Virology 522 (2018) 281–291

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

Nanovirus DNA-N encodes a protein mandatory for aphid transmission

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ARTICLE INFO

Keywords:
Plant virus
Segmented single-stranded DNA genome
Transmission helper factor
Reassortment
Faba bean
Aphis craccivora
Acyrthosiphon pisum

ABSTRACT

Nanoviruses possess a multipartite single-stranded DNA genome and are naturally transmitted to plants by various aphid species in a circulative non-propagative manner. Using the cloned genomic DNAs of faba bean necrotic stunt virus (FBNSV) for reconstituting nanovirus infections we analyzed the necessity of different virus components for infection and transmission by aphids. We found that in the absence of DNA-U1 and DNA-U2 symptom severity decreased, and in the absence of DNA-U1 the transmission efficiency decreased. Most significantly, we demonstrated that the protein encoded by DNA-N (NSP) is mandatory for aphid transmission. Moreover, we showed that the NSP of FBNSV could substitute for that of a distantly related nanovirus, pea necrotic yellow dwarf virus. Altering the FBNSV NSP by adding 13 amino acids to its carboxy-terminus resulted in an infectious but non-transmissible virus. We demonstrate that the NSP acts as a nanovirus transmission factor, the existence of which had been hypothesized earlier.

1. Introduction

Members of the family *Nanoviridae* (nanovirids) infect plants and possess a multipartite genome consisting of six or eight circular single-stranded (ss) DNAs, individually encapsidated in small isometric (~20 nm in diameter) virions that are exclusively transmitted by various aphid species in a persistent (circulative, non-propagative) manner (Vetten et al., 2012). The family is divided into the genera *Nanovirus* and *Babuvirus*. Nanoviruses infect dicotyledonous hosts, predominantly leguminous plants, and possess eight genomic DNAs; babuviruses infect monocotyledonous plants and possess six genomic DNAs. The genomic DNAs or genome components, circular ssDNAs of about 1 kb in size, are named according to the function of the proteins they encode: DNA-R (master replication initiator protein; M-Rep, required for replication initiation of all genomic DNAs) (Timchenko et al., 1999), DNA-S (structural = capsid protein; CP) (Katul et al., 1997), DNA-C (cell cycle link protein; Clink, that modulates the host's cell cycle in favor of nanovirus replication) (Aronson et al., 2000), DNA-M (movement protein; MP) and DNA-N (nuclear shuttle protein; NSP); the names of the latter two were inferred by functional similarities with geminivirus proteins (Krapp et al., 2017; Lazarowitz and Beachy, 1999; Wanitchakorn et al., 2000). These five DNAs are characteristic of all nanovirids, whereas DNA-U1, DNA-U2 and DNA-U4 encoding proteins of unknown function are nanovirus-specific, and DNA-U3 is babuvirus-specific (Vetten et al., 2012). Currently eight nanovirus and three babuvirus species are recognized by the International Committee on Taxonomy of Viruses: *Subterranean clover stunt virus,* the type species (Chu and Helms, 1988), *Black medic leaf roll virus* (Grigoras et al., 2014), *Faba bean necrotic yellows virus* (Katul et al., 1997, 1998), *Faba bean necrotic stunt virus* (Grigoras et al., 2009) *Faba bean yellow leaf virus* (Abraham et al., 2012), *Milk vetch dwarf virus* (Sano et al., 1998), *Pea necrotic yellow dwarf virus* (Grigoras et al., 2010), *Pea yellow stunt virus* (Grigoras et al., 2014), *Banana bunchy top virus* (Burns et al., 1995; Thomas, 2008), *Abaca bunchy top virus* (Sharman et al., 2008) and *Cardamom bushy dwarf virus* (Mandal et al., 2004, 2013). In addition, two further nanoviruses possibly representing new species have been recently identified from the wild legume *Sophora alopecuroides* L. in Iran (Heydarnejad et al., 2017) and *Vicia cracca* L. in France (Gallet et al., 2018).

The other group of plant viruses with an ssDNA genome and a

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https://doi.org/10.1016/j.virol.2018.07.001
Received 11 May 2018; Received in revised form 4 July 2018; Accepted 4 July 2018

persistent (circulative, non-propagative) transmission mode is the family *Geminiviridae* (Brown et al., 2012; Gray et al., 2014; Hogenhout et al., 2008). Nanovirids and geminiviruses resemble each other in genome replication and modulation of the host's cell cycle (Gronenborn, 2004). Whereas the biology of geminiviruses has been well studied (Hanley-Bowdoin et al., 2013), comparable knowledge of nanovirid biology still lags behind (Vetten, 2008). A major reason for that has been the difficulty of applying reverse genetics to nanovirids. By using cloned copies of infectious virus DNAs that problem had been solved to some extent for faba bean necrotic yellows virus (FBNYV) allowing reconstitution of this virus (Timchenko et al., 2006). However, whereas the reconstituted virus induced typical disease symptoms in inoculated plants, it was not transmissible by the pea aphid (*Acyrthosiphon pisum*) or the cowpea aphid (*Aphis craccivora*), the natural insect vectors of FBNYV. With the reconstitution from cloned genomic DNAs of an infectious faba bean necrotic stunt virus (FBNSV) that can be sustainably transmitted by both pea aphids and cowpea aphids a major obstacle for studying nanoviruses was overcome (Grigoras et al., 2009). This finally has lifted a long-standing barrier, which had blocked reverse genetics of these viruses and has meanwhile led to significant novel and unexpected insights into the biology of FBNSV (Sicard et al., 2016, 2013, 2015). Reconstitution from cloned DNAs and subsequent aphid transmission has also been applied successfully to black medic leaf roll virus (BMLRV) and a FBNYV isolate from Spain (Grigoras et al., 2014), as well as to pea necrotic yellow dwarf virus (PNYDV) (Grigoras et al., 2010), as shown below.

While insect transmission of geminiviruses is dependent only on the virus capsid (Brown et al., 2012; Gray et al., 2014; Hogenhout et al., 2008), studies by Franz and co-workers had suggested that aphid transmission of nanoviruses requires a virus-encoded helper factor (Franz et al., 1999). However, there has since been no information on the nanovirus gene(s) encoding such a factor.

By using cloned FBNSV DNAs in agroinoculation experiments we answered the question which of the eight components of the virus genome are essential for infection, symptom development and aphid transmission in faba bean. Whereas DNA-C and DNA-U4 had no influence on disease symptoms and virus transmission, DNA-U1 and DNA-U2 encode proteins that influence symptom severity and virus titer. By contrast, in experiments omitting DNA-N or using a DNA-N mutant encoding a non-functional protein variant we showed that the NSP is mandatory for aphid transmission of the virus but does not influence virion formation, virus titer and disease symptoms.

2. Materials and methods

2.1. Origin and maintenance of virus isolates

Faba bean necrotic stunt virus (FBNSV), isolate FBNSV-[ET:Hol;97], had been identified in diseased faba bean (*Vicia faba*) from Ethiopia and was established and maintained in faba bean by serial vector transmissions using the pea aphid at the JKI-Braunschweig until 2004. A fully infectious and aphid transmissible FBNSV (FBNSV-[ET:Hol;JKI-2000]) was reconstituted from $CaCl_2$-dried infected tissue (Grigoras et al., 2009), (GenBank accession numbers GQ150778 to GQ150785, Supplementary Table S1).

Faba bean necrotic yellows virus (FBNYV), isolate FBNYV-[EG;1–93] was isolated from faba bean (*Vicia faba*) from Egypt (Makkouk et al., 1993). This isolate was established and maintained in faba bean by serial vector transmissions using the pea aphid at JKI Braunschweig until 1998. Viral DNAs were cloned from $CaCl_2$-dried infected tissue (GenBank acc. numbers AJ132179 to AJ132184, AJ132186, AJ749902, Supplementary Table S1) and used by Timchenko and co-workers (Timchenko et al., 2006) for reconstituting infectious virus that was not aphid-transmissible.

Pea necrotic yellow dwarf virus (PNYDV), isolate PNYDV-[DE;15], was originally obtained from pea (*Pisum sativum*) plants collected in Drohndorf, Germany (Grigoras et al., 2010). Total DNA was extracted from symptomatic leaves of infected faba bean plants and circular viral DNA was amplified by RCA and cloned in a same way as described previously (Grigoras et al., 2009). RCA products were digested with restriction enzymes in appropriate buffers. Generated fragments were resolved on 1% agarose gels, extracted and inserted into corresponding plasmids as indicated in Supplementary Table S2. The inserts of recombinant plasmids were sequenced (GenBank acc. numbers GU553134, and JN133279 to JN133285, Supplementary Table S1). Similarly to FBNSV-[ET:Hol;JKI-2000], fully infectious and aphid transmissible PNYDV-[DE;15] was reconstituted from cloned genomic DNAs (see Supplementary Table S2 for details of construction of plasmids used for PNYDV reconstitution). It shall be noted here that wild-type PNYDV reconstituted from cloned DNAs has been maintained by successive pea aphid transmission for more than six years.

The infectious genomes of these viruses, here referred to as FBNSV, FBNYV and PNYDV, were used for agroinoculation and genetic modification. The reconstituted viruses were observed for symptom development and assayed for aphid transmissibility.

2.2. Construction of a modified FBNSV DNA-N

For expression in *E. coli* of FBNSV NSP with a carboxy (C)-terminal hexa-histidine tag, the coding region of DNA-N (GenBank acc No. GQ150782) was PCR-amplified from a DNA-N containing plasmid (Litmus28) using primer pair 5-FB-N-Nde and 3-FB-N-Xho (Supplementary Table S3) and cloned as *Nde*I-*Xho*I insert in pET-22b (+) (Merck Millipore). The resulting plasmid pET22b + NSP-FBNSV encoded an NSP tagged at its C-terminus with the 13 amino acid sequence –GGGGSLEHHHHHH (a hexa-histidine tail preceded by the spacer GGGGSLE). The tagged protein is referred to in the following as NSP-His.

Additionally, a modified FBNSV DNA-N encoding the identical NSP-His was constructed. For this purpose, a *Nde*I site was introduced in the plasmid Litmus28 harboring FBNSV-[ET:Hol:JKI-2000] DNA-N by changing the original nucleotides TAATTA containing the TAA stop codon of the NSP-gene (position 795–800) into CATATG by site directed mutagenesis using the high-fidelity Phusion DNA polymerase (Thermo Scientific) and primer pair Hol_N_Nde_dir2 and Hol_N_Nde_rev2 (Table S3). Fifty microliters of PCR mixture contained 10 nanograms of plasmid DNA, 1 microliter of each primer (at 50 pmol per microliter), 1 microliter of dNTP mix (2.5 mM each) and 1 microliter of Phusion polymerase. After PCR amplification (18 cycles of 98 °C, 10 s; 55 °C, 30 s; 72 °C, 4 min), 1 microliter of *Dpn*I endonuclease was added to the PCR mixture followed by incubation at 37 °C for 2 h. After the digest, 5 microliters were used for transformation of *E. coli* XL1-blue cells (Invitrogen) and plasmid DNA of transformants was tested for the presence of the introduced *Nde*I site by restriction analysis. DNA of *Nde*I-positive candidates was digested by *Sal*I and *Nde*I, and the fragment encoding the C-terminal part of NSP was liberated. This fragment was then exchanged with the fragment encoding the C-terminal part of NSP-His, which was amplified from the plasmid pET22b + NSP-FBNSV using primer pair Hol_N_Saldir and pET22bNdeHisrev (Table S3). The correctness of all constructs was verified by sequencing. The resulting plasmid Litmus28_FBNSV-NSP-His contains a FBNSV DNA-N (1023 nt) encoding NSP with the above-mentioned tag. A head-to-tail dimer of this modified DNA-N was constructed in the *Aat*II site of Litmus28, and the dimeric DNA-N-His was transferred as a *Bam*HI – *Kpn*I fragment into the binary vector pBin19.

2.3. Inoculation of faba bean plants with cloned nanovirus DNAs and insect transmission

Head-to-tail dimer copies of the nanovirus DNAs in pBin19 were transferred by electroporation into *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*) strain COR308 and used in an equimolar