

Faba Bean Necrotic Yellows Virus (Genus *Nanovirus*) Requires a Helper Factor for Its Aphid Transmission

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Purified faba bean necrotic yellows virus (FBNYV; genus *Nanovirus*) alone is not transmissible by its aphid vector, *Acyrtosiphon pisum*, regardless of whether it is acquired from artificial diets or directly microinjected into the aphid's hemocoel. The purified virus contains all of the genetic information required for its infection cycle as it readily replicated in cowpea protoplasts and systemically infected *Vicia faba* seedlings that were biolistically inoculated using gold particles coated with intact virions or viral DNA. The bombarded plants not only developed the typical disease syndrome, thus indicating that FBNYV is the sole causal agent of the disease, but also served as a source from which the virus was readily acquired and transmitted by *A. pisum*. The defect of the purified virus in aphid transmissibility suggests that FBNYV requires a helper factor (HF) for its vector transmission that is either nonfunctional or absent in purified virus suspensions. The requirement for an HF was confirmed in complementation experiments using two distinct isolates of the virus. These experiments revealed that aphids transmitted the purified virus isolate from artificial diets only when they had fed previously on plants infected with the other FBNYV isolate. Also, microinjected FBNYV, which persisted to the same extent in *A. pisum* as naturally acquired virus, was transmissible when aphids had acquired the HF from infected plants. This suggests that one of the functions of the HF in the transmission process is to facilitate virus transport across the hemocoel-salivary gland interface. © 1999 Academic Press

INTRODUCTION

Faba bean necrotic yellows virus (FBNYV; genus *Nanovirus*) causes severe yield losses and crop failure in important food and fodder legumes in African and Asian countries (Makkouk *et al.*, 1994; Franz *et al.*, 1995). The virus has a wide host range, and so far more than 50 plant species, mainly belonging to the *Fabaceae*, have been identified as hosts for the virus (Katul *et al.*, 1993; Franz *et al.*, 1997). All nanoviruses except coconut foliar decay virus (a tentative species in the genus *Nanovirus*; Pringle, 1998) are persistently transmitted in a circulative nonpropagative manner by aphids; coconut foliar decay virus is transmitted by planthoppers (Randles *et al.*, 1986). Efficient vectors of FBNYV are *Aphis craccivora* and *Acyrtosiphon pisum* (Franz *et al.*, 1998). Nanoviruses are phloem limited and not mechanically transmissible from plant to plant using abrasive agents or by seed (Chu and Helms, 1988; Harding *et al.*, 1991; Katul *et al.*, 1993). They consist of small icosahedral particles, 17–20 nm in diameter, that contain circular single-stranded DNA molecules each of about 1 kb in size (Chu and Helms, 1988; Harding *et al.*, 1991; Katul *et al.*, 1993). The viral capsid is composed of a single protein of about 20 kDa.

Recent work has shown that the genomes of two FBNYV isolates as well as that of milk vetch dwarf virus (MDV) consist of 10 different DNA molecules, with each containing one major open reading frame (ORF) coding for a protein (Katul *et al.*, 1998; Sano *et al.*, 1998). Both viruses show striking similarities in their genomic nucleotide sequences. For the other two definite *Nanovirus* species, subterranean clover stunt virus (SCSV) and banana bunchy top virus (BBTV), seven and six different DNA components have been identified, respectively (Boevink *et al.*, 1995; Burns *et al.*, 1995). Karan *et al.* (1997) detected the six different DNA components of BBTV in a number of virus isolates originating from various countries, which led to the suggestion that they represent the integral genome of the virus. Because one DNA component of BBTV does not have a matching counterpart among the other nanoviruses, it is believed that the complete genomes of SCSV, as well as those of FBNYV and MDV, might consist of more DNA components than have been identified. It has been reported that the DNA molecules are separately encapsidated, thus forming the multicomponent structure of the nanoviruses (Chu and Helms, 1988; Katul *et al.*, 1998).

The relatively strong similarity between FBNYV and MDV at the genomic level is also serologically manifested (Franz *et al.*, 1996). Using monoclonal antibodies (MAbs) in ELISA, it was shown that the capsid proteins of

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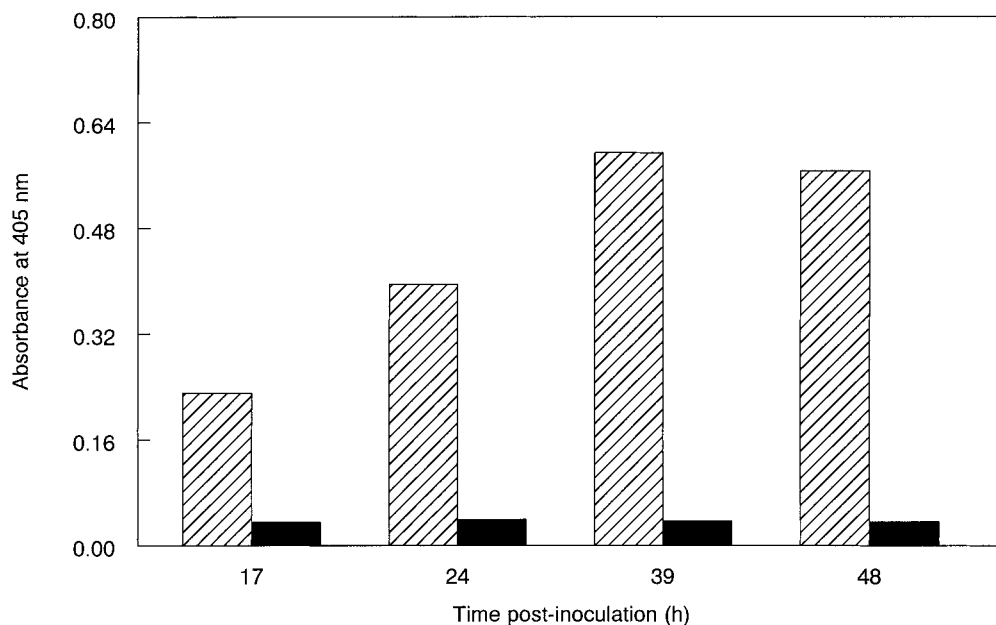


FIG. 1. Serological assessment of the amount of viral capsid antigen in cowpea protoplasts after inoculation with 1 μg of purified FBNYV-Eg. Equivalent amounts of 0.3×10^6 protoplasts were harvested 17, 24, 39, and 48 h after inoculation, and the amount of viral antigen was measured in TAS-ELISA (striped columns). Mock inoculation was performed with H_2O (dark columns).

FBNYV and MDV have several epitopes in common and that the epitope profiles of FBNYV isolates from different geographical regions vary considerably (Franz *et al.*, 1996).

So far, all attempts to demonstrate aphid transmissibility of a purified nanovirus acquired from either artificial diets or sucrose suspensions have failed (Chu and Helms, 1988; Thomas and Dietzgen, 1991; Katul *et al.*, 1993). Chu *et al.* (1993) showed that purified SCSV replicated in protoplasts, leading to *de novo* synthesis of capsid protein. These observations led to the suggestion that the defect in aphid transmissibility might be caused by the lack of a helper factor (HF) of viral origin or a helper virus in the purified virus suspensions (Chu *et al.*, 1993; Katul *et al.*, 1993).

Here, we report that purified FBNYV is infectious to *Vicia faba* but lacks a HF for its transmission by aphids. The requirement of the HF in aphid transmission of the virus was demonstrated in a similar fashion as was instrumental in identifying the helper component (HC) and aphid transmission factor-dependent transmission of potyviruses and caulimoviruses (Govier and Kassanis, 1974; Lung and Pirone, 1974). Moreover, evidence is presented that one of the functions of the HF is to mediate the transport of FBNYV particles across the hemocoel-salivary gland interface of the aphid.

RESULTS

Infectivity of purified FBNYV

So far, all of the methods used to extract nanoviruses from infected plant tissue resulted in the loss of aphid

transmissibility of the purified virus (Chu and Helms, 1988; Thomas and Dietzgen, 1991). We therefore applied several other procedures, including methods previously developed for the isolation of the membrane-bound rhabdoviruses and tospoviruses (Hunter *et al.*, 1990, 1995). However, these gentle isolation procedures did not retain aphid transmissibility of purified FBNYV of two isolates originating from Egypt (FBNYV-Eg) and Ethiopia (FBNYV-Eth). We therefore used an enzyme-assisted purification procedure developed for the isolation of luteoviruses because it gave high yields (400 μg of virus/kg plant material) relative to the procedures previously used.

To test the infectivity of purified FBNYV, cowpea protoplasts were inoculated with purified FBNYV-Eg. Serological analysis clearly showed that the amount of viral capsid antigen increased with time, consistent with the replication of purified FBNYV in these cowpea protoplasts (Fig. 1). To determine whether the genetic information for systemic movement in infected plants and vector transmission is retained in purified FBNYV, *V. faba* seedlings were inoculated by microprojectile bombardment. This revealed that purified virus as well as isolated viral DNA readily infected *V. faba* plants. When using intact virus particles, the infection rate varied between 20% and 33% (Table 1). Higher rates were obtained with higher helium pressure and the omission of spermidine in the coupling procedure. Moreover, the infection rate increased to 67% when 1.5 μg of extracted viral DNA was used as inoculum. Twelve days after bombardment, plants showed strong symptoms typical for the faba bean necrotic yellows syndrome; they were severely stunted

TABLE 1
Biolistic Inoculation of *Vicia faba* Seedlings with FBNYV Isolates

| Inoculum | Amount of purified virus or viral DNA per bombardment | Helium pressure of bombardment | Number of bombardments per plant | Number of plants infected/ Number of plants inoculated |
|---|---|--------------------------------|----------------------------------|---|
| Purified FBNYV-Eg (coated to gold particles with spermidine) ^a | 3.5 μg | 6–10 bar | 9 | 1/5 |
| Purified FBNYV-Eth (coated directly to gold particles) ^b | 4.4 μg | 14 bar | 3 | 2/6 |
| FBNYV-Eth DNA (coated to gold particles with spermidine) ^b | 0.75 μg ^c | 14 bar | 2 | 2/3 |

^a Biolistic inoculation was performed in a custom-made acceleration device with a low-pressure bombardment chamber.

^b Biolistic inoculation was performed with the Helios Gene Gun (BioRad).

^c 0.75 μg of DNA was extracted out of 8.3 μg of purified virus.

and slightly chlorotic with the youngest leaves rolled and not fully developed. Extracts from the bombarded plants reacted strongly with MAbs against FBNYV in triple antibody sandwich (TAS)-ELISA. The genomic DNA of either FBNYV isolate was specifically detected by immunocapture (IC)-PCR (results not shown). To test whether the virus in the bombarded plants was aphid transmissible, 12 test plants were each inoculated with 10 *A. pisum* nymphs that had an acquisition access feeding period (AAP) of 48 h on the bombarded plants. Two weeks after aphid inoculation, it was confirmed by symptomatology, TAS-ELISA, and IC-PCR that the progeny of the biolistically inoculated purified virus and viral DNA of either isolate were readily transmitted by *A. pisum*.

Taken together, these data show that purified FBNYV is highly infectious to *V. faba*, thus harboring the complete set of genetic information to complete the viral infection cycle, including vector transmission. In this way, it was also demonstrated for the first time that FBNYV is the sole causal agent of the faba bean necrotic yellows disease. The defect in aphid transmissibility suggests that the virus requires an additional HF for its vector transmission. The HF is either inactivated or absent in purified virus preparations.

Specific detection of FBNYV isolates by ELISA and IC-PCR

To demonstrate that an HF is required for the aphid transmission of FBNYV, two distinct isolates of the virus are required, which have to be readily distinguishable in plants with mixed infections. The Egyptian isolate of FBNYV could be readily differentiated from FBNYV-Eth by MAbs in TAS-ELISA (Franz *et al.*, 1996). Moreover, using the primer set eg1 in IC-PCR, FBNYV-Eg was specifically detected (Fig. 2). Because the reaction pattern of the MAbs did not enable the specific detection of the Ethiopian isolate in mixed infections, PCR primers to FBNYV-Eth were developed that required part of the genomic DNA of this isolate to be sequenced. The nucleotide

sequences of two different DNA components of the Ethiopian isolate were obtained (Figs. 3A and 3B). Sequence comparisons with FBNYV (Katul *et al.*, 1997) revealed that one of these components displayed 70% similarity to the nucleotide sequence of the putative movement protein-encoding component C4 of the Syrian FBNYV isolate (FBNYV-Sy). According to the nomenclature used for the different DNA molecules of FBNYV by Katul *et al.* (1997, 1998), we named this component of the Ethiopian isolate C4. C4 of FBNYV-Eth consists of 923 nucleotides (Fig. 3A). However, in one of four different clones, an additional stretch of 56 nucleotides at position nucleotide 812 has been identified that possibly represents sequence heterogeneity of the virus. C4 contains one major ORF encoding a putative protein of 13.2 kDa. Its derived amino acid sequence shares similarities of 94% (78% identity), 88% (75% identity), and 63% (45% identity) with that of component C8 of MDV (Sano *et al.*, 1998), C4 of FBNYV-Sy, and C1 of SCSV (Boevink *et al.*, 1995), respectively. In contrast, the major ORF of the other component of FBNYV-Eth showed no similarities with those of any of the DNA molecules of FBNYV-Sy or other nanoviruses characterized. Because 10 components represent the highest number of DNA molecules characterized for a *Nanovirus* species (Katul *et al.*, 1998; Sano *et al.*, 1998) and this component lacks a counterpart among the known nanovirus genomes, we designated it C11. The major ORF of component C11 encodes a putative product of 12.4 kDa for which no function could yet be assigned.

On the basis of the obtained nucleotide sequences, primer sets eth1 and eth2 were developed, enabling the specific detection of FBNYV-Eth (Fig. 2). Using the primer sets eth1 and eth2, 717- and 796-bp DNA fragments were amplified from components C11 and C4 of FBNYV-Eth, respectively.

Complementation of transmission of purified FBNYV

Transmission complementation experiments were performed in which *A. pisum* nymphs were fed on *V. faba*

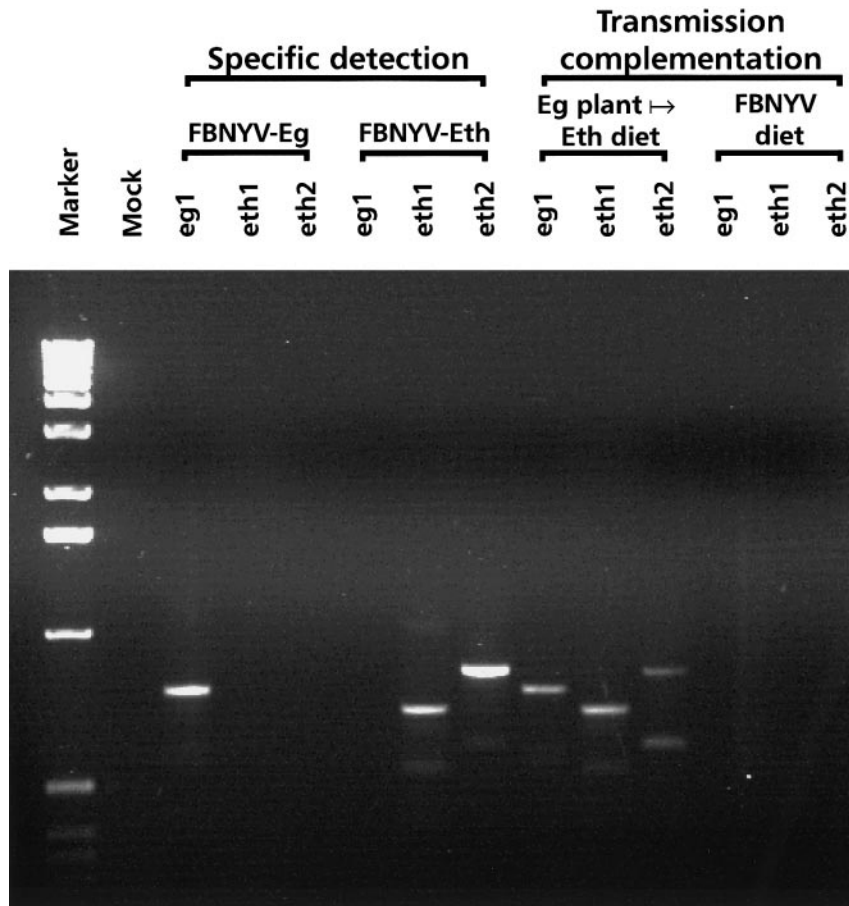


FIG. 2. IC-PCR detection of the Ethiopian and Egyptian FBNYV isolates in inoculated test plants. The primer sets used for detection were eg1 for C5 (Katul *et al.*, 1997) of FBNYV-Eg, eth1 for C11 of FBNYV-Eth, and eth2 for C4 of FBNYV-Eth. To test the specificity of the primer sets, DNA of plant samples singly infected with either FBNYV isolate was used as template. In the transmission complementation experiment, DNA was used from test plants that were inoculated with aphids that had an AAP of 96 h on plants infected with FBNYV-Eg before they were transferred for an AAP of 48 h to artificial diets containing 8 μ g of purified FBNYV-Eth. Alternatively, aphids had an AAP of 48 h on artificial diets only containing purified FBNYV of either isolate.

plants infected with one of the FBNYV isolates before or after they acquired purified virus of the other isolate. The results unequivocally showed that purified FBNYV acquired from artificial diets was transmitted only when aphids had first fed on infected plants. Similarly, the transmission of microinjected FBNYV was complemented in the aphid by the virus isolate acquired from plants (Table 2). Thus the inoculated test plants became infected with both virus isolates. The presence of the purified virus in these mixed infected plants was assessed by TAS-ELISA and by IC-PCR (Fig. 2) and confirmed by sequence analysis of the PCR products. Aphids did not transmit purified FBNYV acquired from artificial diets before feeding on infected plants. This indicates that the putative HF must be acquired before the oral acquisition of purified virus.

It was shown that the length of the AAP on a source plant positively influenced the likelihood of transmission complementation of purified FBNYV. Moreover, the data revealed that although the FBNYV isolates are distinct, their HFs are

functional in the heterologous combination. An AAP of 48 h on source plants infected with FBNYV-Eth resulted in 100% transmission of the purified Egyptian isolate acquired from artificial diets (Table 2). However, when aphids had an AAP of 24 h on infected source plants immediately before acquisition of the purified virus, only 50% of the inoculated plants became infected by the purified FBNYV-Eg. An AAP of 24 h on FBNYV-infected source plants always appeared to be sufficient for 100% transmission complementation of the purified virus if the aphids were held for 3 days on noninfected plants between the two acquisition steps. It was also shown that the transmission of purified FBNYV-Eg was more efficiently complemented by the Ethiopian isolate acquired from plants than vice versa. Nymphs that had an AAP of 48 h on FBNYV-Eth-infected plants transmitted the purified Egyptian isolate acquired from artificial diets in all experiments. In contrast, aphids required an AAP of 96 h on plants infected with FBNYV-Eg before they were able to complement the transmission of the purified Ethiopian isolate (Table 2).

| | | |
|----------|---|-----|
| A | CTGGGGCGGGGCTTAGTATTACCCCGCCCCAGGTTACAGCGGAGTCATCACGTGAGACGC | 60 |
| | ACATGCTATTAAATGAATTATAATTAATAAGTGGGGGATGGTCCCCACTCCGCACATAAC | 120 |
| | TTCAAATACACTGAATATATGCTTTTGCTTCGTACGAAGCAAAGGTAAAGAATAAAAACT | 180 |
| | GGTCCCCACAACGCTATAATGCGTTGTGCTTTAATTTGAAACGAATGGTTGAGATATGTT | 240 |
| | CAACTTTTACTAGCGTACAGAGGACGCTTGAGTATATCTCTATAAACAATGAAGCAGAAG | 300 |
| | TTCATTAATCTCTCGTTTCGTCTTGTTAAATGGCTTCGTCTAGCACTATTCGGATTCTGG | 360 |
| | M A S S S T I P D S G | |
| | GTATTACGATGCGTATCAAGACGACGGAGATGTTAATGCTGAGAAGAGACATCAGGCACCT | 420 |
| | Y Y D A Y Q D D G D V N A E K R H Q A L | |
| | CTATATGATAGGTATTGTTCTGCTAATCATGATCTGTGTTATTATCCTGTGGGTTTGTAT | 480 |
| | Y M I G I V L L I M I C V I I L W V C I | |
| | AATGCTTGCATGCTATCTTCCGGGATTTGTGAAGAAGACAATGGAAGCCTGGTTGAGTTC | 540 |
| | M L A C Y L P G F V K K T M E A W L S S | |
| | TTCATCGTTGATGAAGAGAAGAGTTGCTTCCACTTTAACAAGAACACCCTTTGAAGCTAC | 600 |
| | S S L M K R R V A S T L T R T P F E A T | |
| | TGGTCTGAGAGAGAAAAGGAATGGGATGCTAGAAGACAAGCTAACGCATCTGGTAGTCA | 660 |
| | G P E R E R N W D A R R Q A N A S G S Q | |
| | GCCTGTTAATACATCTGTATTTTAAAGTGTTCAGAAAATATGAAGCTTGTATGTTGTAT | 720 |
| | P V N T S V F * | |
| | ATTCATGTAATATTGTAATTGGGAAATTTAATGAAATTAATATGTTGTTGTTGTTTTGTG | 780 |
| | TAATTGTTGTGTTGTTGAAGAACCAGAGAGTTTGGGTTTACTCCGCGTAGCGGTATGCTT | 840 |
| | CAGCCCAATAGGCCCAATAGTCTTAAGGCCCAATAGAACGACCAATAGGAATGAAGCATG | 900 |
| | ACGTCATTTGATCCCGTGCTGAG | 923 |

FIG. 3. Nucleotide sequences of two DNA components of FBNYV-Eth: components C4 (A) and C11 (B). The deduced amino acid sequence of the major ORF of each component is shown under the nucleotide sequence.

To determine the efficiency of transmission complementation in a more quantitative manner, 15 *V. faba* seedlings were each inoculated with five aphids that had an AAP of 24 h on plants infected with FBNYV-Eth before they acquired purified FBNYV-Eg from artificial diets. A variant of this experiment was performed in parallel with aphids that had an AAP of 48 h on FBNYV-Eth-infected plants. In both experiments, purified FBNYV-Eg was transmitted with an efficiency of 20% compared with 100% transmission of the Ethiopian virus isolate acquired from plants (data not shown). Whether this is due to differences in the amount of HF acquired from infected plants or a reduced affinity of the HF to the heterologous virus remains to be investigated.

Persistence of purified FBNYV in aphids

Purified FBNYV-Eg directly injected into the hemocoel of *A. pisum* nymphs taken from noninfected plants persisted in the hemolymph to the same extent as purified virus injected into aphids that had acquired the Ethiopian isolate from plants before injection (Fig. 4). Over a time span of 7 days in which aphids were placed on noninfected *V. faba* plants, the amount of viral antigen in the aphids gradually decreased to about 50% of the initial amount. This decline might be due to natural degradation of FBNYV particles in the hemolymph of aphids as observed for luteoviruses (van den Heuvel *et al.*, 1997). Combining the persistence data with those of the trans-

B CTGGGGCGGGGCTTAGTATTACCCCGCCCCAGGTTTCAGCGGAGTCATTGAGACTCCATC 60
 TCAGCCGTTTCATTAAATGGACACGTGTACAATCAGGATCTGTGATTTCGTGAAGCGAATCT 120
 GACGGAAGATTGTACACTTTATCTCTTCACGCGTTTTGAAGCTACTGTTGATTATGCAAG 180
 TGGACGATGGTCCCCACATATATATTTAAAACGACAACAGCTGTATACAGCTGTATTGAT 240
 GTGACGTATATAATTAATAATTATTTAATTATGAGGATTGTCCTGCACGCTTATGTTGT 300
 CGTCTATAAAACCTTCTCTACTGAAGAACCTGTCTCTGAATTTACTTTCTCTCTCTCTG 360
 TGTAGAAGAGAGATAAGCAGGTTATGGAATGTAGGTTCTTCATCTCTATTATTTTATTTCG 420
 M E C R F F I S I I L F V
 TCGTGCTGCTGAATCCTTCTCTGATAATTAATATGGTTCTCGGGTATGTTCTTGGCTCTT 480
 V L L N P S L I I N M V L G Y V L G S L
 TATTCAGAAGTAATTATTCCAGATTAAGAAGTTACTGTCAGGTAATAAGAACGAGAACC 540
 F R S N Y S R L K K L L S G N K N E N R
 GCGAAGAAGAAGACGAACATATATCCAGATGAAGAATCCGTTTCGAGGATGCTGAATCAG 600
 E E E D E H I S Q M K N P F E D A E S D
 ATGTATTGCAACATCTAAAGACTCTGGGTCTGGAAACAAAGGTTGAAGGCGATGATCTTG 660
 V L Q H L K T L G L E T K V E G D D L E
 AATATTTACAGCGATTGTGGGAATCTATGAGTAGTAAGAAATAAATATGTTTACCCAAGT 720
 Y L Q R L W E S M S S K K *
 AATTGTAATAGGTAATAACAGCTCACGTCATGCTTCATTCCCTATTGGTCAGTCTATTGG 780
 GCCTTGAGAGCAATTGGGCCCTATTAAGCCATTAAGTCAGTCAGATATATTGTATTTGTA 840
 ATATTGTTAATCAGTCAATAAATATTAATCATTTTTTCAITTTACTCCGCGTAGCGGTATGT 900
 TAAATATTTTATTGCCATATATACCCTTCATTAATGAAGGGGTAATGTCTATTTTGTCTCT 960
 TGTGACGTCATTTGATCCCGTGCTGAG 987

FIG. 3—Continued

mission complementation results (Table 2) suggests that the HF mediates the transport of FBNYV particles across the hemocoel-salivary gland interface because microinjected purified virus was transmissible only when aphids had fed on infected plants.

DISCUSSION

In this study, we demonstrated infectivity for purified FBNYV that provided the first proof that this virus is the sole causal agent of the faba bean necrotic yellows syndrome in *V. faba*. This was achieved by circumventing the aphid vector by bombarding *V. faba* seedlings with the purified virus to reach the phloem vessels of the plant

in which the virus replicates and moves systemically. The particle bombardment technique is commonly used for gene (DNA) transfers into plant tissue (Gordon-Kamm *et al.*, 1990). Here, we report for the first time the successful application of this technique to inoculate plants with virions and purified DNA of a circulatively insect-transmitted plant virus that is not mechanically transmissible from plant to plant using abrasive agents. We demonstrated that purified FBNYV lacks a functional HF for its transmission by aphids as suggested earlier by Katul *et al.* (1993) and by Chu and Helms (1988) for SCSV. The requirement of an HF for the aphid transmission of FBNYV was revealed in complementation experiments that were conducted in accordance with those per-

TABLE 2

Transmission Complementation of Purified FBNYV by Nymphs of *Acyrtosiphon pisum*

| Mode and sequence of FBNYV acquisition by <i>A. pisum</i> using the Egyptian (Eg) and/or Ethiopian (Eth) virus isolates | AAP on infected source plant | Number of transmissions of purified FBNYV/ Number of experiments conducted |
|---|------------------------------|---|
| Purified FBNYV acquired from membranes | | |
| Purified Eg or Eth ^a | | 0/6 |
| Eth from <i>V. faba</i> → purified Eg | 24 h | 3/6 |
| Eth from <i>V. faba</i> → purified Eg | 48 h | 4/4 |
| Eth from <i>V. faba</i> → noninfected plant ^b → purified Eg | 24 h | 3/3 |
| Purified Eg → Eth from <i>V. faba</i> | 24 h | 0/3 |
| Purified Eg → noninfected plant → Eth from <i>V. faba</i> | 24 h | 0/3 |
| Eg from <i>V. faba</i> → purified Eth | 24 h | 0/1 |
| Eg from <i>V. faba</i> → purified Eth | 48 h | 0/2 |
| Eg from <i>V. faba</i> → purified Eth | 96 h | 1/2 |
| Purified FBNYV injected into the hemolymph of aphids | | |
| Purified Eg or Eth | | 0/4 |
| Eth from <i>V. faba</i> → starving ^c → purified Eg | 96 h | 2/2 |
| Eth from <i>V. faba</i> → starving → purified Eg | 48 h | 1/1 |
| Eg from <i>V. faba</i> → starving → purified Eth | 96 h | 1/1 |

Note. In each experiment, one test plant was inoculated with 60–75 aphids that readily transmitted the FBNYV isolate acquired from infected plants.

^a 48 h AAP on a sachet containing 8 μg of purified FBNYV of either isolate in 130 μl of *A. pisum*-specific artificial diet.

^b Aphids were transferred to noninfected plants for 72 h, which were exchanged with noninoculated ones at 24-h intervals.

^c 24 h of starving at 4°C to facilitate microinjection.

formed by Govier and Kassanis (1974) and Lung and Pirone (1974). These researchers discovered the involvement of HF's in the aphid transmission processes of potyviruses and caulimoviruses, which are transmitted in a nonpersistent and semipersistent manner, respectively. Potyviruses and caulimoviruses are acquired dur-

ing probing of an aphid on an infected plant and attach to the inner surface of the stylet food canal and foregut of the aphid. The HC of potyviruses as well as the aphid transmission factor (ATF) of caulimoviruses seem to bridge the binding of the virus particles to their retention sites in the vector (Pirone and Blanc, 1996). Both HC and

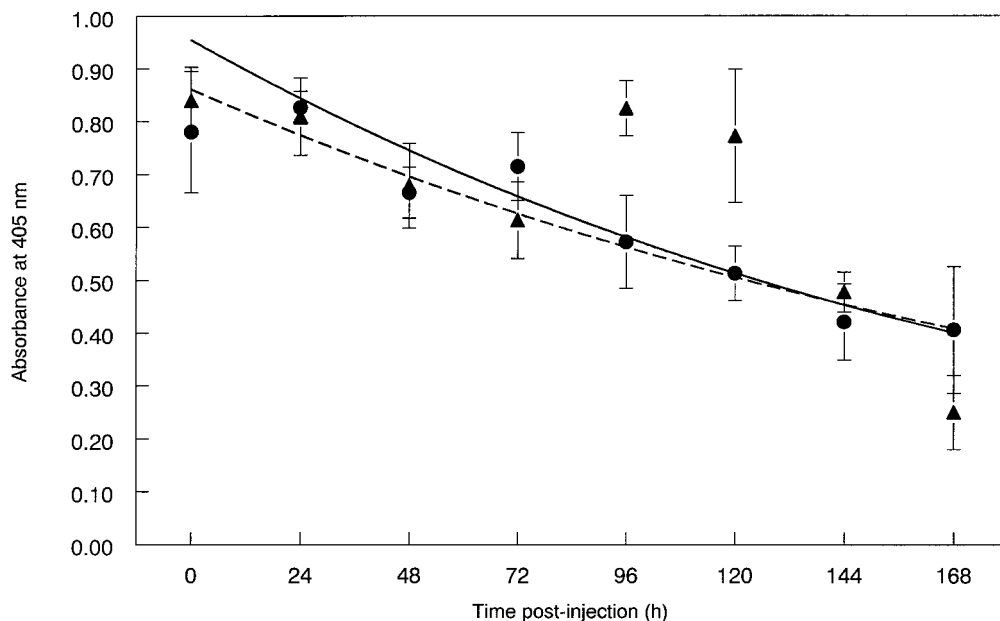


FIG. 4. Persistence of FBNYV-Eg in *A. pisum* tested by TAS-ELISA. About 10 ng of purified FBNYV was microinjected into 5-day-old nymphs that were either reared on noninfected *V. faba* plants (▲ and full curve pattern) or on plants that were infected with the Ethiopian isolate of the virus (● and interrupted curve pattern). Only in the latter case was the microinjected virus transmitted. Two aphids per sample were assayed in five replicates directly after microinjections and at 24-h intervals over a 7-day period, during which aphids were daily transferred to noninfected *V. faba* plants. Each point represents the mean value (\pm standard error).

ATF are virus-encoded proteins that are expressed in infected plants (Pirone and Blanc, 1996). The observation that the progeny of biolistically inoculated DNA isolated from purified FBNYV was aphid transmissible suggests that the HF of FBNYV is a virus-encoded protein as well. The current study did not provide evidence for the involvement of a second virus in FBNYV transmission as previously suggested (Chu and Helms, 1988; Katul *et al.*, 1993).

In our transmission complementation studies, two isolates of the virus were used: FBNYV-Eg (Katul *et al.*, 1998) and FBNYV-Eth. The sequence analysis of two DNA components of FBNYV-Eth showed that this isolate is genetically distinct from FBNYV isolates previously characterized. The deduced amino acid sequence of the putative movement protein of FBNYV-Eth shares higher sequence similarity with that of MDV than with those of other FBNYV isolates. This confirms the previously made observation that FBNYV isolates from different geographical origin are highly variable (Franz *et al.*, 1996). Further molecular data are required to determine whether FBNYV-Eth represents a new nanovirus. Moreover, the coding sequence of component C11 of the Ethiopian isolate appears to be unique among the nanoviruses known at present. This suggests that we may have found a new component of a nanovirus genome that has yet to be revealed in other members of this genus.

Unlike potyviruses and caulimoviruses, FBNYV is transmitted in a circulative manner (Franz *et al.*, 1998). Thus the mode of action of the FBNYV HF in the aphid differs fundamentally from that described for the potyvirus HC and the caulimovirus ATF. The results of the present study have confirmed the circulative mode of FBNYV transmission because purified FBNYV directly injected into the hemocoel of aphids was transmissible when complemented. This justifies a comparison between the aphid transmission of the well-characterized luteoviruses and the transmission mechanisms suggested for FBNYV. Luteovirus particles are ingested by the aphid along with the phloem sap from infected plants and reach the gut system from where they are transported through the epithelium into the hemolymph via receptor-mediated endocytosis (Gildow, 1993). In the hemolymph, which acts as a reservoir for circulatively transmitted viruses, luteoviruses as well as FBNYV persist for several weeks (Franz *et al.*, 1998; van den Heuvel *et al.*, 1997). The persistence of luteoviruses in the hemolymph crucially depends on the presence of a GroEL homolog, expressed by the primary endosymbiotic bacterium of the aphid (Hogenhout *et al.*, 1998, van den Heuvel *et al.*, 1994, 1997). The GroEL homolog protects luteovirus particles from proteolytic degradation, thus explaining their persistent nature. Whether nanoviruses interact with GroEL in a similar fashion as do luteoviruses has yet to be determined.

The HF of FBNYV may be necessary for the transport of

the virus across the hemocoel–salivary gland interface. The basal lamina and plasmalemma of the accessory salivary glands are considered to be the locations for regulation of luteovirus particle uptake by the salivary glands, thus determining vector specificity (Gildow, 1982; Gildow and Rochow, 1980). Gildow and Rochow (1980) suggested that the recognition between virus particles and virus receptors in the salivary gland was the basis of the uptake mechanism. In the case of FBNYV, the HF may facilitate the recognition and binding of virus particles to these putative receptors. The results of the complementation experiments suggest that the HF interacts with the FBNYV particles because the HF of the Ethiopian isolate complemented the transmission of the purified Egyptian isolate with a higher efficiency than the HF-Eg complemented the transmission of the purified Ethiopian isolate.

However, it cannot be excluded that the HF has additional functions such as mediating the transport of FBNYV particles through the epithelial cell linings at the gut of the aphid vector. Further investigation will be required to explain the transport mechanism of FBNYV across the gut–hemocoel barrier.

MATERIALS AND METHODS

Virus isolates and aphids

Two distinct isolates of FBNYV from Egypt (FBNYV-Eg; Katul *et al.*, 1998, formerly named EV1–93, Franz *et al.*, 1996) and Ethiopia (FBNYV-Eth) were used. Both isolates were separately maintained on *V. faba* L. cv. Double White by repeated aphid transfers in climate chambers at 20°C and 16 h light/day. *A. pisum* reared on noninfected *V. faba* was used as aphid vector throughout this study. About 2–3 weeks after inoculation, virus-infected *V. faba* plants were used as source plants for virus acquisition or used for virus purification essentially following the enzyme-assisted procedure previously developed for luteoviruses (van den Heuvel *et al.*, 1991).

Virus acquisition and transmission experiments

To standardize virus acquisition and transmission, only first instar nymphs of *A. pisum* (maximum age of 48 h) were used at the onset of each experiment. All experiments were carried out at a constant temperature of 20°C. Depending on the experiment, the AAP on FBNYV-infected source plants varied between 24 and 96 h. For membrane feeding, about 30–40 nymphs were placed onto a Parafilm sachet containing 8 µg of purified FBNYV in 130 µl of *A. pisum*-specific artificial diet (Akey and Beck, 1971). After an AAP of 48 h, nymphs from three sachets were combined and transferred to one *V. faba* seedling for an inoculation access feeding period of 5 days. At 2–3 weeks after inoculation, leaf samples were tested for the presence of FBNYV by TAS-ELISA (Franz *et al.*, 1996) or IC-PCR.

Microinjection into aphids and persistence of FBNYV in aphids

To circumvent the gut barrier, about 10 ng of purified FBNYV was directly microinjected into the hemocoel of 5-day-old nymphs of *A. pisum*. The aphid's ability to transmit the purified virus and the persistence of injected FBNYV-Eg in the aphid were assessed. For the latter, aphids were collected directly after microinjection and at 24-h intervals over a time span of 7 days, during which they were kept on noninfected test plants. Plants were replaced every 24 h to minimize the risk of reacquisition of the virus. Collected aphids were stored at -80°C before they were tested in TAS-ELISA for the presence of viral antigen of purified FBNYV-Eg. Each ELISA sample consisted of two aphids and 5×2 aphids were analyzed per sampling date.

Biolistic inoculation of FBNYV

Two different systems were used to introduce gold particles coated with either purified FBNYV virions or viral DNA into 2-day-old *V. faba* seedlings. The first system was a custom-made helium-powered acceleration device with a bombardment chamber. The virus-gold suspension was prepared as follows: 60-mg gold particles (0.6 μm in diameter) were washed with 2 ml of 96% (v/v) ethanol, and after centrifugation at 13,000 *g* for 1 min, they were resuspended in 1 ml of H_2O . To 100 μl of this suspension, 100 μl of FBNYV at 60 $\mu\text{g/ml}$ in H_2O , 130 μl of 2.5 M CaCl_2 , and 52 μl of 0.1 M spermidine were added. After a 10-min incubation on ice, the suspension was centrifuged at 13,000 *g* for 1 min. The resulting pellet was washed with 160 μl of 70% (v/v) ethanol, centrifuged at 13,000 *g* for 1 min, and finally resuspended in 50 μl of H_2O . The *V. faba* seedlings were placed into the bombardment chamber of the apparatus in which they were exposed to 50 mbar vacuum. Each bombardment consisted of 5 μl of the virus-gold suspension containing 3.5 μg of purified FBNYV-Eg. When using the Helios Gene Gun (BioRad, Hercules, CA), 67 μg of FBNYV-Eth was coupled to 10-mg gold particles after the above-mentioned procedure with the exception that spermidine was omitted. In addition, viral DNA of FBNYV-Eth was coated onto gold particles in the presence of spermidine. These coated particles were loaded into the cartridges of the Helios Gene Gun according to the manufacturer's instructions. Each bombardment either contained 4.4 μg of purified FBNYV-Eth or 0.75 μg of viral DNA, which was extracted from 8.3 μg of purified FBNYV-Eth.

IC-PCR

DNA of both FBNYV isolates was amplified in IC-PCR. Homogenized leaf material (50 μl) was incubated in PCR tubes coated with FBNYV-specific IgG (kindly provided by H. J. Vetten, BBA, Braunschweig, Germany). After over-

night incubation, the tubes were washed three times with PBS-T (8 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 2 mM KCl, 140 mM NaCl, pH 7.4, and 0.05% Tween 20) and three times with PCR buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl_2 , and 50 mM KCl). For the detection of the Egyptian isolate of FBNYV, we used primer set eg1 consisting of primers P6 and P7 (Katul *et al.*, 1997) specific for component C5 encoding the coat protein of FBNYV-Eg. Component C11 of the Ethiopian isolate of FBNYV was detected with primer set eth1 consisting of primers F30 (5'-CAGTAACTTCTTTAATCTGG-3'; nucleotides 518–499) and F36 (5'-AGCAATTGGGCTATTAAGC-3'; nucleotides 789–808). Primer set eth2, consisting of primers F32 (5'-AGGCTTCCATTGTCTTCTTC-3'; nucleotides 530–511) and F37 (5'-TCAGCCTGTTAATACATCTG-3'; nucleotides 658–677) was specific for component C4 of FBNYV-Eth.

Sequence analysis

The sequences of two components of the FBNYV-Eth genome were deduced by PCR using primers complementary to the highly conserved stem-loop region of the circular DNA molecule of FBNYV and subsequent sequencing of the resulting PCR products. The primers had the following sequences: primer sl1 (5'-TAGTATTAC-CCCCGCCCC-3'; nucleotides 14–31) and primer sl2 (5'-GTAATACTAAGCCCCGCC-3'; nucleotides 22–5). The sequences of the stem-loop region of the two components were confirmed by PCR using the primer sets eth1 and eth2, which were located outside of this region. The resulting PCR products were cloned with the use of the TA Cloning Kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Recombinant clones were purified using the High Pure Plasmid Purification Kit (Roche Products, Welwyn Garden City, UK). Sequence analysis was conducted using an Applied Biosystems model 373 automated sequencer together with a sequencing kit containing AmpliTaq DNA polymerase (Applied Biosystems, Norwalk, CT) and universal or FBNYV-specific primers.

The nucleotide sequences reported here appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under Accession nos. AF159704 (C11) and AF159705 (C4).

Preparation and inoculation of protoplasts

The preparation and incubation of cowpea protoplasts essentially followed the procedure of Rottier *et al.* (1979). About 1×10^6 cowpea protoplasts were inoculated with 1 μg of FBNYV-Eg according to the procedure of van Bokhoven *et al.* (1993). The inoculated protoplasts were incubated for virus multiplication at 25°C under continuous light. Samples of 0.3×10^6 protoplasts were taken at 17, 24, 39, and 48 h after inoculation, sonicated for 30 s, and analyzed for the presence of FBNYV in TAS-ELISA.

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