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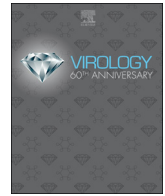
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# Nanovirus DNA-N encodes a protein mandatory for aphid transmission

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## 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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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 (*Acyrtosiphon 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<sub>2</sub>-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<sub>2</sub>-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 *NdeI-XhoI* 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 –GGGGSLHHHHHH (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 *NdeI* 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 HoI\_N\_Nde\_dir2 and HoI\_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 *DpnI* 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 *NdeI* site by restriction analysis. DNA of *NdeI*-positive candidates was digested by *Sall* and *NdeI*, 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 HoI\_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 *AatII* site of Litmus28, and the dimeric DNA-N-His was transferred as a *BamHI* – *KpnI* 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

ratio for agroinoculation of faba bean (*Vicia faba*; varieties Shambat-75, Scirocco or Espresso), as described (Timchenko et al., 2006). Unless otherwise stated, plants that developed symptoms after agroinoculation were pooled and used for virus acquisition and transmission by cowpea or pea aphids to faba bean seedlings as described (Grigoras et al., 2009). Briefly, several hundred individuals of cowpea aphids (of different nymphal stages) reared on faba bean were allowed an acquisition access feeding period (AAP) of 3–5 days on infected faba bean plants, followed by an inoculation access feeding period (IAP) of 3–5 days on young faba bean seedlings using at least 30 individuals per plant. After spraying with a contact insecticide, the plants were kept in an insect-proof glasshouse and observed for symptom development.

To detect viral DNAs in inoculated plants, total DNA was extracted as described (Grigoras et al., 2009), and presence or absence of the respective genome components was confirmed by PCR (35 cycles of 95 °C, 30 s; 53 °C, 40 s; 72 °C, 90 s) using the component-specific primer pairs listed in Supplementary Table S3.

#### 2.4. Production of FBNSV NSP-His protein in *E. coli*

The C-terminally His-tagged FBNSV NSP was expressed in *E. coli* BL21 CodonPlus (DE3)-RIL (Stratagene) cells harboring pET22b+NSP-FBNSV following induction by IPTG. The tagged protein was mainly soluble and was purified using immobilized metal adsorption chromatography (IMAC) on Ni-NTA resin (Qiagen) and imidazole elution. The purified protein served for rabbit and mouse immunization to produce polyclonal- (PAbs) and monoclonal antibodies (MAbs).

#### 2.5. Production of poly- and monoclonal antibodies to NSP-His and serological assays

The production of NSP-specific PAbs and MAbs was carried out using standard methods. IgG of the rabbit PAbs (JKI-1603) was isolated by affinity chromatography using protein A and was labeled with alkaline phosphatase (AP) as described (Clark and Adams, 1977). Double (DAS)- and triple-antibody sandwich (TAS) enzyme linked immunosorbent assay (ELISA) formats were performed with the buffers described by Clark and Adams (Clark and Adams, 1977). AP-labeled rabbit anti-mouse IgG, used at dilution 1:2500, was from Jackson ImmunoResearch Europe Ltd.

The rabbit PAbs obtained gave DAS-ELISA reactions of 0.5–1.0 for FBNSV-infected plants and of < 0.1 for non-infected plants and cross-reacted strongly with heterologous NSPs (several isolates of FBNYV, PNYDV, BMLRV) in various serological tests. To increase specificity and reactivity of the serological tools for NSP detection, we also raised MAbs specific to FBNSV NSP. Immunization of mice, establishment, selection, and cloning of hybridomas as well as typing and preparation of MAbs was done as previously described (Adam et al., 1991; Franz et al., 1996). Supernatant fluids from individual cultures were screened for NSP-specific antibodies by testing against FBNSV-infected and non-infected plant extracts in TAS-ELISA using NSP-specific IgG (concentration 1 mg/ml) at 1:1000 dilution for coating. The properties of the MAbs secreted by seven stable hybridoma lines and used to detect the NSP of FBNSV in TAS-ELISA and TBIA are shown in Table S4.

To detect the capsid protein (CP) in agroinoculated *V. faba* plants we used the FBNSV-specific MAb FBNSV-8-6F8 (Grigoras et al., 2009) and the PNYDV-specific MAb PNYDV-2-4B9 in TAS-ELISA (see above) using rabbit IgG to FBNYV and PNYDV, respectively, for coating of plates (all antibodies from the stock of JKI Braunschweig).

Virus titers in infected faba bean plants agroinoculated with FBNSV, FBNSV- $\Delta$ N, - $\Delta$ U1 or - $\Delta$ U2 were determined by DAS-ELISA (Clark and Adams, 1977) using the rabbit IgG to FBNYV (stock of JKI Braunschweig), which cross-react strongly with FBNSV CP in DAS-ELISA (Franz et al., 1996). The only modification used here was that 100 mg of plant tissue were ground in liquid nitrogen and then dissolved in 500  $\mu$ l of extraction buffer prior to loading on plates. The optical densities

(A<sub>405 nm</sub>) developed at different time points during substrate incubation were measured, with each data set being normalized by subtraction of the A<sub>405 nm</sub> values obtained for non-infected plant tissue. Data obtained for different plant sets from two independent experiments were analyzed using the Kruskal-Wallis test (available in XLSTAT software).

Tissue blot immunoassay (TBIA) detection of nanovirus CP and NSP was essentially done as described (Lin et al., 1990). After blotting fresh stem- or petiole cuttings directly onto nitrocellulose membranes the latter were blocked with polyvinyl alcohol at 1 g/L and developed using a combination of NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt) as AP substrate. Western blot analysis of total protein extractions obtained by precipitation from infected and non-infected plants using trichloroacetic acid (TCA) (Lin et al., 1990) was carried out as described (Franz et al., 1996).

#### 2.6. FBNSV particle purification

One hundred grams of symptomatic *V. faba* plant tissue was ground in liquid nitrogen. The resulting powder was then mixed with 2.5 volumes (w/v) of extraction buffer (0.1 M sodium citrate, pH 6.2, containing 0.2% ascorbic acid, 0.2% Na<sub>2</sub>SO<sub>3</sub>, 0.3%  $\beta$ -mercaptoethanol, 1.5% cellulase, 0.4% macerozyme and 0.1% NaN<sub>3</sub>) and stirred slowly overnight at room temperature. After addition of 0.5 volume of a chloroform/butanol mixture (1:1) and centrifugation (6000 rpm for 20 min in Beckmann JA-14 rotor), the upper aqueous phase was filtered through two layers of Miracloth tissue. Virus particles were precipitated by adding NaCl (2%) and PEG 6000 (8%) and stirring for 2 h at 4 °C. After centrifugation (10,000 rpm for 15 min in Beckmann JA-14 rotor), the pellet was re-suspended in one volume (100 ml) of 0.02 M sodium phosphate buffer (SPB), pH 7.5, containing 1% Triton X-100. Following the removal of cell debris by centrifugation at 8000 rpm, the supernatant was subjected to centrifugation at 35,000 rpm for 180 min in a Beckman 45Ti rotor using a 10-ml sucrose cushion (20% in SPB). After re-suspension of the pellet in SPB buffer, cell debris was sedimented by centrifugation (8000 rpm for 15 min in Beckmann JA-20 rotor) and the supernatant was further centrifuged at 40,000 rpm for 180 min in a Beckman 70 Ti rotor. The light brown/translucent pellet was re-suspended in 0.5 ml of SPB, layered onto 12 ml of a sucrose gradient (prepared by freezing-thawing two times of 25% (w/v) sucrose solution in SPB) and centrifuged at 26,000 rpm for 4 h in Beckman SW41 rotor at 10 °C. Drop-fractions were collected after the centrifugation, fractions with highest absorbance at OD<sub>280</sub> were pooled, diluted 7–10 fold with SPB and centrifuged (40,000 rpm for 14 h in Beckman 70.1 Ti rotor). The pellet was resuspended in 50  $\mu$ l of 50 mM Tris-HCl, pH 7 and kept at 4 °C.

#### 2.7. Transmission electron microscopy

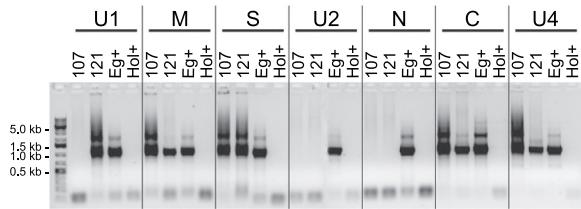
Four  $\mu$ l of virus samples were adsorbed onto the clean face of a carbon film on a mica sheet (carbon/mica interface) and negatively stained with 2% (w/v) sodium silicotungstate, pH 7.5. Images were taken under low-dose conditions with a FEI T12 LaB6 electron microscope working at 120 kV and with a nominal magnification of 49,000 $\times$  using an Orius SC1000.

### 3. Results

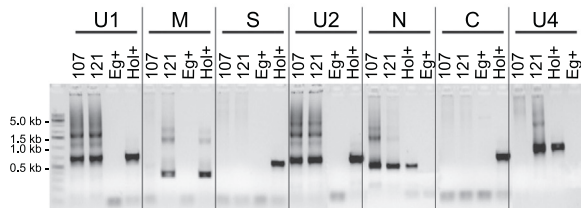
#### 3.1. Selection of aphid transmissible FBNYV-FBNSV reassortants

FBNYV-[EG;1-93] reconstituted by Timchenko and co-workers (Timchenko et al., 2006) was not transmissible by its natural vectors, the pea- or the cowpea aphids, despite producing virions and causing the characteristic FBNYV symptoms on inoculated faba bean plants. To test whether one or several of the cloned genome components of FBNYV might have been affected by mutation(s), we attempted

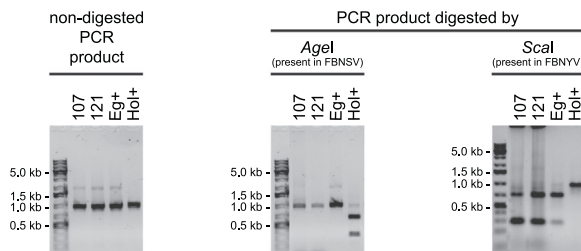
## A. FBNYV-[EG;1-93] specific primers



## B. FBNSV-[ET;Hol] specific primers



## C. Amplification and RFLP analysis of DNA-R with FBNYV-[EG;1-93] specific primers



**Fig. 1.** Differential detection by PCR of genomic DNAs in two lineages (107 and 121) of a mixed infection with FBNYV and FBNSV. Letters on top of each gel designate the respective genome components. Template DNAs are indicated above each slot: total DNA of the lineages 107 and 121, and Eg+ and Ho+ referring to plasmid DNAs of the respective cloned genome components of FBNYV-[EG;1-93] and FBNSV-[ET;Hol], used as positive control templates. PCR primers used for specific detection of the genome components of FBNYV and FBNSV are listed in Table S1. Amplified DNAs and restriction fragments thereof were separated by agarose gel (1%) electrophoresis and visualization by ethidium bromide. The GeneRuler 1 kb Plus DNA ladder (Thermo Scientific) was used as DNA size marker. A. Electrophoresis of amplified DNAs using primers specific for FBNYV-[EG;1-93]. All amplified DNAs correspond to the full-size components of ~1 kb. B. Electrophoresis of amplified DNAs using primers specific for FBNSV-[ET;Hol]. The sizes of the amplified DNAs are 678 bp (DNA-U1), 343 bp (DNA-M), 538 bp (DNA-S), 587 bp (DNA-U2), 413 bp (DNA-N), 619 bp (DNA-C), 995 (DNA-U4), respectively. C. Electrophoresis of amplified DNA-R. Primers were designed for DNA-R of FBNYV-[EG;1-93], but amplify also DNA-R of FBNSV-[ET;Hol]. The left gel shows non-digested PCR products, and the right two gels show analytic digests by the restriction endonucleases *AgeI* and *ScaI*. The *AgeI* recognition site is absent in DNA-R of FBNYV-[EG;1-93] and present in DNA-R of FBNSV [ET;Hol], leading to fragments of 666 bp and 349 bp upon digestion of the 1 kb PCR product with *AgeI*. The *ScaI* recognition site is absent in DNA-R of FBNSV-[ET;Hol] and present in DNA-R of FBNYV-[EG;1-93], leading to fragments of 728 and 287 bp upon digestion of the 1 kb PCR product with *ScaI*.

complementation of the defect by mixing reconstituted FBNYV-[EG;1-93] with a vector maintained FBNSV-[ET;Hol] isolate. Mixing was done by taking two faba bean plants (107 and 121) that showed symptoms following agroinoculation with FBNYV-[EG;1-93] and exposing them for a 3-day inoculation access feeding period to viruliferous pea aphids carrying FBNSV-[ET;Hol]. Three weeks later when these plants were shown to contain both FBNYV and FBNSV based on strong TAS-ELISA reactions with virus-specific MAbs (Franz et al.,

**Table 1**

Presence (+) or absence (-) of nanovirus DNAs in faba bean samples of two independent lineages (107 and 121) established by co-inoculation of FBNYV-[EG;1-93] reconstituted from cloned DNAs and virus of isolate FBNSV-[ET;Hol] followed by successive aphid transfers for over one year.

Sample	107		121	
	FBNYV	FBNSV	FBNYV	FBNSV
R	+	-	+	-
S	+	-	+	-
C	+	-	+	-
M	+	-	+	+
N	-	+	-	+
U1	-	+	+	+
U2	-	+	-	+
U4	+	-	+	+

1999; Grigoras et al., 2009), they were used for vector transmission by pea aphids and maintained as two independent lineages (107 and 121) of mixed viruses by > 20 successive aphid transfers for over a year. Thereafter, we analyzed the virus population using virus- and component-specific PCR for the respective presence or absence of the genomic DNAs of the two viruses in symptomatic plants. Characterization of potential reassortants might identify the transmission-defective genome component(s) of FBNYV-[EG;1-93]. Fig. 1 shows the results of these PCRs (for DNA-R in combination with diagnostic restriction endonuclease digests) and reveals that after about a year of successive transmissions a certain combination of FBNYV and FBNSV DNAs was maintained (Table 1). The master Rep-encoding DNA-R as well as DNA-S and DNA-C of only FBNYV-[EG;1-93] were retained in both 107 and 121 (Fig. 1C; Table 1). In lineage 121, DNA-U1, -M and -U4 of both FBNYV and FBNSV had been simultaneously maintained, whereas in lineage 107 individual DNA components of only one virus, either FBNYV or FBNSV prevailed. Notably, DNA-U2 and -N were from FBNSV-[ET;Hol]. This indicated that one or both of these DNAs are required for aphid transmission, identifying one or both of them as candidates being responsible for the defect in aphid transmission of FBNYV-[EG;1-93].

### 3.2. FBNSV DNA-R, DNA-S and DNA-M are essential for infection of faba bean

Given the outcome of the one year reassortment experiment that had yielded a transmissible FBNYV-FBNSV hybrid from a reconstituted (transmission-defective) FBNYV-[EG;1-93] and a natural FBNSV-[ET;Hol], we used the meanwhile available possibility to reconstitute authentic transmissible FBNSV-[ET;Hol;JKI-2000] (Grigoras et al., 2009) for trying to identify the DNA component(s) involved in aphid transmission. Therefore, we assessed the infectivity of all eight genomic DNAs and various subsets thereof using cloned FBNSV-[ET;Hol;JKI-2000] DNAs (Grigoras et al., 2009) followed by aphid transmission experiments. The results of these infectivity assays, the type of symptoms produced and the transmission behavior of the respective reconstituted FBNSV are summarized in Table 2.

When we used all eight FBNSV genome components to agroinoculate faba bean plants, 40–80% of the inoculated plants developed the characteristic symptoms of a FBNSV infection, i.e. plant stunting and leaf yellowing and distortions (Fig. 2A), and eventual death of the plants after > 30 days. When cowpea aphids were used for virus acquisition from symptomatic plants in two independent transmission assays, virus transmission rates of up to 100% were observed (Table 2, experimental combination 1). In all symptomatic plants obtained after aphid transmission, the full set of eight genomic DNAs was detected by PCR using component-specific primers (data not shown). Also the pea aphid was used to transmit the reconstituted wild-type and mutant

**Table 2**  
Influence of different FBNSV genome components on infectivity, type of symptoms and aphid transmission of the reconstituted virus.

DNA Combination, Experiment	Genome DNAs agroinoculated	Infection efficiency <sup>a</sup>	Type of symptoms	Time of symptom appearance	Transmission efficiency <sup>b</sup>	Time of symptom appearance after transmission
1 a - b	All 8 DNAs	8/10, 5/13	severe	10-14 dpi	19/19, 30/32	10-14 dpi
2 a - b	7 DNAs, no DNA-R (FBNSV-ΔR)	0/10, 0/10	none	-	nt	-
3 a - b	7 DNAs, no DNA-S (FBNSV-ΔS)	0/10, 0/10	none	-	nt	-
4 a - b	7 DNAs, no DNA-M (FBNSV-ΔM)	0/10, 0/10	none	-	nt	-
5 a - b - c	7 DNAs, no DNA-C (FBNSV-ΔC)	2/10, 4/10, 2/13	severe	10-14 dpi	10/10, 11/11, 6/6	10-14 dpi
6 a - b	7 DNAs, no DNA-U4 (FBNSV-ΔU4)	19/25, 7/12	severe	10-14 dpi	21/22, 11/11	10-14 dpi
7 a - b	7 DNAs, no DNA-U2 (FBNSV-ΔU2)	15/23, 3/10	mild	14 dpi	10/15, 13/13	10-14 dpi
8 a - b	7 DNAs, no DNA-U1 (FBNSV-ΔU1)	5/20, 2/20	very mild	22-30 dpi	2/12, 1/10	65 dpi
9 a - b - c - d	7 DNAs, no DNA-N (FBNSV-ΔN)	19/25, 7/10, 19/22, 8/10	severe	10-14 dpi	0/20, 0/10, 0/10, 0/10	no symptoms after > 70 dpi
10 a - b	6 DNAs, no DNA-U1 and DNA-U2 (FBNSV-ΔU1-ΔU2)	1/10, 1/12	very mild	30 dpi	0/10, 0/10	no symptoms after > 70 dpi

The different DNA combinations used for agroinoculation are numbered. Letters a, b, etc. represent independent experiments.

<sup>a</sup> number of symptomatic plants / number of agroinoculated plants,

<sup>b</sup> number of symptomatic plants / number of aphid-inoculated plants, cowpea aphids were used

FBNSV. However, since the transmission efficiency of FBNSV by pea aphids was consistently lower than that by cowpea aphids, only transmission results using cowpea aphids are given here.

Agroinoculation experiments employing seven of the eight genomic FBNSV DNAs revealed that DNA-R, DNA-S and DNA-M are essential for infection and symptom development. This was demonstrated by omission of these DNAs individually from the inoculum (Table 2, DNA combinations 2, 3, 4). In no case did any symptom develop and none of the seven inoculated DNAs were detectable after 2–3 weeks in samples from 4 to 8 individual plants per experiment when assayed by PCR using component-specific primers. The same requirement had been found for FBNSV in a similar experimental set up (Timchenko et al., 2006).

### 3.3. FBNSV DNA-C and DNA-U4 are dispensable for infection of faba bean, aphid transmission and symptom development

When DNA-C or DNA-U4 was individually omitted (Table 2, DNA combinations 5 and 6), severe symptoms developed within the same time period as in inoculations using the full set of all eight genomic DNAs. The transmission efficiency of cowpea aphids for FBNSV infections lacking DNA-C or DNA-U4 was as high as for the virus with a complete genome (8 DNAs). Figs. 2B and 2F show the wild type-like symptoms of FBNSV lacking DNA-C or DNA-U4 after transmission by cowpea aphids. FBNSV lacking DNA-U4 was even maintained for more than two years using the pea aphid for successive transmissions to faba bean at intervals of 3–4 weeks.

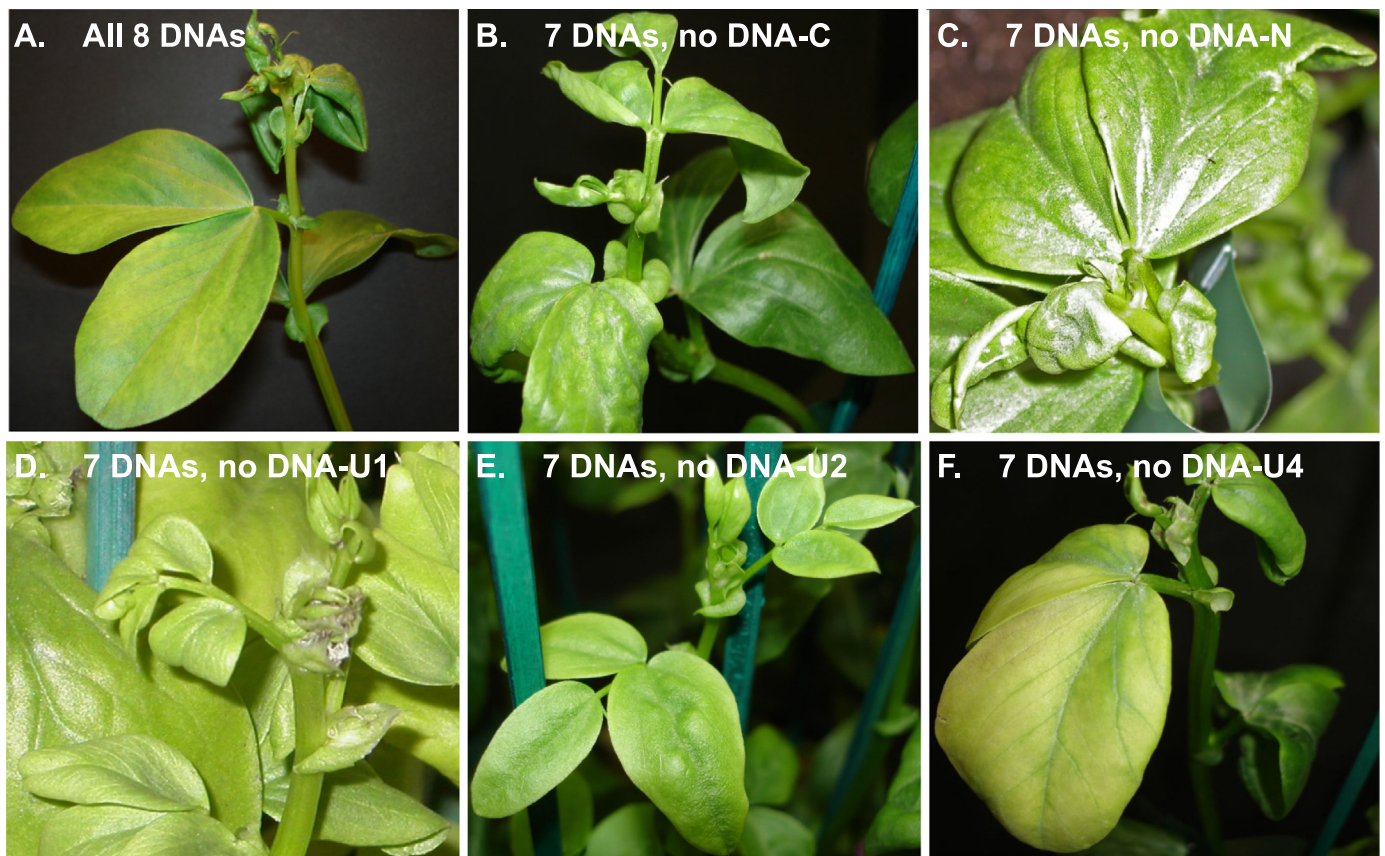
### 3.4. FBNSV DNA-U1 and DNA-U2 are dispensable for infection but influence symptom severity and virus titer

Agroinoculation experiments individually omitting FBNSV DNA-U1 (FBNSV-ΔU1) or DNA-U2 (FBNSV-ΔU2) (Table 2, DNA combinations 7 and 8) resulted in distinct and clearly milder symptoms. Fig. 2E shows the mild symptoms of FBNSV-ΔU2. Whereas the mild symptoms caused by FBNSV-ΔU2 developed within almost the same time period as the severe symptoms of a virus with a complete genome (8 DNAs), the development of the very mild symptoms caused by FBNSV-ΔU1 infections took almost twice as long as that of wild-type virus symptoms. FBNSV-ΔU2 was transmitted as efficiently as wild-type virus by cowpea aphids whereas FBNSV-ΔU1 was transmitted at a much lower efficiency. Also after aphid transmission, the symptoms of FBNSV-ΔU1 developed very slowly and became evident only 12 weeks post inoculation by cowpea aphids (Fig. 2D).

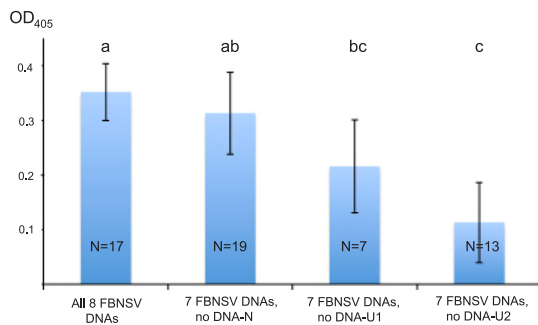
Inoculation with either FBNSV-ΔU1 or FBNSV-ΔU2 resulted in a decrease in the amount of virus produced (Fig. 3). For FBNSV-ΔU1, the virus titer dropped to about 63% of that in wild-type infections and the transmission efficiency was reduced. For FBNSV-ΔU2 the virus titer was only 34% of that in wild-type infections. Nevertheless, transmission efficiency of FBNSV-ΔU2 was hardly affected. When DNA-U1 and DNA-U2 were omitted simultaneously (Table 2, DNA combination 10), symptoms were very mild, and the additive effects of the two missing DNAs abolished vector transmission by aphids, presumably due to a substantial further drop in virus available for transmission.

### 3.5. FBNSV DNA-N is dispensable for infection and symptom development but is mandatory for aphid transmission

The most intriguing effect on aphid transmission of FBNSV occurred following agroinoculations in which DNA-N was omitted (FBNSV-ΔN) (Table 2, DNA combination 9). Whereas symptom severity and symptom development caused by FBNSV-ΔN were indistinguishable from those of wild-type virus (Fig. 2C) FBNSV-ΔN was never transmitted by cowpea (or pea) aphids. Aphid-inoculated plants were kept for more than 70 days and never developed any symptoms of virus infection. Every two weeks these plants were checked by PCR for the



**Fig. 2.** Symptoms on faba bean of FBNSV two to three weeks after agroinoculation with cloned FBNSV DNAs as indicated (A, C). Symptoms on faba bean of reconstituted and transmitted FBNSV three weeks post inoculation (B, E, F) or 12 weeks post inoculation (D) by cowpea aphids.



**Fig. 3.** FBNSV titers in infected *V. faba* plants as determined by DAS-ELISA. Absorbance at 405 nm of each sample was normalized by subtraction of OD obtained for non-infected plant issue. Data obtained for different plant sets from two independent experiments were treated using Kruskal-Wallis test. Significant differences are indicated by letters ( $P$ -value < 0.05). Number of samples tested is indicated in box-plots, vertical bars represent standard deviation.

presence of FBNSV DNAs using component-specific primers; none of the seven inoculated DNAs were detectable (data not shown). In the symptomatic plants used for acquisition access feeding by aphids, all FBNSV DNAs except DNA-N were present as confirmed by PCR using component-specific primers (data not shown). Also, virion-specific MAbs detected CP antigen in symptomatic plants that had been agroinoculated with FBNSV DNAs excluding DNA-N (Table 3, DNA combination 2). This clearly indicated that the presence of DNA-N is essential for aphid transmission of FBNSV. Consequently, the DNA-N may encode the transmission factor, which Franz et al. (1999) suspected to be absent from purified virion preparations.

### 3.6. The lack of DNA-N does not abolish virion formation

To rule out the possibility that the lack of NSP in FBNSV- $\Delta$ N infections negatively affected virion formation and thus abolished virus transmission, we compared virus particles of wild-type virus with virus particles prepared from FBNSV- $\Delta$ N infections. When the preparations were examined by transmission electron microscopy (Fig. 4) no differences in the morphology and substructure of the particles were observed at the resolution used for comparing virions from preparations from both wild-type FBNSV and FBNSV- $\Delta$ N.

Although there were no obvious differences between wild-type virions and virions from FBNSV- $\Delta$ N infections, a difference in the amount of virus particles produced might have indirectly influenced the transmission. We therefore compared the relative virus concentration (viral load) in plant extracts prepared from wild-type infections and infections by FBNSV- $\Delta$ N. Fig. 3 shows that virus titer was about 89%, 61% and 32% of wild-type virus titer for FBNSV- $\Delta$ N, FBNSV- $\Delta$ U1 and FBNSV- $\Delta$ U2 infections, respectively. Thus, there was only a slight drop in virus titer for FBNSV- $\Delta$ N compared to wild-type infections and not as important as that observed for FBNSV- $\Delta$ U1 or FBNSV- $\Delta$ U2 infections.

### 3.7. A FBNSV NSP tagged at the carboxy-terminus loses its activity in virus transmission

To see whether a C-terminally tagged NSP might still be functional for aphid transmission and potentially assist in the identification of plant or aphid proteins that interact with the NSP, we introduced a sequence encoding a 13 amino acid tag (-GGGGGLEHHHHHH) at the end of the 3' end of the NSP gene. We used the mutated genome component (DNA-N-His) along with the other seven genomic DNAs of FBNSV to agroinoculate faba bean plants. In three independent

**Table 3**  
Transmission by pea aphids of virus expressing a heterologous or mutated NSP.

DNA Combination	Virus	Completing DNA	Infection efficiency <sup>b</sup>	DNA detection by PCR <sup>c</sup>			Aphid transmission efficiency <sup>d</sup>	ELISA analysis of symptomatic plants <sup>e</sup>		
				FBNSV DNA-N	PNYDV DNA-N	FBNSV DNA-N-His		Virion-specific antibody		NSP-specific antibody
								PNYDV-Mab 2-4B9 <sup>f</sup>	FBNSV-Mab 8-6F8 <sup>f</sup>	
1 a- b	PNYDV <sup>a</sup> 7 DNAs, no DNA-N	DNA-N of FBNSV	1/10	1/1		13/20	+++	0	+++	
			4/15	4/4		8/19	+++	0	+++	
2 a- b	FBNSV <sup>a</sup> 7 DNAs, no DNA-N	DNA-N of PNYDV	1/10		0/1	0/10, 0/10 <sup>g</sup>	0	+++	0	
			1/10		0/1	0/6	n.t.	n.t.	n.t.	
3 a- b	FBNSV <sup>a</sup> , 7 DNAs, no DNA-N	DNA-N-His of FBNSV	1/10		1/1	0/6	n.t.	n.t.	n.t.	
			2/6		2/2	0/4	0	++	+++	
4 a- b	PNYDV, 7 DNAs, no DNA-N	none	1/6			0/10	+++	0	0	
			2/10			0/6	+++	0	0	
5 a- b	PNYDV, all 8 DNAs		3/6			5/5	+++	0	0	
			4/30			16/20	+++	0	0	

<sup>a</sup> a mixture of cloned DNAs of the corresponding virus, FBNSV-[ET:Hol;JKI-2000] or PNYDV-[DE;15] were used for agroinoculation of faba bean plants: The 7 DNAs are: DNA -R, -M, -S, -C, U1, -U2 and -U4.

<sup>b</sup> number of symptomatic plants / number of agroinoculated plants.

<sup>c</sup> symptomatic plants from (b) were tested by PCR using component specific primers; number of PCR positive / number of tested plants.

<sup>d</sup> number of symptomatic plants / number of aphid-inoculated plants

<sup>e</sup> symptomatic plants from (b) were tested by ELISA, reaction strengths ( $A_{405}$  values) are shown as follows: + + +, > 1.2; + +, 0.35 to 1.2, and 0, < 0.1.

<sup>f</sup> Mab - monoclonal antibody used in triple antibody sandwich (TAS) – ELISA.

<sup>g</sup> transmission assay using cowpea aphids.

experiments the symptoms caused by the mutant DNA-N-His were comparable to those of wild-type virus (Table 3, DNA combination 3). Yet, virus produced in FBNSV infections with the DNA-N-His mutant was never transmitted by aphids. The replication proficiency of the modified DNA-N was confirmed by component-specific PCR (pET22bNdeHisrev and Hol\_N\_EcoRVdir primers, Table S3, which amplify only DNA-N-His). We verified the integrity of the genetic modification of the mutated DNA-N molecules by re-cloning and sequencing four DNA-N-His molecules per infected plant. Furthermore, in two independent experiments we confirmed the expression of the C-terminally tagged NSP by ELISA using the Mab 1-3G9 specific for the NSP of FBNSV (Table 3, DNA combination 3).

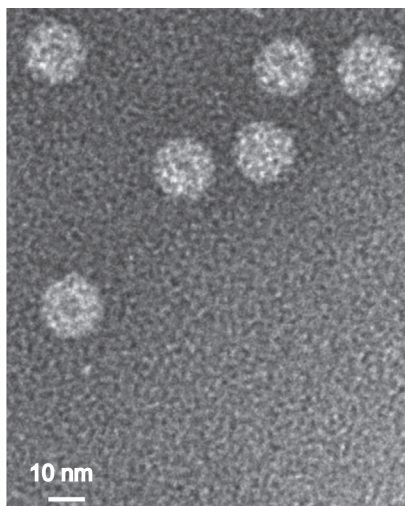
These results show that the C-terminally tagged NSP is expressed in a FBNSV infection, but is not functional for aphid transmission. This provides strong evidence that it is the encoded protein (NSP) and not a replication of DNA-N or a DNA-N encoded RNA that is mandatory for

aphid transmission and that an intact C-terminus of the NSP is needed for vector transmission.

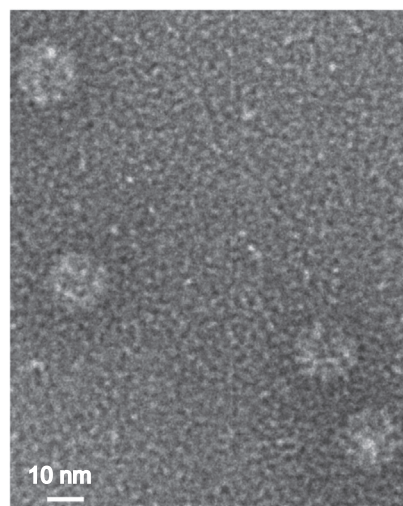
### 3.8. NSP of FBNSV is able to substitute that of a distantly related nanovirus

Since the results of Franz et al. (1999) and the results shown in Table 1 suggest complementation of the transmission factor between two closely related but distinct nanovirus species, FBNSV and FBNSV, we decided to study whether we could genetically exchange the transmission function between two more distantly related nanoviruses, i.e. FBNSV and PNYDV (Grigoras et al., 2014). Whereas the NSP of FBNSV and FBNSV differ by 9% (amino acid identity), those of FBNSV and PNYDV differ by 24%. Agrobacteria harboring the eight PNYDV genome components as head-to-tail dimers in pBin19 served to inoculate faba bean plants, and the virus produced in symptomatic plants was transmitted by pea aphids (Table 3, DNA combination 5). In

#### A. 7 FBNSV DNAs, no DNA-N



#### B. 8 FBNSV DNAs

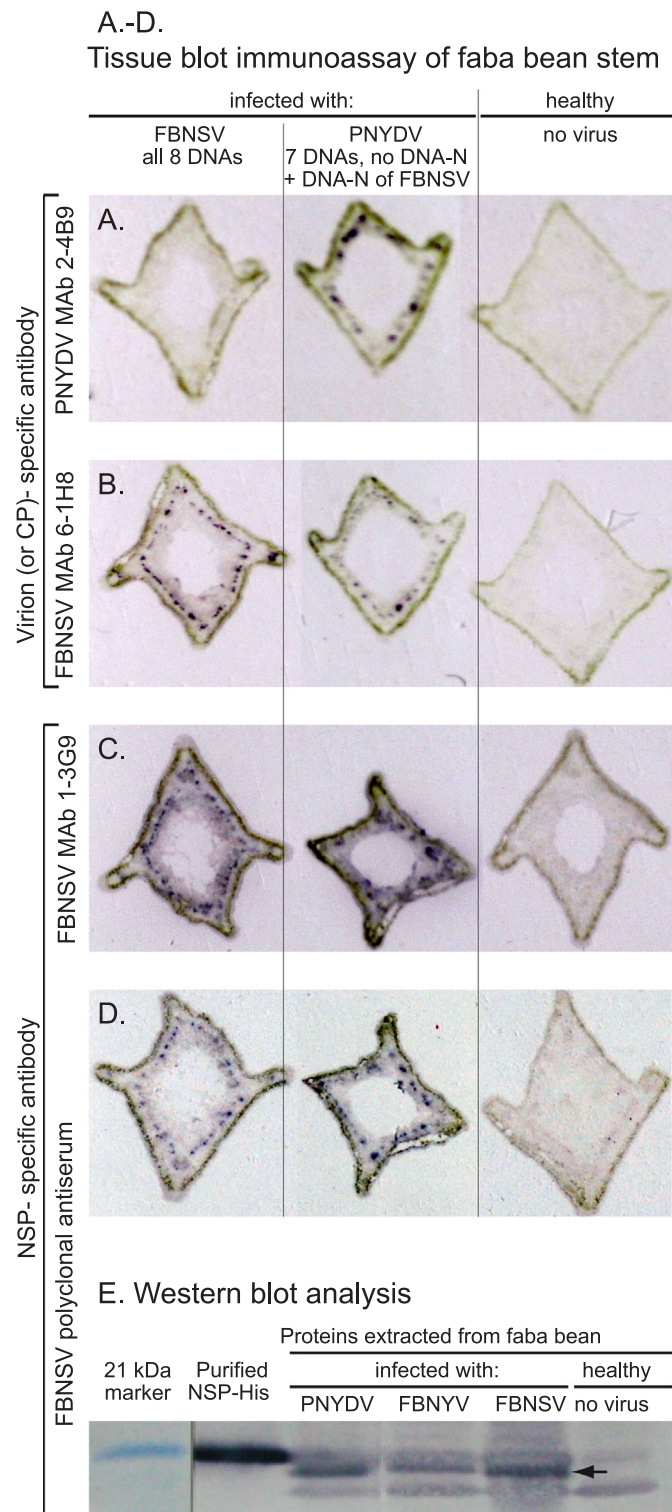


**Fig. 4.** Transmission electron microscopy of purified FBNSV particles negatively stained with 2% (w/v) sodium silico tungstate, pH 7.5. Virion preparations were obtained from *V. faba* plants agroinoculated with cloned FBNSV DNAs, using either all 8 genomic DNAs (B) or only 7 DNAs, no DNA-N (A). Images were taken with a FEI T12 LaB6 electron microscope at a nominal magnification of 49,000 $\times$  using an Orius SC1000. Scale bar equals 10 nm.



contrast, virus reconstituted by using only seven PNYDV DNAs, with DNA-N being omitted, was not transmissible (Table 3, DNA combination 4). This confirmed the results obtained for FBNSV and extends the requirement of DNA-N for aphid transmission to PNYDV, a distantly related nanovirus.

By combining FBNSV DNA-N with the other seven DNAs of PNYDV (Table 3, combination 1) an infection by a reassortant PNYDV with DNA-N of FBNSV was accomplished. The presence of the heterologous FBNSV DNA-N in the context of the PNYDV genome was established by



**Fig. 5.** Serological detection of nanovirus virions (CP) and NSP. Stem prints on nitrocellulose membranes (A to D) of faba bean plants infected by FBNSV, reassorted PNYDV carrying DNA-N of FBNSV and non-infected plants were analyzed by tissue blot immunoassays using the specific antibodies (MAbs and polyclonal IgG) as indicated on the left. Localized staining of phloem tissue is apparent when using virion-specific MAbs as well as NSP-specific MAbs. A. Detection of PNYDV virions or CP by Mab 2–4B9. B. Detection of FBNSV virions by Mab 6-1H8, which also reacts with those of PNYDV. C. Detection of FBNSV NSP by Mab 1–3G9. D. Detection of FBNSV NSP by polyclonal rabbit IgG. E. Western blot analysis of TCA-precipitated total protein extract of faba bean plants infected by PNYDV, FBNYV and FBNSV demonstrating the broad specificity of the polyclonal rabbit antibodies to NSP. Please note the difference in mobility between bacterially expressed NSP tagged by 13 additional C-terminal amino acids (NSP-His) and wild-type NSP from plants (indicated by an arrow).

component-specific PCR and the expression of the heterologous NSP was confirmed by ELISA (Table 3, combination 1). Aphids were able to transmit the artificially reassorted PNYDV for more than ten passages. This shows that a heterologous NSP (24% amino acid difference) functions in the context of a PNYDV infection and suggests that, if the NSP interacted with virions and/or other viral proteins, these interactions were still sufficient to allow transmission. Contrary to the successful transmission of a reassortant PNYDV with FBNSV DNA-N, the reciprocal reassortant consisting of seven FBNSV genomic DNAs and DNA-N of PNYDV, was not transmissible (Table 3, combination 2). Although agroinoculation of this type of reassortant produced typical symptoms in faba bean plants, the PNYDV DNA-N was never detected by component-specific PCR in these symptomatic plants. Apparently, PNYDV DNA-N was not sufficiently replicated or unable to systemically move along with the other FBNSV DNAs in the infected plants.

**3.9. FBNSV NSP is expressed in phloem tissue**

The PABs and MAbs raised here against *E. coli*-expressed FBNSV NSP-His allowed us to demonstrate for the first time that the protein encoded by DNA-N of a nanovirus is expressed in plants, using ELISA and Western blotting of total plant protein extracts (Table 3 and Supplementary Table S4, Fig. 5). Since earlier TBIA experiments using CP-specific antibodies had suggested that nanoviruses are phloem-limited as reactions were only observed in vascular tissues (Kumari et al., 2001), we studied whether expression of the NSP was sufficiently high to be also detectable in tissue blots. Tissue prints of plants infected with genuine FBNSV were compared to plants infected with the experimental reassortant PNYDV carrying DNA-N of FBNSV (Fig. 5). As expected, viral CP could be readily detected in phloem tissue with virion-specific MAbs to PNYDV (Fig. 5A) and FBNSV (Fig. 5B). In addition, FBNSV NSP was also located in phloem tissue using Mab 1–3G9 (Fig. 5C), as well as using the NSP-specific PABs (Fig. 5D). Both types of antibodies detected NSP in plants infected with FBNSV as well as in plants infected with the PNYDV reassortant expressing the heterologous FBNSV NSP. Serological analysis of protein extracts from faba bean plants infected with FBNYV, PNYDV, and FBNSV showed that PABs raised against FBNSV NSP cross-reacted strongly with heterologous NSPs not only in Western blots (Fig. 5E) but also in DAS-ELISA (data not shown), reflecting the fact that NSP is the second most conserved nanovirus protein (Vetten et al., 2012).

**4. Discussion**

The possibility to reconstitute authentic nanovirus from its cloned genomic DNAs allows assessing the requirement of each individual genome component for infection and transmission. Currently four distinct nanoviruses have been reconstituted: FBNSV (Grigoras et al., 2009), FBNYV and BMLRV (Grigoras et al., 2014), and PNYDV (this work). Here we used FBNSV and PNYDV to study the influence of individual viral DNAs on symptom development and aphid transmission.

Earlier we had attempted this for FBNYV but found that the virus reconstituted from the cloned DNAs of FBNYV-[EG;1–93], the isolate we initially used, was not transmissible by cowpea- or pea aphids (Timchenko et al., 2006). This suggested that one or several of the cloned genome components were non-functional or variants with a dominant-negative effect on aphid transmission.

Support for the hypothesis that mutations in the FBNYV-[EG;1–93] DNAs might have rendered the reconstituted virus non-transmissible was provided by the results of a year-long mixing and reassortment experiment of FBNYV-[EG;1–93] with a laboratory maintained FBNSV-[ET;Hol] isolate. In a mixed infection of the two viruses and subsequent repeated aphid transmissions, DNA-U2 and DNA-N maintained in two independent lineages were only those of FBNSV-[ET;Hol] (Table 1). Hence, either one or both of these genome components might have been responsible for the transmission defect of reconstituted FBNYV-[EG;1–93]. The low relative frequency (only 29%) with which DNA-N was maintained in the reconstituted FBNYV-[EG;1–93] infections (Timchenko et al., 2006) had already pointed towards DNA-N as a potential candidate causing the transmission defect.

The availability of an aphid transmissible FBNSV reconstituted from cloned DNAs of FBNSV-[ET; Hol;JKI-2000] (Grigoras et al., 2009), allowed us to use this virus in experimental infections for potentially identifying the DNA(s) responsible for aphid transmission and for assessing the role of individual genome components. In general, the results of agroinoculation experiments using different combinations of viral DNAs corroborated most of our findings obtained with FBNYV-[EG;1–93], yet with some remarkable differences. Whereas we were able to confirm the absolute requirement for DNA-R, DNA-S and DNA-M for any infection as judged by symptom development and confirmed by PCR, we found a slight difference in the requirement of DNA-U1 and DNA-U2. When both DNA-U1 and DNA-U2 had been simultaneously omitted from the inoculum no FBNYV-[EG;1–93] infection at all was obtained (Timchenko et al., 2006). For FBNSV lacking both DNA-U1 and DNA-U2, we observed delayed infections with very mild symptoms (Table 2, DNA combination 10). This suggests that in case of FBNYV-[EG;1–93] the agroinfection rates were generally much lower and the infectivity of a virus lacking both DNA-U1 and DNA-U2 was below the level of detection, which was not the case here for FBNSV-[ET;Hol;JKI-2000]. FBNSV- $\Delta$ U1 and FBNSV- $\Delta$ U2 produced milder symptoms and also less virus than wild-type FBNSV. It is currently unclear whether the symptom phenotype was due to lacking U1 protein function(s) or caused by the reduced viral titer in FBNSV- $\Delta$ U1 infected plants. For FBNSV- $\Delta$ U2 infections, irrespective of the reduced virus titer (34% of wild-type virus) and the milder disease symptoms, the transmission efficiency of FBNSV- $\Delta$ U2 was not negatively affected (Table 2, DNA combination 7). However, when in addition to DNA-U2 also DNA-U1 was omitted, infection efficiency dropped substantially, conceivably by a concomitant substantial drop in virus titer. This in turn would explain the failure to transmit the virus from double-defective infections (lacking both DNA-U1 and DNA-U2), despite the fact that virus transmissions were obtained from plants infected with FBNSV- $\Delta$ U2 or FBNSV- $\Delta$ U1. The U1-protein is not absolutely required for transmission but may indirectly influence virus transmission by modulating the amount of virus or its availability *in planta* for acquisition by vector aphids.

Contrary to FBNSV- $\Delta$ U1 or FBNSV- $\Delta$ U1- $\Delta$ U2, FBNSV- $\Delta$ U4 was transmitted as efficiently as wild-type virus and could even be maintained for more than two years in faba bean by successive transmission using pea aphids. The complete dispensability in faba bean of DNA-U4 for infectivity and prolonged (> 2 years) aphid transmission of FBNSV-[ET;Hol;JKI-2000] is remarkable and may point towards a potential role of DNA-U4 in hosts other than faba bean. It should be noted that all nanoviruses known, whether isolated from cultivated or wild legumes, typically possess a DNA-U4.

It may appear curious that DNA-C, which encodes a protein (Clink) that modulates the cell cycle of infected tissue and causes host genome

endoreduplication (Lageix et al., 2007), is dispensable as Clink is thought to set the cell cycle to a favorable stage for nanovirus replication. Yet, in faba bean, which may not be the principal natural (wild) host of FBNSV, the virus is viable without DNA-C, at least for several consecutive infection cycles. The influence of the plant host on the relative abundance of FBNSV genome components has been demonstrated by the considerable difference in the relative abundance of individual genomic DNAs between faba bean and barrel medic (*Medicago truncatula*), in particular for DNA-C and DNA-N (Sicard et al., 2013). This possibly reflects the respective different necessity of certain nanovirus protein(s) in a specific host environment.

Most striking, however, were the results obtained when omitting DNA-N from the inoculum. Symptoms developed within a similar time period, were as severe as in wild-type infections and virions were detectable at levels comparable to wild-type infections, yet no aphid transmission was ever achieved with virus lacking DNA-N (Tables 2 and 3). It should be noted here that the FBNSV-specific MAb 8-6F8 reacts strongly in TAS-ELISA but not in western blots (Grigoras et al., 2009), suggesting that it reacts only with conformational epitopes present on virions but not present on denatured CP. Hence, we assumed that MAb 8-6F8 detected virions in FBNSV- $\Delta$ N infections. This assumption was substantiated by the fact that intact virions were produced in FBNSV- $\Delta$ N infections and that no difference between these virions and those prepared from wild-type FBNSV infections were observed at the resolution used for electron micrographs (Fig. 4). Together with the successful aphid transmissions of FBNSV variants lacking DNA-C, or DNA-U1, DNA-U2 or DNA-U4 (Table 2), this provides evidence that the presence of DNA-N is mandatory for aphid transmission of the virus. Therefore, we conclude that the DNA-N encoded protein, dubbed nuclear shuttle protein, NSP (Wanitchakorn et al., 2000), functions as a factor required for aphid transmission, first proposed as virus-encoded helper factor by Franz et al. (1999) but to date not identified. The helper factors of closely related nanoviruses, FBNYV and FBNSV, could be complemented *in trans* (Franz et al., 1999). Here we show that the NSP of FBNSV, which differs by 24% in amino acid sequence from that of the more distantly related PNYDV, can substitute for that of PNYDV.

There are no data about any interactions of the helper factor protein with virions outside or inside the vector aphids, mainly due to the fact that a functional helper factor has not been available in purified form. The NSP with a C-terminal extension of 13 amino acids that we used to produce analytical Pabs and Mabs turned out to be dysfunctional in aphid transmission, albeit it was readily detectable in infected plants (Table 3, DNA combination 3).

Currently, we do not know where and how NSP may act as transmission factor, for instance whether it interacts with virions and mediates binding of virions to certain aphid organs like other helper factors for aphid transmission do (Blanc et al., 2011; Hoh et al., 2010; Uzest et al., 2007). The apparently autonomous action of NSP as transmission factor and its complementation *in trans*, in some way resembles that of the non-circulative (stylet-borne) transmission of potyviruses and caulimoviruses, for which a wealth of detailed mechanistic data is available; for review see (Blanc et al., 2011; Froissart et al., 2002). In contrast, details of the circulative transmission of nanovirids by aphids are limited. Apart from the initial experiments by Franz et al. (1999) and the identification here of the NSP as a transmission factor, most information stems from studies of BBTv and its vector, the banana aphid *Pentalonia nigronervosa* (Bressan and Watanabe, 2011; Fu et al., 2009; Watanabe and Bressan, 2013; Watanabe et al., 2013b). For BBTv there is some circumstantial evidence that DNA-N might potentially be involved in aphid transmission. The BBTv strain TW3 causes mild symptoms assumed to be due to a lower titer of its genomic DNAs, except for DNA-U3. Curiously, the amount of BBTv (TW3) DNA-N was below the level of detection and the virus could only be maintained by vegetative propagation but not by aphid transmission (Fu et al., 2009). Unfortunately, reconstitution of BBTv from its DNAs has not yet been established, allowing experimental restoration of aphid transmission by

adding DNA-N. Nevertheless, these data are consistent with our findings that the DNA-N-encoded protein acts as transmission factor for two different nanoviruses and suggest the same being true for also the b-uviruses.

Whereas the circulative, non-propagative transmission of luteovirids and begomoviruses (*Geminiviridae*), for a review see (Gray et al., 2014), appear to require interaction of virions with aphid or sweet potato whitefly (*Bemisia tabaci*) endosymbiont-derived GroEL (Gottlieb et al., 2010), this seems not to be the case for BBTV and at least *Buchnera* GroEL (Watanabe et al., 2013a). Also, the tissue tropism of BBTV in the aphid is different from that of the luteovirids. Similar to begomoviruses (Ghanim, 2014), however, BBTV appears to cross the foregut and anterior midgut lining to reach the haemocoel and was mainly detected in the primary salivary glands (Bressan and Watanabe, 2011). This is again different from luteovirids that cross the posterior midgut or hindgut lining by transcytosis and accumulate in the accessory salivary glands (Gray and Gildow, 2003).

How and where may a protein like the nanovirus NSP act in the process of aphid transmission? In this context it should be recalled that the first functional characterization of the protein encoded by DNA-N stems from it being localized in the cell nucleus and relocated to the cell periphery (membrane) in the presence of the MP (Wanitchakorn et al., 2000), which somehow resembles the concerted action of the NSP and MP in bipartite begomoviruses (Lazarowitz and Beachy, 1999). Whereas the data on begomovirus NSP are supported by studies of nucleic acid binding and phosphorylation of NSP (Pascal et al., 1994), interaction with the MP (Sanderfoot and Lazarowitz, 1995), and identification of sequences responsible for nuclear import (Sanderfoot et al., 1996) and export of NSP (Ward and Lazarowitz, 1999), there are no comparable studies for the respective nanovirus proteins.

Only recently, some functional analyses of nanovirus proteins became available: Krapp et al. (2017) analyzed the cellular localization of PNYDV NSP and abutilon mosaic virus NSP and found that both interact with the stress granule component protein G3BP. However, it remains open how this finding could help to explain the role of a nanovirus NSP in aphid transmission. A further analysis studying interaction of PNYDV proteins in yeast two hybrid assays and bimolecular fluorescence complementation (BiFC) revealed auto-interaction of NSP and interaction with the master Rep protein, but no interaction with any other PNYDV protein, notably not with the virus capsid protein (Krenz et al., 2017). Hence, also these findings provide no further information on the potential mode of action of NSP in the transmission process, but point to a different function than the begomovirus NSP.

The observation that NSP is required for systemic movement and disease symptoms of bipartite begomoviruses, see (Rojas et al., 2005) and references therein, contrasts with our findings that DNA-N and the NSP are dispensable for infection, systemic movement and symptom development of the nanoviruses. Only aphid transmission of nanoviruses is not possible without a functional NSP.

Also the MP, absolutely required for infection, may be involved at some stage in nanovirus transmission. Yet, a formal proof thereof would require infection proficient and transmission deficient MP variants. The same may be true for the CP. However, no transmission deficient nanovirus capsid variants, comparable to the non-transmissible single amino acid CP variants of geminiviruses (Noris et al., 1998), have as yet been found or constructed.

It is to be hoped that, with the availability of four different nanoviruses for reconstitution from cloned DNAs, the functional analysis of nanovirus proteins will advance. A first step towards that goal is the identification here of the elusive nanovirus aphid transmission factor. The identification here of the NSP as an essential factor for aphid transmission will open up a new dimension for studying a unique type of virus-vector interaction because nanoviruses are the only group of circulative-persistently transmitted viruses that require such a protein.

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## Declarations of interest

None.

## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2018.07.001.

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## Supplementary Information

### Nanovirus DNA-N encodes a protein mandatory for aphid transmission

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**Tables S1, S2, S3, S4**

**Table S1.** GenBank accession numbers of the nanovirus DNAs used in this study.

Virus	DNA-R	DNA-S	DNA-C	DNA-M	DNA-N	DNA-U1	DNA-U2	DNA-U4
FBNYV-[EG:1-93]	AJ132180	AJ132183	AJ132179	AJ132182	AJ132186	AJ132181	AJ132184	AJ749902
FBNSV-[ET:Hol:JKL-2000]	GQ150778	GQ150779	GQ150780	GQ150781	GQ150782	GQ150783	GQ150784	GQ150785
PNYDV-[DE:15]	GU553134	JN133279	JN133280	JN133281	JN133282	JN133283	JN133284	JN133285

**Table S2.** Cloning of PNYDV-[DE:15] DNAs used for the virus reconstitution.

DNA type	RCA digest <sup>a</sup>	Monomer <sup>b</sup>	Dimer <sup>c</sup>	transferred as fragment <sup>d</sup>	dimer in pBin19 <sup>e</sup>
R	<i>AatII</i>	Litmus28-DNA-R- <i>AatII</i> -2	Litmus28-DNA-R- <i>AatII</i> -102	<i>EcoRI-XbaI</i>	pBin-DNA-R-1
S	<i>XbaI</i>	pBSIIKS(+)-DNA-S- <i>XbaI</i> -3	pBSIIKS(+)-DNA-S- <i>XbaI</i> -73-1	<i>KpnI-SacI</i>	pBin-DNA-S-2
C	<i>Acc65I</i>	pBSIIKS(+)-DNA-C- <i>Acc65I</i> -3	pBSIIKS(+)-DNA-C- <i>KpnI</i> -77	<i>EcoRI-XbaI</i>	pBin-DNA-C-5
M	<i>XmaI</i>	pUC19-DNA-M- <i>XmaI</i> -1	pUC19-DNA-M- <i>XmaI</i> -63	<i>EcoRI-XbaI</i>	pBin-DNA-M-97
N	<i>SnaI</i>	Litmus28-DNA-N- <i>SnaI</i> -1	Litmus28-DNA-N- <i>SnaI</i> -73	<i>KpnI-SacI</i>	pBin-DNA-N-2
U1	<i>XbaI</i>	pBSIIKS(+)-DNA-U1- <i>XbaI</i> -1	pBSIIKS(+)-DNA-U1- <i>XbaI</i> -7	<i>KpnI-SacI</i>	pBin-DNA-U1-1
U2	<i>PstI</i>	pBSIIKS(+)-DNA-U2- <i>PstI</i> -10	pBSIIKS(+)-DNA-U2- <i>PstI</i> -9	<i>KpnI-SacI</i>	pBin-DNA-U2-1
U4	<i>BamHI</i>	pBSIIKS(+)-DNA-U4- <i>BamHI</i> -1	pBSIIKS(+)-DNA-U4- <i>BamHI</i> -242	<i>KpnI-SacI</i>	pBin-DNA-U4-1

<sup>a</sup> enzyme used to digest RCA product

<sup>b</sup> vector used to clone a given nanovirus DNA into the corresponding cloning site, a last number represents clone number,

pBSIIKS(+): pBluescript IISK(+)

<sup>c</sup> viral DNA from the clone in “b” was used to be cloned as tandem repeat in a given vector, corresponding site;

a last number represents clone number.

<sup>d</sup> viral DNA was liberated from a clone in “c” using a pair of enzymes indicated

<sup>e</sup> clone number of a given nanovirus component in the binary vector

**Table S3.** Oligonucleotides used for PCR amplification of nanovirus DNAs.

Virus	Genomic DNA	Primer name	Sequence (5'-3')	Used in the experiments presented in		
				Figure 1 <sup>a</sup>	Table 2 <sup>b</sup>	Table 3 <sup>c</sup>
FBNSV	DNA-R	C2Holelta142rev	AGCATATAAACTTGCCGAGCC		x	
		C2Holelta806dir	TCGAGGAGTATGTA AACTACGGC		x	
		Hol_R_EcoRI <sub>dir</sub>	TAGAGAAATTCAGAGGAGAACTGAAGAAGAA		x	x
		Hol_R_EcoRI <sub>rev</sub>	CTCTGAATTCCTAAGCGTACTTGCAGACT		x	x
	DNA-S	C5Holelta316rev	TTGCTCACCAATTTTTCGTTG		x	x
		C5Holelta771dir	ACCAGTGAAGCTGTTAAGACTGT		x	x
		Hol_S_PstI <sub>dir</sub>	TGGTCTGCAGCTTTCACAGCACCTGCCTCA		x	x
		Hol_S_PstI <sub>rev</sub>	AAAGCTGCAGACCAATTAACAATAGGTGAA		x	x
	DNA-C	C10Holelta353rev	TAATTCACCCGGGAGAACAG		x	x
		C10Holelta729dir	AACTTGTTCCTGAACCTAGGCCA		x	x
Hol_C_HindIII <sub>dir</sub>		TAAAAAAGCTTCGCTTCCTCGTTGTTTCAT		x	x	
Hol_C_HindIII <sub>rev</sub>		GACGAAGCTTTTTTATAGAAGAGAGAGAGAAA		x	x	
C4Holelta341dir		AGCACTATCCCGGATTCTGG		x	x	
C4Holelta664rev		AGGCTGACTAACAGATGCGTT		x	x	
DNA-M	Hol_M_HindIII <sub>dir</sub>	TATGAAGCTTGTATGTGTATATTCATGT		x	x	
	Hol_M_HindIII <sub>rev</sub>	TAACAAGCTTCATATTTTCTTGAACACTTA		x	x	
	C8Holelta180rev	ATCCAATGAGAGCTTGCCAC		x	x	
	C8Holelta748dir	TGAGAACGGGTTAGATAGTCAGTG		x	x	
	Hol_N_Nde_dir2	AGAATCAAAAGTGCATAATGGTTGTGATGATG				
DNA-N	Hol_N_Nde_rev2	CATCATCACAACCATATGACACTTGTGATTC				
	Hol_N_Sal <sub>dir</sub>	ACGGTGTTCGACCGGATGGAT				
	pET22bNdeHis <sub>rev</sub>	TTTGTAGCAGCCATATGTCAGTGGTGGTGGTG				
	5-FB-N-Nde	ATATCATATGGCAGATTGGTTTCTAG				
	3-FB-N-Xho	TATCTCGAGAGAACCCACCCTTGTGATTCGAG	Used for insertion into pET-22b(+)			
	Hol_N_EcoRV <sub>dir</sub>	GTTGCAGATATCTATGATGGAAGACGCAC	Used for insertion into pET-22b(+)	x	x	
	Hol_N_EcoRV <sub>rev</sub>	CCATCATAGATATCTGCAACTGACCAC		x	x	
DNA-U1	C3Holelta311rev	TGAACGAATTCATCTCTGCAC		x	x	
	C3Holelta619dir	ACCACCTCTGCTCCATCGT		x	x	
	Hol_U1_HindIII <sub>dir</sub>	CATTAAGCTTATATAGCTCTATGCCTTTG		x	x	
	Hol_U1_HindIII <sub>rev</sub>	CTATATAAGCTTAATGTTGATGAGAGAAG		x	x	

	C6Holelta230rev	CAACCACCTCGTTTGAATCCAC	x	x	x
	C6Holelta627dir	ACGATGGCAGTCTAGAAAGTTT	x		x
DNA-U2	Hol_U2_EcoRIdir	TGGTGAATCTGCAGACTCCATGGAAGAAG	x		x
	Hol_U2_EcoRIrev	TGCAGAAATTCACCAAGAACAATCTTCGTGGG	x		x
	C12Holelta192rev	GACCATCGTCCACTTGCATA	x		x
DNA-U4	C12Holelta665dir	TTTACAGCGATTGTGGGAATC	x		x
	Hol_U4_XbaIrev	TTAATCTAGATTAAAGAAGTACTGTTCAGG	x		x
	Hol_U4_XbaIrev	TTAATCTAGAAATAATTAATCTCTGAATAAAG	x		x
DNA-R	EgC2HindIII (+)	ACGAAAGCTTCGAGGAGTATGTTAAATTACGG	x		
	EgC2HindIII (-)	TCGAAAGCTTCGTGGAAGAATCGAAGAGCACT	x		
DNA-S	EgC5SpeI (+)	CGTTTACTAGTCTGCTGTGTAGTGAAGG	x		
	EgC5SpeI (-)	GCAGGACTAGTAAACGCAGCAGACCAACTG	x		
DNA-C	EgC10HincII (+)	ATTGTCAACTGCAGTGAATTTGGATAAAATTA	x		
	EgC10HincII (-)	GCAGTTGACAATCATACCGTCTTCGTATGT	x		
DNA-M	EgC4BamHI (+)	AATACTGGATCCGTTTTTTGAATTTATGTATTG	x		
	EgC4BamHI (-)	TCAAAAACGGATCCAGTATTAGTTGACTTGC	x		
DNA-N	EgC8Xho (+)	TGGATCTCGAGTCTCAGTACTTGAAGAAGG	x		
	EgC8Xho (-)	TGAGACTCGAGATCCAGGTTGAATGTCCCTT	x		
DNA-U1	EgC3EcoRI (+)	GTCTGAAATTCGTTGAAGAAGTCTTCTCC	x		
	EgC3EcoRI (-)	TCAACGAAATTCAGACTTGTGTCTTCTCA	x		
DNA-U2	EgC6PstI (+)	GAAAGCCTGCAGGACTTGTATGATTACGGT	x		
	EgC6PstI (-)	CAAGTCTCTCAGGCCCTTCTGATTTCTGAC	x		
DNA-U4	EgC12XbaI (+)	CTAATCTAGACTGAAAGCAATGATAAGAAG	x		
	EgC12XbaI (-)	TCAGTCTAGAAATAGTTATTCCTGATGATAACA	x		
DNA-R	priPeaRdir	GGAATACCAAGGTGAGTTACGG		x	
	priPeaRrev	TATCCCTGAGAGTCCCGGAC		x	
DNA-S	priPeaSdir	AACCTCCGGATATCACCAGAT		x	
	priPeaSrev	CCGGAGGTTTTTATTTCAAAACCAAC		x	
DNA-M	priPeaMdir	TACCTGAACGTCTCTGTATCTT		x	
	priPeaMrev	TCAGGTACTGAATTACTTGCC		x	
DNA-N	priPeaNdir	GAAGAACCCAGGAAGGTGTTCG		x	
	priPeaNrev	GGTTCITCCAAATTTACCTTTCAATGG		x	
DNA-C	priPeaCdir	GCCGGAAGCTTGCCGGACTGACGGAG		x	
	priPeaCrev	AGTTCGCCGAAGACGGAGTAAATTTG		x	
DNA-U1	priPeaU1dir	TGGTGAAGAAATTCAGAGTGTAT		x	
	priPeaU1rev	TTCACCAAGTTCTCGTCAGAAC		x	
DNA-U2	priPeaU2dir	GATCAAGAACAAAGGTTAGTTATG		x	
	priPeaU2rev	TCTTGATCGGAGACGAACTGGA		x	
DNA-U4	priPeaU4dir	ATCAAGTCTGAAGATGATAG		x	
	priPeaU4rev	GACTTGATTTCAACATCTCTTTCAC		x	



Restriction endonuclease sites used for cloning are shown in bold

<sup>a</sup> mixed infection: plants were agroinoculated with reconstituted FBNYV-[EG;1-93] using cloned viral DNAs and superinfected by laboratory maintained FBNSV isolate [ET;Hol] using pea aphids.

<sup>b</sup> detection of FBNYV-[ET;Hol;JKI-2000] virus in plants agroinoculated with cloned viral DNAs or after aphid transmission.

<sup>c</sup> complementation experiments using cloned DNAs from both FBNSV-[ET;Hol;JKI-2000] and PNYDV-[DE;15].

**Table S4.** Key properties of monoclonal antibodies (MAbs) raised against bacterially expressed NSP-His of FBNSV

clone <sup>a</sup>	IgG type	TAS-ELISA reaction <sup>b</sup> with NSP of		Western blot reaction <sup>c</sup> with FBNSV NSP	
		FBNSV	FBNYV PNYDV		
1-3F9	1, κ	+++	+	0	+
1-3G9	1, κ	+++	0	0	-
1-5F5	1, κ	+++	0	0	+
1-5G11	1, κ	+++	0	0	+
1-5H5	1, κ	+++	+++	0	-
1-6G5	1, κ	+++	+++	+	+
1-6G11	1, κ	+++	0	0	+

<sup>a</sup> Since the culture supernatants of the individual clones had comparable reciprocal titres, they were used at working dilutions of 1:500 to 1:100 in ELISA, TBIA, etc.

<sup>b</sup> TAS-ELISA reaction strengths ( $A_{405}$  values) with extracts from nanovirus-infected plants are shown as follows:

+++ , >1.0; ++ , 0.2 to 1.0; + , <0.2; and 0 , less than two times the  $A_{405}$  value for the non-infected control.

<sup>c</sup> The presence (+) or absence (-) of a specific Western blot reaction was assessed by SDS-polyacrylamide gel electrophoresis of TCA-precipitated total proteins from FBNSV-infected plants.