New Technologies for Studying Negative-Strand RNA Viruses in Plant and Arthropod Hosts

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The plant viruses in the phylum Negarnavirciota, orders Bunyavirales and Mononegavirales, have common features of single-stranded, negative-sense RNA genomes and replication in the biological vector. Due to the similarities in biology, comparative functional analysis in plant and vector hosts is helpful for understanding host–virus interactions for negative-strand RNA viruses. In this review, we will highlight recent technological advances that are breaking new ground in the study of these recalcitrant virus systems. The development of infectious clones for plant rhabdoviruses and bunyaviruses is enabling unprecedented examination of gene function in plants and these advances are also being transferred to study virus biology in the vector. In addition, genome and transcriptome projects for critical nonmodel arthropods has enabled characterization of insect response to viruses and identification of interacting proteins. Functional analysis of genes using genome editing will provide future pathways for further study of the transmission cycle and new control strategies for these viruses and their vectors.

Keywords: virus–plant interactions

Viruses of the phylum Negarnavirciota have increased in numbers with the development and application of high-throughput sequencing (HTS) strategies for studying the viromes associated with insects, plants, and other eukaryotes. HTS technologies have enabled the discovery of new viruses and provide the needed sequence data for better identification and distinction of taxonomic groupings. Coupling HTS with disease biology and symptomology in the field has revealed a plethora of new negative-strand RNA (NSR) viruses associated with long-described diseases and emerging diseases. Plant- and insect-infecting viruses classified in the orders Bunyavirales and Mononegavirales have increased in numbers and are considered emerging virus pathogens (Fig. 1). Plant NSR viruses are classified in the families Rhabdoviridae, Tospoviridae, Aspiviridae, Phenuiviridae, and Fimoviridae and the unassigned genus Coguvirus (Abudurexiti et al. 2019). The majority of these have arthropod vectors that also serve as replicative hosts of the virus (Kormelink et al. 2011). For the plant-infecting bunyaviruses, phylogenetic analysis supports the hypothesis that the plant-infecting orders and genera evolved independently multiple times from invertebrate-infecting viruses (Chen et al. 2019; Junglen 2016; Marklewitz et al. 2015). The plant-infecting genera form distinct clades that are more closely related to other invertebrate viruses than to one another (Chen et al. 2019). Analysis of the rhabdoviruses suggests that there was a single origin of the known plant viruses and arthropod-borne vertebrate viruses, whereas vertebrate- and arthropod-specific viruses arose at least twice (Longdon et al. 2015), and the arthropod origin of the plant-infecting members is supported by accumulating evidence (Whitfield et al. 2018). Longdon and colleagues (2015) proposed that evolutionary evidence suggests that rhabdoviruses have occasionally jumped between distantly related host species and then, from that initial introduction, they spread through related hosts.

The viral particles of NSRs are negative-sense, single-stranded RNA coated in nucleoprotein and, for most of the viruses, these ribonucleoproteins (RNPs) are encapsulated in an envelope. Rhabdoviruses are typically composed of one genomic RNA, except for the dichorhaviruses, with two RNA segments and the tenuiviruses and coguviruses do not appear to form an enveloped particle. When compared with their animal-infecting counterpart viruses, the plant NSRs tend to have an increased number of genome segments and some have lost their ability to form enveloped particles. Essential proteins encoded by these viruses include the RNA-dependent RNA polymerase (RdRp), nucleoprotein, plant movement protein, viral silencing suppressor, and glycoproteins (GPs). Rhabdoviruses have an additional phosphoprotein and matrix protein. The reverse-genetics approaches described later in this review are essential tools that enable functional studies of these proteins and their role in the virus replication cycle in plant and vector hosts.

VECTOR TRANSMISSION OF NSR VIRUSES

Vector transmission of rhabdoviruses and bunyaviruses is mediated by diverse biological vectors, including hemipterans,
thysanopterans, arachnids, and fungi. The common theme in the ability of these diverse organisms to serve as vectors of NSRs is that they can acquire and transmit virus particles from and to living plant cells. Delivery of the infectious particle into a viable cell is the key determinant for successful transmission. Vector specificity of these viruses aligns with their phylogenetic relationships. Hemipteran and thysanopteran transmission of NSRs is well characterized and shares some common features, including replication of the virus in the insect. For insect-transmitted NSRs, the particles are ingested during feeding on the plant and the virus first enters the gut cells, establishes an infection in the epithelial cells, spreads in the gut cells, and then traverses gut escape barriers. The dissemination route in the vector appears to be specific to the virus genus–vector combination. For example, tospoviruses have not been observed in the insect hemocoel whereas tenuiviruses are found in association with host factors that promote dissemination to other insect tissue systems (Huo et al. 2018; Montero-Astúa et al. 2014). For inoculation to a new plant host, the virus must reach and replicate in the salivary gland cells. Some of the NSR viruses are also transovarially transmitted to insect offspring.

**GP FUNCTION IN VECTOR TRANSMISSION**

Recent advancements have been made in understanding the viral and vector proteins that interact for successful transmission of NSR viruses. This progress has been facilitated by the application of genomic, transcriptomic, and proteomic tools to the study of nonmodel insects. The role of viral GPs in binding and interactions with insect vectors have been defined and the host proteins that function in entry, dissemination, and even transovarial transmission identified. Areas for future emphasis include detailed structural analysis of plant NSR vi- rions and resolution of virus GP three-dimensional structures. Of the NSR viruses, perhaps one of the most-studied systems is the *Frankliniella occidentalis*–tomato spotted wilt virus (TSWV) interaction. TSWV was the first identified plant-infecting member of the bunyaviruses (Francki et al. 1991). The role of the two GPs (Gn and Gc) has been inferred by similarity with other bunyaviruses and direct experimentation. The Gn protein can bind to thrips midguts in the absence of other viral proteins, suggesting that it is a viral attachment protein (Whitfield et al. 2004). In transmission-blocking experiments, Gn disrupts virus acquisition and transmission by the thrips vector (Montero-Astúa et al. 2014; Whitfield et al. 2008). Recent structural analysis of animal bunyaviruses agrees with the role of Gn in viral attachment because the structure reveals that Gn resides on the surface of the particle and shields the Gc protein (Haldorsson et al. 2018; Wu et al. 2017). The Gc protein is the fusion protein and, upon acidification, the Gc conformation changes to expose a fusion loop that can insert into the host membrane (Dessau and Modi 2013; Garry and Garry 2004; Guardado-Calvo et al. 2016; Whitfield et al. 2005b).

The tenuiviruses are interesting bunyaviruses because they are phylogenetically placed in the family Phenuiviridae and encode two GPs like the tospoviruses but they do not form enveloped virus particles. The four genome segments are encapsidated in nucleoprotein (N) and are thought to constitute the viral particle, and clear functional roles for N-vector proteins have been defined (Liu et al. 2018). Recent work by Lu et al. (2019) provides strong evidence that the two GPs of the tenuivirus rice stripe virus (RSV) serve functions in the insect vector analogous to those of other bunyaviruses and interact with the RNPs to promote virus entry into the vector. The Gn homolog NSvc2-N binds to planthopper vector midguts and serves as a viral attachment protein (Lu et al. 2019). The Gc homolog NSvc2-C mediates membrane fusion, and both GPs interact with the N protein. The GPs are hypothesized to interact with the nucleocapsid or viral particles and serve as a molecular bridge or helper component for virus acquisition by the planthopper vector (Lu et al. 2019). These findings show functional conservation of the viral GPs despite vast differences in particle structure for tospoviruses and tenuiviruses.

Plant rhabdoviruses encode a single viral GP (G) that is hypothesized to be the viral attachment and entry protein. The G protein is thought to be the determinant of vector specificity for viruses transmitted by diverse vectors (hemipterans, arachnids, and fungi). Comparative functional analysis of NSR GPs will be an interesting topic for future studies enabled by reverse genetics (infectious clones) of these viruses.

**VECTOR PROTEINS THAT INTERACT WITH NSR VIRUS PROTEINS**

The development of insect transcriptome and genome resources has enabled significant advancements in understanding the molecular basis of vector–virus interactions. Due to the importance of orthotospovirus and tenuiviruses as pathogens of agronomic crops, these are the best characterized virus–vector protein interactions for the NSRs. However, the development of reverse-genetics systems for rhabdoviruses and identification of additional important plant-pathogenic NSR viruses will increase the knowledge base for this group of viruses.

The identification of vector proteins that are involved in RSV transmission by the small brown planthopper (SBPH) *Laodelphax striatellus* Fallén (Hemiptera: Delphacidae) were initially identified in yeast two-hybrid screens to identify protein interactions between the nucleocapsid protein (PC3) and *L. striatellus*. PC3 protein served as the bait in the screen and the cDNA library of the planthopper vector as prey, and numerous interesting interactions were documented from this screen (Huo et al. 2014; Liu et al. 2015, 2018). The identification of a PC3–vitellogenin interaction and characterization of the protein role in virus dissemination described a new pathway for viral invasion of insect eggs (Huo et al. 2014). Microscopic examination of PC3 and vitellogenin localization in the planthopper revealed that the two proteins colocalized in the ocellar gerarium and the viral RNPs entered nurse cells of the gerarium and, subsequently, moved into the oocytes via the nutritive cords (Huo et al. 2014; Liu et al. 2018). Additional analysis of this transmission route identified that vitellogenin processed in hemocytes interacts with RSV, leading the authors to hypothesize that this form of

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**Fig. 1.** Plant-infecting viruses in the taxonomic groupings of the orders Bunyavirales and Mononegavirales. Plant rhabdoviruses are divided into four genera and plant bunyaviruses are divided into three distinct families with unique properties. *Genus Coguvirus* (indicated with an asterisk) has not been assigned to a family at this time.
vitellogenin protects viral particles in the hemolymph and aids in invasion of the insect eggs (Huo et al. 2018). This finding is important because tenuiviruses are efficiently transovarially transmitted and the infected eggs may be an important reservoir for the virus. Additional L. striatellus proteins identified to interact with RSV include a cuticular protein, CPR1, that binds to PC3 and colocalizes in hemocytes (Liu et al. 2015). Cuticle proteins of diverse insect vectors have been found to bind directly to viral attachment proteins and are now being recognized as important players in vector competency (Deshoux et al. 2018). More recently, a sugar transporter 6 was identified as a potential receptor for RSV (Qin et al. 2018). The protein was highly expressed in the midgut of L. striatellus and localized to the surface of insect cell membranes, the expected site of a receptor (Qin et al. 2018). The key experiments to show involvement of this insect protein in virus were (i) expression in a heterologous cell system enabled RSV entry and (ii) knock-down of sugar transporter 6 expression by RNA interference (RNAi) reduced virus entry but did not affect virus replication. Functional analysis of interacting proteins has been facilitated by the robust RNAi responses of planthoppers (Yao et al. 2013). Further characterization of tenuivirus–plant hopper interactions is expected to inform basic biology of this system for new disease control strategies that target specific vector molecules involved in the transmission cycle and facilitate transfer of knowledge to more recalcitrant vectors systems such as thrips and mites.

The first thrips proteins that directly interact with the TSWV Gn protein have been identified and they are candidates for further study in this important virus–vector pathosystem. Badillo-Vargas et al. (2019) found three cuticle proteins in larval thrips that interacted with TSWV proteins in gel overlay assays. These results support the earlier work showing that cuticle protein transcripts were dysregulated in virus-infected larval thrips (Schneweis et al. 2017). The endocuticle GP that interacted with Gn in several biochemical assays also colocalized with the viral protein and has a unique virus-interaction domain that is not found in other organisms. Cyclophilin is another protein identified to bind TSWV Gn that continues to appear in diverse virus–vector protein interaction studies (Badillo-Vargas et al. 2019). In experiments with circulative viruses (geminiviruses and luteovirids), cyclophilin was shown to be important in their respective vectors (whiteflies and aphids) (Kanakala and Ghanim 2016; Tamborindeguy et al. 2013). At this time, functional analysis of the proteins in thrips vectors is limited by technological barriers because RNAi has only been successful in adult thrips and larval thrips are the only stage of the insect that can acquire the virus (Badillo-Vargas et al. 2015; Han et al. 2019). The application of genome-editing technologies to thrips vectors may be required for functional analysis of TSWV-interacting proteins of thrips.

REVERSE-GENETICS SYSTEMS FOR NSR VIRUSES

The use of recombinant DNA technologies to generate infectious clones or infectious transcripts derived from the RNA genomes of plant viruses has had an enormous impact on the current state of knowledge concerning their replication, expression strategies, interactions with host components, and mechanisms underlying movement within and between plants. Early success with positive-sense RNA viruses such as brome mosaic virus (Ahluquist et al. 1984), tobacco mosaic virus (Dawson et al. 1986), and cow pea mosaic virus (Vos et al. 1988) presaged the development of infectious clones of viruses in some 18 plant virus families (Boyer and Haenni 1994; Bujarski and Miller 1992). The genomic RNAs of positive-sense RNA viruses encode the viral protein complement in the translatable sense, rendering the unencapsidated RNAs infectious upon introduction into a susceptible cell. NSR viruses have genomes that are either nonsegmented or segmented and, in contrast to positive-sense RNA viruses, their open reading frames (ORFs) are encoded in the noncoding sense, dictating that successful infection must be proceeded by transcription from an RNP complex catalyzed by a viral-encoded RdRp (Jackson and Li 2016). The requirement for the expression of all the essential components in susceptible cells is inherently inefficient and has proven to be an obstacle to the development of reverse-genetics systems. In spite of these challenges, since the first generation of a nonsegmented negative-sense RNA virus entirely from infectious clones (Schnell et al. 1994), reverse-genetics systems have been established for several other nonsegmented (Conzelmann 2004) as well as segmented (Lowen et al. 2004; Neumann and Kawaoka 2004; Neumann et al. 1999; Walpita and Flick 2005) vertebrate-infecting viruses. The generation of reverse-genetics systems for these groups of viruses has proven to be extremely recalcitrant but recent advances in technology have led to the development of robust systems for several NSR plant viruses in the families Rhadoviridae, Tospoviridae, and Fimoviridae and will be reviewed here.

GENERAL APPROACH TO CONSTRUCT REVERSE-GENETICS SYSTEMS

Although each viral reverse-genetics system has its unique attributes, there are a core set of principles and protocols that have been used in their development. A method to deliver multiple plasmids expressing viral genomic RNAs and expression cassettes for core replication proteins is an absolute requirement for such systems. For plant viruses, this is complicated because of the need to breach the cell wall of plant tissues and by the lack of tractable plant or insect cell cultures. Transient or stable expression of proteins and RNA genomic (gRNA) or antigenomic (agRNA) segments from Agrobacterium tumefaciens binary vector systems is the standard way to achieve this goal. Because exact viral 3’ and 5’ ends are presumed to be important for recombinant NSR rescues, vectors for this purpose require a promoter (typically a modified 35S or duplicated 35S, bacteriophage T7, or SP6) to drive transcription of an exact or nearly exact 5’ end and a self-cleaving hepatitis Δ (HDV) ribozyme (HDR) positioned to produce a transcript corresponding closely to the 3’ ends of viral genomic strands. Alternatively, an inducible promoter (such as Pol I or Pol II) has been used to generate a nearly precise transcript or inclusion of hammerhead (HH) ribozyme to cleave the transcribed strand and leave a 5’ terminus representative of the agRNA (Ishibashi et al. 2017). An extremely important antecedent to the development of a reverse-genetics system capable of generating wild-type infections from cDNA clones was the establishment of a minireplicon reporter system (Jackson and Li 2016). Minireplicons comprise a subset of viral RNA such as a defective-interfering particle, a cloned subset of the viral RNA containing reporter genes with appropriate cis-acting elements included, or a small single segment of a segmented viral genome. They optimally include functional cis-acting elements required for replication and transcription of a reporter gene (e.g., green fluorescent protein [GFP], β-glucuronidase [GUS], or luciferase). By assaying for reporter gene expression mediated by the viral core proteins, this approach is useful to test accessory protein functions and cis-acting elements on RNA templates, and optimizing stoichiometry. It is particularly useful for addressing the complications created by
the large and complex RdRp sequences of many NSRs, which can be difficult to clone accurately because of their large size and potential for inclusion of mutations, deletions, cryptic splice sites, or other artifacts that can obviate their functionality.

**REVERSE GENETICS FOR A NUCLEORHABDOVIRUS**

The first reverse-genetics system for a plant NSR virus was developed using sonchus yellow net virus (SYNV), a non-segmented nucleorhabdovirus, aphid-transmitted NSR that completes replication and morphogenesis in association with the plant cell nucleus (Ganesan et al. 2013). Typical of rhabdoviruses, SYNV encodes five structural proteins: a nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), GP (G), viral polymerase (L), and nonstructural protein, sc4, that facilitates cell-to-cell spread (Wang et al. 2015; Zhou et al. 2019). These are arranged on the agRNA in the order 5′-le-N-P-sc4-M-G-L-tr. The agRNA includes a 5′ leader sequence (le) and a trailer sequence (tr) at the 3′ end of the molecule (Fig. 2). Following primary transcription of viral gRNA from the 5′ end to the 3′ end, polyadenylated mRNAs are generated for each gene product in decreasing amounts in a process controlled by cis-acting initiation and termination signals at the gene junctions. After replication, the N, P, and L core proteins assemble with viral gRNA and agRNA in nuclear vioplasts to form nucleocapsids that function in viral replication and secondary transcription in the SYNV-infected cells (Jackson et al. 2005). Near the end of the replication cycle, genomic nucleocapsids interact with the M protein and morphogenesis occurs by envelopment at the nuclear inner membranes studded with viral GPs. (Jackson and Li 2016; Jackson et al. 2005; Sun et al. 2018).

Construction of SYNV minireplicons (Ganesan et al. 2013) was critical for development of a complete SYNV system capable of producing infectious wild-type virus (Wang et al. 2015). One reporter construct consisted of an enhanced GFP (eGFP) gene substituted for the SYNV N gene and a red fluorescent protein (RFP) (DsRed) gene replacement of the SYNV P gene. A second reporter incorporated an N gene substitution with a red-shifted GFP (reGFP) gene and a chloramphenicol acetyltransferase reporter substitution for the P protein. The expression cassette for these constructs, which were inserted between the left and right borders of an Agrobacterium transformation vector, included a double cauliflower mosaic virus (CaMV) 35S promoter; an HH ribozyme to generate a near perfect 5′ end; and the cis-acting signals, including wild-type gene junctions controlling transcription from the bicistronic construct followed by the tr sequence and an HDR predicted to generate an exact viral 3′ end. When the minigenome constructs were agroinfiltrated into Nicotiana benthamiana leaves along with expression vectors encoding the N, P, and L core proteins and three viral-silencing suppressors, clear signals from the fluorescent reporter genes were evident as single-cell foci within the infiltrated regions. Moreover, Western blot analysis demonstrated production of the respective proteins. Importantly, these results were obtained only if all of the core proteins were present in the infiltrated mixture of minireplicon plasmids and the three suppressor proteins enhanced the number of foci by more than 20-fold over mixtures lacking the suppressors. Importantly, the results confirm that the progeny minireplicon agRNAs and gRNAs become associated with the N, P, and L proteins to form nucleocapsids that can be transcribed and translated in a polar manner and replicated in a manner representative of wild-type SYNV. In addition, the minireplicons enabled functional analysis of core protein mutants and cis-acting effects of le and tr sequence modifications on minireplicon rescue and reporter gene expression. Of significance for future attempts with other viruses is that exact HH ribozyme processing of primary 35S transcripts is not absolutely essential for minireplicon rescue because a 16-nucleotide (nt) sequence preceding the 5′ terminus of the le sequence was able to function in substantial minireplicon rescue.

For rescue of wild-type SYNV, constructs were engineered that included the entire viral agRNA containing a GFP reporter between the N and P genes (Wang et al. 2015). Agroinfiltration of this construct, along with the core proteins and silencing suppressors, produced systemic infection that could be monitored by GFP detection and passed from plant to plant by mechanical transmission. Various modifications, including elimination of the 5′ ribozyme, use of a modified 35S promoter to facilitate accurate transcription at the immediate 5′ terminus of the agRNA, and alterations of the agroinfiltration mixtures to increase the abundance of L protein, eventually resulted in up to 25% infection of infiltrated plants (Jackson and Li 2016). Thus, a functional reverse-genetics system capable of rescue of a plant NSR entirely from cloned cDNA was achieved for the first time.

To demonstrate utility of the recombinant virus, proof-of-concept experiments showed that the GP is dispensable for systemic infection but is required for virion morphogenesis and that the deletion of sc4 abrogates cell-to-cell movement. The system was used for a deletion mutant analysis to dissect the specific components of the SYNV matrix and GP interactions required for morphogenesis (Sun et al. 2018). The unique functions of the sc4 protein in SYNV movement provide a model that may be applicable to other NSR viruses (Zhou et al. 2019). Positive-strand RNA virus movement proteins display reciprocal cross-family movement complementarity across a wide range of taxa. In contrast, Zhou et al. (2019) showed that rhabdovirus movement proteins could trans complement positive-sense RNA viruses from two different taxa but that recombinant rhabdoviruses with deletions in the movement protein could only be rescued by their cognate movement proteins. Further support for this specificity was provided by yeast two-hybrid analysis showing that membrane-associated rhabdovirus movement proteins would only interact with their cognate movement protein and phosphoproteins. These data demonstrated that the cell-to-cell movement of plant rhabdoviruses is uniquely specific.

**REVERSE GENETICS FOR A CYTORHABDOVIRUS**

**Barley striate mosaic virus** (BSMV) is a member of the genus Cytorhabdovirus, distinguished by cytoplasmic sites of replication and maturation (Jackson et al. 2005). BSMV is transmitted in a propagative manner (Conti 1980) by L. striatellus to some 26 species of the Gramininae family, resulting in serious food production losses on a worldwide basis (Izadpanah et al. 1991; Makkouk et al. 1996, 2004). The BSMV genome comprises 12,706 nt that encode 10 proteins in an arrangement that is a variation on the typical genome composition of rhabdoviruses. The gene order 3′-N-P-P3-P4/P5-P6-M-G-P9-L-5′ includes four ancillary proteins located in the junctions between the P and M genes that are organized into three transcriptional units, in which gene 5 is nested within gene 4 in an alternative reading frame (Yan et al. 2015).

To create a reverse-genetics system, Gao et al. (2019) cloned the entire BSMV antigenome and inserted it into an A. tumefaciens expression plasmid with a double 35S promoter (2X35S) at the 5′ end and an HDV ribozyme positioned to
cleave the 3’ prime end of the transcript. To monitor virus infection in vivo, the clone was engineered with an RFP reporter gene flanked by the N/P gene junction sequences (required for initiation and termination of transcription) inserted between the N and P genes. To test this for biological activity, the full-length reporter clone along with expression cassettes for the N, P, and L proteins (required for the formation of transcriptionally active ribonucleoprotein structures) and three

Fig. 2. Construction of a sonchus yellow net nucleorhabdovirus (SYNV) minireplicon plasmid (pSYNV-MRGFP-DsRed). Ribozyme cleavage of precursor MRGFP-DsRed antigenomic RNA (P agRNA) transcripts from pSYNV-MRGFP-DsRed for MR nucleocapsid (NC) formation, replication, and reporter gene expression. Comparison of the SYNV antigenomic RNA (agRNA) and pSYNV-MRGFP-DsRed engineered from recombinant SYNV (rSYNV) for Cauliflower mosaic virus (CaMV) double 35S (35S2) promoter-directed transcription of P MR agRNAs. The P MR agRNAs consist of a hammerhead (HH) ribozyme, the SYNV 5’ leader (l) sequence, the 5’ untranslated region (UTR) of the nucleocapsid protein (N) gene, a green fluorescent protein (GFP) open reading frame (ORF) substitution for the N-gene ORF, and a Discosoma sp. red fluorescent protein (DsRed) ORF replacement for the phosphoprotein (P) ORF followed by the 3’ UTR of the large polymerase protein (L) gene, the 3’ sequence, and a hepatitis D virus (H) ribozyme. Cleavage of the P MR agRNAs in cis by the HH and H ribozymes generates SYNV-MRGFP-DsRed agRNAs (MR agRNAs) for encapsidation by the N, P, and L core proteins expressed ectopically from N, P, and L plasmids (35S2 N, 35S2 P, and 35S2 L) to form antigenomic nucleocapsid (agNC) intermediates. The agNCs then serve as templates for replication of MRGFP-DsRed genomic NCs (gNCs) that function in transcription of GFP and DsRed mRNAs. Republished with permission of Annual Review of Phytopathology from Jackson and Li (2016); permission conveyed through Copyright Clearance Center, Inc.
viral RNA silencing suppressors (VSRs) (p19, HcPro, and yb) were agroinfiltrated into *N. benthamiana* leaves. Within 14 days postinoculation (dpi), high-intensity fluorescence was observed in cells inoculated with this mixture of plasmids. As was the case with SYNV described above, this result was totally dependent on the presence of all components of the system. Because *L. striatellus* will not feed directly on *N. benthamiana* plants, to transmit this recombinant virus, expressing plants were ground in buffer, injected into SBPH, and transferred to healthy rice plants. After a 10-day incubation period, RFP fluorescence could be detected in a significant percentage of the *L. striatellus* that survived injection but not in mock injected insects. Furthermore, the insects that had acquired the recombinant virus could transmit to healthy barley plants and generate a robust, systemic infection that produced typical symptoms. Transmission of the BSMV infectious clone by the insect vector is significant and shows that this recombinant virus can be used to study interactions with the insect and plant hosts. Additional experiments showed that the roots and leaves of wheat, barley, foxtail millet, and maize would report the expression of GUS and RFP from a single vector. A significant revelation from this experiment is that the GUS gene is relatively large, indicating that, unlike many positive-sense RNA vectors, BSMV can stably accommodate large inserts.

To demonstrate the expression of multiple gene insertions from a single recombinant virus, constructs were produced that included GFP, RFP, and cyan fluorescent protein reporter genes (in that order from the 5′ end of the molecule) on the same viral RNA clone. Rescue of this viral construct in *N. benthamiana* as above, followed by injection of insects, revealed that all three reporter genes were expressed in insects and, after transfer to healthy plants, resulted in the production of infections that reported all three signals. Interestingly, in agreement with the polarity of mRNA transcription of rhabdoviruses (Jackson and Li 2016; Jackson et al. 2005), fluorescence of the reporter genes decreased progressively relative to the distance from the 5′ end of the molecule. These experiments confirmed an important property of being able to simultaneously express multiple foreign genes and provide a means to regulate their relative levels of abundance.

Given the capacity of this system to deliver multiple and large proteins to cells, experiments were done to test its capacity to deliver CRISPR/Cas 9 nucleases and guide RNAs for genome editing in plants. To accomplish this, a vector was constructed to deliver Cas9 nuclease, a guide RNA, and an RFP reporter to track their cellular location. The guide RNA was designed to target the GFP gene on the chromosome of *N. benthamiana* line 16c plants in such a way as to knock out an *NdeI* restriction digestion so that successful genome modification could be demonstrated by PCR analysis. Obtaining a PCR signal from modified (no restriction site) but not the unmodified (cuts with *NdeI* and abolishes PCR signal) genome demonstrated the expected modification. An additional example of functional genomics application was provided by creating vectors to express RFP and two genes that regulate gibberilic acid biosynthesis (GA5) and inactivation signaling (GA2ox1) that, in turn, regulate cell elongation and morphogenesis. Vectors were constructed that harbored these genes and were rescued in barley plants as above. The effect of overexpression of these genes was the production of dwarf plants in the case of GA2ox1 and longer plants in the case of GA5 compared with plants infected with control virus expressing GFP and RFP as the only nonviral genes. To demonstrate a similar ability in insect vectors, constructs were rescued in plants that express the B2 silencing suppressor from flock house virus and RFP. After injection of sap from *B2*-expressing plants into *L. striatellus*, they detected the RFP gene at 6 dpi, compared with 10 dpi for the insects with virus that did not contain the suppressor construct, confirming that it was expressed, active, and improving the efficiency of gene expression. Taken together, these experiments and observations clearly demonstrate the establishment of a robust and tractable reverse-genetics system to be used in plant hosts of BSMV and its vector.

**A REVERSE-GENETICS SYSTEM FOR AN ORTHOTOPSIVIRUS**

Reverse-genetics systems have been developed for segmented NSR animal-infecting viruses such as for bunyamwera virus (Bridgen and Elliott 1996), which has three gRNA segments; influenza virus (Neumann et al. 1999), which has eight RNA segments; and the bipartite arenavirus (Flatz et al. 2006). The first such system for a plant-infecting NSR virus capable of the complete recovery of infectious virus entirely from cDNA clones based on TSWV (Feng et al. 2019) will be described here. Orthotospoviruses are of increasing importance, with 18 species approved by the International Committee on Taxonomy of Viruses and an additional 11 described viruses that have not been taxonomically approved (Abudurexiti et al. 2019; Rotenberg and Whitfield 2018). TSWV is an NSR with a tripartite genome and is transmitted by thrips, tiny insects in the order Thysanoptera. It is one of the 10 most devastating plant viruses (Scholthof et al. 2011) because of the worldwide distribution of the virus and its vectors (Pappu et al. 2009) and a host range that includes more than a thousand species of plants, many of which are important food, fiber, or ornamental crops, resulting in enormous economic losses (Adkins 2000; German et al. 1992; Whitfield et al. 2005b).

TSWV particles are spherical and enveloped, and contain three RNAs consisting of a large (L), medium (M), and small (S) RNA segment. Each of the segments is encapsidated by multiple copies of nucleoprotein (N). The L segment encodes a single gene, the unusually large (351-kDa) viral RdRp in the viral complimentary sense (Nc) that is required for replication of viral RNA and transcription of subgenomic mRNAs using the nucleocapsids as templates (Adkins et al. 1995; Chen et al. 2019; Kormelink et al. 2011). The M segment encodes the precursor to the two viral GPs (Gn and Gc) in the negative sense; hence, it is ambisense in character. The GPs are not necessary for infection in plants but they play a role in virion maturation and are essential for transmission by thrips vectors. The S segment codes for N protein and the silencing suppressor, NSs, that functions in both plants and the insect vector (Margaria et al. 2014; Takeda et al. 2002).

In spite of numerous failed attempts by many laboratories around the world, emboldened by the establishment of a TSWV based replicon system in yeast (Ishibashi et al. 2017), Feng and colleagues (2019) established a system to generate infectious TSWV entirely from cDNA clones in plants. A minireplicon system was established based on the S RNA template using the gRNA cloned and flanked by self-cleaving HH ribozyme at the 5′ end and an HDR ribozyme at the 3′ terminus and replacement of the N and NSs genes with reporter genes *mCherry* and *eGFP*, respectively. This, in turn, was cloned into an *Agrobacterium* binary vector downstream from 2X3SS. They also generated binary vectors for plant delivery of the viral RdRp, N, and four VSRs, including NSs, P19, HcPro, and yb. However, when all of these constructs were agro-launched into *N. benthamiana* plants, initial experiments failed to produce the expected signals from either *mCherry* or *eGFP*. It was rationalized that, due to the very large size of the RdRp and the quasi species nature of viral infections, the clone being used was likely to have sequence errors, rendering it...
nonfunctional in their assay. To address this, they optimized codon usage and removed many potential intron splicing sites that could be processed in the nucleus during Pol II catalyzed expression by the 35S promoter. Using this optimized TSWV RdRp construct and the same set of core protein vectors, they were now able to observe mCherry and eGFP in infiltrated leaves. These results were confirmed by immunoblot analysis to detect the expressed proteins and Northern blot analysis showing the production of both polarities of the minireplicon RNAs, thereby confirming that ribonucleoprotein cores were assembled and that both replication and transcription of the minireplicon occurred. Importantly, these results were only obtained when all accessory constructs were agroinfiltrated together. After optimization experiments to establish the best ratios of all system components, experiments were done to confirm the importance of using all four silencing suppressors, to confirm the requirement for the 5′ and 3′ cis-acting elements and the role of the A/U rich intergenic region present in the bicistronic replicon.

To work toward a fully functional reverse-transcription system, a movement competent minireplicon was established. A TSWV M gRNA-based replicon maintaining the NSm cell-to-cell movement but with the GP ORF was exchanged for eGFP. Agroinfiltration experiments with all components of the system demonstrated that, in contrast to the S RNA-based replicon, only the M RNA-based replicon showed eGFP expression in many surrounding cells. Having now established an S-based minireplicon system and a movement-competent M-based minireplicon, the next step was to establish expression of wild-type virus from 35S-promoted cDNA clones of L, M, and S RNAs flanked by HH and HDV ribozymes at their 5′ and 3′ ends. When initial experiments with these clones failed, it was again determined that putative splicing sites were the cause. It turned out that the GP ORF contained several such sites that were then removed by codon optimization. Now, upon coexpression of optimized L, optimized M, S RNA eGFP, and three VSRs, a functional and stable M-based minireplicon, the next step was to establish expression of wild-type virus from 35S-promoted cDNA clones of L, M, and S RNAs flanked by HH and HDV ribozymes at their 5′ and 3′ ends. When initial experiments with these clones failed, it was again determined that putative splicing sites were the cause. It turned out that the GP ORF contained several such sites that were then removed by codon optimization. Now, upon coexpression of optimized L, optimized M, S RNA eGFP, and three VSRs, a functional and stable M-based minireplicon, the next step was to establish expression of wild-type virus from 35S-promoted cDNA clones of L, M, and S RNAs flanked by HH and HDV ribozymes at their 5′ and 3′ ends. When initial experiments with these clones failed, it was again determined that putative splicing sites were the cause. It turned out that the GP ORF contained several such sites that were then removed by codon optimization. Now, upon coexpression of optimized L, optimized M, and S gRNA along with three VSRs (P19, HcPro, and yb). At 19 dpi, typical symptoms were observed in systemic leaves of N. benthamiana plants. Complete wild-type infection was confirmed by sequencing of progeny RNA molecules and immunoblot analysis showing the presence in systemically infected leaves of all viral encoded proteins. Furthermore, electron microscopy revealed the presence of typical enveloped virus particles, indicating that, after many attempts and overcoming numerous problems (not all of which are described here), for the first time, infectious virus was successfully rescued from full-length cDNA from gRNA and agRNA of a segmented NSR plant virus.

A REVERSE-GENETICS SYSTEM FOR AN EMARAVIRUS

The genus Emaravirus includes nine species of viruses with multipartite, single-stranded genomes consisting of four to eight negative-sense RNAs (Chen et al. 2019). Rose rosette virus (RRV) causes multimillion-dollar economic losses to the ornamental industry by producing stem elongation, breaking axillary buds, leaflet deformation and wrinkling, bright red pigmentation, phyllody, and hyper-thorniness on commercially important cultivars (Pemberton et al. 2018). Biological transmission is mediated by eriophyid mites, making studies of virus–vector relationships difficult because of their small size and difficulty in manipulation. Further complicating matters, these viruses have proven difficult to passage by mechanical inoculation techniques.

Typical of the genus, RRV has seven negative-sense monocistronic genomic RNA segments. RNAs 1 to 4 encode the core proteins required for the completion of the viral infection cycle: the RdRp, GP precursor, nucleocapsid, and movement proteins, respectively (Mielke-Ehret and Muhlback 2012). Although they have been sequenced, bioinformatics analysis has not revealed a function for the ORFs on RNAs 5 to 7. The recent establishment of a reverse-genetics system for RRV was a significant accomplishment toward developing a better understanding of this group of viruses (Pang et al. 2019) and will be described here.

To overcome the mechanical inoculation problems associated with RRV, Pang and colleagues (2019) developed an inoculation procedure using an air brush to deliver infected plant sap obtained from naturally mite-infected material to the surface of carborundum-dusted leaves of rose plants, a significant advance given that the virus had only been transmitted by mites or grafting previously. PCR analysis demonstrated that this system worked so that they would not have to rely on the tedious process of mite transmission to develop a reverse-genetics system. To establish a reverse-genetics system, agRNA1 through agRNA5 and cDNAs encoding agRNA5 to agRNA7 were directly amplified by reverse-transcription PCR using total RNA isolated from infected rose leaves, then introduced into the pCB301-backbone positioned next to the CaMV 35S promoter and HDR to produce viral transcripts with authentic 5′ and 3′ ends. All constructs were then transformed into A. tumefaciens. Agrobacterium cultures were combined in equal ratios and inoculated to two cultivars of roses with all seven clones encoding RRV antigenomic segments (RRV1 to -7) using an artist airbrush. Between 30 and 40 dpi, 88 to 100% of plants became systemically infected. At 48 dpi, systemic infection was confirmed by diagnostic PCR assays and showed the accumulation of all seven viral RNA species. This result was extremely surprising because, for all negative-strand RNA viruses thus far described, establishment of a reverse-genetics systems requires the inclusion of plasmids expressing the viral core proteins and viral silencing suppressors presumed to reconstitute viral ribonucleoprotein complexes required for replication and transcription (Jackson and Li 2016), as shown in the examples above. Further experiments of this same type revealed that infection could be produced by using only constructs expressing RNAs 1 to 4, confirming that they code all necessary accessory proteins for complete virus recovery. In addition, agroinfiltrating viral constructs in a conventional manner resulted in systemic infections on Arabidopsis thaliana (Col-0) or N. benthamiana plants and produced symptoms specific to each species.

To create a reporter virus to monitor infection and spread, fusions of GFP with the ORF on RNA 4 or RNA 7 were inoculated to Arabidopsis or N. benthamiana plants. The report of these constructs could be seen using an epifluorescence microscope but not by a hand-held UV light. In an attempt to improve visualization of the reporter, the iLOV fluorescent domain of the plant blue light receptor, phototrophin (Chapman et al. 2008), was fused to ORF 4, ORF 5, and ORF 7 constructs, which were able to support infection with constructs 1 to 4 or 1 to 7 in various combinations and be detected by microscopy. The ability to deliver these plasmids to roses by airbrush inoculation (unexpected owing to the belief that the virus is only mite transmissible) and to N. benthamiana and Arabidopsis using agroinfiltration demonstrates the ability of this reverse-genetics system to sustain insertions of foreign genes from one or more genomic segments. This new technology will provide a valuable weapon in the arsenal of tools to investigate the infection of roses with RRV and seek genetic solutions for the disease that it causes.
CONCLUSIONS AND SIGNIFICANCE OF REVERSE-GENETICS SYSTEMS FOR NSR VIRUSES

This review considers the development of four different NSR reverse-genetics systems: two monoparite rhabdoviruses, a tri-partite tospovirus, and a multicomponent fimovirus. There are a number of similarities in the approach taken to construct each of these systems using technology similar to that developed for NSR viruses of vertebrates. At the same time, each system required specific new approaches to solve problems unique to plant and insect viruses. In the case of SYNV (Wang et al. 2015), the use of a modified 35S promoter to ensure the synthesis of a precise 5’ end of transcripts was employed. For BSMV (Gao et al. 2019), the use of SBPH insects injected with plant sap from plants expressing rescued recombinant made it possible to pass the infection to new plants and increased the utility of the system to include insect vectors. In the development of the TSWV system, Feng et al. (2019) succeeded where others had failed by realizing the importance of optimizing the codon usage and removing cryptic splice sites from the very large RdRp and GP segments. In the case of the RRV system (Pang et al. 2019), a key factor was the development of a novel virus transmission and airbrush inoculation protocol for rose plants to replace the need for mite vectors, which provided the remarkable discovery that a reverse-genetics system for an NSR can be established without ectopic expression of the viral accessory proteins.

The establishment of these systems constitutes a major advance in the available technology to study plant viral molecular genetics, interactions of plant and vector host components, screening for resistance genes, and perturbation of plant and insect gene expression using RNAi or CRISPR/Cas9 nucleases. The capacity for multiple gene insertions in a single vector, and the expanded host range provided by the reverse-genetics system described here will provide additional resources for biotechnological applications in the future.

TRANSGENIC APPROACHES FOR VECTORS OF PLANT VIRUSES

The ability to utilize state-of-the-art genomic tools such as CRISPR/Cas9 in insect vectors of plant viruses will facilitate the development of a wide range of new pest control methods and basic studies to understand the basis of vector competence. The CRISPR/Cas9 system is an RNA-mediated, adaptable immune system that protects bacteria from foreign DNA. The power of the system comes from its programmability. Specifically, Cas9 nuclease is directed to its target sequence by a guide RNA with a relatively short (20 nt) recognition sequence which binds the DNA target via base-pair matching (Fig. 3). Cas9 provides added specificity by only creating a double-stranded break in the DNA if the target sequence is adjacent to a nuclease-specific sequence motif (PAM). Such breaks can be repaired through a process called nonhomologous end joining (Fig. 3), which can result in small insertions or deletions that can damage the functionality of a gene. Importantly, these breaks can also be repaired, though less frequently, through homology-directed mechanisms (HDR) (Fig. 3), which normally utilize a sister chromosome as template. However, the HDR system can be hijacked and used to replace or insert DNA segments of our choosing into almost any location we wish by flanking the desired change with approximately 1-kb stretches of DNA sequence that match the sequences flanking our target site. The flanking sequences are recognized as homologous and act as a template to guide the repair of the damaged target site, resulting in integration of the desired change. Taken together, these qualities make CRISPR/Cas9 the most powerful system to date for editing genetic sequences in cell lines and living organisms (Barakate and Stephens 2016).

Despite the undeniable power of CRISPR/Cas9-based genome editing, we are aware of only two published reports of confirmed genome editing in insect vectors of plant viruses. The first is from the brown planthopper, Nilaparvata lugens (Xue et al. 2018), while the other is from the pea aphid, Acyrthosiphon pisum (Le Trionnaire et al. 2019). Although RNAi was rapidly adopted for use in aphids (Jaubert-Possamai et al. 2007; Mutti et al. 2006) and planthoppers (Chen et al. 2010; Liu et al. 2010; Yao et al. 2013), CRISPR/Cas9 and transposon-mediated germline transformation have lagged behind. The problem stems from (i) the fact that double-stranded RNA (dsRNA) easily crosses cell membranes in many insect species, while DNA and proteins cannot, and (ii) the lack of an easily collected egg stage for many vectors. Therefore, although RNAi can be triggered in a wide variety of insect taxa at any life stage through microinjection and, in some cases, ingestion of dsRNAs, the use of CRISPR/Cas9 and transposon-mediated transformation in insects relies on the ability to collect and microinject precellular embryos.

Unlike vertebrates, insect embryos undergo a series of nuclear divisions without cell division, creating a multinucleated cell (Fig. 3), which can result in small insertions or deletions that can damage the functionality of a gene. Importantly, these breaks can also be repaired, though less frequently, through homology-directed mechanisms (HDR) (Fig. 3), which normally utilize a sister chromosome as template. However, the HDR system can be hijacked and used to replace or insert DNA segments of our choosing into almost any location we wish by flanking the desired change with approximately 1-kb stretches of DNA sequence that match the sequences flanking our target site. The flanking sequences are recognized as homologous and act as a template to guide the repair of the damaged target site, resulting in integration of the desired change. Taken together, these qualities make CRISPR/Cas9 the most powerful system to date for editing genetic sequences in cell lines and living organisms (Barakate and Stephens 2016).

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et al. (2018) tested this system in the pea aphid, *A. pisum*, with CRISPR components and, shortly afterward, subjected the method to in vivo electroporation, where individuals are injected with CRISPR/Cas9-based control strategies to bear on these economically important vectors.

Although genetic manipulation of *N. lugens* is far from trivial, they at least readily lay eggs. Aphids, on the other hand, can reproduce without mating, giving birth to live young; therefore, eggs are not easily cross cell membranes, one way to deal with this roadblock is to use a nontraditional delivery method. One such method is in vivo electrotransformation, where individuals are injected with CRISPR components and, shortly afterward, subjected to several electrical pulses to increase cell permeability. Jamison et al. (2018) tested this system in the pea aphid, *A. pisum*, by targeting carotene dehydrogenase (*tor*), which is required for carotenoid pigmentation. Although the authors found that some injectees gave rise to offspring that displayed phototypic changes (white versus pink pigmentation), Cas9-mediated mutation of the target gene could not be verified. Despite this setback, in vivo electrotransformation merits further study.

Another approach for genetic manipulation of aphids is to induce sexual differentiation to employ the standard methodology of collecting and microinjecting precellular embryos. This is exactly what Le Trionnaire and colleagues (2019) did to genetically alter *A. pisum*. The first step in their 7-month-long protocol was the induction of sexual morphs through the use of photoperiod changes, a process that took 2 months. This then enabled the use of “standard methods” for mating, egg collection, and microinjection, which of course were not actually standard because they had to be worked out from scratch. After the required 3-month-long diapause period, the researchers were finally in a position to see the fruits of their labor. The target, a cuticular protein gene (*stylin-01*), the receptor for CaMV, was successfully disrupted in 6 of 17 founding lines.

Despite the time and effort involved in genome manipulation, use of state-of-the-art genomic tools such as CRISPR/Cas9 in these important vectors of plant viruses has the potential for enormous rewards. This is particularly true in light of the discovery of genes such as *sugar transporter 6* and *stylin-01* because loss of function of such genes is expected to result in vector-incompetent insects. Importantly, CRISPR/Cas9 can serve yet another function, both assisting in the creation of vector-incompetent insects as well as catalyzing the replacement of vector-competent insect populations with individuals that can no longer vector the targeted virus, thus offering new approaches for controlling insect vectors as well as the viruses they transmit. As mentioned above, the dsDNA breaks generated by CRISPR/Cas9 can be repaired through HDR. Therefore, with the addition of an HDR construct, the system can be used not only to insert DNA sequences coding for guide RNAs targeting the insert site but also for Cas9 itself, making the insertion, hypothetically, self-replicating, which could “drive” itself into a population. Moreover, if the insertion carries an antiviral gene or disrupts the function of a gene necessary for viral infection, then the gene drive could be used to create a pest population resistant to vectoring the disease.

Although exploration of genetics-based solutions for use in aphids and planthoppers is in its infancy and not yet even applied to thrips and mites, researchers have already made significant progress applying genetic strategies to the control of mosquito-borne diseases. For example, Gantz and Bier (2015) published their groundbreaking manuscript describing the use of CRISPR/Cas9 for gene drive in *D. melanogaster* during spring 2015 and rapidly applied the same technology to *Anopheles stephensi* (Gantz et al. 2015). However, the gene-drive construct used in *A. stephensi* not only carried the DNA sequences coding for the guide RNAs and Cas9 but also included two antimalarial effector genes. Such a system has the potential to replace entire populations of vector-competent insects with those that can no longer transmit the pathogen. CRISPR/Cas9 can also be used for population suppression. If the target site for a gene-drive insertion is in a gene vital for survival, then the system can be used to suppress populations of pest insects as individuals that inherit the insertion begin to die. A similar approach was used in *A. gambiae* (Hammond et al. 2016), where the gene-drive constructs included gRNAs targeting genes required for female reproduction. Such a drive would be short lived in the wild but this would be advantageous for eliminating invasive species without destroying them in their native range, or for short-term control during growing seasons of sensitive crops.

In an effort to aid the development of genetics-based solutions for insect vectors of plant viruses, we have designed and optimized protocols for harvesting and microinjecting precellular embryos from the corn planthopper, *P. maidis* (W. Klobasa, F. Chu, O. Huot, N. Grubbs, A. E. Whitfield, and M. D. Lorenzen unpublished data). To test this system, we attempted to reproduce the work performed in the brown planthopper (Xue et al. 2018), targeting the *P. maidis* white (*Pm-w*) and *innocar* (*Pm-cn*) genes. Interestingly, although CRISPR/Cas9-mediated knockout of *Pm-w* resulted in loss of eye-spot pigmentation in a small percentage of embryos, only embryos that still possessed eye-spot pigmentation hatched, leading us to conclude that *Pm-w* may be vital in this species. On the other hand, individuals possessing loss-of-function mutations in *Pm-cn* survived to adulthood, enabling us to establish a red-eyed *P. maidis* colony. These are merely the first steps required for reaching the true goal, that of bringing game-changing genomic tools to bear on the control of these important agricultural pests.

**CONCLUDING REMARKS**

Recent progress on developing methods for reverse-genetics systems for NSR viruses and genome editing for insect vectors combined with breakthroughs in understanding the viral and vector components required for vector transmission will enable extraordinary new discoveries for these important viruses. For many years, these systems lagged behind their more tractable positive-strand plant-infecting and animal-infecting counterparts. Understanding of NSR viruses will also greatly benefit from the comparative studies that are already revealing conserved functions for viral and vector proteins for related yet diverse virus–vector interactions. Additionally, comparative analyses of viral gene function and conserved responses in plant and vector hosts may enable strategies to mitigate virus infection of both hosts and provide cross-kingdom virus protection.


