mutant-infected plants to accumulate to two to three times the level observed in plants infected with wild-type and sgRNA3 knockout virus. In summary, sgRNA2 appears to play a negative role in virion (or coat protein) accumulation in short-term infection of oat protoplasts and long-term infections of whole plants.

**Discussion**

It is surprising that neither sgRNA2 nor sgRNA3 seem to play a role in symptom development and appear to not be needed for BYDV replication in oat plants. The fact that the sgRNA2 and sgRNA3 knockouts can replicate efficiently without these RNAs deepens
the mystery of why the virus produces these RNAs in the first place. These test results did not clearly show what role sgRNA3 may have in the virus life cycle other than the fact that it appears not to be needed by the virus and offers conflicting results for the role of sgRNA2. Secondly, the results seem to contradict results reported by Shen et al., where subgenomic RNA2 was shown to trans-inhibit translation from gRNA and not sgRNA1 in *in vivo* and *in vitro* studies (27). However, this result may be a direct reflection of the observed weak inhibition of sgRNA1 by sgRNA2. Also, sgRNA1 contains the 3’ BTE and is believed to translate in a manner similar to the genomic RNA. In many viruses, the coat protein sgRNA is highly translatable and in BMV, low sgRNA levels did not affect coat protein levels (4, 28). In the absence of sgRNA2, maybe the lack of interference by sgRNA2, although very weak, could cause a significant increase in coat protein production. Since sgRNA2 seems to play a role in the switch between intermediate and late gene expression, then one would expect to see some deregulation in such viral processes.

The mystery of sgRNA3 deepens considerably given that it does not seem to do anything but accumulate to high levels in virus infections. Perhaps it is a pseudo- DI RNA species. This idea contradicts results reported by Kelly et al (1994), which suggested that sgRNA3 is not a DI RNA but an authentic sgRNA that may have a regulatory role due to its abundance during infections. However, it should be noted that northern blot analysis of this sgRNA knockout consistently appears to show less gRNA compared to other virus samples (see figures 1 and 5 [other unpublished data]). Interestingly, Kelly et al (1994) reported the presence of this RNA in virus particles (14). Perhaps it plays a minor role in virus packaging. Also, in the double knockout mutant, a new RNA band appeared that migrated slightly slower than sgRNA3. It is unclear why this band was produced only in cells infected
with this mutant. These results show that subgenomic RNAs are more than just messengers and that their true function can be hard to define. It is hard to imagine a virus wasting its resources transcribing an RNA that does nothing. In the case of sgRNA2, examining the levels of non-structural proteins will shed more light on its role as a riboregulator of translation.

More rounds of viral passage will be needed to examine the overall fitness of knockout mutants in whole plant infections. Given that the plants were inoculated under controlled conditions with one particular virus, the population of viruses within the plant would not resemble those growing in field conditions, which are usually mixed infections. With such a small population size, along with controlled growing conditions; compensatory mutations may not occur (24). Several rounds of viral passaging from such a small original population of viruses might lead to the accumulation of deleterious mutations that can affect the overall fitness of the virus population over time. This effect is similar to a process known as Muller’s ratchet, in which high mutation rates and the irreversible accumulation of deleterious mutations within a small population decrease the fitness of the virus (21). This process has been reported for several animal viruses (8-12, 18, 22, 23, 30), but has only been recently extended to plant viruses. Tobacco mosaic virus (TMV) was shown to exhibit a decrease in fitness as a result of Muller’s ratchet (5).

Horizontal transmission of the virus by its aphid vector may play a role in overall viral fitness as well. Genetic bottlenecks can occur during aphid transmission of the virus to healthy plants (1). Moury et al (2007) estimated the size of such bottlenecks and showed that the average number of virus particles being transmitted by aphids was between 0.5-3.2 for Potato virus Y (PVY) variants (20). A significant genetic bottleneck in newly infected
squash plants was induced by aphid transmission of CMV into healthy plants (1). In the potyvirus *Tobacco etch virus* (TEV), fitness was reduced due to the effect of accumulated mutations within the viral population. Since BYDV is transmitted by aphids, the actual number of virus particles transmitted to healthy plants would be extremely low compared to the total virus population in the infected source plant. In addition to the point mutations themselves, each time BYDV is passaged into a healthy plant, such a bottleneck could occur and possibly affect the fitness of the virus.

It is unclear as to why the new RNA species appears occasionally in protoplasts and whole plants infected with the double knockout mutant. Plant P11 clearly shows an RNA3 in figure 5 but sequence data obtained from RT-PCR products reveal that both point mutations are maintained. Whether this is due to compensatory mutations in surrounding sequences will have to be examined further. In this study, we show for the first time that sgRNA deficient mutants of a plant RNA virus is able to cause disease in its host. We also show that the point mutations responsible for knocking out the subgenomic RNAs are maintained after passaging the mutant virus twice in healthy oats. The unexpected effect on structural protein levels by the absence of sgRNA2 leads to new roles for the presence of sgRNAs during the virus life cycle and also raises questions to the presence of sgRNAs in virus infections, as demonstrated by sgRNA3 deficient mutants. The effect on fitness of virus populations via repeated horizontal transfer by the aphid vector and/or the potential for reduction in fitness due to the accumulation of deleterious mutations within the population will require further examination. The ability of the virus to maintain a healthy infection in the absence of these sgRNAs is a testament to the flexibility of the genomic structure within virus populations.
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References


Figure Legends

Figure 1. Accumulation of sgRNA knockout mutants in infected protoplasts. (A) Total RNA isolated from a 1 ml aliquot of protoplast cells was analyzed by northern blot hybridization to confirm replication of wild-type and sgRNA knockout mutants before isolation of virus from infected cells for infecting whole plants. Virus mini-preps were performed on infected protoplasts and virus particles were membrane fed to virus-free aphids. RNA species are identified by labels on left side of blot. (B) Northern blot analysis of PAV6 and PAV6-129 infected protoplasts probed with a PAV129-specific probe. The probe, which hybridizes to the last 1500 bases of the PAV-129 genome, is able to detect an sgRNA3 that was not detected with the PAV6-specific probe, SP10. Control lane was inoculated with no RNA. Corresponding genomic and sgRNAs are indicated by labels on left side of blot.

Figure 2. Comparison of virus symptoms induced by PAV6 and the PAV6-PAV129 chimera. Plants are from aphid transmitted virus isolated by the virus mini-prep from infected protoplasts that were analyzed for virus as in Fig. 2. Plants were infected with nonviruliferous “Healthy” aphids (HA cont.), PAV6 (PAV6 #4, PAV6#5), or the PAV6-PAV-129 chimera (PAV6-129 #2 and PAV6-129 #3). Plants were photographed ~7 wpi.

Figure 3. Comparison of virus symptoms induced by PAV6 and sgRNA knockout mutants. Two plants taken from each group of infected plants was compared to healthy aphid control plants (HA cont.). PAV6 and knockout infected plants show similar symptoms while controls show no signs of infection by BYDV at ~7 wpi.
Figure 4. Close-up of virus symptoms on leaves of infected plants. Leaf samples were taken from plant that were successfully infected by membrane fed aphids. (A & B) leaves of plants infected by PAV6 (#4 and #5); (C) leaves of a plant infected with PAV6-129; (D-G) leaves of four plants (#1, #2, #3, and #4) infected with the sgRNA2 knockout mutant; (H & I) infected leaves of plants infected with the sgRNA3 knockout mutant (#2 and #5) and (J) leaves of a plant infected with the double knockout mutant (#4). In all panels, leaves of plants used to feed healthy aphids (HA control) and leaves from plants that were fed no aphids (Control) are shown.

Figure 5. Northern blot analysis of total RNA from infected plants. Total RNA was isolated from plants showing symptoms approximately 7 wpi. Mobilities of genomic and sgRNAs are identified on left side of blot. Specific plants that were tested in the study are identified by the numbers at the top of each lane.

Figure 6. Sequence analysis of progeny virus from infected plants. (A) 3’ half of BYDV genome depicting the region amplified in RT-PCR analysis. Primers amplified a region spanning bases 4439 to 5677 (the 3’ end of the genome) that gave a 1.2 kb cDNA product. (B) Total RNA isolated from infected plants was subjected to RT-PCR analysis using BYDV-specific primers (see methods). An approximately 1.2 kb sized fragment was amplified from each sample, confirming the presence of virus. 2 μl of sample was run on a 0.8% agarose gel stained with ethidium bromide and visualized under UV light. PCR products were sequenced and then aligned using the BOXSHADE 3.21 server web tool. (C) Alignment of sequences surrounding the sgRNA2 and sgRNA3 promoter regions. Bent
arrows represent the sgRNA transcription start sites. Green outlined boxes highlight point mutation base position within the sgRNA promoter. Locations of bases within the promoter region are indicated by small black arrows and nucleotide positions. Short thick red underlines identify wild-type base at that position. Short thick yellow underlines highlight the position of the point mutation in the promoter sequence. Nucleotides with white backgrounds in the sgRNA2 promoter region sequence alignment indicate bases that have the same base as wild-type. Nucleotides with white backgrounds in the sgRNA3 promoter region alignment indicate bases that are different from wild-type.

Figure 7. ELISA detection of BYDV virions in infected protoplasts. At left, calibration curve of BYDV-IL virus standard read at 405 nm. Total coat protein amounts in μg/ml are indicated on the Y axis. Samples are identified on the X-axis.

Figure 8. ELISA detection of BYDV virions in infected plants. At left, calibration curve of BYDV-IL virus standard read at 405 nm. Total coat protein amounts in μg/ml are indicated on the Y axis. Samples are identified on the X-axis.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.
Figure 8.
CHAPTER 4. GENERAL CONCLUSIONS

The information gained from studying viruses continues to surprise us. Understanding the role of sgRNAs during viral infections and the mechanisms by which they are produced would shed great light on how viruses regulate their gene expression. The results presented in this dissertation reveal the complexities of viral gene expression and show that there is still much to be learned in this area. Since sgRNAs represent a major step in controlling viral gene expression, understanding how the virus produces them will aid our understanding about replication.

BYDV is an exceptional virus that uses a series of novel strategies to express all of its genes. It produces three subgenomic RNAs, but only one of them seems to fit the role of a traditional sgRNA, i.e., to encode a 3’ proximal ORF and serve as its messenger during intermediate and late stages of infection. However, sgRNA2 encodes an ORF whose expression product has yet to be detected \textit{in vivo} and sgRNA3 does not encode anything. The surprising findings yielded by this research have led us to question the very role of these RNAs during infections.

The three promoters of BYDV are very different from each other in both structure and primary sequence. Like most RNA viruses, the location of the sgRNA1 promoter is mostly upstream of its transcription site. However, the opposite is true for both the sgRNA2 and sgRNA3 promoters. Their promoters are located almost entirely downstream of their transcription start sites. Out of all three promoters, the sgRNA2 promoter is the most interesting. Not only is it the larger promoter, it is unusual because a significant portion of the promoter region overlaps the 3’ BTE. This \textit{cis}-acting translation element is essential for
virus replication and operates in a cap-independent manner. Analysis of the sgRNA2 promoter revealed that even though the BTE is contained within the sgRNA2 promoter, it was not necessary for promoter function, thus both elements are functionally independent of each other. The promoter was able to function with only a 16 base stem and its core elements seem to be split with the 3’ BTE in the middle. This exemplifies the mastery of how viruses maximize the use of their limited coding capacity. To date, we know of no other virus with such a promoter. The mechanism by which sgRNA2 is generated is still undetermined. An \textit{in vitro} transcription/replication system would greatly aid this effort.

Results from this research also revealed the modular nature of the 3’ BTE. When a wild-type copy of the sgRNA2 promoter was placed in an ectopic location in the BF mutant, it restored translation and replication to near that of wild-type virus. This ectopic promoter mutant could potentially be used for mapping the promoter in its wild-type location, which is normally not possible without the duplicate BTE, due to the low tolerance for mutations by the BTE.

In an effort to determine the roles of these sgRNAs, knockout mutants were examined for possible differences in timing of disease onset and or the ability of these viruses to infect plants. Results showed that all knockout mutants are infectious in both plants and protoplasts. Interestingly, a new RNA band is sometimes observed in the double knockout during infections. I found it very interesting that the virus was able to replicate and cause disease in the absence of either sgRNA or both. The effects on coat protein levels were just as surprising and revealed yet another unexpected role for sgRNA2. The results suggest that sgRNA2 has a slightly negative effect on virion accumulation, given the much higher coat
protein levels seen in the knockout mutant. This further deepens the mystery behind the true role of sgRNA2 during BYDV infections and causes us to re-think sgRNAs.

In future experiments, the stability of the sgRNA knockout mutation should be examined. This can be accomplished by passaging the virus several times in plants and sequencing RT-PCR amplified genome segments encompassing both promoter regions. In this way, revertants and other compensatory mutations can be identified and possibly uncover other aspects of the roles these sgRNAs play. The sgRNA3 promoter still needs further characterization.

Next, the levels of non-structural proteins should be examined. Given the unexpected results of sgRNA2 on coat protein levels, it would be very interesting to if the results support our translation regulation model. Results from this experiment should reveal a more drastic difference in protein levels given the strong trans-inhibition of translation to the gRNA by sgRNA2. Finally, a closer look at possible binding sites by host proteins to the promoter regions should be examined. Understanding how the viral replicase recognizes such divergent promoters and the possible involvement by host proteins could further our understanding of how BYDV replicates and transcribes sgRNAs.
APPENDIX 1. CONTRIBUTION TO PROTOCOL PUBLISHED IN CURRENT PROTOCOLS IN MICROBIOLOGY

UNIT 16D.3 Preparation and Electroporation of Oat Protoplasts from Cell Suspension Culture

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The following procedure was co-written with the first author of this publication. It details a procedure that is routinely used by members of our lab. The support protocol section of this procedure and the critical parameters section of this unit were written entirely by me. All contributed writings were reviewed by my major professor, Dr. W. A. Miller.

Digest oat cell suspension culture

1. Transfer a 7 day-old oat cell suspension subculture (about 40 to 50 ml volume), into a sterile 50 ml conical tube and let cells settle in the tube for 5 min.

For best results, do not use cell suspension cultures that have been subcultured more than 7 days previously. The cell walls of older cultures greatly reduce digestion efficiency.

A satisfactory suspension culture consists of finely growing cells with small clumps.

Expect to obtain approximately 10 ml of packed cells. 50 ml of the enzyme solution is sufficient for the digestion of up to 10 ml of packed cells. If the amount of packed cells exceeds 10ml, adjust volume of cells to 10ml or increase the total amount of enzyme solution.

2. Remove supernatant and gently resuspend cells in 20 ml of freshly prepared enzyme solution

Always use a freshly prepared enzyme solution.
Protoplasts are very fragile to mechanical manipulation. Resuspend cells by gently swirling the tube or by pipeting slowly up and down.

3. Transfer cell/enzyme mix into a large Petri dish (145 x 20 mm) and add the remaining 30 ml of enzyme solution.

If using small Petri dish (100 x 15 mm), transfer the cell/enzyme mix into 3 sterile Petri dishes and aliquot the remaining enzyme solution into each. Final volume in each dish should not exceed 20ml.

4. Seal the Petri dish with parafilm and cover it with aluminum foil to keep out light.

At this point, the cell/enzyme suspension has a granulated appearance.

5. Shake cell/enzyme suspension on a platform shaker at room temperature overnight at 42 rpm.

To avoid over-digestion, do not incubate longer than 16 to 18hrs.

After digestion, cell clumps should no longer be visible with the naked eye, giving the digest a smooth homogenous appearance.

6. Inspect a drop of digested cells under a light microscope to observe isolated protoplasts.

Freely floating protoplasts should have a uniform spherical shape.

Proceed to preparing cells for electroporation only if a large number of healthy protoplasts are observed. A digest that has a few partially digested clumps may be used for replication studies but is not ideal for translation assays.
Wash protoplasts to remove debris

It is recommended to check protoplasts under a light microscope after each wash step. Handle the protoplasts very carefully during the following steps to minimize cell rupture. An electronic pipette controller works best for collecting protoplasts and to dispense media. Protoplasts and all added solution should be released very slowly against the wall of the tube during wash step.

7. Carefully collect the protoplasts with a sterile 10 ml serological pipet and gently dispense the cells into 2 sterile 50 ml conical tubes.

8. Harvest protoplasts by centrifuging at 4°C for 5 min at 100 x g (700 rpm in a SH-3000 Sorvall swinging bucket rotor).

9. Aspirate off supernatant. In each tube, resuspend protoplasts in 10 ml of ASW:0.6M mannitol.

10. Wash cells by centrifuging as in step 8.

Healthy protoplasts should give a yellow pellet in a clear supernatant.

11. Aspirate off supernatant and resuspend cell again in 10 ml of ASW:0.6M mannitol.

12. Repeat wash as in step 8.

If cell debris is still observed under light microscope, one additional wash is recommended. Protoplasts are very sensitive to osmotic pressure. The constant presence of a significant number of broken cells could result from osmotic differences due to poor quality media. If this is observed, discard the preparation.
**Prepare the cells for electroporation**

13. Aspirate off supernatant. Use 10 ml of electroporation buffer containing 0.2 mM spermidine to resuspend and combine protoplasts into one tube.

*Add spermidine (2 ml per 10 ml of electroporation buffer from a 1M stock solution) to the electroporation buffer prior to use.*


15. Aspirate off supernatant. Resuspend the protoplasts in electroporation buffer containing 0.2 mM of spermidine, to approximately $6 \times 10^6$ cells/ml/sample.

*Use a hemocytometer to count cells.*

*Protoplasts generated from approximately 10 ml of packed cells yield about $60 \times 10^6$ cells. The final volume of electroporation buffer used to resuspend protoplasts varies with the type of assay. For replication assays, resuspend protoplasts in up to 10 ml of electroporation buffer (and use 1ml of suspended protoplast per sample). For transient expression, protoplasts can be resuspended in up to 25 ml of buffer.*

16. Inspect a drop of the protoplast suspension under a light microscope. Proceed to step 17 only if protoplast suspension is largely free of cell debris.

*Osmotic difference due to improperly made buffer can contribute to the constant presence of broken cells. Such a protoplast preparation should not be used for electroporation.*

**Add RNA and electroporate protoplasts**

17. Aliquot 1 ml of protoplast suspension into each sterile 4 mm electroporation cuvette and place on ice.
It is recommended to use wide-bore 1ml pipet tips to aliquot protoplasts.

At this point it is recommended to prepare the cell culture plate needed in the final step of electroporation (step 21). According to the total number of samples, aliquot 5 ml of MS + 0.4M mannitol into the appropriate number of wells on a 6 well plate.

18. When ready to electroporate, add RNA to the cells and mix by gently inverting the cuvette.

The amount of RNA to be added depends on the type of assay. For replication studies, it is recommended to use as much as 10 mg of RNA per sample. Higher amount can be used for virion purification and 1mg or less of RNA is sufficient for transient expression.

Co-inoculation can be done by mixing both RNA samples prior electroporation or by a two-step electroporation approach (see Commentary).

19. After adding RNA to the cells, immediately place cuvette into the holder of the electroporator device and electroporate cells at a defined voltage and capacitance setting.

To minimize degradation of the RNA sample, electroporate sample as soon as RNA is added.

If using the BTX electro square porator T820, the optimal setting for electroporating oat protoplast is one pulse for 6 msec at 300V.

If using the Biorad GenePulser Xcell™, the optimal setting is 300V/500µF.

20. After electroporation, put cuvette back on ice until the remaining samples have been electroporated.
21. Under a laminar flow hood, transfer each sample into one well on the 6-well cell culture plate containing 5 ml of MS + 0.4 M mannitol media.

*For maximum recovery of cells, remove 1 ml of MS media from the well prior to transferring the electroporated sample into the well. Use the 1ml aliquot of media to rinse cuvette.*

*White threads of debris resulting from cell lysis after electroporation are commonly observed in the sample.*

*For transient expression assays, it is not critical to work under sterile conditions. The sample can be poured into the MS media immediately after the electroporation.*

22. Seal the plates with parafilm and cover with aluminum foil. Incubate the plates at room temperature for 24 to 48 hr.

*The quality of the protoplast preparation determines the viability of protoplasts for up to 48 hr after electroporation.*

*For transient expression, cells can be harvested 30 min post-inoculation. Optimal expression is observed after 4 hr, although individual experiments may require some optimization.*

**Harvest cells**

23. Check sample in culture plates under light microscope

*A mixture of intact protoplasts and cell debris clumps is commonly observed.*
24. Carefully collect cells from the culture plate and gently transfer each sample into a separate 15 ml centrifuge tube.

25. Pellet cells by centrifuging as in step 8.

*For virion purification, centrifuge cells at 700 x g for 5 min.*

26. Aspirate off supernatant.

*For replication assay, it is recommended to perform at least one additional wash with ASW: 0.6M mannitol to remove cell debris before proceeding to isolation of total RNA.*

*At this stage, the cell pellet can be stored at -80C until needed in an appropriate buffer for downstream applications. However, this is not recommended for recovery of RNA for replication analysis. It is best to isolate total RNA from a fresh sample.*

**Support protocol**

**Subculture oat suspension culture**

It is important to maintain a healthy cell suspension culture. This is accomplished through weekly transfers of 7 day-old subcultures into fresh MS media. Transfers should be carried out under aseptic conditions to avoid contamination. It is recommended to work under a laminar flow hood. To subculture cells, take 10 ml of a 7 day-old oat suspension culture and transfer it into 40 ml of fresh MS media. Seal the flask by plugging the top with sterile cotton wrapped in aluminum foil. Undifferentiated cell suspension cultures are cultured in a 150 ml Erlenmeyer flask in 50 ml of MS media.
Cultures are grown at 20-25°C and are shaken between 160-220 rpm on an orbital shaker. Cells should be subcultured every 7 days. If cells are needed within a few days, transfer 25ml of 7-day-old culture into 40ml of fresh MS media. Oat suspension cultures can then be used for protoplast preparation three days after subculturing.

For storage of cells during shipping, it is recommended that cells be supported on solid media. Prepare fresh MS media containing a solidifying agent such as phytoagar or phytagel. Under sterile conditions, pour cooled media into sterile 50 ml conical tubes. Tilt the tubes so that the media has an angled surface after it solidifies. Pre-cut several pieces of oval shaped whatman paper disks are able to easily fit inside the 50 ml conical tube. Wrap pre-cut disks in foil and sterilize. Once the media solidifies, place the sterile oval paper disk onto the angled media surface using sterile forceps. Collect cells by centrifuging and aspirating off liquid media. Using a sterile spatula, scoop a sample of cells from the pellet and carefully place the sample onto the paper disk in the 50 ml conical tube. Gently secure the scoop of cells by spreading it over the paper disk. Replace the cap and seal with parafilm. Cells are now ready for shipping. To establish a culture, scrape cells from the paper disk and transfer them into 40 ml of fresh MS media. Alternatively, remove the entire paper disk and place it into the flask containing the MS media. Allow the cell suspension to grow and proceed with subculturing into fresh media.

**Critical Parameters**

The use of cell suspension cultures as starting material for making protoplasts, as opposed to using plants, offers the advantage of having a source material that is already
aseptic and already maintained in a controlled environment. For the protocol described above, it is important that these sterile conditions be maintained throughout the course of the experiment. This is especially true for replication assays, which require long incubation periods in sucrose-containing culture media that is an ideal breeding ground for all kinds of contamination. All media should be prepared, autoclaved, and opened only under a laminar flow hood.

Before preparing protoplasts from oat cell suspension cultures, it is vitally important that the protoplasts be generated from a cell suspension culture that has been properly maintained. Therefore, the age of the cell suspension culture is critical when using it as a source for isolating protoplasts. Cultures that are properly maintained ensure reproducible results from the protoplasts generated from them. To minimize the presence of old and dying cells in the culture, oat cell suspension cultures should be sub-cultured on a weekly basis. It is recommended to use 7 day old oat cell suspension cultures for preparation of protoplasts. The yield of protoplasts is greatly reduced if older cultures are used as a source for protoplasts. It has been our observation that cultures older than 7 to 8 days do not digest properly.

Another parameter critical for success of performing an inoculation assay is the quality of protoplasts generated from the overnight enzyme digestion. This is probably the most important critical factor for any experiment requiring the use of protoplasts. Use of a buffer along with the appropriate concentration of enzyme is vital to the success of the digest. We highly recommend the RS cellulase from Onozuka, Yakult. For oat cells, the enzymes are dissolved in artificial sea water (ASW): 0.6 M mannitol with pH adjusted to 5.6-5.7. This one-step approach involves dissolving all of the enzymes in 50 ml of ASW:0.6 M
mannitol and incubation of oat cell suspension cultures in darkness at room temperature overnight. Each enzyme lot varies slightly and differs in activity, so it is important to compare each new batch of enzyme with the older lot. If necessary, the amount of enzyme added may be adjusted to optimize the digestion. This has given us consistent and reproducible results with relatively high yields of viable protoplasts.

Media quality is another important critical parameter that should be addressed when preparing and inoculating protoplasts. Once protoplasts are isolated, maintaining them in the appropriate media ensures that the cells survive and do not rupture during downstream applications. Because protoplasts lack a cell wall, their plasma membranes are extremely sensitive to osmotic differences introduced by the media in which they are maintained. Therefore, correct osmotic conditions must be maintained by the media throughout the entire procedure. Osmotic changes introduced through improperly made media can greatly interfere with the success of the experiment. For example, poor quality protoplasts enhance RNA degradation and result in poor quality Northern blots from extracted RNA. Thus, inert substances such as mannitol are added to the media to equalize the osmotic pressure inside and outside of the cell. Protoplasts should be handled carefully due to their fragile nature and should be siphoned and dispensed slowly during transfers. It is recommended to slowly release the protoplasts and added media against the wall of the tube during the course of the experiment. Avoid sudden jarring or violent shaking of the sample.

Once a healthy sample of protoplasts has been isolated and washed, they are ready for electroporation. It is necessary to resuspend protoplasts in the appropriate electroporation buffer and wash at least once in the buffer before electroporating the sample. Buffers used for electroporation should be made with double deionized water to ensure that the media
is free of any contaminating electrolytes that can affect the electric conductivity of the buffer (Hibi, 1989). The length of the electric pulse and the voltage setting vary for different cell types and are critical parameters for the success of the experiment. Too long a pulse at a given voltage can damage protoplasts due to the dramatic effects on the cell wall (Fromm et al., 1987). For oat protoplasts, we have consistently obtained good results with the BTX electro square porator T820 and the BioRad GenePulser XCell™ (with CE module) when using a one 6 msec pulse at 300V and 300V/500mF, respectively. All samples are electroporated in sterile 4mm gapped electroporation cuvettes. If electroporating protoplasts from cells other than oat, electroporation settings may have to be optimized before a successful transfection can be achieved. Refer to Fromm et al. (1985 and 1987) and Hibi (1989) for more details on how to optimize conditions for successful transfections of protoplasts.

Broken cells within a sample can release nucleases that can degrade the nucleic acid sample. Therefore, samples should always be kept on ice during the electroporation procedure to reduce their activity and electroporated immediately after adding RNA to minimize exposure of the RNA to Rnases (Hibi, 1989). The lower temperature also allow the pores that were induced in the membrane via the DC pulse to remain open longer to allow for more efficient uptake of the nucleic acid (Hibi, 1989). For best results, use freshly prepared transcripts for inoculation experiments. However, properly stored samples can be used several times as inoculum as long as the integrity of the RNA is maintained and monitored. It is recommended to not stop at any stage of the protoplast preparation procedure. All steps should be followed to completion to ensure a successful and reproducible experiment.
Literature cited


*Note: All of the above listed references may or may not be cited in the sections authored by me, but include references cited by other authors of this unit.*
APPENDIX 2. MODIFICATIONS TO THE APHID TRANSMISSION PROTOCOL.

I have made several improvements to the aphid transmission protocol used in our lab. I believe that these changes address areas of the previous procedure that I deemed problematic. Given the scale of the experiment conducted in chapter 3, I found it necessary to use a larger vessel to do the aphid feedings on oat seedlings. Young plants were previously placed in glass test tubes filled with agar to do feedings and only allowed one plant per test tube. I needed a vessel that could hold at least 5 oat seedlings at a time so that the aphids could simultaneously feed on the plants for transmission of the given virus, mutant or wild-type.

The GA-7 vessel (magenta box) was chosen as an alternative because it is large enough to hold several oat seedlings or leaf cuttings in one container and had a capped opening large enough to easily apply aphids onto plants or leaf cuttings. A container of this size allowed me to transmit aphids to as many as 25 plants at a time using only 5 GA-7 vessels, thus greatly increasing my efficiency (figure 1). An agar/water based media was added to the vessel and allowed to solidify to provide support the plants when conducting feeding experiments or passage of the virus from infected oat tissue. After the agar was solidified, moisture was removed with a kimwipe from the sides of the vessel and from the surface of the agar, to prevent the aphids from drowning when applied to plants in the vessel. Oat seedlings and/or leaf blades from infected oat plants were placed directly onto or into the solid agar base (see figure 1A & B) and aphids were
tapped onto young plants (figure 1C & D). I used this vessel for all aphid transmission and passage experiments.

In addition to this, I made a second modification to the protocol that addressed a very important step in the aphid transmission experiment. That is, effectively removing aphids from plants before transferring the plants to soil. This step was very critical for me because each group of tested plants were infected with a different virus construct. Given that all plants in my experiment were grown in the same growth chamber, cross contamination was a real issue. Completely removing the aphids from the plants and maintaining an aphid-free environment became a top priority that had to be addressed. Previously, an insecticidal soap was used, along with removing the aphids from seedlings under running water. I found this to be ineffective at removing aphids from plants completely and experienced several aphid outbreaks in the growth chamber. The insecticidal soap was too harsh for use on the leaves of young plants and was very ineffective at controlling aphid outbreaks.

To address this issue, I used Orthenex Garden Insect & Disease Control Concentrate as an alternative insecticide. It is a systemic insecticide that effectively kills and controls aphids and other insect pests. It did not harm the leaves of the young plants and effectively killed all feeding aphids. The chemical should be used at concentration of 1 fl oz (2 Tbs) per gallon of water. I used 100 ml of diluted Orthenex in a large weigh boat to kill aphids before transferring them to soil (figure 2A). Oat seedlings were placed into the insecticide and allowed to sit for 1 minute. Plants were agitated with forceps to dislodge feeding aphids. After 1 minute, plants were blotted on brown paper towels and then transferred to soil (figure 2B). This practically eliminated aphid problems, but I did
encounter a few aphids on plants during earlier experiments. However, they were spotted quickly and were confined to just one particular plant. To remedy this problem, plants were occasionally sprayed during the first week or two to ensure that no aphids survived. I found that this additional spraying completely solved the problem. I should mention that if you plan to passage the virus into healthy oat plants, allow the effect of the systemic insecticide to wear off before applying aphids. In a few passage experiments, aphids were killed while feeding on infected leaf blades that were previously sprayed. Waiting for at least 2 weeks after application of the orthenex should be sufficient.
Figure 1. A. GA-7 vessel containing agar and cut leaf blades from healthy oats. B. Close-up leaf blades inserted into agar. C. Aphids feeding on oat seedlings in a GA-7 vessel during an aphid transmission experiment. D. Aphids feeding on leaf blades in a GA-7 vessel during a virus passage experiment. Aphids were allowed to either acquire or transmit the virus for 24 to 48 hours.
Figure 2. A. Soaking of oat seedlings in orthenex solution to kill aphids after removal from GA-7 vessel. B. Blotting of excess insecticide from Oat seedlings on brown paper towels before transfer to soil.
APPENDIX 3. Construction of ΔSG2 and ΔSG3 knockout mutant Gateway™ Destination Vectors.

The core promoter for subgenomic RNA2 was previously mapped upstream in ORF 5 at a Kpn I4154 site in the sgRNA2 knockout mutant, pSG2G/C. In experiments conducted in chapter 2, duplicate copies of the promoter were mapped upstream in ORF 5 between the HpaI3785 and KpnI4154 restriction sites (see figure 2A). This deleted additional sequence that would allow the artificial sgRNA2 to migrate ahead of the ribosomal front, which sometimes gives the appearance of a band in northern analysis. The sgRNA2 (SG2G/C) and sgRNA3 (SG3G/C2) knockout mutants were converted into Gateway™ destination vectors for fast and efficient cloning of mutant versions of the sgRNA2 and sgRNA3 promoters at the HpaI3785 and KpnI4154 restriction sites. The resulting gateway vectors are ΔSG2XP2 and ΔSG3XP3, respectively.

Construction of the ΔSG2XP2 and ΔSG3XP3 Gateway destination vectors

The gateway conversion cassette™, frame A, was PCR amplified using the forward primer 5′-AACATCACAAGTTTGTA-3′, which is complementary to bases 1-14 of the frame A cassette sequence and contains half of the HpaI restriction site as a 5′ extension and the reverse primer 5′-GCGCAGGTACCACCTTTGTACAAAGA-3′, which is complementary to bases 1693-1711 of the frame A sequence and includes a KpnI restriction site 5′ extension. The resulting PCR product was digested with KpnI and recovered using the Qiagen PCR clean-up kit. Both SG2G/C and SG3G/C2 plasmids were double digested with Promega brand HpaI and KpnI restriction enzymes and the larger band was gel purified. The KpnI digested “frame A” PCR amplified product was cloned into the double digested SG2G/C and SG3G/C2 vectors to generate the sgRNA2 (ΔSG2XP2) and sgRNA3...
(ΔSG3XP3) knockout gateway destination vectors. However, only the ΔSG2XP2 gateway vector was used for cloning in chapter 2 of this thesis. For future sgRNA3 promoter mapping studies, the ΔSG3XP3 gateway vector can be used in the same manner as ΔSG2XP2.

The TOPO cloning system (Invitrogen) was used to develop entry clones to be used in recombination reactions with the sgRNA knockout destination vectors. It allows for fast and efficient directional cloning of blunt ended PCR into entry vectors and serves as the fast method of entry into the gateway cloning technology through the use of a simple 4 base (CACC) addition to your 5’ primer. A PCR product containing the wt version of the sgRNA2 promoter and a 5’ CACC TOPO extension were cloned into a pENTR/D-TOPO vector. The resulting recombination reaction generated the entry clone that was used in the final recombination reaction (LR) with the newly constructed destination vector.

To create the gateway expression clone (the sgRNA knockout mutant with ectopic promoter insert), in which the wt promoter sequence in the entry clone is transferred to the gateway destination vector, a simple 1 hour LR reaction was performed. 1 μl of this LR reaction was added to 50 μl of DH5α cells and plasmid DNA was isolated from resulting colonies. Potential clones were confirmed through Bam HI restriction digestion and sent for sequencing. The clone was SmaI digested and RNA transcript was made using the Ambion’s MEGAscript T7 transcription kit.

**Testing the Gateway-derived sgRNA2 ectopic promoter mutant**

Sequencing results showed that 45-50 bases of extra sequence were introduced through the TOPO/Gateway cloning process. Mfold analysis revealed a rather long and
stable helix formed by this introduced sequence (figure 1). To test whether the extra sequence introduced by the Topo and Gateway<sup>R</sup> cloning process interferes with translation and replication of the virus, 10 μg of RNA was electroporated into oat protoplasts and incubated for 48 hrs at room temperature. Total RNA was isolated using TRIZOL reagent and analyzed via northern analysis (figure 2C). The results confirmed that the extra sequence introduced by the Gateway<sup>R</sup> cloning process did not interfere with translation and replication of the virus.
Figure 1. Mfold-predicted secondary structure of the remaining minimal stem-loop of the ectopic promoter (highlighted) flanked by sequences remaining from the TOPO/Gateway cloning process. Extra “A”s were added to ensure that the viral stem-loop would remain separate form with the remaining promoter structure. Restriction sites where the gateway conversion cassette was inserted are indicated. RNA secondary structure was determined using CLC combined workbench3 software.
Figure 2. Northern analysis to test the feasibility of using the gateway system to clone mutant sgRNA2 promoter fragments into the ectopic location (between the \textit{HpaI}_3785 and \textit{KpnI}_4154 sites) of sgRNA2 knockout gateway destination vector. (A) \textit{ΔSG2XP2} expression clone with ectopic promoter upstream of wt sgRNA2 promoter. Small squares indicate the 3′BTE and boxes with arrows indicate the sgRNA2 promoter region. Larger colored boxes indicate the six ORFs encoded by BYDV. (B) SgRNA2 knock-out mutant. Labels
identifying the RNAs are located on the left. (C) Northern analysis of gateway-derived clone. Lane 1: Pav6 wt. Lane 2: Control construct, ΔSG2XP2wt, which was made by ligating a PCR product containing the wt sgRNA2 promoter into the ectopic location HpaI\textsubscript{3785} and KpnI\textsubscript{4154} restriction sites. This produces a new artificial sgRNA2 (sgRNA2a). Lane 3: Gateway-derived ΔSG2XP2wt, which is essentially the same construct as in lane 2 with the exception of additional sequence left over from the TOPO/Gateway cloning process. Lane 4: the gateway destination vector. Insertion of the gateway conversion cassette into the ectopic location in ΔSG2 completely disrupted viral replication to undetectable amounts.
APPENDIX 4. Evidence for PAV6-129 having an sgRNA3 and possibly a fourth sgRNA.

Figure 1. Northern blot analysis of RNA from PAV6 and PAV6-129 infected protoplasts probed with a PAV6-129 specific probe. Lane 1 shows that this probe does not detect PAV6 as well as it does PAV6-129 in lane 2. An extra sgRNA appears to be present in PAV6-129-infected cells and would be a new sgRNA species produced by a luteovirus. The genomic RNA appears to be reduced in amount relative to sgRNAs and PAV6 RNAs.
APPENDIX 5. Additional images of BYDV and BYDV mutant virus symptoms in infected oats

The following photographs catalog virus symptoms observed in infected oat plants aphid transmitted with wild-type and subgenomic knockout mutant viruses. Plants in figures 2-5 catalog virus symptoms observed in plants that did not show typical reddening and/or yellowing of the leaves. Instead, various forms of leaf deformation were observed. These plants were part of an attempt to passage the virus from plants what were infected by aphids that were fed wild-type and mutant virus through membranes. Presence of the virus in these aphid transmitted plants (plants used to passage from) was confirmed via Northern blot analysis and virus symptoms were observed. Leaf blades from these plants were then used to passage the virus into healthy oat plants, thus being the first passage. Interestingly, plants passaged from a few of these virus positive plants failed to show symptoms. I then attempted to re-passage the virus again into healthy oat plants from these same plants that were previously confirmed to have the virus. These are the plants shown in figures 2-5. These plants never exhibited the usual reddening or yellowing of leaves, but displayed unusual leaf deformations instead. They were confirmed to be virus positive via RT PCR and all photographed symptoms can be found in digital format in the 10-25-05 re-passaged plant file folder on a lab archival disk in the Miller lab. Plants are indentified in the following tables for each figure and include the original file name of each photograph and the date of the file folder where each are located.

**Digital photo key for figure 1:** Virus symptoms in PAV-129 infected plants

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Figure 1.
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