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Reconstitution of Authentic Nanovirus from Multiple Cloned DNAs▽†

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We describe a new plant single-stranded DNA (ssDNA) virus, a nanovirus isolate originating from the faba bean in Ethiopia. We applied rolling circle amplification (RCA) to extensively copy the individual circular DNAs of the nanovirus genome. By sequence analyses of more than 208 individually cloned genome components, we obtained a representative sample of eight polymorphic swarms of circular DNAs, each about 1 kb in size. From these heterogeneous DNA populations after RCA, we inferred consensus sequences of the eight DNA components of the virus genome. Based on the distinctive molecular and biological properties of the virus, we propose to consider it a new species of the genus *Nanovirus* and to name it faba bean necrotic stunt virus (FBNSV). Selecting a representative clone of each of the eight DNAs for transfer by T-DNA plasmids of *Agrobacterium tumefaciens* into *Vicia faba* plants, we elicited the development of the typical FBNSV disease symptoms. Moreover, we showed that the virus thus produced was readily transmitted by two different aphid vector species, *Aphis craccivora* and *Acyrthosiphon pisum*. This represents the first reconstitution of a fully infectious and sustainably insect-transmissible nanovirus from its cloned DNAs and provides compelling evidence that the genome of a legume-infecting nanovirus is typically comprised of eight distinct DNA components.

Circular single-stranded DNA (ssDNA) viruses that belong to the family *Nanoviridae* have recently emerged as important pathogens which often severely affect leguminous crops in West Asia, North Africa (40), and Spain (46). One of the most important nanoviruses is faba bean necrotic yellows virus (FBNYV), which infects a range of legume crops in this geographic region. Also, some nonleguminous dicotyledonous plant species have been reported as hosts for FBNYV in natural or experimental conditions (19, 32, 57). FBNYV belongs to the genus *Nanovirus*, which comprises at least two other legume-infecting members, milk vetch dwarf virus (MDV) from Japan (48) and China (36) and subterranean clover stunt virus (SCSV) from Australia (10).

A second taxon of the family *Nanoviridae* is the genus *Babuvirus*, with species infecting monocotyledonous plants. Banana bunchy top virus (BBTV) infects bananas (*Musa* spp.) and is present in banana-growing countries in the Asia-Pacific region and Africa (24, 52). Recently, abacá bunchy top virus (ABTV) from Southeast Asia has been molecularly characterized (50), and also a single DNA component of cardamom bushy dwarf virus from India was described (41). These two viruses have been proposed as members of the genus *Babuvirus* (www.ictvonline.org).

Both nano- and babuviruses are transmitted by several aphid species in a persistent nonpropagative manner, with *Aphis craccivora* and *Acyrthosiphon pisum* being efficient vectors of the three nanoviruses, whereas BBTV and ABTV are naturally transmitted by *Pentalonia nigronervosa* (39, 45).

Viruses of the family *Nanoviridae* have a multipartite genome consisting of six to eight ssDNA molecules (58). Each circular ssDNA component is about 1 kb in size and is encapsidated in individual icosahedral particles. Experimental data and sequence evidence suggest that each DNA molecule, with a single exception, encodes only one protein (4, 58). A set of five homologous genome components are common to both nano- and babuviruses: DNA-R encoding the master Rep (M-Rep) protein, which initiates rolling circle replication of all genomic DNAs of a nano- or babuvirus (25, 53, 55); DNA-S encoding the capsid protein (11, 30, 61); DNA-C encoding Clink, a cell-cycle link protein (3); DNA-M encoding a movement protein (60); and DNA-N encoding a nuclear-shuttle protein (60). Three other DNAs (DNAs-U1, -U2, and -U4) encoding proteins of as yet unknown functions have been identified from the nanoviruses FBNYV and MDV, and one further DNA (DNA-U3) was identified from the babuviruses BBTV (58) and ABTV (50). An additional putative protein of unknown function appears to be encoded by a small internal open reading frame (ORF) embedded within the *m-rep* gene of BBTV, the sole exception to the "one DNA, one protein" rule for nano- and babuviruses (4). In addition to these bona fide integral genome components, additional Rep-encoding DNAs have been found associated with several nano- and babuvirus isolates (26, 58). They encode related, but distinct Rep proteins (para-Rep) that, in contrast to the M-Rep, are capable of initiating the replication only of their respective cognate DNAs (53).

A prerequisite to studying viruses by reverse genetics is an

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TABLE 1. Properties, reactivities, and specificities of MAbs raised against FBNSV virions

MAb	IgG type	Reciprocal titer of culture supernatant (working dilution)	TAS-ELISA reaction with:[a]			Western blotting reaction[b]
			FBNSV-[ET:Hol:97]	FBNYV-[DZ:Bis:00]	FBNYV-[EG:93]	
5-1G8	2b, κ	>25,600 (1:500)	+++	+	+	NT
8-2G10	1, κ	>25,600 (1:100)	+++	0	0	+
8-3G11	2a, κ	>25,600 (1:300)	+++	0	0	−
8-4A2	2a, κ	>25,600 (1:500)	+++	+	+	+
8-4F9	1, κ	>25,600 (1:5,000)	+++	(+)	(+)	−
8-6F8-A5	2a, κ	>25,600 (1:500)	+++	0	0	−
8-6F8-D9	1, κ	>25,600 (1:5,000)	+++	(+)	(+)	NT

[a] TAS-ELISA reaction strengths (A_{405} values) 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 noninfected control; (+), weak cross-reactions with FBNYV isolates from Algeria [DZ:Bis:00] and Egypt [EG:93], if culture supernatants were used at concentrations equal to, and higher than, the working dilutions indicated.

[b] The presence (+) or absence (−) of a specific Western blot reaction following sodium dodecyl sulfate-polyacrylamide gel electrophoresis of trichloroacetic acid-precipitated proteins (62) from FBNSV-infected plants is indicated. NT, not tested.

artificial infection system, which has been accomplished only for FBNYV (54). Cloned viral DNAs were shown to be infectious, yet the virus thus obtained was not transmitted by aphid species known to be efficient vectors of FBNYV. The reason for this failure may be the existence of not yet identified genomic component(s) or a suboptimal sequence of the eight cloned FBNYV DNAs. Hence, there was a need for a rigorous and exhaustive search to detect and characterize all DNAs of a given nanovirus from infected plants.

An ideal method for such a comprehensive study is the rolling circle amplification (RCA) technique (27). It has been successfully used to detect and molecularly characterize geminiviruses, the other family of plant ssDNA viruses (9, 21, 23, 28, 33–35, 37, 49, 56), and also the babuvirus ABTV (50).

To sufficiently cover the genetic variability within a nanovirus isolate, we applied RCA to analyze and clone a hitherto little-characterized nanovirus from Ethiopia. Limited information on this Ethiopian isolate suggested that it is closely related to, but genetically distinct from, FBNYV and MDV (20). Here, we report the comprehensive sequence analysis of the DNA genome of this nanovirus from Ethiopia. Based on its DNA sequence and distinct serological and biological characteristics, we propose to assign it as a new species to the genus *Nanovirus* and to name it faba bean necrotic stunt virus (FBNSV). Moreover, we established clone banks of all essential genomic DNAs of FBNSV, used representative molecules for artificial infection, and succeeded in reconstituting for the first time an authentic and aphid-transmissible nanovirus.

MATERIALS AND METHODS

Origin and maintenance of virus isolates. A symptomatic faba bean plant was collected from a field near Holetta, Ethiopia, and kindly sent to Braunschweig, Germany, by K. M. Makkouk in October 1997. From this sample a pure nanovirus isolate, FBNSV-[ET:Hol:97], was established in faba bean using single individuals of the aphid *A. pisum* Harris for repeated acquisition and transmission, followed by serological analysis of inoculated plants (see below) in order to separate the nanovirus from chickpea chlorotic stunt virus, a polerovirus also present in the field sample. It was maintained under greenhouse conditions in *Vicia faba* seedlings of cultivar Condor and/or Scirocco by vector inoculation at intervals of 3 to 4 weeks until August 2004, when it was inadvertently lost due to the use of a systemic insecticide for controlling a massive thrips infestation in faba bean.

For FBNSV transmission by aphids, *A. pisum* reared on faba bean was used by giving several hundred individuals (at different larval stages) an acquisition access feeding period (AAP) of 2 to 3 days on infected faba bean plants, followed by an inoculation access feeding period of 2 to 3 days on young faba bean seedlings using about 30 individuals per plant (20, 32). After plants were sprayed with a contact insecticide (e.g., pyrethrine), they were kept in an insect-proof glasshouse and observed for symptom development.

The FBNYV isolate from Egypt (FBNYV-[EG:93]) was available in frozen faba bean leaves, whereas an FBNYV isolate from Biskra, Algeria (FBNYV-[DZ:Bis:00]) (57), was maintained by vector transmission in the same fashion as FBNSV-[ET:Hol:97].

Serology and MAb production and characterization. For serological analysis of inoculated plants, triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) was performed as described previously (18), using high-binding polystyrene plates (Greiner Bio-One GmbH, Germany), the buffers described by Clark and Adams (12), and the nanovirus-specific monoclonal antibodies (MAbs) 1-1F2, 2-1A1, and 3-4F2 (18) and the broad-spectrum luteovirid-specific MAb B-2-5G4 (29) as detecting antibodies. For trapping of nanovirus and luteovirid antigen in TAS-ELISA, we coated plates with immunoglobulin G (IgG) to FBNYV and turnip yellows virus, respectively.

MAbs specific to FBNSV-[ET:Hol:97] were produced using virion preparations purified as described previously (54) from FBNSV-infected leaves sampled in 1998. Each of two female BALB/c mice (2 to 3 months old) received a subcutaneous injection of ~75 μg of FBNSV virions in 150 μl containing 50% Freund's incomplete adjuvant. Booster injections, fusion experiments, selection of hybridomas, and cloning were essentially conducted as described previously (1, 18). For hybridoma selection by TAS-ELISA, however, FBNYV IgG was used for the trapping of antigen from extracts of FBNYV- and FBNSV-infected faba bean plants as well as from noninoculated plants. Isotyping of MAbs was done using an Hbt Mouse Monoclonal Antibody Isotyping kit (HyCult Biotechnology, The Netherlands) essentially following the manufacturer's instructions. Seven stable hybridoma lines obtained from two fusion experiments secreted FBNSV-specific antibodies that were typed as IgG1 (three), IgG2a (three), and IgG2b (one) (Table 1). Serial dilutions of culture supernatants ranging from 1:10 to 1:25,600 were tested in TAS-ELISA for determining MAb titers. For examining the reactivity and specificity of MAbs, they were used at appropriate dilutions in Western blot analyses and incubated with trichloroacetic acid-precipitated proteins (62) from FBNSV- and FBNYV-infected faba bean plants as well as with proteins from noninoculated faba bean plants.

DNA extraction, RCA, cloning, and sequencing. From FBNSV-infected *V. faba* tissue that had been frozen in 2000, total DNA was extracted according to a modified Edwards protocol described previously (15, 54) with an additional step of phenol-chloroform extraction.

Circular DNAs present in 1 μl of nondiluted or 10-fold diluted total DNA preparations were amplified by RCA using an illustra TempliPhi Amplification Kit (GE Healthcare) following the manufacturer's instructions. RCA products were obtained after 18 to 20 h of incubation at 30°C followed by 10 min at 65°C.

Tenfold diluted RCA products were digested with restriction enzymes in appropriate buffers. Fragments generated by AatII or MluI restriction endonucleases were resolved on 1% agarose gels, extracted, and inserted into plasmids Litmus28 or Litmus38 (New England Biolabs), respectively. Candidate bacteria for harboring recombinant plasmids (i.e., forming white colonies on indicator plates) were boiled in 20 μl of H_2O, and 1 μl thereof was directly subjected to PCR in a total volume of 20 μl containing 10 pmol each of M13 direct and reverse sequencing primers, 67 mM Tris-HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 1.5 mM $MgCl_2$, 50 μM of each deoxynucleoside triphosphate, and 0.5 units of *Taq* II DNA polymerase (Eurobio). To avoid

excessive redundant sequencing of FBNSV DNAs, additional PCRs (35 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 90 s) using component-specific primers as specified in Table S1 in the supplemental material were run. The inserts of recombinant plasmids were sequenced.

For the identification of putative additional Rep-encoding DNAs associated with FBNSV, total DNA of this sample was amplified by PCR (35 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 90 s) using primers specified in Table S1 in the supplemental material. Dilutions (5×10^7 molecules/μl) of cloned additional Rep DNAs from FBNYV-[SY] and FBNYV-[EG] (53) were used as positive controls.

To obtain consensus sequences of the different genomic DNAs in the viral population, RCA products were diluted (7.5- or 3.3-fold) and directly sequenced (23, 49) at GATC Biotech or Eurofins MWG GmbH, respectively, using component-specific primers (see Table S1 in the supplemental material).

Sequence analysis. Sequences were analyzed with the Wisconsin Package, version 10.0 (Genetics Computer Group, Madison, WI) (13) and DNASTAR Lasergene, version 8.0.2 (DNASTAR, Inc., Madison, WI). Phylogenetic analyses were conducted in MEGA, version 4 (51).

qPCR analysis. Quantitative PCR (qPCR) reactions were performed using the LightCycler FastStart DNA Master Plus SYBR green I kit (Roche) on a Roche LightCycler according to the manufacturer's instructions. The reaction mixture (10 μl) consisting of 0.5× Master Mix, 2 mM $MgCl_2$, 5 pmol of each primer (see Table S1 in the supplemental material) or 10 pmol in the case of DNA-S, and 1 μl of DNA sample was cycled as follows: 95°C for 10 min and 45 cycles each consisting of 10 s at 95°C, 5 s at 65°C (70°C in the case of DNA-S and DNA-N), 15 s at 72°C, and 5 s at 82°C (80°C in the case of DNA-N) for single-mode acquisition. The last step was omitted in the case of DNA-S and DNA-M, and the single-mode acquisition was performed at the end of the extension step. PCR amplification specificity was verified by a dissociation curve (65 to 95°C). Serial dilutions to 5×10^3 to 5×10^7 molecules per sample of Litmus28 containing the respective FBNSV DNAs in the AatII site served to establish standard curves for calibration. Negative controls without nanovirus template (water and uninfected plant DNA extract) or a mixture containing all other seven DNAs at 5×10^7 molecules per sample each, except the one to be quantified, were included. At least two different dilutions of each sample were quantified.

Inoculation of *V. faba* plants with cloned nanovirus DNAs and insect transmission. FBNSV DNAs corresponding to the respective consensus sequences were chosen for introduction into plants except for DNA-M, DNA-U1, and DNA-U4. In the case of the these three, sequences deviating by a single nucleotide from the consensus were used: DNA-M T175G, DNA-U1 G201A, and DNA-U4 C267T. All but DNA-U4 were dimerized as direct repeats in the AatII site of Litmus28 and subsequently transferred as KpnI-BamHI fragments to the binary T-DNA vector pBin19 (6). Copies of DNA-U4 in tandem repeats were generated in the MluI site of Litmus38 and transferred to pBin19 as a SalI-BamHI fragment.

For agroinoculation of *V. faba* (variety Shambat-75), derivatives of pBin19 carrying the respective nanovirus DNAs were transferred by electroporation into *Agrobacterium tumefaciens* COR308 and used as described previously (54). Plants that developed symptoms after agroinoculation were pooled and used for virus acquisition and transmission by *A. craccivora* or *A. pisum* to *V. faba* seedlings (cultivar Shambat-75 or Scirocco) as described previously (54). First, all symptomatic plants were combined and exposed to >100 *A. craccivora* individuals. After an AAP of 3 days, the insects were brushed off the plants, mixed, and allowed to redistribute to the same plants for an additional AAP of 4 days. Subsequently, the aphids were distributed on 1-week-old *V. faba* (cultivar Shambat-75 or Scirocco) seedlings for a 2-day inoculation access feeding period. To detect FBNSV DNAs in inoculated plants, total DNA was extracted; the component-specific primer pairs listed in Table S1 in the supplemental material were used, and 35 PCR cycles were run (95°C for 30 s, 53°C for 40 s, and 72°C for 90 s). To assess aphid transmission rates, inoculated plants were observed for symptom development at intervals of 2 to 3 days and analyzed at 3 to 4 weeks after inoculation by TAS-ELISA using polyclonal IgG to FBNYV as the trapping antibody and the FBNSV-specific MAbs 8-2G10 and 8-4F9 (Table 1) and/or the broad-spectrum, nanovirus-specific MAbs 1-1F2, 2-1A1, and 3-4F2 as the detecting antibodies.

Detection of FBNSV virions in infected plants. Purification of FBNSV virions and electron microscopy of negatively stained virion preparations were done essentially as described previously (54). However, virions were purified from only about 30 g of infected leaves, and the initial leaf extract was not stirred overnight at room temperature as the extraction buffer did not contain a macerating enzyme.

Nucleotide sequence accession numbers. The FBNSV sequences reported here were deposited in GenBank under accession numbers GQ150778 to GQ150785.

RESULTS

Symptoms and serology. Upon establishment in the faba bean cultivar Condor, FBNSV-[ET:Hol:97] consistently produced symptoms different from those of FBNYV isolates (32). Since it caused leaf rolling and severe plant stunting which was often associated with apical necrosis in faba bean as early as 2 to 3 weeks after inoculation, the name FBNSV was coined. Symptomatic plants reacted only with the nanovirus-specific MAbs 1-1F2, 2-1A1, and 3-4F2 (18) but not with the broad-spectrum luteovirid-specific MAb B-2-5G4 (32) in TAS-ELISA. Moreover, FBNSV-[ET:Hol:97] failed to react with the FBNYV MAbs 1-3D8, 2-5H9, and 3-4A5. For instance, MAbs 2-5H9 and 3-4A5 reacted in TAS-ELISA with FBNYV but not with MDV and a nanovirus in faba bean samples originating from Ambo, Ethiopia (18). This suggested that FBNSV-[ET:Hol:97] is serologically distinct from Syrian and Egyptian isolates of FBNYV as well as from MDV. Furthermore, the newly produced MAbs (Table 1) reacted strongly with FBNSV and only weakly or not at all with FBNYV isolates. In Western blotting experiments, only MAbs 8-2G10 and 8-4A2 reacted with a trichloroacetic acid-precipitated protein of ~20 kDa (capsid protein) from FBNSV-infected leaves (results not shown). MAb 8-4A2 reacted also with a ~20-kDa protein from FBNYV-infected tissue, confirming its weak TAS-ELISA reactions with FBNYV isolates (Table 1).

Although the titers of all the MAb-containing culture supernatants exceeded 1:25,600 in TAS-ELISA, culture supernatant dilutions of 1:100 to 1:5,000 were found to give the best signal-to-noise ratio for routine use in TAS-ELISA (Table 1). At these working dilutions, all homologous reactions of the FBNSV MAbs were strong (A_{405} values of >1.0 after substrate reaction of 1 h), whereas no reactions or only very weak heterologous reactions were observed with FBNYV isolates from Egypt and Algeria (Table 1). MAbs 8-2G10 and 8-4F9, which consistently reacted slightly more strongly with FBNSV and did not cross-react with FBNYV isolates, were selected for routine use as detecting antibodies in TAS-ELISA.

Comprehensive analysis of FBNSV by extensive RCA, cloning, and sequencing. FBNYV-[EG] is the only nanovirus for which artificial infection with cloned DNAs has been achieved (54). However, the fact that the virus produced in artificially infected and symptomatic faba bean plants was not aphid transmissible had indicated a negative influence of potentially "suboptimal" sequences used although a high-fidelity proofreading DNA polymerase from *Pyrococcus furiosus* (*Pfu* Pol) had been used for amplification prior to cloning (54). Therefore, a rigorous de novo cloning appeared necessary. Here, we used a different nanovirus (FBNSV) and applied an alternative technique (RCA) prior to cloning its DNAs.

Total DNA was extracted from FBNSV-infected leaves dating from 2000 and submitted to RCA using the proofreading ϕ29 DNA polymerase (16). Amplified circular DNA-derived products were then restricted by endonucleases, cloned, and sequenced. The endonuclease used for restricting nanovirus RCA products was chosen based on available sequence infor-

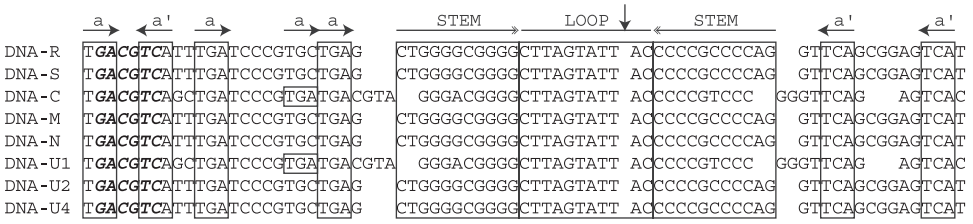

FIG. 1. Nucleotide sequence alignment of the common region stem-loop of FBNSV DNAs. Conserved sequences are boxed. The inverted repeat sequences at the origin of replication potentially forming a stem-loop structure are marked by open horizontal arrows. A vertical arrow indicates the position of cleavage by the M-Rep protein (53). Filled arrows indicate the iteron-like sequences (a) and their respective inversions (a′). The universally conserved AatII site is shown in bold italics.

mation (55). One of the iteron-like sequences (TGAY), in conjunction with its respective inversion, represents an AatII restriction site (GACGTC) (Fig. 1). This AatII site is a conserved and unique site in all bona fide integral genomic nanovirus DNAs, except for two genomic DNAs of SCSV (55). We used AatII to restrict the RCA product into one major population of DNA fragments of about 1-kb, which were cloned and sequenced. This way, we analyzed 143 individually cloned AatII-restricted DNAs, 124 of which were completely sequenced (see Table S2 in the supplemental material). A minor fragment of about 1.5 kb represented *V. faba* mitochondrion plasmid 3 DNA (59) as revealed by sequences of eight clones of this fragment.

In order to have at least 20 independent and completely sequenced representatives of each genomic DNA of FBNSV for a pertinent statistical analysis, we used the endonuclease EcoRV or HindIII prior to cloning to eliminate from the pool of 1-kb AatII fragments genomic DNAs that were already well represented. Alternatively, endonuclease MluI was used to restrict the RCA product. This enzyme has unique sites only in DNA-R, DNA-N, and DNA-U4, increasing their probability of being cloned.

In summary, more than 208 individual, cloned FBNSV genomic DNAs ranging from 979 to 1,003 nucleotides were sequenced (see Table S2 in the supplemental material), and a consensus sequence was established for the genomic DNAs and deposited in GenBank under accession numbers GQ150778 to GQ150785. Moreover, we directly sequenced the RCA products using component-specific primers (see Table S1 in the supplemental material) to obtain a population consensus sequence. All sequences converged into eight distinct DNA components (Fig. 2).

No previously undescribed genomic DNAs and no additional Rep-encoding DNAs were found in the FBNSV sample from 2000 using RCA combined with AatII or MluI restriction. An additional 20 enzymes (AfeI, ApaI, AvrII, BamHI, BclI, BglII, BssHII, BstBI, ClaI, EagI, KpnI, MscI, NarI, NheI, NruI, NsiI, PvuI, SacII, SpeI, and XhoI), which do not cut the eight genomic components of the FBNSV-[ET:Hol:97] genome, were also used for digesting the RCA product to potentially identify additional FBNSV DNAs. No restriction products were observed. To specifically search for additional Rep-encoding DNAs, the FBNSV-infected sample was subjected to PCR analysis using several primer pairs specific for known para-Rep-encoding DNAs associated with FBNYV isolates (see Table S1 in the supplemental material). No amplification products were obtained, confirming earlier results from dot blot hybridization assays for para-Rep-encoding

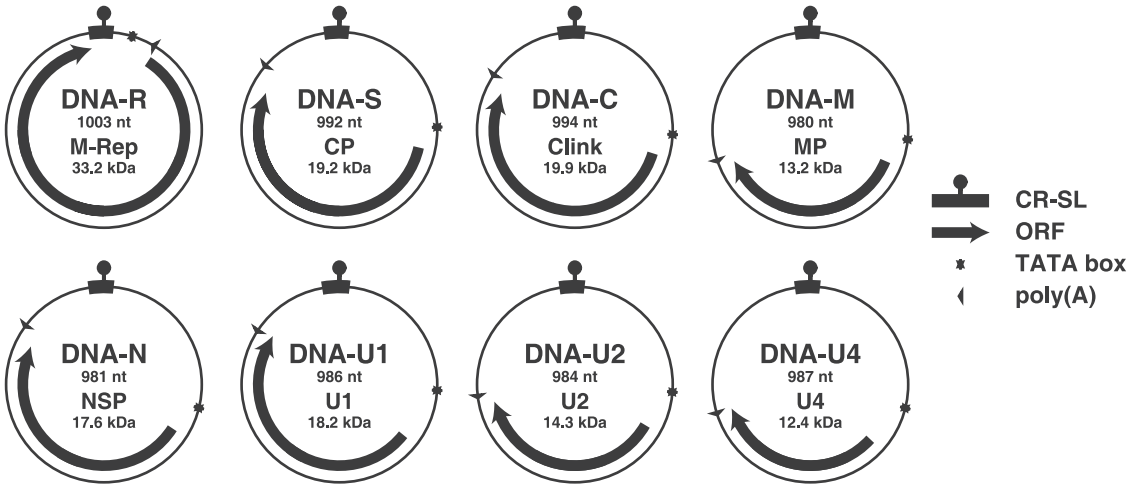

FIG. 2. Genome organization of FBNSV. The eight genomic DNAs are shown as circles and designated according to current nomenclature (58). Arrows represent protein-encoding ORFs. The sizes of the individual DNAs (consensus sequences) and the corresponding proteins are indicated in nucleotides (nt) and in kDa, respectively. CP, capsid protein; MP, movement protein; NSP, nuclear shuttle protein; CR-SL, common region stem-loop.

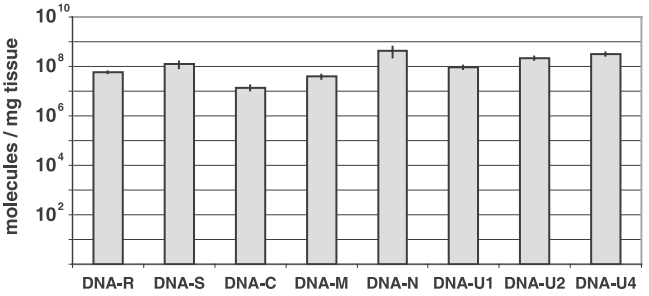

FIG. 3. Quantification of FBNSV DNAs. Bars represent the amount of each genomic DNA component in leaf tissue of infected plants. Mean standard deviations are indicated by vertical lines.

DNAs in FBNSV-[ET:Hol:97] (53). Based on these results, we consider it highly unlikely that Rep-encoding DNAs other than DNA-R were present in the sample of FBNSV.

qPCR to determine the natural relative abundance of FBNSV DNAs. As not all FBNSV genome components were equally represented in the differently prepared recombinant plasmids (see Table S2 in the supplemental material) (e.g., 197 full-length clones of DNA-N compared to only 16 of DNA-U4), we directly determined the relative abundance of each genomic DNA in infected tissue by qPCR on total DNA (for qPCR primers, see Table S1 in the supplemental material). As shown in Fig. 3, DNA-C had the lowest titer (1×10^7 molecules/mg of leaf tissue) while DNA-N was the most abundant DNA (4×10^8 molecules/mg of leaf tissue). It is noteworthy that the copy numbers of DNA-N and DNA-U4 in infected tissue are about equal (Fig. 3). The same relative abundances of all eight FBNSV DNAs were found in qPCR analyses after RCA or AatII restriction of the RCA product (data not shown), ruling out an RCA or restriction bias.

The FBNSV genome. RCA, extensive cloning, and sequencing identified eight distinct DNAs of about 1 kb as the FBNSV genome (Fig. 2). Each DNA bears a single ORF of virion-sense polarity, which is preceded by TATA sequences and followed by AATAAA-like polyadenylation addition signals. On DNA-R, these signals are arranged in the vicinity of the inverted repeat (IR) sequences at the origin of replication in the unique and characteristic order for DNA-R (22): the first poly(A) addition signal lies 3′ of the IR sequences between the TATA box and the ATG of the *m-rep* gene.

Like all nano- and babuviruses, the genomic DNAs of FBNSV also have a common region stem-loop (Fig. 1), composed of iteron-like sequences flanking the IR sequences at the origin of replication with the potential of forming a stem-loop structure (8, 31, 50, 55).

At least 20 complete sequences of each individually cloned FBNSV DNA, with DNA-U4 as an underrepresented exception, were determined (Table 2). Dividing the total number of deviations from the consensus by the number of nucleotides sequenced, a mutation frequency of 6.0×10^{-4} was determined. A detailed assessment of intra- and interisolate variability of FBNSV genomes including base substitution rates and comparison with those of other ssDNA and RNA viruses will be presented elsewhere (unpublished data).

Of the DNAs in the FBNSV sample from 2000, DNA-C is the most conserved genome component. Fourteen of 20 DNA-C sequences were identical to the cloning consensus, and six had single base deviations from the cloning consensus (Table 2). There were no polymorphisms between the cloning consensus of DNA-C and the population consensus determined by direct sequencing of the RCA product.

DNA-M is one of the most polymorphic genome components. None of 27 DNA-M sequences corresponded to the cloning consensus. Up to eight nucleotide variations (transitions, transversions, and single nucleotide deletions) or recombination events occurred per cloned molecule. Some of the polymorphic sites were also apparent in the population consensus sequence of DNA-M.

In summary, based on their respective mutation frequencies (Table 2), the FBNSV DNAs can be ranked from the most conserved to the most variable as follows: DNA-C, DNA-R, DNA-N, DNA-S, DNA-U2, DNA-M, DNA-U4, and DNA-U1.

Phylogenetic analyses. Comparing the eight FBNSV DNAs with the homologous DNAs of all other available nanoviruses (Fig. 4), we found that FBNSV is more closely related to FBNYV and MDV than to SCSV. In general, FBNSV, FBNYV, and MDV are about equally distant from each other (72% to 75% overall identity). The percent identity between the three viruses varies from 56% (DNA-U2) to 89% (DNA-R), depending on component; all three share identities ranging

TABLE 2. Variability of the FBNSV genome

DNA type	No. of clones sequenced	No. of consensus clones	No. of different clone types	No. of polymorphic sites						Mutation frequency
				Total	Noncoding region	Coding region		Maximum per clone	In population consensus	
						Synonymous	Nonsynonymous			
DNA-R	21	10	8	8	2	5	1	3	1	3.80×10^{-4}
DNA-S	29	12	10	16	10	4	2	6	5	5.56×10^{-4}
DNA-C	20	14	6	5	3	0	2	1	0	2.52×10^{-4}
DNA-M	27	0	14	21	15 + 1[a]	3	2	8	9	7.94×10^{-4}
DNA-N	40	17	17	19	10	4	5	6	3	4.84×10^{-4}
DNA-U1	29	0	18	24	13	5	5 + 1[b]	7	9	8.39×10^{-4}
DNA-U2	26	11	11	18	13	0	5	5	3	7.04×10^{-4}
DNA-U4	16	5	9	13	11	0	2	5	4	8.23×10^{-4}
Genome	208	69	93	124	78	21	25	41	34	6.04×10^{-4}

[a] Recombinant; ≥29 nucleotides exchanged between DNA-M and DNA-U2 and counted as a single event.
[b] Nonsynonymous nucleotide change leading to a stop codon.

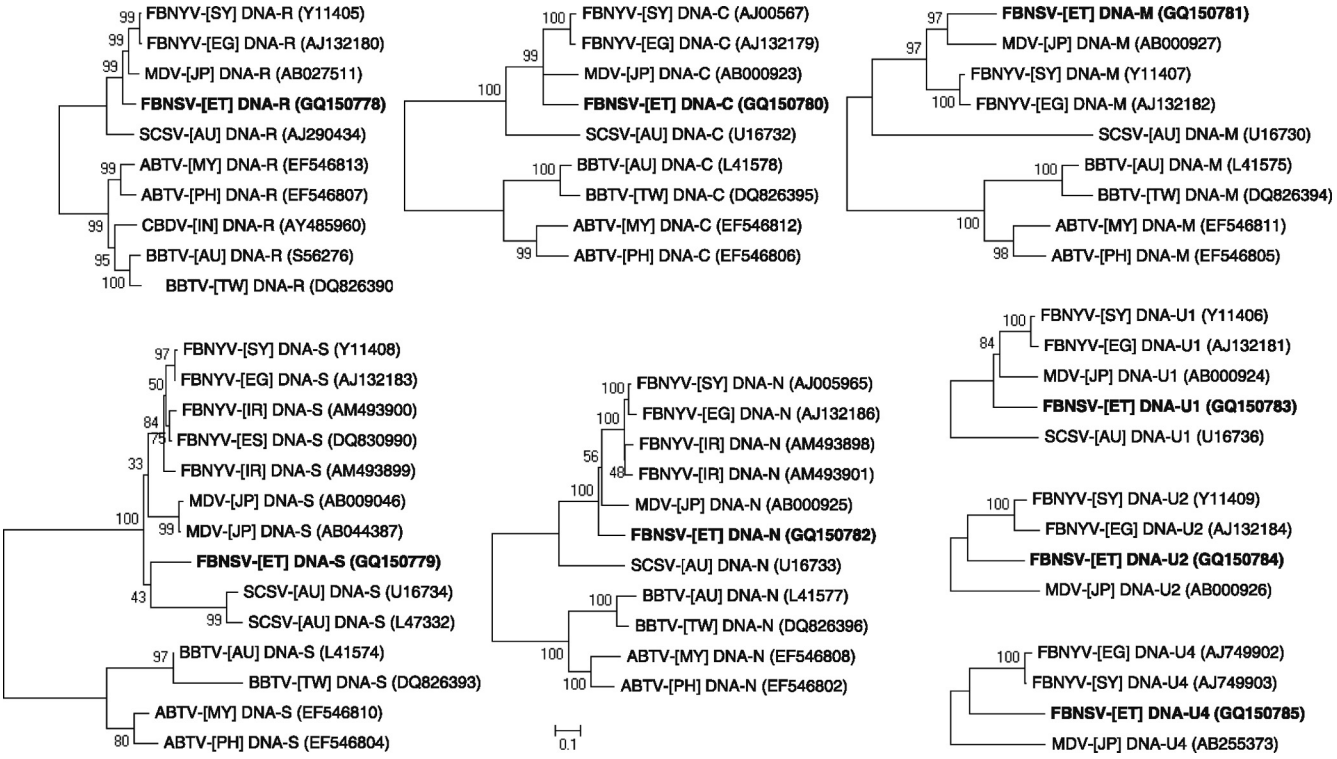

FIG. 4. Neighbor-joining dendrograms illustrating the phylogenetic relationships between FBNSV-[ET] genome DNAs (displayed in bold) with those of other nanoviruses and their isolates. Horizontal branch lengths are scaled according to the number of base substitutions per site. Vertical branch lengths are arbitrary. Bootstrap percentages (500 replicates) are shown next to the branches. GenBank accession numbers are shown after the virus acronyms. CBDV, cardamom bushy dwarf virus.

from 36% (DNA-M) to 78% (DNA-R) with SCSV while all four legume-infecting nanoviruses are only 35% (DNA-M) to 59% (DNA-R) identical to BBTV, ABTV, or cardamom bushy dwarf virus.

FBNSV, FBNYV, and MDV appear to be similarly equidistant when only their protein amino acid sequences (coding sequences represent 49% of the total genome) are considered. The M-Rep protein is the most conserved protein between FBNSV, FBNYV, and MDV (93 to 97% amino acid sequence identity) as well as among all nanoviruses (83% amino acid sequence identity with SCSV and 54 to 57% with babuviruses). FBNSV, FBNYV, and MDV share amino acid sequence identities of 80 to 84% in the capsid protein and less than 75% in the proteins with unknown functions (encoded by DNA-U1, -U2, and -U4).

Reconstitution of FBNSV symptoms in *V. faba* plants infected with cloned viral DNAs. Infection of *V. faba* by cloned nanovirus DNAs via agroinoculation requires copies of each genomic DNA in tandem repeats within the T-DNA of an *A. tumefaciens* plasmid, followed by delivery of the bacteria to the plant. This way, the first and, so far, only demonstration of infectivity of cloned yet aphid-nontransmissible nanovirus DNAs was accomplished for FBNYV-[EG] (54).

To minimize the risk of using suboptimal cloned FBNSV sequences for infectivity and transmission studies, we chose only clones representing the respective consensus sequences of DNA-R, -S, -C, -N, and -U2. At the time when the clones for infectivity studies were chosen, none representing the consensus sequence was available for DNA-M, -U1, and -U4. Therefore, we used molecules whose sequence deviated from the respective consensus only by a single nucleotide in the noncoding region.

We used mixtures of agrobacteria carrying the eight FBNSV DNAs, inserted as complete direct repeats in the pBin19 T-DNA, to inoculate 1-week-old *V. faba* cv. Shambat-75 seedlings. Symptoms such as leaf rolling and brittleness, as well as stunted growth, developed 10 to 14 days postinoculation. Within three weeks postinoculation, plants also showed yellowing and later became necrotic. In two independent experiments, 69% (40/58) (Table 3) and 100% (10/10) of the agroinoculated plants developed symptoms within 3 weeks.

To confirm that the observed disease phenotype was due to an FBNSV infection, we assessed the presence in the youngest part of each *V. faba* plant of all DNA components by PCR using the component-specific primers listed in Table S1 in the supplemental material. The results summarized in Table 3 indicate that FBNSV had replicated and systemically infected the symptomatic plants.

Moreover, the symptomatic plants agroinoculated with the eight cloned FBNSV DNAs contained typical nanovirus particles, as demonstrated by electron microscopy of partially purified virions from symptomatic faba bean plants (Fig. 5A). The yield and the sucrose gradient sedimentation rate of purified virions from agroinoculated plants were comparable to those from plants infected by aphids with FBNSV-[ET:Hol:97].

TABLE 3. Detection of the reconstituted FBNSV in *V. faba* plants following agroinoculation with eight cloned virus DNAs and subsequent aphid transmission

Infection and transmission scheme	Vector	*V. faba* cultivar	No. of symptomatic plants/no. of inoculated plants	PCR result by DNA clone (no. of positive plants/no. of symptomatic plants analyzed)								ELISA (no. of positive plants/no. of inoculated plants)[b]
				DNA-R	DNA-S	DNA-C	DNA-M	DNA-N	DNA-U1	DNA-U2	DNA-U4	
Agroinoculation	*A. tumefaciens*	Shambat-75	40/58	40/40	40/40	40/40	40/40	39/40	40/40	40/40	40/40	
Transmission 1	*A. craccivora*	Shambat-75	19/19	19/19	19/19	19/19	19/19	19/19	19/19	19/19	19/19	
	A. craccivora	Scirocco	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	
Transmission 2[a]	*A. craccivora*	Shambat-75	11/12	11/11	11/11	11/11	11/11	11/11	11/11	11/11	11/11	
	A. pisum	Scirocco	16/17									16/17
Transmission 3	*A. craccivora*	Shambat-75	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	
	A. pisum	Scirocco	6/6									6/6
Transmission 4	*A. pisum*	Scirocco	6/6									6/6
Transmission 5	*A. pisum*	Scirocco	6/6									6/6
Transmission 6	*A. pisum*	Scirocco	3/3									3/3
Transmission 7	*A. pisum*	Scirocco	6/6	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	6/6

[a] Symptomatic plants of transmission 1 served for subsequent serial transmission by *A. pisum*.
[b] Number of ELISA-positive plants using FBNSV-specific MAbs in TAS-ELISAs.

Aphid transmission of FBNSV from artificially infected *V. faba* plants. Vector transmissibility of the reconstituted virus was assessed using the aphid species *A. craccivora* and *A. pisum* (20, 32) and carried out as described in Materials and Methods.

All the plants exposed to aphids for inoculation developed disease symptoms (stunting, leaf rolling, and brittleness) within 2 weeks postinoculation (Fig. 5B). Three weeks postinoculation, the *V. faba* cv. Shambat-75 plants showed yellowing and necrosis (Fig. 5C) while we observed no necrosis yet on the *V. faba* cv. Scirocco plants (Fig. 5D). We confirmed the presence of all eight FBNSV DNAs in the youngest part of each *V. faba* plant by component-specific PCR (Table 3). We serially continued virus transmission by *A. craccivora* for another two cycles of transmission to 1-week-old *V. faba* cv. Shambat-75 plants.

A. pisum also proved to be an efficient vector of the reconstituted FBNSV. We obtained high transmission rates of the reconstituted FBNSV to faba bean cultivar Scirocco seedlings in seven serial transmission experiments using 10 to 20 *A. pisum* larvae per plant (Table 3) and detected by PCR all eight genomic DNAs. Moreover, we revealed the presence of capsid protein antigen by TAS-ELISA. Symptomatic tissue reacted with both a mixture of broad-spectrum nanovirus-specific MAbs and with the FBNSV-specific MAbs 8-2G10 and 8-4F9. The latter indicated that the epitope(s) recognized by these FBNSV-specific MAbs were encoded by the consensus DNA-S. When using single *A. pisum* larvae for inoculation of individual faba bean seedlings (cultivar Scirocco) with the reconstituted FBNSV, we observed transmission rates ranging from 20% (5/24) to 88% (21/24), which is comparable to those obtained with the original isolate FBNSV-[ET:Hol:97] during maintenance from 1997 to 2004.

In contrast to FBNYV (19), host range studies of the original FBNSV-[ET:Hol:97] isolate were limited. However, the reconstituted virus was transmitted by aphids not only to *V. faba* but also to bean (*Phaseolus vulgaris*), chickpea (*Cicer arietinum*), cowpea (*Vigna unguiculata*), pea (*Pisum sativum*),

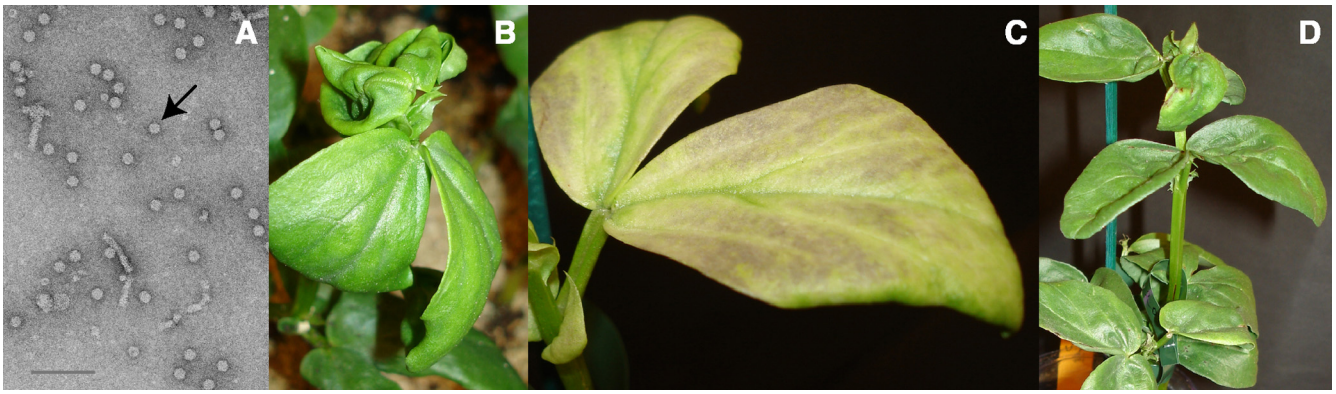

FIG. 5. FBNSV particles from agroinoculated plants and disease symptoms produced by the reconstituted virus after aphid transmission. (A) Electron micrograph of a partially purified virion preparation from symptomatic *V. faba* cv. Shambat-75 following agroinoculation with cloned FBNSV DNAs. The arrow points at a typical nanovirus particle measuring about 20 nm in diameter; the other spherical and filamentous structures appear to be phytoferritin and host membranes, respectively. Bar, 100 nm. (B and C) Symptoms of the reconstituted FBNSV on *V. faba* cv. Shambat-75 at 2 and 3 weeks postinoculation by *A. craccivora*. (D) Symptoms of the same reconstituted FBNSV on *V. faba* cv. Scirocco at 3 weeks postinoculation by *A. craccivora*.

and soybean (*Glycine max*), in which it caused characteristic disease symptoms.

The recovery of fully infectious and readily transmissible FBNSV has been repeated in four independent agroinoculation and transmission experiments, proof of the successful reconstitution of an authentic nanovirus from eight cloned DNAs.

DISCUSSION

Here, we describe an Ethiopian isolate of a legume-infecting nanovirus that we named FBNSV. Since FBNSV, FBNYV, and MDV not only are serologically and biologically closely related but also have strikingly similar ssDNA components, we cannot rule out the possibility that they are genetically deviating strains of one virus species that evolved in Africa, the Near East (Syria, Egypt, or Iran), and Japan, respectively. More likely, however, the data reported here provide evidence for the occurrence of a distinct nanovirus species infecting legumes in Ethiopia, based on the current International Committee for the Taxonomy of Viruses criteria for nanovirus species demarcation (58).

Extensive sequence analysis of FBNSV from Ethiopia based on RCA and subsequent cloning of viral DNAs from infected plant material showed that the genome of this nanovirus consists of a single M-Rep-encoding DNA and the seven other genomic DNAs identified from most legume-infecting nanoviruses. One particular FBNSV genome component (DNA-U4) was underrepresented in the clone banks. Incidentally, the same DNA happened to be identified last for FBNYV from Egypt and Syria, for MDV, and not at all (yet?) for SCSV. This prompted us to quantitate the relative abundance of each of the eight individual FBNSV DNAs in a natural infection. The qPCR results clearly indicated that DNA-U4 was as abundant as DNA-U1, -U2, and -N. The titer of the individual FBNSV DNAs varied from 1×10^7 molecules to 4×10^8 molecules/mg of leaf tissue (Fig. 3). DNA-N was the most abundant molecule, and DNA-C was the least abundant (Fig. 3). The underrepresentation of DNA-U4 in the present set of clones and the fact that it so far has not been found for SCSV is probably due to a potential toxicity to bacteria of the U4 protein. Now that a component-specific qPCR for a nanovirus has been established, it will be possible to assess the quantitative distribution of particular genomic DNAs in the course of an infection, both in time and space (tissue).

The intrapopulation variability of a particular FBNSV DNA that became apparent from our extensive sequence analysis ranged from five single polymorphic sites per genomic DNA in the case of DNA-C to a total of 21 polymorphisms (one single to up to eight different concomitant polymorphisms per cloned molecule) in DNA-M, one of the most variable genome components (Table 2). Interestingly, DNA-M differed also from a DNA-M sequence described previously (20) (GenBank accession number AF159705) by an additional 56 nucleotides at position 812, adjusting its size closer to that of the majority of the legume nanovirus DNAs. As DNA-M is essential for infectivity (54), the impact on its function of the deletion in the DNA-M sequence of AF159705 remains to be seen. About 27% of the polymorphisms that we identified as sequence variants in the clone population were also apparent as polymorphic sites in the population consensus. This indicates that by sequencing at least 20 individually cloned molecules, we analyzed a reasonably representative sample of the different genomic FBNSV DNAs in the population (14).

In the FBNSV sample of 2000, we detected a total of 46 sequence variants in the coding regions, which included 21 (45.7%) synonymous and 25 (54.3%) nonsynonymous changes. As a whole, the FBNSV genome appeared as a swarm of eight variable molecules, with consensus sequences of DNA-R, -C, -S, -N, -U2, and -U4 being well represented among the cloned representatives of these genomic DNAs. For the most variable DNA-M and -U1, no representative consensus molecules were among the clones ($n = 27$ and $n = 29$, respectively).

Although transmission by aphids may represent bottlenecks for certain plant viruses that possess mono- or multipartite genomes (2, 5, 38, 42), the most extreme bottleneck is in cloning a single individual genome representative, whether it consists of one or a combination of several molecules, and the risk of selecting one or more suboptimal representatives is high. The FBNYV DNAs used previously for infectivity studies had been selected on the basis of limited sequence information on two to four cloned molecules (54). Therefore, we cannot rule out the possibility that one or more suboptimal FBNYV genomic DNAs had been used for infectivity and aphid transmission assays. It is therefore conceivable that this may have been the reason for the futile attempts to transmit progeny virus resulting from agroinoculation of these cloned DNAs (54). RCA and extensive sequencing of FBNSV have helped us to overcome the experimental limitations associated with such a cloning bottleneck.

Assuming that the identified consensus sequences represented biologically reasonably fit examples of the respective genomic FBNSV DNAs, clones of these genomic DNAs were used for further infectivity and insect transmission studies. For DNA-M, -U1, and -U4, clones with only single consensus deviations in the noncoding sequences were chosen. When used for agroinoculation, these FBNSV DNAs elicited the development of the viral disease as rapidly as did the natural virus population inoculated by aphid vectors. This indicates that these particular genomic DNAs do not deviate strikingly in fitness from those of the population. More importantly, for the first time the virus derived from this set of cloned FBNSV DNAs was readily transmissible by two natural aphid vector species, *A. craccivora* and *A. pisum*. Symptoms of FBNSV infection appeared as early as 10 days after inoculation and developed in the characteristic way (Fig. 5B, C, and D). Moreover, a faba bean genotype dependence of the disease was consistently observed, with symptoms being more severe in cultivar Shambat-75 than in cultivar Scirocco. All eight genomic FBNSV DNAs were detected in symptomatic tissue of aphid-inoculated plants after multiple successive serial transmissions, spanning a period of about 8 months.

By using RCA of the heterogeneous population of circular DNAs prevailing in an infection by the nanovirus FBNSV and subsequent direct sequencing or cloning of a representative sample of each individual genomic DNA of the virus, we were able to derive workable and biologically active physical representatives of consensus sequences for each of the eight genomic DNAs. No additional as yet undescribed nanovirus sequences were found associated with FBNSV in the sample

from the year 2000, nor were any additional Rep-encoding molecules detected in this sample.

The established system now allows reverse genetics of a nanovirus. Other examples of a successful application of cloned copies of multiple virus genome segments for reverse genetics include influenza A virus (*Orthomyxoviridae*) (17, 43, 44) and recently also bluetongue virus (*Reoviridae*) (7), a considerable achievement for this virus (47).

With our set of cloned genuine and integral DNAs of a nanovirus, we are now able to address questions on all aspects of the virus's biological cycle including vector transmission. For instance, the identity and nature of the helper factor required for aphid transmission (20) or the respective roles in infection of the DNAs encoding the proteins of as yet unknown function and their potential significance as host specificity factors, some of which appeared to be nonessential in *V. faba* (54), can now be analyzed by reverse genetics.

Finally, the long-standing question of how many DNAs constitute a nanovirus genome has, at least for FBNSV, been answered: eight. We predict that the same is true for the nanoviruses FBNYV and MDV and very likely also for SCSV, from which DNA-U2 and -U4 have not been identified.

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