Advances in Tetravirus Research: New Insight into the Infectious Virus Life Cycle and an Expanding Host Range

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Abstract

Tetraviruses are a group of relatively unknown small RNA viruses with particles that display a characteristic $T=4$ capsid architecture. Tetraviruses are classified into three families, the *Alphatetraviridae*, *Permutotetraviridae* and *Carmotetraviridae*, according to the divergent characteristics of their respective viral replicases. Tetraviruses generally infect the larvae of lepidopteran insect species, many of which are important agricultural pests and, until recently, were thought to have an unusually narrow host range and tissue tropism. The development of experimental systems for studying the viral infectious life cycle in tissue culture has permitted the extension of the virus host range to mammalian cells and plants. This chapter will review recent advances in the understanding of the biology of tetraviruses, highlighting new information on the expression and functional characterization of viral proteins and the development of biological systems for elucidating the molecular mechanisms of infection, viral replication and host range.

Introduction

Tetraviruses are small, RNA viruses that infect lepidopteran (butterflies and moths) insects, notably Saturniid, Limacodid and Noctuid moths, many of which are important agricultural pests. Tetravirus particles are characterized by the unique $T=4$ quasi-symmetry of their capsids (the name derived from the Greek *téttares*, which means 'four') encapsidating one or two single-stranded (ss), positive sense (+) genomic RNAs. The viral genome may encode up to four genes translated directly into proteins that may be processed further into smaller, functionally distinct peptides (Dorrington *et al.*, 2011; Mendes *et al.*, 2015).

The first described tetravirus was isolated from diseased larvae of the pine emperor moth (*Nudaurelia cytherea capensis*) in South Africa (Hendry *et al.*, 1968). The larvae were infected with at least five structurally distinct small RNA viruses including *Nudaurelia capensis β virus* (NβV). NβV was the most plentiful and extensively characterized of these viruses and was found to have a single coat protein of approximately 60 kDa and a (+) ssRNA genome (Struthers and Hendry, 1974; Juckes, 1979). Structural analysis of the NβV particles subsequently revealed a new type of virus capsid with unusual $T=4$ icosahedral symmetry (Finch *et al.*, 1974) and this virus became the type-member of a new group of viruses named the Nudaurelia β-like viruses (Moore *et al.*, 1981; Matthews, 1982), later classified as the *Tetraviridae* family (Francki *et al.*, 1991).

For more than 35 years, tetravirus research focused on the structure and assembly of their unique capsids, the molecular characterization of

their genomes and on developing their potential as biocontrol agents for important agricultural pests (Christian *et al.*, 1993, 2005; Hanzlik *et al.*, 1999). However, research on tetravirus pathogenicity and replication biology lagged behind due to a lack of tissue culture cell lines susceptible to infection and difficulties in establishing laboratory colonies of most of the host species. The discovery of *Providence virus* (PrV) as a persistent infection in a *Helicoverpa zea* midgut cell line (Pringle *et al.*, 2003) provided new opportunities for studying tetravirus replication and virus–host interactions *in vivo*. This chapter will review recent advances in the understanding of the biology of tetraviruses, highlighting new information on the expression and functional characterization of viral proteins and the development of biological systems for elucidating the molecular mechanisms of infection, viral replication and host range.

Taxonomy

The distinguishing feature of all tetraviruses is the characteristic $T = 4$ icosahedral symmetry of their capsids. Tetraviruses are generally identified by the size of their capsids (approximately 40 nm in diameter) and the presence of major and minor capsid proteins of 58–64 kDa and 6–8 kDa in size, respectively (Dorrington *et al.*, 2011). These common structural features, together with serological relationships led to their classification within a single family, the *Tetraviridae*, (Francki *et al.*, 1991) comprising two genera according to their genome organization, namely *Betatetravirus* with monopartite genomes and *Omegatetravirus*, which have bipartite genomes (Fauquet *et al.*, 2005). Most of the betatetraviruses, with the exception of *Pseudoplusia includens virus* (PiV), were serologically related to some degree (Reinganum and Scotti, 1976; Reinganum *et al.*, 1978; Greenwood and Moore, 1981; Chao *et al.*, 1983; Hendry and Agrawal, 1994). However, this was not the case for the omegatetraviruses (Hanzlik *et al.*, 1993), despite significant sequence conservation between their capsid proteins (Hanzlik *et al.*, 1995; Yi *et al.*, 2005), highlighting the limitations of using serological criteria for distinguishing between tetravirus species.

Phylogenetic analyses revealed that the RNA-dependent RNA polymerase (RdRp) domains of tetravirus replicases are clustered into three distinct groups, each belonging to a different superfamily of RNA viral replicases (Walter *et al.*, 2010). The permuted RdRp domains of *Thosea asigna virus* (TaV) and *Euprosterna elaeasa virus* (EeV) form a group distantly related to the picorna-like birnaviruses (Gorbalenya *et al.*, 2002; Zeddam *et al.*, 2010), while the alpha-like RdRps of NβV and *Helicoverpa armigera stunt virus* (HaSV) form a second, distinct cluster. The PrV replicase is isolated from other tetraviruses, being more closely related to the tombusvirus, *Pelargonium chlorotic ring pattern virus* (PCRPV), and other carmo-like plant viruses (Walter *et al.*, 2010; Dorrington *et al.*, 2011). Therefore, based upon the diverse characteristics of their viral replicases, the tetraviruses were reclassified into three new families, namely the *Permutotetraviridae*, the *Alphatetraviridae* and the *Carmotetraviridae* (ICTV, 2011). There are currently 14 known tetravirus species, the majority of which are classified within the *Alphatetraviridae* family (Table 7.1). With the exception of NβV, the remaining viruses within the *Betatetravirus* genus of this family have been classified based upon their monopartite genome organization and not on the characteristics of their viral replicase. Thus, some of these viruses may well be reclassified into the *Permutotetraviridae or Carmotetraviridae* families in the future, when their genome sequence becomes available.

The tetravirus capsid

Tetravirus virions have non-enveloped, spherical particles exhibiting $T = 4$ icosahedral shell quasi-symmetry, ranging in diameter from 38–41 nm (Dorrington *et al.*, 2011). The particles assemble as procapsids composed of 240 identical capsid protein precursor (CP) subunits that adopt different conformations arranged in four quasi-equivalent positions in the mature virus particle (Munshi *et al.*, 1996). During maturation, autoproteolytic cleavage of the CP (α subunit) at its C-terminus results in the production of the β and γ subunits (Agrawal and Johnson, 1995; Hanzlik *et al.*, 1995; Gordon *et al.*, 1999; Pringle *et al.*, 1999, 2003). The internal helical domain (N-terminus and γ subunit) of the CP interacts with viral RNA forming the molecular switch that determines the $T = 4$ icosahedral symmetry of the virus particle. The

Table 7.1 Taxonomic classification of known tetraviruses

Virus species	Acronym	Host family	Geographical location	GenBank Accession number	References
Family: *Alphatetraviridae*					
Genus: *Omegatetravirus*					
Nudaurelia capensis ω virus[1]	NωV	*Saturniidae*	South Africa	[S43937.1]	Hendry *et al*. (1985); du Plessis *et al*. (2005)
Helicoverpa armigera stunt virus	HaSV	*Noctuidae*	Australia	[KX423453.1]; [L37299]	Hanzlik *et al*. (1993); Hanzlik *et al*. (1995); Gordon *et al*. (1995)
Dendrolimus punctatus Tetravirus	DpTV	*Lasiocampidae*	China	[AY594352]; [AY594353]	Yi *et al*. (2005)
Genus: *Betatetravirus*					
Nudaurelia capensis β virus[1]	NβV	*Saturniidae*	South Africa	[AF102884]	Juckes *et al*. (1973); Struthers and Hendry (1974); Gordon *et al*. (1999)
Antheraea eucalypti virus[2]	AeV	*Saturniidae*	Australia	NA	Grace and Mercer (1976)
Darna trima virus	DtV	*Lymacodidae*	Malaysia	NA	Reinganum *et al*. (1978); King and Moore (1984)
Dasychira pudibunda virus *Calliteara pudibunda virus*[3]	DpV CpV	*Lymantriidae*	UK	NA	Reinganum *et al*. (1978); King and Moore (1984)
Philosamia cynthia × ricini virus	PxV	*Saturniidae*	UK	NA	Reinganum *et al*. (1978)
Pseudoplusia includens virus	PiV	*Noctuidae*	USA	NA	Chao *et al*. (1983)
Trichoplusia ni virus	TnV	*Noctuidae*	USA	NA	Morris *et al*. (1979)
Family: *Permutotetraviridae*					
Genus: *Alphapermutotetravirus*					
Thosea asigna virus[1]	TaV	*Lymacodidae*	Malaysia, USA	[AF282930], [AF062037]	Pringle *et al*. (1999)
Euprosterna elaeasa virus	EeV	*Zygaenidae*	Peru	[AF461742]	Gorbalenya *et al*. (2002); Zeddam *et al*. (2010)
Family: *Carmotetraviridae*					
Genus *Alphacarmotetravirus*					
Providence virus[1]	PrV	Noctuidae	USA	[AF062037]	Pringle *et al*. (2003); Walter *et al*. (2010)

[1]Type strain for the genus
[2]Serologically indistinguishable from NβV (Grace and Mercer, 1976)
[3]Host renamed *Calliteara pudibunda*.

capsid shell comprises a β-barrel and the external immunoglobulin-like domain believed to function in receptor binding during infection of host cells (Munshi *et al.*, 1996; Helgstrand *et al.*, 2004). In the alphatetraviruses, autoproteolytic cleavage releases the γ peptide, resulting in stabilization of the mature virus particle, which is important for infectivity (Munshi *et al.*, 1996; Taylor *et al.*, 2002; Helgstrand *et al.*, 2004; Domitrovic *et al.*, 2012). In TaV and EeV (*Permutotetraviridae*) and PrV (*Carmotetraviridae*) the CP undergoes additional co-translational processing at its amino terminus prior to assembly

(Pringle *et al.*, 1999, 2003; Gorbalenya *et al.*, 2002; Zeddam *et al.*, 2010).

The molecular mechanism of the assembly and maturation of alphatetravirus capsids is well characterized (Canady *et al.*, 2000; Banerjee *et al.*, 2010; Tang *et al.*, 2014). At neutral pH, NωV and HaSV virus-like particles (VLPs) purified as immature procapsids undergo maturation *in vitro* at pH 5.0 (Canady *et al.*, 2000; Tomasicchio *et al.*, 2007). Maturation of NωV capsids involves conformational changes promoting autoproteolytic cleavage of CP, releasing the γ peptide to stabilize the molecular switch that supports the flat contacts of the virus particle (Canady *et al.*, 2001). Not all subunits are cleaved at the same rate and the γ peptides of the A and D subunits positioned at the five-fold and three-fold axes, respectively, are stabilized and cleaved within 30 minutes of incubation at pH 5.0. Cleavage of the B and C subunits follows more slowly (Matsui *et al.*, 2010). Lowering the pH is proposed to result in protonation of glutamate and aspartate side chains that facilitate a conformational change and shrinking of the capsid shell enabling autoproteolytic cleavage of the γ peptide. The conformational change is reversible, provided autoproteolytic cleavage of the γ peptide is inhibited (Taylor *et al.*, 2002). How the conformational changes observed *in vitro* relate to the infectious life cycle of tetraviruses will be addressed below.

Genome organisation and regulation of viral gene expression

Alphatetraviridae

Alphatetraviruses may have either monopartite (Genus: *Betatetravirus*) or bipartite (Genus: *Omegatetravirus*) genomes (Fig. 7.1). The genomic RNAs are capped at their 5′ ends and not polyadenylated at their 3′ ends, which instead, terminate at their 3′ ends with tRNA-like structures (Gordon *et al.*, 1995; Hanzlik *et al.*, 1995, du Plessis *et al.*, 2005; Yi *et al.*, 2005). In plant viruses, tRNA-like structures play a role in promoting replication of the minus strand RNAs, encapsidation of viral RNAs and maintaining the integrity of the 3′ end of the viral RNA (Dreher, 2009). They are also involved in the recoding of host translational machinery and evading the host antiviral response (Ariza-Mateos and Gómez, 2017).

The type member of the *Betatetravirus* genus, NβV, encodes a large replicase (REP) open reading frame (ORF) that almost entirely overlaps the CP coding sequence located at the 3′ end of the gRNA (Fig. 7.1). The CP is translated from a subgenomic (sg) RNA originating just upstream of the CP coding sequence (Gordon *et al.*, 1999). In omegatetraviruses the smaller RNA2 encodes two ORFs. At the 5′ end is the non-structural p17 ORF, which overlaps with the start of the second ORF encoding the CP, which ranges between 70 and 71 kDa in size (Hanzlik *et al.*, 1995; Mendes *et al.*, 2015).

The omegatetravirus REP is translated from RNA1, which also encodes three additional small ORFs (p11, p15 and p8), all three of which overlap with the 3′ end of the REP ORF in HaSV (Fig. 7.1). This arrangement is not conserved in the other sequenced omegatetravirus, *Dendrolimus punctatus tetravirus* (DpTV) (Yi *et al.*, 2005). In both HaSV and DpTV the p11 stop codon is immediately upstream of and in-frame with the AUG start of p15. In HaSV the p8 AUG is three nucleotides downstream of the p15 stop codon (de Bruyn *et al.*, 2016), while the DpTV p8 is in a different reading frame and is 99 nucleotides downstream of the stop codon of p15 (Yi *et al.*, 2005).

The alphatetravirus replicases include three conserved functional domains, namely methytransferase (MT), helicase (HEL) and RdRp domains, in an N- to C-terminal modular arrangement (Fig. 7.1) that are characteristic of all members of the alpha-like virus superfamily (van der Heijden and Bol, 2002). The RdRp domain of the DpTV replicase has been expressed in *E. coli* and shown to initiate primer-independent synthesis of viral RNA *in vitro* (Zhou *et al.*, 2008). Although there are a number of insect-borne animal (alphaviruses) and plant viruses (marafiviruses) within the alpha-like virus family, the alphatetraviruses represent the only insect-specific family within the group (van der Heijden and Bol, 2002). Another feature of the alphatetravirus REPs that is unusual among the alpha-like viruses, is the apparent lack of co- or post-translational processing, a characteristic shared with a small group of plant viruses that includes the *Foveavirus* and *Potexvirus* genera (van der Heijden and Bol, 2002).

Of the alphatetraviruses, the best studied is HaSV, which employs diverse strategies to modulate expression of viral genes including

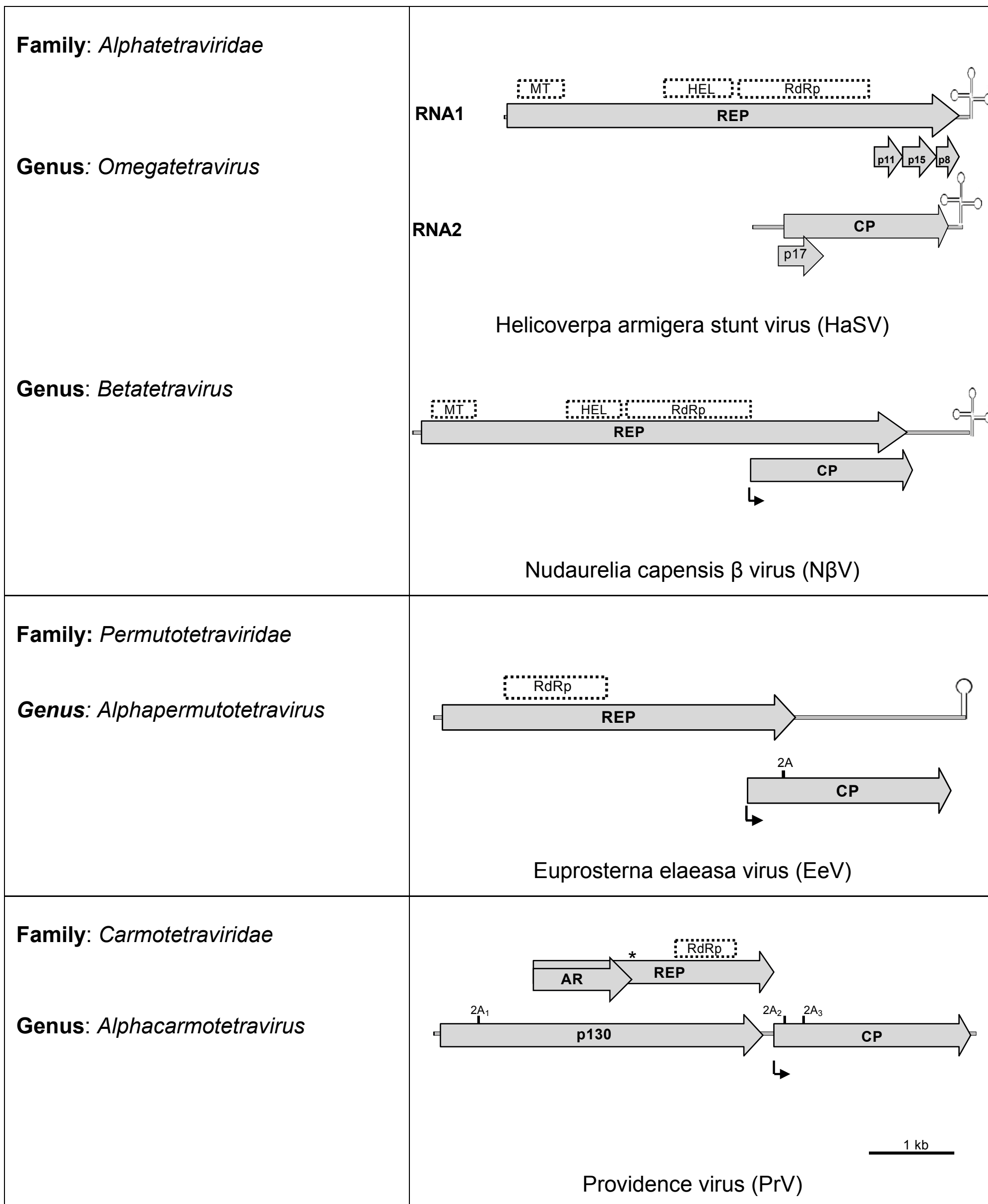

Figure 7.1 Genome organization of tetraviruses representative of the *Alphatetraviridae*, *Permutotetraviridae* and *Carmotetraviridae* families. Viral genes encoding the replicase (REP), Capsid Protein precursor (CP) and replication accessory protein of PrV (p40) are indicated. Putative tRNA-like (HaSV and NβV) and pseudoknot-like (EeV) structures are shown at the 3′ ends of the genomic RNAs. The positions of 2A-like processing sequences (2A) and read-through stop codon (*) are indicated as are the functional domains of the viral replicases (HEL, helicase; MT, methyltransferase; RdRp, RNA-dependent RNA polymerase). The bent arrow shows the start of the subgenomic RNAs of the monopartite viruses (NβV, EeV and PrV) from which the viral CP is translated. GenBank Accession numbers: KX423453.1 (HaSV RNA1), L37299 (HaSV RNA2), AF102884 (NβV), AF461742 (EeV) and AF062037 (PrV).

temporal regulation of the replication of the two viral genomic RNAs, translational control and regulation of protein turnover. In HaSV-infected *Helicoverpa armigera* larvae, RNA1 can be detected three days after infection, while small amounts of RNA2 appear after a further two days (Bawden *et al.*, 1999). This implies that RNA1, encoding the REP, is replicated during the early stages of

infection and that replication of RNA2 occurs during the later stages of infection. This would ensure that expression of the CP and p17, encoded by RNA2, would commence later in the virus life cycle. This phenomenon has been well documented in the nodavirus, *Flock House virus* (FHV). Nodaviruses are small, non-enveloped insect RNA viruses that are structurally related to tetraviruses and also have a bipartite genome. Nodavirus RNA1 encodes the viral REP and two small ORFs (B1 and B2) that overlap with the 3′ end of the REP ORF (Thiéry *et al.*, 2011). B1 and B2 are translated from a sgRNA (RNA3) originating from the 3′ end of RNA1 (Eckerle *et al.*, 2003). Mutations in B2 suppress the replication of *Nodamura virus* (Johnson *et al.*, 2004) and the FHV B2 protein has been shown to inhibit Dicer function in infected cells (Galiana-Arnoux *et al.*, 2006). FHV RNA3 is required for promoting replication of RNA2 during the later stages of infection (Eckerle *et al.*, 2003).

Alphatetraviruses also encode small ORFs that overlap with the 3′ end of REP on RNA1 that could play a similar role to nodavirus B2 in the infectious virus life cycle (discussed in detail below). The three ORFs are expressed in the midgut cells of HaSV-infected *H. armigera* larvae as 11 kDa (p11) and 8 kDa (p8) proteins, together with a 34 kDa polyprotein comprising p11, p15 and p8 (de Bruyn *et al.*, 2016). Given the organization of the three ORFs in a tandem in-frame arrangement, separated by a UGA stop (p11 and p15) and UAA stop followed by GGG (p15 and p8), it is likely that the translation of these three ORFs involves an as yet uncharacterised read-through stop mechanism (de Bruyn *et al.*, 2016).

In HaSV the expression of the non-structural p17 is down-regulated relative to CP via translation regulation and the control of protein turnover (Mendes *et al.*, 2015). The p17 ORF is found upstream of and overlapping with the 5′ end of the CP ORF of RNA2 in all three omegatetraviruses for which genome sequence is available (Hanzlik *et al.*, 1995; du Plessis *et al.*, 2005; Yi *et al.*, 2005). In HaSV-infected *H. armigera* larval midgut tissues p17 is expressed at levels several orders of magnitude lower than that of the viral CP (Mendes *et al.*, 2015). The most likely explanation for low levels of expression of p17 is the poor context of the translational start (AUUUAUGA in HaSV and conserved in DpTV and NωV). This is in contrast to the translational context of the CP ORF downstream of p17 as compared with the consensus for invertebrates (A/CAAA/CAUGG, Cavener, 1987). In addition, high level of PEST amino acids predicted to be present in all three omegatetravirus p17 translation products are thought to promote rapid turnover of the p17 protein *in vivo* (Mendes *et al.*, 2015).

Permutotetraviridae

The monopartite genomes of EeV and TaV encode two overlapping ORFs, with the viral replicase located at the 5′ end and the CP at the 3′ end (Gordon *et al.*, 1999; Gorbalenya *et al.*, 2002; Zeddam *et al.*, 2010). Bioinformatic analysis suggests the presence of a pseudoknot at the 3′ ends of the EeV and TaV genomic RNAs, which may be involved in the regulation of genome replication in these two permutotetraviruses or they may play a role in preventing degradation of the genomic RNA by nucleases (Zeddam *et al.*, 2010). The replicase ORF of EeV and TaV overlaps CP by 536 nt and 529 nt, respectively in contrast to the NβV replicase ORF, which is significantly larger (Fig. 7.1).

As with the alphatetravirus, NβV, the permutotetravirus CP is translated from a sgRNA originating just upstream of the CP coding sequence (Gordon *et al.*, 1999; Pringle *et al.*, 1999). The TaV and EeV CP ORFs encode proteins with a predicted molecular mass of 82.5 kDa (Pringle *et al.*, 1999; Zeddam *et al.*, 2010). There is a 2A-like processing site located near the N-terminus of the TaV CP, the activity of which, in TaV, results in the production of a 17 kDa amino-terminal peptide (p17) as well as the CP of 65 kDa (Pringle *et al.*, 1999). The CP is then autoproteolytically cleaved at its C-terminus during particle maturation to produce the 58 kDa and 7 kDa proteins found in mature capsids (Pringle *et al.*, 1999). This 2A-like site is conserved in the same position in the EeV capsid protein precursor ORF (Zeddam *et al.*, 2010).

The replicases of TaV and EeV are structurally related to those of two members of the double-stranded (ds)RNA birnaviruses and *Drosophila A virus* (DAV), and are distantly related to members of the picorna-like virus superfamily (Gorbalenya *et al.*, 2002; Ambrose, *et al.*, 2009; Zeddam *et al.*, 2010). This distinct cluster of replicases encode an internally-permuted C-A-B arrangement of the canonical A, B and C motifs found in the palm

subdomain of all template-dependent polynucleotide polymerases (Poch *et al.*, 1989; Gorbalenya *et al.*, 2002; Ambrose *et al.*, 2009). A putative nucleotidylation (VPg) signal is present upstream of the TaV and EeV RdRp domains, a feature shared with the *Birnaviridae* and absent from other tetravirus replicases (Zeddam *et al.*, 2010). The TaV and EeV replicases both lack discernible MT and HEL domains (Gorbalenya *et al.*, 2002), which is unusual for picorna-like replicases that usually encode a HEL domain (van der Heijden and Bol, 2002).

The structure of the TaV RdRp domain encoded within an N-terminal peptide was solved at 2.1 Å resolution (Ferrero *et al.*, 2012) and is to date the only tetravirus replicase RdRp domain for which the structure is known. Data obtained from the crystal structure of the TaV RdRp domain implies that the TaV REP likely dimerizes via a region within the RdRp domain (Ferrero *et al.*, 2015). These data confirm the bioinformatic predictions of structural conservation with the RdRp domains of the *Birnaviridae* but interestingly also revealed similarities with an extended loop known to be involved in the initiation of RNA replication in the RdRp domains of flaviviruses and in bacteriophage ϕ6 suggesting an evolutionary relationship between these distantly related virus groups (Ferrero *et al.*, 2015).

Carmotetraviridae

PrV has a monopartite genome encoding three viral genes. The CP ORF is located at the 3′ end of the genome and upstream of CP is an ORF encoding the viral REP, which ends with a stop codon (UGA) four nucleotides upstream of the AUG start of the CP coding sequence. As in the other monopartite tetraviruses, the (p81) is translated from an sgRNA originating just upstream of the CP AUG (Pringle *et al.*, 2003). There are two 2A-like processing sites (PrV-$2A_2$ and PrV-$2A_3$) located near the amino terminus of the CP coding sequence, the activity of which result in the translation of two small peptides of 7 kDa and 8 kDa along with the capsid α protein of 68 kDa (Pringle *et al.*, 2003). As with all other tetraviruses, p68 also undergoes assembly-dependent autoproteolytic cleavage at its carboxyl terminus during particle maturation to produce the β (p62) and γ (p6) subunits, respectively (Pringle *et al.*, 2003). In addition to REP and CP, PrV encodes a third, large ORF (p130) at its 5′ end and upstream of the replicase ORF, which is not found in any other tetraviruses (Fig. 7.1). A third 2A-like processing site is present in the N-terminus of p130, the activity of which is predicted to result in the translation of two products of 17 kDa and 113 kDa (Walter *et al.*, 2010).

The PrV REP ORF is distinguished from other tetravirus replicases in that it encodes a read-through stop codon that results in the translation of a smaller product (p40) in addition to REP, designated p104 (Walter *et al.*, 2010). The only similarity between the replicases of PrV and those of the other tetraviruses is the absence of MT and HEL domains, as is the case with TaV and EeV. This along with the sequence similarity of the RdRp domain with the replicases of the tombus- and umbraviruses [both (+)ssRNA plant viruses] places PrV within the carmo-like supergroup. The PrV RdRp domain carries yet another unusual arrangement of the canonical A-B-C palm subdomain, with an additional A and (poorly conserved) B motif spatially arranged in a similar manner to the permuted RdRps of TaV and EeV (Walter *et al.*, 2010).

The PrV REP ORF contains an in-frame Group 1 read-through stop codon following amino acid 359. This results in leaky read-through by the ribosome (for a review on viral ribosomal recoding, see Dabrowski *et al.*, 2015) and the production of a shorter translation product (p40) in addition to the full-length REP (p104) at a ratio of approximately 10:1 (Walter *et al.*, 2010). The PrV signal, which shares little homology with read-through stop signals found in insect and animal viruses, was the first example of a Group 1 read-through signal that is active in an animal virus, raising interesting questions about the origin of PrV.

The use of co-translational modification via 2A-like processing is a strategy shared by both permutotetraviruses and PrV with biologically active 2A-like sites at the amino-termini of the CP of TaV (TrV-2A), EeV (EeV-2A), two in the PrV (PrV-$2A_2$, PrV-$2A_3$) CP as well as in the PrV p130 (PrV-$2A_1$) (Pringle *et al.*, 2001, Luke *et al.*, 2008; Walter *et al.*, 2010). In contrast to PrV-$2A_1$ and PrV-$2A_3$, processing at the PrV-$2A_2$ site is less efficient *in vitro* (Luke *et al.*, 2008). These data are consistent with *in vitro* translation experiments that show translation products of approximately 15 kDa and 68 kDa,

corresponding to a peptide with a predicted molecular mass of 13.2 kDa (p13) resulting from processing only at PrV-$2A_3$ and the capsid protein precursor p68 (Walter *et al.*, 2010). The 13 kDa peptide does not cross-react with antibodies raised against PrV virus particles nor does it co-purify with wild-type PrV virus particles, as is the case with the N-terminal 2A-like processing product of TaV, p17 (Pringle *et al.*, 1999, 2001, 2003).

2A sequences were first identified in the *Picornaviridae*, but they are present in other mammalian viruses (e.g. human type C rotaviruses) as well as a wide range of insect viruses (Luke *et al.*, 2008) providing the viruses with yet another mechanism to translate multiple peptides with potentially different functions, from a single ORF (Roulston *et al.*, 2016). The mechanism of 2A processing has been well characterized *in vitro* and a *Saccharomyces cerevisiae* experimental system has been developed to identify host-cell (ribosomal translation) factors that interact with these 2A sequences (Doronina *et al.*, 2008a,b).

Elucidating the infectious virus life cycle

For many years, the absence of tissue culture cell lines permissive to tetravirus replication and the difficulties associated with studies on whole animals (Bawden *et al.*, 1999; Brooks *et al.*, 2002) meant that most research on the tetraviruses was focused on the structure, assembly and maturation of the viral capsid, molecular characterization of their genomes and their potential uses as biocontrol agents. Tetraviruses were believed to infect only lepidopteran insects, with host ranges confined to a few closely related species replicating only in larval midgut cells (Fauquet *et al.*, 2005). The lack of insect tissue culture cell lines supporting tetravirus replication was attributed to the absence of host factors required for replication of HaSV and the lack of suitable receptors to mediate virus binding and cell entry (Bawden *et al.*, 1999). The discovery of PrV as a persistent infection in a *H. zea* midgut cell line (Pringle *et al.*, 2003) and recent breakthroughs in establishing alphatetravirus replication in tissue culture (Penkler *et al* 2016), have dispelled this notion and resulted in significant advances in our understanding of the infectious viral life cycle.

Binding and entry

The capsids of alphatetraviruses are unique amongst non-enveloped viruses because they are stabilized under acidic conditions and release their membrane-lytic peptides under alkaline conditions (Canady *et al.*, 2002; Domitrovic *et al.*, 2012). Precisely how the unusual biophysical properties relate to the infectious viral life cycle was revealed in a ground-breaking study in which conformational changes in the HaSV particles induced by alkaline pH promote binding to *Spodoptera frugiperda* (Sf9) cells. Binding was followed by the initiation of infection and RNA replication (Fig. 7.2) and the production of infectious HaSV particles in these cells (Penkler *et al.*, 2016). These results were confirmed *in vivo*, showing that an alkaline midgut pH was required for HaSV infection of *H. armigera* larvae. This study provided for the first time, an experimental system for studying the alphatetravirus infectious life cycle in cell culture.

Not all tetraviruses use an alkaline pH to induce the conformational changes required for binding and entry. The carmotetravirus, PrV, readily infects tissue culture cells in the neutral to slightly acidic conditions of tissue culture medium (Jiwaji *et al.*, 2016). The CP termini of PrV are structurally and functionally distinct from those of the alphatetraviruses: in contrast to the alphatetraviruses, the N-terminal and not the γ peptides of the PrV CP serves as the molecular switch. The arrangement of peptide termini in the PrV capsid shares an evolutionary relationship with nodaviruses (Speir *et al.*, 2010), in which infectivity is triggered by acidic pH conditions (Odegard *et al.*, 2010). HaSV and PrV both infect and replicate in the non-host *S. frugiperda* (Sf9) cell line (Fig. 7.2), which was derived from *S. frugiperda* ovarian tissue.

Virus replication

Tetraviruses replicate in the host cell cytoplasm in association with host cell membranes. Transient expression of HaSV REP fused to EGFP at its C terminus, in *S. frugiperda* Sf9 and mammalian (HeLa and BHK-21) cells, results in the subcellular localization of fluorescence to punctate cytoplasmic structures (Short *et al.*, 2010) that are associated with detergent-resistant membranes (Short and Dorrington, 2012). The punctate pattern is abolished when EGFP is fused to the N-terminus of the

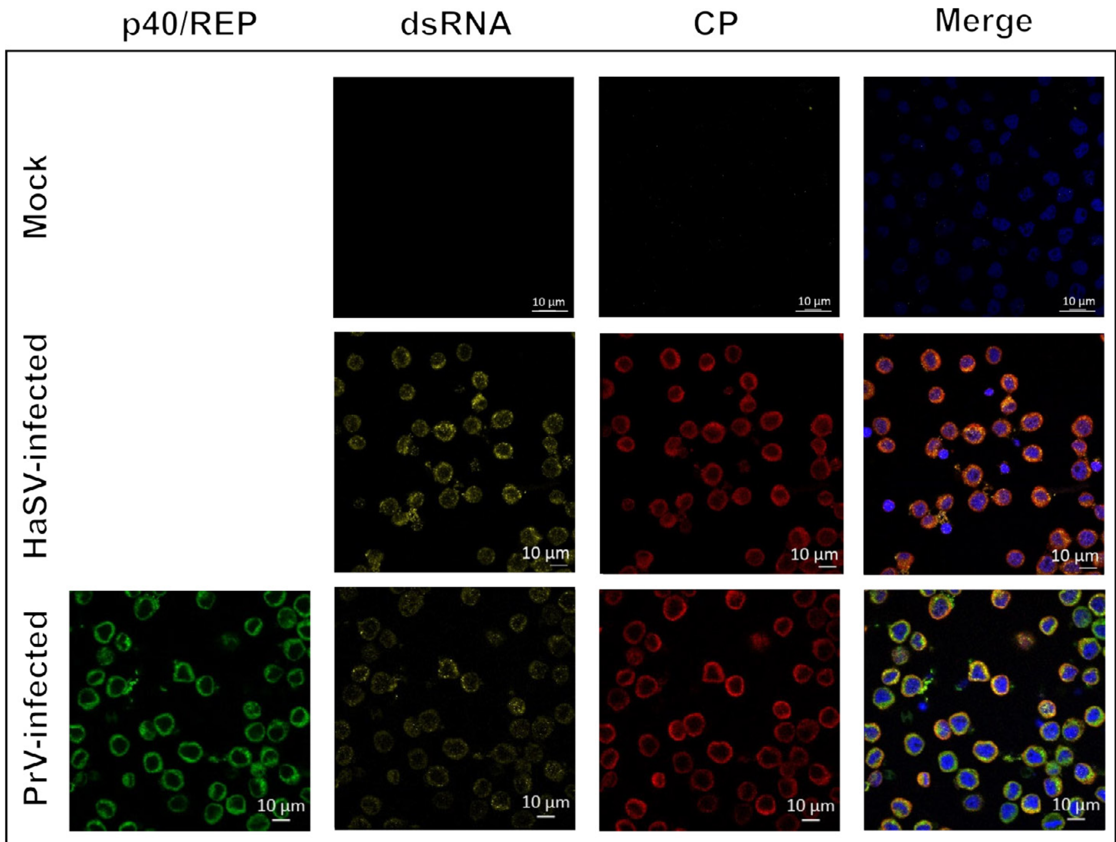

Figure 7.2 Subcellular localization of HaSV and PrV replication complexes in *Spodoptera frugiperda* (Sf9) cells. HaSV infection was carried out as described by Penkler *et al*. (2016) and the cells collected for immunofluorescence staining 8-hour post infection. PrV infection was carried out by incubating confluent Sf9 cells with filtered supernatant from *H. zea* MG8 cultures for 4 hours. Thereafter the supernatant was replaced with fresh growth medium and the cells incubated for 8h. Cells were probed with mouse monoclonal anti-dsRNA antibodies (English and Scientific Consulting; 10020500) and anti-mouse AF546 (Invitrogen; A11003) as described in Short *et al*. (2013). Viral replicase was stained with biotin-conjugated anti-PrV p40 and streptavidin AF488 (Invitrogen; S11223), while rabbit polyclonal anti-HaSV CP or anti-PrV CP antiserum and anti-rabbit AF647 (Invitrogen; A20170) were used to detect the CP. All images represent 1 µm optical slices taken using a Zeiss LSM 780 laser scanning confocal microscope using a 63, 0.75 NA objective. Scale bar: 10 µm.

replicase, implying a function for the N-terminus in subcellular localization of the replicase (Short *et al.*, 2010). Two domains, located at the N-terminus and within the C-terminal half of the HaSV replicase are both required for targeting the HaSV REP–EGFP fusion to punctate structures within the cytoplasm (Short and Dorrington, 2012).

Immunofluorescence microscopy studies have shown that PrV REP localizes to similar punctate structures in the cytoplasm of persistently infected *H. zea* MG8 cells (Short and Dorrington, 2012) and also in *S. frugiperda* cells expressing EGFP–REP fusion proteins (Short *et al.*, 2013). Co-localization of the PrV REP with replicating viral RNA confirmed that these punctate structures are the site of viral replication in PrV-infected infected *H. zea* MG8 (Short *et al.*, 2013) and *S. frugiperda* cells (Fig. 7.2) and that as with HaSV, virus replication occurs in association with detergent-resistant membranes (Short *et al.*, 2012).

Initially, studies with HeLa cells expressing HaSV REP-EGFP showed that EGFP fluorescence partially co-localized with CD63, a marker that is cycled between the endocytic and secretory pathways (Kobayashi *et al.*, 2000; Short *et al.*, 2010). Subsequently, recombinant organellar markers for insect cells (Maroniche *et al.*, 2011) were used to show that both the PrV and HaSV REP co-localize

with vesicles originating from the Golgi and secretory vesicles (Short *et al.*, 2013; de Bruyn *et al.*, 2016). Thus, both PrV and HaSV share the same site of replication and replicate in association with detergent-resistant membranes of vesicles derived from the Golgi and secretory pathway. Given the differences between their replicases and the presence of additional virus-specific non-structural proteins, it is likely that each virus uses a distinct mechanism to initiate infection and assemble the virus replication factory.

As mentioned previously, the PrV REP ORF encodes a read-through stop codon, the activity of which results in the production of a truncated accessory replication protein, p40 and the full-length REP (p104). p40 and REP co-localize with dsRNA and are involved in the establishment of the replication complex (Short *et al.*, 2013). Located within the N-terminal region of REP and within p40 are membrane spanning domains that anchor both proteins in the target membrane. A protein–protein interaction domain facilitates self-interaction between p40 proteins and interaction with REP, which encodes the RdRp domain at its C-terminal end (Nakayinga, 2013). p40 and REP are trafficked to the site of replication where they interact with the target membranes and each other to form the replication complex (Short *et al.*, 2013). The function of the non-structural protein, p130, is unclear. However, preliminary data suggest that expression of p130 is required for translation of the PrV REP, implying that p130 is likely involved in initiation of viral replication (J.A. Awando and R.A. Dorrington, unpublished results).

There is biochemical evidence that suggests that the HaSV REP also self-interacts, via a region located near to or within the RdRp domain (Short and Dorrington, 2012). The assembly of a replication complex in HaSV-infected cells likely also involves accessory proteins in addition to the REP. The translation products of two of the three small ORFs located at the 3′ end of HaSV RNA1 co-localize with REP and are trafficked to secretory vesicles providing strong evidence that they play a role in the assembly of the viral replication complex (de Bruyn *et al.*, 2016). Precisely what their function is in the viral life cycle is unclear but over-expression of all three ORFs in a recombinant baculovirus appears to be toxic to *S. frugiperda* cells (de Bruyn *et al.*, 2016).

Packaging of tetraviral RNAs

There have been several apparently conflicting reports whether tetravirus VLPs are produced in the absence of viral replication package viral RNAs. Agrawal and Johnson (1995) demonstrated the assembly of VLPs in insect cells with evidence that these VLPs packaged viral capsid (VCAP) mRNA. However, in other studies, omegatetravirus VLPs produced by recombinant baculovirus expression of VCAP do not specifically encapsidate viral RNAs (Taylor, 2003; Gordon and Waterhouse, 2006; Routh *et al.*, 2012). This is also the case with VLPs produced in the yeast, *Saccharomyces cerevisiae* (Tomasicchio, 2007). These VLPs package mostly cellular RNAs as well as some mRNA expressing the capsid protein precursor. Gordon *et al.* (2001) reported the replication independent assembly of infectious HaSV particles in tobacco protoplasts. Their experimental system was based on the co-expression of HaSV RNA1 and RNA2 cDNAs fused at their 5′ ends to the (–1) nucleotide of the Cauliflower mosaic virus promoter and flanked at their 3′ ends by a *cis*-acting ribozyme, producing precise copies of viral RNA. In addition, the plant protoplasts were co-transfected with a third plasmid expressing HaSV VCAP message after which the protoplasts were fed to neonate *H. armigera* larvae. Evidence for assembly of infectious particles was the recovery of infectious HaSV from these larvae and detection of virus-like particles by immuno-electron microscopy, but the yields of virus particles were too low to confirm successful encapsidation of viral RNAs (Gordon *et al.*, 2001).

The same experimental system was used to demonstrate delivery of non-viral RNA encoding EGFP to larval midgut cells by recombinant HaSV VLPs assembled in tobacco protoplasts (Gordon and Waterhouse, 2006). This was achieved by transfecting protoplasts with plasmids expressing RNA1, VCAP and a third with RNA2 in which VCAP had been replaced with EGFP just downstream of the p17 stop codon. The presence of EGFP fluorescence in larval midgut cells up to 10 days after ingestion suggested replication of recombinant RNA2-EGFP transcripts in the midgut cells. However, the low level of VLP assembly in the system precluded analysis to show specific encapsidation of the viral RNAs (Gordon and Waterhouse, 2006).

The observation that p17 is expressed in infected insect cells and that the protein is present at low

concentrations in wild-type virus particles purified form HaSV infected insects, has led to the proposal that p17 is involved in packaging of viral RNA during capsid assembly (Mendes *et al.*, 2015). To test this hypothesis, p17 was co-expressed with VCAP in *S. frugiperda* cells, the resulting VLPs were purified and analysed for the presence of viral RNA2. VLPs assembled in cells co-expressing VCAP and p17 contained viral mRNA, but when p17 was absent, no viral RNA could be detected in purified capsids. VLPs produced by recombinant baculoviruses package vRNA2 at detectable levels even in the absence of p17 and apparently excluded baculoviral transcripts. Deletion analysis showed that the 5′ and 3′ UTR sequences of HaSV RNA2 were not required for packaging (Mendes *et al.*, 2015). These data, together with those of Gordon and Waterhouse (2006), suggest that a sequence within the p17 ORF or immediately downstream of the p17 ORF may be required for packaging HaSV RNA2.

Are p17-like homologues present in other tetraviruses? Analysis of the amino acid sequence of p13, produced by 2A-like processing of the PrV VCAP, reveals a high incidence of PEST amino acid residues, raising the possibility that this peptide might have a similar function in facilitating packaging of viral RNAs by PrV particles. Similarly, a peptide of approximately 17 kDa is also produced by 2A-like processing of the TaV (and probably EeV) capsid protein precursor. The observation by Pringle *et al.* (1999) that the presence of TaV p17 appears to stabilize TaV VLPs suggests that this peptide may serve a similar function as the omegatetraviral p17 in the betatetraviruses TaV and EeV. There is no evidence for the presence of a p17-like ORF on the NβV genome sequence (Gordon *et al.*, 1999), which may either indicate an alternative packaging mechanism in this virus, or mutations in cDNA fragments used to assemble the NβV genome sequence.

Redefining the host range of tetraviruses

For many years, the most striking feature of the tetraviruses other than their distinctive capsid structure, was their narrow host range, often confined to a single species or selected species within one family (Dorrington *et al.*, 2011). This was a characteristic that made tetraviruses prime candidates for the development of biopesticides to control agricultural pests (Christian *et al.*, 1993; Hanzlik *et al.*, 1999). However, there are several exceptions to this rule. Trichoplusia ni virus and Dasychira pudibunda virus infect species in families other than that of their normal host (Greenwood and Moore, 1981). The host range of the omegatetravirus HaSV has been extended to several Noctuid species, including *H. armigera*, *H. zea*, *Heliothis virescens*, *Heliocheilus pallida*, (Subfamily *Heliothenae*) as well as *Earias huegeliana* and *Chrysodeixis argentifera* (Christian *et al.*, 2001) and recently, *S. frugiperda* (Penkler *et al.*, 2016).

HaSV infectivity requires the induction of conformational changes within the virus particle by an alkaline pH (Penkler *et al.*, 2016) suggesting that the requirement for an alkaline gut pH might be the barrier to infection that restricts HaSV and other alphatetraviruses such as NωV, with their conserved capsid structures and the shared biophysical properties of their VLPs to noctuid insects. The alkaline environment of the midgut of herbivorous insects is believed to be an adaptation to the chemical defence mechanisms of plants (Appel and Martin, 1990), which in turn has required their pathogens to evolve strategies to overcome some of the highest pH environments known in living systems.

There appear to be no such constraints on the host range of the carmotetravirus, PrV, which does not require an alkaline environment for infectivity. The distinct biophysical characteristics of its capsids allow PrV to infect and replicate in mammalian tissue culture cells (Fig. 7.3), including baby hamster kidney and human cancer cell lines (Jiwaji *et al.*, 2016; M. Jiwaji, unpublished data). Also unique to PrV is the ability to establish asymptomatic, persistent infections in both insect and mammalian cells (Pringle *et al.*, 2003; Jiwaji *et al.*, 2016). This implies conservation of the host cell receptor(s) used by PrV for binding and entry as well as host factors required to support virus replication in the invertebrate and vertebrate host cells. A PrV-like virus with 85% sequence identity was recently discovered in the guano of a female western barbastelle bat (*Barbastella barbastellus*) in Hungary (Kemenesi *et al.*, 2016). Western barbastelle bats are insectivorous with a predominantly lepidopteran diet (Zeale *et al.*, 2010), so the presence of PrV was most likely due to ingestion of PrV-infected prey (Kemenesi *et*

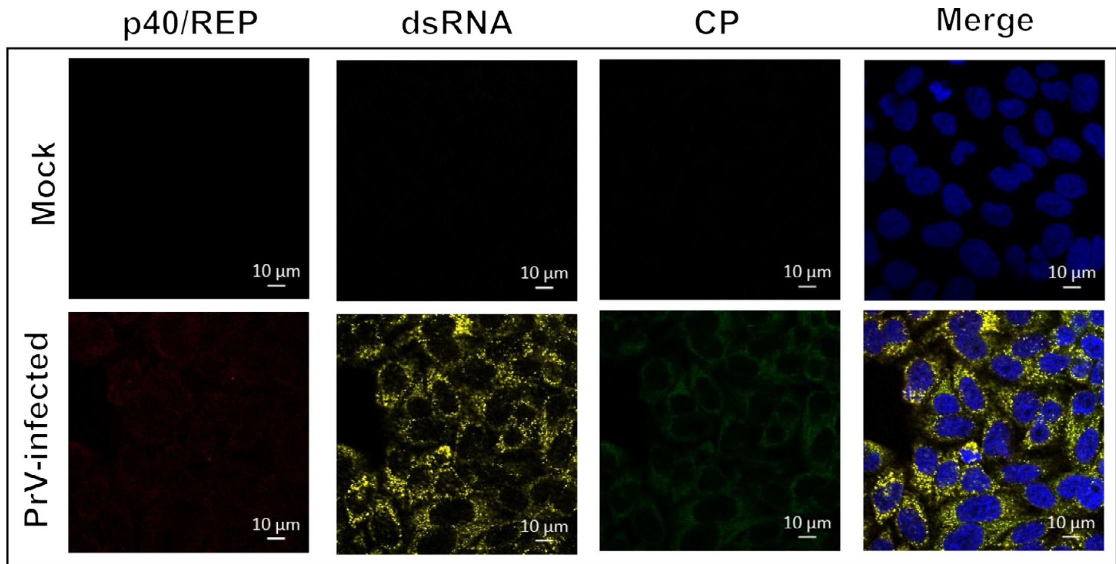

Figure 7.3 Replication of PrV in mammalian cells. PrV infections were carried out as described in Jiwaji *et al*. (2016). HeLa cells were probed with mouse monoclonal anti-dsRNA antibodies (English and Scientific Consulting; 10020500) and anti-mouse AF546 (Invitrogen; A11003) as described in Short *et al*. (2013). Viral replicase was stained with biotin-conjugated anti-PrV p40 and streptavidin AF488 (Invitrogen; S11223), while rabbit polyclonal anti-CP and anti-rabbit AF647 (Invitrogen; A20170) were used to detect PrV CP. All images represent 1 μm optical slices taken using a Zeiss LSM 780 laser scanning confocal microscope using a 63, 0.75 NA objective. Bar, 10 μm.

al., 2016). However, it is possible that the bat could have become infected with PrV.

PrV is the only tetravirus with a replicase that is more closely related to those of plant-infecting tombus- and umbraviruses, (Family: *Tombusviridae*). Not only is there conservation between their RdRp domains, but the translation strategy of PrV and tombusviruses is also conserved, with the presence of a read-through stop that results in the expression of a truncated accessory replication protein (Walter *et al*., 2010). This has given rise to the hypothesis that PrV may have arisen via horizontal transmission from a plant to a phytophagous insect and recombination with a tetravirus in the insect gut. The observation that PrV replicates in plant cell-free extracts (Jiwaji *et al*., 2016) raised the question of whether the virus may still be able to replicate in plants. PrV particles isolated from persistently infected *H. zea* MG8 cells were used to mechanically infect the leaves of *Vigna unguiculata* (cowpea) plants. After two weeks, *V. unguiculata* plants infected with PrV showed lesions and yellowing compared to uninfected control plants (Fig. 7.4A) and PrV virus purified from plants (Fig. 7.4B) was able to infect mammalian

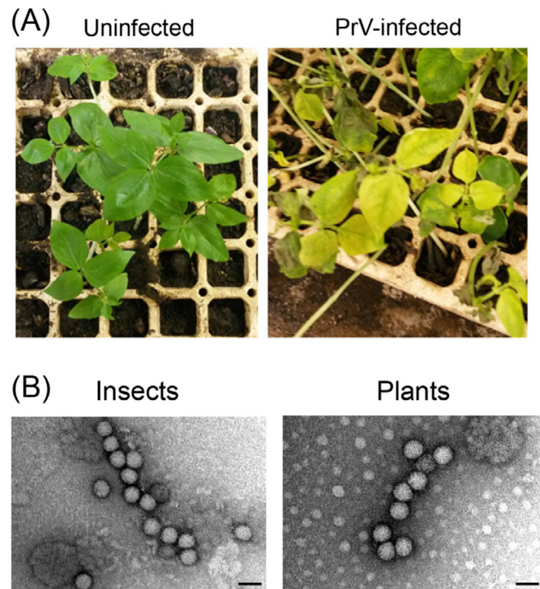

Figure 7.4 PrV infection of cowpea plants. (A) Comparison of leaves from mock infected and PrV-infected cowpea plants. (B) Transmission electron microscopic visualization of the virus particles purified from *H. zea* MG8 cells and cowpea plants as described in Jiwaji *et al*. (2018). Scale bar: 50 nm.

tissue culture cells. Remarkably, the virus had moved from the site of infection to the new leaves at the top of the plant (Figure 7.4A, M. Jiwaji, unpublished data), demonstrating that not only has PrV retained the ability to infect and replicate in plants, but it has also retained the ability to move from cell to cell, like umbraviruses. These findings provide strong evidence that PrV has indeed evolved from an ancestral plant virus via horizontal transmission to an insect. Thus, not only is PrV able to infect and replicate in invertebrate and vertebrate animal cells, but it can also initiate a productive infection in plants, implying that the host factors required to support the PrV infectious life cycle are conserved across the plant and animal Kingdoms.

Are there new tetraviruses yet to be discovered? A study by Water *et al.* (2008) suggests that this is likely to be the case. A survey of the viruses in a wild population of *Nudaurelia cytherea capensis* (pine emperor moths) over a 3-year period resulted in the isolation of three distinct small RNA viruses, including NβV, which had not been found in wild South African *Nudaurelia* populations for almost 30 years. The other two viruses, designated NζV and NψV, were serologically related to NβV and NωV, respectively, but were distinct in terms of virion diameter, surface morphology and the sizes of their major capsid proteins.

Metagenomic studies have reported the discovery of tetravirus-like genomes, but with the exception of PrV, discovered in the virome of an insectivorous bat (Kemenesi *et al.*, 2016) no bona fide tetraviruses have been discovered. One of the first metagenomic studies reported the discovery of a tetravirus-like sequence, encoding a 984 aa protein, assembled from virus-derived siRNAs isolated from the *Drosophila melanogaster* S2 cell line (Wu *et al.*, 2010). The encoded protein shared 29% identity with the RdRp of EeV and TaV, leading the authors to propose that this sequence represented a new permutotetravirus that was named *Drosophila* tetravirus. Subsequently, it was determined that that this sequence likely originated from a strain of the unclassified ssRNA virus, DAV (Bronkhorst and van Rij, 2014), the RdRp of which shares homology with that of the permutotetraviruses (Ambrose *et al.*, 2009). Metagenomic sequencing of mosquito viromes resulted in the assembly of several ssRNA virus genomes including Sarawak virus, which is proposed to be a new tetravirus, due to sequence homology to the RdRp of the DpTV replicase (Sadeghi *et al.*, 2017). The classification of Sarawak virus as a tetravirus, however, will require further investigation into the biophysical characteristics of the virus capsid.

Concluding remarks

Tetraviruses have long been considered to have relatively simple genomes, encoding just the viral replicase and a single CP sequence (Hanzlik *et al.*, 1999). However, the apparent complexity of their genomes together with the identification of additional non-structural viral proteins has challenged the notion of genomic simplicity and focused attention on the mechanisms by which tetraviruses express multiple viral proteins from their relatively compact genomic RNAs.

The tetraviruses are a diverse group of small non-enveloped RNA viruses unified by the distinct $T=4$ icosahedral architecture of their capsids. However, the distinguishing characteristics of the RdRp domains of their replicases led to their classification in three separate families. It is reasonable to propose that the three tetravirus lineages are the result of genome re-assortment with (+)ssRNA viruses contributing the replication machinery and ancestral tetraviruses contributing the structural genes, on co-infection of the lepidopteran host gut. This hypothesis is supported by observations that (1) tetravirus VLPs package non-viral RNAs; (2) viruses belonging to two of the three families (*Alphatetraviridae* and *Carmotetraviridae*) replicate in association with secretory vesicles; and (3) PrV as an extant example of such a cross-Kingdom re-assortment.

In recent years, there have been significant breakthroughs in tetravirus research, most notably the establishment of virus replication in tissue culture that have provided the tools to elucidate the infectious virus life cycle and to develop a fundamental understanding of the interactions between virus and host that determine host range and infectivity. It is becoming clear that the biology of these viruses is very different, despite structural conservation of their capsid architecture and a shared site of replication in the host cell. To our knowledge, PrV is the only (+)ssRNA virus that infects and productively replicates in invertebrate and vertebrate cells as well as in plants, providing an unprecedented

opportunity to study the fundamental mechanisms of RNA virus replication in eukaryotic cells.

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